UNIVERSITAT DE BARCELONA

Interplay of virulence, antibiotic resistance and epidemiology in *Escherichia coli* clinical isolates

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"Interplay of virulence, antibiotic resistance and epidemiology in *Escherichia coli* clinical isolates"

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Barcelona, Setembre 2018

El Dr. JORDI VILA ESTAPÉ, Catedràtic del Departament de Fonaments Clínics de la Facultat de Medicina de la Universitat de Barcelona, Cap del Servei de Microbiologia de l'Hospital Clínic de Barcelona i Research Professor i Director de la Iniciativa de Resistències Antimicrobianes de l'Institut de Salut Global de Barcelona (ISGlobal) i la Dra. SARA M. SOTO GONZÁLEZ, Professora Associada del Departament de Fonaments Clínics de la Universitat de Barcelona i Associate Research Professor d' ISGlobal,

CERTIFIQUEN:

Que el treball de recerca titulat **"Interplay of virulence, antibiotic resistance and epidemiology in *Escherichia coli* clinical isolates**", presentat per ELISABET GUIRAL VILALTA, ha estat realitzat al Laboratori de Microbiologia de l'ISGlobal, dins les dependències de l'Hospital Clínic de Barcelona, sota la seva direcció i compleix tots els requisits necessaris per la seva tramitació i posterior defensa davant del Tribunal corresponent.

Barcelona, Setembre 2018,

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Abbreviation	Explanation
AAC	N-acetyltransferase
AAF	Aggregative adherence fimbriae
ADP	Adenosine diphosphate
AE	Attaching and effacing
aEPEC	Atypical EPEC
AGs	Aminoglycoside
AMC	Amoxicillin-clavulanic acid
AME	Aminoglycoside-modifying enzyme
AMR	Antimicrobial resistance
ArmA	Aminoglycoside resistance methyltransferase A
BFP	Bundle-forming pilus
CC	Clonal complex
CDC	Center for Disease Control and Prevention of the United States of America
CF	Colonisation factor
CFA	Colonisation factor antigen
cGMP	Cyclic guanosine monophosphate
CNF	Cytotoxic necrotizing factor
CS	Coli surface antigen
CSF	Cerebrospinal fluid
DAEC	Diffusely-adherent *E. coli*
DEC	Diarrhoeagenic *E. coli*
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
ECDC	European Centre for Disease Prevention and Control
EHEC/STEC	Enterohaemorrhagic (*Shiga* toxin-producing) *E. coli*
EIEC	Enteroinvasive *E. coli*
EONS	Early-onset neonatal sepsis
EPEC	Enteropathogenic *E. coli*
ERIC	Enterobacterial repetitive intergenic consensus
ESBL	Extended-spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
FQX	Fluoroquinolone
GBS	Group B *streptococci* or *Streptococcus agalactiae*
GEI	Genomic island
HC	Haemorrhagic colitis

Abbreviation	Explanation
HGT	Horizontal gene transfer
HlyA	α-haemolysin
HUS	Haemolytic uremic syndrome
HUVEC	Human umbilical vein endothelial cell
IAI	Intra-amniotic infection
Inc	Plasmid incompatibility group
IR	Inverted repeat
IS	Insertion sequence
IUTI	Invasive UTI
kb	Kilobase(s)
LMIC	Low- and middle-income countries
LONS	Late-onset neonatal sepsis
LPS	Lipopolysaccharide
LT	Heat-labile enterotoxin
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
MDR	Multidrug resistance / multidrug resistant
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
mRNA	Messenger RNA
NMEC	Neonatal meningitis-causing *E. coli*
OMP	Outer membrane protein
PAI	Pathogenicity-associated island
PBP	Penicillin-binding proteins
PBRT	PCR-based replicon typing
PCR	Polymerase chain reaction
pEAF	EPEC adherence factor plasmid
PFGE	Pulse-field gel electrophoresis
QRDR	Quinolone resistance-determining region
REP-PCR	Repetitive Extragenic palindromic sequence-based PCR
RMTase	RNA methyltransferase
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
SEPEC	Sepsis-causing *E. coli*
ShET	*Shigella* enterotoxin
SMZ	Sulfamethoxazole
SPATE	Serine Protease Autotransporters Toxins in *Enterobacteriaceae*
ST	Sequence type
ST	Heat-stable enterotoxin
stx	Shiga-toxin

Abbreviation	Explanation
SXT	Trimethoprim/sulfamethoxazole
TD	Traveller's diarrhoea
tEPEC	Typical EPEC
TMP	Trimethoprim
tRNA	Transfer RNA
TTSS	Type II secretion system
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
VEC	Vaginal *Escherichia coli*
VF	Virulence factor
VFG	Virulence factor gene
WHO	World Health Organization

II. INTRODUCTION

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1. General characteristics of *Escherichia coli*

Escherichia coli are one of the microorganisms most frequently studied worldwide. They are Gram-negative bacilli, facultative anaerobic, rod-shaped bacterium, which are most often found in the gastrointestinal tract as a normal coloniser of warm blood organisms (mainly in mammals, but are also present in birds, reptiles and fish). Taxonomically, *E. coli* belong to the *Enterobacteriaceae* family and are an important component of the intestinal microbiota, being involved in some essential metabolic processes such as the production of vitamin K and vitamin B12 (1). *E. coli* also help to maintain the anaerobic environment needed for most of the microbiota by consuming oxygen that enters the gut and competitively exclude pathogens from the lower intestine of their hosts (2).

E. coli can also adapt to life in an external environment outside hosts, such as soil, water, plants and food, due to their hardiness and metabolic flexibility (3).

Despite establishing symbiotic relationships, this well-known microorganism can also have an important pathogenic role within their hosts, especially from a human point of view. It is able to cause a wide variety of infections that can reach a high prevalence and even cause significant morbidity and mortality (4). According to a recent report on this topic, it is predicted that three million people will die from a drug-resistant *E. coli* infection by 2050 if no measures are taken to tackle the problem of antimicrobial resistance (5). Although this figure exceeds that expected in a developed continent, it demonstrates the presence of an emerging health threat - antimicrobial resistance.

1.1 Specific features

E. coli (*Fig. 1*) has the following phenotypic and metabolic characteristics which make it a unique bacterium (2):

- It measures approximately 1 µm in length and 0.35 µm in width, although it can vary depending on the strain and the environmental conditions.
- It is facultative anaerobe, meaning that it can grow with or without oxygen, but it is not able to grow at extreme temperatures or pH.
- It is able to use lactose, glucose and saccharose as a carbon source for fermentation.
- It is catalase-positive, oxidase-negative, indole-positive, and able to reduce nitrate to nitrite.
- Its optimal temperature for growth is 37ºC (similar to that of warm-blood organisms), but it can grow within a range of 10ºC to 45ºC (6).
- It can have either flagella or pili, which facilitates the motility and the capacity to attach to surfaces or other cells, respectively.
- It is non-spore forming.

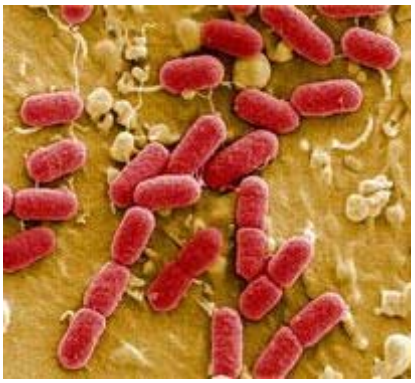

Figure 1: *Escherichia coli*. Adapted from *Nature newsblog* (288).

1.2 Discovery and applications of *E. coli*

E. coli was discovered in 1885 by the German-Austrian bacteriologist and paediatrician Theodore von Escherich (7). While focusing his studies on infant gut microbes and their role in digestion and disease, he discovered a fast-growing bacterium in the faeces of infants which he named *Bacterium coli commune*. Dr. Escherich concluded that this microorganism could be considered to be commensal, since the function of the digestive tract in breast-feeding neonates remained undisturbed (8). After his death, scientists renamed this bacterium *Escherichia coli* in honor of its discoverer.

Taking into account the availability and ease of working with this species, *E. coli* became the bacteria of first-choice for microbiology studies and teaching laboratory collections in the early 20th century, and was thereafter established as a bacterial model for the molecular biology revolution in the 1950s. Scientists of the standing of Francis H. C. Crick, who was awarded the Nobel Prize in Physiology of Medicine in 1962, used *E. coli* for his studies on the genetic code. Nowadays, *E. coli* is widely used in a variety of branches of science such as commercial genetic engineering, pharmaceutical production, and biotechnology industry (2).

1.3 Niches and relationships: commensal and pathogenic *E. coli*

The *E. coli* niche *par excellence* is the gastro-intestinal tract of mammals. Although the gut microbiome has an anoxic environment and is dominated by obligate anaerobes bacteria such as members of the Bacteroidetes and Firmicutes phyla, *E. coli* is the most common aerobe in the lower intestine, accounting for 0.1-5% of the total microbiota (9). *E. coli* grows in the mucus layer of the gut (one of the ecological niches of the intestine defined by its nutrient availability) within a complex biofilm together with other species, competing for nutrients. Paradoxically, very little is known about the acquisition and metabolism of nutrients which enable the growth and persistence of colonised microorganisms, but several studies have shown that the mucus layer is the most suitable niche for *E. coli*, because it is not able to synthetize polysaccharide-degrading enzymes and the mucus releases monosaccharides and other glycoproteins to allow its growth (10). The *E. coli* population in mammals varies constantly due to different environmental factors, with the host organism regulating its microbiome according to the impact on its health.

Since *E. coli* is a gut microorganism and its mucus lining is constantly detached, it is regularly excreted to the external environment together with other faecal matter. This life cycle forces *E. coli* to adapt to life outside the host, which is often threatening. Nonetheless, the genomic plasticity of *E. coli* enables it to survive under adverse conditions long enough to enter another host. Environmental *E. coli* populations develop the capacity of persistence, a general

adaptation that allows cell survival in very variable conditions and is apparently based on the metabolic inactivity status.

Thus, this species can survive in soil, manure, irrigation water, plants such as radishes or lettuces as well as meat, among others. It can even colonise roots or leaf surfaces and internal plant compartments (and therefore cannot be removed by simple washing or the addition of disinfectants). *E. coli* can also contaminate consumable products during food processing or packaging, resulting in dissemination throughout the food production chain and closing the cycle by infecting a host again (11).

From the host perspective, *E. coli* can establish two main relationships depending on the advantage or disadvantage of hosting these bacteria: commensal or pathogenic. While commensal strains innocuously colonise the colon contributing to several metabolic advantages already cited, they can acquire several virulence traits from other species or different strains and cause intestinal pathology resulting in diarrhoea, thereby making them pathogenic. Moreover, they can also translocate the intestinal epithelium and reach other parts of the body depending on the virulence traits they possess, causing extraintestinal diseases.

1.4 Pathogenic *E. coli*: virulence and antimicrobial resistance

Bacterial pathogenicity is defined as the genetic capacity of the bacterium to cause disease, based on the virulence and resistance traits it possesses. Unfortunately, *E. coli* can easily acquire this capacity and is considered a major cause of human infectious disease worldwide. In order to better understand the double component of the pathogenicity of *E. coli*, it is important to accurately define two concepts: virulence and resistance.

Virulence is the pathogenic ability to cause damage in a host and is mediated by the expression of combined virulence factors.

Resistance is the capacity to persist and grow in a determinate environment taking into account different variables such as temperature, pH conditions or antimicrobial concentrations. The antimicrobial resistance of *E. coli* is a worrisome aspect, which is very important and difficult to manage.

1.4.1 Virulence factors: Virulence factor genes and pathogenicity-associated islands

Virulence factors are specific determinants that contribute to pathogen virulence. There are several categories of virulence factors including adhesins, toxins, invasins, secretion systems, iron uptake systems or siderophores which are codified by virulence factor genes (VFGs) and are not specific for syndromes, hosts or strains. A combination of factors determines whether a bacterium can cause infection since the presence of a single VF rarely makes an organism virulent. Moreover, the combinations of VFGs that each bacteria possesses for causing infections at a specific site are very diverse (12). These genes can be located in different parts of the bacterial genome including chromosomal DNA, though they are mostly located on mobile genetic elements such as bacteriophages, plasmids and genomic islands (GEIs). In the *E. coli* K-12 model strain, 18% of the genome has been estimated to represent horizontally acquired DNA, elucidating the high capacity to retain foreign information, and thus, the genomic plasticity of the species (13).

The presence of distinct GEIs encoding different VFGs, designated as pathogenicity-associated islands (PAIs), has led to a broad variety of *E. coli* pathotypes. Up to 13 PAI-like genetic entities providing significant structural and functional diversity have been identified in a single pathogenic *E. coli* strain (14). VFGs associated with each *E. coli* pathotype will be further explained further on.

1.4.2 Antimicrobial therapy and the threat of resistance

Antimicrobials are probably one of the most successful forms of chemotherapy in the history of medicine. They have significantly contributed to the control of infectious diseases that have been the leading causes of human morbidity and mortality throughout most of human existence (15). However, the so-called term **"antibiotic paradox"**, reported by Levy in 1992 (16) has become a fact - while antibiotics are the solution to fight infectious diseases they are also the cause of the selection of resistant microorganisms.

Nowadays, the use and abuse of antimicrobial agents can easily select these resistant pathogens, increasing the prevalence of multidrug resistant (MDR) microorganisms. A report published in 2016 by Lord O'Neill indicates that over 700,000 people die annually worldwide due to infections caused by MDR pathogens, and it is predicted that this number will reach 10 million by 2050 unless new policies and actions are implemented (5). A One Health approach is needed to tackle this problem as it is not only limited to humans; the use of antimicrobial agents is important for the prevention and treatment of infections in the animal industry and are even used as "growth promoters" for animal fattening in some countries, further increasing the problem of resistance (17).

In the last years, the number of available antimicrobial agents active against resistant microorganisms has decreased, thereby reducing the therapeutic options to treat infections due to these pathogens. Although MDR microorganisms mainly cause infection in hospital settings, the figures regarding these infections in the community are on the rise. When resistance to first-line drugs develops, infections last longer and become more expensive to treat, requiring hospitalisation in many cases. This situation increases health costs and poses a serious risk to the progress made in global health by countries, communities and individuals in the past decades.

With regard to *E. coli*, it is expected that more than three million people will lose their lives by 2050 due to MDR *E. coli* strains, the most worrisome being carbapenem-resistant strains, which are already spreading throughout the world, and the only treatment available for eradicating them (the colistin) is already losing effectiveness (5).

For these reasons, research on the molecular bases of antimicrobial resistance and its relationship with virulence as well as the search for new antimicrobial agents are a focal point of interest in order to establish a surveillance system and adapt antimicrobial therapy guidelines to prevent the emergence and dissemination of MDR strains. In Europe, the European Centre for Disease Prevention and Control (ECDC) is responsible for editing periodic reports on the prevalence of resistant strains in each country of the continent, providing tools for antimicrobial resistance surveillance among a wide spectrum of activities regarding infectious diseases (18).

This PhD dissertation is focused on the study of the molecular bases of the most prevalent antimicrobial resistance mechanisms in *E. coli* as well as the prevalence of different VFGs in various intestinal and extraintestinal *E. coli* infections in order to better understand the pathogenic aspects of this bacterium in human hosts.

2. *E. coli* as a human pathogen

E. coli are one of the most important pathogens in humans. They cause of a wide variety of infections including diarrhoea, bloodstream infections and urinary tract infections (UTIs), among others. In order to classify the pathogenesis of the species, several types of pathogenic *E. coli* have been described according to the location of the infection and the genomic specificities they possess.

2.1 Types of *E. coli* in humans

This section will focus on the *E. coli* pathotypes causing infection in different human locations, but also on specific commensal *E. coli*.

2.1.1 Diarrhoeagenic *E. coli* (DEC)

Although most *E. coli* strains live innocuously in the intestines and rarely cause intestinal disease in healthy individuals, some strains have evolved by acquiring virulence determinants that enable them to cause tissue damage in the intestinal tract, producing diarrhoeal illnesses and known as diarrhoeagenic *E. coli* (DEC). The syndromes vary in clinical presentation, preferential host colonisation sites and the distinctive virulence traits of the strains, leading to their classification into different pathotypes: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and diffusely-adherent *E. coli* (DAEC).

E. coli are one of the main aetiological agents of diarrhoeal illnesses. These infections are a severe public health problem and a major cause of morbidity and mortality mainly in infants and young children. Indeed, according to the World Health Organization (WHO) data repository, in 2016, 525,977 children under 5 years of age died due to diarrhoea (19). Low- and middle-income countries (LMIC) in Africa, Asia and Latin America are the most affected

regions with diarrhoeal diseases occurring more often with lethal outcomes mainly due to poor living conditions (inadequate water supplies, poor environmental hygiene and sanitation, and insufficient education) (20).

E. coli are also one of the most common causes of **traveller's diarrhoea** (TD), especially the EAEC and ETEC pathotypes. It is acquired primarily through the ingestion of contaminated food or drinks and affects over 50% of travellers to specific destinations with poor public health infrastructure and hygiene practices, particularly in warmer climates. TD is defined as the passage of three or more unformed stools over 24h and is usually mild and self-limiting, although 10% of affected patients experience persistent diarrhoea or other complications.

The different intestinal *E. coli* pathotypes are classified according to the virulence-related properties they possess, which have several implications in their pathogenic pathways as well as in the treatment required.

All the categories of diarrhoeagenic *E. coli* described have been shown to carry at least one virulence-related property upon a plasmid. EIEC, EHEC, EAEC, and EPEC strains typically harbour highly-conserved plasmid families, each encoding multiple virulence factors (21).

2.1.1.1 Enteropathogenic *E. coli* (EPEC)

The term enteropathogenic *E. coli* (EPEC) is defined as *E. coli* strains which have the ability to cause diarrhoea, to produce histopathology of the intestinal epithelium known as attaching and effacing (AE) lesions, and the inability to produce Shiga toxins and heat-labile (LT) or heat-stable (ST) enterotoxins (21).

Nowadays, EPEC strains are sub-classified into typical EPEC (tEPEC) and atypical EPEC (aEPEC) in accordance with the presence of a large virulent plasmid known as the EPEC adherence factor plasmid (pEAF). tEPEC strains harbour this virulence determinant which encodes for type IV fimbria called bundle-forming pilus (BFP), while aEPEC strains do not possess the plasmid.

The BFP enables a localised-adherence pattern of the *E. coli* to the epithelial cells (*Fig. 2*) and other organ cultures *in vitro*. This fimbria also contributes to antigenicity, autoaggregation, and biofilm formation.

Typical EPEC strains also present:

- A large surface protein, lymphocyte inhibitory factor (LifA), which inhibits the expression of multiple lymphokines and the lymphocyte proliferation.
- Intimin, a 94-kDa protein encoded by the *eae* gene and required for intimate adherence of EPEC to host cells at the sites of AE lesions. The *eae* gene is used for the detection of EPEC by DNA probes or PCR assays.

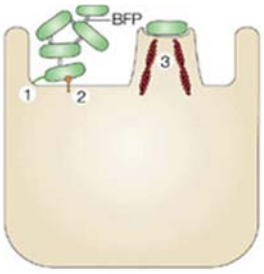

Figure 2: Enteropathogenic *E. coli*. Adapted from Kaper *et al.* (37).

Diarrhoea due to tEPEC decreases with age, and infections in adults are rarely reported. This apparent resistance in adults and older children has been attributed to the loss of specific receptors with age or the development of immunity (20).

2.1.1.2 Enterohaemorrhagic (or Shiga toxin-producing) *E. coli* (EHEC/STEC)

Enterohaemorrhagic (Shiga toxin-producing) *E. coli* (*Fig. 3*) constitutes a well-known group of foodborne pathogens distributed worldwide. The ability to produce one or more of the Shiga toxin (Stx) family cytotoxins is the main virulence attribute of this pathotype of *E. coli*. EHEC/STEC can cause a wide variety of infections ranging from almost unapparent diarrhoea to more serious manifestations such as haemorrhagic colitis (HC) and the development of a life-threatening syndrome known as haemolytic uremic syndrome (HUS), which mainly affects infants and children.

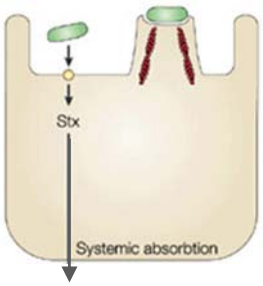

Figure 3: Enterohaemorrhagic *E. coli*. Adapted from Kaper *et al.* (37).

The *stx* operon is usually found within the sequence for an inducible, lysogenic, lambda-like bacteriophage. Stx toxins inhibit protein synthesis as well as act in cell signal transduction and immune modulation causing proinflammatory and pro-apoptotic responses.

The ability to adhere, colonise and form biofilm in food and on several types of surfaces may be an important source and/or vehicle of EHEC/STEC transmission. In addition, its biofilm forming capacity may also act as bacterial protection against adverse environmental conditions (20).

2.1.1.3 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* strains are currently defined as *E. coli* strains that adhere in an stacked-brick arrangement to the surface of epithelial cells (*Fig. 4*) and also to the coverslip between cells. They produce a characteristic histopathologic lesion in the intestine and several specific virulence factors.

EAEC strains characteristically enhance mucus secretion by the mucosa, with trapping of the bacteria in a bacterium-mucus biofilm. Thus, the diarrhoea produced is predominantly watery, often mucoid, with or without blood and inducing abdominal pain, vomiting and low fever. Acute self-limiting diarrhoea is the usual pathology, but some patients may develop protracted diarrhoea, lasting up to more than 14 days. The formation of a heavy biofilm may be related to its ability to cause persistent colonisation and diarrhoea (22). In addition, EAEC infection is accompanied by cytotoxic effects on the intestinal mucosa, with shortening of the villi, haemorrhagic necrosis of the villous tips, and a mild inflammatory response with oedema and mononuclear infiltration of the submucosa.

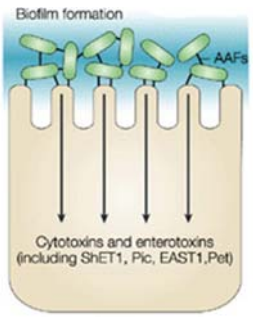

Figure 4: Enteroaggregative *E. coli*. Adapted from Kaper *et al.* (37).

Only some EAEC strains carrying specific virulence factors are able to cause diarrhoea. These putative virulence factors are mostly plasmid-borne and include adhesins, secreted proteins and toxins, but not all of these factors are present in all EAEC strains. The typical EAEC strains

are defined by the presence of *aggR*, a gene that encodes for a transcriptional factor acting as a global regulator of EAEC virulence genes such adherence factors (20,23).

The adherence capacity of the EAEC strains is explained by a family of aggregative adherence fimbriae named AAF, which includes five types of fimbriae, designated from I to V. Another plasmidic VFG, *aap*, encodes for the dispersin, an antiaggregation protein which is secreted and linked to lipopolysaccharide, neutralizing the negative charge of the bacterial surface leading to AAF projection and consequent dispersion along the intestinal mucosa.

Several **toxins** and other proteins have also been described in EAEC in association with the cytotoxic or enterotoxic effects:

- The heat-stable toxin enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST-1) was the first toxin characterized in the EAEC pathotype. This toxin is a 38 amino-acid peptide codified by the *astA* gene which induces the production of high levels of cyclic guanosine monophosphate (cGMP) in the cell, inhibiting the Na/Cl cotransport system and reducing the absorption of electrolytes and water from the intestine at the villus tips, resulting in an elevated secretion of Cl^- and water in crypt cells (24).
- *Shigella* enterotoxin 1 (ShET-1) is a toxin that causes the accumulation of fluid in ileal loops and induces a secretory response (25). Its subunits are encoded by the *set1* genes, located in the *she* pathogenicity island (PAI) (26).
- *Shigella* enterotoxin 2 (ShET-2) is encoded by the *sen* gene located on a 140-MDa invasiveness plasmid (27).
- The TolC antiaggregation protein transporter is located on the outer membrane and encoded by the *aatA* gene. This protein has also been associated with the secretion of a factor that contributes to aggregation. The *aatA* gene has been empirically used as a probe, named pCVD432, to identify EAEC (28).
- Pet and Pic are two immunogenic proteins which are members of the serine protease autotransporters of *Enterobacteriaceae*, also known as SPATEs. Pet is a cytotoxin that modifies the cytoskeleton of enterocytes, leading to rounding and cell detachment. Pic is a multitask protein encoded by *pic* gene (originally the *she* gene and harboured in *she* PAI) that mediates haemagglutination, mucus cleavage and hypersecretion,

intestinal colonisation in mice, cleavage of surface glycoproteins involved in leukocyte trafficking and cleavage of key complement molecules.

- Cytopathic autotransporter protein encoded by the *sigA* gene and also harboured in *she* PAI, contributes to fluid accumulation (29).

Several studies have reported EAEC as the predominant agent of persistent diarrhoea in children younger than 5 years old living in LMICs (30,31).The persistence of EAEC may induce chronic intestinal inflammation, even in the absence of diarrhoea, reducing its absorptive function and leading to malnutrition (32). Considering the high number of asymptomatic EAEC-colonised children in LMICs, this pathotype has an important impact on public health, being a cause of impaired physical and cognitive development.

2.1.1.4 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* strains are characterised by the production of colonisation factors (CFs) and at least one of following two enterotoxins: heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST), which are located in transmissible plasmids. These enterotoxins are produced after the adhesion of the ETEC strains to the intestinal mucosa and, throughout the activation of the adenylate and guanylate cyclases for LT and ST, respectively (*Fig. 5*), cause deregulation of membrane ion channels in the epithelial membrane, leading to a massive loss of ions and water.

On the other hand, the main groups of CFs harboured in genetic mobile elements of ETEC strains are:

- Colonisation factor antigen I (CFA/1)-like group, the most clinically relevant being CS1, CS2, CS4, CS14, CS17 and CS19.
- Coli surface antigen 5 (CS5)-like group, mainly harbouring CS5 and CS7 in clinical strains.
- The class 1b group, the most important being the CS12, CS18,CS20, CS26-28 and CS30 types (33,34).

The ETEC group represents an epidemiologically highly diverse pathotype of diarrhoeagenic *E. coli*. This diversity has been evaluated by both phenotypic and molecular approaches such as serotyping, multilocus sequence typing (MLST), phylogrouping and whole-genome sequencing. These methods demonstrate that ETEC strains can belong to more than 100 somatic serogroups (O) and at least 34 flagellar types (H), although only a limited number of O:H serotypes are associated with infections. The 5 major phylogenetic groups are represented in the ETEC group, as well as several distinct sequence types (ST). However, some clonally related ETEC lineages of worldwide distribution share the same serotype, CFs and toxin profiles (35).

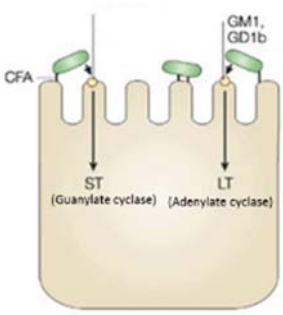

Figure 5: Enterotoxigenic *E. coli*. Adapted from Kaper *et al.* (37).

ETEC represent one of the most common causes of diarrhoea in children in LMIC and in travellers to these regions, with infection being more frequent during warm periods of the year (36). The clinical features of ETEC-infected patients are a watery stool often accompanied by vomiting without fever. Massive loss of fluids and electrolytes can lead to rapid dehydration. The diarrhoea is usually self-limited to 3-4 days, but in some cases it can persist and cause other complications. It is estimated that ETEC cause more than 200 million diarrhoeal episodes annually and approximately 75,000 deaths due to dehydration, mainly among infants and children in tropical areas with poor sanitary conditions. ETEC is also an economic burden to farmers and industry, in which it is an important pathogen for broilers, swine, cattle and other farm animals.

2.1.1.5 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* are one of the most common causes (together with *Shigella* spp.) of dysentery in humans, especially in LMIC, causing fever, abdominal cramps and diarrhoea containing blood and mucous (37).

EIEC are characterised by their capacity of invasion, penetration and multiplication in the enterocytes (*Fig. 6*), leading to their subsequent destruction and an important proinflammatory response. The bacteria specifically bind to the large intestine mucosa and invade the cells by endocytosis. This invasive capacity is dependent on the presence of large plasmids (approximately 220 kb) known as *pInv*, encoding the production of several outer membrane proteins involved in invasiveness by cell spreading (encoded by the *icsA* and *icsB* genes), inhibition of autophagy, regulation of host immune response (encoded by the *osp* gene), and the type II secretion system (TTSS) (38).

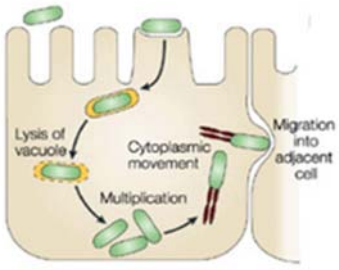

Figure 6: Enteroinvasive *E. coli*. Adapted from Kaper *et al.* (37).

EIEC often resemble *Shigella* by being non-motile and unable to ferment lactose, and the proteins involved in invasiveness are antigenically closely related (in some cases identical), but the infectious dose of EIEC needed to cause dysentery is much higher than that of *Shigella*, and it appears to be a milder and self-limiting form of the disease.

This *E. coli* diarrhoeagenic pathotype belongs to well-defined serotypes (such as O28ac:H-, O124:H30, O136:H- or O167:H-) that have produced large outbreaks worldwide since its first description in 1947 (39,40). Although there are few reports on the routes of transmission and distribution in nature, water and milk products as well as direct person-to-person transmission has been described as potential sources of transmission (41,42).

2.1.1.6 Diffusely-adherent *E. coli* (DAEC)

Diffusely-adherent *E. coli* are characterised by adhering to monolayers of epithelial cells HEp-2 in a diffused pattern. This group has been reported to cause diarrhoea in children older than 12 months of age by inducing a cytopathic effect - long cellular extensions are developed and wrap around the bacteria adhered. Approximately 75% of DAEC strains produce a fimbrial adhesin called F1845, belonging to the family of adhesins. This adhesin binds to an epithelial cellular receptor called DAF enhancing the cytopathic effect (*Fig. 7*). Infection by DAEC can be proinflammatory, inducing inflammatory bowel diseases. However, this pathotype requires further epidemiological studies and is difficult to identify and classify.

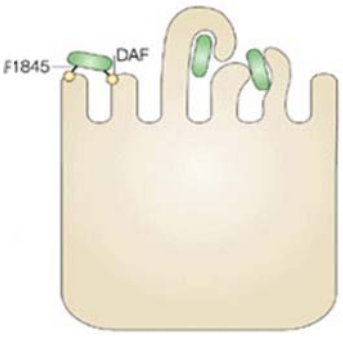

Figure 7: Diffusely-adherent *E. coli*. Adapted from Kaper *et al.* (37).

A summary of the most important virulence factors harboured by the different DEC pathotypes is shown in *Table 1*.

	Type of virulence factor		
DEC Pathotype	**Adhesion, invasion or colonisation factors**	**Toxins**	**Others**
EPEC	-BFP (bundle-forming pilus) -Intimin		- LifA (lymphocyte inhibitory factor)
EHEC/STEC		- Stx (Shiga toxin)	
EAEC	- AAF (aggregative adherence fimbriae) - Dispersin encoded by *aap*	- EAST-1 (EAEC heat-stable enterotoxin) - ShET-1 and 2 (*Shigella* enterotoxin) -Pet (cytotoxin from SPATE family)	-Transcriptional factor AggR -TolC (antiaggregation protein encoded by *aatA*) - Pic (SPATE family) - Cytopathic autotransporter protein encoded by *sigA*
ETEC	- CFA/1-like group (colonisation factor antigen) -CS5-like group (coli surface antigen) - Colonisation factor class 1b	- LT (heat-labile enterotoxin) - ST (heat-stable enterotoxin)	
EIEC	- Invasive proteins encoded by *icsA* and *icsB*		-Host immune response regulative protein encoded by *osp* -TTSS (type III secretion system)
DAEC	- F1845 fimbrial adhesin		

Table 1: Summary of the most important virulence factors in DEC pathotypes.

2.1.1.7 Diagnosis and treatment of EAEC and ETEC

As the diarrhoeal *E. coli* pathotypes studied in the present PhD dissertation are EAEC and ETEC, it is important to highlight the diagnosis and treatment of these pathotypes.

Taking into account that diarrhoea can also be caused by other bacterial and viral agents and the clinical symptoms are not sufficient to differentiate these agents, the use of antimicrobials for the treatment of diarrhoea is often problematic. For that reason, it is important to carry out the promptest and achieve the most accurate **diagnosis**. Multiple PCR-based protocols to detect EAEC plasmidic VFGs have been described to date, mainly amplifying for *aggR* and other AAFs, but the sensitivity and specificity of these protocols never reaches 100% due to the heterogeneity of the pathotype. On the other hand, the diagnosis of ETEC is much easier, since it only depends upon identifying either LT and/or ST encoding genes by PCR or toxin production by immunological assays.

The **treatment** of diarrhoeal disease caused by *E. coli* is based on the rehydration of the patient with oral rehydration solutions until the diarrhoea ceases, or even using intravenous fluids such as Ringer's lactate for patients with severe dehydration. Up to now, cotrimoxazole or ciprofloxacin have been the first antimicrobial choice for empiric treatment of TD and acute cases of diarrhoea mostly produced by EAEC and ETEC, chosen depending of the country visited. However, the increasing prevalence of quinolone resistance strains has led to the inclusion of the non-absorbable rifaximin as one of the most effective drugs for diarrhoea in adults (which is mainly caused by *E. coli*), and azithromycin for children and patients coming from endemic areas of *Campylobacter* spp. (especially South-East Asia), thus rifaximin is not active against intracellular bacteria (36,43,44).

2.1.2 Extraintestinal *E. coli*

Although the main population of *E. coli* in humans remains in the gut and constitutes part of the gut microbiota, its presence in other sites of the human body is not negligible, although it may not always be pathogenic.

2.1.2.1 Commensal extraintestinal *E. coli*

Commensal extraintestinal *E. coli* can commonly be found in the anterior urethra and vagina, being more rare in the skin, conjunctiva, nose, pharynx and mouth (< 5%) (45). These strains cohabit within the host and are unable to cause infections, and may even sometimes play a beneficial role.

An example of commensal *E. coli* related to the subject of the present thesis is vaginal *E. coli* (VEC).

2.1.2.1.1 Vaginal *E. coli* (VEC)

E. coli are the enteric Gram-negative bacilli most frequently found in the genital tract of women (normally made up of 5-15 different species), with the potential to cause vaginal or endocervical colonisation as well as different infections in pregnant women, including intra-amniotic and puerperal infection, and subsequent neonatal infections, such as early or late neonatal sepsis.

The presence and the possible beneficial effects on the host of commensal *E. coli* in the vagina have been poorly characterized, in contrast to other anatomic sites, despite this species being in the genital tract of 9-28 % of non-pregnant women and 24-31% of pregnant women (46). Although its presence does not always mean infection, VEC share virulence profiles, phylogenetic groups and serotypes with extraintestinal pathogenic *E. coli* (ExPEC) (47).

2.1.2.2 Extraintestinal pathogenic *E. coli* (ExPEC): microbiology and virulence factors

There is a distinctive group of *E. coli* strains that possess an enhanced ability to overcome host defences and cause extraintestinal disease in healthy hosts to thereafter become important pathogens in humans. They are known worldwide as extraintestinal pathogenic *E. coli* (ExPEC) and receive a more specific designation depending on the niche they colonise, the most important being sepsis-causing *E. coli* (SEPEC), uropathogenic *E. coli* (UPEC) causing urinary tract infections (UTIs), and neonatal meningitis-causing *E. coli* (NMEC), collectively representing a huge public health problem. Even though they have classically been categorized into subgroups, there are commonalities across syndromes, anatomical sites and/or hosts. For example, the recognized O18:K1:H7 clonal group associated with neonatal meningitis is also the most prevalent among women with acute uncomplicated cystitis (48). Consequently, a few researchers have proposed the use of these more-restrictive terms only in relation to a specific site or syndrome, and they have suggested that these microorganisms should be globally referred to as ExPEC because of their ability to cause disease in multiple anatomical sites (49).

There is no clear distinction between non-pathogenic *E. coli* and ExPEC, since the latter are able to asymptomatically colonise the human intestinal tract and certain ExPEC and commensal *E. coli* have a similar genome content (50,51). However, ExPEC differ epidemiologically and phylogenetically from non-pathogenic *E. coli* by the presence of specific virulence determinants and clonal backgrounds (50,52). Indeed, the virulence capability of these strains is determined by a combination of distinctive accessory traits, such as the source, the virulence factors or the O:K:H serotype, in conjunction with their distinctive phylogenetic background and experimental virulence in an animal model (53,54).

2.1.2.2.1 Bacteraemia or sepsis-causing *E. coli* (SEPEC)

E. coli bacteraemia can appear secondarily from primary sites of infection such as UTI, abdominal and pelvic infection, pneumonia, surgical site infection or meningitis, skin infection,

from percutaneous intravascular devices, or from increased intestinal mucosal permeability. According to international studies, *E. coli* accounts for 17–37% of clinically positive blood isolates (4). This bacteraemia can lead to sepsis when the presence of the bacteria in blood triggers an important systemic response causing sepsis syndrome, severe sepsis (sepsis-induced dysfunction of at least one organ or system), or septic shock (55). Septicaemia was ranked the 10th cause of mortality in the US in 2000 and was responsible for 1.3% of deaths overall (56).

Sepsis-causing *E. coli* (SEPEC) are phylogenetically and epidemiologically different from both intestinal pathogenic *E. coli* and commensal *E. coli* (57). A variety of virulence factors have been related to human SEPEC, especially secreted toxins such as HlyA (α-haemolysin), Sat (secreted autotransporter toxin), and CNF-1 (cytotoxic necrotizing factor 1) (58). These toxins can change the host cell shape and/or function, thereby contributing to the biological processes stimulated by the pathogen. In a study carried out by Soares Tibo *et al.* in 2016, it was observed that the supernatant of SEPEC strains was cytotoxic to human umbilical vein endothelial cells (HUVECs), causing the loss of intracellular junctions, cellular elongation and death, possibly indicating how SEPEC can reach the bloodstream during sepsis (59). Other virulence factors that may not have been related to SEPEC strains, such as enterotoxins, could play an important role in the pathogenicity of septicaemia.

Antimicrobial therapy has become the *sine qua non* of sepsis management. However, several aspects of antimicrobial prescription deserve specific consideration taking into account the need for rapid administration of adequate treatment. The initial antimicrobial choice for sepsis is always empiric, as the diagnosis for the pathogen-directed treatment is not immediately available. Local surveillance of the most prevalent pathogens causing sepsis and the prevalence of antimicrobial resistance reported, together with the identification of the source of infection help in selecting the most adequate empiric treatment. The prescription of broad-spectrum antimicrobials such as piperacillin/tazobactam and third-generation cephalosporins is the most common practice, but it is strongly associated with the increasing appearance of antimicrobial resistance (60). Once the pathogen has been identified, its antimicrobial susceptibility profile has been established and/or adequate clinical improvement is noted,

narrow-spectrum antimicrobial treatment is recommended in order to avoid the appearance of resistance (61).

2.1.2.2.2 Uropathogenic *E. coli* (UPEC)

Uropathogenic *E. coli* (UPEC) are the main aetiological agent of UTIs and are associated with substantial morbidity and mortality rates. UPEC are responsible for 70-95% of community-acquired UTIs and approximately 50% of nosocomial UTIs, being recurrent and relapsing episodes specially problematic (58).

The primary reservoir of UPEC is believed to be the human intestinal tract, where several virulence factors are engaged by these bacteria to colonise and infect the urinary tract in an ascending pathway (62), although there is also evidence of transmission via contaminated food and sexual activities (63).

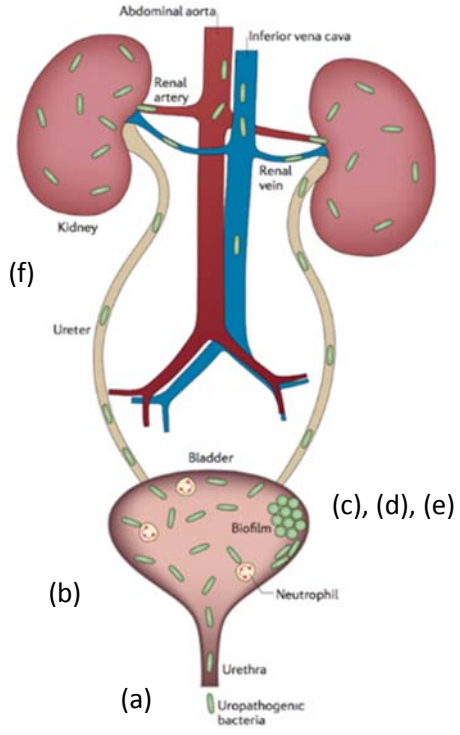

Figure 8: Pathogenesis of urinary tract infection. Adapted from Flores-Mireles *et al.* (289).

The pathogenic process of UPEC during UTI includes the following steps (*Fig. 8*): (a) UPEC colonisation of the periurethral and vaginal areas with subsequent colonisation of the urethra; (b) ascension into the bladder lumen and growth as planktonic cells in urine; (c) adherence to the bladder surface and interaction with the bladder epithelium defence system (see Figure); (d) biofilm formation; (e) invasion and replication by forming bladder intracellular bacterial communities where quiescent intracellular reservoirs form and reside in the underlying urothelium; (f) kidney colonisation and host tissue damage with increased risk for bacteraemia or septicaemia (64).

The most important virulence factor families of UPEC implicated in UTI are several surface structural

components such as lipopolysaccharide (LPS), flagella, pili, non-pilus adhesins, as well as toxins and iron-acquisition systems which are described below (64,65):

- **LPS** are amphipathic molecules consisting of fatty acids with an oligosaccharide core, which is, in turn, bound to a long polysaccharide chain commonly called O antigen. They mediate multiple aspects of the UPEC life cycle, including their ability to colonise the bladder, to evoke immune responses in the host and to modulate their antimicrobial resistance potential.
- **Flagella** play an important role in UPEC isolates causing acute, asymptomatic or recurrent UTIs by conferring adhesive and invasive properties as well as having a key role in biofilm dynamics.
- **Pili** are encoded by several operons such as *fim* (type 1 pili), expressing a mannose-sensitive hemmaglutination and *pap* (from pyelonephritis-associated pili), which encodes for P- or Pap-pili, and are able to interact with the blood antigens of the host. The *fim* operon is constitutive in UPEC clinical isolates but *pap* is part of a PAI that includes other virulence factors. Generally, these type of pili are heteropolymeric with minor subunit proteins at the distal end acting as actual adhesins.
- **Adhesins** not linked to pili are also important virulence factors prevalent in UPEC. The adhesin TosA is encoded in 30% of *E. coli* isolates causing UTI, and FdeC is involved in the colonisation of the bladder and kidneys in a mouse model of infection. The iron-regulated adhesin Iha mediates adherence to the bladder epithelial cells.
- UPEC **toxins** can play different pathogenic roles during UTI. The α-haemolysin induces cation oscillations in renal tubular epithelial cells enhancing ascension and colonisation of ureters and kidney parenchyma, being highly associated with renal damage. The cytotoxic necrotizing factor is an important cytotoxin in UPEC, affecting fundamental cellular processes, including cytoskeletal dynamics, cell cycle progression, transcriptional regulation and cell survival and migration (66).

The abovementioned virulence factors are not released as soluble molecules but are associated with outer-membrane vesicles or endosomes, becoming an efficient delivery system to protect these pathogenic determinants.

- As the urinary tract is iron-limited, adequate **iron acquisition systems** are critical for UPEC survival. For this purpose, several siderophores acting as iron chelators are produced by *E. coli* strains: yersiniabactin, salmochelin and aerobactin. In addition, siderophore receptors are present on the surface of these bacteria.

Other VFGs not typically associated with UPEC such as intestinal *E. coli* virulence determinants can also be harboured in the genome of these strains due to the plasticity of these bacteria.

There are several **treatment** guidelines for UTIs caused by UPEC depending on the site of infection, the severity and the population affected. In general lines, the first-choice empiric antibiotic treatment for UTIs includes fosfomycin, nitrofurantoin, fluoroquinolones, aminoglycosides and beta-lactams (amoxicillin-clavulanic acid, cefuroxime, ceftibuten and cefixime). Despite being a very effective agent against this infection, cotrimoxazole is not recommended for empiric treatment in Spain, because the resistance rate in UPEC strains is higher than 20% (67).

2.1.2.2.3 *E. coli* causing obstetric infections. Intra-amniotic infection.

Infections developing during pregnancy, childbirth and the postpartum period are considered obstetric infections. Extraintestinal *E. coli* is the second most prevalent etiologic agent causing obstetric infections (68). *E. coli* possess several VFGs that enhance vaginal and/or endocervical colonisation in pregnant women. This colonisation can lead to different infections in obstetric patients, such as intra-amniotic infection (IAI), endometritis or intrauterine infection, sometimes accompanied by sepsis, becoming a relevant public health problem. Prospective studies have reported an incidence of IAI of 0.2%-10.5% of all deliveries (69). Puerperal infections are frequently associated with IAI, accounting for 13% of maternal deaths, and have been described as the fourth leading cause of maternal mortality in the United States (70).

The rectum and the urinary tract are the main reservoir of *E. coli* that colonise the genital tract, via the 'faecal-vaginal-urinary' transmission route (47,71). Vaginal and/or endocervical colonisation in pregnant women can lead to the development of intra-amniotic, puerperal and neonatal infections (71,72).

The physiological conditions that bacteria have to adapt along this route are very different. In fact, changes in vaginal flora can be related to the risk of preterm birth. Thus, it has been determined that acquisition of *E. coli* is more strongly associated with preterm delivery than any other factor (73).

In the case of IAI (infection of the amniotic fluid, membranes, placenta, or uterus (74)), the pathogenic process mainly develops by an ascending route of the bacteria, commonly after prolonged rupture of membranes and labour in patients with multiple examinations, but it can also occur in patients with labour but without membrane rupture. Other routes of IAI are haematogenous spread in mothers with bacteraemia or the introduction of the bacteria during invasive procedures such as amniocentesis or intrauterine transfusions, in which the risk of acute infection is estimated as being less than 5% of cases (75).

Intrauterine infection by *E. coli* is developed in four stages (*Fig. 9*):

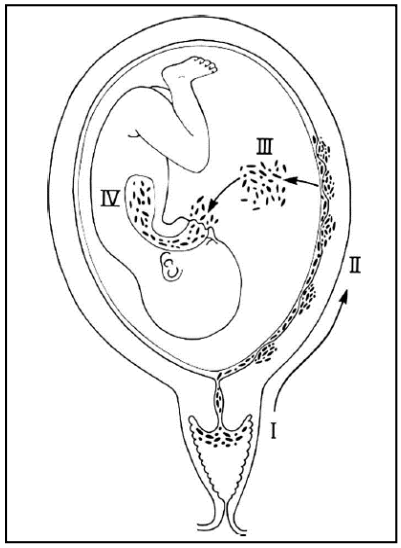

Figure 9: Ascending route by *E. coli*. Adapted from *Romero et al.* (290).

I. The presence of *E. coli* colonising the vagina/cervix.

II. *E. coli* may reside in the choriodecidual space.

III. *E. coli* may cross the chorioamnion, reaching the amniotic cavity, thereby producing chorioamnionitis.

IV. In some cases, *E. coli* may be aspirated or ingested by the foetus and may cause infection (76,77).

As the *E. coli* strains causing obstetric infections mainly originate from the urinary tract, the virulence factors they possess are similar to those of UPEC strains, with adhesins, fimbriae, and toxins being the most important.

The treatment of choice for obstetric infections includes the administration of different antimicrobial agents depending on the focus of infection, being limited by the low number of antimicrobial agents considered to be safe for the foetus (78). In our hospital, the treatment

of choice in patients with IAI consists of ceftriaxone, ampicillin-gentamicin or ampicillin-cefoxitin (79).

E. coli causing obstetric infections can also lead to perinatal sepsis, which despite being underreported in developing countries due to the lack of post-natal follow-up, is an infection accounting for up to 13% of maternal deaths globally. According to the WHO data from 2000, in North America approximately 3 women die from perinatal sepsis for every 100,000 deliveries, and complications are presented by 1% to 8% of all deliveries (78).

2.1.2.2.4 Neonatal sepsis and meningitis-causing *E. coli* (NMEC)

E. coli can spread from the mother to the foetus/infant by vertical transmission. This vertical transmission may be intrauterine or congenital via ascending infection, or perinatal, which takes place at delivery and is caused by contact with the microbiota of the birth canal and perineal area. The prevalence of perinatal transmission of *E. coli* during delivery ranges from 21 to 50% (80) being a clear predisposing factor for the development of neonatal infections (81).

Neonatal sepsis is divided into two types according to the time the symptoms are manifested: early-onset neonatal sepsis (EONS) which occurs within the first 72 hours of life (or 7 days depending on the hospital/authors) and late-onset neonatal sepsis (LONS) which takes place after the first 72 hours of life (or 7 days depending on the hospital/authors). LONS is very frequently the trigger of many complications such as intraventricular haemorrhage and meningitis (82).

Studies on the virulence of *E. coli* causing neonatal sepsis are scarce (83). In this sense, IbeA (invasion of brain endothelium factor) has been proposed as a virulence factor that could play an important role in the translocation of *E. coli* through the amniotic membrane (84). The gene encoding this protein is located in the pathogenicity island GimA that contributes to the invasion of the blood-brain barrier through a carbon-regulated process. Studies carried out in our laboratory have shown that two *E. coli* toxins could also be involved in translocation

through the amniotic membrane and in the development of neonatal sepsis (Sáez-López et al., unpublished data).

E. coli is the second cause of **neonatal meningitis** leading to high mortality rates (20%–29%) and morbidity among neonates, with an incidence of around 0.1/1000 live births among industrialized countries (85). The development of neonatal meningitis by *E. coli* comprises three steps: (i) translocation from the intestinal lumen to the bloodstream (as well as from the urinary tract or the uterus); (ii) intravascular survival and multiplication; and (iii) passage of the bacteria through the blood–cerebrospinal fluid (CSF) barrier and invasion of the arachnoidal space.

The virulence factors associated with the ability of these *E. coli* strains to cause neonatal meningitis are related to outer membrane proteins (capsular antigen K1, OmpA protein), siderophores (encoded by the *iroN*, *fyuA* and *iucC*/*iutA* genes), adhesins (P-fimbriae, S-fimbriae and type-1-fimbriae), and invasion (encoded by the *ibeA* and *cnf1* genes) (86).

Antibiotic treatment of neonatal infections is highly dependent on the type of infection. For neonatal sepsis, ampicillin and gentamicin are the first choice, leaving third generation cephalosporins for special cases in order to prevent the rapid development of drug-resistance microorganisms. Nevertheless, neonatal meningitis is treated with cefotaxime due to its excellent penetration into the CSF (87).

A summary of the most important virulence factors harboured by the different ExPEC pathotypes is shown in *Table 2*.

	Type of virulence factor		
ExPEC Pathotype	**Adhesion, translocation or colonisation factors**	**Toxins**	**Iron acquisition systems**
SEPEC		- HlyA (α-haemolysin) -Sat (secreted autotransporter toxin) - CNF-1 (cytotoxin necrotizing factor)	
UPEC / Obstetric infection-causing *E. coli*	- LPS (lipopolysaccharide) - Type I pili encoded by *fim* operons -P- or Pap- pilli encoded by *pap* operon (pyelonephritis-associated pili) -TosA adhesin - Iha adhesin	- HlyA (α-haemolysin) - CNF-1 (cytotoxin necrotizing factor)	- Yersiniabactin - Salmochelin - Aerobactin - Various siderophore receptors
NMEC	-IbeA (invasion of brain endothelium factor) -K1 capsular antigen -OmpA protein -P-fimbriae -S-fimbriae -Type-1-fimbriae	- CNF-1 (cytotoxin necrotizing factor)	- Siderphores encoded by *iroN* , *fyuA* , *iucC* and *iutA*

Table 2: Summary of the most important virulence factors in ExPEC pathotypes.

3. Antimicrobial resistance mechanisms in *E. coli*

Antimicrobial resistance is a concern worldwide. Microorganisms can be resistant to specific antimicrobial drugs due to two main reasons: (i) the innate mechanisms of resistance of the strains, and (ii) the ability of these strains to acquire resistance mechanisms by different means.

The pressure induced by the misuse and abuse of antibiotics has led to easy selection of bacterial-resistant strains and their mechanisms of resistance can, in turn, be rapidly spread both intra- and interspecifically (88–90). Taking all of this into account in depth and updated surveillance studies are needed on the prevalence of microorganisms causing specific infections as well as their antimicrobial susceptibility and mechanisms of resistance. The results of these studies guide the choice of the most adequate empirical treatment and the implementation of interventions to fight the dissemination of MDR bacteria.

This raises several questions such as - What are the most prevalent antimicrobial resistance mechanisms in *E. coli*? Are they disseminated worldwide? How can they spread between strains? This section will focus on the strategies of antimicrobial resistance to the most commonly used antibiotic families to treat *E. coli* infections and their main mechanisms of resistance to the aforementioned antibiotics.

3.1 Strategies of antimicrobial resistance

Several strategies have been defined and classified concerning the mechanisms of antimicrobial resistance in bacteria.

From an evolutionary perspective, bacteria use two major genetic strategies to fight against the effects of antibiotics:

1. Mutations in genes associated with the mechanism of action of the antibiotic by one of the following mechanisms (*Fig. 10*):

i) Modifications of the antimicrobial target (thus decreasing the affinity for the drug).

ii) Reduction in drug uptake by alterations in bacterial cell permeability.

iii) Over-expression of efflux mechanisms to extrude the harmful molecule from the bacterial cell.

iv) Global changes in important metabolic pathways via modulation of regulatory networks that overpass the mechanism of action of the antibiotic.

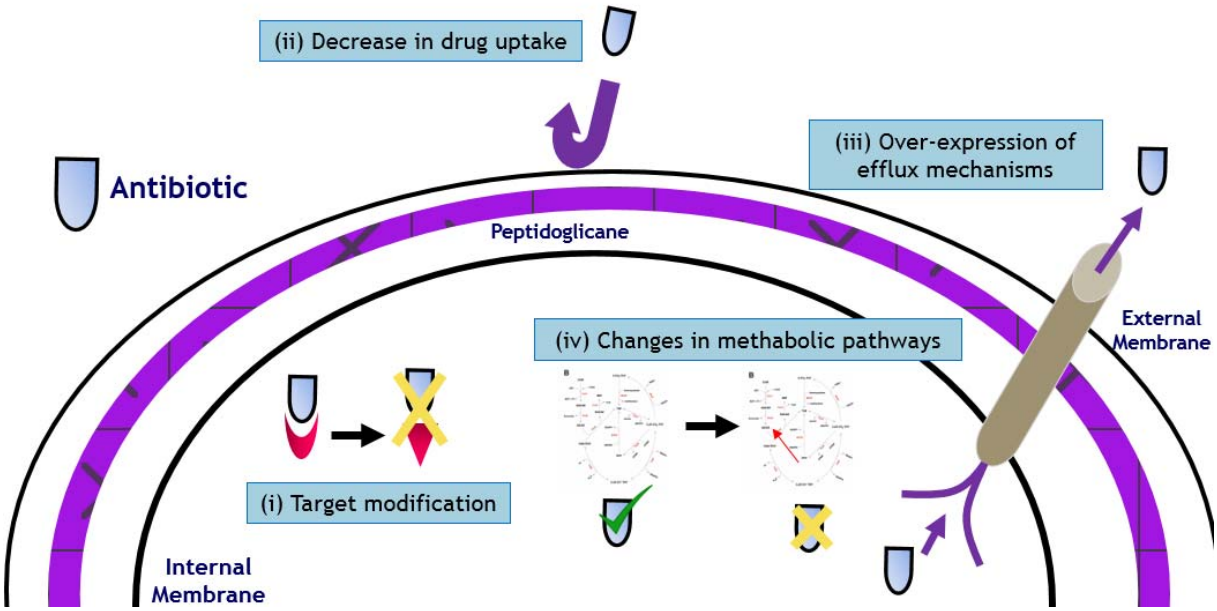

Figure 10: Antimicrobial resistance genetic strategies associated with the mechanism of action of the antibacterial agent.

2. Acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT) (91).

   Classically, bacteria acquire external genetic material through three main strategies:

i) *Transformation:* natural incorporation of naked DNA, the simplest way of HGT. Only a few clinically relevant bacterial species are able to incorporate DNA in this way to acquire genes encoding for resistance mechanisms.

ii) *Transduction:* foreign DNA is introduced into the bacteria by a phage. This method is highly employed for transferring genetic material *in vitro*, but it is not the most prevalent strategy in clinical bacterial strains.

iii) *Conjugation:* also commonly known as bacterial "sex". Conjugation is the most frequent strategy to spread antimicrobial resistance mechanisms in bacteria. It involves cell-to-cell contact by a pilus produced by the donor cell which attaches to the recipient cell (*Fig. 11*). This phenomenon occurs at high rates in the human intestinal tract under antibiotic treatment. Although direct transfer from chromosome to chromosome has been well characterised (92), conjugation usually uses mobile genetic elements (MGEs), mainly plasmids, as vehicles to share antimicrobial resistance determinants.

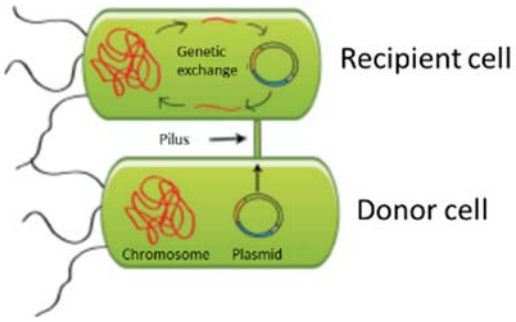

Figure 11: Illustration of bacterial conjugation. Adapted from *Brolund et al.* (95)

3.2 Horizontally transferred genetic elements carrying antimicrobial resistance (AMR) mechanisms

The most important horizontally transferred genetic elements harbouring AMR determinants in clinically relevant bacteria are transposable elements and plasmids. Other genetic elements such as integrons cannot be transferred by themselves, but may be located in conjugative plasmids allowing their dissemination.

3.2.1 Transposable elements

The genome of living organisms contains DNA sequences with mobility capacity, able to integrate in different sites of the genome without depending on large homology regions between the transposable elements and its insertion site. The so-called transposition movement plays an important role in the genetic reorganisation of the organisms. These elements encode for their own recombinase enzymes (transposases), having independent activity from the bacteria that harbours them.

The main transposable elements containing AMR mechanisms are:

- **Insertion sequences (IS)** are the simplest transposable elements. They are short DNA segments constituted by two identical sequences with inverted orientations in the extremes (inverted repeats [IRs]) and a central region only containing the genes encoding for the transposases (the enzymes necessary for their mobilization) (*Fig. 12.A*). Transposases are DNA recombinases that specifically recognise the inverted repeats in the transposable elements extremes and promote mobilization (93). IS can be found in both the chromosome and in plasmids, upstream from AMR determinants, thereby promoting their capacity to spread.

- **Transposons** are larger transposable elements than IS, as they can contain other genes (such as AMR genes) in addition to those necessary for transposition. Transposons can contain two identical IS flanking resistance genes (composed transposon or Class I)

(*Fig. 12.B*), without an IS but with an IR on both sides of the transposase and other genes (non-composed transposons or Class II) or transposons with conjugative capacities by a circular molecule that must insert into the chromosome or plasmid of the receptor cell.

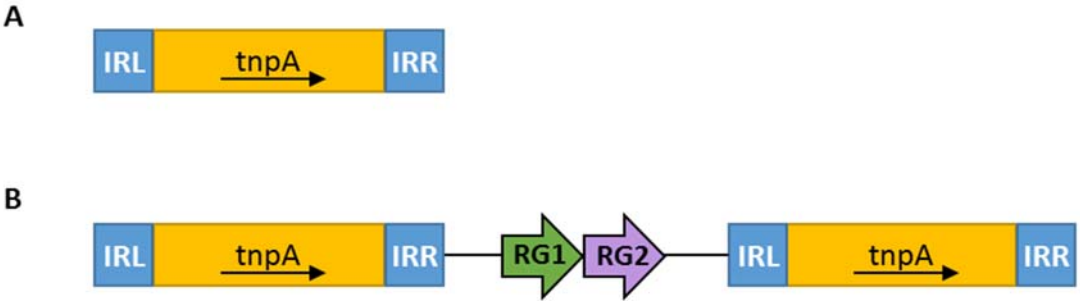

Figure 12: Schematic representation of (A) an insertion sequence (IS): gene encoding for transposase (*tnpA*) and inverted repeats left and right (IRL and IRR), and (B) Composed tranposon containing two IS flanking two resistance genes (RG)

3.2.2 Plasmids

Plasmids are double-stranded circular extra-chromosomal DNA capable of autonomous replication in a host cell that can be disseminated by horizontal gene transfer between bacteria from the same or different species by transformation or conjugation processes. Plasmids do not carry essential genes for the survival of bacteria, but they have a highly conserved part called **replicon** or **core genome**, where genes essential for their maintenance such as the initiation and control of replication are harboured (*Fig. 13*).

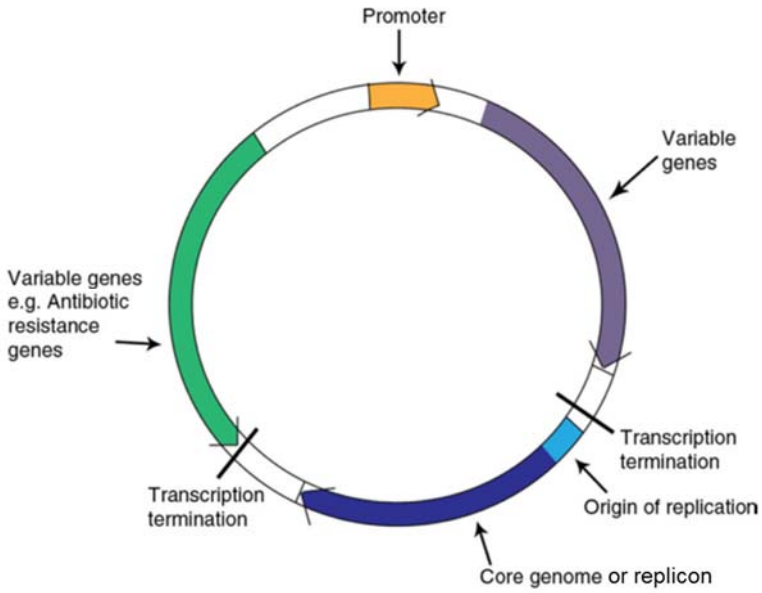

Figure 13: Schematic illustration of a plasmid. Adapted from *Brolund et al.* (95)

Additionally, they may present other genes that may be useful for the plasmids themselves or even for the host bacterial cell, such as antibiotic resistance or virulence genes. These genes are assembled by transposition (transposable elements and IS) and site-specific recombination mechanisms (94). Consequently, plasmids are highly diverse and the plasmid genome is often scattered with mobile genetic elements that can move genes around within the plasmid as well as between the chromosome and other plasmids (95). Mobile genetic elements found in plasmids include transposons, integrons, and IS common regions.

The size of the plasmids can be extremely variable, as they may only carry their own replication essential genes or even 400 additional genes (94). Many resistance plasmids are conjugative (when encoding the functions necessary to promote their own transfer) and others are mobilizable (when helped by a conjugative plasmid co-resident in the cell). Accordingly, mobilizable resistance plasmids tend to be relatively small, often less than 10 kb in size, whereas conjugative plasmids are larger, from 30 kb to more than 100 kb (94).

Plasmids can be classified according to incompatibility groups (Inc) by replicon typing, which is based on the principle that plasmids with the same replicon cannot be stably propagated in the same bacterial cell as they share the same replication or copy segregation mechanisms (96,97). Carattoli *et al.* developed a multiplex PCR-based replicon typing (PBRT) protocol in 2005 (98) for the classification of plasmids occurring in members of the *Enterobacteriaceae* family. A simplified version of this procedure for commensal and pathogenic *E. coli*, requiring only three multiplex panels to identify 18 plasmid replicons was described two years later by Johnson and colleagues (99).

3.2.3 Integrons

Integrons are not strictly MGE, but rather are site-specific recombination systems capable of recruiting open reading frames in the form of mobile gene cassettes. They provide an efficient mechanism for the addition of new genes into bacterial chromosomes, along with the necessary machinery to ensure their expression (91).

All integrons characterised to date are composed of three key elements necessary for the capture of exogenous genes: a gene (*intI*) encoding an integrase belonging to the tyrosine-

recombinase family; a primary recombination site (*attI*); and an outward-orientated promoter (Pc) that directs transcription of the captured resistance genes (*Fig. 14*).

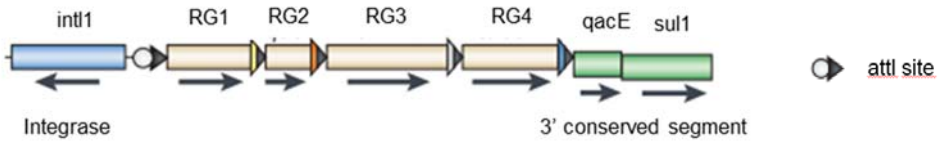

Figure 14: Schematic representation of a class I integron. RG: Resistance gene. Adapted from Mazel D. (100).

Up to now, five classes of mobile integrons are known to have a role in the dissemination of antibiotic-resistance genes. These classes have historically been defined based on the sequence of the encoded integrases, which show 40–58% identity. All five classes are physically linked to mobile DNA elements, such as ISs, transposons or conjugative plasmids, all of which can serve as vehicles for both intra- and interspecific transmission of genetic material. Multidrug resistant isolates classically harbour Class 1, 2 and 3 integrons (100).

3.3 Main antimicrobial agents and AMR mechanisms in *E. coli*

The main antimicrobial agents used in the treatment of community and hospital infections caused by *E. coli* are aminoglycosides, macrolides, quinolones, rifaximin, cotrimoxazole and β-lactams. For that reason, we will focus on the AMR mechanisms of the abovementioned antibiotic families.

The most prevalent antimicrobial resistance mechanisms in *E. coli* clinical isolates are listed below:

- Genes encoding for enzymes capable of introducing chemical changes or destructing the antimicrobial molecule.
- Alterations in membrane permeability.
- Efflux pumps.
- Mutations of the antimicrobial target site maintaining its functionality.

3.3.1 Aminoglycosides and AMR mechanisms

Aminoglycosides (AGs) include a group of drugs which are characterised by the presence of an aminocyclitol ring linked to amino sugars in their structure and have a broad spectrum of activity against bacteria (101). This group includes streptomycin, kanamycin, gentamycin, tobramycin, and amikacin, which are commonly used in the treatment of infections by both Gram-negative and Gram-positive organisms. AGs bind to the 16S RNA of the 30S bacterial ribosomal subunit inhibiting protein synthesis. They exert a concentration-dependent killing effect that can be bacteriostatic or bactericidal. Aminoglycoside drugs require therapeutic-drug monitoring to achieve the correct dose and limit toxicity, mainly ototoxicity and nephrotoxicity (102).

Resistance to AGs is highly diverse, being the main mechanisms present in *E. coli* the following (103):

- **Modifications of the ribosome AG-binding site.** The AG-binding site may be modified enzymatically by acquired 16S ribosomal RNA methyltransferases (RMTases) such as ArmA (aminoglycoside resistance methyltransferase A) which can also co-exist with endogenous ribosomal methyltransferases, for example RsmH and RsmI.

- **Aminoglycoside-modifying enzymes (AMEs).** This is a large family of enzymes divided into three subclasses depending on the type of chemical modification they apply to their AG substrates: acetylases, phosphotransferases or adenylases. The most prevalent and clinically relevant class in *E. coli* are the AG N-acetyltransferases (AACs), in particular AAC(6')-Ib (104). AMEs are highly mobile as their genes can be harboured in plasmids, integrons, transposons and other mobile genetic elements, together with other resistance genes.

- **Cell membrane modification and efflux pumps.** Due to their cationic, hydrophilic structures, it has been hypothesized that AGs penetrate bacterial cell walls through porin channels rather than direct diffusion through the phospholipid bilayer. Indeed, a mechanism of resistance to AG may be the reduced uptake of the antibiotic by reducing the number of porins in the cell membrane. It is thought that the *E. coli* porin OmpF may be involved in kanamycin resistance, although there is no clear evidence. Regarding active expulsion systems, AcrAD, a member of the resistance-nodulation-division (RND) family, is the main AG efflux pump in Gram-negative bacteria. Intrinsic AcrAD-TolC-type efflux pumps have been identified in *E. coli* (105).

3.3.2 Macrolides and AMR mechanisms

The chemical structure of macrolide antibiotics is characterised by a large lactone ring which can vary from 12 to 16 atoms, with one or more sugar chains attached (106). Macrolides have been widely used to combat respiratory, skin and soft tissue infections caused by Gram-positive pathogens, as they offer good activity and are relatively safe. Although the first macrolides showed modest potency against Gram-negative bacteria (specifically *Enterobacteriaceae*), the second-generation macrolide azithromycin (derived from

erythromycin), has a broader spectrum of activity and improved pharmacokinetic properties (107) and is now recommended for the treatment of intestinal infections caused by *E. coli*, *Shigella* or *Salmonella* (108).

Macrolides inhibit bacterial protein synthesis by reversibly binding to subunit 50S of the bacterial ribosome and preventing translocation of peptidyl-tRNA (109).

The most important macrolide resistance mechanisms in *E. coli* are acquired and include:

- **Modification of the target site** by methylases encoded by *erm* genes.
- **Modification of enzymes** such as esterases encoded by the *ere* genes or phopshotransferases encoded by the *mph* genes (*mphA* being the most prevalent in *E. coli*) (108).
- **The *mef(A)* gene encoding for an efflux pump** is the most prevalent macrolide resistant determinant found in a collection of Gram-negative bacteria (110).

3.3.3 Quinolones and AMR mechanisms

The first quinolone with antibacterial activity (nalidixic acid) was discovered in 1962. Since then, several derivatives have become available on the market, the most important being the fluoroquinolones (FQX): ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin. FQX are broad spectrum antibiotics exhibiting a bactericidal effect against Gram-positive and Gram-negative bacteria as well as anaerobes (111). FQX are one of the most important antibiotics used in the treatment of UTI in both community and hospital settings worldwide due to their affordability and availability (112). These antimicrobial agents are also widely used for the treatment of *E. coli* causing TD, although an increase in the prevalence of resistant strains has been reported in the last years (113).

Quinolones are heterocycles with a bicyclic core structure, and their mechanism of action is based on the inhibition of two enzymes essential for bacteria viability (the DNA gyrase and topoisomerase IV) by interfering with DNA segregation and supercoiling (114).

The acquisition of quinolone resistance in *E. coli* is related to (115):

- **Chromosomal mutations in genes encoding the target enzymes.** Point mutations within the quinolone-resistance determining region (QRDR) of the chromosomal *gyrA* and *gyrB* genes, encoding for subunits A and B of the DNA gyrase, respectively, and the *parC* and *parE* genes, encoding for subunits A and B of topoisomerase IV, which increase the resistance to quinolones by inhibiting the ability of the antimicrobial agent to bind to its target. Clinically relevant FQX resistance frequently requires an accumulation of several genetic changes over time, with the first mutation producing minor increases in the minimum inhibitory concentration (MIC) with the subsequent need for at least 2 point mutations (one in *gyrA* and the other in *parC*, typically) to acquire a high level of resistance (91).

- **Mutations causing reduced drug accumulation**, either by decreased uptake through a lower expression of porins or by increased efflux through the up-regulation of efflux systems. However, only four efflux pumps have been shown to have a clear implication in quinolone efflux by overexpression from a plasmid: AcrAB, AcrEF, MdfA, and YdhE.

- **The OqxAB multidrug efflux pump**, which belongs to the RND family and confers low-level resistance to quinolones, is encoded by chromosomal-located *oqxA* and *oqxB* genes and has also been found in *E. coli* clinical isolates (116).

- **Plasmid-mediated quinolone resistance genes**. These plasmids include the *qnr* genes, which protect the DNA gyrase and topoisomerase IV from the action of quinolones and the *aac(6')-Ib-cr* gene, the *cr* (ciprofloxacin resistance) variant of the aminoglycoside acetyltransferase that acetylates ciprofloxacin, conferring reduced susceptibility to this antimicrobial agent.

3.3.4 Rifaximin and AMR mechanisms

Rifaximin is a semisynthetic non-absorbable rifamycin-derivative specifically licensed for the treatment of TD caused by non-invasive bacterial pathogens. Considering its minimal absorption by the intestine (<1% of oral dose) this drug offers an alternative to other antimicrobial agents such as quinolones (with a growing prevalence of resistant isolates causing TD) with fewer systemic effects.

Rifamycins inhibit RNA synthesis by binding to the β-subunit of DNA-dependent mRNA polymerase, chromosomally encoded by the *rpoB* gene.

Although low MIC levels are found in *E. coli* isolates causing TD, resistance to rifamycins can entail: (i) **target modification** arising from mutations within four highly conserved regions of *rpoB* (117), (ii) **antibiotic modification** via acquisition of plasmid-mediated *arr* genes, which encode ADP-ribosyltransferases that inactivate rifamycins (118), and (iii) a reduction in cellular accumulation of the drug by **efflux systems**. The latter mechanism has been shown to play a relevant role in the resistance of *E. coli* clinical isolates to rifaximin (119).

As several studies have demonstrated, the MIC levels to rifaximin are similar to those to other widely used rifamycins such as rifampicin, elucidating that there are cross-resistance mechanisms between these antimicrobial agents (118,119).

3.3.5 Thrimethoprim /sulfamethoxazole and AMR mechanisms

Thrimethoprim/sulfamethoxazole (SXT), also known as cotrimoxazole, is a synthetic combined antibacterial product that seems to have a synergistic effect. It consists of one part trimethoprim (TMP) to five parts sulfamethoxazole (SMZ), which, together cover a wide antibacterial spectrum (120). Sulfonamides and TMP interfere with bacterial folic acid synthesis by inhibiting two essential enzymes: dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), respectively (121). Thus, two consecutive steps of the biosynthesis of nucleic acids that are essential for bacterial growth are blocked.

Bacterial resistance to TMP and SMZ in *E. coli* is mainly mediated by the following four main mechanisms (121):

- **The permeability barrier and/or efflux pumps.** Resistance mediated by the permeability barrier and efflux pumps has recently been shown to mediate resistance to both SMZ and TMP, even simultaneously.
- **Regulational changes in the target enzymes.** *E. coli* have shown to present an overproduction of chromosomal DHFRs caused by promoter mutations producing TMP resistance.
- **Mutational or recombinational changes in the target enzymes.** Single amino acid mutations in the chromosomal *dhps* genes of *E. coli* are easily found, mediating resistance to SMZ.
- **Resistance acquired by horizontally transferred genetic elements.** A large family of genes encoding for DHFR enzymes resistant to TMP has been described to be harboured mainly in plasmids, but they can also be found in integrons or transposable elements. These genes mediate a high level of resistance to TMP. Transferable resistance to SMZ is widespread and is mainly mediated by 2 drug-resistant DHPS enzymes, which are encoded by the *sulI* and *sulII* genes (120).

3.3.6 β-lactams and AMR mechanisms

β-lactam antibiotics are a group of antibiotics which are characterised by the possession of a β-lactam ring that includes penicillins, cephalosporins, carbapenems and monobactams. β-lactams constitute the largest family of antimicrobial agents and are the antibiotics currently most extensively used in clinical practice. Penicillins used to be the antibiotics most commonly employed worldwide, especially in LMIC because of their ready availability and relatively low cost (101). However, due to the increasing resistance for the abovementioned antimicrobial

agent, cephalosporins are currently widely used in surgical prophylaxis and severe community-acquired infections, being cefotaxime the preferred agent for meningitis. As a last option, carbapenems are the choice for mixed nosocomial and multiresistant bacterial infections (122).

β-lactams disrupt peptidoglycan biogenesis by inactivating the enzymes that enhance the transpeptidation of the peptidoglycan precursors, called penicillin-binding proteins (PBPs) or transpeptidases, thus generating a loss of wall integrity accompanied by bacterial cell lysis (123).

Resistance to β-lactam antibiotics may be due to four mechanisms:

- **Increased efflux**. The multidrug efflux pump AcrB, which resides in the inner membrane and forms a tripartite complex with a periplasmic adaptor protein (AcrA) and an efflux porin (TolC), is one of the major mechanisms of resistance to β-lactams in *E. coli* (124).
- Modification of **penicillin-binding proteins (PBPs)**. Some transferable genes encode modified PBPs that have a low affinity for β-lactams and are not inactivated by them or that use different ways to construct the cell wall (89).
- **Reduced permeability**. The down-regulation of outer-membrane porins expression such as OmpF and OmpC as well as the emergence of mutations in porin encoding genes of *E. coli* leads to reduced permeability of the outer membrane and limits the entry of the β-lactam into the bacterial cell. These mechanisms are mainly induced under antibiotic exposure (124).
- Hydrolysis by **β-lactamases** (see next section).

3.3.6.1 β-lactamases

The most common mechanism of resistance to the β-lactam antimicrobial family in clinically important Gram-negative bacteria is the beta-lactamase enzymes (125). β-lactamases constitute a large family of hydrolases that catalyse the hydrolysis of the amide bond in the β-lactam ring of penicillins and cephalosporins (126). In Gram-negative bacteria, β-lactamases are intracellular and have a periplasmic location; they can be intrinsic (such as AmpC) or transferable (TEM, SHV, CTX-M), and may be produced constitutively or hyper-produced by mutations in the promoter region (89).

Two classification schemes are currently in use for these enzymes:

- The molecular classification established by Ambler in 1980 (127) divides β-lactamases into four classes and is based on their encoding gene amino acid sequence. Classes A, C and D enzymes utilize serine for β-lactam hydrolysis, and class B metallo-enzymes require divalent zinc ions for substrate hydrolysis.

- The functional classification scheme proposed by Bush in 1989 (128), extended by Bush-Jacoby-Medeiros in 1995 (129) and updated in 2010 (125), takes into account substrate and inhibitor profiles of the β-lactamases in an attempt to group the enzymes in ways that can be correlated with their phenotype in clinical isolates.

The *E. coli* β-lactamases studied in the present thesis according to both classification schemes are summarized in *Table 3*.

Bush-Jacoby group	Ambler molecular class	Distinctive substrate(s)	Defining characteristics	Representative enzyme(s)
1	C	Cephalosporins	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins. Not inhibited by clavulanic acid.	*E. coli* AmpC, ACT-1, CMY-2, FOX-1, MIR-1
2b	A	Penicillins, early cephalosporins	Similar hydrolysis of benzylpenicillin and cephalosporins. Inhibited by clavulanic acid.	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam). Inhibited by clavulanic acid.	TEM-3, SHV-2, SHV-12, CTX-M-15, CTX-M-27, CTX-M-37, PER-1, VEB-1
2d	D	Cloxacillin	Increased hydrolysis of cloxacillin or oxacillin. Variable inhibition by clavulanic acid.	OXA-1, OXA-10

Table 3: Classification schemes for E. coli β-lactamases. Adapted from Jacoby *et al.* (125).

3.3.6.1.1 Extended spectrum beta-lactamases (ESBLs)

Extended spectrum beta-lactamases (ESBLs) are defined as enzymes produced by certain bacteria that are able to hydrolyse extended spectrum cephalosporins. They are therefore effective against beta-lactam antibiotics such as ceftazidime, ceftriaxone, cefotaxime and oxyiminomonobactam (130).

According to the functional classification scheme proposed by Bush et al. in 2010, ESBLs are included in group 2be serine β-lactamases as they have serine in the active site needed for β-lactam hydrolysis. Moreover, these enzymes present increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam) and remain sensitive to inhibition by clavulanic acid, a feature used in their phenotypical detection by clinical laboratories.

The most representative ESBL enzymes are: TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1 encoded by the genes named *bla* (for β-lactamase) and the enzyme they encode for in subscript (125).

ESBLs are plasmid-mediated and are often located in MDR regions containing different transposons and ISs (131). These plasmids frequently carry aminoglycoside, tetracycline, sulfonamide and/or fluoroquinolone resistance genes, and are able to facilitate the spread of *bla* genes by co-selection processes (94,132). In addition, ESBLs are usually found in well-established plasmids in different environments, having the ability to persist and mediate rapid and efficient inter-replicon and cell-to-cell dissemination, resulting in highly successful lineages and subsequently limiting therapeutic options (131,133).

3.3.6.1.1.1 CTX-M enzymes (CTX-M-15)

Over the last two decades CTX-M enzymes have become the most prevalent extended-spectrum β-lactamases, both in nosocomial and in community settings (134). They were initially reported in *E. coli* strains in the second half of the 1980s, and their rate of dissemination among bacteria worldwide has dramatically increased since 1995 (135).

The potential origin of the $bla_{CTX\text{-}M}$ genes has been described as chromosomally-located in the environmental bacteria *Kluyvera* spp., which is also presumed to act as a reservoir for plasmid-encoded CTX-M enzymes. Nonetheless, this has yet to be definitely elucidated (135–137).

Most CTX-M enzymes exhibit powerful activity against cefotaxime and ceftriaxone but not ceftazidime. However, some CTX-Ms, such as CTX-M-15, CTX-M-16 and CTX-M-19, have enhanced catalytic efficiencies against ceftazidime (136).

CTX-M enzymes are plasmid-mediated acquired cefotaximases that constitute a rapidly growing family of ESBLs with significant clinical impact (134,135). *In vitro* studies have shown that CTX-M-encoding plasmids are transmissible by conjugation with a frequency of transfer from 10^7 to 10^2 per donor cell. This property explains the easy dissemination of $bla_{CTX\text{-}M}$-harbouring plasmids (135). Among the plasmid replicon types harbouring $bla_{CTX\text{-}M}$ genes, plasmids belonging to incompatibility group IncF are the most commonly associated with ESBL-producing *E. coli* (95).

Up to now, more than one hundred CTX-M enzymes have been described (138). These cefotaximases can be classified by amino acid sequence similarities. Phylogenetic studies reveal five major groups of acquired CTX-M enzymes (the members of each group share 94% identity, whereas 90% identity is observed between the members belonging to distinct groups): CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, CTX-M-9 group, and CTX-M-25 group (135). The distribution of these enzymes among the abovementioned groups varies greatly depending on the geographic area (139).

It is of note that, among the CTX-M-1 group of enzymes, CTX-M-15 has been highly associated with clinically relevant epidemic *E. coli* clones distributed worldwide. This enzyme was first

detected in isolates from India in 2001 (140). It differs from CTX-M-3 by one amino acid substitution, which apparently confers an increased catalytic activity to ceftazidime (139).

Molecular epidemiologic studies have suggested that the sudden increase in CTX-M-15–producing *E. coli* worldwide was mainly caused by a single high risk clone (ST131) and that foreign travel to high-risk areas, such as the Indian subcontinent, might play a partial role in the spread of this clone across continents (141).

The $bla_{\text{CTX-M-15}}$ gene is usually found downstream from the insertion sequence IS*Ecp1*, which may be involved in clonal dissemination and expression (142).

This phenomenon has contributed to a rise in the prevalence of resistant isolates to third generation cephalosporins worldwide, making the infections caused by these microorganisms more difficult to treat, with the subsequent associated morbidity, mortality and economic problems. In Europe, for example, the resistant rates have dramatically increased in the last two decades, as shown in *Figure 15*.

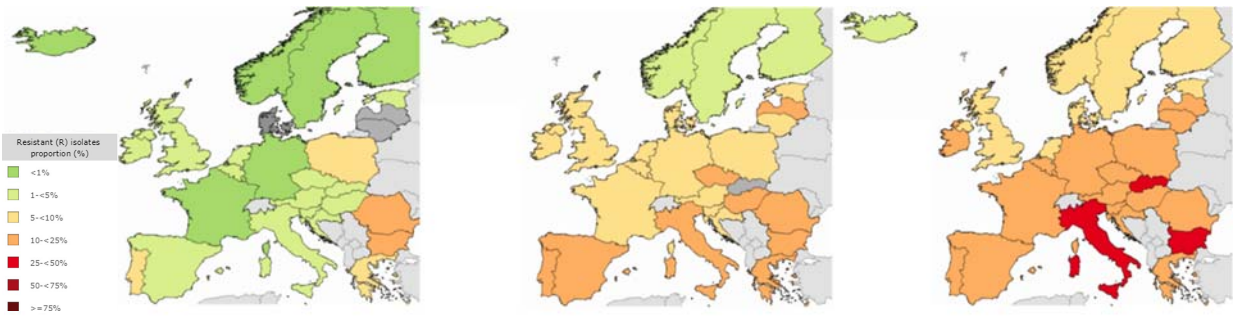

Figure 15: Evolution of the proportion of third generation cephalosporins resistant *E. coli* isolates between 2002 (A), 2010 (B) and 2016 (C) among the countries partnering the European Surveillance System. Adapted from ECDC Surveillance Atlas – Antimicrobial Resistance (291)

4. Relationship between virulence and resistance in *E. coli* clinical isolates

On analysing the virulence carriage of *E. coli* isolates causing UTI at the beginning of the 21st century, researchers in the field of clinical microbiology realised that there was something characteristic of their quinolone susceptibility profile: isolates presenting resistance to quinolones (with a steadily increased prevalence from the previous decade) harboured fewer VFGs related to the invasive capacity of the urinary tract (143). They found this intriguing, and several studies with UPEC collections have been carried out since then (143–150). Further studies were then extended to other ExPEC and commensal *E. coli* collections, causing obstetric infections or even infection or colonisation in domestic animals (72,151) and the results were in accordance with the previously mentioned studies. This relationship has even been elucidated with VFGs not typical from ExPEC but rather from diarrhoeal *E. coli* (152).

The main explanation proposed and supported by *in vitro* studies (153) is:

The UPEC virulence factors haemolysin, cytotoxic necrotizing factor-1 (CNF-1), aerobactin, *sat*, as well as the enterotoxin ShET-1 encoding gene, are chromosomally-located into PAIs. However, PAIs are easily and spontaneously deleted from the chromosome (154). During the development of quinolone resistance, probably facilitated by quinolone exposure, these antimicrobial agents can act by increasing the deletion and transposition of DNA regions. The PAIs share some features with bacteriophages. It has been shown that pro-phages hidden within chromosomal DNA are excised by the activation of SOS, a DNA repair mechanism. Because quinolones activate SOS system, these antimicrobial agents likely contribute to the partial or total excision of PAIs in a SOS-dependent way (155).

Thus, it has been observed that quinolone-resistant *E. coli* is less able to cause invasive UTIs (IUTI) as the acquisition of resistance may be associated with phenotypic changes in bacteria, including the loss of virulence factors that might affect the invasion of renal and prostatic parenchyma by *E. coli* (143). Moreover, it has been observed that the percentage of quinolone-resistant *E. coli* isolates causing pyelonephritis is lower than that of those causing

cystitis; thus, the more invasive the infection, the lower the prevalence of quinolone-resistant isolates causing it. Although it appears that quinolone resistance impairs the capacity of *E. coli* to invade local tissue of the kidney and prostate, it does not disrupt the ability to produce bacteraemia once local invasion has taken place (143).

5. Epidemiology of *E. coli*

Epidemiology is the method used to find the causes of health outcomes and diseases in populations. By definition, epidemiology is the study of the distribution and determinants of health-related states and events in specific populations (156).

Nevertheless, in epidemiological studies related to clinical microbiology (such as those presented in this thesis), host-related characteristics are not taken into account, but rather the features of only the bacteria are studied, including genomic content and virulence and antimicrobial resistance patterns.

5.1 Epidemiological typing strategies

Globalization has expanded the threat of the epidemic spread of infectious diseases. For this reason, it is essential to establish a method to classify *E. coli* isolates based on their genotypic or phenotypic traits. This approach must be capable of differentiating or assembling the strains in order to determine if they have a common origin or not and to establish the pathogenicity or antimicrobial resistance potential they may have. Since the classical phenotypic typing methods were not sufficiently discriminative, molecular typing techniques have evolved and significantly advanced over last decades, providing useful data for epidemiological surveillance and the prevention and control of infections and/or outbreaks among populations (157–159).

Nowadays, phylogenetic methods are able to determine the genetic evolution of the *E. coli* species thereby associating certain clonal lineages with the virulence potential of ExPEC or determining the origins of pathogenic *E. coli*. Although recombination has played a significant role in the evolution of *E. coli* due to its genome plasticity, it has not occurred at a sufficient level to disrupt the phylogenetic signal present in whole genome. The current availability of hundreds of complete *E. coli* genomes represents an invaluable resource for the study of the

diversity of *E. coli* (158). These phylogenetic studies have become an essential tool to better understand the molecular epidemiology of the species (160).

Unfortunately, a rapid, precise and reliable epidemiological technique able to differentiate among all the types of pathogens has not yet been established, and thus combinations of several molecular methods should be carried out (161).

Although there are many methods for the epidemiological typing of *E. coli*, three are relevant to the present dissertation: phylogenetic grouping, multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE).

5.1.1 Phylogenetic grouping

Upon confirmation of the genetic substructure of *E. coli* at the end of the 20th century, researchers in this field found that *E. coli* strains were not randomly distributed, and multilocus enzyme electrophoresis and ribotyping techniques were developed to determine the phylogenetic groups of *E. coli* (162). Nevertheless, these methodologies were complex and time-consuming. For this reason, in 2000, Clermont and colleagues described a simple PCR-based method that enabled an *E. coli* isolate to be assigned to one of four main phylogenetic groups: A, B1, B2 or D (163). The methodology consisted of a multiplex-PCR of three fragments encoding a siderophore (*chuA*), a putative virulence gene (*yjaA*) and an anonymous DNA fragment (TspE4.C2), which was later discovered to be a putative lipase esterase gene (164). The phylogenetic group is determined according to the presence or absence of the three fragments as detailed in *Figure 16*. Using this method of classification, most clinically relevant ExPEC with a high potential of virulence were assigned to phylogenetic group B2 (165). The second highest number of ExPEC belonged to group D, which presented

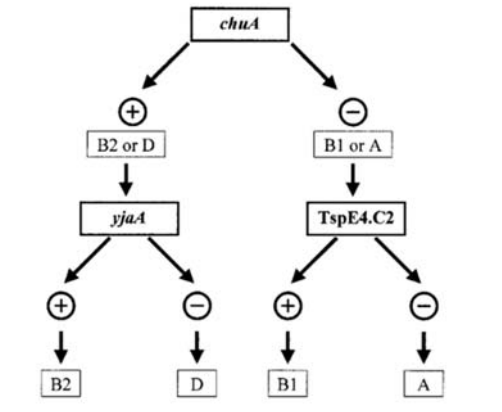

Figure 16: Dichotomous decision tree for determining *E. coli* phylogenetic group by Clermont *et al.* methodology (163).

different and a lower number of virulence factors than group B2. Finally, *E. coli* strains belonging to groups A and B1 did not frequently cause extraintestinal infections and presented fewer virulence factors (166). With the ever growing body of multilocus sequence data and genome data for *E. coli* the understanding of phylogroup structure of *E. coli* was refined, and in 2013 Clermont and colleagues updated the methodology (167) by establishing eight phylogroups: seven (A, B1, B2, C, D, E, F) belonging to *E. coli sensu stricto*, with the eighth being *Escherichia* cryptic clade I (Figure 17). This new quadruplex PCR method was validated, correctly assigning over 95% of *E. coli* isolates to a phylogroup. Incorrect distribution of the remaining 5% was due to two main reasons: (i) the high genetic variability due to the gain or loss of genes, and (ii) the presence of extremely rare phylogroups or results of large-scale recombination events.

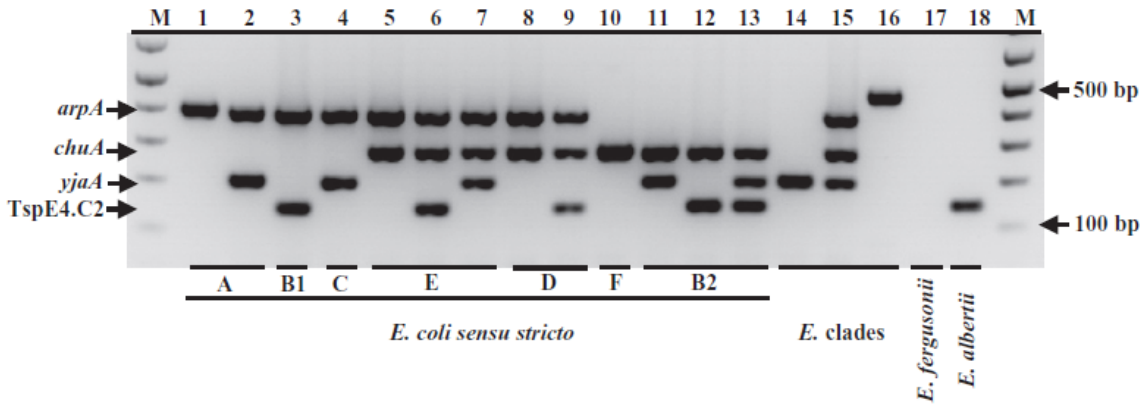

Figure 17: Quadruplex PCR profiles of the new Clermont *E. coli* phylotyping method (167).

The majority of ExPEC strains belong to B2 (161,167), including *E. coli* isolates causing UTIs, obstetric infections and neonatal septicaemia or meningitis (46,168,169). However, in some bacteraemia and sepsis-causing *E. coli* collections studied, phylogroup A was predominant (170,171). The four main phylogroups are represented in intestinal *E. coli* strains (both commensal and pathogenic) (172–174). Overall, these data indicate that phylogrouping alone is not adequate for predicting pathogenic potential. Indeed, it has been elucidated that socioeconomic and geographic factors are presumably more relevant in phylogenetic group distribution (158,174,175).

5.1.2 Multilocus sequence typing (MLST)

In 1998 Maiden et al. developed **multilocus sequence typing (MLST)** as an epidemiological tool to characterise pathogenic strains with the *Neisseria meningitidis* model, in which sequences of multiple genes are compared for nucleotide base changes (176). This methodology allowed higher levels of discrimination between isolates than the previous multilocus enzyme electrophoresis (MLEE) approach on which the technique is based (177).

Regarding *E. coli*, three MLST schemes providing very similar results are currently available. However, the method specifically set up in our laboratory due to its probable persistence and use worldwide is that created by Mark Achtman and hosted at the Warwick Medical School (Coventry, UK) (178,179). The strain associations obtained with this technique are consistent with previously determined clonal grouping by MLEE.

The Mark Achtman MLST method is not focused on any particular group of *E. coli* and is based on the determination of the nucleotide sequence of seven housekeeping genes: *purA, adk, icd, fumC, recA, mdh,* and *gyrB*. For each locus, unique sequences (alleles) are assigned arbitrary numbers, and a sequence type (ST) is determined based on the allelic profile, which is the combination of the alleles identified. Isolates sharing at least 6 out of the 7 loci are assigned to the same clonal complex (CC) and named as the ancestral genotype ST. Isolates not included in any CC are called singletons.

With the MLST data, relationships between closely-related isolates of a bacterial species can be displayed using the BURST algorithm (180), which unlike cluster diagrams, trees or dendrograms, uses a simple model of bacterial evolution in which an ancestral genotype increases in frequency in the population, and while doing so, begins to diversify to produce a cluster of closely-related genotypes that are all descended from the founding genotype. This cluster of related genotypes corresponds to a specific CC. The first implementation of this algorithm, capable to cope with very large data sets and offer crude graphical outputs is the

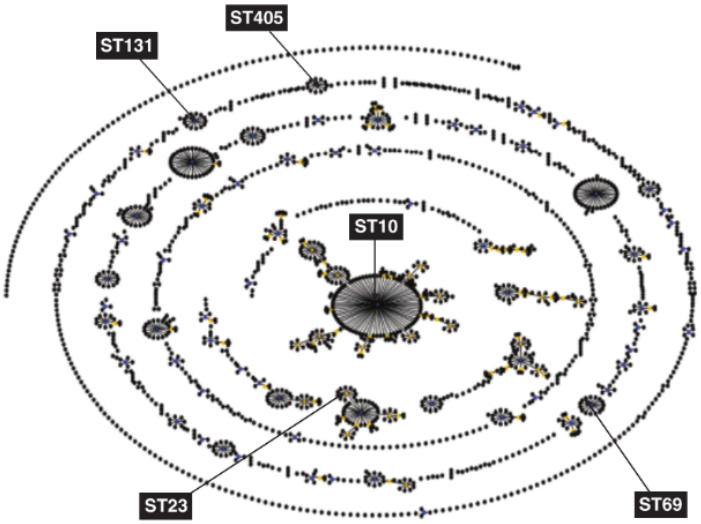

Figure 18: Population Snapshot determined by eBURST analysis showing the clusters of linked STs and unlinked STs in the *Escherichia coli* MLST database ('Achtman' scheme). Adapted from eBURSTv2 webpage (180).

eBURST (*Fig. 18*), which divides an MLST data set of any size into groups of related isolates and clonal complexes, predicts the ancestral genotype of each clonal complex, and computes the bootstrap support for the assignment (181).

The amount of MLST data for *E. coli* recovered from a variety of hosts and habitats has rapidly expanded since 2000, as this technique allows laboratories to exchange molecular typing data for global epidemiology via Internet. Nevertheless, this method cannot distinguish between commensal and pathogenic *E. coli* or among ExPEC subtypes. The only approach able to do this was made by Köhler et al. (161) by establishing a relationship between a few CCs and ExPEC isolates according to the increasingly expanding tree of concatenated MLST sequences. These CCs were 95, 73, 131, 127, 141, 17, 14, 12, and 144, some of which have been confirmed as successful ExPEC clones (139,182).

5.1.3 Pulsed-field gel electrophoresis (PFGE)

The **pulsed-field gel electrophoresis (PFGE) method** is the most common genotyping method used for the typing of a number of bacterial species. The technique consists in cutting the DNA previously immobilized in agarose using low frequency cleaving enzymes, thus obtaining a particular band pattern or DNA fingerprint for epidemiologically related isolates (183) (*Fig. 19*).

Since the bacterial chromosome is typically a circular molecule, its digestion yields several linear molecules of DNA, which are separated based on size using an electric field. The DNA

fingerprints obtained are analysed with a software programme and compared to determine the epidemiological relationship between isolates (*Figure 19*).

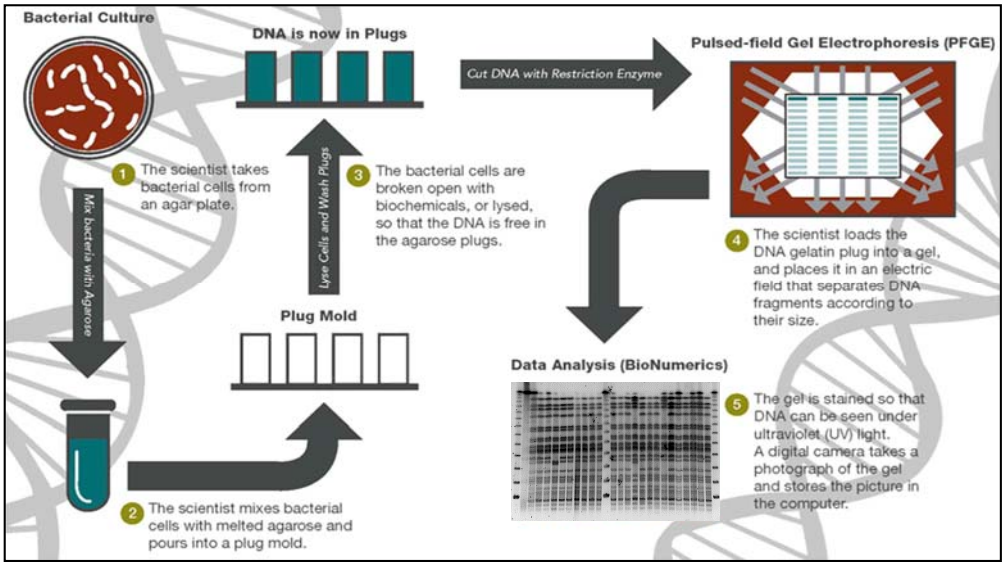

Figure 19: The PFGE process. Adapted from *Pulsenet, CDC* (292).

Although the PFGE procedure is cost effective, it is more labour-intensive than the other methods, requiring 2-4 days to perform the procedure and the analysis of the results (184). Nevertheless, PFGE is a reproducible and versatile method which is very suitable for the epidemiological analysis of outbreaks and has been successfully employed in tracking diseases and outbreaks caused by different bacterial pathogens including *E. coli* (159,185). This method is currently the "gold standard" in epidemiological analysis due to its discriminatory power and global comparability. Indeed, the use of PFGE has had a major impact on pathogen subtyping and outbreak investigation through the establishment of PulseNet, a network of state and local health departments belonging to the Center for Disease Control and Prevention (186).

5.1.4 Other typing methods

Apart from the typing methods described above, other techniques have historically been used for the classification and epidemiological study of *E. coli*. The most important of these methodologies are serotyping and REP-PCR.

Serotyping is one of the first typing methods established and consists in the use of antibodies to test for bacterial surface antigens: the LPS (O), the capsular (K) and the flagellar (H). In the 1940s, Kauffmann (187) and later Ørskov and Ørskov (188) described the antigenic composition of *E. coli,* and since then, standardised procedures have been developed. The current serotyping scheme comprises 182 O-groups, 53 H antigens and 60 different K antigens(189).

The serotyping technique has several disadvantages: it requires a high level of expertise and access to cross-absorbed antisera and it is expensive (190). However, O:H serotyping has become the gold standard to determine the ecology of the isolate and to discriminate among intestinal pathogenic *E. coli* and/or even ExPEC, since many of these strains belong to a limited number of well-known serotypes (161).

For instance, about 80% of the *E. coli* strains causing meningitis belong to the capsular serotype K1 (86), and from 10 to 12 O serotypes account for approximately 90% of meningitis isolates and 60% of bacteraemia isolates (191).

Another molecular typing technique that lacks in reproducibility but is an affordable, simple and useful first-screening method to determine the potential clonal relationship between isolates is **REP-PCR**, a repetitive extragenic palindromic sequence-based PCR. This method, first proposed by Woods and colleagues in 1993 (192), enables the generation of DNA fingerprints through a simple PCR, allowing the discrimination between bacterial species and strains (*Figure 20*). Several REP-PCR variants have been developed using this strategy, such as ERIC-PCR, which is designed with enterobacterial

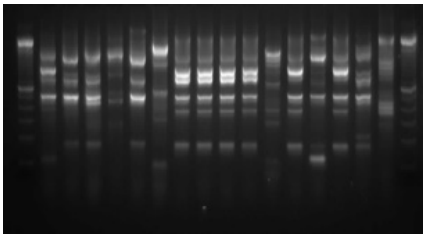

Figure 20: REP-PCR gel

repetitive intergenic consensus (ERIC) sequences (193). Some epidemiological studies have even used available commercial software to automatically carry out this technique (194,195).

5.1.5 Comparison of *E. coli* typing methodologies

The different *E. coli* typing methodologies described have several advantages and disadvantages, demonstrating that the most appropriate molecular typing method for a specific epidemiological study should be chosen based on the particular characteristics of the study.

Since the MLST, PFGE and serotyping techniques allow global comparisons between isolates, they represent a very reliable method for worldwide dissemination studies. However, they are expensive, time-consuming and require a high level of expertise. MLST is excellent for evolutionary studies, and for readily comparing isolates, but may lack the discrimination required for outbreak analysis. On the other hand, PFGE provides exceptional discrimination and has been used widely for typing of a range of bacterial species, but does not describe the isolates numerically, thereby making global comparison difficult (196).

On the other hand, phylogrouping and REP-PCR methodology are not discriminative enough and are sometimes not reproducible to universally compare strains, but they are very useful and inexpensive tools for the first screening of the genetic clonality in large scale collections, or even for identifying outbreaks in specific locations. An overall comparison of these molecular typing methodologies is summarised in *Table 4*.

Method	Cost	Rapidness	Simplicity	Globally comparable	Discrimination power
Phylogenetic group	€	++	++	++	+
PFGE	€€	-	-	++*	++
MLST	€€€	-	+	+++	+++
Serotyping	€€	+++	+++	++	-
REP-PCR	€	++	++	-	+

Table 4: Overall comparison of *E. coli* molecular typing methodologies.
*Only when Standard Operating Procedures are available (i.e. *PulseNet*).

5.2 *E. coli* high-risk clones

Some clinically relevant *E. coli* clones have successfully spread worldwide, while others have caused large outbreaks causing bloody diarrhoeas, UTIs or other extraintestinal infections.

These clones are often multidrug resistant, undermining empirical treatment regimens and reducing the options of appropriate antimicrobial treatment.

Overall, however, to what phenotypic and genotypic combination can this victory be accredited? Unless there is a clear epidemiological trail, conclusions to this question must be made with caution.

In *E. coli* species, several elements have shown to be essential for the success of high-risk clones:

- **Plasmids:** Plasmids have largely been found to contain antimicrobial resistance determinants for the most frequently used antimicrobial families. Upon the selective pressure of beta-lactams, for instance, plasmids harbouring the $bla_{\text{CTX-M-15}}$ gene that belong to incompatibility groups IncF, IncN, and IncK have spread worldwide.
- **Transposable elements:** Resistance genes have been highly associated with certain ISs or transposons. These elements are able to capture and effectively mobilize the resistance genes among members of the *Enterobacteriaceae* and can also act as strong promoters for their high-level expression. The most important example of this success is the $bla_{\text{CTX-M}}$ gene associated with IS*Ecp1* or IS*CR1*.
- **Virulence factors:** Some clones present a typical virulence profile (a combination of toxins, outer membrane proteins, adhesion factors or siderophore receptors) that provides them a successful combination for causing disease and becoming a high-risk clone. For instance, the uropathogenic specific protein (*usp*), outer membrane protein (*ompT*), secreted autotransporter toxin (*sat*), aerobactin receptor (*iutA*), and pathogenicity island marker (*malX*), specifically correspond to ST131.

It is thought that the multidrug resistance associated with some virulence factors in the **O25b-ST131** ExPEC clone provides the best match for success in terms of bacterial fitness,

pathogenicity and antimicrobial resistance traits. Following its first description in the community setting in different countries in the mid-2000s, this clone suddenly appeared worldwide probably in relation to international travel to the Indian subcontinent (197). The O25b-ST131 clone causes UTI, bacteraemia, obstetric infection and other extraintestinal diseases. The combination of plasmids harbouring multiple antibiotic resistance determinants with the increased fitness of the high-risk clone due to several virulence factors, enabled ST131 to spread easily within the community, hospitals, and long-term-care facilities worldwide.

Another important clone causing intestinal diseases worldwide is the EHEC Shiga toxin-producing **O157:H7** (198). Since its first description as a human pathogen in 1982, this clone has become an important food- and waterborne pathogen causing diarrhoea, haemorrhagic colitis, and HUS. Outbreaks of *E. coli* 0157:H7 have involved communities, nursing homes, schools, and day care facilities.

In the last decades, other *E. coli* outbreaks produced by specific high-risk clones have occurred. Some of the most notable outbreaks are described below:

- Denmark, 1991: An EAEC strain of serotype **O78:H10** caused a community-acquired UTI outbreak. It was a multidrug resistant strain belonging to ST10 and phylogenetic group A. This was the first time that EAEC was implicated as an agent of an outbreak of extraintestinal disease. The uropathogenic properties of this EAEC strain were conferred by specific virulence factors, including AAF/I fimbriae (199).
- Japan, 1997: An EAEC **O untypeable:H10** caused a foodborne outbreak affecting 2697 school children in Japan with gastrointestinal infection after the consumption of school lunches (200).
- Europe (Germany), 2011: an intestinal STEC/EAEC strain belonging to the serotype **O104:H4** caused a foodborne outbreak of bloody diarrhoea and HUS traced to contaminated bean sprouts. The clone harboured a wide variety of VFG typical of STEC, including toxins (*stx1*, *stx2*, *hlyA*, among others), serine proteases, adhesins (*eae*, *iha*, among others), iron acquisition systems encoded on a pathogenicity island (marker genes *irp2* and *fyuA*), as well as a virulence loci typical of other intestinal pathogenic *E.*

coli including EPEC, ETEC, EIEC and EAEC. The clone was found to be multidrug resistant and harboured the ESBL enzyme CTX-M-15 (182,201).

Overall, the resistance, virulence and epidemiological characterisation of *E. coli* clinical isolates leads to a better understanding and management of the infections caused by this bacterium and thus, to an improvement in global health.

III. WORK JUSTIFICATION AND HYPOTHESES

III. WORK JUSTIFICATION AND HYPOTHESES

Escherichia coli are ubiquitous bacteria in the human body (as they are throughout the world). Depending on their genotypic and phenotypic pattern, they can colonise specific niches, playing a beneficial or prejudicial role in the human body. *E. coli* have several factors that can determine the severity of the infection, such as virulence potential, resistance mechanisms to antimicrobial agents or the epidemiology of the isolates. These factors may or may not be interrelated. Nonetheless, is there a universal rule to predict if a single isolate will cause infection? Regarding the great concern related to the infections caused by these bacteria worldwide and the attributed morbidity and mortality, it is important to study these microorganisms in depth as a holistic process, taking into account each and every factor of their biology, their ability to survive, and their success in causing human infections.

This PhD dissertation is an attempt to provide an integrated approach to the virulence, antimicrobial resistance and epidemiology of *E. coli*, considering the following hypotheses:

- **Virulence:**

Several **virulence factors** confer *E. coli* the capacity to cause specific infections which have, to date, not been well characterised, or have been wrongly associated with specific pathotypes. The prevalence of virulence determinants typical of diarrhoeal *E. coli* has not been studied in ExPEC isolates, but may also play an important role in these types of infections. Some VFGs may specifically be present depending on the site of the infection, while others offer transversal skills for colonisation.

- **Antimicrobial resistance:**

As the emergence of *Enterobacteriaceae* antimicrobial resistant isolates worldwide is worrisome, it is important to elucidate the main **resistance mechanisms** belonging to *E. coli* isolates causing infections. The mechanisms allowing *E. coli* to avoid the effects of antimicrobials in the infections studied are mainly enzymatic, the most important being the beta-lactamase CTX-M group, and more specifically, the CTX-M-15 enzyme disseminated

worldwide. Although the rise of other enzymes (such as carbapenemases) has been important in the last 10 years, it is still relevant to follow and characterise CTX-M enzymes due to their ubiquity and genomic location plasticity.

Moreover, the therapeutic guidelines available must be updated according to the **rates of resistance** to the different antibiotic families found in the isolates causing infections in each geographic area. It is therefore important to characterise the prevalence of resistance among *E. coli* isolates causing each kind of infection collected in reference hospitals of every city or district, as well as the prevalence of multidrug resistant *E. coli* clinical isolates, which are likely to be steadily increasing.

The virulence potential and antimicrobial resistance are not isolated properties of the bacteria; there may be a relationship between the two and a biological explanation for this relationship in *E. coli* clinical isolates in regards of the resistance profile to quinolones.

- **Epidemiology:**

A description of the **epidemiological relationship** among *E. coli* isolates causing different types of infections will provide information about the **spreading capacity** of specific clones in order to establish surveillance programmes, if appropriate. Some phylogenetic groups have shown to be more pathogenic than others, or they may cause specific extraintestinal or intestinal infections. On the other hand, intestinal *E. coli* pathotypes cannot be phylogenetically related on a worldwide scale, but rather other important factors must also be taken into account.

IV. OBJECTIVES

IV. OBJECTIVES

According to the previously described hypotheses, the **objectives** of this doctoral thesis are:

A. GENERAL OBJECTIVE:

The main aim of the present work is to **study the versatile bacteria *Escherichia coli* as an organism with clinical implications** in terms of virulence, antimicrobial resistance and epidemiology.

B. SPECIFIC OBJECTIVES:

- **Virulence of *E. coli* clinical isolates:**

    1. Determine the **prevalence of virulence factor genes typical from enteroaggregative *E. coli*** in *E. coli* isolates causing extraintestinal infections (*Papers 1* and *2*).

    2. Determine the **prevalence and potential environmental specialisation of particular virulence factor genes** from vaginal *E. coli* potentially causing obstetric infections *(Papers 3* and *4)*.

    3. Investigate **virulence factors carriage among multidrug resistant** *E. coli* isolates causing extraintestinal and intestinal infections *(Papers 4* and *7)*.

    4. Elucidate the **possible relationship between virulence and resistance to specific antimicrobial agents** in *E. coli* isolates causing different extraintestinal infections *(Papers 1, 3* and *4)*.

- **<u>Antibiotic resistance of *E. coli* clinical isolates:</u>**

    5. Determine the **prevalence of antimicrobial resistance** in *E. coli* isolates causing extraintestinal and intestinal infections *(Papers 4, 5, 6* and *Additional Results I)*.

    6. Study the **evolution of the prevalence of antimicrobial resistance** in *E. coli* isolates causing extraintestinal and intestinal infections in order to determine if changes in the therapeutic guidelines are required *(Paper 5 and Additional Results I)*.

    7. Investigate the **molecular basis of resistance** to the antimicrobial agents most frequently used in the clinical treatment of infections by *E. coli (Papers 5, 6, 7 and Additional Results I)*.

    8. Determine the prevalence and identify the most important **enzymatic resistance mechanisms** to β-lactam antibiotics *E. coli* causing extraintestinal and intestinal infections *(Papers 4, 5, 6, 7 and Additional Results I)*.

- **<u>Epidemiology of *E. coli* clinical isolates:</u>**

    9. Establish the **epidemiological relationship** between *E. coli* isolates sharing resistance mechanisms and/or virulence factors causing extraintestinal and intestinal infections *(Papers 1, 2, 3, 6, 7 and Additional Results I)*.

    10. Determine the **epidemiology** of *E. coli* strains causing traveller's diarrhoea worldwide in order to elucidate a possible clonal dissemination *(Additional Results II)*.

V. RESULTS

V. RESULTS

The results of the present PhD dissertation have been divided into three sections depending on the type of infections caused by the *E. coli* clinical isolates and the main property/ies studied in each collection:

- SECTION 1. Virulence in ExPEC: Enterotoxins.
- SECTION 2. Virulence and antibiotic resistance in ExPEC: Women, neonates and children.
- SECTION 3. Antibiotic resistance and epidemiology of traveller's diarrhoea.

SECTION 1. Virulence in ExPEC: enterotoxins

This section includes the following studies:

- PAPER 1: Prevalence of enterotoxins among *Escherichia coli* isolates causing bacteraemia.
- PAPER 2: Prevalence of the *set-1B* and *astA* genes encoding enterotoxins in uropathogenic *Escherichia coli* clinical isolates.

PAPER 1:

Prevalence of enterotoxins among *Escherichia coli* isolates causing bacteraemia

Authors:

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Journal, volume (issue): pages, date of publication:

FEMS Microbiology Letters, 306 (2): 117-21, 2010 May.

Impact Factor: 2.04 - Q3 (2010)

Hypothesis:

Virulence factors typical of diarrhoeagenic *Escherichia coli* can also be found in ExPEC isolates causing bacteraemia.

There may be a relationship between the acquisition of quinolone resistance and loss of VFGs integrated in PAI.

Objectives:

Determine the presence and spread of the genes encoding the ShET-1, ShET-2 and EAST-1 toxins (*set1*, *sen* and *astA*) and AggR factor (typical of diarrhoeagenic *E. coli*) in *E. coli* isolates causing bacteraemia and their possible relationship with both clinical and microbiological characteristics in order to elucidate the possible role of these enterotoxins in the pathogenicity of bacteraemia.

Establish possible relationships between quinolone-susceptibility, biofilm-forming capacity and the phylogenetic group of the isolates studied.

Material and methods:

174 *E. coli* blood isolates from patients with bacteraemia in the Hospital Clinic of Barcelona in 2002 were included**.**

The presence of enterotoxin encoding genes, *aggR* and the phylogenetic group were determined by PCR.

The antimicrobial susceptibility of quinolones was tested by disk diffusion following CLSI guidelines.

In vitro biofilm-producing capacity was tested by staining with cristal violet.

Results:

The *set1* gene (contained in *she* PAI) was presented significantly more frequently among quinolone-susceptible isolates, in phylogenetic group B2 isolates and among biofilm-forming isolates. In contrast, the *sen* gene was significantly more frequent among nalidixic acid-resistant isolates from patients who received quinolone treatment and among phylogenetic group B1. The gene encoding for the EAST-1 toxin was significantly found among isolates causing septic shock and non-B2 isolates. The AggR transcriptional factor was not associated with any major phylogenetic group but was more present in isolates causing chronic renal insufficiency and pneumonia. The *aggR* gene was associated with biofilm-forming isolates.

Conclusions:

There seems to be a relationship between the presence of the *set1* gene and nalidixic acid susceptibility, possibly due to the integration site structure of the *she* PAI that may also be involved in the excision promoted by quinolones. The ShET-1 encoding gene is mainly associated with isolates belonging to phylogenetic group B2, indicating a higher capacity of these strains to acquire VFGs from other bacteria. On the other hand, ShET-2 was related to phylogenetic group B1, suggesting a possible increase in the virulence of these commensal strains. A trend was found between the presence of the *aggR* gene and biofilm formation, which might explain the relationship between this capacity and chronic renal insufficiency, as one of the functions of this transcriptional factor is to facilitate the colonisation and persistence of this bacteria in the kidney.

RESEARCH LETTER

Prevalence of enterotoxins among *Escherichia coli* isolates causing bacteraemia

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Received 3 February 2010; revised 23 February 2010; accepted 23 February 2010.
Final version published online April 2010.

DOI:10.1111/j.1574-6968.2010.01945.x

Editor: Rob Delahay

Keywords
Escherichia coli; bacteraemia; enterotoxins; transcriptional factor; gene transfer.

Abstract

The most frequent cause of bacteraemia among Gram-negative bacteria is *Escherichia coli*. Analysis of the genes encoding the *Shigella* enterotoxin 1 (ShET-1), ShET-2, enteroaggregative heat stable toxin 1 (EAST-1) toxins and AggR factor in *E. coli* strains causing bacteraemia revealed that *set*1 genes were presented significantly more frequently among quinolone-susceptible strains ($P < 0.0001$), in phylogenetic group B2 ($P = 0.0004$) and in biofilm strains ($P = 0.02$). In contrast, *sen* genes were significantly more frequent among nalidixic acid-resistant isolates (15% vs. 6%, $P = 0.046$) and in phylogenetic group B1 ($P = 0.0001$). This is the first study in which ShET1, ShET2 and EAST-1 have been found in *E. coli* collected from blood.

Introduction

The most frequent cause of bacteraemia among Gram-negative bacteria is *Escherichia coli*. These isolates possess specialized virulence factors (VFs) such as adhesins, toxins, iron-acquisition systems, polysaccharide coats and invasines that are not present in commensal and intestinal pathogenic strains (Sannes *et al.*, 2004).

The *Shigella* enterotoxin 1 (ShET-1) toxin has been described in *Shigella flexneri* 2a. This toxin is encoded by chromosomal *set* genes, and these genes have been found on the antisense strand of a mucinase gene in *S. flexneri*, as well as in enteroaggregative *E. coli* (EAEC) (Vila *et al.*, 2000; Henderson & Nataro, 2001). The active toxin of ShET-1 has a configuration of one A subunit and several B subunits ($A_1 - B_n$) (Noriega *et al.*, 1995; Vargas *et al.*, 1999; Niyogi *et al.*, 2004). The *set*1 genes are located in the *she* pathogenicity island (PAI). This PAI is a 46 kb chromosomal element that carries a number of genes with established or potential roles in bacterial virulence (Al-Hasani *et al.*, 2001). In addition to *set* genes, this PAI includes the *sig*A gene, which encodes a cytopathic autotransporter protein that contributes to fluid accumulation in ligated rabbit ileal loops (Al-Hasani *et al.*, 2000) and also contains the *pic* gene (originally *she* gene), which encodes an autotransporter protein that cleaves mucin and complement and plays a role in inflammation (Henderson & Nataro, 2001). This PAI has been detected in other diarrhoeal pathogens such as *Yersinia enterocolitica*, *Salmonella typhimurium* and pathogenic strains of *E. coli* (Al-Hasani *et al.*, 2001), but has not been sought in *E. coli* associated with bacteraemia. The ShET-1 toxin induces fluid accumulation in the rabbit ileal loop and may account for the initial watery diarrhoea that can occur in early steps of *S. flexneri* infections (Fasano *et al.*, 1995).

The ShET-2 toxin is encoded by the *sen* gene located on the 140-MDa invasiveness plasmid (Fasano *et al.*, 1995). This toxin has been reported in different species of *Shigella* causing traveller's diarrhoea (Vargas *et al.*, 1999) and increases transepithelial conductance in an *in vitro* model, although the relevance of the toxin in clinical disease is unknown (Nataro *et al.*, 1995).

The enteroaggregative heat stable toxin 1 (EAST-1) toxin is encoded by the *ast*A gene (Savarino *et al.*, 1996). This toxin is thought to play a role in EAEC pathogenicity.

Epidemiological studies have associated this gene with *E. coli* pathotypes other than EAEC such as ETEC and EHEC and with other bacterial genera including *Salmonella* (Vargas *et al.*, 1999; Paiva de Sousa & Dubreuil, 2001). EAST-1 is a 38 amino-acid peptide, and the *ast*A gene is detected in commensal and diarrheic *E. coli* strains (Kaper *et al.*, 2004). EAST-1 induces the production of high levels of cGMP in the cell, inhibiting the Na/Cl cotransport system and reducing the absorption of electrolytes and water from the intestine at villus tips (Dreyfus & Robertson, 1984), resulting in an elevated secretion of Cl^- and water in crypt cells. However, the role of this toxin in the development of diarrhoea has yet to be defined.

AggR is a transcriptional factor encoded by the *agg*R gene, which controls expression of not only adherence factors (AAFI and AAFII) but also chromosomal genes (Nataro *et al.*, 1994).

Relationships between susceptibility to several antimicrobial agents and virulence have been demonstrated. Thus, exposure to subinhibitory concentrations of quinolones induces a loss of VFs contained within PAIs (Soto *et al.*, 2006).

The transference of VFs contained in PAIs and other mobile genetic elements among different species plays an important role in bacterial pathogenicity, and thus the aim of this study was to determine the presence and spread of the genes encoding the ShET-1, ShET-2 and EAST-1 toxins and AggR factor in *E. coli* strains causing bacteraemia and their possible relationship with both clinical and microbiological characteristics in order to elucidate whether these enterotoxins could play a role in the pathogenicity of these infectious diseases.

Materials and methods

Bacterial isolates

A total of 174 *E. coli* blood isolates collected from patients with bacteraemia in the Hospital Clinic of Barcelona during 2002 were included. The uropathogenic *E. coli* (UPEC) clinical strain HC91255 was used as a control for biofilm assay.

Clinical characteristics

Clinical variables collected from patients with bacteraemia included: demographics, place of acquisition (hospital or community), primary source of infection, selected extrinsic risk factors (bladder catheterization, corticosteroids or other immunosuppressive therapy), selected clinical aspects (fever and shock), antibiotic therapy-related issues (previous administration of antibiotics), in-hospital mortality, HIV infection, neutropenia, patients considered immunocompromised, liver cirrhosis, alcoholism, diabetes, heart disease and pneumonia.

PCR procedures

The *E. coli* phylogenetic group was determined by a three-locus PCR-based method (Clermont *et al.*, 2000). The epidemiological relationship was analysed by REP-PCR as described elsewhere (Vila *et al.*, 1996). The presence of the *set*1, *sen*, *ast*A and *agg*R genes was determined by PCR using specific primers and PCR conditions described in Mendez-Arancibia *et al.* (2008).

Antimicrobial resistance

In order to determine if a relationship similar to that in uropathogenic *E. coli* exits between nalidixic acid resistance and virulence, nalidixic acid susceptibility was analysed by disc diffusion following CSLI recommendations (Clinical and Laboratory Standards Institute, 2008).

In vitro biofilm assay

The biofilm assay was carried out using minimal glucose medium (M63) (Danese *et al.*, 2000). The strains were grown overnight in Luria–Bertani (LB) medium at 37 °C without shaking. An aliquot (1.25 μL) of the overnight culture was subcultured in 125 μL of M63 medium with 1% of LB in each well of a polystyrene microtitre plate and incubated at 30 °C overnight without shaking. Then, 1.25 μL of each culture was subcultured again in 125 μL of M63 medium in a new polystyrene microtitre plate, and incubated as cited above. After 24 h, the culture was removed from the plate and the biofilm was stained with 175 μL of violet crystal for 1 min, washed with 1 × phosphate-buffered saline and air dried for about 1 h. The colourant was solubilized in dimethyl sulphoxide to measure the absorbance at λ of 550 nm in an automatical spectrophotometer (Anthos Reader 2001, Innogenetics, Spain). The result was considered positive when the absorbance was greater than fourfold the value obtained in the well containing bacteria-free medium.

Statistical analysis

The association between the different variables was assessed using the χ^2-test and Fisher's exact test.

Results

The presence of the *set*1, *sen*, *ast*A and *agg*R genes, encoding the ShET-1, ShET-2 and EAST-1 toxins and the AggR transcriptional factor, respectively, was studied in 174 *E. coli* isolates collected from blood.

Thirty-two (18%), 18 (10%), 18 (10%) and 23 (13%) isolates were positive for the *set*1, *sen*, *ast*A and *agg*R genes, respectively. No isolate showing the *set*1 gene had the *sen* gene; however, six isolates carried the *set*1 together with the

*agg*R gene. The *ast*A gene together with the *set*1, *sen* or *agg*R genes was shown by two, one and three isolates, respectively.

When each toxin was analysed separately, the ShET-1 toxin was presented more frequently among patients who had not previously received quinolone treatment ($P = 0.01$). Accordingly, only 2.6% of isolates showing the ShET-1 toxin were nalidixic acid resistant in contrast to the 30.6% among susceptible isolates ($P < 0.0001$). The ShET-1 toxin was significantly more frequent among isolates belonging to phylogenetic group B2 ($P = 0.0004$). Moreover, the ShET-1 toxin was more frequently found among the isolates forming *in vitro* biofilm ($P = 0.02$) (Table 1). To determine if these isolates showed the *she* PAI associated with the *set*1 gene, the presence of other genes contained in this PAI, the *pic*, *sig*A and *sap* genes, was studied. Only two isolates carried the three genes indicating the presence of the whole island, 22 showed the *pic* and *sap* genes and eight only the *pic* gene. This indicates the high variability in the structure of this PAI.

In contrast to the ShET-1 toxin, the ShET-2 toxin encoded by the *sen* gene was more frequent among isolates collected from patients who had taken quinolones before isolation of the bacteria. This toxin was significantly more frequent among nalidixic acid-resistant isolates (15% vs. 6%, $P = 0.046$), and 35% of ShET2-positive isolates belonged to phylogenetic group B1 ($P = 0.0001$).

The EAST-1 toxin was more frequently found in the *E. coli* isolates collected from patients with septic shock (19% vs. 8%, $P = 0.07$). No B2 isolates had this toxin; it was more frequently found among isolates belonging to the A, B1 and D phylogenetic groups ($P = 0.02$).

Finally, the AggR transcriptional factor encoded by the *agg*R gene was more frequently found among isolates collected from patients with chronic renal insufficiency (37.8% vs. 12%, $P = 0.03$) and from patients with pneumonia (33% vs. 12%, $P = 0.09$). The presence of this transcriptional factor was not associated with any phylogenetic group, and it was more frequently found among isolates forming biofilm (18% vs. 9%, $P = 0.08$) (Table 1).

Discussion

The presence of genes encoding enterotoxins and a transcriptional factor involved in virulence were analysed in *E. coli* isolates collected from patients with bacteraemia.

Table 1. Features of isolates containing the different toxins

	ShET-1		ShET-2		EAST-1		AggR	
Feature (number of cases)	+ (32)	− (142)	+ (18)	− (156)	+ (18)	− (156)	+ (23)	− (151)
> 65 years old (106)	18 (56%)	88 (62%)	12 (67%)	94 (60%)	12 (67%)	94 (60%)	12 (52%)	94 (62%)
Hospital-acquired infection (30)	7 (22%)	23 (16%)	1 (6%)	29 (19%)	5 (28%)	25 (16%)	6 (26%)	24 (16%)
Any prior antibiotic (41)	3 (9%)	38 (27%)	4 (22%)	37 (24%)	7 (39%)	34 (22%)	6 (26%)	35 (23%)
Prior quinolones (21)	0*	21 (15%)	4 (22%)	17 (11%)	4 (22%)	17 (11%)	4 (17%)	17 (11%)
Probe (28)	4 (13%)	24 (17%)	4 (22%)	24 (15%)	4 (22%)	24 (15%)	3 (13%)	25 (17%)
Urological abnormalities (30)	4 (13%)	26 (18%)	2 (11%)	28 (18%)	4 (22%)	26 (17%)	5 (22%)	25 (17%)
Chronic renal insufficiency (8)	1 (3%)	7 (5%)	1 (6%)	7 (4%)	0	8 (5%)	3 (13%)	5 (3%)
Diabetes (39)	8 (25%)	31 (22%)	3 (17%)	36 (23%)	5 (28%)	34 (22%)	7 (30%)	32 (21%)
Liver cirrhosis (15)	1 (3%)	14 (10%)	2 (11%)	13 (8%)	3 (17%)	12 (8%)	3 (13%)	12 (8%)
Obstructive pulmonary disease (10)	1 (3%)	9 (6%)	0	10 (6%)	0	10 (6%)	2 (7%)	8 (5%)
Immunocompromising condition (72)	10 (31%)	62 (44%)	9 (50%)	63 (40%)	7 (39%)	35 (22%)	10 (43%)	62 (41%)
Neutropenia (15)	3 (9%)	12 (8%)	1 (6%)	14 (9%)	2 (11%)	13 (8%)	3 (13%)	12 (8%)
Inmunosuppressed (37)	5 (16%)	32 (23%)	6 (33%)	31 (20%)	3 (17%)	34 (22%)	4 (17%)	33 (22%)
HIV (3)	1 (3%)	2 (1%)	0	3 (2%)	0	3 (2%)	0	3 (2%)
Alcoholism (3)	2 (6%)	1 (0.5%)	0	3 (2%)	1 (6%)	2 (1%)	1 (4%)	2 (1%)
Heart disease (22)	4 (13%)	18 (13%)	3 (17%)	19 (12%)	3 (17%)	19 (12%)	4 (17%)	18 (12%)
Shock (31)	3 (9%)	28 (20%)	3 (17%)	28 (18%)	6 (33%)	25 (16%)	3 (13%)	28 (19%)
Urinary tract infection (100)	21 (66%)	79 (56%)	8 (44%)	92 (59%)	9 (50%)	91 (58%)	15 (65%)	85 (56%)
Pneumonia (9)	1 (3%)	8 (6%)	2 (11%)	7 (4%)	2 (11%)	7 (4%)	3 (13%)	6 (4%)
Venous catheter (5)	1 (3%)	4 (3%)	0	5 (3%)	1 (6%)	4 (3%)	2 (7%)	3 (2%)
Death (19)	2 (6%)	17 (12%)	3 (17%)	16 (10%)	3 (17%)	16 (10%)	3 (13%)	16 (11%)
Nalidixic acid resistance (76)	2 (6%)	74 (52%)*	12 (67%)*	64 (41%)	11 (61%)	65 (42%)	12 (52%)	64 (42%)
Phylogenetic group A (28)	1 (3%)	27 (19%)	0	28 (18%)	5 (28%)	23 (15%)	4 (17%)	24 (16%)
Phylogenetic group B1 (20)	0	20 (14%)	7 (39%)*	13 (8%)	2 (11%)	18 (12%)	2 (7%)	18 (12%)
Phylogenetic group B2 (34)	14 (44%)*	20 (14%)	1 (6%)	33 (21%)	0*	34 (22%)	4 (17%)	30 (20%)
Phylogenetic group D (92)	17 (53%)	75 (53%)	10 (56%)	82 (53%)	11 (61%)	81 (52%)	13 (56%)	79 (52%)
Biofilm (77)	20 (63%)*	57 (40%)	8 (44%)	69 (44%)	7 (39%)	70 (45%)	14* (61%)	63 (42%)

*Statistically significant.

The ShET-1 toxin has been described in *S. flexneri* 2a and has also been detected in other bacterial taxa such as *Y. enterocolitica*, *S. typhimurium* and *E. coli* (Al-Hasani *et al.*, 2001). This toxin has been found in EAEC causing diarrhoea (Mohamed *et al.*, 2007; Mendez-Arancibia *et al.*, 2008). In both of these studies, an association was observed between the presence of the *set*1 gene and biofilm production. Thus, 43% of biofilm producers presented this gene in contrast to 6% of nonbiofilm producers ($P = 0.0004$). These results are in agreement with those obtained in the present study. This ability to form biofilm is a trait that is closely associated with bacterial persistence and virulence, and many persistent and chronic bacterial infections are now believed to be linked to the formation of biofilm (Mohamed *et al.*, 2007).

There seems to be a relationship between the presence of the *set*1 gene and nalidixic acid susceptibility. In fact, *set*1 was more frequent among nalidixic acid-susceptible isolates. A possible explanation for this phenomenon may be that this gene is contained in the *she* PAI. This PAI is a chromosomal, laterally acquired, integrative element of *S. flexnerii* that carries genes with established or putative roles in virulence (Mohamed *et al.*, 2007). One of the two phe-tRNA genes is specifically integrated in the 3′ termini of the *she* PAI. Integration occurs via recombination between similar sequences in the chromosome target and episomal circle. This PAI is flanked by direct repeat sequences, suggesting that it may also adopt a circular intermediate form that is essential for its integration into the chromosome. It has been suggested that this excision is mediated by a PAI-borne integrase gene (*int*) related to the integrase gene of P4, a satellite element of phage P2 (Sakellaris *et al.*, 2004). These structures may be involved not only in horizontal transference of the PAI but also in the excision promoted by quinolones as occurs in uropathogenic *Escherichia coli* (UPEC). In this bacterium, quinolones induce the loss of a PAI by activation of the SOS system, which promotes the excision of phage-related sequences (Soto *et al.*, 2006).

Closely related islands that vary in structure can be found in a wide range of *Shigella* species and enteroinvasive *Escherichia coli* (EIEC) (Al-Hasani *et al.*, 2001). These islands are the result of the instability of the *she* PAI. In our isolates, we found diverse structures of this PAI, similar to the results obtained by Al-Hasani *et al.* (2001). This variation suggests that the right end of the *she* PAI may be unstable and undergoes deletions of varying lengths to yield a variety of structural forms of the PAI.

The presence of ShET-2 enterotoxin in *E. coli* shows that horizontal transference of VFs among bacteria belonging to different species had taken place. The presence of this toxin could increase the virulence potential of these strains allowing them to cause more severe infections, although further investigation is needed to prove this hypothesis.

Paiva de Sousa & Dubreuil (2001) studied the distribution of the *ast*A gene among 358 strains of *Enterobacteriaceae*. The gene was found in 32.6% of *E. coli*. Most *E. coli* EAST-1-positive strains were found among EHEC (88%), EAEC (86.6%), A-EPEC (58.3%) and EPEC (13.7%). This toxin has also been detected in 15.1% EAEC (Mendez-Arancibia *et al.*, 2008) in which in a plasmid of 60–65 MDa has been located.

Analyses have shown that *E. coli* strains fall into four main phylogenetic groups (A, B1, B2 and D) and that virulent extraintestinal strains mainly belong to groups B2 and D, whereas most commensal strains belong to groups A and B1 (Clermont *et al.*, 2000). A relationship between the presence of ShET-1 enterotoxin and phylogenetic group B2 has been observed, indicating the higher capacity of these strains to acquire VFs from other bacteria and reinforces the hypothesis that this enterotoxin plays a role as a VF in this phylogenetic group. On the other hand, ShET-2 was related to phylogenetic group B1, suggesting a possible increase in the virulence of these commensal strains.

Finally, we found a relationship between the presence of the *agg*R gene and biofilm formation, with this gene being more frequent among biofilm-producing isolates. This association has also been found in several previous studies. Mohamed *et al.* (2007) observed that 40% of biofilm-producing strains showed the *agg*R gene vs. 11% of the nonbiofilm producers ($P = 0.008$). In our study, this tendency could be observed but without statistical significance. The presence of AggR was also related to chronic renal insufficiency, which could be due to the function of this transcriptional factor regulating adherence factors that allow the bacteria to colonize and to persist in the kidney.

In conclusion, this is the first study on the presence of enterotoxins from *Shigella* and EPEC collected from blood. ShET-1 and EAST-1 have previously been found in *E. coli* but not in ShET-2. In addition, a relationship between quinolone resistance and the presence of the ShET-1 toxin has been demonstrated, although further studies are needed to determine whether quinolones induce this excision.

Acknowledgements

This work was supported by the projects FIS05/0068 of Fondo de Investigaciones Sanitarias of the Ministry of Health, the Spanish Network for the Research in Infectious Diseases (REIPI RE06/0008) and SGR050444 from the Departmanet d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya, Spain. S.M.S. is recipient of a contract from the 'Sistema Nacional de Salud' (CP05/00140) from Fondo de Investigaciones Sanitarias from the Ministry of Health of Spain. This work has also been supported by funding from the European Community (TROCAR contract HEALTH-F3-2008-223031). M.T. has a

fellowship from Federation of European Microbiological Societies.

References

Al-Hasani K, Henderson IR, Sakellaris H, Rajakumar K, Grant T, Nataro JP, Robins-Browne R & Adler B (2000) The *sig*A gene which is borne in the *she* pathogenicity island of *Shigella flexneri* 2a encodes an exported cytopathic protease involved in intestinal fluid accumulation. *Infect Immun* **68**: 2457–2463.

Al-Hasani K, Adler B, Rajakumar K & Sakellaris H (2001) Distribution and structural variation of the *she* pathogenicity island in enteric bacterial pathogens. *J Med Microbiol* **50**: 780–786.

Clermont O, Bonacorsi S & Bingen F (2000) Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microb* **66**: 4555–4558.

Clinical and Laboratory Standards Institute (2008) *Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement M100-S17*. CLSI, Wayne, PA.

Danese PN, Pratt LA, Dove SL & Kolter R (2000) The outer membrane protein, antigen-43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. *Mol Microbiol* **37**: 424–432.

Dreyfus LA & Robertson DC (1984) Solubilization and partial characterization of the intestinal receptor for *Escherichia coli* heat-stable enterotoxin. *Infect Immun* **46**: 537–543.

Fasano A, Noriega FR, Maneval DR Jr, Chanasongcram S, Russell R, Guandalini S & Levine MM (1995) *Shigella* enterotoxin 1: an enterotoxin of *Shigella flexneri* 2a active in rabbit small intestine *in vivo* and *in vitro*. *J Clin Invest* **95**: 2853–2861.

Henderson IR & Nataro JP (2001) Virulence factors of autotransporter proteins. *Infect Immun* **69**: 1231–1243.

Kaper JB, Nataro JP & Mobley HL (2004) Pathogenic *Escherichia coli*. *Nat Rev Microbiol* **2**: 123–140.

Mendez-Arancibia E, Vargas M, Soto S, Ruiz J, Kahigwa E, Schellenberg D, Urraza H, Gascón J & Vila J (2008) Prevalence of different virulence factors and biofilm formation production in enteroaggregative *Escherichia coli* isolates causing diarrea in children in Ifakara (Tanzania). *Am J Trop Med Hyg* **78**: 985–989.

Mohamed JA, Huang DB, Jiang ZD, Dupont HL, Nataro JP, Belkind-Gerson J & Okhuysen PC (2007) Association of putative enteroaggregative *Escherichia coli* virulence genes and biofilm production from travelers to developing countries. *J Clin Microbiol* **45**: 121–126.

Nataro JP, Yikang D, Yingkang D & Walker K (1994) AggR, a transcriptional activator of aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli*. *J Bacteriol* **176**: 4691–4999.

Nataro JP, Seriwatana J, Fasano A, Maneval DR, Guers LD, Noriega F, Dubovsky F, Levine MM & Morris JG Jr (1995) Identification and cloning of a novel plasmid-encoded enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains. *Infect Immun* **63**: 4721–4728.

Niyogi SK, Vargas M & Vila J (2004) Prevalence of the *sat*, *set* and *sen* genes among diverse serotypes of *Shigella flexneri* strains isolated from patients with acute diarrhoea. *Clin Microbiol Infec* **10**: 574–576.

Noriega FR, Liao FM, Formal SB, Fasano A & Levine MM (1995) Prevalence of *Shigella* enterotoxin 1 among *Shigella* clinical isolates of diverse serotypes. *J Infect Dis* **172**: 1408–1410.

Paiva de Sousa C & Dubreuil JD (2001) Distribution and expression of the *ast*A gene (EAST1 toxin) in *Escherichia coli* and *Salmonella*. *Int J Med Microbiol* **291**: 15–20.

Sakellaris H, Luck SN, Al-Hasani K, Rajakumar K, Turner SA & Adler B (2004) Regulated site-specific recombination of the *she* pathogenicity island of *Shigella flexneri*. *Mol Microbiol* **52**: 1329–1336.

Sannes MR, Kuskowski MA, Owens K, Gajewski A & Johnson JR (2004) Virulence factor profiles and phylogenetic background of *Escherichia coli* isolates from veterans with bacteraemia and uninfected control subjects. *J Infect Dis* **190**: 2121–2128.

Savarino SJ, McVeigh A, Watson J, Cravioto A, Molina J, Echevarria P, Bhan MK, Levine MM & Fasano A (1996) Enteroaggregative *Escherichia coli* heat-stable enterotoxin is not restricted to enteroaggregative *E. coli*. *J Infect Dis* **173**: 1019–1022.

Soto SM, Jimenez de Anta MT & Vila J (2006) Quinolones induce partial or total loss of pathogenicity islands in uropathogenic *Escherichia coli* by SOS-dependent or -independent pathways, respectively. *Antimicrob Agents Ch* **50**: 649–653.

Vargas M, Gascón J, Jiménez de Anta MT & Vila J (1999) Prevalence of *Shigella* enterotoxins 1 and 2 among *Shigella* strains isolated from patients with traveller's diarrhea. *J Clin Microbiol* **37**: 3608–3611.

Vila J, Marcos MA & Jiménez de Anta MT (1996) A comparative study of different PCR-based DNA fingerprinting techniques for typing of the *Acinetobacter calcoaceticus–A. baumannii* complex. *J Med Microbiol* **44**: 482–489.

Vila J, Vargas M, Henderson IR, Gascón J & Nataro JP (2000) Enteroaggregative *Escherichia coli* virulence factors in traveller's diarrhea strains. *J Infect Dis* **182**: 1780–1783.

PAPER 2:

Prevalence of the *set-1B* and *astA* genes encoding enterotoxins in uropathogenic *Escherichia coli* clinical isolates

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Journal, volume (issue): pages, date of publication:

Microbial Pathogenesis, 47 (6): 305-7, 2009 December.

Impact Factor: 2.324 – Q3 (2009)

Hypothesis:

Virulence factors typical of diarrhoeagenic *Escherichia coli* can also be found in ExPEC isolates causing urinary tract infections (UTI).

The *she* PAI containing the enterotoxin ShET-1 encoding genes is an unstable chromosomal locus and spontaneously deletes, totally or partially.

Objectives:

Determine the presence of the genes encoding for ShET-1, ShET-2 and EAST-1 toxins (*set1B*, *sen* and *astA*) in UPEC clinical isolates causing an increase in the virulence background of the strains and acquiring properties from other bacteria.

Determine the presence of other VFGs (*sigA*, *pic* and *sap*) located in the *she* PAI, such as the *set1B* gene, in order to elucidate the presence or not of the whole island.

Material and methods:

170 *E. coli* clinical isolates causing UTI and 35 *E. coli* from faeces of healthy humans (as controls) from the Hospital Clinic of Barcelona were included.

The presence of enterotoxin encoding genes as well as other genes belonging to the *she* PAI and phylogenetic group was determined by PCR and further sequencing.

Results:

The *set1B* gene was found in 16% of the UPEC, *astA* in 8% whereas the *sen* gene was not found in any isolate.

The isolates collected from faeces did not present the *set1* gene, and 14% of these presented the *astA* gene.

The presence of genes belonging to the *she* PAI was variable, and only three strains presented the three PAI markers, and therefore, the whole island.

Most of the isolates presenting enterotoxins encoding genes belonged to phylogenetic group B2, in agreement with the higher virulence pattern presented by this group.

Conclusions:

The variability in the presence of other *she* PAI gene markers could be due to the high instability shown by this PAI.

This is the first time that enterotoxins from *Shigella* have been found in UPEC isolates. Further studies are needed to determine the horizontal transference of these enterotoxin-encoding genes and the effect of their expression on damaging the urinary epithelium.

Microbial Pathogenesis 47 (2009) 305–307

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Prevalence of the *set*-1B and *ast*A genes encoding enterotoxins in uropathogenic *Escherichia coli* clinical isolates

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ARTICLE INFO

Article history:
Received 19 June 2009
Received in revised form
7 September 2009
Accepted 9 September 2009
Available online 13 September 2009

Keywords:
Uropathogenic
Escherichia coli
Enterotoxins
Pathogenicity island

ABSTRACT

One hundred seventy human uropathogenic *Escherichia coli* (UPEC) clinical isolates were compared with 35 *E. coli* strains isolated from feces of a control group to determine the presence of the *set*1, *sen* and *ast*A genes encoding the ShET-1, ShET-2, and EAST toxins, respectively. Overall, 27 (16%), 8 (8%) and 0 UPEC isolates presented the *set*1B, the *ast*A, and the *sen* genes, respectively. This is the first time the *set* gene has been found in UPEC clinical isolates.

1. Introduction

Escherichia coli is one of the microorganisms most frequently involved in urinary tract infections (UTIs). Uropathogenic *E. coli* (UPEC) show different virulence factors, such as adhesins, invasins and toxins, which allow colonization and invasion of the urinary epithelium and infection [1].

The ShET-1 (*Shigella* enterotoxin 1) toxin has been described in *Shigella flexneri* 2a and it is encoded by the *set*1A and *set*1B chromosomal genes which are located in the *she* pathogenicity island [2–4]. Similar to other PAIs, it is an unstable chromosomal locus and spontaneously deletes at a frequency of 10^{-5} to 10^{-6} per cell per generation. This toxin induces fluid accumulation in the rabbit ileal loop and may account for initial watery diarrhea that can occur in early stages of *S. flexneri* infections [5].

The ShET-2 toxin is encoded by the *sen* gene located on the 140MDa invasiveness plasmid pINV [5]. This toxin increases transepithelial conductance in an *in vitro* model [6], however, the relevance of the toxin in clinical disease is unknown.

The EAST-1 (EnteroAggregative heat Stable Toxin 1) toxin is encoded by the *ast*A gene [7]. This toxin is thought to play a role in enteroaggregative *E. coli* (EAEC) pathogenicity. It has been proposed that the mechanism of action of EAST-1 is identical to that of STa. The toxin binds to the receptor and activates guanylate cyclase, which stimulates production of cyclic GMP (cGMP). High levels of cGMP in the cell inhibit the Na/Cl cotransport system and reduce the absorption of electrolytes and water from the intestine at villus tips and result in an elevated secretion of Cl^- and water in crypt cells [8]. However, the role of this toxin in the development of diarrhea has yet to be defined.

Within *E. coli*, four phylogenetic groups or "subspecies" (A, B1, B2 and D) have been defined, and isolates belonging to these groups differ in their phenotypical characteristics, their antimicrobial resistance patterns, growth-rate/temperature relationships, as well as their ecological niches and propensity to cause disease [9]. The phylogentic groups B1 and A are considered less virulent than B2 and D, and they are associated with *E. coli* commensal strains, while B2 and D phylogenetic groups are associated with pathogenic strains [10].

The genes encoding these toxins are located in mobile elements such as plasmid or in PAIs, which often have clear indications of being mobile elements, therefore they can be transferred from one clone to another or from one pathotype of *E. coli* to another favouring the evolution of this microorganism.

The aim of this study was to determine the presence of the genes encoding for ShET-1, ShET-2 and EAST-1 toxins in UPEC clinical isolates causing an increase in the virulence background of the strains and acquiring properties from other bacteria.

2. Results

The 170 clinical isolates of UPEC collected from patients in our hospital were analyzed by PCR in order to study the presence of

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doi:10.1016/j.micpath.2009.09.007

the three genes encoding enterotoxins found in some diarrheagenic *E. coli* isolates. Twenty-seven isolates presented the *set*1B gene (16%), and eight isolates were positive for the *ast*A gene (8%). None presented the *sen* gene. Six (18%), 14 (14%), and seven (19%) isolates positive for the *set*1B gene were collected from cystitis, pyelonephritis and protatitis, respectively (Table 1). Moreover, the eight isolates that presented the *ast*A gene were collected from patients with pyelonephritis. Only one UPEC isolate, collected from pyelonephritis and belonging to phylogenetic group A, presented both genes.

The isolates collected from feces did not present the *set*1 gene and five of these (14%) presented the *ast*A gene (Table 1). The *set*1 gene was significantly more frequent among isolates collected from urinary tract infections (16% in UTI vs. 0% in feces; $p = 0.004$), whereas the *ast*A gene was significantly more frequent among isolates collected from feces (14% in feces vs. 5% in UTI; $p = 0.03$) (Table 1). In order to determine if the *set* gene was found in the *she* PAI, the *sig*A, *pic* and *sap* genes also in this PAI were detected by PCR, with the presence of these genes varying. Only three strains presented the three PAI markers and, therefore, the whole island.

Twenty-two (82%) UPEC isolates, presenting the ShET-1B toxin, belonged to phylogenetic group B2, two to phylogenetic group A and B1 each (7%), and only one to phylogenetic group D (3%). Among the eight *ast*A positive isolates, five (63%) belonged to phylogenetic group B2 and one to phylogenetic groups A, B1, and D each. The high prevalence of both genes in *E. coli* isolates belonging to phylogenetic group B2 is in agreement with the higher virulence presented by this group in comparison with the other.

3. Discussion

The ShET-1 toxin has been found in *Shigella* species such as *S. flexneri*, *Shigella sonnei*, and *Shigella. dysenteriae* [5]. A study carried out by Noriega FR et al. [4], analyzing the presence of the ShET-1 toxin in 172 *Shigella* clinical isolates and 10 enteroinvasive *E. coli* (EIEC), found that no EIEC presented this toxin. Vila et al. [11] found the *set* gene in 8% of enteroaggregative *E. coli* (EAEC) strains. Paiva de Sousa and Dubreuil [12] found EAST toxin in enterohemorragic *E. coli* (EHEC) (58.3%) and in enteroaggregative *E. coli* (EAEC) (88%) as well as in several strains of *Salmonella* but they did not analyze UPEC strains. Vila J et al. [11] found this toxin in EAEC (2%) clinical isolates, whereas Mendez-Arancibia *et al.* [13] detected the presence of the ShET-1 and EAST-1 toxins in 16.3% and 15.1%, respectively, among 348 EAEC collected from children with diarrhea in Tanzania. On analyzing several virulence factors specific for both urinary and diarrheagenic *E. coli*, Abe CM. *et al.* [14] have recently found the *astA* gene in 7.1% of 225 UPEC isolates suggesting that the UPEC strains which have acquired these toxins could become a potential agent of diarrhea. This result is in accordance with that found in the present study, although they did not analyze a control group. The EAST-1 toxin has been detected in EAEC clinical isolates and also in *E. coli* strains isolated from animal hosts including pigs, cattle and sheep [15].

Table 1
Features of strains presenting the ShET-1 and/or EAST-1 toxins.

Origin (No. isolates)	ShET-1 positive isolates	Phylogenetic group	EAST-1 positive isolates	Phylogenetic group
Cystitis (33)	6 (18%)	B2 (6)	0	
Pyelonephritis (100)	14[a] (14%)	B2 (13), A (1)	8[a] (8%)	B2 (5), B1 (1), D (1), A (1)
Prostatitis (37)	7 (19%)	B2 (3), B1 (2), A (1), D (1)	0	
Total UPEC (170)	27 (16%)	B2 (22), A (2) B1(2), D (1)	8 (5%)	
Feces (35)	0		5 (14%)	

UPEC, uropathogenic *E. coli*.
[a] One strain presented both toxins.

Table 2
Primers used in this study.

Gene	Primer forward sequence	Primer reverse sequence	Reference
set-1	GTGAACCTGCTGCCGATATC	ATTTGTGGATAAAAATGACG	[14]
Sen	ATGTGCCTGCTATTATTTAT	CATAATAATAAGCGGTCAGC	[14]
astA	ATGCCATCAACACAGTATAT	GCGAGTGACGGCTTTGTAGT	[14]
sigA	TCCTCGGTATTATTTTATCC	CGTAACCCCTGTTGTTTCCAC	This work
sap	TACCCTCCACAACAGAGAATG	TACCCTCCACAACAGAGAATG	This work
pic	ACTGGATCTTAAGGCTCAGGAT	GACTTAATGTCACTGTTCAGCG	20

The variability in the presence of the other *she* PAI gene markers could be due to the high instability shown by this PAI. Al-Hasani K. *et al.* [16] found this phenomenon among *Shigella* strains suggesting that the right end of the *she* PAI may be unstable and undergo deletions of varying lengths to yield a variety of structural forms of the PAI.

In conclusion, this is the first time that enterotoxins from *Shigella* have been found in UPEC clinical isolates. Further studies are needed to determine the horizontal transference of this genetic information and the effect of the expression of these toxins on damaging the urinary epithelium.

4. Material and methods

4.1. Bacteria

One hundred seventy uropathogenic *E. coli* (UPEC) clinical isolates were included in this study. These isolates were collected from patients with cystitis (33 isolates), pyelonephritis (100 isolates), and prostatitis (37 isolates) in the Hospital Clinic of Barcelona, Spain. In addition, 35 *E. coli* isolates collected from feces of healthy humans were used as controls.

4.2. PCR procedures

The phylogenetic group was determined by multiplex-PCR as described elsewhere [21]. The presence of the genes encoding ShET-1, ShET-2 and EAST-1 was detected by PCR using gene-specific primers (Table 2) and the following PCR conditions: an initial step of 94 °C for 3 min, followed by 30 cycles to 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, and a final step of 72 °C for 5 min. In addition, other genes belonging to the *she* pathogenicity island (*pic*, *sig*A, *sap*) were detected (Table 2). The DNA products obtained were sequenced using the 3.1 AbiPrism kit (Amersham), and analyzed using the BLAST database.

Acknowledgements

This work was supported by the projects FIS05/0068 of Fondo de Investigaciones Sanitarias of the Ministry of Health, the Spanish Network for the Research in Infectious Diseases (REIPI RE06/0008) and SGR050444 from the Departmanet d'Universitats, Recerca I Societat de la Informació de la Generalitat de Catalunya, Spain. Sara M. Soto is recipient of a contract from the "Sistema Nacional de Salud" (CP05/00140) from Fondo de Investigaciones Sanitarias from Ministry of Health of Spain. This work has also been supported by funding from the European Community (TROCAR contract HEALTH-F3-2008-223031).

References

[1] Donnenberg MS, Welch RA. In: Mobley HLT, Warren JW, editors. Urinary tract infection: pathogenesis and clinical management. Washington D.C.: American Society for Microbiology Press; 1996.
[2] Niyogi SK, Vargas M, Vila J. Prevalence of the *sat*, *set* and *sen* genes among diverse serotypes of *Shigella flexneri* strains isolated from patients with acute diarrhoea. Clin Microbiol Infect 2004;10:574–6.

[3] Noriega FR, Liao FM, Formal SB, Fasano A, Levine MM. Prevalence of *Shigella* enterotoxin 1 among *Shigella* clinical isolates of diverse serotypes. J Infect Dis 1995;172:1408–10.
[4] Vargas M, Gascón J, Jiménez de Anta MT, Vila J. Prevalence of *Shigella* enterotoxins 1 and 2 among *Shigella* strains isolated from patients with travelleŕs diarrhea. J Clin Microbiol 1999;37:3608–11.
[5] Fasano A, Noriega FR, Maneval Jr DR, Chanasongcram S, Russell R, Guandalini S, et al. *Shigella* enterotoxin 1: an enterotoxin of *Shigella flexneri* 2a active in rabbit small intestine in vivo and in vitro. J Clin Invest 1995;95:2853–61.
[6] Nataro JP, Seriwatana J, Fasano A, Maneval DR, Guers LD, Noriega F, et al. Identification and cloning of a novel plasmid-encoded enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains. Infect Immun 1995:4721–8.
[7] Savarino SJ, McVeigh A, Watson J, Cravioto A, Molina J, Echevarria P, et al. Enteroaggregative *Escherichia coli* heat-stable enterotoxin is not restricted to enteroaggregative *E. coli*. J Infect Dis 1996;173:1019–22.
[8] Dreyfus LA, Robertson DC. Solubilization and partial characterization of the intestinal receptor for *Escherichia coli* heat-stable enterotoxin. Infect Immun 1984;46:537–43.
[9] Gordon DM, Clermont O, Tolley H, Denamur E. Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. Environ Microbiol 2008;10:2484–96.
[10] Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. App Environ Microbiol 2000;66: 4555–8.
[11] Vila J, Vargas M, Henderson IR, Gascón J, Nataro JP. Enteroaggregative *Escherichia coli* virulence factors in traveller's diarrhea strains. J Infect Dis 2000;182:1780–3.
[12] Paiva de Sousa C, Dubreuil JD. Distribution and expression of the *ast*A gene (EAST1 toxin) in *Escherichia coli* and *Salmonella*. Int J Med Microbiol 2001;291:15–20.
[13] Mendez-Arancibia E, Vargas M, Soto S, Ruiz J, Kahigwa E, Schellenberg D, et al. Prevalence of different virulence factors and biofilm production in enteroaggregative *Escherichia coli* isolates causing diarrhea in children in Ifakara (Tanzania). Am J Med Hyg 2008;78:985–9.
[14] Abe CM, Salvador FA, Falsetti IN, Vieira MAM, Blanco J, Blanco JE, et al. Uropathogenic *Escherichia coli* (UPEC) strains may carry virulence properties of diarrhoeagenic *E. coli*. FEMS Immunol Med Microbiol 2008;52:397–406.
[15] Veilleux S, Dubreuil JD. Presence of *Escherichia coli* carrying the EAST1 toxin gene in farm animals. Vet Res 2006;37:3–13.
[16] Al-Hasani K, Adler B, Rajakumar K, Sakellaris H. Distribution and structural variation of the *she* pathogenicity island in enteric bacterial pathogens. J Med Microbiol 2001;50:780–6.

SECTION 2. Virulence and antibiotic resistance in ExPEC: Women, neonates and children

This section includes the following studies:

- PAPER 3: Prevalence of *Escherichia coli* among samples collected from the genital tract in pregnant and nonpregnant women: relationship with virulence.
- PAPER 4: Antimicrobial resistance and virulence characterization among *Escherichia coli* clinical isolates causing severe obstetric infections in pregnant women.
- PAPER 5: Antimicrobial resistance of *Escherichia coli* strains causing neonatal sepsis between 1998 and 2008.
- PAPER 6: Epidemiology and molecular characterization of multidrug-resistant *Escherichia coli* isolates harboring $bla_{CTX\text{-}M}$ group 1 extended-spectrum β-lactamases causing bacteremia and urinary tract infection in Manhiça, Mozambique.

PAPER 3:

Prevalence of *Escherichia coli* among samples collected from the genital tract in pregnant and nonpregnant women: relationship with virulence

Authors:

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Journal, volume (issue): pages, date of publication:

FEMS Microbiology Letters, 314 (2): 170-3, 2011 January.

Impact Factor: 2.044 – Q3 (2011)

Hypothesis:

Data on the features and virulence factors of infection-causing or commensal *E. coli* strains in pregnant women are scarce. However, the virulence profile of these strains might be different from those infecting nonpregnant women, as they are capable of causing severe infections including neonatal sepsis. Quinolone susceptibility may be related to the virulence profile.

Objectives:

Analyse the prevalence of *E. coli* in obstetric samples and compare the virulence factors present in *E. coli* isolates from the genital tract of pregnant and nonpregnant women in order to detect possible differences in the virulence profile that could explain their differential capacity to cause severe infection.

Elucidate a possible relationship between virulence factor burden and the quinolone susceptibility profile.

Material and methods:

648 vaginal and endocervical samples from 321 pregnant and 327 nonpregnant women visiting the Gynaecology Department of the Hospital Clinic of Barcelona were studied.

E. coli isolates were detected by growth in MacConkey agar plates and subsequent biochemical identification. Haemolysin expression was detected by the spread of

the isolates in blood agar plates, and the virulence genes and phylogenetic grouping were analysed by PCR. Nalidixic acid susceptibility was tested by the disk diffusion method following CLSI recommendations.

Results:

Out of the total of 648 samples, 86 were positive for *E. coli*: 15% from pregnant women and 12% from nonpregnant women. The virulence factors *hly*, *cnf*, *papC* and iroN were more frequent in isolates from pregnant women. In contrast, the gene encoding for adhesin *iha* was more frequent in nonpregnant women. Phylogenetic group B2 was the most frequent among the collection, being significantly more prevalent in isolates obtained from pregnant women. Some virulence factors were significantly more prevalent among the isolates susceptible to nalidixic acid.

Conclusions:

The results of the most prevalent virulence factors are in accordance with other studies. The *hly*, *cnf* and *pap* genes, which are all associated with PAIs, were significantly more prevalent in *E. coli* from pregnant women, and most of these isolates belonged to phylogenetic group B2, confirming the greater virulence of *E. coli* isolates collected from pregnant women. The lower frequency of these genes among the nalidixic acid-resistant isolates could be explained by the partial loss of PAIs associated with the acquisition of quinolone resistance.

RESEARCH LETTER

Prevalence of *Escherichia coli* among samples collected from the genital tract in pregnant and nonpregnant women: relationship with virulence

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Received 17 October 2010; revised 2 November 2010; accepted 5 November 2010.
Final version published online 6 December 2010.

DOI:10.1111/j.1574-6968.2010.02160.x

Editor: William Wade

Keywords
genital tract; pregnant women; *Escherichia coli*; virulence factors.

Abstract

Escherichia coli are enteric Gram-negative bacilli that can colonize the female genital tract and become implicated in different infections in pregnant women, including intra-amniotic infection, puerperal infections and neonatal infections. The virulence profiles of *E. coli* isolates from vaginal swabs from pregnant and nonpregnant women were compared. The *hly*-, *cnf*-, *pap*- and *iro*N-genes were found significantly more frequently in *E. coli* isolated from pregnant women in comparison with those isolated from nonpregnant women. *Escherichia coli* from pregnant women seem to be more virulent than from nonpregnant women developing severe infections, thereby increasing possible neonatal sepsis.

Introduction

Escherichia coli are enteric Gram-negative bacilli found most frequently in the genital tract of women. These microorganisms possess several virulence factors that allow them to cause vaginal and/or endocervical colonization and have been implicated in different infections in pregnant women, as well as in intra-amniotic, puerperal and neonatal infections both early and late neonatal sepsis, presenting sometimes with meningitis or urinary tract infections. The transmission of maternal *E. coli* colonizing the newborn can occur after colonization or infection of amniotic fluid, after membrane rupture or on passage of the neonate through the vaginal canal during delivery, and may cause early neonatal infection.

Data on the features and virulence factors of infection-causing *E. coli* strains in mothers and babies, and colonization of genital tracts of pregnant women by this microorganism are scarce. Neonatal sepsis by *E. coli* is related to a limited number of phylogenetic groups B2 and D, both considered as virulent. The pathogenicity of these groups is associated with the presence of several virulence factors, some of which are contained into pathogenicity islands (PAIs) (Soto *et al.*, 2008).

The study of these *E. coli* strains is necessary to understand the potential risk factors for vertical transmission of neonatal infection by pregnant women and to design interventions to address such risk factors adequately.

The aim of this study was to compare the virulence factors present in *E. coli* isolates from the genital tract of pregnant women with those of *E. coli* from nonpregnant women in order to shed light on the possible differences in the virulence profiles that could explain their capacity to cause severe infections.

Materials and methods

Clinical samples

The study included 648 vaginal and endocervical samples from 321 pregnant and 327 nonpregnant women followed either at the antenatal visits or at the Gynecology Department of the Hospital Clinic of Barcelona. Samples from each woman were collected using sterile swabs.

Table 1. Primers used in this study

Gene	Sequence 5′–3 (F/R)	PCR product (bp)	Reference
hly	AACAAGGATAAGCACTGTTCTGGCT/ACCATATAAGCGGTCATTCCCGTCA	1177	Yamamoto *et al*. (1995)
cnf1	AAGATGGAGTTTCCTATGCAGGAG/CATTCAGAGTCCTGCCCTCATTATT	498	Yamamoto *et al*. (1995)
sat1	ACTGGCGGACTCATGCTGT/AACCCTGTAAGAAGACTGAGC	387	Vila *et al*. (2002)
papA	ATGGCAGTGGTGTCTTTTGGTG/CGTCCCACCATACGTGCTCTTC	720	Johnson & Stell (2000)
papC	GACGGCTGTACTGCAGGGTGTGGCG/ATATCCTTTCTGCAGGGATGCAATA	328	Yamamoto *et al*. (1995)
papEF	GCAACAGCAACGCTGGTTGCATCAT/AGAGAGAGCCACTCTTATACGGACA	336	Johnson & Stell (2000)
focG	CAGCACAGGCAGTGGATACGA/GAATGTCGCCTGCCCATTGCT	360	Johnson & Stell (2000)
fyu	TGATTAACCCCGCGACGGGAA/CGCAGTAGGCACGATGTTGTA	880	Johnson & Stell (2000)
hra	CAGAAAACAACCGGTATCAG/ACCAAGCATGATGTCATGAC	260	Bingen-Bidois *et al*. (2002)
sfaS	AGAGAGAGCCACTCTTATACGGACA/CCGCCAGCATTCCCTGTATTC	240	Johnson & Stell (2000)
ibeA	AGGCAGGTGTGCGCCGCGTAC/TGGTGCTCCGGCAAACCATGC	170	Johnson & Stell (2000)
iha	CTGGCGGAGGCTCTGAGATCA/TCCTTAAGCTCCCGCGGCTGA	827	Takahashi *et al*. (2006)
iucD	TACCGGATTGTCATATGCAGACCGT/AATATCTTCCTCCAGTCCGGAGAAG	602	Yamamoto *et al*. (1995)
iutA	GGCTGGACATCATGGGAACTGG/CGTCGGGAACGGGTAGAATCG	300	Johnson & Stell (2000)
iroN	AAGTCAAAGCAGGGGTTGCCCG/GACGCCGACATTAAGACGCAG	665	Takahashi *et al*. (2006)
ag43	ACGCACAACCATCAATAAAA/CCGCCTCCGATACTGAATGC	600	Mendez-Arancibia *et al*. (2008)

Escherichia coli detection

The samples were spread in chocolate agar (PVX, BioMèrieux, Spain). Colonies with an *E. coli* appearance were grown in McConkey agar (MCK, BioMèrieux) with subsequent biochemical identification using the β-glucuronidase/indol test (DIATABS, Rosco Diagnostica, Taastrup).

Hemolysin expression

Escherichia coli isolates were grown in blood agar plates (COS, Oxoid) to study their hemolytical capacity.

Molecular characterization

The virulence profile was analyzed by PCR using gene-specific primers for 17 virulence genes such as hemolysin (*hly*), cytotoxic necrotizing factor (*cnf*1), autotransporter (*sat*1), P-fimbriae (*pap* genes), type 1C fimbriae (*foc*G), yersiniabactin (*fyu*), heat-resistant hemagglutinin (*hra*), S-fimbriae (*sfa*S), invasin (*ibe*A), adhesin (*iha*), aerobactin (*iuc*D), siderophores (*iut*A, *iro*N) and antigen 43 (ag43) (Table 1). PCR conditions were 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, the corresponding annealing temperature (55–63 °C) for 30 s, 72 °C for 2 min and a final elongation of 72 °C for 5 min. Samples were run in 1.5% agarose gels and stained with SYBR Safe DNA gel stain (Invitrogen, Spain). The *E. coli* phylogenetic group was determined using a three-locus PCR-based method (Clermont *et al*., 2000). The epidemiological relationship was studied by REP-PCR (Vila *et al*., 1996).

Nalidixic acid susceptibility

Nalidixic acid susceptibility was tested using the disk-diffusion method following CLSI recommendations (CLSI, 2008).

Statistical analysis

Data were statistically analyzed using the Fisher exact test due to the small size of the sample.

Results

We studied 331 vaginal samples (114 from pregnant and 217 from nonpregnant women from 16 to 50 years old) and 317 endocervical samples (271 and 46, respectively). Eighty-six (86/648, 13%) samples were positive for *E. coli*: 48 (15%) from pregnant and 38 (12%) from nonpregnant women. REP-PCR did not show any epidemiological relationship between isolates (data not shown). Table 2 summarizes the different virulence factors and the phylogenetic characteristics among *E. coli* strains in general and stratified by pregnancy status. Phylogenetic group B2 was the most frequent among the strains (51%), followed by groups D (34%), A (12%) and B1 (3%). Sixty percent of the strains from pregnant women were phylogenetic group B2 vs. 39% of those from nonpregnant women ($P = 0.043$). The *iro*N, *fyu*, *pap* and *iut*A genes were the virulence factors found most frequently (57%, 53%, 51% and 41%, respectively). However, only the *hly*, *cnf*, *pap* and *iroN* genes occurred significantly more frequently when comparing the strains from pregnant women (48) with those from nonpregnant women (38) (Table 2). In contrast, the adhesin *iha* occurred more frequently among strains from nonpregnant women (17% vs. 39%, $P = 0.017$). The *iuc*D and *iut*A genes tended to be more frequent among strains from nonpregnant women (Table 2), but the differences were not statistically significant.

No statistically significant differences were found in nalidixic acid susceptibility between *E. coli* strains collected from pregnant and nonpregnant women, although the

Table 2. Features from the *Escherichia coli* isolates studied

Virulence factor	Total (86)	Pregnant (48)	Nonpregnant (38)	*P*
NAL resistant	19 (22%)	8 (17%)	11 (29%)	0.298
NAL susceptible	67 (78%)	40 (83%)	27 (71%)	0.135
hly expression	23 (27%)	17 (35%)	6 (16%)	**0.034**
hly	28 (33%)	20 (42%)	8 (21%)	**0.035**
cnf 1	26 (30%)	20 (42%)	6 (16%)	**0.0007**
sat 1	13 (15%)	6 (13%)	7 (15%)	0.321
papC	37 (43%)	25 (52%)	12 (25%)	**0.045**
foc G	13 (15%)	9 (19%)	4 (10%)	0.227
fyu	46 (53%)	25 (52%)	21 (55%)	0.47
hra	27 (31%)	18 (38%)	9 (24%)	0.098
sfa S	17 (20%)	12 (25%)	5 (13%)	0.136
ibeA	16 (19%)	9 (19%)	7 (18%)	0.397
iha	23 (27%)	8 (17%)	15 (39%)	**0.016**
iut A	35 (41%)	16 (33%)	19 (50%)	0.089
iuc D	34 (40%)	16 (33%)	18 (47%)	0.135
papEF	38 (44%)	23 (46%)	15 (39%)	0.286
papA	44 (51%)	25 (52%)	19 (50%)	0.168
iroN	49 (57%)	32 (67%)	17 (45%)	**0.034**
Ag43	32 (37%)	16 (33%)	16 (42%)	0.125
Phylogenetic group B2	44 (51%)	29 (60%)	15 (39%)	**0.043**
Phylogenetic group B1	3 (3%)	2 (4%)	1 (3%)	0.5
Phylogenetic group A	10 (12%)	3 (6%)	7 (18%)	0.079
Phylogenetic group D	29 (34%)	14 (29%)	15 (39%)	0.219

Numbers in bold are statistically significant.

strains from pregnant women presented a lower resistance to this antimicrobial agent than those from nonpregnant women.

The comparison between nalidixic acid-susceptible (67) and -resistant (19) strains showed that those that were resistant presented *hly*, *cnf*1 and *foc*G less frequently (Table 3). It is also of note that among nalidixic acid-susceptible strains, phylogenetic group B2 was significantly more frequent, confirming greater virulence. On the other hand, phylogenetic group D was the most frequent among nalidixic acid-resistant strains (Table 3).

Discussion

The predominant flora in the vagina consists of *Lactobacillus* and *Streptococcus* species; however, the presence of other bacteria such as *E. coli* may be very important, albeit not necessarily synonymous with infection. Vaginal *E. coli* may cause symptomatic infections and is associated with neonatal sepsis (Percival-Smith *et al.*, 1983). These strains possess several virulence factors allowing vaginal and/or endocervical colonization. We analyzed the prevalence of *E. coli* in vaginal and endocervical samples among pregnant and nonpregnant women and the virulence characteristics of the *E. coli* found.

Table 3. Comparison between nalidixic acid susceptible and nalidixic acid resistant strains

Virulence factor	NAL resistant (19)	NAL susceptible (67)	*P*
hly expression	3 (16%)	20 (30%)	0.177
hly	3 (16%)	25 (37%)	0.06
cnf 1	3 (16%)	23 (34%)	0.09
sat 1	3 (16%)	10 (15%)	0.688
papC	9 (47%)	28 (42%)	0.757
foc G	0	13 (19%)	**0.029**
fyu	10 (53%)	36 (54%)	0.635
hra	6 (32%)	21 (31%)	0.624
sfa S	2 (11%)	15 (22%)	0.21
ibeA	5 (26%)	11 (16%)	0.90
iha	8 (42%)	16 (24%)	0.965
iut A	13 (68%)	22 (33%)	**0.006**
iuc D	13 (68%)	21 (31%)	**0.004**
papEF	10 (53%)	28 (42%)	0.28
papA	10 (53%)	34 (51%)	0.54
iroN	11 (58%)	38 (57%)	0.57
Ag43	7 (37%)	25 (37%)	0.596
Phylogenetic group B2	6 (32%)	38 (57%)	**0.046**
Phylogenetic group B1	2 (11%)	1 (1%)	0.12
Phylogenetic group A	3 (16%)	7 (10%)	0.852
Phylogenetic group D	8 (42%)	21 (31%)	0.874

Numbers in bold are statistically significant.

Cook *et al.* (2001) studied the presence of several virulence factors among 50 strains of *E. coli* causing vaginitis in nonpregnant women, with findings similar to ours. However, both studies differed considerably compared with the results of Birõsová *et al.* (2004), who found higher percentages of the virulence factors studied (*hly*, *cnf*, *pap*, *sfa*, *iuc*D genes) among *E. coli* isolates from vaginal samples of nonpregnant and pregnant women than in our study. Obata-Yasuoka *et al.* (2002) compared *E. coli* isolates from pregnant women with isolates from nonpregnant women, with different results.

The *hly*, *cnf* and *pap* genes, all associated with PAIs, were significantly more frequent among strains from pregnant women presenting a higher percentage of nalidixic acid-susceptible strains. In spite of the lack of significant differences in the levels of nalidixic acid resistance between *E. coli* strains from pregnant and nonpregnant women, statistically significant differences were found in the frequency of several virulence factors among nalidixic acid-resistant and -susceptible strains.

The lower frequency of the *hly*, *cnf*1 and *pap* genes among the nalidixic acid-resistant isolates could be explained by the partial loss of PAI associated with acquisition of quinolone resistance, as has been demonstrated in a previous study carried out in our laboratory (Soto *et al.*, 2006), in which a quinolone-susceptible *E. coli* strain was grown in culture media with subinhibitory concentrations of ciprofloxacin, observing the loss of the *hly* and *cnf*1 genes.

In urinary tract infections, P-fimbriae mediate the specific attachment of uropathogenic *E. coli* to kidney tissue and elicit a cytokine response in these cells (Johnson, 1991; Johnson, 2005). Nevertheless, the role of P-fimbriae in genital tract infection remains unknown.

Notably, 60% of isolates from pregnant women belonged to phylogenetic group B2, considered the most virulent, and a high percentage of nonpregnant isolates were phylogenetic group A, considered as commensal, thereby confirming the greater virulence of *E. coli* isolates from pregnant women.

In summary, *E. coli* strains isolated from vaginal and/or endocervical samples of pregnant women are more virulent than those from nonpregnant women. The presence of hemolysin, cytotoxic necrotizing factor and P-fimbriae, all in a PAI, may allow the bacteria to cause severe infections during pregnancy, thereby increasing the possibility of neonatal sepsis. Further studies are needed in order to analyze the role of each virulence factor in the transmission of microorganisms between mother and baby.

Acknowledgements

Thanks are due to Quique Bassat (CRESIB) for the revision and suggestions. This work was supported by the Spanish Network for the Research in Infectious Diseases (REIPI RE06/0008), SGR091256 from the Department d'Universitats, Recerca I Societat de la Informació de la Generalitat de Catalunya, Spain, and by funding from the European Community (TROCAR contract HEALTH-F3-2008-223031). S.M.S. is a recipient of a contract 'Miguel Servet' (CP05/00140) from 'Fondo de Investigaciones Sanitarias' from the Spanish Ministry of Health.

References

Bingen-Bidois M, Clermont O, Bonacorsi S, Terki M, Brahimi N, Loukil C, Barraud D & Bingen E (2002) Phylogenetic analysis and prevalence of urosepsis strains of *Escherichia coli* bearing pathogenicity island-like domains. *Infect Immun* **70**: 3216–3226.

Birõsová E, Siegfried L, Kmet'ová M *et al.* (2004) Detection of virulence factors in α-haemolytic *Escherichia coli* strains isolated from various clinical materials. *Clin Microbiol Infec* **10**: 569–573.

Clermont O, Bonacorsi S & Bingen E (2000) Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microb* **66**: 4555–4558.

Clinical and Laboratory Standards Institute (2008) *Performance Standards for Antimicrobial Susceptibility Testing. Seventeenth Informational Supplement M100-S17*. CLSI, Wayne, PA.

Cook SW, Hammill HA & Hull RA (2001) Virulence factors of *Escherichia coli* isolated from female reproductive tract infections and neonatal sepsis. *Infect Dis Obstet Gynecol* **9**: 203–207.

Johnson JR (1991) Virulence factors in *Escherichia coli* urinary tract infection. *Clin Microbiol Rev* **4**: 80–128.

Johnson JR (2005) Virulence factors in *Escherichia coli*. *J Clin Microbiol* **43**: 6221.

Johnson JR & Stell AL (2000) Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. *J Infect Dis* **181**: 261–272.

Mendez-Arancibia E, Vargas M, Soto S, Ruiz J, Kahigwa E, Schellenberg D, Urassa H, Gascon J & Vila J (2008) Prevalence of different virulence factors and biofilm production in enteroaggregative *Escherichia coli* isolates causing diarrhea in children in Ifakara (Tanzania). *Am J Trop Med Hyg* **78**: 985–989.

Obata-Yasuoka M, Ba-Thein W, Tsukamoto T, Yoshikawa H & Hayashi H (2002) Vaginal *Escherichia coli* share common virulence factor profiles, serotypes and phylogeny with other extraintestinal *E. coli*. *Microbiology* **148**: 2745–2752.

Percival-Smith R, Bartlett KH & Chow AW (1983) Vaginal colonization of *Escherichia coli* and its relation to contraceptive methods. *Contraception* **27**: 497–504.

Soto SM, Jimenez de Anta MT & Vila J (2006) Quinolones induce partial or total loss of pathogenicity islands in uropathogenic *Escherichia coli* by SOS-dependent or -independent pathways, respectively. *Antimicrob Agents Ch* **50**: 649–653.

Soto SM, Bosch J, Jimenez de Anta MT & Vila J (2008) Comparative study of virulence traits of *Escherichia coli* clinical isolates causing early and late neonatal sepsis. *J Clin Microbiol* **46**: 1123–1125.

Takahashi A, Kanamaru S, Kurazono H, Kunishima Y, Tsukamoto T, Ogawa O & Yamamoto S (2006) *Escherichia coli* isolates associated with uncomplicated and complicated cystitis and asymptomatic bacteriuria possess similar phylogenies, virulence genes, and O-serogroup profiles. *J Clin Microbiol* **44**: 4589–4592.

Vila J, Marcos MA & Jiménez de Anta MT (1996) A comparative study of different PCR-based DNA fingerprinting techniques for typing of the *Acinetobacter calcoaceticus–A. baumannii* complex. *J Med Microbiol* **44**: 482–489.

Vila J, Simon K, Ruiz J, Horcajada JP, Velasco M, Barranco M, Moreno A & Mensa J (2002) Are quinolone-resistant uropathogenic *Escherichia coli* less virulent? *J Infect Dis* **186**: 1039–1042.

Yamamoto S, Terai A, Yuri K, Kurazono H, Takeda Y & Yoshida O (1995) Detection of urovirulence factors in *Escherichia coli* by multiplex polymerase chain reaction. *FEMS Immunol Med Mic* **12**: 85–90.

PAPER 4:

Antimicrobial resistance and virulence characterization among *Escherichia coli* clinical isolates causing severe obstetric infections in pregnant women

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Journal, volume (issue): pages, date of publication:

Journal of Clinical Microbiology, 53 (5): 1745-7, 2015 May.

Impact Factor: 3.631 – Q2 (2015)

Hypothesis:

The treatment of choice in patients with obstetric infections caused by *E. coli* administered in our hospital is correct regarding the antimicrobial susceptibility rates of the isolates causing these infections.

The prevalence and roles of virulence factor genes (VFGs) in *E. coli* isolates causing obstetric infections of different severity are still unknown, but there may be a relationship between their presence and the type of infection they cause.

Virulence carriage may be related to the resistance phenotype of the *E. coli* isolates.

Objectives:

Analyse and compare the antimicrobial susceptibility phenotype and the virulence pattern of *E. coli* isolates causing obstetric infections accompanied or not by sepsis in pregnant women.

Material and methods:

78 *E. coli* isolates causing sepsis or non-bacteraemic intra-amniotic infection (IAI) recovered from pregnant women attending the Hospital Clinic of Barcelona from 1987 to 2010 were studied.

Antimicrobial resistance profiles were determined by disk diffusion and interpreted according to CLSI guidelines.

The presence of VFGs was analysed by PCR using gene-specific primers.

The phylogenetic group was determined by 3-locus PCR and Achtman scheme multilocus sequence typing (MLST) methodology to determine the epidemiology of O25b serotype isolates.

Results:

26% of the isolates had a multidrug-resistant phenotype, although the prevalence of resistance to amoxicillin-clavulanic acid (AMC), 3rd generation cephalosporins and aminoglycosides was low. The carriage of VFGs was higher among susceptible isolates and isolates causing sepsis.

Regarding VFGs, *hly* and *cnf1* were more prevalent among isolates causing sepsis. Iron recruitment systems were specifically found depending on the type of infection: *fyuA* and the genes encoding for the siderophore receptors *iha* and *iroN* were more prevalent in isolates causing sepsis, whereas *iutA* was more frequent among intra-amniotic infection-causing isolates.

Conclusions:

Low rates of resistance to AMC and 3rd generation cephalosporins indicate that the treatment guidelines applied in our hospital are correct.

Regarding VFGs, *hly* and *cnf1* were more prevalent among isolates causing sepsis possibly in relation to the tissue damage involved in this infection, and iron recruitment systems were specifically found depending on the type of infection.

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Antimicrobial Resistance and Virulence Characterization among *Escherichia coli* Clinical Isolates Causing Severe Obstetric Infections in Pregnant Women

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The virulence markers and the antimicrobial resistance profiles of 78 *Escherichia coli* isolates causing obstetric infections accompanied by sepsis or not were studied. Adhesion-related virulence factors were the most prevalent markers. Low rates of resistance to the antimicrobial agents used as first-line therapy suggest their correct implementation in stewardship guidelines.

Escherichia coli is the enteric Gram-negative bacillus most frequently found in the genital tract of women. Despite its commensal role, this microorganism can become pathogenic, colonizing new environments. Extraintestinal *E. coli* is the second most prevalent etiologic agent causing obstetric infections (1). *E. coli* possesses several virulence factor genes (VFG) that enhance vaginal and/or endocervical colonization in pregnant women. This colonization can lead to different infections in obstetric patients, such as intra-amniotic infection (IAI) or endometrial and urinary tract infections (UTIs), sometimes accompanied by sepsis. In addition, these microorganisms can cause neonatal infections, leading to maternal and fetal morbidity and mortality (2, 3). It has been estimated that 15% of pregnant and 12% of nonpregnant women in our hospital present *E. coli* in the genital tract (4).

The treatment of choice for maternal sepsis includes the administration of different antimicrobial agents, depending on the infection focus, being limited by the low number of antimicrobial agents considered to be safe to the fetus (5). In our hospital, the treatment of choice in patients with IAI consists of ceftriaxone, ampicillin-gentamicin, or ampicillin-cefoxitin, while the treatment of endometritis involves the use of ampicillin-gentamicin-metronidazole.

Briefly, among the virulence factors involved in UTIs, it is well known that adhesins, fimbriae, and toxins are the most important, as they allow the bacteria to adhere to the uroepithelium and cause tissue damage. However, further knowledge is necessary regarding their prevalences and the roles of other families of virulence factors in the specific field of obstetric infections derived from UTIs.

For this purpose, 78 *E. coli* isolates obtained from pregnant women attending the Hospital Clinic of Barcelona from 1987 to 2010 were included in the study; 56 were isolated from the blood samples of patients with sepsis from a genital or urinary origin, and 22 were isolated from amniotic fluid or placenta samples of patients with nonbacteremic IAI.

The resistance profiles were determined using the disk diffusion method. The antimicrobial agents tested are listed in Table 1 and include the first therapeutic options used to treat UTIs and genital infections. The results were interpreted according to CLSI guidelines (6), and the *E. coli* ATCC 25922 strain was used as the control.

The VFG profiles of the isolates were analyzed by PCR using gene-specific primers for the virulence genes coding for the adhesins, toxins, and invasins most prevalent in the uropathogenic *E. coli* (UPEC) isolates described, from which the isolates causing the obstetric infections studied potentially come. The isolates were also screened for 5 specific virulence markers for extraintestinal pathogenic *E. coli* (ExPEC) or non-ExPEC classification (7). The PCR conditions used were 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, with the corresponding annealing temperature (55 to 63°C) for 30 s, 72°C for 2 min, and a final elongation cycle of 72°C for 5 min. The samples were run in 1.5% agarose gels and stained with SYBR Safe DNA gel stain (Invitrogen, Spain). The *E. coli* phylogenetic group was determined using the 3-locus PCR-based method described previously (8). In order to determine if any isolate belonged to sequence type 131 (ST131), serotype O25b was identified in the collection, according to the methodology proposed by Clermont et al. (9), and the multilocus sequence typing (MLST) methodology was carried out with these isolates using the University of Warwick database for assigning sequence types (ST).

Statistical analysis was performed using Stata version 13.1 (Stata Corp., TX, USA). *P* values of <0.05 were accepted as significant, and statistical correction for multiple comparisons was applied.

Twenty isolates (26%) were resistant to three or more antimicrobial classes, presenting a multidrug-resistant (MDR) phenotype. Sixty-three percent of all the isolates were resistant to ampicillin, whereas only 13% were resistant to amoxicillin-clavulanic

Received 20 February 2015 **Returned for modification** 20 February 2015
Accepted 23 February 2015

Accepted manuscript posted online 4 March 2015

Citation Guiral E, Sáez-López E, Bosch J, Goncé A, López M, Sanz S, Vila J, Soto SM. 2015. Antimicrobial resistance and virulence characterization among *Escherichia coli* clinical isolates causing severe obstetric infections in pregnant women. J Clin Microbiol 53:1745–1747. doi:10.1128/JCM.00487-15.

Editor: R. Patel

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doi:10.1128/JCM.00487-15

TABLE 1 Resistance to antimicrobial agents in *E. coli* isolates, according to the clinical features

Antimicrobial agent	No. (%) resistant in isolate group			
	Non-sepsis causing ($n = 22$)	Sepsis causing ($n = 56$)	Total ($n = 78$)	*P* value
Ampicillin	13 (59)	36 (64)	49 (63)	0.6692[a]
Amoxicillin-clavulanic acid	3 (14)	7 (12)	10 (13)	1.0000[b]
Cefazolin	3 (14)	14 (25)	17 (22)	0.3680[b]
Cefuroxime	1 (5)	2 (4)	3 (4)	1.0000[b]
Cefoxitin	0 (0)	1 (2)	1 (1)	1.0000[b]
Cefotaxime	1 (5)	1 (2)	2 (3)	0.4872[b]
Ceftazidime	0 (0)	0 (0)	0 (0)	
Imipenem	0 (0)	0 (0)	0 (0)	
Piperacillin-tazobactam	0 (0)	1 (2)	1 (1)	1.0000[b]
Nalidixic acid	5 (23)	9 (16)	14 (18)	0.5216[b]
Ciprofloxacin	0 (0)	2 (4)	2 (3)	1.0000[b]
Chloramphenicol	2 (9)	5 (9)	7 (9)	1.0000[b]
Gentamicin	0 (0)	3 (5)	3 (4)	0.5547[b]
Amikacin	0 (0)	1 (2)	1 (1)	1.0000[b]
Kanamycin	1 (5)	5 (9)	6 (8)	0.6697[b]
Tetracycline	7 (32)	20 (36)	27 (35)	0.7448[a]
Trimethoprim-sulfamethoxazole	6 (27)	16 (29)	22 (28)	0.9087[a]

[a] Chi-square test.
[b] Fisher's exact test.

acid. Most of the isolates were susceptible to second- and third-generation cephalosporins, imipenem, aminoglycosides, ciprofloxacin, and chloramphenicol, with higher rates of resistance for tetracycline, trimethoprim-sulfamethoxazole, cefazolin, and nalidixic acid. The isolates causing sepsis had a lower prevalence of resistance to nalidixic acid, with a higher percentage of resistance to cefazolin being observed (Table 1).

The most prevalent VFG found among the isolates were adhesion related, with prevalences between 56 and 86%. The isolates harboring the greatest number of VFG were those causing sepsis, with a significantly higher percentages of *hlyA*, *cnf1*, *papA*, *iha*, *fyuA*, or *papG*II, all of them contained in pathogenicity islands. Regarding virulence factors related to iron recruitment, the *iutA* gene was found significantly more frequently in IAI-causing isolates ($P = 0.0001$), whereas the *iroN* gene was the most common in sepsis-causing isolates ($P = 0.0284$). A multivariate analysis of VFG showed the presence of the *fimA*, *iucC*, *iroN*, *iutA*, *iha*, and *hra* genes as being independent predictors of sepsis-causing isolates (Table 2). Seventy-eight percent of the isolates (with no significant differences between the sepsis- and non-sepsis-causing isolates) were classified as ExPEC according to the virulence markers harbored, and only two of these isolates belonged to ST131.

An analysis of the presence of each VFG among the resistance profiles of the isolates to each of the antimicrobial agents tested was carried out, showing that susceptible isolates had a higher carriage of VFG.

The phenotypic results of antimicrobial resistance observed in the present study indicated high levels of ampicillin-resistant isolates in the collection, in accordance with those found in *E. coli* isolates causing neonatal sepsis and in extraintestinal *E. coli* in general (10). On the other hand, the low rates of resistance to amoxicillin-clavulanic acid and second- and third-generation cephalosporins observed in the present study are in contrast with the increasing appearance of strains carrying extended-spectrum

TABLE 2 Prevalence of virulence factor genes according to the clinical features[a]

Virulence factor	No. (%) with VFG in isolate group				Univariate analysis			Multivariate analysis		
	Non-sepsis causing ($n = 22$)	Sepsis causing ($n = 56$)	Total ($n = 78$)	*P* value	Odds ratio[d]	95% confidence interval	*P* value	Odds ratio[d]	95% confidence interval	*P* value
hlyA	**5 (23)**	**28 (50)**	**33 (42)**	**0.0282**[b]	**3.40**	**(1.10, 10.49)**	**0.0332**			
cnf1	**2 (9)**	**19 (34)**	**21 (27)**	**0.0261**[b]	**5.14**	**(1.08, 24.32)**	**0.0392**			
sat1	9 (41)	23 (41)	32 (41)	0.9895[b]	1.01	(0.37, 2.74)	0.9895			
fimA	17 (77)	50 (89)	67 (86)	0.2757[c]	2.45	(0.66, 9.07)	0.1792	**32.20**	**(1.28, 809.78)**	**0.0349**
papA	**8 (36)**	**40 (71)**	**48 (62)**	**0.0042**[b]	**4.37**	**(1.54, 12.43)**	**0.0056**			
papC	11 (50)	33 (59)	44 (56)	0.4742[b]	1.43	(0.53, 3.86)	0.4752			
papEF	12 (55)	38 (68)	50 (64)	0.2701[b]	1.76	(0.64, 4.83)	0.2727			
*papG*I	0 (0)	0 (0)	0 (0)		1.00					
*papG*II	**8 (36)**	**42 (75)**	**50 (64)**	**0.0014**[b]	**5.25**	**(1.82, 15.13)**	**0.0021**			
*papG*III	9 (41)	14 (25)	23 (29)	0.1656[b]	0.48	(0.17, 1.37)	0.1697			
prs	15 (68)	36 (64)	51 (65)	0.7448[b]	0.84	(0.29, 2.40)	0.7450			
fyuA	**4 (18)**	**29 (52)**	**33 (42)**	**0.0069**[b]	**4.83**	**(1.45, 16.10)**	**0.0103**			
hra	8 (36)	11 (20)	19 (24)	0.1216[b]	0.43	(0.14, 1.27)	0.1270	**0.13**	**(0.02, 0.96)**	**0.0452**
sfa	5 (23)	18 (32)	23 (29)	0.4118[b]	1.61	(0.51, 5.06)	0.4142			
ibeA	5 (23)	9 (16)	14 (18)	0.5216[c]	0.65	(0.19, 2.22)	0.4926			
iucC	14 (64)	44 (79)	58 (74)	0.1740[b]	2.10	(0.71, 6.16)	0.1787	**53.38**	**(2.31, 1,233.37)**	**0.0130**
iutA	**15 (68)**	**10 (18)**	**25 (32)**	**<0.0001**[b]	**0.10**	**(0.03, 0.31)**	**0.0001**	**0.01**	**(0.00, 0.13)**	**0.0016**
iha	**4 (18)**	**26 (46)**	**30 (38)**	**0.0210**[b]	**3.90**	**(1.17, 13.00)**	**0.0267**	**20.61**	**(1.77, 240.12)**	**0.0157**
iroN	**8 (36)**	**36 (64)**	**44 (56)**	**0.0252**[b]	**3.15**	**(1.13, 8.79)**	**0.0284**	**6.47**	**(1.30, 32.15)**	**0.0225**
ag43	10 (45)	27 (48)	37 (47)	0.8261[b]	1.12	(0.42, 3.01)	0.8262			
malX	**9 (41)**	**38 (68)**	**47 (60)**	**0.0286**[b]	**3.05**	**(1.10, 8.44)**	**0.0319**			

[a] Statistically significant results are in bold type.
[b] Chi-square test.
[c] Fisher's exact test.
[d] Odds ratio for present versus absent.

β-lactamases (ESBLs) in the last years and causing infections from other sources, suggesting that the implementation of these antimicrobial agents as first-line therapy in these types of infections is correct (11). Nonetheless, the treatment administered should still be chosen depending on the rates of resistance in each hospital to gentamicin and cephalosporins in *E. coli* causing obstetric infections, as well as the prophylaxis or previous treatment with these antimicrobial agents, which have led to the development of resistant bacteria.

Regarding the VFG present in *E. coli* involved in the obstetric infections studied, it was found that adhesins and fimbriae may play an important role in the development of these infections, allowing the bacteria to colonize different environments. The higher prevalence of *hlyA* and *cnf1* among the isolates causing sepsis might be related to the tissue damage involved with these infections. Concerning the iron recruitment systems, the yersiniabactin receptor encoded by *fyuA* and the genes encoding the siderophore receptors Iha and IroN were also more prevalent among the isolates causing sepsis, due to the need for UPEC to capture iron from the host within the hostile environment of urine. These virulence factors have been largely described as characteristic of UPEC (12). On the other hand, *iutA* was more frequently found in isolates causing IAI, elucidating a high adaptation capacity according to the particular microenvironmnent colonized.

A specific relationship was found between tetracycline-resistant isolates and the lower presence of several VFG included in pathogenicity islands (PAIs), similar to the previously described relationship between the acquisition of quinolone resistance and the loss of VFG (13).

In conclusion, to date, *E. coli* isolates causing obstetric infections present similar rates of antimicrobial resistance to those described for extraintestinal *E. coli* infections, except for a lower prevalence of resistance to third-generation cephalosporins, thereby those not carrying ESBLs. These results demonstrate that the administration of antimicrobials in our hospital is correct. However, it is important to establish surveillance networks specific for these kinds of infections in order to adapt stewardship programs when appropriate.

ACKNOWLEDGMENTS

This work was supported by the Spanish Network for Research in Infectious Diseases (grant REIPI RD06/0008), from Instituto de Salud Carlos III (ISCIII), by the Instituto de Salud Carlos III (grant FIS 10/01579), cofinanced by the European Regional Development Fund (ERDF) 'A way to achieve Europe.' It was also funded by the grant for research groups support (SGR09-1256) of the Agència de Gestió d'Ajuts Universitaris i de Recerca from Generalitat de Catalunya and by European Commission funding (TROCAR contract HEALTH-F3-2008-223031). Sara M. Soto has a fellowship from program I3 of the ISCIII.

REFERENCES

1. **Wait RB.** 1984. Urinary tract infection during pregnancy. Asymptomatic bacteriuria, acute cystitis, and acute pyelonephritis. Postgrad Med **75:**153–157, 161.
2. **Andreu A, Sanfeliu I, Vinas L, Barranco M, Bosch J, Dopico E, Guardia C, Juncosa T, Lite J, Matas L, Sanchez F, Sierr M, Grupo de Microbiólogos pare el Esduio de las Infecciones de Transmisión Vertical, Societat Catalana de Malaties Infeccioses I Microbiologia Clínica.** 2003. Decreasing incidence of perinatal group B streptococcal disease (Barcelona 1994–2002). Relation with hospital prevention policies. Enferm Infecc Microbiol Clin **21:**174–179. (In Spanish.) http://dx.doi.org/10.1157/13045447.
3. **Connolly A, Thorp JM, Jr.** 1999. Urinary tract infections in pregnancy. Urol Clin North Am **26:**779–787. http://dx.doi.org/10.1016/S0094-0143(05)70218-4.
4. **Guiral E, Bosch J, Vila J, Soto SM.** 2011. Prevalence of *Escherichia coli* among samples collected from the genital tract in pregnant and nonpregnant women: relationship with virulence. FEMS Microbiol Lett **314:**170–173. http://dx.doi.org/10.1111/j.1574-6968.2010.02160.x.
5. **Burke C.** 2009. Perinatal sepsis. J Perinat Neonatal Nurs **23:**42–51. http://dx.doi.org/10.1097/JPN.0b013e31819614ed.
6. **Clinical and Laboratory Standards Institute.** 2008. Performance standards for antimicrobial susceptibility testing: 17th informational supplement. CLSI document M100-S17. Clinical and Laboratory Standards Institute, Wayne, PA.
7. **Johnson JR, Stell AL.** 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis **181:**261–272. http://dx.doi.org/10.1086/315217.
8. **Clermont O, Bonacorsi S, Bingen E.** 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol **66:**4555–4558. http://dx.doi.org/10.1128/AEM.66.10.4555-4558.2000.
9. **Clermont O, Dhanji H, Upton M, Gibreel T, Fox A, Boyd D, Mulvey MR, Nordmann P, Ruppe E, Sarthou JL, Frank T, Vimont S, Arlet G, Branger C, Woodford N, Denamur E.** 2009. Rapid detection of the O25b-ST131 clone of *Escherichia coli* encompassing the CTX-M-15-producing strains. J Antimicrob Chemother **64:**274–277. http://dx.doi.org/10.1093/jac/dkp194.
10. **Guiral E, Bosch J, Vila J, Soto SM.** 2012. Antimicrobial resistance of *Escherichia coli* strains causing neonatal sepsis between 1998 and 2008. Chemotherapy **58:**123–128. http://dx.doi.org/10.1159/000337062.
11. **García-Hernández AM, García-Vázquez E, Hernández-Torres A, Ruiz J, Yague G, Herrero JA, Gomez J.** 2011. Bacteraemia due to *Escherichia coli* producing extended-spectrum beta-lactamases (ESBL): clinical relevance and today's insights. Rev Esp Quimioter **24:**57–66. (In Spanish.)
12. **Porcheron G, Garenaux A, Proulx J, Sabri M, Dozois CM.** 2013. Iron, copper, zinc, and manganese transport and regulation in pathogenic *Enterobacteria*: correlations between strains, site of infection and the relative importance of the different metal transport systems for virulence. Front Cell Infect Microbiol **3:**90. http://dx.doi.org/10.3389/fcimb.2013.00090.
13. **Soto SM, Jimenez de Anta MT, Vila J.** 2006. Quinolones induce partial or total loss of pathogenicity islands in uropathogenic *Escherichia coli* by SOS-dependent or -independent pathways, respectively. Antimicrob Agents Chemother **50:**649–653. http://dx.doi.org/10.1128/AAC.50.2.649-653.2006.

PAPER 5:

Antimicrobial resistance of *Escherichia coli* strains causing neonatal sepsis between 1998 and 2008

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Journal, volume (issue): pages, date of publication:

Chemotherapy, 58 (2): 123-8, 2012.

Impact Factor: 2.066 – Q3 (2012)

Hypothesis:

After the introduction of preventive measures against Group B *streptococci or Streptococcus agalactiae* (GBS) causing neonatal sepsis, the prevalence of *E. coli* causing this infection in developed countries is increasing. For this reason, it is important to characterise the resistance phenotype of these isolates, causing early-onset neonatal sepsis (EONS) or late-onset neonatal sepsis (LONS) in order to duly prevent or treat these infections.

Objectives:

Evaluate the antimicrobial resistance phenotype of *E. coli* isolates causing EONS and LONS and their evolution.

Material and methods:

Sixty-one *E. coli* isolates causing neonatal sepsis in neonates collected at the Hospital Clinic of Barcelona between 1998 and 2008 were analysed.

The minimum inhibitory concentrations were determined using the MicroScan-Negative MIC Panel Type 37 (NM37, Siemens).

Antimicrobial resistance genes were detected by PCR with gene-specific primers with further DNA Sanger sequencing.

Results:

No statistically significant differences in resistance profiles were found between strains causing EONS and LONS. An increase in resistance to all the antimicrobial agents studied was observed in the period 2000–2008 in comparison with the 1985–

1999 period. The resistance genotype of the isolates was analysed, and two isolates were found to carry a *bla*$_{CTX-M}$ enzyme.
Conclusions: The increase in ampicillin and gentamicin resistance (antimicrobials used as first choice to treat neonatal sepsis) requires a change in the treatment guidelines of neonatal sepsis, suggesting cephalosporins as an alternative.

Microbiology

Chemotherapy

Chemotherapy 2012;58:123–128
DOI: 10.1159/000337062

Received: August 9, 2011
Accepted after revision: February 5, 2012
Published online: May 10, 2012

Antimicrobial Resistance of *Escherichia coli* Strains Causing Neonatal Sepsis between 1998 and 2008

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Key Words
Antimicrobial resistance mechanisms · Neonatal sepsis · *Escherichia coli*

Abstract
Background: Bloodstream infections are a significant cause of neonatal morbidity and death. An increase in the incidence of early neonatal sepsis due to *Escherichia coli* has been reported. The objective was to evaluate the antimicrobial resistance of *E. coli* strains causing early-onset neonatal sepsis (EONS) and late-onset neonatal sepsis (LONS) and their evolution. ***Methods:*** *E. coli* strains from EONS and hospital-acquired LONS collected at the Hospital Clinic of Barcelona were included in the study. ***Results:*** No statistically significant differences in resistance profiles were found between strains causing EONS and LONS. An increase in the resistance to all the antimicrobial agents studied was observed for the period 2000–2008 in comparison with the 1985–1999 period, with the increase in resistance to gentamicin, piperacillin and tobramycin being statistically significant. Two strains carried the $bla_{\text{CTX-M}}$ genes ($bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$). ***Conclusion:*** The increase in ampicillin and gentamicin resistance makes a change in the treatment of neonates necessary.

Introduction

Despite careful hygiene and powerful broad-spectrum antibiotic treatment, neonatal septicemia remains an unsolved problem associated with high mortality [1]. Neonatal sepsis may be subdivided into early-onset neonatal sepsis (EONS) and late-onset neonatal sepsis (LONS). The former is caused by microorganisms acquired from the mother before or during birth, is vertically transmitted and perinatally acquired. LONS is infection presenting 4 or more days after birth and is generally caused by environmentally or nosocomially acquired microorganisms or by horizontal transmission, rather than from the mother [2]. Group B *Streptococcus* (GBS) is considered to be the most common microorganism causing EONS, but there have been reports of an increase in the incidence of early neonatal sepsis due to *Escherichia coli,* especially in premature or very low-birth-weight neonates [3, 4]. Stoll et al. [3] studied early onset neonatal sepsis from 1998 to 2000 and found a reduction in group B streptococcal sepsis (from 5.9 to 1.7 per 1,000 live births of very low-weight infants, $p < 0.001$) and an increase in *E. coli* (from 3.2 to 6.8 per 1,000 live births, $p = 0.004$). The use of maternal intrapartum antibiotics for the prevention of GBS infection in the neonate is becoming increasingly common in

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developed countries [5], but most of these antibiotics are administered in a term pregnancy. Maternal prophylactic antibiotics are also used in cases of preterm premature rupture of membranes and if chorioamnionitis is suspected [6]. Ampicillin and an aminoglycoside are the two antibiotics recommended for initial empiric therapy in the case of neonates with suspected bacterial sepsis and/or meningitis [7, 8], but may no longer be effective in treating many newborns with sepsis due to increased ampicillin resistance among EONS cases occurring in low-birth-weight and premature neonates [9]. Pathogens causing neonatal infections and their antimicrobial profiles may change over time and differ between countries [10].

The objective of the present work was to evaluate the antimicrobial resistance of *E. coli* strains causing EONS and LONS as well as their evolution.

Materials and Methods

Bacteria

Sixty-one *E. coli* strains collected at the Hospital Clinic of Barcelona between 1995 and 2008 (27 from EONS and 34 from intrahospital LONS) were included in the study.

Antimicrobial Resistances

Minimal inhibitory concentrations were determined using the MicroScan-Negative MIC Panel Type 37 (NM37, Siemens). The antimicrobial agents tested were: amikacin (Ak), amoxicillin/K clavulanate (Aug), ampicillin (Am), ampicillin/sulbactam (A/S), aztreonam (Azt), cefazolin (Cfz), cefepime (Cpe), cefotaxime (Cft), cefotaxime/K clavulanate (Cft/CA), cefoxitin (Cfx), cefpodoxime (Cpd), ceftazidime (Caz), ceftazidime/K clavulanate (Caz/CA), cefuroxime (Crm), ciprofloxacin (Cp), chloramphenicol (C), colistin (Cl), ertapenem (Etp), fosfomycin (Fos), gentamicin (Gm), imipenem (Imp), levofloxacin (Lvx), meropenem (Mer), mezlocillin (Mz), moxifloxacin (Mxf), nitrofurantoin (Fd), norfloxacin (Nxn), piperacillin/tazobactam (P/T), piperacillin (Pi), tetracycline (Te), tigecycline (Tgc), tobramycin (To), and trimethoprim/sulfamethoxazole (T/S). The results were interpreted following CSLI guidelines [11] and *E. coli* ATCC25922 strain was used as the control.

Detection of Resistance Genes

We detected the following selected antimicrobial resistance genes: $bla_{\text{TEM1-like}}$, $bla_{\text{OXA1-like}}$, bla_{PSE1} in all ampicillin-resistant strains; $bla_{CTX\text{-}M}$ in ceftiofur-resistant strains; *qnrA, B, C, D, S* in all isolates; *aac(3)-II, aac(3)-IV* and *aac(6)-Ib* in gentamicin-resistant strains; *catA*, *cmlA* and *floR* in choramphenicol-resistant strains; *dfrI*-like, *dfrA7-dfrA17*, *dfrA12*, *dfrA5-dfrA14* and *dfrA17* in trimethroprim-resistant strains; *sul1, sul2* and *sul3* in sulfadiazine (Sd)-resistant strains, and *tet(A), tet(B), tet(C), tet(D), tet(E)* and *tet(G)* in tetracycline-resistant strains [12–15]. Mutations in the quinolone resistance-determining region of the genes encoding the essential enzymes DNA gyrase and topoisomerase IV are the primary cause of clinically relevant levels of fluoroquinolone resistance in both gram-negative and gram-positive microorganisms [16]. Detection of these mutations and class 1 integrons were carried out by specific PCR amplification and sequencing as previously described [13]. The sequences obtained were compared to those registered in GenBank.

Statistical Analysis

Data were statistically analyzed using the Fisher exact test due to the small sample size.

Results

Among the *E. coli* strains causing EONS, 78% were collected from premature neonates (birth before 37 weeks of pregnancy and weighing less than 2.5 kg). All were born after prolonged premature membrane rupture and/or chorioamnionitis. Antimicrobial resistance was tested in 61 *E. coli* strains collected from neonatal sepsis. No statistically significant differences were found between resistance profiles among strains causing EONS and those causing LONS. Strains from EONS tended to be more resistant to the ampicillin, mezlocillin and piperacillin β-lactamics and to gentamicin than strains from LONS. On the other hand, strains causing LONS tended to be more resistant to chloramphenicol, moxifloxacin and tetracycline (table 1).

The evolution of the resistance was studied and, for this purpose, the *E. coli* strains causing neonatal sepsis, except those collected from infants >28 days of age, were divided into two groups depending on the year of isolation (1985–1999 and 2000–2008). An increase in the resistance to all the antimicrobial agents studied was observed in the second period of time. Moreover, the increase in resistance to gentamicin (0–26%), piperacillin (40–78%) and tobramycin (4–30%) between the two groups was statistically significant ($p = 0.01$, 0.01 and 0.02, respectively; fig. 1).

All ampicillin-resistant strains (42 isolates) were tested by PCR for the presence or absence of three different families of *bla*-genes. $Bla_{TEM1\text{-like}}$ genes were found in 31 isolates (74% of the ampicillin-resistant strains). Of these, 19 (70%) were collected from EONS and only 16 (47%) were from LONS. The β-lactam resistance phenotype of two strains (ampicillin-aztreonam-cefazolin-cefepime-cefotaxime-cefpodoxime-ceftazidime-cefuroxime), one from EONS and the other from LONS, indicated the presence of extended-spectrum β-lactamase enzymes. The $bla_{\text{CTX-M-15}}$ gene was detected in the strain causing EONS and the $bla_{\text{CTX-M-14}}$ gene was detected in the strain caus-

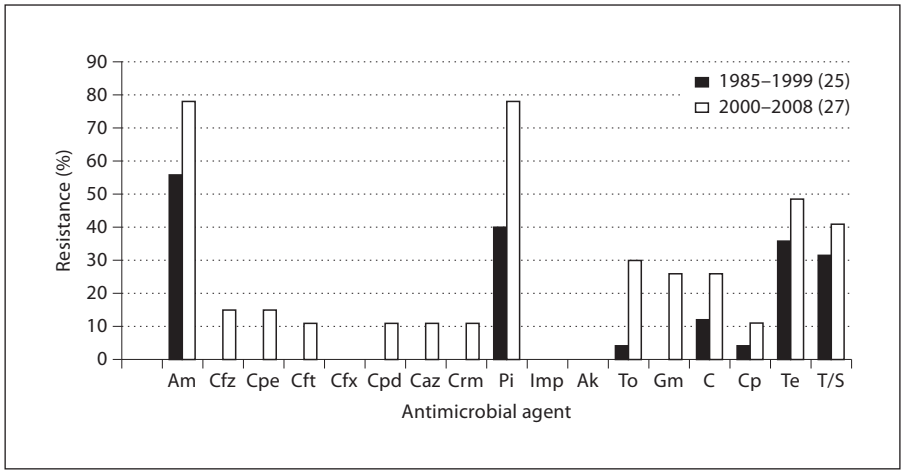

Fig. 1. Evolution of the resistance among *E. coli* causing neonatal sepsis.

ing LONS. In addition, one strain causing LONS presented the AmpC enzyme although it was not overexpressed (table 2).

The *aac(3)-II* gene was present in 75% (6/8) of the gentamicin-resistant strains, making it the most frequent among the two strain groups (EONS and LONS; table 2). Six determinants of tetracycline resistance were studied *(tetA, B, C, D, E* and *tetG)*. The *tetA* gene was more frequently found among the strains from LONS (35%) than from EONS (11%). On the other hand, the *tetB* gene was more frequent among strains from EONS (19%) than from LONS (9%). The *tetC* and *tetD* genes were only found in two strains causing LONS. The *tetE* gene was found in one strain causing EONS, and the *tetG* gene was found in the same proportion of strains among the two groups (table 2).

Among the trimethoprim-resistant strains, the *dfrA1* gene was the most frequently found (10 strains, 50%), followed by *dfrA17* (3 strains) and *dfrA12* (2 strains). No tetracycline resistance determinants were detected in 5 strains.

It is important to note the high percentage of strains that presented the *sul-II* gene (81%, 18 strains) among the sulfadiazine-resistant strains (22 strains), in contrast with the *sul-I* gene (7%, 2 strains from EONS) and *sul-III* (4%, 1 strain from EONS).

The quinolone resistance-determining regions of the *gyrA* and *parC* genes of all isolates were analyzed by PCR amplification/sequencing. All susceptible strains presented the same amino acid codons in both the *gyrA* and *parC* genes as the *E. coli* K12 strain MG1655 (GenBank accession numbers AE000312 and AE000384). The only Cp-resistant *E. coli* strain from EONS presented a single

Table 1. Percentages of resistance to different antimicrobial agents

Antimicrobial agent	EONS (n = 27)	Intrahospital LONS (n = 34)
β-Lactamics		
Amoxicillin/K clavulanate, Aug	6 (22%)	9 (26%)
Ampicillin/sulbactam, A/S	16 (59%)	20 (59%)
Ampicillin, Am	21 (78%)	21 (62%)
Aztreonam, Azt	2 (7%)	2 (6%)
Cefazolin, Cfz	4 (15%)	4 (12%)
Cefepime, Cpe	2 (7%)	1 (3%)
Cefotaxime, Cft	2 (7%)	2 (6%)
Cefoxitin, Cfx	–	1 (3%)
Cefpodoxime, Cpd	2 (7%)	2 (6%)
Ceftazidime, Caz	2 (7%)	2 (6%)
Cefuroxime, Crm	2 (7%)	2 (6%)
Mezlocillin, Mz	18 (67%)	20 (59%)
Piperacillin/tazobactam, P/T	2 (7%)	2 (6%)
Piperacillin, Pi	18 (67%)	20 (59%)
Aminoglycosides		
Gentamicin, Gm	5 (19%)	3 (9%)
Tobramycin, To	5 (19%)	4 (12%)
Quinolones		
Ciprofloxacin, Cp	1 (4%)	3 (9%)
Levofloxacin, Lvx	1 (4%)	1 (3%)
Moxifloxacin, Mxf	1 (4%)	4 (12%)
Others		
Chloramphenicol, C	5 (19%)	10 (29%)
Tetracycline, Te	10 (37%)	19 (56%)
Trimethoprim/sulfamethoxazole, T/S	9 (33%)	11 (32%)

Table 2. Resistance genes found in *E. coli* strains causing EONS and LONS

Resistance/gene	EONS (n = 27)	LONS (n = 34)
Am-resistance		
teml	19 (70%)	16 (47%)
shvl	1 (4%)	0
carb	0	1 (3%)
CTX-M-14	0	1 (3%)
CTX-M-15	1 (4%)	0
AmpC	0	1 (3%)
Unknown	0	2 (6%)
C-resistance		
catA2	5 (19%)	6 (18%)
cmlA	0	2 (6%)
floR	0	1 (3%)
Unknown	0	2 (6%)
Gm-resistance		
aac(3)-IV	2 (7%)	0
aac(3)-II	3 (11%)	3 (9%)
Te-resistance		
tetA	3 (11%)	12 (35%)
tetB	5 (19%)	3 (9%)
tetC	0	2 (6%)
tetD	0	2 (6%)
tetE	1 (4%)	0
tetG	1 (4%)	2 (6%)
T-resistance		
dfrAla	4 (15%)	6 (18%)
dfrB	0	0
dfrA12	0	2 (6%)
dfrA17	2 (7%)	1 (3%)
Unknown	3 (11%)	2 (6%)
Sd-resistance		
sul-l	2 (7%)	0
sul-II	8 (30%)	10 (29%)
sul-III	1 (4%)	0
Unknown	0	1 (3%)
Quinolone resistance		
qnrA	0	0
qnrB	0	0
qnrC	0	0
qnrD	0	0
qnrS	0	1 (3%)
qepA	0	0
acc(6)-Ib-cr	0	0
Integrons	5 (19%)	8 (24%)

mutation in *gyrA* (Asp87→Lys87) and two mutations in *parC* (Ser80→Ile80 and Glu84→Val84). Two strains from LONS presented only a single mutation in *gyrA* (Ser83→Leu83); however, the third Cp-resistant strain from this group presented two mutations in *gyrA* (Ser83→Leu83 and Asp87→Lys87) and one single mutation in *parC* (Ser80→Ile80).

One strain causing LONS carried the *qnr*S1 gene. This strain showed an MIC of ≤1 mg/l for ciprofloxacin, an MIC of ≤32 mg/l for nalidixic acid and no presented mutations in *gyrA* or *parC* genes. No *qnrA, B, C* or *D* genes were detected. The *aac-(6′)-Ib* gene with the *'cr'* mutation that confers an increase in quinolone resistance and the *qep*A gene were not found among the strains.

To determine the prevalence of class 1 integrons among the *E. coli* strains, all resistant strains were tested for the presence or absence of class 1 and class 2 integrons by PCR. Class 1 integrons were present in 13 strains. The analysis of the variable region of these integrons by PCR sequencing using the 5′CS/3′CS primers allowed four different integron types to be defined. Integrons generating amplicons of about 1,000 bp and carrying the *aadA1* gene cassette were the most frequently found (5 strains: 3 from EONS and 2 from LONS), followed by integrons with variable regions of 700 bp, carrying *dfrA1a* (4 strains from LONS). Other integrons found had variable regions of 1,700 bp with *dfrA17-aadA5* (2 strains from EONS) and 1,600 bp carrying *dfrA1-aadA1a* (2 strains from LONS).

Discussion

Neonatal sepsis is one of the most important causes of infant morbidity and mortality [17]. Bacterial neonatal sepsis remains a significant problem for pediatricians in spite of the prevention of EONS due to *Streptococcus agalactiae* by intrapartum antibiotic prophylaxis in developed countries such as Spain. In fact, these prophylactic policies, which were developed in the 1990s [18, 19], have decreased the incidence of *S. agalactiae* infections (>70% in perinatal invasive GBS disease incidence in the United States) [20]; however, the role of Gram-negative bacteria in newborn infection has gained importance. *E. coli* is a significant cause of mortality among newborns, particularly among those of very low weight [3, 4]. There are few studies on the antimicrobial resistance of *E. coli* strains causing neonatal sepsis.

Ampicillin, gentamicin or cephalosporins are chosen as treatments in newborns with sepsis. In the present study a higher percentage of resistance to ampicillin and gentamicin among strains causing EONS was observed in comparison with those causing LONS. In addition, the percentages of resistance to all the antimicrobial agents studied have increased between 1985 and 2008. A possible explanation for these results may be the use of ampi-

cillin and gentamicin in the treatment of premature preterm membrane rupture and chorioamnionitis, which has led to the selection of more resistant strains.

Several studies have reported a relationship between intrapartum therapy and the presence of ampicillin-resistant *E. coli* in neonates. Joseph et al. [21] observed an increase in the proportion of infections caused by ampicillin-resistant *E. coli* in the period between 1988 and 1993 (67%) compared to the earlier period between 1982 and 1987 (25%) which was concomitant with the fact that in the second period 61% of the mothers received intrapartum ampicillin in contrast with the first period when only 17% received this therapy. Terrone et al. [22], Bizarro et al. [17] and Kunh et al. [23] found a possible association between antenatal antibiotic treatment, prolonged antepartum exposure to ampicillin and infection with ampicillin-resistant *E. coli*. However, there are also several studies that have demonstrated that this relationship does not exist [20]. Friedman et al. [5] postulated that the presence of prolonged rupture of the fetal membranes and an elevated maternal temperature during labor, which is suggestive of chorioamnionitis, are also perinatal variables associated with the emergence of resistant *E. coli* isolates. In addition, a lower gestational age and birth weight are neonatal variables associated with the appearance of these resistant strains.

The percentage of ampicillin-resistant *E. coli* collected from neonates found in the present study is similar to that found in other studies. However, differences in the percentages of Gram-resistant isolates from 3% [3] to 50% [5, 24] among EONS strains and from 0% [19] to 16% [4] among LONS strains seem to be present among the different studies [3, 5, 17, 24]. Quinolone resistance is frequently associated with extended-spectrum cephalosporin resistance in Enterobacteriaceae [25]; however, this association was not found in the present study.

Studies on resistance mechanisms among *E. coli* strains collected from neonatal sepsis are scarce. However, several studies about determinants of resistance among *E. coli* strains collected from children have been reported. Karami et al. [26, 27] studied the occurrence of phenotypic tetracycline and ampicillin resistance and the carriage of resistance genes in intestinal *E. coli* strains obtained from Swedish infants followed over their first year of life. They found that 12% of strains were resistant to tetracycline. In contrast with the present study, *tet*B was the resistance gene most frequently found followed by *tet*A and *tet*C. This group only found 12% of ampicillin resistance among the strains analyzed, with the $bla_{\text{TEM-1}}$ resistance gene being the most frequently found demonstrating ampicillin resistance. This gene was also the most prevalent among our series.

In conclusion, despite the limitation of the study due to the small number of strains, the observation of an increase in ampicillin and gentamicin resistance makes a change in the treatment of neonates necessary. Cephalosporins are one of the antibiotics suggested taking into account the lower percentages of resistance to these antimicrobial agents, despite the recent appearance of *E. coli* strains with extended-spectrum β-lactamase. The increase in *E. coli* ampicillin- and piperacillin-resistant strains causing neonatal sepsis could be due to the selection of resistant strains due to exposure to antibiotics. Further studies are needed to elucidate the role of intrapartum antibiotic prophylaxis in the emergence of resistant strains.

Acknowledgements

This work was supported by the Spanish Network for the Research in Infectious Diseases (REIPI RE06/0008), SGR091256 from the Department d'Universitats, Recerca I Societat de la Informació de la Generalitat de Catalunya, Fondo de Investigaciones Sanitarias (PI10/01579) of Spain, and by funding from the European Community (TROCAR contract HEALTH-F3-2008-223031). Sara M. Soto is the recipient of a contract 'Miguel Servet' (CP05/00140) from the 'Fondo de Investigaciones Sanitarias' of the Spanish Ministry of Health.

References

1 Placzek MM, Whitelaw A: Early and late neonatal septicaemia. Arch Dis Child 1983; 58:728–731.

2 Soto SM, Bosch J, Jimenez de Anta MT, Vila J: Comparative study of virulence traits of *Escherichia coli* clinical isolates causing early and late neonatal sepsis. J Clin Microbiol 2008;46:1123–1125.

3 Stoll BJ, Hansen N, Fanaroff AA, et al: Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. N Engl J Med 2002;347:240–247.

4 Towers CV, Carr MH, Padilla G, Asrat T: Potential consequences of widespread antepartal use of ampicillin. Am J Obstet Gynecol 1998;179:879–993.

5 Friedman S, Shah V, Ohlsson A, Matlow AG: Neonatal *Escherichia coli* infections: concerns regarding resistance to current therapy. Acta Paediatr 2000;89:686–689.

6 Amstey MS: Group B streptococcal prophylaxis. Am J Obstet Gynecol 1994;171:1675.

7 Klein JO: Antimicrobial therapy issues facing pediatricians. Pediatr Infect Dis J 1995; 14:415–419.

8 Wiese G: Treatment of neonatal sepsis with ceftriaxone/gentamicin and with azlocillin/gentamicin: a clinical comparison of efficacy and tolerability. Chemotherapy 1988;34:158–163.
9 Moore MR, Schrag SJ, Schuchat A: Effects of intrapartum antimicrobial prophylaxis for prevention of group-B-streptococcal disease on the incidence and ecology of early-onset neonatal sepsis. Lancet Infect Dis 2003;3:201–213.
10 Vergnano S, Menson E, Kennea N, et al: Neonatal infections in England: the NeonIN surveillance network. Arch Dis Child Fetal Neonatal Ed 2011;96:F9–F14.
11 Clinical and Laboratory Standards Institute: Performance standards for antimicrobial susceptibility testing. 16th informational supplement, M100-S18. Wayne, CLSI, 2008.
12 Perreten V, Boerlin P: A new sulfonamide resistance gene (*sul3*) in *Escherichia coli* is widespread in the pig population of Switzerland. Antimicrob Agents Chemother 2003;47:1169–1172.
13 Guerra B, Junker E, Miko A, Helmuth R, Mendoza MC: Characterization and localization of drug resistance determinants in multidrug-resistant, integron-carrying *Salmonella enterica* serotype Typhimurium strains. Microb Drug Resist 2004;10:83–91.
14 Akinbowale OL, Peng H, Barton MD: Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. J Appl Microbiol 2007;103:2016–2025.
15 Ozgumus OB, Celik-Sevim E, Alpay-Karaoglu S, Sandalli C, Sevim A: Molecular characterization of antibiotic resistant *Escherichia coli* strains isolated from tap and spring waters in a coastal region in Turkey. J Microbiol 2007;45:379–387.
16 Sanfilippo CM, Heske CK, Haas W, Morris TW: Topoisomerase mutations that are associated with high-level resistance to earlier fluoroquinolones in *Staphylococcus aureus* have less effect on the antibacterial activity of besifloxacin. Chemotherapy 2011;57:363–371.
17 Bizzarro MJ, Dembry LM, Baltimore RS, Gallagher PG: Changing patterns in neonatal *Escherichia coli* sepsis and ampicillin resistance in the era of intrapartum antibiotic prophylaxis. Pediatrics 2008;121:689–696.
18 American Academy of Pediatrics Committee on Infectious Diseases and Committee on Fetus and Newborn: Guidelines for prevention of group B streptococcal (GBS) infection by chemoprophylaxis. Pediatrics 1992;90:775–778.
19 Centers for Disease Control and Prevention: Prevention of perinatal group B streptococcal disease: a public health perspective. MMWR Recomm Rep 1996;45:1–24.
20 Schrag SJ, Stoll BJ: Early-onset neonatal sepsis in the era of widespread intrapartum chemoprophylaxis. Pediatr Infect Dis J 2006;25:939–940.
21 Joseph TA, Pyati SP, Jacobs N: Neonatal early-onset *Escherichia coli* disease: the effect of intrapartum ampicillin. Arch Pediatr Adolesc Med 1998;152:35–40.
22 Terrone DA, Rinehart BK, Einstein MH, Britt LB, Martin JN Jr, Perry KG: Neonatal sepsis and death caused by resistant *Escherichia coli*: possible consequences of extended maternal ampicillin administration. Am J Obstet Gynecol 1999;180:1345–1348.
23 Kuhn P, Dheu C, Bolender C, et al: Incidence and distribution of pathogens in early-onset neonatal sepsis in the era of antenatal antibiotics. Paediatr Perinat Epidemiol 2010;24:479–487.
24 Thaver D, Ali SA, Zaidi AK: Antimicrobial resistance among neonatal pathogens in developing countries. Pediatr Infect Dis J 2009;28:S19–21.
25 Seo MR, Park YS, Pai H: Characteristics of plasmid-mediated quinolone resistance genes in extended-spectrum cephalosporin-resistant isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Korea. Chemotherapy 2010;56:46–53.
26 Karami N, Nowrouzian F, Adlerberth I, Wold AE: Tetracycline resistance in *Escherichia coli* and persistance in the infantile colonic microbiota. Antimicrob Agents Chemother 2006;50:156–161.
27 Karami N, Hannoun C, Adlerberth I, Wold AE: Colonization dynamics of ampicillin-resistant *Escherichia coli* in the infantile colonic microbiota. J Antimicrob Chemother 2008;62:703–708.

PAPER 6:

Epidemiology and molecular characterization of multidrug-resistant *Escherichia coli* isolates harboring *bla*$_{CTX-M}$ group 1 extended-spectrum β-lactamases causing bacteremia and urinary tract infection in Manhiça, Mozambique

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Journal, volume (issue): pages, date of publication:

Infection and Drug Resistance, 2018 (11): 927-936, 2018 July.

Impact Factor: 3.443 – Q2 (2017)

Hypothesis:

The emergence and spread of extended-spectrum β-lactamases (ESBLs), especially CTX-M, is an important public health problem with serious implications for low-income countries where second-line treatment is often unavailable. Knowledge of the local prevalence of ESBL is critical to define appropriate empirical therapeutic strategies for multidrug resistant (MDR) organisms, and data from the Sub-Saharan African countries is scarce.

Objectives:

Characterise at an epidemiological and molecular level the resistance phenotype of *Escherichia coli* isolates carrying ESBL *bla*$_{CTX-M}$ group 1 from patients with bacteraemia and urinary tract infection (UTI) in Manhiça, Mozambique.

Material and methods:

151 *E. coli* isolates from bacteraemia and UTI in children attending the Manhiça District Hospital were screened for β-lactamases by antimicrobial susceptibility testing, PCR and sequencing. Isolates carrying CTX-M group 1 β-lactamases were further studied. Resistance to other antibiotic families was determined by

phenotypic and genotypic methods, and the location of the *bla*$_{CTX-M}$ gene as well as the epidemiology of the isolates and extensive plasmid characterisation were performed.

Results:

12 of the isolates carrying a CTX-M group 1 ESBL were further characterised. The CTX-M-15 enzyme was the most frequently detected (75% of the total isolates characterised). The *bla*$_{CTX-M}$ gene was located in different plasmids belonging to different incompatibility groups and was found in non-epidemiologically related isolates, indicating the high capacity of this resistance determinant to spread widely.

Conclusions:

The data obtained suggest the presence of a co-selection of third-generation cephalosporin-resistant determinants in the study area despite limited access to these antibiotics. This highlights the importance of continuous surveillance of antimicrobial resistance of both genetic elements of resistance and resistant isolates in order to monitor the emergence and trends of ESBL-producing isolates to promote adequate therapeutic strategies for the management of MDR bacterial infections.

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ORIGINAL RESEARCH

Epidemiology and molecular characterization of multidrug-resistant *Escherichia coli* isolates harboring bla_{CTX-M} group 1 extended-spectrum β-lactamases causing bacteremia and urinary tract infection in Manhiça, Mozambique

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Background: The emergence and spread of extended-spectrum β-lactamases (ESBLs), especially CTX-M, is an important public health problem with serious implications for low-income countries where second-line treatment is often unavailable. Knowledge of the local prevalence of ESBL is critical to define appropriate empirical therapeutic strategies for multidrug-resistant (MDR) organisms. This study aimed to assess and characterize the presence of ESBL and especially CTX-M-producing *Escherichia coli* MDR isolates from patients with urinary tract infections (UTIs) and bacteremia in a rural hospital in Mozambique.

Materials and methods: One hundred and fifty-one *E. coli* isolates from bacteremia and UTI in children were screened for CTX-M, TEM, SHV and OXA β-lactamases by polymerase chain reaction and sequencing. Isolates carrying CTX-M group 1 β-lactamases were further studied. The resistance to other antibiotic families was determined by phenotypic and genotypic methods, the location of the bla_{CTX-M} gene and the epidemiology of the isolates were studied, and extensive plasmid characterization was performed.

Results: Approximately 11% (17/151) of *E. coli* isolates causing bacteremia and UTI were ESBL producers. CTX-M-15 was the most frequently detected ESBL, accounting for 75% of the total isolates characterized. The bla_{CTX-M} gene is located in different plasmids belonging to different incompatibility groups and can be found in non-epidemiologically related isolates, indicating the high capacity of this resistance determinant to spread widely.

Conclusion: Our data suggest the presence of a co-selection of third-generation cephalosporin-resistant determinants in the study area despite limited access to these antibiotics. This highlights the importance of continuous surveillance of antimicrobial resistance of both genetic elements of resistance and resistant isolates in order to monitor the emergence and trends of ESBL-producing isolates to promote adequate therapeutic strategies for the management of MDR bacterial infections.

Keywords: CTX-M-15, multidrug-resistance, Enterobacteriaceae, resistance determinant location

Introduction

Infections caused by members of the Enterobacteriaceae family are among the major causes of hospital admission and associated morbidity and mortality in children,

particularly in Africa.[1,2] Infections caused by these microorganisms in low- and middle-income countries (LMIC) have been successfully treated with the inexpensive antibiotics available. Nevertheless, with the widespread development of multidrug-resistant (MDR) strains, the usefulness of the early effective antibiotics has greatly decreased,[3,4] leading to the introduction of broad-spectrum antibiotics such as fluoroquinolones or third-generation cephalosporins (cefotaxime, ceftriaxone, or ceftazidime). Unfortunately, these agents are often unaffordable in most LMIC, especially in remote rural areas.

On the other hand, since their first description in 1983, extended-spectrum β-lactamases (ESBLs) produced by enteric pathogens have spread worldwide.[5]

The emergence and spread of ESBLs, especially those included in the CTX-M group, is an important public health problem.[6] In fact, it has been considered that ESBL-carrying Enterobacteriaceae cause >1700 deaths yearly in the USA alone,[7] and these pathogens have had a tremendous impact on the treatment of severe or MDR-associated infections, particularly in LMIC where second-line antibiotics are often unaffordable or unavailable. In addition, few new antibiotics against Gram-negative bacteria have been marketed in the last decades[8] which may favor the emergence of new resistances, further challenging the management of infectious diseases in this setting. This may play a role in the high morbidity and mortality observed in these countries, particularly in children <5 years of age.

Although different types of ESBLs have been reported among the Enterobacteriaceae family, CTX-M-15, a community-acquired ESBL that was originally described in India in the 1990s, is one of the most frequent type I CTX-M disseminated worldwide.[9] The genes encoding ESBL enzymes are usually located in plasmids but can also be found in the chromosomal DNA as described elsewhere.[10] It has been reported that the $bla_{\text{CTX-M-15}}$ gene is usually found downstream from the insertion sequence IS*Ecp1* that may be involved in their dissemination and expression.[11] Plasmid-mediated ESBL genes are of special interest due to their capability of getting transferred between strains or even species, favoring their dissemination among the bacterial population and from region to region. Moreover, these plasmids usually carry other antibiotic resistance determinants, resulting not only in the spread of ESBL but also in the dissemination of other resistance genes.[12] The selection of one resistance gene due to environmental pressure harbored in the same genetic element as another resistance gene or genes is known as the co-selection of resistance genes phenomenon. Since ESBL-producing microorganisms are also often resistant to other commonly available antibiotics, including fluoroquinolones, especially in most LMIC,[13] knowledge of their prevalence and characterization is important for defining local empirical stewardship programs for infections caused by MDR organisms.

In Africa, ESBLs have increasingly been reported.[14,15] In Mozambique, the prevalence of these pathogens is extremely high, although the data available are limited to only a few studies.[16] Herein, we report the prevalence of *Escherichia coli* harboring the ESBL gene $bla_{\text{CTX-M}}$ group I as well as its molecular characterization and epidemiology among isolates recovered from blood cultures and urine in a rural hospital in Southern Mozambique.

Materials and methods

Study population and clinical isolates

The study was conducted by the Centro de Investigação em Saúde de Manhiça (CISM) at the Manhiça District Hospital, a rural referral hospital of the Manhiça district, located 80 km north of Maputo, in Southern Mozambique. Invasive bacterial disease surveillance has been conducted in the pediatric population in this area since 1997. The full description and characteristics of the study area are detailed elsewhere.[17] As described previously in standard clinical protocols, blood cultures are systematically collected upon admission of all children up to 14 years of age with an axillary temperature ≥37.5°C or meeting criteria of severe infection.[2] We analyzed *E. coli* isolates recovered from children with community-acquired bacteremia between August 2004 and December 2009. Urine samples were also collected during the same study period from patients (adults and children) visited at the outpatient department or admitted to the hospital with clinical suspicion of urinary tract infection (UTI). All the isolates included in the present study were recovered from different patients.

Bacterial culture and identification

Blood culture tubes were incubated in an automated system (BACTEC® 9050; Becton Dickinson, Franklin Lakes, NJ, USA). Positive blood cultures were subcultured in solid media after Gram staining as appropriate. Urine samples were microscopically screened after centrifugation, and those with pathologic sediment (presence of leucocytes or bacteria) were cultured in MacConkey and blood agar media. Pathogens were identified according to conventional microbiology protocols. Among the Enterobacteriaceae isolates identified, ceftriaxone susceptibility was tested by disk diffusion in Mueller–Hinton agar (Oxoid®, Basingstoke, Hampshire, UK)

according to the Clinical and Laboratory Standard Institute (CLSI) 2013 guidelines.[18] The selected ceftriaxone non-susceptible *E. coli* isolates were screened for the ESBL enzyme CTX-M group 1 and the positive isolates were included in the study. The isolates were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) prior to further analysis.[19]

Antimicrobial susceptibility testing

The susceptibility phenotype for ampicillin, chloramphenicol, ceftriaxone, gentamicin, tetracycline, trimethoprim-sulfamethoxazole, rifampicin, and amikacin was determined by a conventional disk diffusion method and for nalidixic acid and ciprofloxacin by minimum inhibitory concentration (MIC). The interpretative category of resistance for disk diffusion and MIC were done according to the CLSI 2013 guidelines.[18] Antimicrobial susceptibility testing of the isolates was also performed by Siemens MicroScan panels NEG MIC TYPE 37. The *E. coli* American Type Culture Collection 25922 strain was used as the quality control. Multidrug resistance was defined as resistance to 3 or more unrelated antibiotic families.[20] Resistance genes to quinolones and rifampicin were also studied by polymerase chain reaction (PCR) and sequencing methods using primers for *aac (6')-Ib-cr*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *gyrA*, *parC*, *arr2,3*, *arr4*, *arr5*, *arr6*, *arr7*, *arr3*, and *rpoB* already described.[21–29]

ESBL phenotype detection

All ceftriaxone non-susceptible *E. coli* isolates were phenotypically screened for the presence of ESBL. Phenotypic confirmation of ESBL expression was carried out using the ESBL disk synergy test with disks containing cefotaxime, amoxicillin with clavulanate, and ceftazidime on Mueller–Hinton agar (Oxoid) as described elsewhere.[30] *E. coli* isolates with an ESBL phenotype were tested by PCR for the presence of genes encoding β-lactamases and were further characterized as follows.

β-lactamases analysis

The presence of bla_{CTX-M} was detected by PCR using universal primers, while the bla_{CTX-M} groups 1, 2, 8, 9, and $bla_{CTX-M-15}$ were determined using specific CTX-M group primers and subsequently sequenced.[31] Moreover, the presence of bla_{SHV}, bla_{TEM}, $bla_{OXA-1-like}$, $bla_{OXA-2-like}$, and $bla_{OXA-5-like}$ genes was also determined by PCR and sequencing as described elsewhere.[32] The presence of the insertion sequence IS*Ecp1* upstream from the bla_{CTX-M} genes was determined by PCR and sequencing as previously described.[11] Sequencing of the genes was performed by the Macrogen® DNA Sequencing Service (Macrogen, Amsterdam, the Netherlands) using sets of consecutive primers specific for each gene type.

Class I integron analysis

The presence of class I integrons was analyzed in all the *E. coli* isolates. A PCR was carried out with primers 3′CS and 5′CS as described by Lévesque et al[33] and the amplicons obtained were sequenced by Beckman Coulter Sequencing Genomics® sequencing facilities (Takeley, UK).

Typing

Pulsed-field gel electrophoresis (PFGE) was performed with the *XbaI* restriction enzyme (New England Biolabs, Beverly, MA, USA) as described previously.[34] PFGE profiles were analyzed with InfoQuest FP software version 4.5 (Bio-Rad Laboratories Inc., Hercules, CA, USA). In order to establish the epidemiological relationship among the isolates from the electrophoretic patterns, the Dice coefficient was used and clustering was based on the unweighted pair group method with arithmetic mean with a 1% tolerance in band position differences. The isolates were considered to belong to the same epidemiological group when the PFGE-*XbaI* profiles showed ≥80% of homology, adapting the criteria described by Tenover et al.[35] Multi-locus sequence typing (MLST) was carried out by amplification and sequencing of the 7 *E. coli* housekeeping genes as described previously.[36] The database available at http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/ was used for assigning sequence types (STs) and clonal complexes (CCs). Classification of isolates into *E. coli* phylogenetic groups was done using a previously described triplex PCR-based protocol.[37]

Plasmid transferability analysis

Conjugation assays were carried out with all the isolates in order to determine if the bla_{CTX-M} group 1 gene was located in a conjugative plasmid adapting the protocol described elsewhere.[38] The *E. coli* K7 759 lac$^-$ kanamycin-resistant isolate was used as the recipient strain. Both parental and recipient strains were cultured over-night (ON) with Luria Bertani (LB) broth (Laboratorios Conda, Barcelona, Spain). The parental isolates were grown in LB medium supplemented with 32 μg/mL of cefotaxime in order to force resistance determinant replication. An aliquot of 500 μL of a parental isolate subcultured for 2 hours was mixed with the same volume of the recipient strain and cultured ON at 37°C. Transconjugant strains were finally grown in MacConkey agar plates supplemented with 256 μg/mL of kanamycin and 32 μg/mL of cefotaxime. Repetitive extragenic palindromic

PCRs and a bla_{CTX-M} group 1 PCR were performed to ensure the correct selection of the transconjugants.[39]

The bla_{CTX-M} gene location

Considering the previously described large size of the plasmids carrying bla_{CTX-M} group 1 ESBL genes,[40] a S1 nuclease (Promega, Madison, WI, USA) digestion followed by PFGE analysis were performed in the 12 *E. coli* isolates and the transconjugants obtained as described elsewhere.[41] To determine the plasmid or chromosomal location of the ESBL-encoding gene, a Southern blot of the PFGE gel followed by hybridization with a bla_{CTX-M} group 1 probe was carried out.

PCR-based Replicon Typing (PBRT)

Plasmids from both parental and transconjugant isolates were assigned to incompatibility groups depending on the presence of specific replicon sequences identified by PCR using the primers designed by Carattoli et al in 2005 but employing the adapted amplification protocols for commensal and pathogenic *E. coli* isolates described by Johnson et al.[42]

Ethical clearance

The strains characterized here were isolated from the ongoing invasive bacterial surveillance system that included several research protocols reviewed and approved by the Mozambican National Bioethics Committee for Health (IR00002657) and by Institutional Review Boards of Hospital Clinic of Barcelona, Spain; the US Centers for Disease Control and Prevention; and the School of Medicine, University of Maryland. Written informed consent was obtained from parents or caretakers of the eligible children.

Results

Study population and clinical isolates

During the study period, a total of 15,057 blood cultures were collected and 1325 (8.8%) were found to be positive for any pathogen evaluable. Of these, 27.7% were identified as belonging to the Enterobacteriaceae family, with *E. coli* being the second most frequent after non-typhoidal *Salmonella*, accounting for 29% (106/368). Among these, 8 out of 12 *E. coli* isolates non-susceptible to ceftriaxone and positive for the ESBL disk synergy test (11.3% of total *E. coli* isolates) were found to carry a bla_{CTX-M} group I gene.

Of the 298 urine samples cultured for bacterial isolation, 35% (n=103) were positive for pathogenic bacteria. Among these, 81 (78.6%) corresponded to Enterobacteriaceae with *E. coli* being the most prevalent species with 45 isolates of which 5 (11.1%) were non-susceptible to ceftriaxone and positive for the ESBL double-disk synergy test. Four were found to carry a bla_{CTX-M} group I gene.

A total of 12 *E. coli* isolates from bacteremia and UTI carrying a bla_{CTX-M} group I gene were selected for further characterization.

β-lactamases analysis

Gene amplification sequencing revealed that 92% (n=11) of the isolates harbored $bla_{CTX-M-15}$, while the remaining strain presented the ESBL gene $bla_{CTX-M-37}$. The non-ESBL resistance genes also detected, bla_{TEM-1} and bla_{OXA-1}, were found in 100% and 58.3% of the isolates, respectively. Another ESBL-encoding gene detected was bla_{SHV-12}, which was found in 2 of the isolates also presenting $bla_{CTX-M-15}$. In all cases, the insertion sequence IS*Ecp1* was found upstream from the bla_{CTX-M} group 1 gene. The overall results are summarized in Table 1.

Antimicrobial susceptibility testing

All the isolates were MDR, presenting not only resistance to third-generation β-lactams (ceftriaxone) but also to other classes of antimicrobial agents. All the isolates were resistant to rifampicin, gentamicin, chloramphenicol, and trimethoprim-sulfamethoxazole, while 66.7% were resistant to quinolones (Table 1). Regarding the resistance genotype of the *E. coli* isolates to quinolones, among the isolates with a MIC=1 μg/mL of ciprofloxacin, 4 showed the presence of the *qnrB* gene without mutations in the *gyrA* and *parC* genes; 1 isolate showed only a mutation in amino acid codon Ser83 of *gyrA* and 1 did not show any of the resistance determinants studied. The isolate with a MIC=64 μg/mL of ciprofloxacin showed 3 mutations (2 in *gyrA* and 1 in *parC*) and the other isolate with a MIC >256 μg/mL has the same mutations plus the presence of the *qnrB* gene (Table 2).

The resistance genotype to rifampicin was not well elucidated as *arr* genes were not detected and any significant mutations in *rpoB* gene were observed in any isolate.

All the isolates were susceptible to fosfomycin, nitrofurantoin, and carbapenems.

Class 1 integron analysis

Seven isolates were found to carry class 1 integrons. Two isolates harbored an integron of ~1000 bp carrying the resistance gene *aadA1*, conferring resistance to streptomycin and spectinomycin. One isolate had a 2000 bp class 1 integron carrying 2 resistance genes: *dfrA12* and *aadA2* that confer resistance to trimethoprim and streptomycin-spectinomycin, respectively. Four isolates presented 2 integrons of ~800 and

Table 1 Study strains, β-lactamase analysis, resistance profile and class 1 integron characterization

Isolate	Source	Isolation date	ESBL enzymes	Other β-lactamases	Non-β-lactamic resistance profile	Integron class 1	Resistance genes in Integron class 1
E12	Blood	09/10/2008	CTX-M-15	TEM-1	CHL-GM-TET-SXT-RIF	–	–
E15	Blood	15/04/2009	CTX-M-15	TEM-1	CHL-GM-TET-SXT-RIF	–	–
E11	Blood	15/09/2008	CTX-M-15	TEM-1/OXA-1	CHL-GM-CIP-TET-SXT-RIF	1000 bp	*aadA1*
E4	Blood	04/05/2007	CTX-M-15/SHV-12	TEM-1	CHL-GM-NAL-TET-SXT-RIF	–	–
E3	Blood	14/12/2006	CTX-M-15	TEM-1	CHL-GM-TET-SXT-RIF	–	–
E16	Blood	29/03/2009	CTX-M-15	TEM-1/OXA-1	CHL-GM-NAL-CIP-TET-SXT-RIF	1000 bp	*aadA1*
E18	Blood	10/11/2008	CTX-M-15	TEM-1	CHL-GM-SXT-RIF	2000 bp	*dfrA12* + *aadA2*
E2	Urine	23/11/2005	CTX-M-15	TEM-1/OXA-1	CHL-GM-NAL-CIP-TET-SXT-RIF	–	–
E7	Blood	26/12/2007	CTX-M-15	TEM-1/OXA-1	CHL-GM-NAL-CIP-TET-SXT-RIF	800 bp/1000 bp	*dfrA16/aadA1*
E14	Urine	13/02/2009	CTX-M-15	TEM-1/OXA-1	CHL-GM-NAL-CIP-TET-SXT-RIF	800 bp/1000 bp	*dfrA16/aadA1*
E8	Urine	30/07/2008	CTX-M-37	TEM-1/OXA-1	CHL-GM-NAL-CIP-TET-SXT-RIF	800 bp/1000 bp	*dfrA16/aadA1*
E17	Urine	20/03/2009	CTX-M-15/SHV-12	TEM-1/OXA-1	CHL-GM-NAL-CIP-TET-SXT-RIF	800 bp/1000 bp	*dfrA16/aadA1*

Abbreviations: CHL, chloramphenicol; CIP, ciprofloxacin; ESBL, extended-spectrum β-lactamases; GM, gentamicin; NAL, nalidixic acid; RIF, rifampicin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.

Table 2 Quinolone susceptibility phenotype and genotype

Isolate	Minimum inhibitory concentration (μg/mL)				Resistance genes							Aminoacidic mutations			
	NAL		CIP		*qnrA*	*qnrB*	*qnrC*	*qnrD*	*qnrS*	*qepA*	*aac (6') Ib-cr*	*gyrA*		*parC*	
												83 (*wt* Ser)	87 (*wt* Asp)	80 (*wt* Ser)	84 (*wt* Glu)
E2	>256	R	64	R	–	–	–	–	–	–	–	Leu	Asn	Ile	Glu
E3	4	S	0.03	S	–	–	–	–	–	–	–	–	–	–	–
E4	128	R	1	I	–	–	–	–	–	–	–	Leu	Asp	Ser	Glu
E7	16	I	1	I	–	+	–	–	–	–	–	Ser	Asp	Ser	Glu
E8	16	I	1	I	–	–	–	–	–	–	–	Ser	Asp	Ser	Glu
E11	>256	R	>256	R	–	+	–	–	–	–	–	Leu	Asn	Ile	Glu
E12	1	S	0.007	S	–	–	–	–	–	–	–	–	–	–	–
E14	16	I	1	I	–	+	–	–	–	–	–	Ser	Asp	Ser	Glu
E15	4	S	0.015	S	–	–	–	–	–	–	–	–	–	–	–
E16	16	I	1	I	–	+	–	–	–	–	–	Ser	Asp	Ser	Glu
E17	16	I	1	I	–	+	–	–	–	–	–	Ser	Asp	Ser	Glu
E18	8	S	0.5	S	–	–	–	-	–	–	–	–	–	–	–

Abbreviations: CIP, ciprofloxacin; NAL, nalidixic acid; R, resistant; I, intermediate; S, susceptible; Leu, leucine; Ser, serine; Asn, asparagine; Asp, aspartic acid; Ile, isoleucine; Glu, glutamic acid.

1000 bp containing the *dfrA16* and *aadA1* genes, respectively, conferring the same resistances as those mentioned earlier (Table 1).

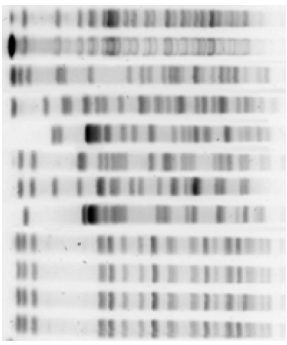
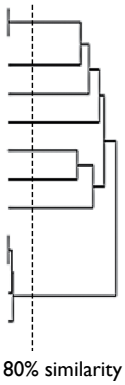
Molecular typing

According to the PFGE analysis constructed from the electrophoresis patterns of the *XbaI* restriction and considering the same profile of ≥80% of similarity, there were 8 different epidemiological groups among the 12 isolates studied. The analysis showed 4 and 2 other isolates to be in the same epidemiological group, thereby being epidemiologically related isolates. This association, however, involved grouping isolates harboring different bla_{CTX-M} group 1 genes. The MLST analysis also showed the same number of ST groups as epidemiologically unrelated isolates (singletons). This data correlates 100% with the epidemiological grouping established by the PFGE analysis and with the genetic characterization of non-β-lactam resistance genes and the antimicrobial susceptibility profiles. Only 2 out of the 8 STs described belonged to the same CC (ST10). Four *E. coli* phylogenetic groups were represented in the collection of isolates (A, B1, B2, and D), with none having a statistically significant prevalence taking into account the epidemiological associations. All the isolates causing UTI belonged to phylogenetic group A (Table 3).

Plasmid characterization

Transconjugants were obtained from 10 parental isolates as shown in Table 4. S1 endonuclease digestion allowed visualizing the plasmid profile of each isolate. The parental isolates

Table 3 Typing and epidemiological relationship between the *Escherichia coli* isolates

Isolate	Phylogenetic group	Sequence type	Clonal complex	PFGE profile	Dendrogram
E12	B1	ST3	ST3		
E15	B1	ST3	ST3		
E11	D	ST405	ST405		
E4	D	ST38	ST38		
E3	B2	ST2451	Singleton		
E16	A	ST10	ST10		
E18	B1	ST453	ST86		
E2	A	ST617	ST10		
E7	A	ST216	Singleton		
E14	A	ST216	Singleton		
E8	A	ST216	Singleton		
E17	A	ST216	Singleton		

80% similarity

Abbreviations: PFGE, pulsed-field gel electrophoresis; ST, sequence type.

Table 4 Plasmid transferability assay and characterization

Donor isolates			Transconjugants	
Isolate	No of plasmids	Plasmid replicon types	Transconjugant	Plasmid replicon types
E12	3	FIIA	E12T	FIIA
E15	3	FIIA	E15T	FIIA
E11	3	HI2/FIB	E11T	HI2
E4	2	HI2	Not obtained	–
E3	3	FIB	E3T	None
E16	1	HI2	E16T	HI2
E18	1	FIB	E18T	None
E2	1	FIA	Not obtained	–
E7	2	HI2	E7T	HI2
E14	2	HI2	E14T	HI2
E8	2	HI2	E8T	None
E17	2	HI2	E17T	HI2

carried 1, 2, or even 3 plasmids each, ranging from <48.5 kb to ~380 kb (Figure 1). Hybridization with the bla_{CTX-M} group 1 probe allowed the localization of the plasmid carrying the antimicrobial resistance determinant in each isolate, which appeared to be of 3 different plasmid sizes among the 8 epidemiological groups (Figure 2). However, epidemiologically related isolates carried plasmids with different sizes harboring the resistance determinant. The conjugative plasmid in isolate E8 carrying $bla_{CTX-M-37}$ was the smallest (<48.5 Kb), whereas in the epidemiologically related isolates carrying $bla_{CTX-M-15}$, the conjugative plasmid was the largest (~290 Kb). The transconjugant E11T showed a hybridization signal in a larger-sized plasmid from its donor isolate. Isolates in which conjugation was not possible (E2 and E4) only showed a hybridization signal in the chromosome.

The plasmid incompatibility groups amplified in the PBRT analysis of the parental isolates were IncFIIA, IncFIA, IncHI2, and IncFIB, but only the incompatibility groups IncFIIA and IncHI2 were found in the plasmids carrying the bla_{CTX-M} group 1 gene (such as those amplified in the transconjugants; Table 4).

Discussion

This is one of the few studies on the prevalence of CTX-M group 1 ESBLs in *E. coli* causing both bacteremia and UTIs among children in Mozambique. The prevalence of ESBLs reported here is a matter of concern as MDR pathogens causing infectious diseases are common in this area, limiting the therapeutic options for treating severe infections often associated with a poor outcome. The rates of ESBLs and other antibiotic resistances observed in this study may be associated with the high prevalences of other infectious diseases, such as tuberculosis, respiratory infections, malaria, and human immunodeficiency virus, which requires the frequent use of antibacterial agents.[43–45] Despite the high prevalence of ESBLs reported in this study, it is lower compared with the prevalence of those causing UTIs in children admitted to the malnutrition and pediatric wards described in the central region of the country (Beira City).[46]

Regarding the resistance mechanisms to non-β-lactamic antibiotic families found among the *E. coli* isolates studied, the resistance determinants to quinolones correspond to those described so far,[47] whereas the lack of detection of resistance determinants to rifampicin suggests other mechanisms of resistance, such as the effect of efflux pumps, as described elsewhere.[29]

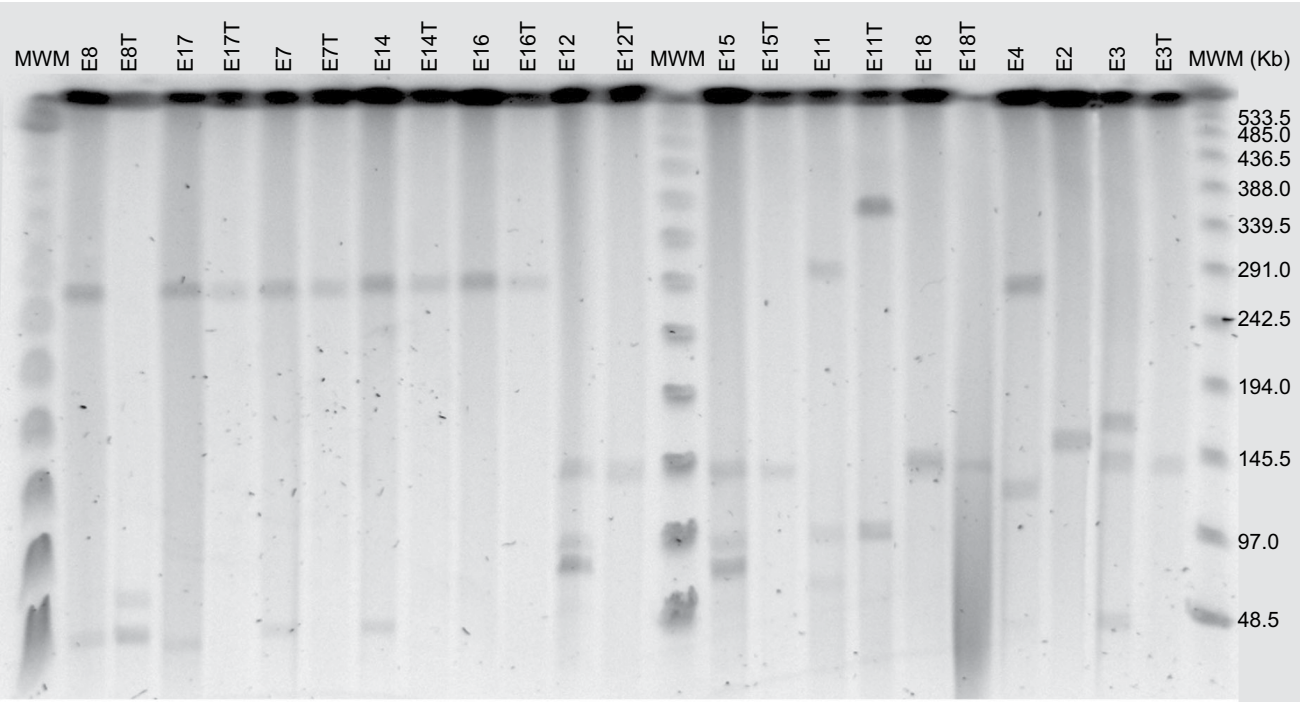


Figure 1 S1 endonuclease pulsed-field gel electrophoresis (PFGE).
Abbreviation: MWM, molecular weight marker.

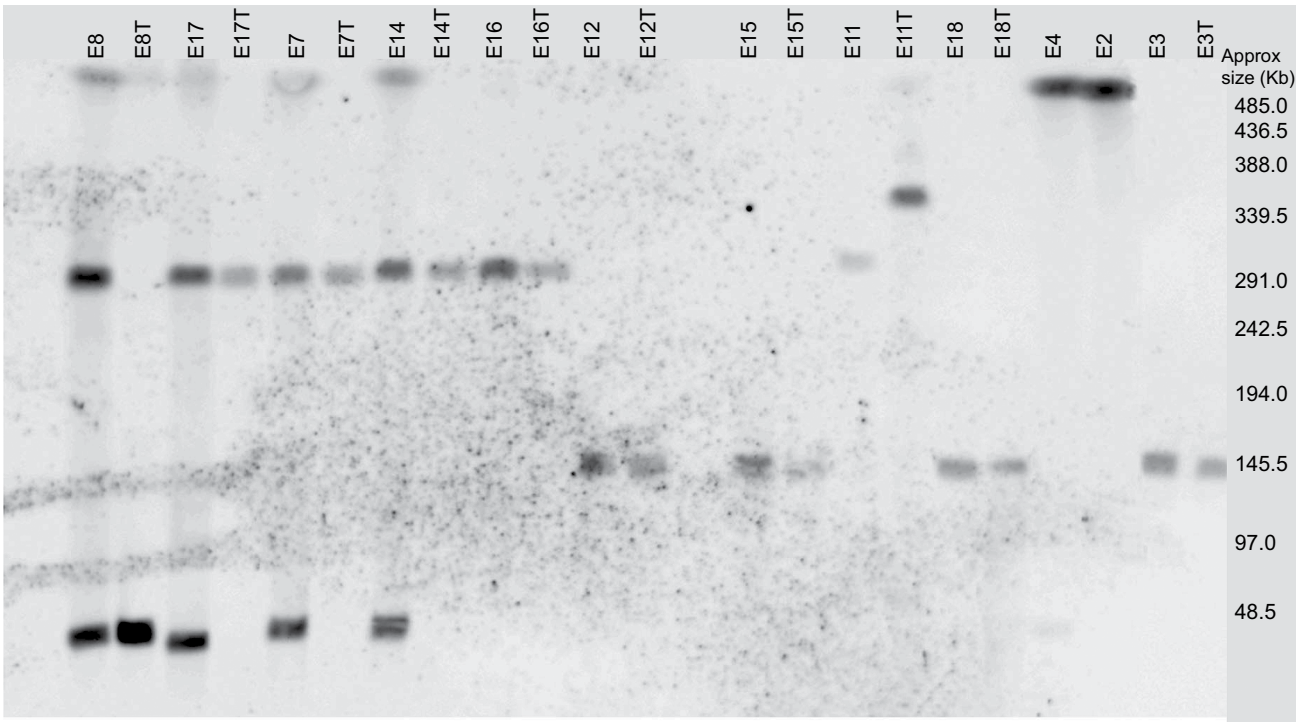


Figure 2 Hybridization of S1 endonuclease pulsed-field gel electrophoresis (PFGE) with bla_{CTX-M} group 1 probe.

The most prevalent resistance mechanism to third-generation cephalosporins found in the collection of MDR isolates studied is the bla_{CTX-M} gene belonging to a sublineage or group I (accounting for 70.6% of the ESBL-carrying isolates), which is consistent with other reports[5] showing isolates harboring a bla_{CTX-M} group I gene with almost 92% being $bla_{CTX-M-15}$. Within the CTX-M-1 group, $bla_{CTX-M-15}$ is the most frequently described resistance gene in isolates causing both community-acquired bacteremia and UTI.[6,16] In fact, $bla_{CTX-M-15}$ is currently the most common variant detected worldwide in clinically important Gram-negative bacteria together with $bla_{CTX-M-14}$.[9] According to the current data and the widely reported $bla_{CTX-M-15}$ dissemination, this is not the first description of this gene in the area since it has previously been described in ESBL-carrying *Klebsiella pneumoniae* isolates.[16]

Regarding the other bla_{CTX-M} group I gene detected, this is the first description of $bla_{CTX-M-37}$ in Mozambique. This infrequently detected CTX-M was first described in an *Enterobacter cloacae* isolated in Mongolia in 2002, and to our knowledge, it has only been previously reported in 1 other African country, the neighboring South Africa, as well as in the chromosome of an isolate of *Kluivera cryocrescences* from Argentina (GenBank access No: FN813246.1).[48]

Whereas the mechanism of resistance to third-generation cephalosporins in the collection of isolates of our study was the same, the isolates showed low relatedness at an epidemiological level, being distributed in 4 phylogenetic groups and 8 epidemiological groups. Although the range of phylogenetic groups represented is wide, it is important to highlight that all the isolates from UTIs were phylogenetic group A. This phylogenetic group has been associated with MDR strains causing UTI.[49] Based on PFGE and MLST results, 4 isolates within the same epidemiological group belonged to ST ST216. However, these isolates showed some divergent evolution concerning the resistant determinants, as one harbored CTX-M-37 (isolate E8) instead of CTX-M-15 and another (isolate E17) also showed SHV-12. Furthermore, isolates E12 and E15 belonged to the same epidemiological group although they were isolated at a different period. The non-related isolates belonged to different STs and even different CCs, indicating that it was not a clonal dissemination.

Regarding the plasmid analysis to determine the location of bla_{CTX-M} group 1, the collection presented a wide range of different plasmid incompatibility groups. Furthermore, 2 epidemiologically unrelated isolates harbored the resistance determinant in the chromosome and, therefore, no transconjugants were obtained. The transconjugant E11 showed a hybridization signal in a larger plasmid from its donor isolate, but its size corresponded to the recombination of the 2 plasmids harbored by the parental isolate, a common phenomenon in conjugation assays. Moreover, together with E16, which belongs to ST ST10, this isolate harbored the same plasmid (in terms of size and incompatibility group) as the isolates belonging to the clone ST216, suggesting a potential dissemination of the same plasmid among *E. coli* strains belonging to different STs.

The location of the bla_{CTX-M} gene upstream from the insertion sequence IS*Ecp1* in all the isolates and in a conjugative plasmid in most of the isolates implies a high potential of dissemination of this ESBL, suggesting that this is not a result of the dissemination of particular clones but rather is due to the spread of multiple specific clones and/or mobile genetic elements. However, it is interesting that these strains presented such resistance to third-generation cephalosporins, as these antimicrobial agents are little used in Mozambique.

With regard to the resistance phenotype, 100% of the isolates were resistant to gentamicin, chloramphenicol, and trimethoprim-sulfamethoxazole. The first 2 antibiotics are used in the empirical treatment of bacteremia (chloramphenicol or penicillin plus gentamicin) whereas ceftriaxone is reserved for MDR cases,[2] which would not be effective in the isolates studied. The transconjugant isolates showed not only β-lactam resistance but also the same resistance profile to other antibiotic families, which may have an impact on the clinical management of the patients in this setting. There are 3 main non-exclusive explanations for the finding of CTX-M group 1 ESBL resistance genes in these isolates: 1) the use of third-generation cephalosporins as second-line treatment may play a role in the emergence and subsequent dissemination of β-lactamase resistance; 2) the resistance to β-lactam antibiotics is the result of a co-selection from another family of antibiotic resistance mechanisms located in the same genetic mobile element; 3) globalization may influence the dissemination of ESBLs-carrying isolates in the community similar to what has been shown with New Dehli metallo-β-lactamase.[50] The historical movement of the population between Manhiça and neighboring South Africa,[17] as well as the increasingly more frequent presence of international travelers to the Manhiça District may support the international dissemination of these strains, as third-generation cephalosporins are not commonly used in this community.

Conclusion

As observed in this study, continuously increasing resistance among Gram-negative bacteria associated with the emergence and spread of MDR isolates, including ESBL producers, is an important public health problem with serious implications in low-income countries. Taking into account that the availability of effective antibiotics is a challenge worldwide and is of special concern in LMIC due to limited resources, clinical microbiology services in Mozambique – and in all the Sub-Saharan African countries – need to be reinforced in order to perform coordinated antimicrobial resistance surveillance and establish national policies to control this public health problem.

Acknowledgments

The authors thank all the clinical and laboratory staff from the CISM for their contribution in different stages of the study, specifically for Dinis Jantilal, Oscar Fraile, Tacilta Nhampossa, and Pedro Aide. Special thanks to the bacteriology laboratory technicians for their excellent support in performing antimicrobial susceptibility testing and ESBL phenotyping. The authors are also grateful to Alessandra Carattoli for providing the control strains for the PBRT and to Donna Pringle for language correction.

This study received funding from Planes Nacionales de I+D+i 2008–2011/2013–2016 and the Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD12/0015/0013 and REIPI RD16/0016/0010) and was co-financed by European Development Regional Fund "A way to achieve Europe" and operative program Intelligent Growth 2014–2020. The CISM received core funding from the Agencia Española de Cooperación Internacional y Desarrollo (AECID). This work was also supported by grant 2009 SGR 1256 from the Agència de Gestió d'Ajuts Universitaris i de Recerca of the Generalitat de Catalunya. JR had a fellowship from program I3 of the Instituto de Salud Carlos III (ISCIII) (grant number: CES11/012).

ISGlobal is a member of the Centres de Recerca de Catalunya (CERCA) Programme, Generalitat de Catalunya.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Berkley JA, Lowe BS, Mwangi I, et al. Bacteremia among children admitted to a rural hospital in Kenya. *N Engl J Med*. 2005;352(1):39–47.
2. Sigaúque B, Roca A, Mandomando I, et al. Community-acquired bacteremia among children admitted to a rural hospital in Mozambique. *Pediatr Infect Dis J*. 2009;28(2):108–113.
3. Mandomando I, Sigaúque B, Morais L, et al. Antimicrobial drug resistance trends of bacteremia isolates in a rural hospital in southern Mozambique. *Am J Trop Med Hyg*. 2010;83(1):152–157.
4. Okeke IN, Laxminarayan R, Bhutta ZA, et al. Antimicrobial resistance in developing countries. Part I: recent trends and current status. *Lancet Infect Dis*. 2005;5(8):481–493.
5. Rossolini GM, D'Andrea MM, Mugnaioli C. The spread of CTX-M-type extended-spectrum β-lactamases. *Clin Microbiol Infect*. 2008;14(Suppl 1):33–41.
6. Peirano G, Pitout JD. Molecular epidemiology of *Escherichia coli* producing CTX-M beta-lactamases: the worldwide emergence of clone ST131 O25:H4. *Int J Antimicrob Agents*. 2010;35(4):316–321.
7. Centers for Disease Control and Prevention. US Department of Health and Human Services. *Antibiotic Resistance Threats in the United States, 2013*. Atlanta, GE: 2013.
8. Fair RJ, Tor Y. Perspectives in medicinal chemistry antibiotics and bacterial resistance in the 21th century. *Perspect Medicin Chem*. 2014;6:25–64.
9. Coque TM, Novais Â, Carattoli A, et al. Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. *Emerg Infect Dis*. 2008;14(2):195–200.
10. Rodríguez I, Thomas K, Van Essen A, et al; SAFEFOODERA-ESBL consortium. Chromosomal location of bla_{CTX-M} genes in clinical isolates of *Escherichia coli* from Germany, The Netherlands and the UK. *Int J Antimicrob Agents*. 2014;43(6):553–557.
11. Chouchani C, El Salabi A, Marrakchi R, Ferchichi L, Walsh TR. Characterization of IncA/C conjugative plasmid harboring bla_{TEM-52} and $bla_{CTX-M-15}$ extended-spectrum β-lactamases in clinical isolates of *Escherichia coli* in Tunisia. *Eur J Clin Microbiol Infect Dis*. 2012;31(6):1081–1087.
12. Carattoli A. Plasmids and the spread of resistance. *Int J Med Microbiol*. 2013;303(6–7):298–304.
13. Tumbarello M, Sali M, Trecarichi EM, et al. Bloodstream infections caused by extended-spectrum-beta-lactamase-producing *Escherichia coli*: risk factors for inadequate initial antimicrobial therapy. *Antimicrob Agents Chemother*. 2008;52(9):3244–3252.
14. Gray KJ, Wilson LK, Phiri A, Corkill JE, French N, Hart CA. Identification and characterization of ceftriaxone resistance and extended-spectrum beta-lactamases in Malawian bacteraemic Enterobacteriaceae. *J Antimicrob Chemother*. 2006;57(4):661–665.
15. Blomberg B, Jureen R, Manji KP, et al. High rate of fatal cases of pediatric septicemia caused by gram-negative bacteria with extended-spectrum beta-lactamases in Dar es Salaam, Tanzania. *J Clin Microbiol*. 2005;43(2):745–749.
16. Pons MJ, Vubil D, Guiral E, et al. Characterisation of extended-spectrum β-lactamases among *Klebsiella pneumoniae* isolates causing bacteraemia and urinary tract infection in Mozambique. *J Glob Antimicrob Resist*. 2015;3(1):19–25.
17. Sacoor C, Nhacolo A, Nhalungo D, et al. Profile: Manhiça health research centre (Manhiça HDSS). *Int J Epidemiol*. 2013;42(5):1309–1318.
18. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Third Informational Supplement*. M100-S23. Wayne, PE: Clinical and Laboratory Standards Institute; 2013.
19. Sogawa K, Watanabe M, Sato K, et al. Use of the MALDI BioTyper system with MALDI-TOF mass spectrometry for rapid identification of microorganisms. *Anal Bioanal Chem*. 2011;400(7):1905–1911.
20. Magiorakos AP, Srinivasan A, Carey RB, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect*. 2012;18(3):268–281.
21. Park CH, Robicsek A, Jacoby GA, Sahm D, Hooper DC. Prevalence in the United States of *aac(6')-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob Agents Chemother*. 2006;50(11):3953–3955.

22. Cattoir V, Poirel L, Rotimi V, Soussy CJ, Nordmann P. Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing enterobacterial isolates. *J Antimicrob Chemother*. 2007;60(2):394–397.
23. Kim HB, Park CH, Kim CJ, Kim E-C, Jacoby GA, Hooper DC. Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. *Antimicrob Agents Chemother*. 2009;53(2):639–645.
24. Zhao J, Chen Z, Chen S, et al. Prevalence and dissemination of *oqxAB* in *Escherichia coli* isolates from animals, farmworkers, and the environment. *Antimicrob Agents Chemother*. 2010;54(10):4219–4224.
25. Heisig P. Genetic evidence for a role of *parC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. *Antimicrob Agents Chemother*. 1996;40(4):879–885.
26. Heisig P, Kratz B, Halle E, et al. Identification of DNA gyrase a mutations in ciprofloxacin-resistant isolates of *Salmonella typhimurium* from men and cattle in Germany. *Microb Drug Resist*. 1995;1(3):211–218.
27. Hopkins KL, Mushtaq S, Richardson JF, et al. In vitro activity of rifaximin against clinical isolates of *Escherichia coli* and other enteropathogenic bacteria isolated from travellers returning to the UK. *Int J Antimicrob Agents*. 2014;43(5):431–437.
28. Lee K, Yum JH, Yong D, et al. Novel acquired metallo-β-lactamase gene, bla_{SIM-1}, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. *Antimicrob Agents Chemother*. 2005;49(11):4485–4491.
29. Pons MJ, Mensa L, Gascón J, Ruiz J. Fitness and molecular mechanisms of resistance to rifaximin in in vitro selected *Escherichia coli* mutants. *Microb Drug Resist*. 2012;18(4):376–379.
30. Drieux L, Brossier F, Sougakoff W, Jarlier V. Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: review and bench guide. *Clin Microbiol Infect*. 2008;14(Suppl 1):90–103.
31. Woodford N, Fagan EJ, Ellington MJ. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. *J Antimicrob Chemother*. 2006;57(1):154–155.
32. Medina AM, Rivera FP, Pons MJ, et al. Comparative analysis of antimicrobial resistance in enterotoxigenic *Escherichia coli* isolates from two paediatric cohort studies in Lima, Peru. *Trans R Soc Trop Med Hyg*. 2015;109(8):493–502.
33. Lévesque C, Piché L, Larose C, Roy PH. PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob Agents Chemother*. 1995;39(1):185–191.
34. Ribot EM, Fair MA, Gautom R, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathog Dis*. 2006;3(1):59–67.
35. Tenover FC, Arbeit RD, Goering RV, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol*. 1995;33(9):2233–2239.
36. Wirth T, Falush D, Lan R, et al. Sex and virulence in *Escherichia coli* : an evolutionary perspective. *Mol Microbiol*. 2006;60(5):1136–1151.
37. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol*. 2000;66(10):4555–4558.
38. Wachino J, Kurokawa H, Suzuki S, et al. Horizontal transfer of bla_{CMY}-bearing plasmids among clinical *Escherichia coli* and *Klebsiella pneumoniae* isolates and emergence of cefepime-hydrolyzing CMY-19. *Antimicrob Agents Chemother*. 2006;50(2):534–541.
39. Eckert C, Gautier V, Saladin-Allard M, et al. Dissemination of CTX-M-type β-lactamases among clinical isolates of Enterobacteriaceae in Paris, France. *Antimicrob Agents Chemother*. 2004;48(4):1249–1255.
40. Wang J, Stephan R, Karczmarczyk M, Yan Q, Hächler H, Fanning S. Molecular characterization of bla_{ESBL}-harboring conjugative plasmids identified in multi-drug resistant *Escherichia coli* isolated from food-producing animals and healthy humans. *Front Microbiol*. 2013;4:188.
41. Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. *Anal Biochem*. 1995;226(2):235–240.
42. Johnson TJ, Wannemuehler YM, Johnson SJ, et al. Plasmid replicon typing of commensal and pathogenic *Escherichia coli* isolates. *Appl Environ Microbiol*. 2007;73(6):1976–1983.
43. González R, Munguambe K, Aponte JJ, et al. High HIV prevalence in a southern semi-rural area of Mozambique: a community-based survey. *HIV Med*. 2012;13(10):581–588.
44. García-Basteiro A, Miranda Ribeiro R, Brew J, et al. Tuberculosis on the rise in southern Mozambique (1997–2012). *Eur Respir J*. 2017;49(3):1601683.
45. Maltha J, Guiraud I, Kaboré B, et al. Frequency of severe malaria and invasive bacterial infections among children admitted to a rural hospital in Burkina Faso. *PLoS One*. 2014;9(2):e89103.
46. Van der Meeren BT, Chhaganlal KD, Pfeiffer A, et al. Extremely high prevalence of multi-resistance among uropathogens from hospitalised children in Beira, Mozambique. *S Afr Med J*. 2013;103(6):382–386.
47. Hopkins KL, Davies RH, Threlfall EJ. Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. *Int J Antimicrob Agents*. 2005;25(5):358–373.
48. Lee K, Yong D, Jeong SH, et al. Genetic and biochemical characterisation of CTX-M-37 extended-spectrum β-lactamase from an *Enterobacter cloacae* clinical isolate from Mongolia. *J Glob Antimicrob Resist*. 2017;10:3–7.
49. Ejrnæs K. Bacterial characteristics of importance for recurrent urinary tract infections caused by *Escherichia coli*. *Dan Med Bull*. 2011;58(4):B4187.
50. Lee CR, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: epidemiology, genetic context, treatment options, and detection methods. *Front Microbiol*. 2016;7:895.

SECTION 3. Antibiotic resistance and epidemiology of traveller's diarrhoea

This section includes the following papers:

- PAPER 7: CTX-M-15-producing enteroaggregative *Escherichia coli* as cause of travelers' diarrhea.
- ADDITIONAL RESULTS I: Antimicrobial susceptibility and mechanisms of resistance to quinolones and β-lactam antibiotics in enteroaggregative and enterotoxigenic *Escherichia coli* causing traveller's diarrhoea.
- ADDITIONAL RESULTS II: Epidemiology of enteroaggregative and enterotoxigenic *Escherichia coli* causing traveller's diarrhoea from South-East Asia, Latin America and Africa.

PAPER 7:

CTX-M-15-producing enteroaggregative *Escherichia coli* as cause of travelers' diarrhea

Authors:

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Journal, volume (issue): pages, date of publication:

Emerging Infectious Diseases, 17 (10): 1950-3, 2011 October.

Impact Factor: 6.169 – Q1, **D1** (2011)

Hypothesis:

Although ESBL production has mainly been shown in extraintestinal *E. coli* infections, studies concerning the prevalence and characterisation of ESBLs in intestinal *E. coli* infections causing traveller's diarrhoea (TD) are scarce.

Objectives:

Describe the molecular epidemiology and plasmid analysis of CTX-M-15 producing EAEC isolates from patients with TD who had travelled to India.

Material and methods:

51 enteroaggregative *E. coli* isolates from patients with diarrhoea coming from India and visiting the Tropical Medicine Unit of Hospital Clinic of Barcelona were screened for resistance to 3rd generation cephalosporins. ESBL carriage as well as the environment of the resistance gene were studied by PCR and Sanger sequencing.

The epidemiologic relationship between the isolates was analysed by REP-PCR, MLST, phylogenetic grouping and pulsed-field gel electrophoresis (PFGE). Plasmid analysis by S1 digestion and plasmid extraction was carried out, and subsequent hybridization with the $bla_{\text{CTX-M-15}}$ probe was done to locate the resistance gene.

Results:

Five ESBL-producing isolates according to their resistance phenotype were further studied. All the isolates carried the CTX-M-15 enzyme and were non-epidemiologically related, although three isolates belonged to clonal complex ST38.

Three isolates belonged to phylogenetic group D and two isolates to B2. Typical VFGs of EAEC were found in all the isolates, mainly *aatA*. The insertion sequence IS*Ecp1* was found upstream from $bla_{CTX\text{-}M\text{-}15}$ in all the isolates. Only three isolates harboured plasmids ranging from 93 kb to 170 kb. The $bla_{CTX\text{-}M\text{-}15}$ was plasmid-located in three isolates and chromosomally located in two isolates.

Conclusions:

The phylogenetic groups represented in this study (B2 and D) are the most commonly described in pathogenic intestinal *E. coli*.

Other clonal complexes of *E. coli* different from ST131 which has spread worldwide may play an important role in causing intestinal infections.

The $bla_{CTX\text{-}M\text{-}15}$gene is not only harboured in plasmids but can also have a chromosomal location, although it is always related to IS*Ecp1*, meaning that the latter might have originated from a previous plasmidic location. This resistance gene is located in different types of plasmids, demonstrating the high flexibility of this ESBL and highlighting the importance of standardised epidemiologic surveillance and the correct use of antibiotics to prevent the increase of resistance worldwide.

CTX-M-15–producing Enteroaggregative *Escherichia coli* as Cause of Travelers' Diarrhea

Elisabet Guiral, Eva Mendez-Arancibia, Sara M. Soto, Pilar Salvador, Anna Fàbrega, Joaquim Gascón, and Jordi Vila

Travelers' diarrhea is a major public health problem. From patients in whom diarrhea developed after travel to India, 5 enteroaggregative *Escherichia coli* strains carrying β-lactamase CTX-M-15 were identified; 3 belonged to clonal complex sequence type 38. This β-lactamase contributes to the multidrug resistance of enteroaggregative *E. coli*, thereby limiting therapeutic alternatives.

Travelers' diarrhea remains a major public health problem, causing substantial illness and disability. Almost 50% of patients with travelers' diarrhea require treatment with antimicrobial drugs because of persistence or severity of signs and symptoms (*1*). Enteroaggregative *E. coli* (EAEC) is among the most common diarrheagenic *E. coli* pathotypes recognized (*2*). The first-choice agents for treating EAEC infections are quinolones, rifaximin, azithromycin, and cephalosporins. However, the number of pathogenic *E. coli* strains resistant to multiple antimicrobial agents has increased, and resistance to third-generation cephalosporins (e.g., ceftazidime, ceftriaxone, or cefotaxime) associated with production of extended-spectrum β-lactamases (ESBLs) limits therapeutic options (*3*).

Although ESBL production has mainly been shown in extraintestinal *E. coli* infections, studies concerning effects of ESBLs in intestinal *E. coli* infections are scarce. The worldwide spread of CTX-M-15 type ESBLs has led these β-lactamases to replace TEM- and SHV-type ESBLs in Europe, Canada, and Asia and become one of the major groups of ESBLs studied. Of the different CTX-M–type ESBLs, CTX-M-15 has become the most widely distributed enzyme worldwide. It was first identified in an isolate from India in 1999 and thereafter became prevalent around the world (*4*). CTX-M-15 enhances hydrolytic activity against ceftazidime (*5*). A particular clone of CTX-M-15–producing *E. coli*, characterized by phylogenetic type (phylotype) B2 and sequence type 131 (ST131), seems to be largely responsible for international epidemics of CTX-M–producing *E. coli* (*6*). Sequence types (STs) are grouped into clonal complexes by their similarity to a central allelic profile.

ST131 is a singleton and therefore does not belong to a clonal complex (*7*). Molecular epidemiologic studies have suggested that the sudden increase in CTX-M-15–producing *E. coli* worldwide was mainly caused by this single clone (ST131) and that foreign travel to high-risk areas, such as the Indian subcontinent, might play a partial role in the spread of this clone across continents (*8*). The $bla_{\text{CTX-M-15}}$ gene is usually found downstream from the insertion sequence IS*Ecp1*, which may be involved in the clone's dissemination and expression (*9*). We describe molecular epidemiology and plasmid analyses of 5 CTX-M-15–producing EAEC isolates from patients with travelers' diarrhea who had traveled from Spain to India.

The Study

The study included all patients with diarrhea who visited the Tropical Medicine Unit of Hospital Clinic in Barcelona, Spain, during 2005 and 2006. Patients with diarrhea that started during or shortly after (<5 days) a stay in a developing country were eligible. After the participants provided informed consent, clinical and epidemiologic data were collected.

Among all eligible participants, infection with EAEC and no other enteropathogen was found for 51. Of these 51 EAEC isolates, 5 from patients who had traveled to India were resistant to third-generation cephalosporins. Resistance phenotypes indicated ESBL production. MICs for antimicrobial agents and susceptibility class were determined by using the Clinical and Laboratory Standards Institute breakpoints guideline (Table 1). All strains were resistant to penicillins; second-, third-, and fourth-generation cephalosporins; and all β-lactamase–inhibitor combinations except piperacillin/tazobactam. Apart from β-lactam susceptibility, the strains showed resistance to other classes of antimicrobial agents, such as fluoroquinolones, tetracyclines, and monobactams (aztreonam). Positive amplification with specific primers and sequencing for the $bla_{\text{CTX-M-15}}$ gene provided positive genotypic confirmatory test results for ESBL production.

The epidemiologic relationships among the 5 strains were studied by repetitive sequence–based PCR, pulsed-field gel electrophoresis, and multilocus sequence typing (*10*,*11*). The PCR and pulsed-field gel electrophoresis genomic fingerprinting showed that the 5 strains were not epidemiologically related (Figure 1). However, multilocus

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DOI: http://dx.doi.org/10.3201/eid1710.110022

Table 1. Susceptibility of 5 enteroaggregative *Escherichia.coli* strains that produced diarrhea in patients returning from India, 2005–2006*

Strain	Antimicrobial agent																							
	AM	PR	AG	P/T	A/S	FU	FOX	FZ	PIM	CTX	CAZ	GN	AK	TB	F	IMI	ME	AZ	CIP	NOR	LEV	TE	SXT	CL
HC19	R	R	R	S	R	R	I	R	R	R	R	R	S	R	S	S	S	R	R	R	R	S	R	S
HC64	R	R	R	S	R	R	I	R	R	R	R	S	S	R	S	S	S	R	R	R	R	R	R	S
HC67	R	R	R	S	R	R	I	R	R	R	I	R	R	R	S	S	S	R	R	R	R	R	R	S
HC74	R	R	R	S	R	R	I	R	R	R	R	S	R	R	S	S	S	R	R	R	R	R	R	R
HC76	R	R	I	S	R	R	S	R	I	R	S	S	S	S	S	S	S	I	R	R	R	R	R	S

*AM, ampicillin; PR, piperacillin; AG, amoxiclavulanic acid/augmentin; P/T, piperacillin/tazobactam; A/S, ampicillin/sulbactam; FU, cefuroxime; FOX, cefoxitin; FZ, cefazoline; PIM, cefepime; CTX, cefotaxime; CAZ, ceftazidime; GN, gentamicin; AK, amikacin; TB, tobramycin; F, fosfomycin; IMI, imipenem; ME, meropenem; AZ, aztreonam; CIP, ciprofloxacin; NOR, norfloxacin; LEV, levofloxacin; TE, tetracycline; SXT, cotrimoxazole; CL, chloramphenicol; R, resistant; S, sensitive; I, intermediate.

sequence typing identified 2 clonal complexes: ST38 (3 strains) and ST10 (1 strain). The fifth strain could not be classified into any clonal complex (Table 2).

E. coli strains were classified into phylogenetic groups by multiplex PCR, described by Clermont et al. (*12*). The 3 strains in clonal complex ST38 belonged to the potentially virulent phylogenetic group D; the other 2 belonged to group B2 (Table 2).

A PCR method was used to detect genes encoding for typical EAEC virulence factors (*2*). These genes include *aggA* and *aafA* (encoding for adhesions); *aap* (for dispersin); *aatA* (for TolC); *aggR* (for regulation of aggregation); *astA*, *set1A*, and *sen* (for toxins), *fyuA* (for iron recruitment); *agn43* (for antigen 43); and genes encoding for serine protease autotransporter toxins such as *pet* and *sat*. Gene *aatA* was detected in the 5 strains, whereas *aap*, *aggR*, and *aggA* had positive amplification for only 2 of the strains belonging to ST38. The other genes detected are shown in Table 2. EAEC was also identified by typical adherence to HEp-2 cells.

To determine the genetic environment of the $bla_{\text{CTX-M-15}}$ gene, we designed an inverse PCR. We designed the primers by studying the gene sequence and were directed outside the gene. The IS*Ecp1* insertion sequence was upstream from the $bla_{\text{CTX-M-15}}$ gene, which was also confirmed by PCR of the specific insertion sequence. To confirm the possible relationship between IS*Ecp1* and the resistance $bla_{\text{CTX-M-15}}$ gene, we conducted a PCR with the forward primer for the IS*Ecp1* and the reverse primer for the $bla_{\text{CTX-M-15}}$ gene.

For plasmid extraction of the 5 isolates, we used the method of Kado and Liu (*13*). Only 3 strains had plasmids ranging from 93 kb to 170 kb (Figure 2, panel A). To confirm the absence of plasmids in the 2 strains, we conducted S1 digestion of the strains, resolving chromosomal DNA from plasmidic DNA. Southern blot of this digestion showed that the $bla_{\text{CTX-M-15}}$ gene was chromosomally located in these 2 strains, as was the *aatA* gene (usually found in the plasmid contained in EAEC strains) (data not shown). Finally, the location of the $bla_{\text{CTX-M-15}}$ gene in the 3 plasmid-containing strains was analyzed by using Southern blot from the plasmid extraction. The $bla_{\text{CTX-M-15}}$ gene was located in a plasmid in the 3 strains. The size of the plasmid containing CTX-M-15 varied in each strain (Figure 2, panel B). Plasmids with specific known molecular weight were used to provide a range of the size of the plasmids studied.

Conclusions

We identified several features concerning the molecular epidemiology of CTX-M-15–producing EAEC isolates in India. First, all strains belonged to phylogenetic groups D and B2, the 2 groups most commonly found with *E. coli* infections (*14*). Second, not finding ST131 suggests that ST131 might not be the most common ST among EAEC strains from India and that clonal complex ST38 might play a large role in causing infectious intestinal diseases. Third, the $bla_{\text{CTX-M-15}}$ gene is not only located in the plasmid but may also be in the chromosome. However, previous reports have shown that $bla_{\text{CTX-M-15}}$ is consistently linked with IS*Ecp1*, which means that the chromosomal location might have originated from a previous plasmid location that was part of either a transposon or a cassette within an integron (*9*). It is also worth noting that the size of the plasmids containing the $bla_{\text{CTX-M-15}}$ gene was not the same in all strains, indicating that this gene may be located in different types of plasmids.

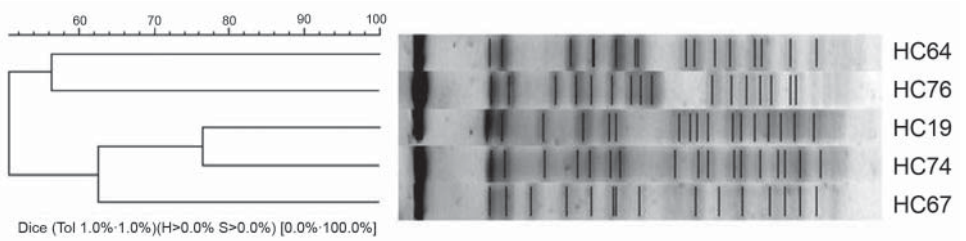

Figure 1. Cluster analysis of the enteroaggregative *Escherichia coli* strains from the pulsed-field gel electrophoresis fingerprinting.

Table 2. Analysis results for 5 enteroaggregative *Escherichia coli* strains that produced travelers' diarrhea in patients returning from India, 2005–2006*

Strain	PFGE type	MLST clonal complex	Phylotype	Genes encoding for virulence factors	$bla_{CTX-M-15}$ location
HC19	A	ST38	D	*aat, aap, aggR, aggA*	Chromosome
HC64	B	None	B2	*aat, astA,sat*	Plasmid
HC67	C	ST38	D	*aat, astA*	Plasmid
HC74	A_1	ST38	D	*aat, aap, aggR, aggA, afn43, fyuA*	Chromosome
HC76	D	ST10	B2	*aat, fyuA*	Plasmid

*PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence type; ST, sequence type.

This evidence of widespread distribution and flexibility of the $bla_{CTX-M-15}$ gene highlights the need to develop appropriate means to control dissemination of this gene and associated resistance genes. Epidemiologic surveillance and correct use of antimicrobial agents will help prevent the steady increase of antimicrobial drug resistance worldwide.

Acknowledgments

We thank R. Rodicio and I. Montero for their help with the plasmid extraction method.

S.M.S. received funding from contract "Miguel Servet" (CP05/00140) from "Fondo de Investigaciones Sanitarias" from the Spanish Ministry of Health. This study was supported by the Generalitat de Catalunya, Departament d'Universitats, Recerca i Societat de la Informació (2009 SGR 1256), by the Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Spanish Network for the Research in Infectious Diseases (REIPI RE06/0008), by the European Community (TROCAR contract HEALTH-F3-2008-223031).

Ms Guiral is a PhD student with the Microbiologist Research Team at the August Pi i Sunyer Biomedical Research Institute in Barcelona. Her research interests include the genetic characterization of antimicrobial drug–resistant bacteria, especially all *E. coli* pathotypes.

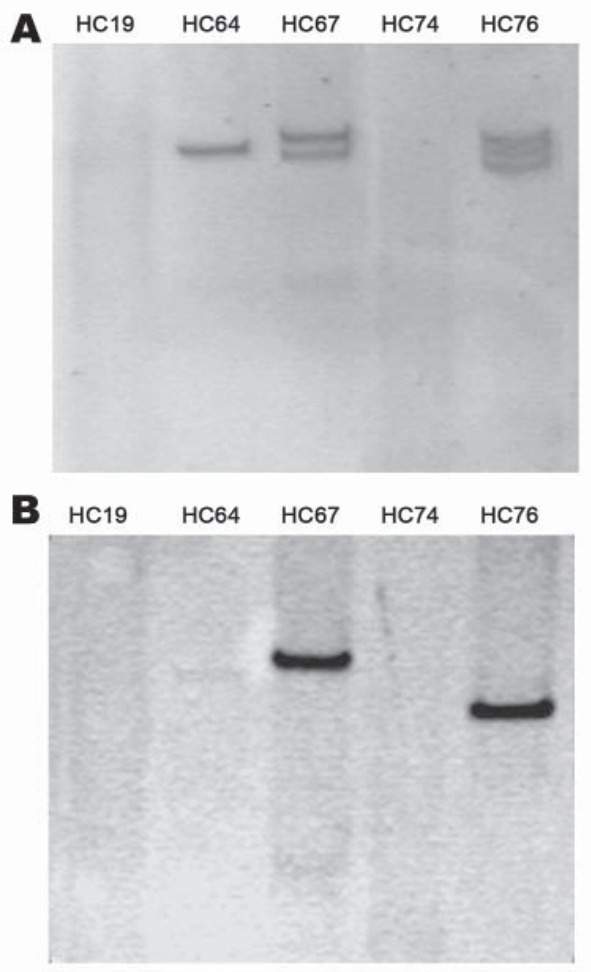

Figure 2. Plasmidic profile of the enteroaggregative *Escherichia coli* strains (A) and Southern blotting of the $bla_{CTX-M-15}$ gene (B).

References

1. Mendez Arancibia E, Pitart C, Ruiz J, Marco F, Gascón J, Vila J. Evolution of antimicrobial resistance in enteroaggregative *Escherichia coli* and enterotoxigenic *Escherichia coli* causing traveller's diarrhoea. J Antimicrob Chemother. 2009;64:343–7. doi:10.1093/jac/dkp178
2. Flores J, Okhuysen PC. Enteroaggregative *Escherichia coli* infection. Curr Opin Gastroenterol. 2009;25:8–11. doi:10.1097/MOG.0b013e32831dac5e
3. Zahar JR, Bille E, Schnell D, Lanternier F, Mechai F, Masse V, et al. Extension of β-lactamases producing bacteria is a worldwide concern [in French]. Med Sci (Paris). 2009;25:939–44. doi:10.1051/medsci/20092511939
4. Cantón R, Coque TM. The CTX-M β-lactamase pandemic. Curr Opin Microbiol. 2006;9:466–75. Epub 2006 Aug 30. doi:10.1016/j.mib.2006.08.011
5. Poirel L, Gniadkowski M, Nordmann P. Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum beta-lactamase CTX-M-15 and of its structurally related β-lactamase CTX-M-3. J Antimicrob Chemother. 2002;50:1031–4. doi:10.1093/jac/dkf240
6. Coque TM, Novais A, Carattoli A, Poirel L, Pitout J, Peixe L, et al. Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum β-lactamase CTX-M-15. Emerg Infect Dis. 2008;14:195–200. doi:10.3201/eid1402.070350
7. Oteo J, Diestra K, Juan C, Bautista V, Novais A, Pérez-Vázquez M, et al. Extended-spectrum β-lactamase-producing *Escherichia coli* in Spain belong to a large variety of multilocus sequence typing types, including ST10 complex/A, ST23 complex/A and ST131/B2. Int J Antimicrob Agents. 2009;34:173–6. doi:10.1016/j.ijantimicag.2009.03.006

8. Peirano G, Pitout JD. Molecular epidemiology of *Escherichia coli* producing CTX-M β-lactamases: the worldwide emergence of clone ST131 O25:H4. Int J Antimicrob Agents. 2010;35:316–21. doi:10.1016/j.ijantimicag.2009.11.003
9. Eckert C, Gautier V, Saladin-Allard M, Hidri N, Verdet C, Ould-Hocine Z, et al. Dissemination of CTX-M–type β-lactamases among clinical isolates of *Enterobacteriaceae* in Paris, France. Antimicrob Agents Chemother. 2004;48:1249–55. doi:10.1128/AAC.48.4.1249-1255.2004
10. Durmaz R, Otlu B, Koksal F, Hosoglu S, Ozturk R, Ersoy Y, et al. The optimization of a rapid pulsed-field gel electrophoresis protocol for the typing of *Acinetobacter baumannii, Escherichia coli* and *Klebsiella* spp. Jpn J Infect Dis. 2009;62:372–7.
11. Tartof SY, Solberg OD, Manges AR, Riley LW. Analysis of a uropathogenic *Escherichia coli* clonal group by multilocus sequence typing. J Clin Microbiol. 2005;43:5860–4. doi:10.1128/JCM.43.12.5860-5864.2005
12. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol. 2000;66:4555–8. doi:10.1128/AEM.66.10.4555-4558.2000
13. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol. 1981;145:1365–73.
14. Saeed MA, Haque A, Ali A, Mohsin M, Bashir S, Tariq A, et al. Relationship of drug resistance to phylogenetic groups of *E. coli* isolates from wound infections. J Infect Dev Ctries. 2009. 22;3(9):667–70.

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ADDITIONAL RESULTS I:

Antimicrobial susceptibility and mechanisms of resistance to quinolones and β-lactam antibiotics in enteroaggregative and enterotoxigenic *Escherichia coli* causing traveller's diarrhoea

Authors:

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Hypothesis:

Information in the scientific literature regarding the antimicrobial susceptibility of enteroaggregative *Escherichia coli* (EAEC) and enterotoxigenic *E. coli* (ETEC) is scarce, and therefore, it is not clear whether the guidelines recommending empiric treatment currently remain valid. The prevalence of ESBLs as a mechanism of resistance to 3rd generation cephalosporins has mainly been investigated in extraintestinal pathogenic *E. coli* (ExPEC), but few studies have been carried out in diarrhoeagenic *E. coli.*

Objectives:

Assess the antimicrobial resistance of EAEC and ETEC causing traveller's diarrhoea (TD) and investigate the mechanisms of resistance to third-generation cephalosporins, chepamycins and quinolones.

Material and methods:

39 EAEC and 43 ETEC clinical isolates were studied. The susceptibility of EAEC and ETEC against ampicillin, amoxicillin-clavulanic acid, cefotaxime, imipenem, chloramphenicol, tetracycline, cotrimoxazole, nalidixic acid, ciprofloxacin, azithromycin and rifaximin was determined.

All genes encoding resistant determinants were detected by polymerase chain reaction (PCR) and DNA sequencing. The epidemiology of extended-spectrum β-lactamase (ESBL)-producing EAEC and ETECwas studied using multilocus sequence typing (MLST), following the Achtman scheme.

Results:

The resistance of EAEC and ETEC strains causing TD has significantly increased to quinolones, mainly in patients travelling to India and sub-Saharan Africa. ST38 and ST131 carrying the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}27}$ genes, respectively, are highly prevalent among ESBL-producing EAEC and ETEC.

The cephamycinase ACT-20 has also been described for the first time in EAEC and ETEC strains causing TD in patients who had travelled to Central America.

The percentages of resistance to azithromycin in EAEC and ETEC isolates from patients to South-East Asia / India and Africa are greater than 25%. Meanwhile, rifaximin is still active against EAEC and ETEC and does not show a high prevalence of resistant strains.

Conclusions:

Fluoroquinolones should no longer be considered the drugs of choice for the treatment of TD for travellers to India and Africa. Azithromycin and rifaximin are still good alternatives for the treatment of TD caused by EAEC or ETEC.

Emergence of resistance to quinolones and β–lactam antibiotics in enteroaggregative and enterotoxigenic *Escherichia coli* causing traveler's diarrhea.

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Running head: Emergence of antimicrobial resistance in EAEC and ETEC

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E.G. and M.G.Q. contributed equally to this work.

Abstract.

The objective of this study was to assess the antimicrobial resistance of enteroaggregative *Escherichia coli* (EAEC) and enterotoxigenic *E. coli* (ETEC) causing traveler's diarrhea (TD) and investigate the molecular characterization of antimicrobial resistance genes to third generation cephalosporins, chepamycins and quinolones. Overall, 39 EAEC and 43 ETEC clinical isolates were studied. The susceptibility of EAEC and ETEC against ampicillin, amoxicillin-clavulanic acid, cefotaxime, imipenem, chloramphenicol, tetracycline, cotrimoxazole, nalidixic acid, ciprofloxacin, azithromycin and rifaximin was determined. All genes encoding resistant determinants were detected by PCR or PCR and DNA sequencing. The resistance to quinolones of EAEC and ETEC strains causing TD has significantly increased especially in patients traveling to India and sub-Saharan Africa. The epidemiology of extended-spectrum β-lactamase (ESBL)–producing EAEC and ETEC strains was studied using MLST. The ST38 and ST131 carrying the $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-27}}$ genes, respectively, are highly prevalent among these strains. The cephamycinase ACT-20 is described in the present study for the first time in EAEC and ETEC strains causing TD in patients who had traveled to Central America. The percentages of resistance to azithromycin in EAEC and ETEC isolates from patients to South-East Asia/India and Africa are above 25%. Meanwhile, rifaximin is still active against EAEC and ETEC with the prevalence of resistant strains not being high. In conclusion, fluoroquinolones should no longer be considered the drugs of choice for the prevention or treatment in TD for travelers traveling to India and Africa. Azithromycin and rifaximin are still a good alternative to treat TD caused by EAEC or ETEC.

Introduction.

Traveler's diarrhea is the most frequent infection presented by travelers attending a Travel Medicine Unit following a trip to low or middle-income countries (1,2). Enteroaggregative (EAEC) and enterotoxigenic (ETEC) *Escherichia coli* are one of the most frequent bacteria causing traveler's diarrhea (TD) together with *Shigella* spp. and *Campylobacter* spp. (3-5). Other non-bacterial enteric pathogens identified as etiological agents of TD in minor proportions (between 28% and 35%) are norovirus, *Giardia* and *Cryptosporidium* (5). In the cases of TD where antimicrobial treatment is suggested, fluoroquinolones, azithromycin and rifaximin are the antibiotics recommended (6,7). In addition, fluoroquinolones have been considered as an option in the prevention of TD in travelers with high-risk such as immunocompromised patients in whom chemoprophylaxis is considered essential. However, other views concerning the antibiotic use for TD (especially for mild and moderate diarrhea) have emerged recently, as antimicrobials have been shown to be an independent risk factor that predisposes travelers to contracting resistant strains such as extended-spectrum β-lactamase (ESBL)–producing *Enterobacteriaceae* (8).

Information in the scientific literature regarding the antimicrobial susceptibility of EAEC and ETEC is scarce, therefore we do not know whether the guidelines recommending empiric treatment still remain valid. In addition, the prevalence of extended spectrum β-lactamases (ESBLs) as a mechanism of resistance to third generation cephalosporins has mainly been investigated in extraintestinal *E. coli* (ExPEC) but there is little research into diarrheagenic *E. coli.* The main purpose of this study was to assess the antimicrobial

resistance of EAEC and ETEC causing traveler's diarrhea during the period 2011-2017 and investigate the mechanisms of resistance to third generation cephalosporins, cephamycins and quinolones.

Results.

The susceptibility of 39 EAEC and 43 ETEC clinical isolates was determined by disk diffusion, Etest or microdilution and is shown in Figure 1 and also in Supplementary Material (Table 1S). Overall, EAEC showed greater resistance than ETEC, without significant differences. EAEC presented the following percentages of resistance: ampicillin (AMP), 56.4%; amoxicillin-clavulanic acid (AMC), 12.8%; cefotaxime (CTX), 12.8%; cotrimoxazole (SXT), 59%; chloramphenicol (CHL), 7.7%; tetracycline (TET), 51.3%; nalidixic acid (NAL), 43.6%; ciprofloxacin (CIP), 23% and azithromycin (AZT), 23%. Meanwhile the percentages of resistance of the ETEC clinical isolates were: AMP, 48.9%; AMC, 7%; CTX, 14%; SXT, 44.2%; CHL, 11.6%; TET 39,5%; NAL, 44.2%; CIP, 21% and AZT, 14%. All EAEC and ETEC clinical isolates were susceptible to imipenem. Since no breakpoints are defined for rifaximin we determined the MIC_{50} and MIC_{90} for EAEC and ETEC, being 8 and 16, respectively, for the two *E. coli* pathotypes.

The distribution of the percentages of resistance according to the *E. coli* pathotype and the geographical area visited is shown in Table 1. The percentages were similar between EAEC and ETEC, with levels of resistance to cefotaxime greater than 33% in strains isolated from patients traveling to South-East Asia / India. The prevalence of strains resistant to cotrimoxazole was higher in Africa compared to South-East Asia / India and Latin America.

92 The prevalence of nalidixic acid resistant strains was greater than 27% in all areas, being
93 above 64% in South-East Asia / India. The high level (>40%) of strains resistant to
94 ciprofloxacin in South-East Asia / India is worthy of mention, while in Africa the
95 percentage was intermediate (between 11 and 19%) and in Latin America it was below
96 10%. Azithromycin-resistant strains were also more frequent in South-East Asia / India
97 than in Africa and Latin America, with percentages of resistance of 33.3%, 25% and 9.1%
98 respectively for EAEC and 28.6%, 11.1% and 0%, respectively for ETEC. It is important to
99 highlight that 58% of the patients with TD from South-East Asia / India visited India and
100 among these the percentage of resistance to nalidixic acid was 75% and 71.4% for EAEC
101 and ETEC, respectively; ciprofloxacin resistance was 62.5% and 43% for EAEC and ETEC,
102 respectively and the resistance to azithromycin was 37.5% and 28.6% for EAEC and ETEC,
103 respectively (data not shown). However, statistical analysis was not performed due to the
104 low population size obtained when stratifying the strains according to the pathotype and
105 the geographical origin.
106 The mechanisms of resistance to third generation cephalosporins are usually associated
107 with the production of ESBLs. This analysis was determined in all 11 isolates presenting
108 cefotaxime resistance by the disk diffusion test (diameters obtained ranged between 8
109 and 18 mm, considering the following criteria, R ≤ 22mm, I: 23-25mm and S≥) and a
110 positive result by double-disk synergy test. The MIC of cefotaxime was determined,
111 showing a range between 6 and >256 mg/L was found in both EAEC and between 6 and
112 96 µg/ml in ETEC. All the isolates were positive for CTX-M ESBL; eight of them belonged
113 to CTX-M-15 and three to the CTX-M-27. The MLST analysis generated a high
114 heterogeneity of types. Indeed, it was of note that three strains from India belonging to
115 the ST38 (two EAEC and one ETEC) carried the *bla*$_{\text{CTX-M-15}}$ gene; however, the plasmid

116 typing was K for one EAEC strain, FIB and FII for the other EAEC strain and Y for the ETEC
117 strain. In addition, two ETEC strains carrying the *bla*$_{\text{CTX-M-27}}$ gene belonging to the high-risk
118 clone ST131 had the same plasmid type profile (FIB and FIA). One of the patients with TD
119 caused by the ST131 clone had visited India, while another had traveled to Vietnam and
120 Cambodia (Table 2). Moreover, the *bla*$_{\text{CTX-M-27}}$ gene was also found in a singleton (ST1193)
121 of EAEC carrying plasmid replicon types FIB and FIA. Five EAEC and three ETEC isolates
122 were resistant to amoxicillin-clavulanic acid with a MIC from 12 to 64 mg/L (data not
123 shown). The presence of genes encoding OXA, TEM, SHV and plasmid mediated AmpC-
124 type β-lactamases was determined. The *bla*$_{\text{ACT-20}}$ gene was detected in two strains, one
125 EAEC and one ETEC, showing the highest MICs of amoxicillin-clavulanic acid of 24 and 64
126 mg/L, respectively. These patients had visited Guatemala and the Dominican Republic
127 (Table 1S). The remaining six strains (four EAEC and two ETEC) with a MIC of 12 mg/L
128 presented the *bla*$_{\text{OXA-1-like}}$ gene that explains the moderate level of resistance to
129 amoxicillin-clavulanic acid. Two EAEC strains isolated from patients who had traveled to
130 India were also harboring the *bla*$_{\text{SHV-like}}$ gene, and none of the overall strains were found
131 to be carrying the *bla*$_{\text{TEM}}$ gene.
132 Two phenotypes could be defined among quinolone-resistant EAEC (17 strains) and ETEC
133 (20 strains). One was nalidixic acid resistant but ciprofloxacin intermediate or susceptible
134 (NAL^{R}, CIP^{I-S}, considering the following criteria, R <24mm, I: 24-25mm and S$\geq$ 26mm). The
135 second phenotype corresponded to the strains which were resistant to both nalidixic acid
136 and ciprofloxacin (NAL^{R}, CIP^{R}). Both chromosomal- and plasmid-mediated quinolone
137 resistances were found (Table 3). All eight EAEC strains with the NAL^{R}, CIP^{I-S} phenotype
138 showed a mutation in the *gyrA* gene, whereas only one EAEC strains with NAL^{R}, CIP^{R}
139 phenotype presented a mutation in amino acid codon Ser-83 of the *gyrA* gene. The

140 remaining eight strains with the NAL^R, CIP^R phenotype showed the following mechanisms
141 of resistance: Four strains had a mutation in the same position in the *gyrA* gene and in the
142 amino acid codon Ser-80 of the *parC* gene; two strains had the same double mutation plus
143 the *qnrS* gene and two strains also had this double mutation and the *aac(6')-Ib-cr* gene.
144 In the 11 ETEC strains with the Nal^R, CIP^{I-S} phenotype, the mechanisms of resistance to
145 quinolones found were: eight strains with a mutation in the amino acid codon Ser-83 of
146 the *gyrA* gene; one with a mutation in the amino acid codon Asp-87 and only the *qnrS*
147 gene was detected in the last strain (the only one NAL^I, CIP^I). The mechanisms of
148 resistance to quinolones in the nine ETEC strains with the NAL^R, CIP^R phenotype were: one
149 strain with a mutation in the amino acid codon Ser-83 of the *gyrA* gene; three strains with
150 a double mutation in the *gyrA* and *parC* genes as mentioned above; two strains with a
151 *gyr*A gene mutation and the presence of the *qnrS* gene, and finally, three strains with the
152 a double mutation and the *aac(6')-Ib-cr* gene.
153
154
155 **Discussion.**
156
157 ETEC and EAEC cause not only TD but also a high morbidity in children in developing
158 countries, mainly in those under five years of age (9). More than 50% of the patients
159 attending the Tropical Medicine Unit of our hospital presented TD and antimicrobial
160 therapy is needed due to the severity or persistence of the symptoms in around 35% of
161 those in whom diarrhea is caused by ETEC or EAEC (10, 11). Nowadays with the
162 incorporation of rapid diagnostic tests based mainly on multiplex PCR the etiology of the
163 TD can be determined on the same working day, and therefore a more adequate

164 treatment can be implemented (12). Knowledge of the antimicrobial susceptibility of the
165 most frequent etiological agents causing TD such as EAEC or ETEC will help in
166 administering the most adequate treatment before the antimicrobial susceptibility of the
167 bacteria isolated is generated.
168 Overall, in this study, the antimicrobial resistance of EAEC was slightly higher than that of
169 ETEC without significant differences. However, for both, the resistance to the classical and
170 cheaper antibiotics used in developing countries such as ampicillin, cotrimoxazole and
171 tetracycline was greater than 39%. On stratifying the ETEC and EAEC according to the
172 geographical area visited by the patient with TD, it was of note that the strains from Latin
173 America were less resistant than those from South-East Asia / India or Africa, but
174 significance could not be calculated since the population was not large enough. This
175 reflects the situation of antimicrobial resistance in different countries in Latin America
176 versus South-East Asia / India. The latter countries present high rates of resistance to the
177 most available and inexpensive antibiotics, including quinolones, whereas in Latin
178 America ETEC and EAEC strains remain susceptible to these antimicrobial agents (13-19).
179 The high prevalence of quinolone-resistance EAEC and ETEC isolates from South-East Asia
180 / India is worthy of mention with percentages higher than 40% of resistance to
181 ciprofloxacin for both EAEC and ETEC. In a previous study performed by our group during
182 the period 2001-2007, the overall percentage of resistance to nalidixic acid for EAEC and
183 ETEC was 15 and 22%, respectively, and to ciprofloxacin for EAEC and ETEC was 4 and 8%,
184 respectively (20). Therefore, a significant increment ($p<0.0001$ for both nalidixic acid and
185 ciprofloxacin among EAEC strains and $p=0.0013$ for nalidixic acid and $p=0.0062$ for
186 ciprofloxacin among ETEC strains) has been observed, being more dramatic in strains
187 isolated from patients who had traveled to South-East Asia / India, especially India, where

188 53.3% of the total strains were resistant to ciprofloxacin.

189 Four (44%) out of nine EAEC strains and two (33%) out of six ETEC strains with a MIC of
190 azithromycin greater than 16 μg/ml showed a MIC $\geq$ 256 μg/ml. Azithromycin reaches
191 rectal concentrations of a mean of 133 μg/g with a single 1g dose (21), therefore it is
192 above the MICs of most EAEC (89%) and ETEC (95%) strains with a MIC of azithromycin
193 less than 256 μg/ml. The activity of rifaximin against EAEC and ETEC remained unchanged
194 compared to previous studies (22, 23). Rifaximin is a non-absorbable antibiotic reaching a
195 faecal concentration of 7,961 μg/g with a dose of 800 mg daily for three days (24), which
196 is far above the MIC_{90} that we found for EAEC and ETEC.

197 The main mechanisms of resistance to cefotaxime are ESBLs. Different ESBLs have been
198 described to date, being the main types TEM-type, SHV-type, and CTX-M-type, with the
199 latter being the most currently extended ESBL at a global level (25-28). Travelers have
200 been shown to be potential carriers of the ESBL-producing *Enterobacteriaceae* in the
201 intestinal tract, facilitating the dissemination of these microorganisms between countries
202 (8, 28-33). The two most prevalent STs detected were ST38 carrying CTX-M-15 and ST131
203 carrying CTX-M-27. CTX-M-15 producing EAEC strains belonging to ST38 from India
204 causing TD have described previously, demonstrating that ST38 is a successful EAEC group
205 (34,35); however, in our study this ST-type was also found in ETEC strains. In a recent study
206 performed in *E. coli* isolated from diarrheic patients from China, the ST5584 carrying the
207 $bla_{CTX\text{-}M\text{-}15}$ gene isolated in our study in a traveler to China was not reported (36). Three
208 different replicon types (FIB/FII, K and Y) were found in these ST38 EAEC strains,
209 demonstrating the heterogeneity of these genetic elements. In addition, CTX-M-15 or
210 other types of CTX-M-producing *E. coli* ST38 clone, mainly ExPEC, has been detected in
211 Saudi Arabia. In fact, some UPEC ST38 strains were also described as carrying the *aggR*

212 gene, a main feature of EAEC (37). In China, the UK, Bangladesh and Nigeria, ST38 was
213 found among the most frequent STs in a collection of EAEC strains (38, 39).
214 CTX-M-27 producing *E. coli* ST131 has been described in several countries (40), and the
215 $bla_{CTX\text{-}M\text{-}27}$ gene has been detected in EPEC and EIEC strains isolated in China (36). This
216 $bla_{CTX\text{-}M\text{-}27}$ gene has also been detected in one EAEC strain isolated from surface water
217 (41). However as far as we know its presence in ETEC strains has not been reported. In
218 this study, both ETEC strains came from South-East South-East Asia / India, and the $bla_{CTX\text{-}M\text{-}27}$
219 gene was located in an IncF plasmid, as expected (42-44).
220 The main enzymatic mechanisms of *E. coli* associated with the acquisition of resistance to
221 AMC include: 1. Hyperproduction of a plasmid-mediated class A β–lactamases such as
222 TEM-1 and SHV-1; 2. Plasmid-mediated AmpC-type β–lactamase (p-AmpC); 3.
223 Chromosomal AmpC β–lactamase (c-AmpC); 4. Production of inhibitor-resistant TEM (IRT)
224 β–lactamases; and 5. Plasmid-mediated β–lactamase OXA-1 (45). Among the EAEC and
225 ETEC strains resistant to AMC a plasmid-mediated AmpC (ACT-20) was detected only in
226 the two strains with the highest MIC. This type of p-AmpC has previously been found in a
227 strain of *Enterobacter hormaechei* isolated from dog faeces (46), but so far it has not been
228 described in bacteria causing infections in humans. The EAEC and ETEC with moderate
229 resistance to AMC presented an OXA-1 enzyme that is currently the most frequently found
230 mechanism of resistance to AMC (47).
231 The acquisition of resistance to quinolones in *E. coli* can be either chromosomal or
232 plasmid-mediated. Chromosomal mutations generating resistance to quinolones are
233 those associated mainly with the *gyrA* and *parC* genes encoding the A subunits of DNA
234 gyrase and topoisomerase IV, respectively, which are the protein targets of these
235 antibacterial agents. In addition, mutations that produce an overproduction of an efflux

pump or a decreased expression of a gene encoding a porin can also reduce the accumulation of the quinolone, and hence, increase resistance. The plasmid-mediated mechanisms of resistance to quinolones are related to the presence of three genes: i. The *qnr* genes, which protect the protein target of the binding of the quinolones; ii. The *aac(6')-Ib-cr* gene, which produces the acetylation of a radical group of some quinolones generating a decrease in activity and iii. The *qepA* or *opxAB* genes, which are quinolone efflux pumps (48).

In this study the NAL^{R}, CIP^{I-S} phenotype shown by both ETEC and EAEC was mainly associated with a mutation in the *gyrA* gene with the exception of one ETEC strain, showing a NAL^{I}, CIP^{I} phenotype that did not have any mutation in the *gyr*A gene but it presented the *qnrS* gene. The *qnr* gene was not detected in a previous study performed with ETEC and EAEC strains resistant to quinolones (20). In addition, in the present study the NAL^{R}, CIP^{R} phenotype was related to a double mutation in the *gyrA* and *parC* genes alone or together with the *qnrS* or *aac(6')Ib-cr* genes. In a study performed in India, the main mechanisms of resistance to quinolone in ETEC were also amino acid changes in GyrA and ParC. They did not find any Qnr determinant but 65% of the strains presented the *aac(6')Ib-cr* gene (49).

In summary, our results strengthen the message that resistance to quinolones and third generation cephalosporins has increased in EAEC and ETEC strains causing of TD, mainly in patients traveling to India and Africa, and especially sub-Saharan Africa. In addition, the ST38 and ST131 carrying the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}27}$ genes, respectively, are highly prevalent in ESBL-producing EAEC and ETEC. The cephamycinase ACT-20 has also been described for the first time in EAEC and ETEC strains causing TD in patients who had traveled to Central America. The percentages of resistance to azithromycin in EAEC and

ETEC isolates from patients to South-East Asia / India and Africa are above 25%; however, the high concentration of azithromycin reached in the intestinal tract can surpass the MIC of most of azithromycin-resistant strains. Meanwhile, rifaximin is still active against EAEC and ETEC and strains with a MIC > 32 μg/ml were not found. However, it is not recommended as empirical treatment for inflammatory febrile diarrhea due to its non-absorbable nature. The preliminary data obtained regarding the prevalence of resistance to quinolones challenge the recommendation of use of this antibiotic in the treatment of TD in patients visiting or coming from the geographical areas studied, especially India and Africa, although further studies must be done in order to elucidate the prevalence of resistance to fluoroquinolones in larger collections of EAEC and ETEC causing TD as well as in other etiological agents of this infectious disease. In addition, it must be also taken into account that *in vitro* susceptibility testing does not always correlate with lack of success in the clinical practice (50).

Materials and Methods.

Bacterial isolates.

EAEC and ETEC clinical isolates causing TD were investigated in this study. The bacterial isolates were collected from 2011 to 2017. These strains were isolated from patients who were travelers and had diarrhea at the time they visited at the Tropical Medicine Unit in our hospital. None of the patients required hospital admission. The stool samples were collected during the acute phase of diarrhea and were processed within 2 h of collection. The stool specimens were cultured for *E. coli* and other bacterial enteropathogens by

284 conventional methods. Single colony subcultures of all different colonial morphotypes
285 growing on MacConkey agar were identified by conventional criteria (51). These colonies
286 were tested by polymerase chain reaction (PCR) to detect EAEC and ETEC as described
287 elsewhere (52).
288
289 ***Antimicrobial susceptibility testing.***
290 The susceptibility of EAEC and ETEC against ampicillin, amoxicillin-clavulanic acid,
291 cefotaxime, imipenem, chloramphenicol, tetracycline, cotrimoxazole, nalidixic acid, and
292 ciprofloxacin was determined by disk diffusion following the European Committee on
293 Antimicrobial Susceptibility Testing (EUCAST) recommendations. Meanwhile, the
294 minimum inhibitory concentrations (MICs) of amoxicillin-clavulanic acid, cefotaxime and
295 azithromycin were determined by the Etest method, and the MIC of rifaximin was
296 obtained using the microdilution method according to EUCAST guidelines (53). *E. coli*
297 ATCC 25922 and *E. coli* ATCC 35218 were used as controls. The Clinical and Laboratory
298 Standards Institute (CLSI) and EUCAST breakpoints were used to define resistance to
299 nalidixic acid and ciprofloxacin, respectively. The breakpoints of azithromycin considered
300 were those described by EUCAST for *Salmonella* Typhi (MIC ≤16 mg/L for wild type
301 isolates).
302
303 ***Detection of β–lactam and quinolone resistance mechanisms.***
304 Double-disk synergy test was carried out in the cefotaxime-resistant isolates in order to
305 confirm the extended spectrum beta-lactamase (ESBL) carriage (54). The detection of
306 ESBL genes ($bla_{\text{CTX-M}}$, bla_{SHV}, bla_{OXA} and bla_{TEM} genes) was carried out by PCR and DNA
307 sequencing using previously described condition (55). In addition, strains resistant to

amoxicillin-clavulanic acid were also tested to detect the presence of cephamycinases also by PCR and DNA sequencing using specific primers as described previously (56).

To determine the quinolone resistance mechanisms, mutations in the quinolone-resistance determining region of the *gyrA* and *parC* genes were detected by PCR and sequencing was performed as described elsewhere (57, 58). The purified PCR products visualized in gels were processed for DNA sequencing and analyzed in an automatic DNA sequencer (ABI 377; Perkin-Elmer, Emeryville, CA, USA) using the BigDye terminator cycle sequencing kit (v3.1; Perkin-Elmer). Detection of the *qnr* genes screening for the *qnrA, qnrB, qnrC, qnrD and qnrS* genes was performed by multiplex PCR using a combination of specific primers (59). Bacterial strains positive for each *qnr* gene were used as positive controls and were run in each batch of samples tested. Detection of the *aac(6')-Ib-cr* gene was performed using specific described previously (60).

Plasmid typing and multilocus sequence typing (MLST).

Replicon typing was then performed in the strains carrying the bla_{CTXM} genes to know the potential plasmids carrying this resistance gene using the primers designed by Carattoli *et al.* in 2005 but employing the adapted amplification protocols for commensal and pathogenic *E. coli* isolates described by Johnson *et al.* (61) or also using the PCR based replicon typing kit (Diatheva, Cartoceto, Italy). In the same set of strains, the multilocus sequence typing (MLST) was determined analyzing by amplification seven housekeeping genes (*adk, fumC, icd, pur A, gyr B, recA,* and *mdh*) (62). The database available at www.enterobase.warwick.ac.uk was used for assigning sequence types (STs) and clonal complexes (CCs).

Statistical analysis.

Data of the present study is presented as frequencies. The prevalence of resistance was compared to previous data using the Binomial test (63). Proportions were compared using Chi-squared test or Fisher's exact test if the application conditions of the former where not met. Significance was set at 0.05. The analysis was carried out using Stata (64).

Acknowledgements.

This study received funding from the Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD16/0016/0010) and was co-financed by European Development Regional Fund “A way to achieve Europe”. This work was also supported by award 2017 SGR 0809 from the Agència de Gestió d’Ajuts Universitaris i de Recerca of the Generalitat de Catalunya.

We want to thank ALFASIGMA S.p.A. for providing us with the rifaximin-α.

Transparency declarations.

None to declare.

Supplementary data.

Table 1S is available as Supplementary data.

References.

1. Leder K, Torresi J, Libman MD, et al. 2013. GeoSentinel surveillance of illness in returned travelers, 2007-2011. Ann Intern Med 158: 456–68.

2. Hagmann SHF, Han P V., Stauffer WM, et al. 2014. Travel-associated disease among US residents visiting US GeoSentinel clinics after return from international travel. Fam Pract 31: 678–87.

3. Steffen R, Hill DR, DuPont HL. 2015. Traveler's Diarrhea. JAMA 313(1):108.

4. Zboromyrska Y, Hurtado JC, Salvador P, et al. 2014. Aetiology of traveler's diarrhea: evaluation of a multiplex PCR tool to detect different enteropathogens. Clin Microbiol Infect 20: O753–9.

5. Jiang ZD, DuPont HL. Etiology of travelers' diarrhea. 2017. J Travel Med 2017 24: S13–6.

6. Statement on Travelers' Diarrhea - Canada.ca. Available at: https://www.canada.ca/en/public-health/services/travel-health/about-catmat/statement-travellers-diarrhea.html#a55.

7. Riddle MS, Connor BA, Beeching NJ, et al. 2017. Guidelines for the prevention and treatment of travelers' diarrhea: a graded expert panel report. J Travel Med 24: S63–80.

8. Kantele A, Laaveri T, Mero S et al. 2015. Antimicrobials Increase Travelers' Risk of Colonization by Extended-Spectrum Betalactamase-Producing *Enterobacteriaceae*. Clin Infect Dis. 2015 Mar;60(6):837–46.

9. Kotloff KL, Nataro JP, Blackwelder WC, et al. 2013. Burden and aetiology of diarrheal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382: 209–22.

10. Vila J, Vargas M, Ruiz J, Corachan M, Jimenez De Anta MT, Gascon J. 2000. Quinolone

377 resistance in enterotoxigenic *Escherichia coli* causing diarrhea in travelers to India in
378 comparison with other geographical areas. Antimicrob Agents Chemother 44: 1731–3.
379 11. Vila J,Gascón J, Anta MTJ de, et al. 2001. Susceptibility patterns of enteroaggregative
380 *Escherichia coli* associated with traveler's diarrhea: emergence of quinolone resistance. J
381 Med Microbiol 50: 996–1000.
382 12. Zboromyrska Y, Vila J. 2016. Advanced PCR-based molecular diagnosis of
383 gastrointestinal infections: challenges and opportunities. Expert Rev Mol Diagn 16: 631–
384 40.
385 13. Aslani MM, Alikhani MY, Zavari A, Yousefi R, Zamani AR. 2011. Characterization of
386 enteroaggregative *Escherichia coli* (EAEC) clinical isolates and their antibiotic resistance
387 pattern. Int J Infect Dis 15: e136-139.
388 14. Ali MMM, Ahmed SF, Klena JD, Mohamed ZK, Moussa TAA, Ghenghesh KS. 2014.
389 Enteroaggregative *Escherichia coli* in diarrheic children in Egypt: molecular
390 characterization and antimicrobial susceptibility. J Infect Dev Ctries 8: 589–96.
391 15. Guerra JA, Romero-Herazo YC, Arzuza O, Gómez-Duarte OG. 2014. Phenotypic and
392 Genotypic Characterization of Enterotoxigenic *Escherichia coli* Clinical Isolates from
393 Northern Colombia, South America. Biomed Res Int 2014: 1–11.
394 16. Nguyen T V., Le Van P, Le Huy C, Gia KN, Weintraub A. 2005. Detection and
395 Characterization of Diarrheagenic *Escherichia coli* from Young Children in Hanoi, Vietnam.
396 J Clin Microbiol 43: 755–60.
397 17. Medina AM, Rivera FP, Pons MJ, et al. 2015. Comparative analysis of antimicrobial
398 resistance in enterotoxigenic *Escherichia coli* isolates from two paediatric cohort studies
399 in Lima, Peru. Trans R Soc Trop Med Hyg 109: 493–502.

400 18. Begum YA, Talukder KA, Azmi IJ, et al. 2016. Resistance Pattern and Molecular
401 Characterization of Enterotoxigenic *Escherichia coli* (ETEC) Strains Isolated in Bangladesh
402 PLoS One 11: e0157415.
403 19. Odetoyin BW, Hofmann J, Aboderin AO, Okeke IN. 2015. Diarrheagenic *Escherichia coli*
404 in mother-child Pairs in Ile-Ife, South Western Nigeria. BMC Infect Dis 16: 28.
405 20. Mendez Arancibia E, Pitart C, Ruiz J, Marco F, Gascon J, Vila J. 2009. Evolution of
406 antimicrobial resistance in enteroaggregative *Escherichia coli* and enterotoxigenic
407 *Escherichia coli* causing traveler's diarrhea. J Antimicrob Chemother 64: 343–7.
408 21. Kong FYS, Rupasinghe TW, Simpson JA, et al. 2017. Pharmacokinetics of a single 1g
409 dose of azithromycin in rectal tissue in men. Winston A, ed. PLoS One 12: e0174372.
410 22. Sierra JM, Ruiz J, Navia MM, Vargas M, Vila J. 2001. *In vitro* activity of rifaximin against
411 enteropathogens producing traveler's diarrhea. Antimicrob Agents Chemother 45: 643-4.
412 23. Ruiz J, Mensa L, O'Callaghan C, et al. 2007. *In vitro* antimicrobial activity of rifaximin
413 against enteropathogens causing traveler's diarrhea. Diagn Microbiol Infect Dis 59: 473–
414 5.
415 24. Jiang ZD, Ke S, Palazzini E, Riopel L, Dupont H. 2000. *In vitro* activity and fecal
416 concentration of rifaximin after oral administration. Antimicrob Agents Chemother 44:
417 2205–6.
418 25. Woodford N, Turton JF, Livermore DM. 2011. Multiresistant Gram-negative bacteria:
419 the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol
420 Rev 35: 736–55.
421 26. Pitout JDD. 2012. Extraintestinal Pathogenic *Escherichia coli:* A Combination of
422 Virulence with Antibiotic Resistance. Front Microbiol 3: 9.
423 27. Doi Y, Iovleva A, Bonomo RA. 2017. The ecology of extended-spectrum β-lactamases

424 (ESBLs) in the developed world. J Travel Med 24: S44–51.
425 28. Woerther P-L, Burdet C, Chachaty E, Andremont A. 2013. Trends in human fecal
426 carriage of extended-spectrum β-lactamases in the community: toward the globalization
427 of CTX-M. Clin Microbiol Rev 26: 744–58.
428 29. Sole M, Pitart C, Oliveira I, et al. 2014. Extended spectrum β-lactamase-producing
429 *Escherichia coli* faecal carriage in Spanish travelers returning from tropical and subtropical
430 countries. Clin Microbiol Infect 20: O636–9.
431 30. Kuenzli E, Jaeger VK, Frei R, et al. 2014. High colonization rates of extended-spectrum
432 β-lactamase (ESBL)-producing *Escherichia coli* in Swiss Travelers to South South-East Asia
433 / India– a prospective observational multicentre cohort study looking at epidemiology,
434 microbiology and risk factors. BMC Infect Dis 14: 528.
435 31. Yaita K, Aoki K, Suzuki T, et al. 2014. Epidemiology of Extended-Spectrum β-Lactamase
436 Producing *Escherichia coli* in the Stools of Returning Japanese Travelers, and the Risk
437 Factors for Colonization. PLoS One 9: e98000.
438 32. Lääveri T, Vlot JA, van Dam AP, et al. 2018. Extended-spectrum beta-lactamase-
439 producing *Enterobacteriaceae* (ESBL-PE) among travellers to Africa: Destination-specific
440 data pooled from three European prospective studies. BMC Infect Dis. 18(1):1–12.
441 33. Arcilla MS, van Hattem JM, Haverkate MR, et al. 2017. Import and spread of extended-
442 spectrum β-lactamase-producing *Enterobacteriaceae* by international travelers (COMBAT
443 study): a prospective, multicentre cohort study. Lancet Infect Dis 17:78–85.
444 34. Guiral E, Mendez-Arancibia E, Soto SM, et al. 2011. CTX-M-15–producing
445 Enteroaggregative *Escherichia coli* as Cause of Travelers' Diarrhea. Emerg Infect Dis 17:
446 1950–3.

447 35. Chattaway MA, Jenkins C, Ciesielczuk H, et al. 2014. Evidence of evolving
448 extraintestinal enteroaggregative *Escherichia coli* ST38 clone. Emerg Infect Dis 20: 1935–
449 7.
450 36. Bai L, Wang L, Yang X, et al. 2017. Prevalence and Molecular Characteristics of
451 Extended-Spectrum β-Lactamase Genes in *Escherichia coli* Isolated from Diarrheic
452 Patients in China. Front Microbiol 8: 144.
453 37. Alghoribi MF, Gibreel TM, Farnham G, Al Johani SM, Balkhy HH, Upton M. 2015.
454 Antibiotic-resistant ST38, ST131 and ST405 strains are the leading uropathogenic
455 *Escherichia coli* clones in Riyadh, Saudi Arabia. J Antimicrob Chemother 70: 2757–62.
456 38. Okeke IN, Wallace-Gadsden F, Simons HR, et al. 2010. Multi-locus sequence typing of
457 enteroaggregative *Escherichia coli* isolates from Nigerian children uncovers multiple
458 lineages. PLoS One **5**: e14093.
459 39. Chattaway MA, Jenkins C, Rajendram D, et al. 2014. Enteroaggregative *Escherichia coli*
460 Have Evolved Independently as Distinct Complexes within the *Escherichia coli* Population
461 with Varying Ability to Cause Disease. PLoS One 9: e112967.
462 40. Matsumura Y, Pitout JDD, Gomi R, et al. 2016. Global *Escherichia coli* Sequence Type
463 131 Clade with bla $_{CTX\text{-}M\text{-}27}$ Gene. Emerg Infect Dis 22: 1900–7.
464 41. Franz E, Veenman C, van Hoek AHAM, Husman A de R, Blaak H. 2015. Pathogenic
465 *Escherichia coli* producing Extended-Spectrum β-Lactamases isolated from surface water
466 and wastewater. Sci Rep 5: 14372.
467 42. Carattoli A, Miriagou V, Bertini A, et al. 2006. Replicon typing of plasmids encoding
468 resistance to newer beta-lactams. Emerg Infect Dis 12: 1145–8.

469 43. Coque TM, Novais Â, Carattoli A, et al. 2008. Dissemination of Clonally Related
470 *Escherichia coli* Strains Expressing Extended-Spectrum β-Lactamase CTX-M-15. Emerg
471 Infect Dis 14: 195–200.
472 44. Mshana SE, Imirzalioglu C, Hossain H, Hain T, Domann E, Chakraborty T. 2009.
473 Conjugative IncFI plasmids carrying CTX-M-15 among *Escherichia coli* ESBL producing
474 isolates at a University hospital in Germany. BMC Infect Dis 9: 97.
475 45. Jacoby GA. AmpC B-Lactamases. 2009. Clin Microbiol Rev 22: 161-82.
476 46. Smet A, Vaes R, Praud K, et al. 2014. New broad-spectrum β-lactamases emerging
477 among Enterobacteriaceae from healthy cats and dogs: a public health concern? Int J
478 Antimicrob Agents 44: 81–2.
479 47. Ortega A, Oteo J, Aranzamendi-Zaldumbide M, et al. 2012. Spanish Multicenter Study
480 of the Epidemiology and Mechanisms of Amoxicillin-Clavulanate Resistance in *Escherichia*
481 *coli*. Antimicrob Agents Chemother 56: 3576–81.
482 48. Fàbrega A, Madurga S, Giralt E, Vila J. 2009. Mechanism of action of and resistance to
483 quinolones. Microb Biotechnol 2: 40–61.
484 49. Pazhani, GP; Chakraborty, S; Fujihara, et al. 2011. QRDR mutations, efflux system &
485 antimicrobial resistance genes in enterotoxigenic *Escherichia coli* isolated from an
486 outbreak of diarrhea in Ahmedabad, India. Indian J Med Res 134: 214–23.
487 49. Vargas M, Gascón J, Gallardo F, Jimenez De Anta MT, Vila J. 1998. Prevalence of
488 diarrheagenic *Escherichia coli* strains detected by PCR in patients with travelers' diarrhea.
489 Clin Microbiol Infect 4: 682–8.
490 50. Cavallo J, Mérens A. 2008. Spectre d'activité antibactérien d'un antibiotique et
491 catégorisation clinique. Pathol Biol (Paris) 56(5):300–4.
492 51. Álvarez M, Buesa J, Castillo J, Vila J. Diagnóstico microbiológico de las infecciones

493 gastrointestinales. 2008. Vol. 30, Sociedad de Enfermedades Infecciosas y Microbiología
494 Clínica.
495 52. Ruiz-Blázquez J, Vargas M, Nataro JP, Vila J, Gascón i Brustenga J. 2005. Validation of
496 a PCR technique for the detection of enteroagreggative *Escherichia coli* causing traveler's
497 diarrhea. Enferm Infecc Microbiol Clin 23: 479–81.
498 53. EUCAST. 2018.Eur Comm Antimicrob Susceptibility Testing Break tables Interpret MICs
499 Zone diameters, version 80.
500 54. Calvo J, Cantón R, Fernández-Cuenca F, Mirelis B, Navarro F. Procedimientos en
501 microbiología clínica. 2011. Sociedad Española de Enfermedades Infecciosas y
502 Microbiología Clínica.
503 55. Woodford N, Fagan EJ, Ellington MJ. 2006. Multiplex PCR for rapid detection of genes
504 encoding CTX-M extended-spectrum β-lactamases. J Antimicrob Chemother 57: 154–5.
505 56. Chuong L Van, Prachayasittikul V, Isarankura Na Ayudhya C, Lawung R. 2018. Multiplex
506 PCR scheme for variant plasmid mediated class C β-lactamase typing. J Clin Lab Anal 32:
507 e22298.
508 57. Vila J, Ruiz J, Marco F, et al. 1994. Association between double mutation in gyrA gene
509 of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. Antimicrob Agents
510 Chemother 38: 2477–9.
511 58. Vila J, Ruiz J, Goñi P, De Anta MT. 1996. Detection of mutations in parC in quinolone-
512 resistant clinical isolates of *Escherichia coli*. Antimicrob Agents Chemother 40: 491–3.
513 59. Cattoir V, Poirel L, Rotimi V, Soussy C-J, Nordmann P. 2007. Multiplex PCR for detection
514 of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial
515 isolates. J Antimicrob Chemother 60: 394–7.

516 60. Park CH, Robicsek A, Jacoby GA, Sahm D, Hooper DC. 2006. Prevalence in the United
517 States of *aac(6')-Ib-cr* Encoding a Ciprofloxacin-Modifying Enzyme. Antimicrob Agents
518 Chemother 50: 3953–5.
519 61. Johnson TJ, Wannemuehler YM, Johnson SJ, et al. 2007. Plasmid Replicon Typing of
520 Commensal and Pathogenic *Escherichia coli* Isolates. Appl Environ Microbiol 73: 1976–83.
521 62. Wirth, T., Falush, D., Lan R, et al. 2006. Sex and virulence in *Escherichia coli*: An
522 evolutionary prespective. Mol Microbiol 60: 1136–51.
523 63. Hoel, P. G. 1984. Introduction to Mathematical Statistics. 5th ed. New York: Wiley.
524 64. StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp
525 LLC.
526

527

528 **FIGURE 1** Percentages of resistance to different antibacterial agents in EAEC and ETEC strains.

529 AMP: Ampicillin; AMC: Amoxicillin-clavulanic acid; CTX: Cefotaxime; IMI: Imipenem; SXT: Cotrimoxazole;

530 CHL: Chloramphenicol; NAL: Nalidixic acid; CIP: ciprofloxacin; TET: Tetracycline; AZT: Azithromycin

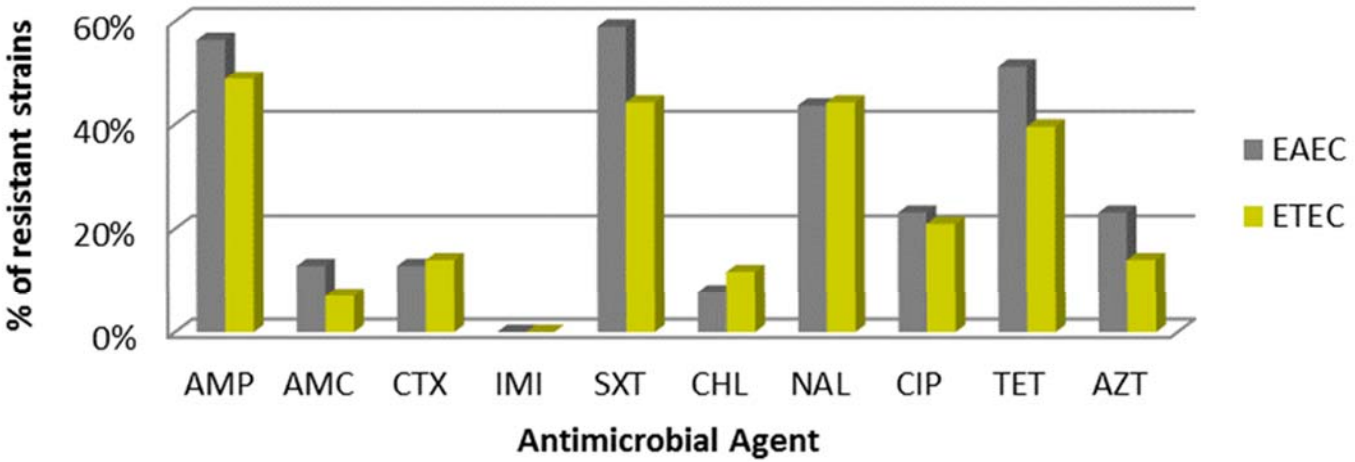

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TABLE 1 Percentage of resistance of EAEC and ETEC to different antimicrobial agents according to three geographical areas.

Antimicrobial Agent	% EAEC – resistant isolates (n)			%ETEC – resistant isolates (n)		
	South-East Asia - India (n=12)	Africa (n=16)	Latin America (n=11)	South-East Asia - India (n=14)	Africa (n=18)	Latin America (n=11)
Cefotaxime	33.3 (4)*	6.3 (1)*	0 (0)	42.9 (6)*	0 (0)	0 (0)
Cotrimoxazole	50 (6)	81.3 (13)	36.4 (4)	35.7 (5)	61.1 (11)	27.3 (3)
Nalidixic acid	66.7 (8)	37.5 (6)	27.3 (3)	64.3 (9)	38.9 (7)	27.3 (3)
Ciprofloxacin	41.7 (5)	18.8 (3)	9.1 (1)	42.9 (6)	11.1 (2)	9.1 (1)
Azithromycin	33.3 (4)	25 (4)	9.1 (1)	28.6 (4)	11.1 (2)	0 (0)

* ESBL–producing strains

TABLE 2 Main features of the EAEC and ETEC clinical isolates carrying ESBLs.

Isolate Number	Geographical origin	MLST ST	MLST CC	Plasmid typing	CTX-M-type	MIC of CTX (µg/ml)
EAEC						
5	India	ST1193	Singleton	FIB, FIA	CTX-M-27	8
11	India	ST38	ST38	FIB, FII	CTX-M-15	>256
20	India	ST7615	Singleton	I1, K, B/O	CTX-M-15	6
70	India	ST38	ST38	K	CTX-M-15	128
84	Togo	ST44	ST10	I1, FIA	CTX-M-15	>256
ETEC						
36	India/Nepal	ST23	ST23	FIB	CTX-M-15	64
38	India	ST1284	Singleton	FIA	CTX-M-15	96
39	India	ST131	ST131	FIA, FIB	CTX-M-27	6
43	Vietnam/Cambodia	ST131	ST131	FIA, FIB	CTX-M-27	12
102	China	ST5584	Singleton	Y	CTX-M-15	12
107	India	ST38	ST38	Y	CTX-M-15	64

TABLE 3 Mechanisms of resistance to quinolones in EAEC and ETEC strains.

	Quinolone-resistant EAEC isolates		**Quinolone-resistant ETEC isolates**	
Quinolone resistance mechanisms	**Nal^R Cip^{I-S} (n=8)**	**Nal^R Cip^R (n=9)**	**Nal^R Cip^{I-S} (n=11)**	**Nal^R Cip^R (n=9)**
gyrA mutation	8	1	10	1
gyrA + parC mutations	0	4	0	3
gyrA mutations + *qnrS*	0	0	0	2
gyrA + parC mutations + *qnrS*	0	2	0	0
gyrA + parC mutations + *aac(6')-Ib-cr*	0	2	0	3
qnrS	0	0	1 (Nal^I Cip^I)	0

TABLE 1S. Overall results.

Isolate	Country/ies visited	Geographical area	Antimicrobial Agent											β-lactamases	Plasmid /s Replicon Type/s	MLST		Quinolone resistance mechanism
			AMP	AMC	CTX	IMP	SXT	CHL	NAL	CIP	TET	AZT	RFX*			ST	CC	
EAEC																		
1	Indonesia, Japan, Singapore, India	Asia	S	S	S	S	S	S	R	I	S	S	8	-	-	-	-	*gyrA* S83L
2	Sudan	Africa	S	S	S	S	R	S	S	S	S	S	16	-	-	-	-	-
3	Ethiopia	Africa	S	S	S	S	S	S	S	S	S	S	4	-	-	-	-	-
4	Peru	Latin America	S	S	S	S	S	S	R	I	S	S	8	-	-	-	-	*gyrA* S83L
5	India	Asia	R	S	R	S	R	S	R	R	R	R	8	CTX-M-27	FIB, FIA	ST1193	NONE	*gyrA* S83L / *parC* S80I
9	Guatemala	Latin America	S	R	S	S	S	S	S	S	I	S	16	ACT-20	-	-	-	-
11	India	Asia	R	R	R	S	R	S	R	R	R	S	16	CTX-M-15, OXA-1	FIB, FII	ST38	ST38	*gyrA* S83L / *parC* S80I /*aac(6´)-Ib-cr*
13	Burkina Faso	Africa	R	S	S	S	R	S	R	I	R	S	8	-	-	-	-	*gyrA* S83L
15	Madagascar	Africa	R	R	S	S	R	R	R	R	R	R	16	-	-	-	-	*gyrA* S83L / *parC* S80I
16	Morocco	Africa	S	S	S	S	S	S	S	S	S	S	16	-	-	-	-	-
17	Peru	Latin America	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
18	Morocco	Africa	S	S	S	S	S	S	S	S	S	S	16	-	-	-	-	-
19	Laos	Asia	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
20	India	Asia	R	S	R	S	R	S	R	R	R	S	16	CTX-M-15, SHV	I1, K, B/O	ST7615	NONE	*gyrA* S83L
21	Burkina Faso	Africa	R	S	S	S	R	S	R	S	R	S	16	-	-	-	-	*gyrA* S83A
25	Sri Lanka	Asia	R	S	S	S	I	S	S	S	S	R	16	-	-	-	-	-
26	Senegal	Africa	R	S	S	S	R	S	S	S	R	S	16	-	-	-	-	-
27	Cuba	America	S	S	S	S	S	S	S	S	S	S	16	-	-	-	-	-

61	Thailand, India	Asia	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
64	Guinea	Africa	R	S	S	S	R	S	S	S	R	S	8	-	-	-	-	-
65	Bolivia	America	R	S	S	S	R	S	S	S	R	S	8	-	-	-	-	-
66	Senegal	Africa	R	S	S	S	R	S	S	S	R	S	16	-	-	-	-	-
67	Bolivia	America	R	S	S	S	S	S	R	S	R	S	16	-	-	-	-	*gyrA* S83L
69	Haiti	America	R	S	S	S	R	S	S	S	R	S	16	-	-	-	-	-
70	India	Asia	R	R	R	S	S	S	R	R	R	S	16	CTX-M-15, OXA-1, SHV	K	ST38	ST38	*gyrA* S83L / *parC* S80I / *aac(6´)-Ib-cr*
72	Cambodia	Asia	R	S	S	S	R	S	R	I	R	S	4	-	-	-	-	*gyrA* S83L
73	Senegal	Africa	S	S	S	S	R	S	S	S	S	R	8	-	-	-	-	-
74	Bolivia, Ecuador	America	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
75	Sudan	Africa	R	S	S	S	R	S	R	R	R	R	16	-	-	-	-	*gyrA* S83L / *parC* S80I
77	Thailand	Asia	R	S	S	S	R	S	R	S	S	S	32	-	-	-	-	*gyrA* S83A
78	India	Asia	R	S	S	S	S	S	R	R	R	R	8	-	-	-	-	*gyrA* S83L / *parC* S80I / *qnrS*
79	India	Asia	S	S	S	S	R	S	I	S	S	R	8	-	-	-	-	-
82	Colombia	America	R	S	S	S	R	S	I	S	R	S	8	-	-	-	-	-
83	Sierra Leone	Africa	R	S	S	S	R	R	S	S	R	S	16	-	-	-	-	-
84	Togo	Africa	R	R	R	S	R	S	R	R	R	R	16	CTX-M-15, OXA-1	I1, FIA	ST44	ST10	*gyrA* S83L / *parC* S80I / *qnrS*
85	Chad	Africa	S	S	S	S	R	R	S	S	R	S	8	-	-	-	-	-
86	Mozambique	Africa	S	S	S	S	R	S	R	S	S	S	8	-	-	-	-	*gyrA* S83L
87	Ecuador	America	R	S	S	S	R	S	R	R	S	R	16	-	-	-	-	*gyrA* S83L / *parC* S80I
88	Ecuador, Peru	America	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
ETEC																		
30	Indonesia	Asia	R	S	S	S	S	R	S	S	R	S	16	-	-	-	-	-
31	Senegal, Cameroon	Africa	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
32	Senegal	Africa	R	S	S	S	R	S	R	R	R	R	32	-	-	-	-	*gyrA* S83L / *qnrS*

33	Morocco	Africa	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
34	Cape Verde	Africa	R	S	S	S	S	S	S	S	S	S	16	-	-	-	-	-
35	Kenya	Africa	R	S	S	S	I	S	S	S	R	S	32	-	-	-	-	-
36	India, Nepal	Asia	R	S	R	S	S	S	R	S	S	S	8	CTX-M-15	FIB	ST23	ST23	*gyrA* S83V
38	India	Asia	R	R	R	S	S	S	R	R	R	S	32	CTX-M-15, OXA-1	FIA	ST1284	NONE	*gyrA* S83L / *parC* S80I / *aac(6´)-Ib-cr*
39	India	Asia	R	S	R	S	R	S	R	R	R	R	16	CTX-M-27	FIB, FIA	ST131	ST131	*gyrA* S83L / *parC* S80I
40	Thailand	Asia	S	S	S	S	S	S	S	S	R	S	32	-	-	-	-	-
41	Dominican Republic	America	S	S	S	S	S	S	R	R	S	S	4	-	-	-	-	*gyrA* S83L
42	Uzbekistan	Asia	R	S	S	S	R	R	R	R	R	R	16	-	-	-	-	*gyrA* S83L / *parC* S80I / *aac(6´)-Ib-cr*
43	Vietnam, Cambodia	Asia	R	S	R	S	R	S	R	R	R	R	16	CTX-M-27	FIB, FIA	ST131	ST131	*gyrA* S83L / *parC* S80I
44	Senegal	África	S	S	S	S	R	R	S	S	S	S	16	-	-	-	-	-
46	Senegal, Cameroon	Africa	S	S	S	S	R	S	S	S	S	S	4	-	-	-	-	-
47	Ghana	Africa	R	S	S	S	R	R	R	I	R	S	4	-	-	-	-	*gyrA* S83L
48	Zimbabwe	Africa	R	S	S	S	S	S	R	R	S	S	8	-	-	-	-	*gyrA* S83L / *parC* S80I
49	Egypt	Africa	S	S	S	S	S	S	I	I	S	S	8	-	-	-	-	*qnrS*
50	Kenya	Africa	S	S	S	S	R	S	R	S	R	S	16	-	-	-	-	*gyrA* S83L
51	Thailand	Asia	S	S	S	S	S	S	S	S	S	S	16	-	-	-	-	-
52	Peru	América	S	S	S	S	S	S	R	I	S	S	16	-	-	-	-	*gyrA* D87Y
53	Senegal	Africa	R	S	S	S	R	S	S	S	R	S	4	-	-	-	-	-
54	Morocco	Africa	S	S	S	S	S	S	R	S	S	S	8	-	-	-	-	*gyrA* S83A
55	Argentina, Brazil	America	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
57	India	Asia	R	S	S	S	R	S	S	S	R	S	4	-	-	-	-	-
58	Tanzania	Africa	R	S	S	S	R	S	S	S	S	S	4	-	-	-	-	-
59	Dominican Republic	America	R	R	S	S	S	S	S	S	I	S	16	ACT-20	-	-	-	-
94	India	Asia	S	S	S	S	S	S	R	S	S	S	4	-	-	-	-	*gyrA* S83L

95	Nepal, India, Philippines	Asia	S	S	S	S	S	S	S	S	R	S	8	-	-	-	-	-
97	Peru	America	R	S	S	S	R	S	S	S	S	S	8	-	-	-	-	-
98	Costa Rica	America	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
99	Peru, Colombia	America	R	S	S	S	R	S	R	S	S	S	8	-	-	-	-	*gyrA* S83L
100	Nigeria	Africa	R	S	S	S	R	S	R	S	S	R	8	-	-	-	-	*gyrA* S83L
102	Republic of China	Asia	R	S	R	S	S	S	R	R	S	S	8	CTX-M-15	Y	ST5584	NONE	*gyrA* S83L / *qnrS*
103	Paraguay	America	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
105	Philippines	Asia	S	S	S	S	S	S	R	S	S	S	4	-	-	-	-	*gyrA* S83L
106	Kenya	Africa	S	S	S	S	R	S	R	S	R	S	16	-	-	-	-	*gyrA S*83L
107	India	Asia	R	R	R	S	R	R	R	R	R	R	8	CTX-M-15, OXA-1	Y	ST38	ST38	gyrA *S83L* / *parC* S80I / *aac(6´)-Ib-cr*
110	Nicaragua	America	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
111	Haiti	America	R	S	S	S	R	S	S	S	R	S	8	-	-	-	-	-
112	Mexico	America	S	S	S	S	S	S	S	S	S	S	8	-	-	-	-	-
113	Burkina Faso	Africa	S	S	S	S	R	S	S	S	R	S	8	-	-	-	-	-
115	Senegal, Cameroon	Africa	S	S	S	S	R	S	S	S	S	S	4	-	-	-	-	-

AMP: ampicillin; AMC: amoxicillin-clavulanic acid; CTX: cefotaxime; IMI: imipenem; SXT: cotrimoxazole; CHL: chloramphenicol; NAL: nalidixic acid; CIP: ciprofloxacin; TET: tetracycline; AZT: azithromycin; RFX: rifaximin; S: susceptible; I: intermediate; R: resistant.

* Expressed in µg/ml as no breakpoints are stablished for rifaximin.

ADDITIONAL RESULTS II:

Epidemiology of enteroaggregative and enterotoxigenic *Escherichia coli* causing traveller's diarrhoea from South-East Asia, Latin America and Africa

Authors:

Elisabet Guiral, Marta Marí-Almirall, Jennifer García, Ignasi Roca, Pilar Salvador, Sara M. Soto, Francesc Marco, Jordi Vila.

Hypothesis:

There is an important lack on studies on the epidemiologic relationship of EAEC an ETEC clinical isolates causing TD in low and middle income countries in the literature.

Objectives:

Detect the possible epidemiologic relationship of EAEC and ETEC isolates causing TD worldwide and determine, if it is possible, the best epidemiological technique in order to stablish a gold standard protocol to compare the EAEC and ETEC strains isolated worldwide.

Material and methods:

Ninety-six *E. coli* isolates (49 EAEC and 47 ETEC) from patients with diarrhoea attended at the Tropical Medicine Unit of Hospital Clínic of Barcelona from 2001 to 2014 were included in the collection. VFGs encoding for the toxins LT and ST, the *aggR* global regulator and the *aatA* gene were detected by PCR in order to characterise the diarrhoeagenic pathotype of the isolates. Multilocus sequence typing (MLST) was carried out by amplification and sequencing of the 7 *E. coli* housekeeping genes using the Achtman scheme and sequence types (ST), and clonal complexes (CC) were assigned according to the eBURST program. Classification of isolates into *E. coli* phylogenetic groups was done using the Clermont *et al.* quadruplex PCR-based protocol. Cluster analysis of all the *E. coli* STs included in the study based on the concatenated partial sequences of the genes used for MLST was carried out.

Results:

Phylogenetic group A was the most prevalent among the EAEC and ETEC collection (39%), followed by the B1 and E phylogenetic groups (around 20% each). None of the isolates belonged to phylogenetic group B2. Phylogenetic group A was less prevalent in South-East Asia but more frequently found among Latin American isolates.

Forty-seven different STs and 21 different CCs were assigned among the isolates studied. The most prevalent STs among the isolates studied were ST10 (14.4%) and ST4 (8.3%), both belonging to CC10. The composition of the other STs found in each geographical area was very heterogeneous.

The two pathotypes were not grouped separately in the cluster analysis of the *E. coli* STs of the collection.

Conclusions:

EAEC and ETEC strains may not be associated with the B2 lineage, mainly belonging to phylogenetic group A.

There exists a high clonal diversity among the EAEC and ETEC isolates causing TD. EAEC and ETEC strains are simply any *E. coli* lineage that can acquire, express and retain plasmids harbouring colonisation factors and/or toxins, and therefore, the classification of pathotypes is not related to the *E. coli* phylogeny.

MLST and phylogenetic grouping are very reliable tools for epidemiological studies of EAEC and ETEC isolates causing TD or intestinal disease.

Epidemiology of enteroaggregative and enterotoxigenic *Escherichia coli* causing traveller's diarrhoea from South-East Asia, Latin America and Africa

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Traveller's diarrhoea (TD) is an infection affecting over 50% of travellers returning from low and middle-income countries (LMIC) (1,2). Together with *Shigella* spp., enteroaggregative *Escherichia coli* (EAEC) and enterotoxigenic *E. coli* (ETEC) are the most frequent bacteria causing TD (3,4). Only a few studies on the epidemiological relationship of EAEC an ETEC clinical isolates causing TD in LMIC are available to date (5–10).These intestinal *E. coli* pathotypes are classified according to the virulence-related properties they possess, the determinants of which are usually located in plasmids. The aim of this study is to epidemiologically characterise EAEC and ETEC isolates causing TD in travellers to LMIC using two different typing methodologies: phylogenetic grouping and multilocus sequence typing (MLST).

The study

A total of 96 *E. coli* isolates (49 EAEC and 47 ETEC) from patients with diarrhoea attended at the Tropical Medicine Unit of Hospital Clínic of Barcelona from 2001 to 2014 were included in the collection. The stool specimens were cultured for *E. coli* and other bacterial enteropathogens by conventional methods (11). Single colony subcultures of all different colonial morphotypes growing on MacConkey agar were identified by conventional criteria (12), and identification at the species level was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) prior to further analysis (13). In order to classify the isolates into the diarrhoeagenic *E. coli* pathotypes EAEC and ETEC, the *aatA* gene (previously referred to as CVD432 or AA probe), the *aggR* global regulator, and the genes encoding for heat-labile (LT) and heat-stable (ST) enterotoxins were detected by polymerase chain reaction (PCR) as described elsewhere (12,14,15). Two different approaches for determining the possible epidemiological relationship between EAEC and ETEC isolated in different continents were carried out: (i) the updated methodology of phylogenetic grouping established by Clermont *et al.* in 2013 (16), based on quadruplex PCR, with subsequent duplex PCRs if required, and (ii) multilocus sequence typing (MLST) by amplification and sequencing of the partial sequences of 7 *E. coli* housekeeping genes according to the Achtman scheme (17). The Enterobase database (http://enterobase.warwick.ac.uk/) was used to assign sequence types (STs) among the 8,498 STs

described up to now and the goeBURST software (http://www.phyloviz.net/goeburst) was used to evaluate the population structure of all STs as well as to allocate them to specific clonal complexes (CCs). For each ST described, the concatenated sequences of the 7 *E. coli* housekeeping genes were aligned and compared using cluster analysis. Cluster analysis was performed using the MEGA 5.1 software (18). The MUSCLE program was used for sequence alignment (19) and phylogenetic trees were reconstructed using the neighbour-joining method with genetic distances computed by the Kimura 2-parameter model using bootstrap values based on 1000 replicates (20). STs that were identified in both EAEC and ETEC isolates were also reflected in the cluster analysis.

Results

The phylogenetic grouping of all 96 isolates revealed that the phylogenetic group A was the most prevalent among the EAEC and ETEC collection (39%), followed by the B1 and E phylogenetic groups (23% and 17%, respectively). None of the isolates belonged to phylogenetic group B2 (*Fig. 1*).

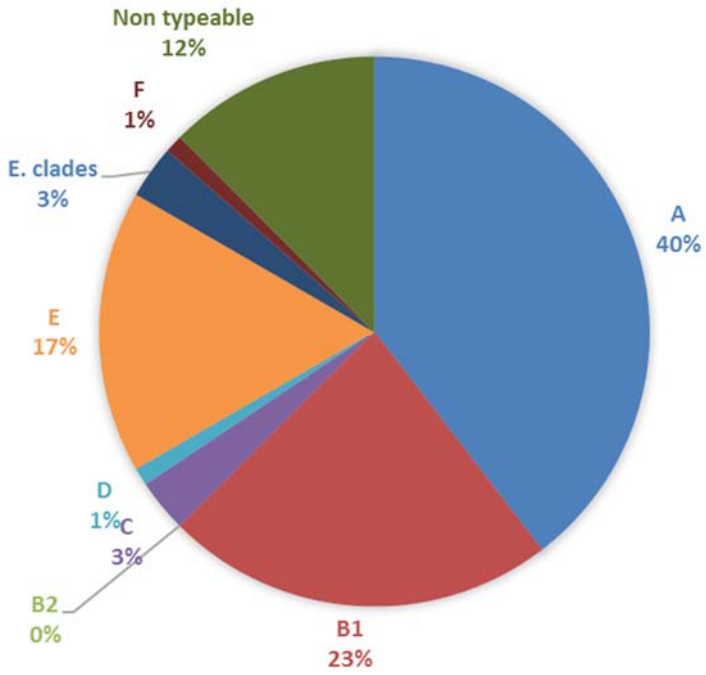


Figure 1. Overall distribution of the phylogenetic groups.

Figure 2 shows the distribution of phylogenetic groups according to the EAEC (*Fig. 2A*) or ETEC (*Fig. 2B*) pathotypes. Phylogenetic group A was still predominant in both pathotypes but while ETEC isolates can be classified in all phylogenetic groups but B2, we did not find isolates belonging to phylogenetic groups C, D or F (B2 as well) among EAEC isolates. Likewise, the phylogenetic group B1 was more prevalent among EAEC (33%) than among ETEC (13%) isolates. We did not observe major differences regarding the distribution of phylogenetic groups E and *E*. clades or the percentage of non-typeable isolates.

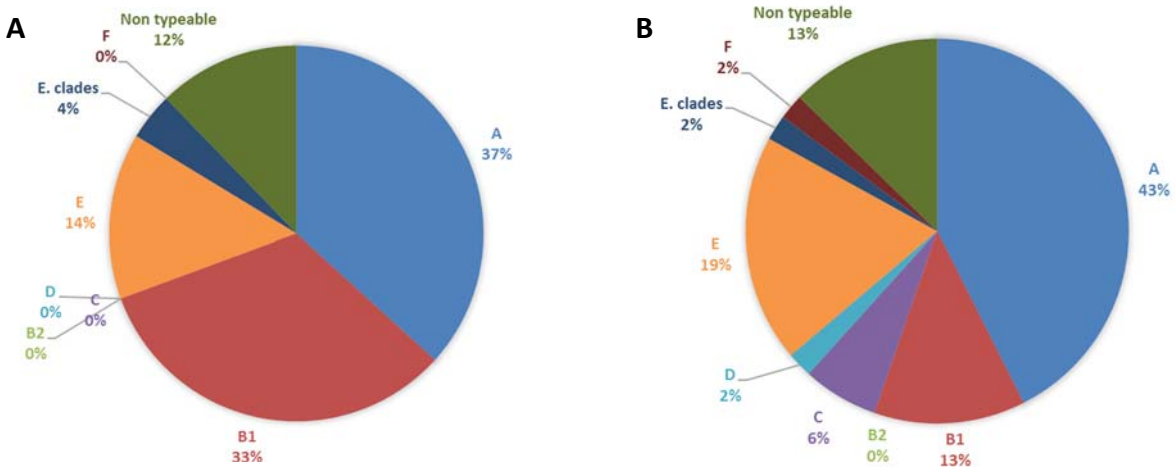


Figure 2. Phylogenetic group distribution by pathotype. **A**, Enteroaggregative *E. coli*; **B**, Enterotoxigenic *E. coli.*

Regarding the geographical distribution of the phylogenetic groups according to the travel destination, phylogenetic group A was more frequently recovered from travellers returning from Latin American countries, followed by those returning from Africa and finally South-East Asia. We did not find important differences in the distribution of B1, E or non-typeable isolates. In our study, isolates from phylogenetic group C were missing from Africa and those of group D and group F were only recovered from Latin America and Africa, respectively, although there were just too few isolates from this phylogenetic groups for these results to be conclusive (*Figure 3*).

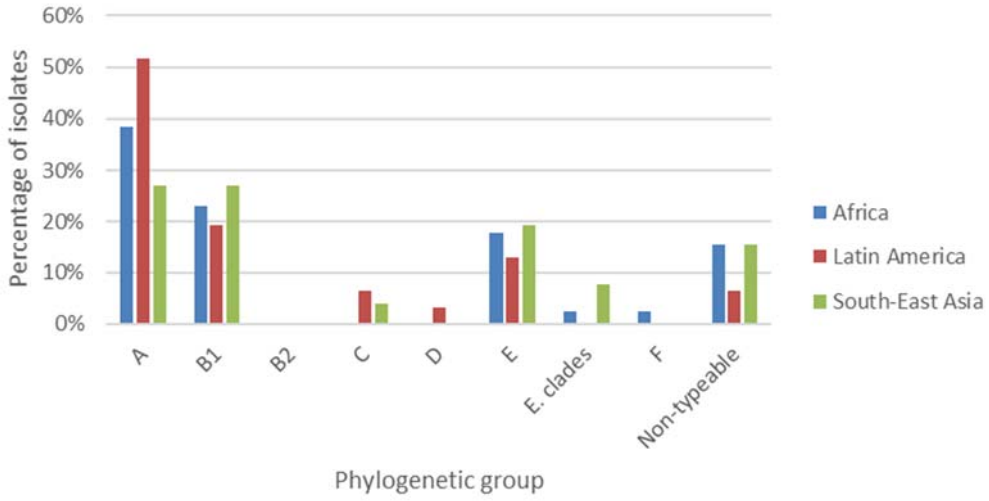


*Figure 3.*Geographical distribution of the *E. coli* phylogenetic groups.

To further understand the evolution of phylogenetic groups belonging to the EAEC and ETEC pathotypes overtime, all isolates were clustered into two groups according to their isolation period: 2001-2007 or 2008-2014. There were no remarkable differences regarding the prevalence of isolates from phylogenetic group A between both periods but isolates from phylogenetic group B1 were considerably more prevalent between 2001-2007 than between 2008-2014, while the prevalence of phylogenetic groups C, E and F notably increased during the latter period (*Figure 4*). Of note, since B1 isolates were highly associated to the EAEC pathotype, this pathotype is more prevalent during the first isolation period (56%) than during the second isolation period (41%), which may suggest a shift in the predominant *E. coli* pathotypes causing traveller's diarrhoea.

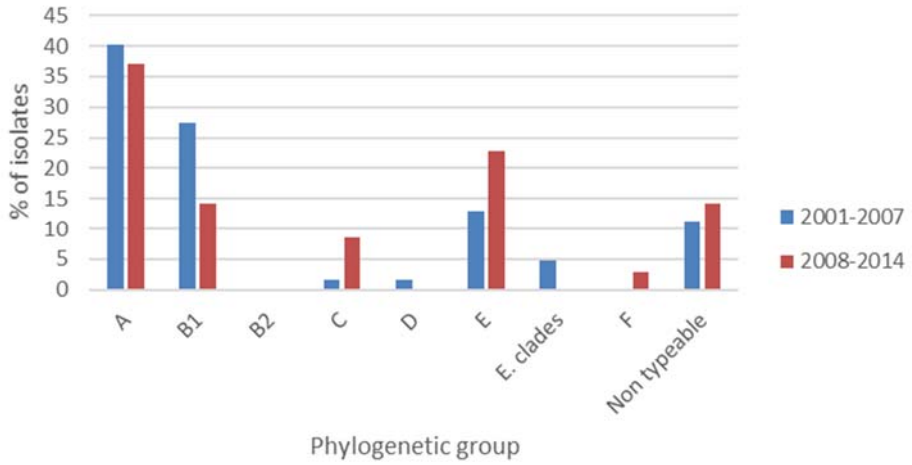


Figure 4. Distribution of the *E. coli* phylogenetic groups overtime.

On the other hand, using the MLST-based approach we were able to identify 47 different STs that were further grouped into 21 different CCs, out of 96 isolates included in the study. There were nine STs that could not be allocated to any CCs. In addition, there were 6 isolates that presented novel allelic combinations and, thus, represent novel STs, two of them being related to major CCs.

As shown in *Figure 5*A, ST10 was the most prevalent ST in our collection (14.4%), followed by ST4 (8.3%). Both STs belong to CC10, being ST10 the ancestral genotype for ST4, sharing 6 out of 7 loci. Likewise, the most prevalent CC was CC10 (29.9%) (*Figure 5*B).

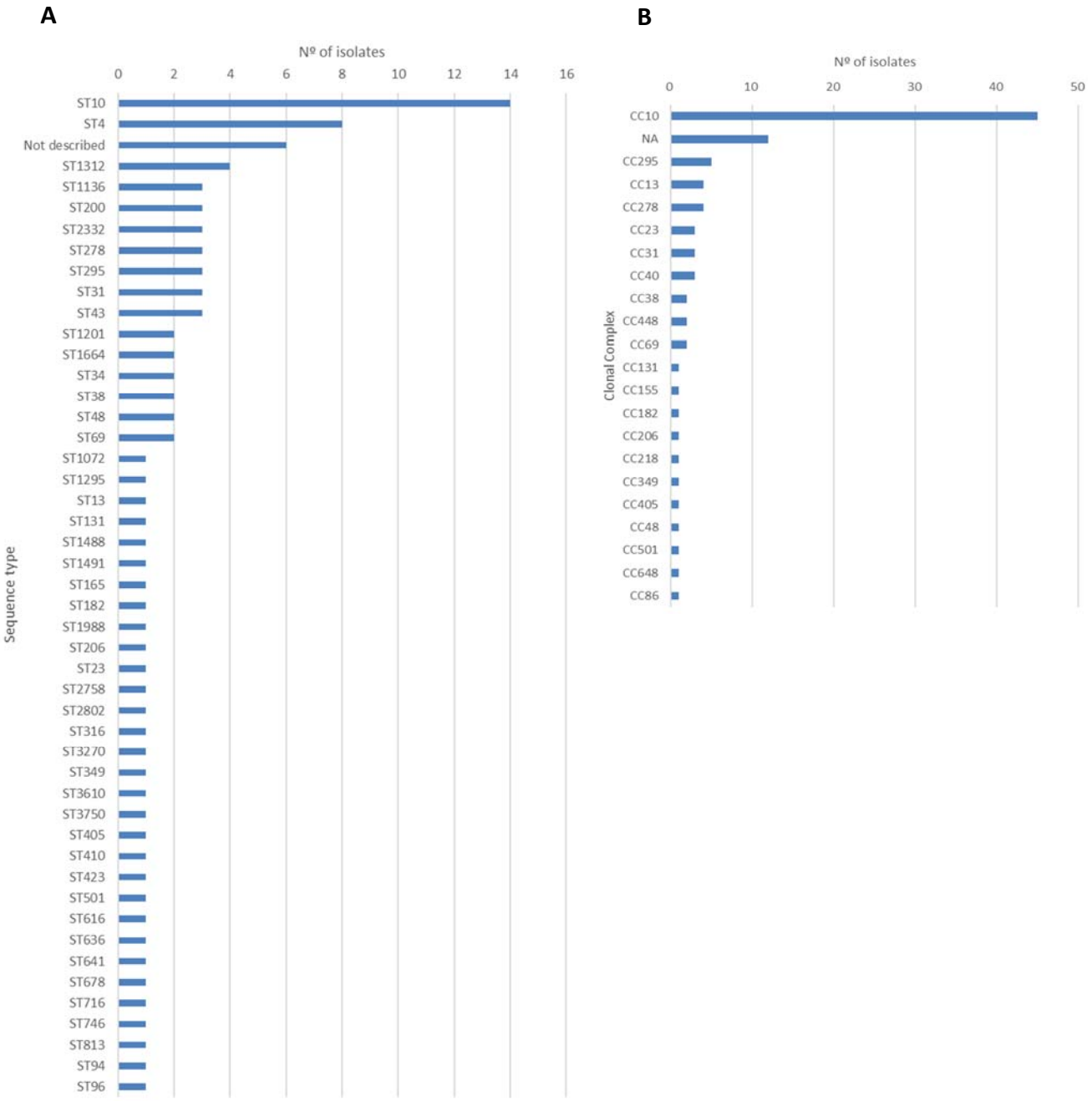


Figure 5. Overall distribution of sequence types (**A**) and clonal complexes (**B**).

Taking into account the different pathotypes, 23 different STs were found among EAEC isolates, and the most prevalent ST was ST10 (24.5%). On the other hand, 28 different STs were described for ETEC isolates, being ST4 (16.7%) the most frequent (*Figure 6*A *and 6*B). Interestingly, all isolates belonging to ST4 were classified as ETEC, and the majority of isolates within ST10 (85.7%) were EAEC. The composition of the least frequent STs of each pathotype was highly diverse.

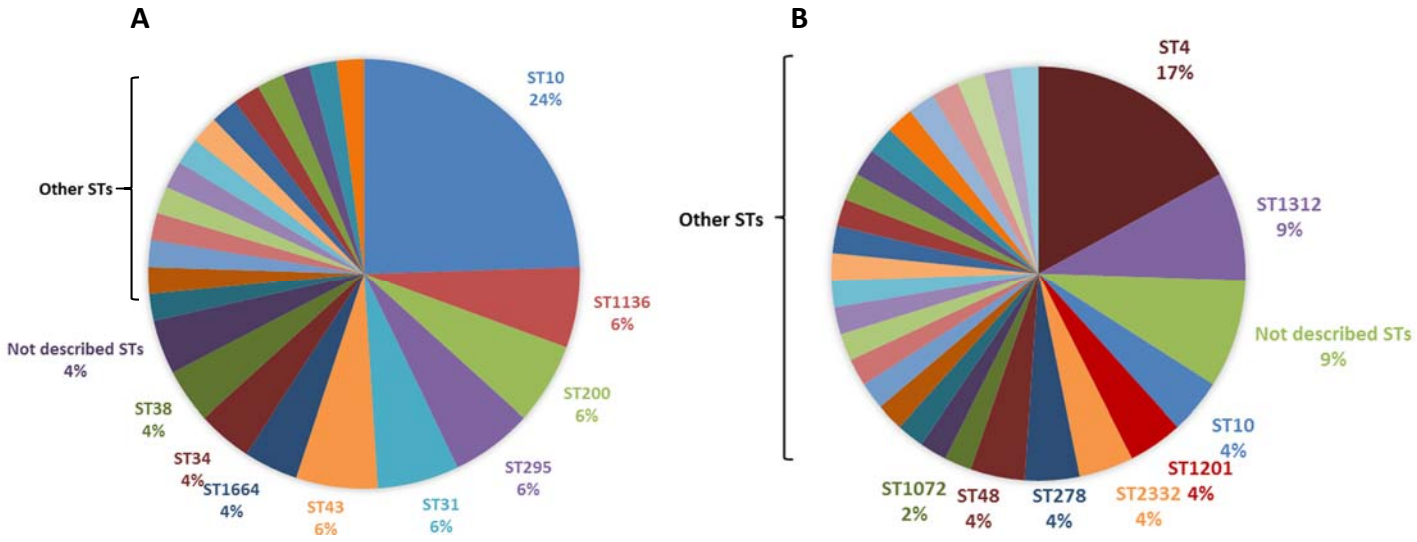


Figure 6. Distribution of sequence types (ST) by pathotype. **A**, Enteroaggregative *E. coli*; **B**, Enterotoxigenic *E. coli*.

The distribution of the STs in terms of percentage per geographical area varied greatly: whereas no ST was highlighted in South-East Asia, the most prevalent ST in Africa was ST4, and in Latin America ST10 was the most frequently found. The composition of the other STs found in each geographical area was very heterogeneous (*Figure 7*). Twenty-two different STs were described in South-East Asia, and the most prevalent were ST10, ST1136 and ST23 (7.4% each). In Africa, 24 different STs were described and ST4 accounted for 12.8% of the total African isolates, followed by ST10 and unknown STs (7.7% each). Among the 18 different STs found in the Latin American isolates, ST10 was identified in 29% of the isolates followed by ST4 and ST278 (9.7% each). It was of note that while ST10 was found in the three geographical areas, none of the isolates belonging to ST4 were present in South-East Asia.

Figure 7. Percentual distribution of *E. coli* sequence types (STs) by geographical area.

On arranging the STs by CCs in terms of percentage (*Figure 8*), the most prevalent CC in the three geographical areas was CC10, followed by isolates not assigned to any CC and CC295 and CC13 in Africa and South-East Asia, respectively. CC10 accounted for 46% in Africa, 58% in Latin America and 35% in South-East Asia.

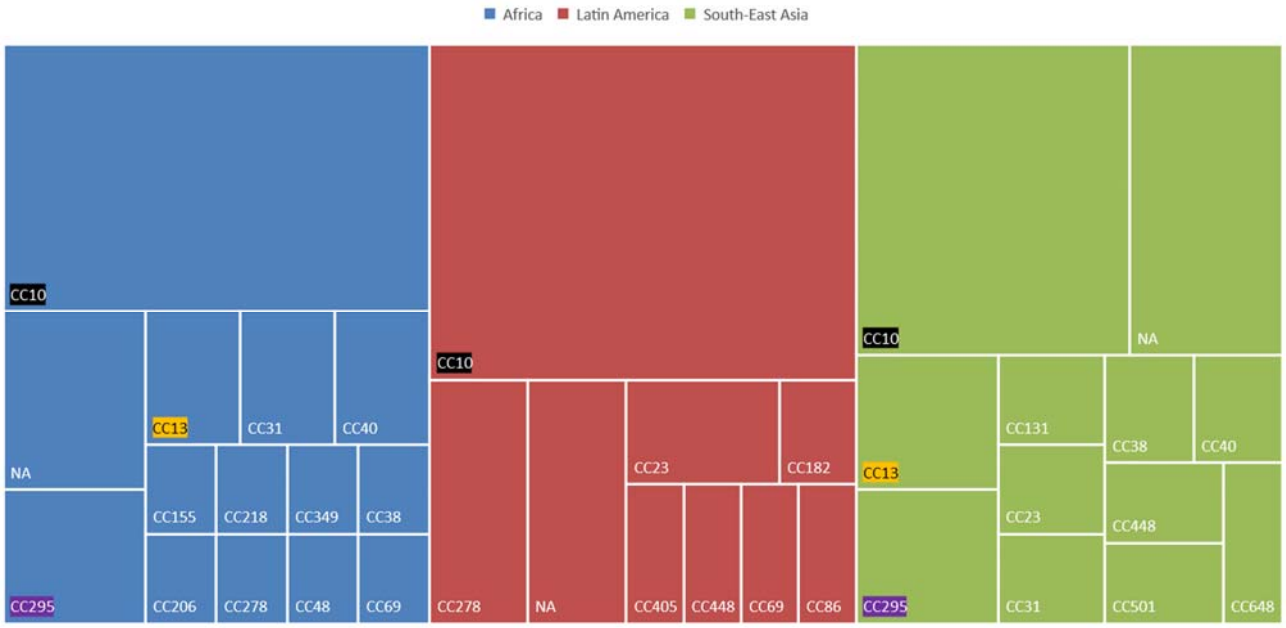


Figure 8. Percentual distribution of *E. coli* clonal complexes (CCs) by geographical area.

On dividing the TD-causing isolates into the two isolation periods specified above, the distribution of the STs of EAEC and ETEC isolates was shown to evolve. As observed in *Figure 9*, the variability of the STs was much greater in the first isolation period. Among the 35 different STs obtained in the first period, the most prevalent was ST10, accounting for 17.7% of the total isolates in that period. However, in the second isolation period, ST4 was the most frequent ST, being identified in 17.6% of the isolates, among the 20 different STs described in this period. Concerning the periodic distribution of CCs, since ST10 is the ancestral genotype for ST4, the most prevalent CC was CC10, being around 47% in both periods (*Figure 10*). Another CC mostly identified during the first period was CC278, whereas in the second period CC23 was the second most prevalent CC. Isolates not assigned to any CC were highly prevalent in both periods.

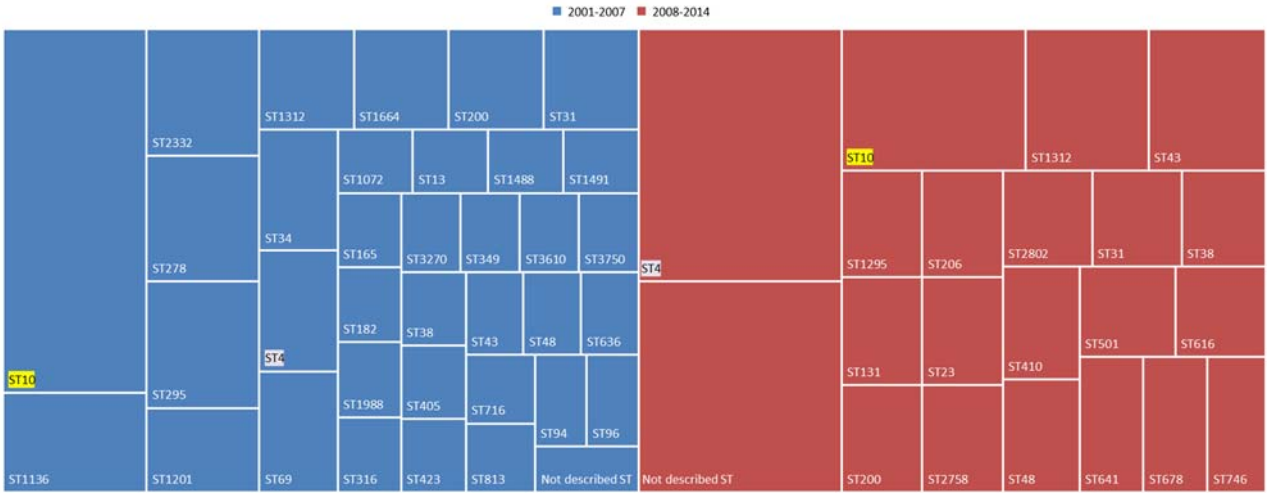


Figure 9. Percentual distribution of E. coli sequence types (STs) by isolation period.

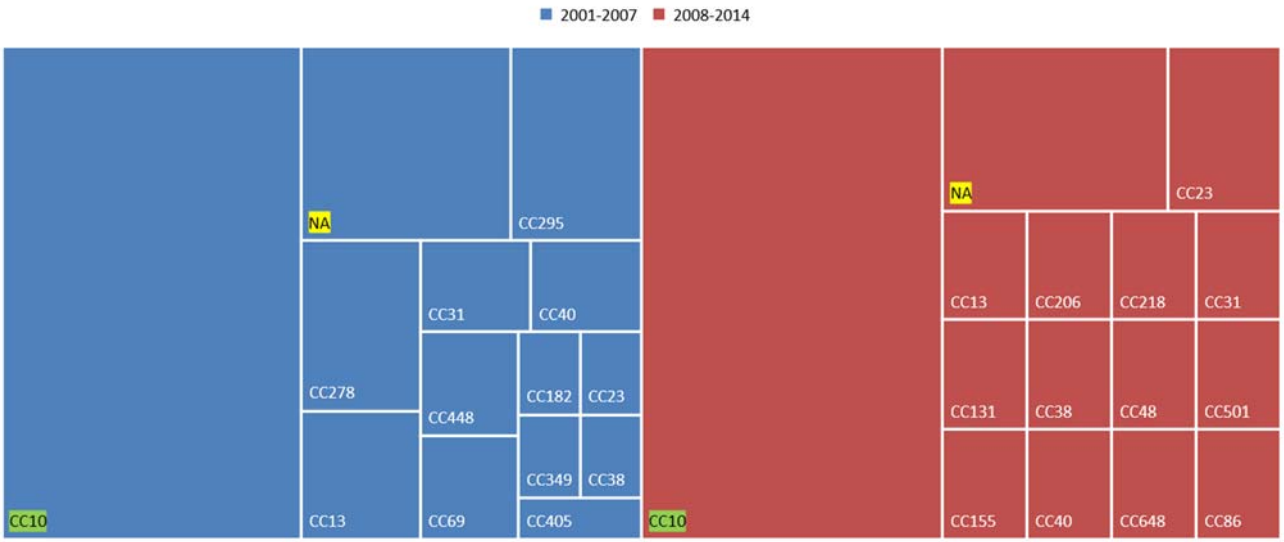


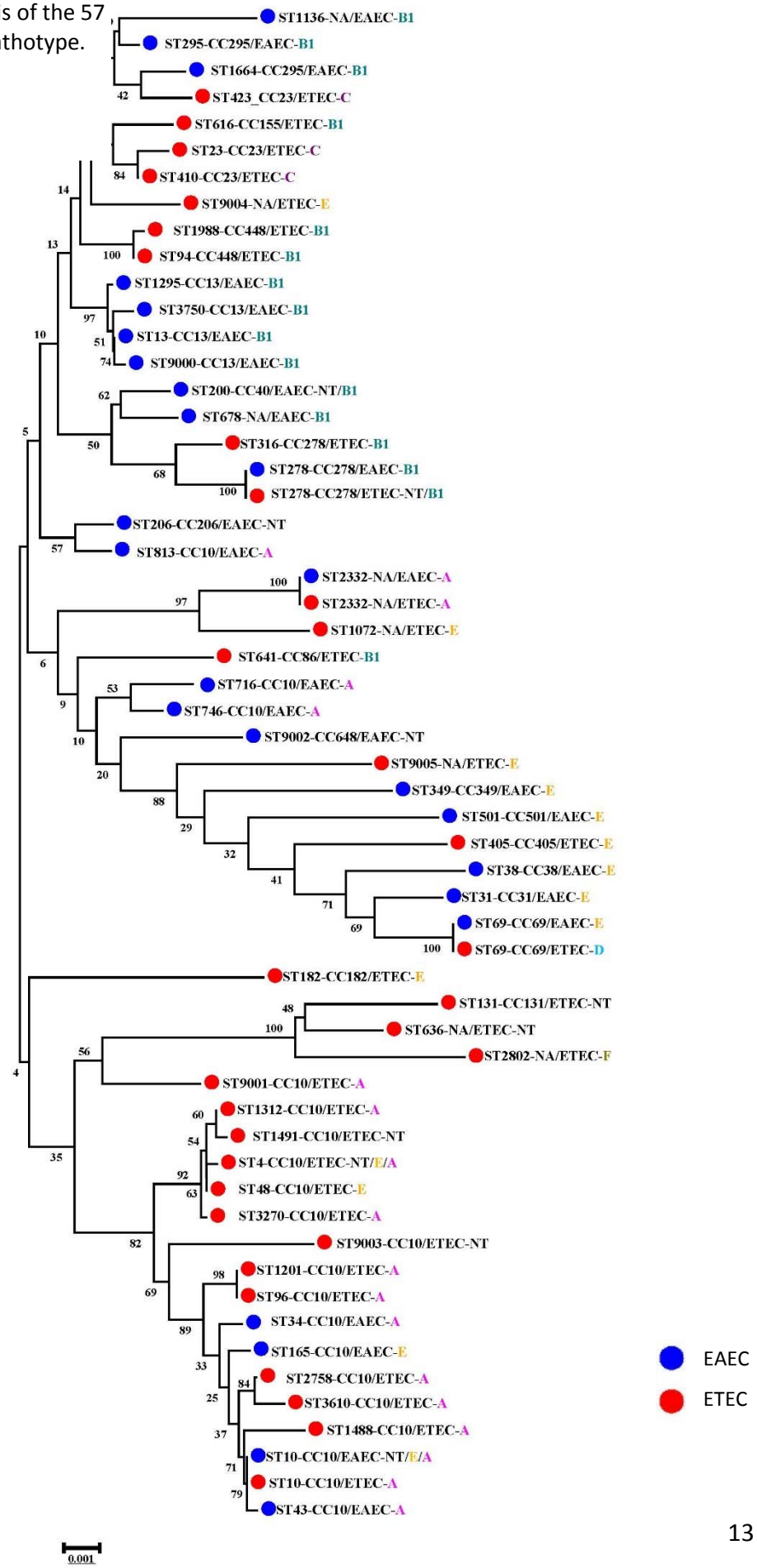
Figure 10. Percentual distribution of *E. coli* clonal complexes (CCs) by isolation period. NA: Not assigned

The results of the MLST gene-based sequence analysis of the 57 isolates among the EAEC and ETEC collection causing TD worldwide (*Figure 11*) showed that the two pathotypes were not grouped separately; indeed, the same ST was found in both ETEC and EAEC isolates (as is the case of ST278, ST2332, ST69 and ST10). As expected, STs belonging to the same CC were grouped separately as was the case for CC10, which was the most prevalent CC among the collection in terms of number of isolates as well as in the number of different STs belonging to this group. Some isolates not assigned to any CC were highly related and separated from the other established groups, including ST1072-ST2332 and ST2802-ST636, which were, in turn, related to ST131. Isolates with non-described allelic combinations and which therefore did not belong to any ST were not epidemiologically related to each other or to the other STs described in the collection.

Concerning the most prevalent phylogenetic groups among the collection, STs belonging to groups B1 and E formed two distinct groups in the cluster analysis, whereas a proportion of the STs classified as phylogenetic group A were clustered separately, as observed in *Figure 11*.

When studying the phylogenetic tree in regard to the geographical origin of each ST, no epidemiological relationship was found, with the three geographic areas not being grouped with related STs (data not shown).

Figure 11. Cluster analysis of the 57 different *E. coli* STs by pathotype.



Discussion and conclusions

Epidemiological studies of EAEC and ETEC *E. coli* causing TD are scarce. These bacteria are one of the main causes of intestinal diseases not only in traveller's to LMIC but also in the general population of these geographical areas, causing high morbidity rates in children under five years of age (21). Given the importance and lack of surveillance related to this disease, the present study provides the first approach to determine the epidemiological relationships among the E. Coli pathotypes by typing the isolates causing TD in patients attending the Tropical Medicine Unit of our hospital between 2001 and 2014 after returning from Latin America, South-East Asia and Africa.

The most prevalent phylogenetic group among the EAEC and ETEC isolates studied was group A, followed by B1 and E. None of the isolates was clustered in phylogenetic group B2, a group that is largely comprised of extraintestinal pathogenic *E. coli* (22) together with group D, the prevalence of which was also low in this collection. Phylogenetic group clustering differed slightly on analysing the two pathotypes separately, and the distribution of the EAEC phylogenetic groups was in accordance with the study carried out by Okeke *et al.*(6) in Nigeria. These authors also studied EAEC isolates (mainly from Nigeria but also from other locations), showing a low prevalence of phylogroup B2 (4.6%). Another report by von Mentzer *et al.* on the distribution of phylogenetic groups of ETEC isolated from different countries showed similar percentages of groups A, B1 and E, while B2 was very infrequent (5). Okeke and collaborators suggested a possible differential geographic distribution of EAEC isolates belonging to phylogenetic group B2, considering that a previous French study had reported this group to be more commonly found in Asia than in Africa (23). However, the present study strongly supports the contention that, on a worldwide scale, EAEC strains (also ETEC) may not be associated with the B2 lineage. Nonetheless, further studies should be made in this regard. Among the other most prevalent phylogenetic groups, no major differences were found in the geographical distribution except for group A, which was more frequently found in the Latin American isolates. This result is in accordance with the French study mentioned above, in which phylogenetic group A was more frequently found in intestinal *E. coli* from Latin

American populations, with percentages of around 60% (23). When dividing the EAEC and ETEC isolates into two isolation periods, the distribution of the phylogenetic groups seems to have evolved; while the prevalence group B1 decreased almost 50% in isolates from the latter period, the prevalence of phylogenetic groups C, E and F increased. Periodic changing in the distribution of phylogenetic groups has been reported for commensal *E. coli* isolated from human populations; however, the greatest reduction was not found in group B1 but rather group A, mainly being replaced by group B2 (24).

In general lines, the MLST approach showed high clonal diversity among the EAEC and ETEC isolates causing TD. The most frequent STs and CCs in EAEC isolates were ST10 and CC10, respectively, again in accordance with the Nigerian study (6). Nonetheless, taking into account that ST10 is the one of the largest STs in the MLST database in terms of prevalence, this ST may be overrepresented among the EAEC collection.

The ETEC isolates presented a greater number of STs than the EAEC group; ST4 (also belonging to CC10) was the most frequent, being ST10 poorly clustered. The abovementioned ETEC study by von Mentzer *et al.* also detected isolates belonging to ST4, although this ST was not the most predominant of the collection. Nonetheless, similar to our results, it was also detected ST1312 as one of the most prevalent STs among the ETEC isolates studied (5).

With regard to the distribution of the STs per geographical area not taking into account the pathotype, ST4 was the most prevalent in Africa and ST10 in Latin America, and no isolate from South-East Asia belonged to ST4. Isolates belonging to ST10 and ST4 are highly described in the *E. coli* species, and the STs found in the three areas varied greatly suggesting that there might be a geographical distribution of STs belonging to multiple lineages causing intestinal disease. Moreover, the distribution of STs and CCs for the two isolation periods was very variable, observing a significant decrease of isolates belonging to ST10 as well as an increase of the ST4 lineage, indicating the evolution of strains causing TD probably related to fitness cost of the EAEC or ETEC-determining plasmid carriage. Nevertheless, larger studies are necessary to strengthen this hypothesis, as there is a limitation in the isolates number per geographical area in the present study.

Since the cluster analysis of the STs of the study isolates found no evidence of any epidemiological relationship between the EAEC and ETEC pathotypes, we hypothesised that EAEC and ETEC strains are simply any *E. coli* lineage that can acquire, express and retain plasmids harbouring colonisation factors and/or toxins, and therefore, the classification of pathotypes is not related to the *E. coli* phylogeny. Nevertheless, MLST and phylogenetic grouping are very reliable tools for epidemiological studies of EAEC and ETEC isolates causing TD or intestinal disease, and their use is widely encouraged for surveillance networks over time.

References

1. Leder K, Torresi J, Libman MD, Cramer JP, Castelli F, Schlagenhauf P, et al. GeoSentinel surveillance of illness in returned travelers, 2007-2011. Ann Intern Med. 2013;158(6):456–68.

2. Al-abri SS, Beeching NJ, Nye FJ. Traveller's diarrhoea. 2005;5(June):349–60.

3. Jiang ZD, DuPont HL. Etiology of travellers' diarrhea. J Travel Med. 2017;24(suppl_1):S13–6.

4. Zboromyrska Y, Hurtado JC, Salvador P, Alvarez-Mart Inez MJ, Valls ME, Mas J, et al. Aetiology of traveller's diarrhoea: evaluation of a multiplex PCR tool to detect different enteropathogens. Clin Microbiol Infect. 2014;20:753–9.

5. von Mentzer A, Connor TR, Wieler LH, Semmler T, Iguchi A, Thomson NR, et al. Identification of enterotoxigenic *Escherichia coli* (ETEC) clades with long-term global distribution. Nat Genet. 2014;46(12):1321–6.

6. Okeke IN, Wallace-Gadsden F, Simons HR, Matthews N, Labar AS, Hwang J, et al. Multi-locus sequence typing of enteroaggregative *Escherichia coli* isolates from nigerian children uncovers multiple lineages. PLoS One. 2010;5(11):e14093.

7. Li Y, Luo Q, Shi X, Lin Y, Qiu Y, Lv D, et al. Phenotypic and genotypic characterization of clinical enterotoxigenic *Escherichia coli* isolates from Shenzhen, China. Foodborne Pathog Dis. 2017;14(6):333–40.

8. Khalil U, Younus M, Asghar N, Siddiqui F, Gómez-Duarte OG, Wren BW, et al. Phenotypic and genotypic characterization of enteroaggregative *Escherichia coli* isolates from pediatric population in Pakistan. Apmis. 2016;124(10):872–80.

9. Imuta N, Ooka T, Seto K, Kawahara R, Koriyama T, Kojyo T, et al. Phylogenetic analysis of enteroaggregative *Escherichia coli* (EAEC) isolates from Japan reveals emergence of CTX-M-14-producing EAEC O25:H4 clones related to sequence type 131. J Clin Microbiol. 2016;54(8):2128–34.

10. Pan H, Zhang J, Kuang D, Yang X, Ju W, Huang Z, et al. Molecular analysis and antimicrobial susceptibility of enterotoxigenic *Escherichia coli* from diarrheal patients. Diagn Microbiol Infect Dis. 2015;81(2):126–31.

11. Álvarez M, Buesa J, Castillo J, Vila J. Diagnóstico microbiológico de las infecciones gastrointestinales. Vol. 30, Enfermedades Infecciosas y Microbiologia Clinica. 2008.

12. Vargas M, Gascón J, Gallardo F, Jimenez De Anta MT, Vila J. Prevalence of diarrheagenic *Escherichia coli* strains detected by PCR in patients with travelers' diarrhea. Clin Microbiol Infect. 1998;4(12):682–8.

13. Sogawa K, Watanabe M, Sato K, Segawa S, Ishii C, Miyabe A, et al. Use of the MALDI BioTyper system with MALDI-TOF mass spectrometry for rapid identification of microorganisms. Anal Bioanal Chem. 2011;400(7):1905–11.

14. Ruiz-Blázquez J, Vargas M, Nataro JP, Vila J, Gascón I Brustenga J. Validación de la técnica de PCR para la detección de *Escherichia coli* enteroagregativa causante de diarrea del viajero. Enferm Infecc Microbiol Clin. 2005;23(8):479–81.

15. Mendez-Arancibia E, Vargas M, Soto S, Ruiz J, Kahigwa E, Schellenberg D, et al. Prevalence of different virulence factors and biofilm production in enteroaggregative *Escherichia coli* isolates causing diarrhea in children in Ifakara (Tanzania). Am J Trop Med Hyg. 2008;78(6):985–9.

16. Clermont O, Christenson JK, Denamur E, Gordon DM. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep. 2013;5(1):58–65.

17. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in *Escherichia coli* : an evolutionary perspective. Mol Microbiol. 2006;60(5):1136–51.

18. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28(10):2731–9.

19. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792–7.

20. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980;16(2):111–20.

21. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet. 2013 Jul;382(9888):209–22.

22. Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P, et al. Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. PLoS Genet. 2009;5(1):e1000344.

23. Escobar-Paramo P, Grenet K, Le Menac'h A, Rode L, Salgado E, Amorin C, et al. Large-scale population structure of human commensal *Escherichia coli* isolates. Appl Environ Microbiol. 2004;70(9):5698–700.

24. Tenaillon O, Skurnik D, Picard B, Denamur E. The population genetics of commensal *Escherichia coli*. Nat Rev Microbiol. 2010;8(3):207–17.

V. DISCUSSION

VI. DISCUSSION

Regarding the heterogeneity of the results of this PhD dissertation, the Discussion has been structured following the sections established by the specific objectives.

1. Virulence of *E. coli* clinical isolates

The virulence potential of *E. coli* is measured by the carriage of identified virulence factor genes (VFGs) present in their genome. The different pathotypes of intestinal and extraintestinal *E. coli* have specific VFGs depending on the type of infection they cause, whereas other VFGs can be found in several pathotypes, providing a transversal property to cause disease. Some VGFs have been classified as belonging to specific pathotypes, but can also be harboured among others, playing an important role in their pathogenesis.

1.1 Prevalence of virulence factor genes typical of EAEC in *E. coli* isolates causing extraintestinal infections *(Papers 1* and *2)*

The presence of genes encoding for enterotoxins was analysed in *E. coli* isolates collected from patients with bacteraemia and urinary tract infection (UTI) in *Papers 1* and *2*, respectively. These VFGs have not been described in ExPEC but rather are typical of diarrhoeagenic *E. coli*, specifically EAEC, with little literature in this respect. In *Paper 1*, the VFGs *set1*, *sen* and *astA* were found in 18%, 10% and 10%, respectively of the *E. coli* isolates causing bacteraemia analysed. Although these percentages are not negligible, no further studies have been made on the prevalence of these VFGs in *E. coli* isolates causing bacteraemia.

However, other enterotoxins such as the Shiga-toxin *stx2* gene have been found in *E. coli* clinical isolates causing bacteraemia (202). Furthermore, an EAEC strain harbouring the *astA*

gene isolated from a patient in Germany returning from the Philippines, was reported as the cause of UTI and bacteraemia leading to sepsis (203).

The uropathogenic *E. coli* (UPEC) collection from *Paper 2* presented the *set1B* gene in 16% of the isolates, and the *astA* gene was harboured by 8% of the isolates. The first gene, encoding for the ShET-1 toxin, has been reported in EAEC causing diarrhoea as well as in *Shigella* spp. clinical isolates (demonstrating the horizontal gene transfer between species) (204,205), but its prevalence has only been studied in a Mexican UPEC collection, in which 31.4% of the isolates harboured this gene (206). On the other hand, the *astA* gene encoding for the EAST toxin, has not only been found in EAEC diarrhoeagenic *E. coli* (205,207,208), but also in 30.9% of the abovementioned Mexican UPEC collection as well as in 7.1% of a Brazilian UPEC collection (209), suggesting that UPEC strains acquiring these enterotoxins could become potential agents of diarrhoea. In another study conducted with a collection of ESBL-producing ExPEC isolates from human and animal sources in Germany, the Netherlands and the United Kingdom, 11 EAEC isolated from human blood and urine were found, mainly belonging to ST38, a steadily increasing clonal group among EAEC strains. Since the presence of EAEC VF may enhance the adherence of *E. coli* to bladder epithelial cells, it has been hypothesised that a ST38 *E. coli* strain adapted to EAEC plasmid carriage has acquired UPEC virulence factors, facilitating the exploitation of extraintestinal niches (210). Moreover, a Brazilian EAEC collection was characterised as EAEC strains with UPEC markers suggesting that diarrhoeagenic strains may have uropathogenic abilities, thereby making intestinal carriers of these strains potentially at risk of developing UTI (211).

Overall, these studies emphasise the need to increase the understanding of the extraintestinal virulence potential of diarrhoeagenic *E. coli* or the diarrhoeagenical potential of ExPEC, as well as to track the acquisition of this virulence potential.

1.2 Prevalence and potential environmental specialisation of virulence factor genes from vaginal *E. coli* potentially causing obstetric infections (*Papers 3* and *4*)

In *Papers 3* and *4*, the carriage of VFGs in *E. coli* found in the genital tract of pregnant and nonpregnant women as well as in *E. coli* clinical isolates causing sepsis of obstetric origin or IAI was determined, respectively. The VFGs tested in both collections included genes encoding for the most prevalent adhesins, toxins, invasins and iron recruitment systems in UPEC strains, which have been hypothesised to be the main source of these obstetric infections.

In *Paper 3*, VFGs associated with iron recruitment systems (*iroN*, *fyu* and *iutA*) and adhesion (*pap* genes) were the most frequently found among the collection, with percentages between 41% and 57%. In particular, the *fyu* gene was found in 53% of the strains, which is in accordance with a Mexican study with UPEC causing UTI in women (44,4%) (212), but much lower than the prevalence of this VF reported in an Italian UPEC collection (86.7%) (213). The prevalence of the *iutA* gene found in nonpregnant women in our study (33%) is similar to that found in a recent study among vaginal *E. coli* (VEC) (28%) (214) but lower than in UPEC collections from other studies in women (213). In 2001, Cook *et al.* studied the presence of several VFs among 50 strains of *E. coli* causing vaginitis in nonpregnant women and obtained findings similar to ours (215). However, both studies considerably differed compared with the high percentages of the *cnf1*, *pap* and *sfa* genes described in 2004 by Birošová *et al*. in vaginal α-haemolytic *E. coli* isolates (216).

On comparing VFG carriage among pregnant versus nonpregnant women, much higher percentages were found in the group of pregnant women. For instance, the toxin encoding genes *hly* and *cnf1*, P-fimbriae encoding the *papC* gene and the *iroN* gene were significantly more frequent in strains from pregnant than from nonpregnant women (42% vs. 21%, 42% vs. 16%, 52% vs. 25%, and 67% vs. 45%, respectively). The *hly* and *cnf1* genes were also the most prevalent VFGs found in a study of *E. coli* causing vaginitis from Chile in 2014 (217). Watt et

al. found a similar prevalence of the *hly* gene (48%) but a lower percentage of *papC* (32%) in a collection of vaginal *E. coli* from pregnant women (83). However, a Japanese study carried out by Obata-Yasuoka *et al.* in 2002 comparing *E. coli* isolates from pregnant women with isolates from nonpregnant women, obtained different results, with the prevalences of *hly*, *cnf1* and *papC* being lower in pregnant than in nonpregnant women. Overall, there is a tendency to these genes being more prevalent in pregnant women, leading to severe infections during pregnancy, and thereby increasing the possible development of neonatal sepsis.

In *Paper 4*, the most prevalent VFG found among all the *E. coli* isolates of obstetric origin were adhesion related (*pap* genes and *fimA*), with a prevalence of between 56% and 86%. On comparing isolates causing sepsis with those causing IAI, the first harboured the greatest number of VFGs, with significantly higher percentages (from 34% to 75%) of *hlyA*, *cnf1*, *papA*, *iha*, *fyuA*, and *papG*II, all of which were contained in pathogenicity islands (PAIs). Regarding the two toxin-encoding genes, *hly* and *cnf1*, several studies have reported a high prevalence of these genes in *E. coli* isolates causing UTIs and vaginal infections (46,215,217,218). With respect to the high prevalence of the *papGII* gene, a study from Iraq found this allele to be the most prevalent among vaginal *E. coli* isolates from pregnant and nonpregnant women (219) and a French study of *E. coli* causing sepsis showed that this allele was more prevalent than other *papG* alleles (220).

Overall, these results demonstrate that the VFGs *hly*, *cnf1* and *papGII,* are more frequently harboured in more pathogenic groups of strains, possibly related to the tissue damage induced by these infections (as occurs in UTIs) and that they could be potential markers of virulence for obstetric *E. coli* infection.

Regarding VFs related to iron recruitment, the *iutA* gene was significantly more frequently found in isolates causing IAI in *Paper 4*, whereas the *fyuA*, *iroN* and *iha* genes were more common in sepsis-causing isolates. In a study carried out in our lab by Sáez-López *et al.*, the *iutA* VFG was more prevalent in *E. coli* isolates causing obstetric infections than in commensal

vaginal *E. coli* from pregnant women (46). However, this VFG was also significantly more prevalent in vaginal *E. coli* recovered from pregnant women from different geographical sites (221). These results suggest the presence of an environmental-related specialisation of iron recruitment systems harboured in the ExPEC genome.

Thus, not only does antibiotic pressure promote the spread of plasmids with resistance determinants (and often also VFs), but certain environments encourage bacterial interchange and the distribution of these VFGs, providing the bacteria with the ability to colonise new niches and cause infection in different human body sites (222).

1.3 Virulence factor carriage among multidrug resistant *E. coli* isolates causing extraintestinal and intestinal infections *(Papers 4* and *7)*

In *Paper 4*, twenty isolates (26%) were resistant to three or more antimicrobial classes, presenting a multidrug resistant (MDR) phenotype. Seventy percent of these isolates were sepsis-causing, having a variable virulence score (total number of VFGs detected) from 2 to 19. The most prevalent VFG in the MDR isolates causing sepsis were the *pap*, *fim*, *fyu*, *ag43*, *iucC* and *iroN* genes, whereas in IAI-causing *E. coli* the *prs*, *iut* and *iuC* genes were more frequently found. The VF profile of the strains causing these two different infections seems to be specialised, with the presence of genes for adhesion and iron recruitment systems being more prevalent in the most virulent ExPEC MDR isolates –that is, those causing sepsis.

In *Paper 7*, the five CTX-M-15-producing EAEC isolates were screened for VFGs typical of EAEC, including the *aggA* and *aafA* genes (encoding for adhesins), *aap* (for dispersin), *aatA* (for TolC), *aggR* (for regulation of aggregation), *astA*, *set1A*, and *sen* (for toxins), *fyuA* (for iron recruitment); *agn43* (for antigen 43), and genes encoding for serine protease autotransporter toxins such as *pet* and *sat*. The *aatA* gene was detected in the 5 strains, whereas *astA*, *aap*, *aggR*, and *aggA* had positive amplification for only 2 of the strains belonging to ST38.

Since molecular classification into the EAEC pathotype is based on the detection of VFGs and taking into account the genetic heterogeneity of this pathotype, these results reinforce the contention that the pCVD432 probe (derived from the *aatA* gene) - the first molecular tool for EAEC diagnosis - remains one of the most useful and reliable molecular methods for screening stool specimens for the presence of EAEC strains. However, no study has demonstrated a 100% correlation with the HEp-2 cell adhesion assay and the presence of a single VFG, which is why other EAEC specific genes must be identified to duly diagnose this pathotype (28).

1.4. Relationship between virulence and resistance to specific antimicrobial agents in ExPEC *(Papers 1, 3* and *4)*

In *Paper 1*, the *set1* gene encoding for the ShET1 toxin was more frequent among nalidixic acid-susceptible isolates. In *Paper 3*, the *hly*, *cnf1* and *pap* genes were more prevalent among the nalidixic acid-susceptible isolates, whereas in *Paper 4* a specific relationship was found between tetracycline-resistant isolates and a lower presence of several VFGs included into PAIs.

Several studies have shown the same pattern, with the majority of VFGs being more prevalent among quinolone-susceptible isolates (151,223–225). A Portuguese study found this relationship in a UPEC collection, in which the VFGs carried in PAIs were more frequently found not only in quinolone-susceptible strains but also in β-lactam, gentamicin and cotrimoxazole-susceptible isolates. In contrast, the VFGs that are frequently harboured in plasmids such as the *iutA* or *eae* genes, encoding for the aerobactin receptor and for intimin adhesin, respectively, were more prevalent in resistant strains, suggesting that they are located in the same plasmid as resistance determinants (226).

This phenomenon has even been demonstrated *in vitro* with a quinolone-susceptible *E. coli* strain by Soto *et al.* (2006). The bacterium was grown in culture media with subinhibitory concentrations of ciprofloxacin, observing the loss of the *hly* and *cnf1* genes in a multistep selection of the mutants obtained (153).

Since all the VFGs shown to be more prevalent in susceptible strains are contained in PAIs, the most reasonable explanation for this phenomenon is that antibiotic exposure contributes to the partial or total loss of PAIs in a SOS-dependent way by increasing the deletion or transposition of DNA regions, and that PAIs are flanked by direct repeat sequences which are, in turn, susceptible to excision (153).

2. Antibiotic resistance of *E. coli* clinical isolates

Although medical practice has limited the development and spread of pathogens, this has led to a global increase in antibiotic resistance. Resistance prevalence in *E. coli* clinical isolates have steadily increased over the last two decades, especially to specific antibiotic families and/or particular pathotypes due to overexposure to antimicrobial agents. Surveillance of the prevalence of resistance is essential in order to update the therapeutic guidelines for specific infections on not only a global but also a local scale, since recommendations may vary according to the geographical area and the prevalence of different bacteria.

Understanding of the molecular basis of antibiotic resistance mechanisms to the antimicrobial families most commonly used against *E. coli* infections is also important in order to: (i) achieve more adequate and effective empiric treatment, decreasing therapeutic failures and patient recovery time, (ii) limit (as far as possible) the spread of these resistance determinants to thereby avoid the steady increase in the prevalence of antimicrobial resistance, and (iii) find new targets or mechanisms of action in the search for new antibiotics.

2.1. Prevalence of antimicrobial resistance in *E. coli* isolates causing extraintestinal and intestinal infections (*Paper 4, 5, 6* and *Additional Results I*)

The present PhD dissertation addresses the prevalence of resistance to the antimicrobial agents most commonly used against recurrent *E. coli* clinical isolates in different infections

(obstetric, neonatal, bloodstream infections, UTIs and TD), taking into account the great importance of the significant problems related to antimicrobial resistance today.

In *Paper 4*, almost two thirds of the isolates were resistant to ampicillin, in accordance with the results found in *E. coli* isolates causing neonatal sepsis and in extraintestinal *E. coli* in general (227,228). By contrast, only 13% were resistant to AMC, and most of the isolates were susceptible to second- and third-generation cephalosporins in comparison with the increasing appearance of strains carrying ESBLs in the last years causing infections by other sources, suggesting that the current implementation of these antimicrobial agents as first-line therapy in obstetric infections is adequate (229,230).

In *Paper 5*, the comparison of the prevalence of antimicrobial resistance of *E. coli* isolates causing early-onset neonatal sepsis (EONS) and late-onset neonatal sepsis (LONS) showed a high prevalence of resistance to ampicillin (78% and 62%, respectively) as well as to gentamicin (19% and 9%, respectively), in accordance with results reported worldwide for *E. coli* causing neonatal sepsis (231–234). The same studies as well as *Paper 5* showed a low prevalence of resistance to third-generation cephalosporins. Taking into account that the treatment of neonates with sepsis is ampicillin, gentamicin or cephalosporins and that the first two are also used for the treatment of premature preterm membrane rupture and chorioamnionitis, the resistance of *E. coli* causing neonatal sepsis is high; therefore, cephalosporins are the antibiotics most recommended to treat this infection, especially cefotaxime due to its excellent penetration into cerebrospinal fluid (CSF). Nonetheless, further studies are needed to elucidate whether the intrapartum therapy given as prophylaxis is related to the increasing prevalence of resistance to these antimicrobial agents in neonatal sepsis caused by *E. coli*. It is of note that following the results of this study, the therapeutic guidelines in our hospital were changed by neonatologists, thereby avoiding the administration of ampicillin and gentamicin as empiric treatment for neonatal sepsis when intrapartum prophylaxis had been given to the mother.

In *Paper 6*, among the 11.3% of ESBL-producing *E. coli* isolates causing UTI or bloodstream infection in children in Mozambique, 70.6% were found to be carrying the CTX-M enzyme and also presenting a MDR phenotype. All these isolates were resistant to rifampicin, gentamicin, chloramphenicol, and cotrimoxazole, and susceptible to fosfomycin and carbapenems, and 66.7% were resistant to quinolones. The rates of ESBLs and other antibiotic resistances observed in this study may be associated with possible cross-resistance acquisition phenomenon (the resistance determinants are located in the same plasmid or other horizontally transferred genetic element) or with the frequent use of these antibacterial agents for the treatment of other infectious diseases highly prevalent in this geographical area, such as tuberculosis, respiratory infections, and additional bacterial infections associated with the immunocompromised status of patients with malaria or the human immunodeficiency virus (235–237). Despite the high prevalence of ESBLs reported in this study, it is lower compared with the prevalence of those causing UTIs in children in other regions of the country (238). For that reason, the treatment administered for each infection should still be chosen depending on the rates of resistance to the antimicrobial agent used as first-line therapy in each hospital, as well as the prophylaxis or previous treatment with these antimicrobial agents, which have led to the development of resistant bacteria.

In *Paper 7*, 10% of the EAEC isolates recovered from patients with TD were resistant to third-generation cephalosporins and had travelled to India, whereas in *Additional Results I*, 15.4% were ESBL-producing isolates, with four out of six also being from India. Among all the collection studied in *Additional Results I*, including 39 EAEC and 43 ETEC clinical isolates causing TD, EAEC showed greater resistance than ETEC. Overall, the resistance to the classical and cheaper antibiotics used in developing countries such as ampicillin, cotrimoxazole and tetracycline was greater than 39%. When stratifying the ETEC and EAEC according to the geographical area visited by the patient with TD, it was of note that the strains from Latin America were less resistant to the abovementioned antibiotics including quinolones and cefotaxime than those from South-East Asia / India or Africa, similar to data from previous studies (239–245). The prevalence of resistance to azithromycin and rifaximin was low, demonstrating that these drugs are still active and are recommended as therapy against EAEC and ETEC causing TD.

2.2 Evolution of the prevalence of antimicrobial resistance in *E. coli* isolates causing extraintestinal and intestinal infections (*Paper 5* and *Additional Results I*)

There is a clear trend to an increasing prevalence of antimicrobial resistance of clinical bacteria worldwide, and *E. coli* is not an exception. Under antimicrobial selective pressure, bacteria easily develop resistance traits that can be rapidly disseminated between the same or even different species. Over the last decades, *E. coli* have been exposed to particular antimicrobial families to which percentages of resistance are now worrisome.

The evolution of the resistance pattern of *E. coli* isolates causing neonatal sepsis was studied in *Paper 5*, showing there was an increase in the prevalence of resistance to all the antimicrobial agents studied in the second time period (2000-2008), being statistically significant for gentamicin (0-26%) and with a clear trend for ampicillin, two of the first-line therapeutic options for this infection as well as for intrapartum prophylaxis and cases of preterm premature rupture of membranes and suspected chorioamnionitis (102,246,247). Very similar results were described by Mendoza-Palomar *et al.* (2017) in another Spanish hospital, being especially of note for the latter study period (2010-2014) in which the prevalence of resistance among EONS-causing *E. coli* reached 92.8% for ampicillin and 28.6% for gentamicin (248). The studies in our setting clearly suggest the need for a change of empirical treatment, especially in the most susceptible neonates. In order to avoid the predictive failure of the two abovementioned antimicrobial agents, cefotaxime would be the most recommended as the first-choice therapeutic alternative for empirical treatment due to its broad-spectrum activity, low percentage of resistant strains and excellent penetration in the CSF in cases in which the sepsis evolves to meningitis. However, another study from the United States on EONS and *E. coli* causing meningitis published in 2016, concluded that the same empiric antibiotic combinations appeared to remain adequate in this country (although continuous surveillance is needed), and highlighted the importance of local epidemiology and surveillance since the guidelines should differ depending on the geographical area (249).

Regarding diarrheoagenic *E. coli*, the first antimicrobial choice for the empiric treatment of TD used to be ciprofloxacin and azithromycin, depending on the geographical area visited (43). However, in the last years, rifaximin has been added to the therapeutic guidelines against TD caused by *E. coli,* but it is not adequate for enteroinvasive pathogens, because of its non-absorbable nature. Information in the scientific literature regarding the antimicrobial susceptibility of EAEC and ETEC is scarce and has not been updated, thereby leading to questioning the validity of the current guidelines on empiric treatment. In *Additional Results I*, resistant rates for different antimicrobial agents are studied in a collection of EAEC and ETEC causing TD in travellers to South-East Asia, Africa and Latin America during the period 2011-2017, and further compared to previously established resistance rates (250). The prevalence of EAEC and ETEC isolates resistant to ciprofloxacin increased from 4% to 23% and from 8% to 21%, respectively. This significant increment is more dramatic in strains isolated from patients who had travelled to South-East Asia, especially India, where 63% of the strains were resistant to ciprofloxacin. Regarding azithromycin, the percentages of resistance in EAEC and ETEC isolates from patients to South-East Asia / India and Africa were above 25%; however, the high concentration of this antimicrobial agent reached in the intestinal tract surpass the minimal inhibitory concentration (MIC) of most azithromycin-resistant strains. Meanwhile, the activity of rifaximin against EAEC and ETEC remained unchanged compared to previous studies (251,252), indicating that it is still active against these pathogens. The prevalence of resistance to third-generation cephalosporins has significantly increased in EAEC and ETEC strains causing TD, mainly in patients travelling to sub-Saharan Africa. Overall, these data totally invalidate the use of ciprofloxacin in the prevention or treatment of TD in patients visiting or returning from South-East Asia, and recommend that the therapeutic guidelines for TD be changed to azithromycin or rifaximin, depending on the geographical area visited and the diagnosis obtained.

2.3 Molecular basis of the resistance to β-lactams in clinically relevant *E. coli* isolates (*Papers 5, 6, 7* and *Additional Results I*)

β-lactam antibiotics are the most widely used in current medical practice due to their broad therapeutic spectrum and low toxicity. Nevertheless, the progressive emergence of acquired resistance has limited the empirical use and efficacy of β-lactams. Despite this, penicillin is still the treatment of choice for a large number of classic infections, cephalosporins are widely used in surgical prophylaxis and severe community-acquired infections, and carbapenems are the first choice for mixed nosocomial and multiresistant bacterial infections. It is therefore important to elucidate the mechanisms of resistance to β-lactams that clinical *E. coli* possess and how to detect these mechanisms promptly in order for the diagnosis and resulting treatment to be the most accurate and effective.

Regarding the neonatal sepsis-causing *E. coli* characterised in *Paper 5*, the ampicillin-resistant strains harboured mainly $bla_{\text{TEM-1-like}}$ genes, in accordance with other studies (253). Two of these strains, one causing EONS and the other LONS, also presented an ESBL-producing phenotype, and the $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ genes were detected, respectively. In addition, one strain causing LONS presented the AmpC enzyme, although it was not overexpressed. As the emergence of ESBL-producing *E. coli* in neonates makes epidemiological surveillance for vertical transmission of neonatal sepsis necessary (254,255), these strains were further characterised by our research group (256), finding both strains to be MDR, as observed in most ESBL-producing *E. coli* (133,257). These resistance enzymes were detected in conjugative plasmids, and the $bla_{\text{CTX-M-15}}$ gene was harboured in an IncFIA incompatibility group plasmid, in agreement with several previous reports (95,258), suggesting that one of the main mechanisms of the spread of the resistance (not only to β-lactams but also to other antibiotic families, because they were MDR strains) are plasmids.

E. coli causing bacteraemia and UTI in children are among the major causes of hospital admission and associated morbidity and mortality in children in Africa (259,260). Although

these infections have been successfully treated with inexpensive antibiotics such as chloramphenicol and ampicillin, the global spread of MDR strains have made these antibiotics useless in many cases (261), thus requiring unavailable or unaffordable antibiotics for LMIC (such as FQX or third-generation cephalosporins), especially in remote rural areas. Moreover, the emergence and spread of ESBL-producing *E. coli* and the lack of new antibiotic families against Gram-negatives marketed in the last decades have further aggravated this situation. Consistent with other reports, the data in *Paper 6* on the most prevalent resistance mechanism to third-generation cephalosporins found in the collection of MDR isolates causing bacteraemia or UTI show that the $bla_{\text{CTX-M}}$ gene belonging to sublineage or group I is the most frequent, and is mainly $bla_{\text{CTX-M-15}}$ (262,263). The gene encoding for this β-lactamase is mainly found in conjugative plasmids belonging to incompatibility groups IncFIIA or IncHI2, although two unrelated strains were found to be carrying the resistance determinant in the chromosome. The location of CTX-M-15 encoding gene in IncF plasmids has been largely reported, although few reports on its location in IncH plasmids are available (264), possibly due to the scarce data available from this geographical area.

All the isolates presented the $bla_{\text{CTX-M}}$ gene located downstream from the insertion sequence IS*Ecp1*, which together with its mainly plasmidic location implies a high potential of dissemination of this ESBL, suggesting that this is the result of the spread of mobile genetic elements.

In relation to diarrhoeagenic *E. coli*, similar results regarding the molecular basis of the resistance to third-generation cephalosporins were found in an EAEC collection causing TD characterised in *Paper 7*, in which all the isolates presented the $bla_{\text{CTX-M-15}}$ gene downstream from insertion sequence IS*Ecp1,* mainly in conjugative plasmids but also in a chromosomic location. In *Additional Results I*, several ESBL-producing EAEC and ETEC isolates causing TD to travellers to LMIC were also characterised and almost all were carrying CTX-M enzymes (CTX-M-15 and CTX-M-27). The $bla_{\text{CTX-M-15}}$ gene has been largely described in diarrhoeagenic *E. coli* (265–267), whereas the $bla_{\text{CTX-M-27}}$ gene has been detected in EPEC, EIEC and EAEC strains (268,269), but has never been reported in ETEC. Among the EAEC and ETEC strains resistant

to AMC, a plasmid-mediated AmpC (ACT-20) was detected in only the two strains with the highest MIC values. This type of plasmidic AmpC has previously been found in a strain of *Enterobacter hormaechei* isolated from dog faeces (270), but so far it has not been described in bacteria causing infections in humans. The EAEC and ETEC showing moderate resistance to AMC presented an OXA-1 enzyme, that is currently the most frequently found mechanism of resistance to AMC (271).

The plasmid incompatibility groups described in the ESBL-producing isolates were very diverse, including IncF, IncY, IncK, Inc B/O and IncI, all reported to be carrying a CTX-M-15 encoding gene, except the last one (harboured in isolates carrying other incompatibility groups reported to harbour the resistance determinant) (264,272,273).

The low diversity of antimicrobial resistance mechanisms to β-lactams detected in the collections characterised in the present thesis as well as in agreement with the literature available, suggests that few mechanisms of resistance (mainly enzymatic) are involved in the worldwide resistance to β-lactam antibiotics in clinically relevant *E. coli*, and that resistance is mainly disseminated by heterogeneous plasmids or transposable elements such as IS*Ecp1*.

2.4 Prevalence of enzymatic resistance mechanisms to β-lactam antibiotics in *E. coli* causing extraintestinal and intestinal infections (*Papers 5, 6, 7* and *Additional Results I*)

Diagnosis of the most prevalent resistance mechanism to β-lactam antibiotics (β-lactamases) in *Enterobacteriaceae* is one of the main targets of clinical microbiology laboratories nowadays. In order to improve diagnostic methodologies, three approaches are necessary: (i) identification of the most prevalent enzymatic mechanisms responsible for this resistance, (ii) demonstration of the prevalence of the resistance mechanism in the pathotype(s) of interest, and (iii) identification of the mechanisms of resistance to antimicrobial agents included in the

current therapeutic guidelines for specific infections, prior to designing the most susceptible, sensitive, rapid and inexpensive technique for their detection.

For instance, among the *E. coli* isolates causing neonatal sepsis characterised in *Paper 5*, 69% were ampicillin-resistant, 74% of which harboured $bla_{\text{TEM-1-like}}$ genes, in accordance with other studies (253). These resistant strains represented 70% of the *E. coli* causing EONS, compared to only 47% of the collection causing LONS. In this sense, knowledge on TEM enzyme carriage is relevant for the diagnosis of neonatal sepsis, as ampicillin is used as empirical treatment for this infection, and the prevalence of this enzymatic mechanism is high enough to allow its rapid identification in order to avoid therapeutic failure. On the other hand, only two isolates of the collection were ESBL-producing and harboured a $bla_{\text{CTX-M}}$ gene. Although this represents a low prevalence among the total number of *E. coli* isolates in this study, medical practitioners and microbiology specialists should determine, according to the surveillance data available locally, whether it is worthwhile to diagnose this mechanism in neonatal sepsis early.

In Mozambique, data obtained from *E. coli* causing bacteraemia and UTIs in children showed that 11.3% of the *E. coli* isolates were ESBL-producers, of which 70.6% were harbouring a $bla_{\text{CTX-M}}$ group 1 gene (92% being the CTX-M-15 encoding gene). The prevalence of ESBL-producing *E. coli* in bacteraemia is lower than in other European countries (274) but higher than others such as Israel (275). CTX-M has been reported to be harboured in 78% of the ESBL-producing *E. coli* isolates causing bacteraemia in a multicentre European study, also suggesting higher rates of community transmission of this enzyme in association with *E. coli* (276). These results demonstrate that percentages of ESBL-producing ExPEC *E. coli* can substantially differ depending on the geographical area. Nonetheless, its surveillance is mandatory in order to redirect infection control policies if necessary.

Data on the prevalence of enzymatic β-lactam resistance mechanisms in diarrhoeagenic *E. coli* is scarce. As shown in *Paper 7*, in the first decade of 2000, 9.8% of EAEC causing TD in patients travelling to LMIC were ESBL-producing, $bla_{\text{CTX-M-15}}$-carrying and were from India. The results

from the next decade (*Additional Results I*) showed 15.4% of ESBL-producing EAEC and 14% of ESBL-producing ETEC. These latter isolates were further characterised and were shown to be harbouring $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}27}$ (72% and 18%, respectively). These percentages of ESBL-producing EAEC and ETEC isolates are not negligible, and therefore, more studies of the molecular mechanisms of resistance to β-lactams in diarrhoeagenic *E. coli* are needed to elucidate the carriage rates, potential dissemination and origin of this resistance.

3. Epidemiology of *E. coli* clinical isolates

Given the heterogeneity and genomic plasticity of *E. coli*, epidemiological studies based on typing methodologies are essential to better understand clonal dissemination and the spread of resistance or virulence determinants.

Phylogenetic group classification has been well-defined, arranging *E. coli* strains by phenotypical characteristics, antimicrobial resistance patterns, ecological niches and even the propensity to cause disease in specific environments. Phylogenetic groups B1 and A are considered less virulent and are associated with *E. coli* commensal strains, while the B2 and D phylogenetic groups are commonly associated with pathogenic extraintestinal strains (163). Since Clermont and colleagues updated this methodology in 2013, the existence of phylogenetic group E, a small set of unassigned strains, is now well recognized. Phylogenetic group F is also now documented and consists of strains that form a sister group to group B2; and phylogenetic group C has been proposed for a group of strains closely related to, but distinct from group B1 (167). In the present thesis, the updated methodology was only employed in *Additional Results II*, the time at which it was implemented in our research laboratory.

Other important typing strategies such as MLST (Achtman scheme) and pulsed-field gel electrophoresis (PFGE) have been used in limited collections sharing the type of infection or the molecular resistance mechanism to β-lactams, being restricted to only MLST for larger collections with scarce literature available, such as EAEC and ETEC causing TD.

3.1. Epidemiological relationship between *E. coli* isolates sharing resistance mechanisms and/or virulence factors causing extraintestinal and intestinal infections (*Papers 1, 2, 3, 6, 7* and *Additional Results I*)

With regard to the collection characterised in *Paper 1*, *E. coli* strains causing bacteraemia fall into the four main phylogenetic groups (11.5% B1, 16% A, 19.5% B2 and 53% D), phylogenetic group D being the most prevalent. Although this phylogenetic group distribution does not correlate with that reported for ExPEC strains, on analysing the strains positive for the enterotoxin VFs the following was observed: (i) a relationship between the presence of the ShET-1 enterotoxin and phylogenetic group B2, possibly indicating the higher capacity of these strains to acquire VFs from other bacteria, and (ii) the ShET-2 encoding gene was related to strains belonging to phylogenetic group B1. These results suggest that despite being typical of intestinal *E. coli,* enterotoxin ShET-1 could play an important role as a VF in phylogenetic group B2, which is typically formed by ExPEC strains. Regarding the prevalence of phylogenetic group B1 within the ShET-2 gene-carrying isolates, there might be an increase in the virulence of these commensal phylogenetic group. Nevertheless, further studies are needed to reinforce these hypotheses.

The UPEC strains positive for the enterotoxin encoding genes ShET-1B and EAST-1 studied in *Paper 2* also presented the four main phylogenetic groups, being phylogenetic group B2 the most prevalent. The majority of the UPEC isolates presenting the ShET-1B toxin (82%) belonged to phylogenetic group B2, two to phylogenetic group A and B1 each (7%), and only one to phylogenetic group D (3%). Among the eight *astA*-positive isolates, five (63%) belonged to phylogenetic group B2 and one to phylogenetic groups A, B1, and D. The high prevalence of both genes in *E. coli* isolates belonging to phylogenetic group B2 is in agreement with the higher virulence reported by this phylogenetic group. In a Korean study carried out by Lee *et al.* with a UPEC collection, the overall phylogenetic group distribution was similar, and the number of VFs exhibited was also significantly higher in strains from phylogenetic groups B2

and D (277). Since the phylogenetic group of the strains carrying the enterotoxin VFGs has not, to date, been explored in any study with UPEC strains, specific results of this section cannot be adequately discussed.

Among the collection of *E. coli* vaginal isolates from pregnant and nonpregnant women studied in *Paper 3*, phylogenetic group B2 was the most frequent among the strains (51%), followed by groups D (34%), A (12%) and B1 (3%). Among the nalidixic acid-susceptible strains (which in turn present greater VF carriage than the resistant counterpart), phylogenetic group B2 was significantly more frequent. Strains isolated from pregnant women were also mainly classified into phylogenetic group B2, and a high percentage of isolates from nonpregnant women were phylogenetic group A, thereby confirming the greater virulence of *E. coli* isolates from pregnant women and the commensal role of the isolates colonising nonpregnant women. Similar results on the phylogenetic distribution were found in another study with vaginal *E. coli*, although the prevalence of phylogenetic group B2 was found to be greater in the group of nonpregnant women (47).

The 12 *E. coli* isolates carrying a $bla_{\text{CTX-M}}$ group1 gene and causing bloodstream infection and UTI in children in Manhiça were characterised in *Paper 6* by three typing methods. PFGE allowed the epidemiological associations that were further considered for treating the typing results by epidemiological groups. The final eight non-epidemiologically related isolates fell into the four main phylogenetic groups, none being notably prevalent. All the strains causing UTI belonged to phylogenetic group A, in accordance with a Russian UPEC collection (278). On the other hand, this result was completely opposite to the literature available on the distribution of the UPEC phylogenetic group, especially that of an Estonian study with UPEC isolated from children with recurrent UTIs (279), although it has been associated with MDR strains causing UTI (280). This phenomenon may be due to three causes: (i) the possible differential geographical distribution of phylogenetic groups of UPEC, (ii) UPEC virulent strains have a commensal microbiota origin and have acquired virulence and resistance determinants, or (iii) a study population with a greater number of clinically compromising

conditions (174). The MLST results classified all the non-epidemiologically related isolates into different ST, and only two belonged to the same clonal complex (CC10). The overall results indicated that, whereas the mechanism of resistance to third-generation cephalosporins in the collection of isolates of our study was the same, the isolates showed low relatedness at an epidemiological level, thus not being a clonal dissemination or plasmid spreading, as demonstrated above.

In *Paper 7*, the epidemiologic relationship among the 5 EAEC strains causing TD in patients returning from India and carrying the $bla_{\text{CTX-M 15}}$ was studied by PFGE and MLST. Cluster analysis of the PFGE indicated that the strains were non-epidemiologically related, and MLST classified the strains into two CCs: 3 strains belonging to ST38 (phylogenetic group D), one to ST10 (phylogenetic group B2) and the last strain was a singleton. The distribution of clonal complex CC38 has globally expanded among ESBL-producing ExPEC (281), but it was rarely identified in intestinal strains until 2010 when Okeke *et al.* found this CC to be the potential cause of a diarrhoeal outbreak in Nigerian children (282). Recently, epidemiological studies with EAEC have shown that CC38 may also be associated with multidrug resistance and with UTIs in humans and that the UPEC/EAEC pathotype may be an evolving clonal group (210). Concerning CC10, it was the second most prevalent among *E. coli* clinical isolates of all specimen types in a multicentre French study after CC131. This CC10 was a highly heterogeneous group (made up of different STs), and it was formed by more CTX-M producing strains than non-ESBL producers (283). Regarding epidemiological studies on EAEC, CC10 was the most common CC found in the collection of intestinal EAEC recovered from Nigerian children (282). Since none of the isolates of *Paper 7* were found to be the successful ST131 clone, this suggests that CC38 might also play an important role in causing infectious intestinal diseases in India.

In the EAEC and ETEC collection causing TD in patients travelling to LMIC characterised in *Additional Results I*, ESBL-producing isolates were typed by MLST, and the two most prevalent STs detected were ST38 carrying CTX-M-15 (all from India) and ST131 carrying CTX-M-27 (from South-East Asia). ST38 has been demonstrated to be a successful EAEC causing intestinal

disease as well as ESBL-producing group (282,284), although this ST was also found in one ETEC strain in the present study and has not been described in previous reports. CTX-M-27-producing ExPEC ST131 has been described in several countries (285), but as far as we know its presence in ETEC strains has not been reported previously. These results indicate that further epidemiological studies are needed on clinical intestinal *E. coli* isolates at a global scale, in order to elucidate whether we are facing a clonal or a resistance traits dissemination. These analyses will help to focus to develop more accurate diagnostic approaches to identify the best predictive features of the pathogenesis of diarrhoeagenic *E. coli* as well as the epidemiology and the molecular basis of antimicrobial resistance.

3.2. Epidemiology of *E. coli* strains causing traveller's diarrhoea worldwide (*Additional Results II*)

In *Additional Results II*, an approach to determine the epidemiological relationships among EAEC and ETEC isolates is done, by typing the isolates causing TD in patients attending the Tropical Medicine Unit of our hospital between 2001 and 2014 after returning from Latin America, South-East Asia and Africa. As these bacteria are also causing intestinal disease in the general population of these geographical areas, it is important to determine the epidemiological relationships among these *E. coli* pathotypes.

The most prevalent phylogenetic group among the EAEC and ETEC isolates studied was group A, followed by B1 and E. None of the isolates was clustered in phylogenetic group B2, a group that is largely comprised of extraintestinal pathogenic *E. coli* (286) together with group D, the prevalence of which was also low in this collection. Phylogenetic group clustering differed slightly on analysing the two pathotypes separately, and the distribution of the EAEC phylogenetic groups was in accordance with the study carried out by Okeke *et al.* (282) in Nigeria. These authors also studied EAEC isolates (mainly from Nigeria but also from other locations), showing a low prevalence of phylogroup B2 (4.6%). Another report by von Mentzer *et al.* on the distribution of phylogenetic groups of ETEC isolated from different countries

showed similar percentages of groups A, B1 and E, while B2 was very infrequent (35). Okeke and collaborators suggested a possible differential geographic distribution of EAEC isolates belonging to phylogenetic group B2, considering that a previous French study had reported this group to be more commonly found in Asia than in Africa (287). However, the present study strongly supports the contention that, on a worldwide scale, EAEC strains (also ETEC) may not be associated with the B2 lineage. Nonetheless, further studies should be made in this regard. Among the other most prevalent phylogenetic groups, no major differences were found in the geographical distribution except for group A, which was more frequently found in the Latin American isolates. This result is in accordance with the French study mentioned above, in which phylogenetic group A was more frequently found in intestinal *E. coli* from Latin American populations, with percentages of around 60% (287). When dividing the EAEC and ETEC isolates into two isolation periods, the distribution of the phylogenetic groups seems to have evolved. While the prevalence group B1 decreased almost 50% in isolates from the latter period, the prevalence of phylogenetic groups C, E and F increased. Periodic changing in the distribution of phylogenetic groups has been reported for commensal *E. coli* isolated from human populations; however, the greatest reduction was not found in group B1 but rather group A, mainly being replaced by group B2 (175).

In general lines, the MLST approach showed high clonal diversity among the EAEC and ETEC isolates causing TD. The most frequent STs and CCs in EAEC isolates were ST10 and CC10, respectively, again in accordance with the Nigerian study (282). Nonetheless, taking into account that ST10 is the one of the largest STs in the MLST database in terms of prevalence, this ST may be overrepresented among the EAEC collection.

The ETEC isolates presented a greater number of STs than the EAEC group; ST4 (also belonging to CC10) was the most frequent, being ST10 poorly clustered. The abovementioned ETEC study by von Mentzer *et al.* also detected isolates belonging to ST4, although this ST was not the most predominant of the collection. Nonetheless, similar to our results, it was also detected ST1312 as one of the most prevalent STs among the ETEC isolates studied (35).

With regard to the distribution of the STs per geographical area not taking into account the pathotype, ST4 was the most prevalent in Africa and ST10 in Latin America, and no isolate

from South-East Asia belonged to ST4. Isolates belonging to ST10 and ST4 are highly described in the *E. coli* species, and the STs found in the three areas varied greatly suggesting that there might be a geographical distribution of STs belonging to multiple lineages causing intestinal disease. Moreover, the distribution of STs and CCs for the two isolation periods was very variable, observing a significant decrease of isolates belonging to ST10 as well as an increase of the ST4 lineage, indicating the evolution of strains causing TD probably related to fitness cost of the EAEC or ETEC-determining plasmid carriage. Nevertheless, larger studies are necessary to strengthen this hypothesis, as there is a limitation in the number of isolates per geographical area in the present study.

Since the cluster analysis of the STs of the study isolates found no evidence of any epidemiological relationship between the EAEC and ETEC pathotypes, we hypothesised that EAEC and ETEC strains are simply any *E. coli* lineage that can acquire, express and retain plasmids harbouring colonisation factors and/or toxins, and therefore, the classification of pathotypes is not related to the *E. coli* phylogeny. Nevertheless, MLST and phylogenetic grouping are very reliable tools for epidemiological studies of EAEC and ETEC isolates causing TD or intestinal disease, and their use is widely encouraged for surveillance networks over time.

VII. CONCLUSIONS

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1. There is a transference of virulence factor genes (VFGs) between intestinal and extraintestinal *E. coli* leading to the finding of enterotoxins typical of *Shigella* spp. and enteroaggregative *E. coli* (EAEC) among clinical isolates causing bloodstream and urinary tract infections (UTIs).

2. *E. coli* strains isolated from vaginal and endocervical samples of pregnant women present more VFGs than those from nonpregnant women. Among these, haemolysin, cytotoxic necrotizing factor and P-fimbriae could play an important role in the subsequent development of neonatal sepsis.

3. Some VFGs are more prevalent in *E. coli* isolates causing specific extraintestinal infections, and may even show an environmental-dependent distribution in relation to iron recruitment systems for *E. coli* causing obstetric infections. The presence of these VFGs usually corresponds to the virulence potential of the bacteria.

4. A relationship between *E. coli* quinolone-susceptible isolates and the presence of certain VFGs such as the *set1* gene (in bloodstream infections) and the *hly*, *cnf* and *pap* genes (in the genital tract of women) has been demonstrated, possibly due to induction of the partial or total loss of the pathogenicity islands (PAIs) in which these genes are located.

5. The prevalence of antimicrobial resistance among *E. coli* causing obstetric infections was similar to that found in other extraintestinal pathogenic *E. coli* (ExPEC), except with lower rates of resistance to third-generation cephalosporins. This demonstrates that the empirical treatment of this infection with ceftriaxone or ampicillin-cefoxitin in our hospital is adequate.

6. High percentages of resistance to ampicillin and gentamicin among *E. coli* isolates causing neonatal sepsis were observed, making a change in the empirical treatment of neonates in our setting necessary. The lower prevalence of resistance to cephalosporins among

these isolates suggests that these antimicrobial agents should be included as first-line therapy for this infection.

7. Since the increase of the resistance to ampicillin among *E. coli* isolates causing neonatal sepsis has been associated with the use of this antibiotic for intrapartum prophylaxis, the current empirical treatment of this infection in our hospital has been revised and now takes in account the previous administration of intrapartum prophylaxis to the mother.

8. A significant percentage of MDR isolates carrying extended spectrum β-lactamases (ESBL) was found among *E. coli* causing bloodstream infections and UTIs in children from Mozambique, despite cephalosporins not being widely used for these infections in this setting. This may be attributed to a cross-resistance acquisition phenomenon or the use of this family of antimicrobial agents to treat other bacterial infections highly prevalent in this geographical area.

9. Resistance to quinolones and third-generation cephalosporins has significantly increased over the last decade in enteroaggregative (EAEC) and enterotoxigenic (ETEC) *E. coli* strains causing traveller's diarrhoea (TD), mainly in patients travelling to India and sub-Saharan Africa. For that reason, fluoroquinolones (the current therapy) should no longer be considered the drugs of choice for travellers to high-risk areas.

10. The low prevalence of azithromycin- and rifaximin-resistant EAEC and ETEC isolates obtained demonstrates that these antimicrobial agents remain adequate for the treatment of TD, being azithromycin recommended for children and patients travelling to endemic areas of *Campylobacter* spp. such as South-East Asia.

11. The main molecular mechanism of resistance to β-lactams in clinically relevant *E. coli* isolates is CTX-M ESBL. The gene encoding this enzyme is mostly located in conjugative plasmids and downstream from the insertion sequence IS*Ecp1*, thereby allowing rapid and easy dissemination of this resistance determinant.

12. The most prevalent plasmid incompatibility group described in the ESBL-producing *E. coli* isolates of the collections studied was IncF.

13. Although *E. coli* phylogenetic groups B2 and D are described as the most virulent among ExPEC isolates, phylogenetic groups A and B1 were also found in the clinically relevant *E. coli* isolates studied.

14. Although several *E. coli* clinical isolates carrying the $bla_{CTX\text{-}M}$ group 1 gene among the collections studied belonged to the clonal complexes CC10 and CC38, the remaining isolates were not epidemiologically related.

15. The clonal groups ST38 and ST131 carrying the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}27}$ genes, respectively, are highly prevalent in ESBL-producing EAEC and ETEC isolates causing TD.

16. EAEC and ETEC isolates causing TD worldwide are epidemiologically very heterogeneous, mainly belonging to CC10 and being differently distributed among the geographical areas.

17. Due to the scarce data on virulence, antimicrobial resistance and the clonal prevalence of *E. coli* causing the infections compiled in the present thesis, it is important to establish or maintain specific surveillance networks for these kinds of infections in order to adapt therapeutic guidelines as needed.

VIII. RESUM DE LA TESI

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8.1 Introducció

8.1.1 Característiques generals d'*Escherichia coli*

Escherichia coli és un dels bacteris més estudiats arreu del món des del seu descobriment l'any 1885 pel bacteriòleg i pediatra alemany-austríac Theodore von Escherich. És un bacil Gram-negatiu, anaeròbic facultatiu, que es troba majoritàriament com a colonitzador normal del tracte intestinal d'animals de sang calenta, tot i que també es pot adaptar a ambients externs a l'hoste (aigua, sòl, plantes i aliments) donada la seva robustesa i flexibilitat metabòlica. Taxonòmicament pertany a la família *Enterobacteriaceae*, essent un component important de la microbiota intestinal i involucrat en alguns processos metabòlics essencials com la producció de vitamina K i vitamina B12. L'*E. coli* també ajuda a mantenir l'ambient anaeròbic necessari per la majoria de microbiota tot consumint l'oxigen que entra als intestins i exclou competitivament patògens del còlon dels seus hostes. Tot i establir relacions simbiòtiques, aquest conegut microorganisme també pot tenir un paper patogènic en els seus hostes, especialment des del punt de vista humà. L'*E. coli* pot adquirir varis trets o factors de virulència (FV), ja sigui d'altres espècies o d'altres soques d'*E. coli*, que li confereixen la capacitat de causar patologies intestinals resultants en diarrea. A més, aquests trets també els poden ajudar a travessar la barrera intestinal, tot arribant a altres parts del cos, essent capaços de causar gran varietat d'infeccions com urinàries, genitals i obstètriques o septicèmia en adults i nounats. Aquestes infeccions poden presentar gran prevalença i fins i tot arribar a altes taxes de morbiditat o mortalitat. Com a exemple, la càrrega associada a les infeccions del torrent sanguini degudes a *E. coli* a Europa fou de 17.000 morts el 2016, de les quals 6.000 van ser causades per bacteris resistents a antibiòtics. Tot i que aquestes xifres excedeixen les esperades en un continent desenvolupat, demostren la presència d'un repte de salut emergent –la resistència antimicrobiana. Segons un informe recent sobre aquesta problemàtica, es prediu que moriran 3 milions de persones per infeccions degudes a *E. coli* resistent a antibiòtics abans del 2050 si no es prenen mesures per afrontar el tema en qüestió.

La patogenicitat bacteriana es defineix com la capacitat genètica del bacteri per a causar malaltia, basada en els trets de virulència i resistència que posseeixi. Desafortunadament, l'*E. coli* pot adquirir fàcilment aquesta capacitat donada la seva plasticitat genètica (s'ha estimat que fins a un 18% del seu genoma és representat per ADN adquirit horitzontalment) i és considerat una de les majors causes d'infeccions bacterianes en humans arreu del món. Per tal d'entendre millor el doble component de la patogenicitat de l'espècie, cal definir acuradament els conceptes de virulència i resistència.

La **virulència** és l'habilitat patogènica per a causar dany a l'hoste i és controlada per l'expressió de factors de virulència combinats. Aquests factors estan codificats pels anomenats gens de factors de virulència (GFV) i poden incloure adhesines i invasines per a la colonització de teixits o ambients específics, toxines, sistemes de secreció i sistemes d'absorció de ferro o sideròfors. Els GFV no són específics de cap infecció, de cap soca d'*E. coli* ni de cap hoste. La seva combinació determinarà la seva capacitat de causar infecció en una localització específica de l'hoste. Aquests gens es poden trobar a diferents llocs del genoma bacterià, ja sigui en el cromosoma o en elements genètics mòbils com bacteriòfags, plasmidis o illes genòmiques (illes associades a patogenicitat –IAPs– quan estan formades per GFV).

La **resistència** és la capacitat de persistir i créixer en un determinat ambient, tenint en compte diferents variables com la temperatura, les condicions de pH o les concentracions d'antibiòtic presents. La resistència antimicrobiana en *E. coli* és un aspecte preocupant, la gestió de la qual és important però complicada; mentre els antibiòtics són la solució per a lluitar contra les malalties infeccioses, també són la causa de la selecció de bacteris resistents. El mal ús i abús dels antibiòtics fa augmentar la prevalença d'aquests últims, pel que ara s'estan prenent moltes mesures a nivell global (no només en humans sinó en animals i en l'agricultura) per a evitar que els medicaments que ara curen esdevinguin inefectius en uns anys. En aquesta línia, s'estan dedicant molts esforços en millorar o implementar sistemes de vigilància, revisar les guies terapèutiques, millorar el diagnòstic i impulsar el desenvolupament de noves famílies d'antimicrobians. Per a desenvolupar totes aquestes accions és essencial el coneixement en les bases moleculars de la resistència antimicrobiana així com la seva relació amb la virulència.

La present tesi està enfocada a l'estudi de les bases moleculars dels mecanismes de resistència a antibiòtics més comunament usats en la teràpia d'*E. coli*, així com de la prevalença dels diferents GFV en diverses infeccions d'*E. coli* intestinals i extraintestinals, per tal de comprendre millor els aspectes de la patogènia del bacteri en l'hoste humà.

8.1.2 L'*E. coli* com a patogen humà

L'*E. coli* és un dels patògens humans més importants. Per tal de classificar la patogènia de l'espècie, s'han establert diverses designacions als diferents patotips segons la localització de la infecció i de les especificitats genòmiques que posseeixen:

***E. coli* intestinal o diarreogènic:**

L'*E. coli* és un dels principals agents etiològics de la diarrea comú o de la diarrea del viatger. Aquesta infecció és un greu problema de salut pública que causa altes taxes de morbiditat i mortalitat, majoritàriament en infants de països de renta mitjana o baixa. Tots els *E. coli* causants de diarrea es caracteritzen per la possessió d'un tret genètic relacionat amb la virulència que està localitzat en un plasmidi.

L'*E. coli* causant de diarrea pot presentar diverses manifestacions clíniques, preferències en els llocs de colonització i trets de virulència distintius, derivant la seva classificació en vàries categories o patotips:

a. *E. coli* enteropatogènic (ECEP)

   Els ECEP són soques que produeixen dany a l'epiteli intestinal però no excreten cap toxina. Les soques típiques d'aquest patotip es caracteritzen per la presència d'un plasmidi virulent molt gran anomenat plasmidi de factor d'adherència, que conté GFV que faciliten l'adherència i inhibeixen vies del sistema immunitari de l'hoste.

b. *E. coli* enterohemorràgic (o productor de toxina Shiga) (ECEH/ECTS)

Aquest patotip és molt robust i sol trobar-se en aliments contaminats, donada la seva capacitat per formar biopel·lícula. Es caracteritza per la producció de toxina/es Shiga i pot causar manifestacions molt diverses de diarrea, des de pràcticament inaparents fins al síndrome urèmic-hemolític, que afecta bàsicament a infants.

c. *E. coli* enteroagregatiu (ECEA)

L'ECEA es caracteritza per la capacitat d'adherir-se a la superfície de les cèl·lules epitelials de l'intestí en forma de "maons apilats", produint una lesió molt característica del teixit intestinal que fa augmentar la secreció de mucus, en el qual es queden els bacteris atrapats. La diarrea que provoca és molt aquosa, sovint mucosa i pot arribar a ser persistent. Els GFV més característics d'aquest patotip codifiquen per factors d'adherència agregatius (FAA) que inclouen 5 famílies de fímbries, dispersines, toxines (EAST, ShET-1, ShET-2, Pet i Pic) i proteïnes d'antiagregació.

Aquest patotip té un impacte molt important en la salut pública, essent l'agent predominant de diarrea persistent en infants menors de 5 anys.

d. *E. coli* enterotoxigènic (ECET)

Els ECET es caracteritzen per la producció de factors de colonització i d'almenys una de les següents enterotoxines: enterotoxina termo-làbil (LT) i enterotoxina termo-estable (ST), ambdues localitzades en plasmidis transferibles. Aquestes es produeixen després de l'adhesió a la mucosa de l'intestí i causen la desregulació dels canals iònics a la membrana epitelial, produint una pèrdua massiva d'ions i aigua. Es tracta d'un patotip molt divers a nivell epidemiològic i juntament amb l'ECEA és una de les principals causes de diarrea en infants en països de renta mitjana o baixa així com de diarrea del viatger.

e. *E. coli* enteroinvasiu (ECEI)

Aquest patotip és una de les causes més comuns de disenteria en humans, provocant febre, rampes abdominals i diarrea sanguinolenta i mucosa. L'ECEI envaeix i penetra als enteròcits, amb la subseqüent destrucció i resposta proinflamatòria important. La capacitat invasiva li confereix un plasmidi gran (d'unes 220 kb) anomenat *pInv*, que

conté gens d'invasivitat, de regulació de la resposta immunitària de l'hoste i de sistema de secreció tipus II. Les soques d'aquest patotip pertanyen a un serotip concret que ha causat brots importants arreu del món des de que es va descriure per primer cop el 1947.

f. *E. coli* difusament adherent (ECDA)

L'ECDA es caracteritza per l'adhesió a la monocapa de cèl·lules epitelials en un patró difús, causant diarrea en infants de més de 12 mesos induint un efecte citopàtic (es desenvolupen extensions cel·lulars que rodegen al bacteri adherit). La majoria de soques d'ECDA produeixen una adhesina fimbrial anomenada F1845 que s'uneix a un receptor epitelial induint la inflamació intestinal. Aquest patotip és difícil de diagnosticar i la seva epidemiologia ha estat poc estudiada.

E. coli extraintestinal patogènic (ECExP):

Existeixen algunes soques d'*E. coli* que tenen l'habilitat de sobrepassar les defenses de l'hoste i causar malalties extraintestinals en persones sanes, esdevenint importants patògens humans. Es coneixen com a *E. coli* extraintestinals patogènics (ECExP), tot i que reben una designació més concreta en funció del nínxol que colonitzen. Els grups més importants d'*E. coli* extraintestinal patogènics són:

a. *E. coli* causant de bacterièmia o septicèmia (ECSEP)

La infecció del torrent sanguini o bacterièmia per *E. coli* pot aparèixer posteriorment a una infecció primària del sistema urinari, infecció abdominal, pneumònia o altres infeccions. Quan la presència del bacteri a la sang desencadena una resposta sistèmica important, la malaltia es designa com a septicèmia, una de les 10 primeres causes de mortalitat en països de renta alta. Les soques d'ECSEP són filogenèticament diferent a les d'*E. coli* intestinal o comensal, i posseeixen gran varietat de GFV, com hemolisines o factors necrotitzants, tot i que també poden contenir altres GFV no tan habituals que els hi confereixin capacitats especials. Aquesta infecció requereix tractament antibiòtic

immediat, i el tractament empíric que es prescriu habitualment ja presenta elevades taxes de resistència.

b. *E. coli* uropatogènic (ECUP)

E. coli és el principal agent etiològic de les infeccions urinàries, les quals solen presentar recidives. Entre els factors de virulència més importants de les soques d'ECUP destaquen el lipopolisacàrid (que permet colonitzar la bufeta, entre altres funcions), flagels i pilis, adhesines, toxines i sistemes d'adquisició de ferro. Un ampli ventall de famílies d'antibiòtic s'utilitza pel tractament d'infeccions produïdes per ECUP.

c. E. coli causant d'infeccions obstètriques

E. coli és el segon agent etiològic d'infeccions obstètriques. Les soques que les produeixen posseeixen varis GFV (com adhesines, fímbries i toxines) que faciliten la colonització vaginal i endocervical de les dones embarassades. Aquesta colonització pot provocar infeccions importants com endometritis, infecció intraamniòtica i fins a septicèmia, que alhora pot passar al fetus causant-li infeccions greus. El tractament d'aquestes infeccions està limitat pels antibiòtics considerats segurs pel desenvolupament fetal, principalment β-lactàmics o aminoglicòsids.

d. *E. coli* causant de septicèmia neonatal i meningitis (ECMN)

L'*E. coli* pot propagar-se des de la mare fins al fetus o nounat (segons es doni abans o en el moment del part, respectivament) per transmissió vertical. La septicèmia neonatal es divideix en precoç o tardana, segons el temps de vida del nounat en el moment que els primers símptomes es manifesten. La virulència de les soques causants d'aquesta infecció és bastant desconeguda, tot i que s'ha proposat que el factor de virulència IbeA pot jugar un paper important en la translocació de l'*E. coli* a través de la membrana amniòtica.

La meningitis neonatal causada per *E. coli* té una taxa de mortalitat alta (20-29%) i una incidència de 0,1/1000 naixements en països de renta alta. Els factors de virulència

relacionats amb les soques que causen aquesta infecció són les proteïnes de membrana externa K1 i OmpA, sideròfors, adhesines i factors d'invasió.

El tractament antibiòtic en infeccions neonatals sol basar-se en combinacions de β-lactàmics i aminoglicòsids per la septicèmia i en cefotaxima per la meningitis, donada la seva excel·lent penetració al líquid cefaloraquidi.

8.1.3 Mecanismes de resistència antimicrobiana en *E. coli*

La resistència antimicrobiana és una problemàtica global. Els bacteris poden ser resistents als antibiòtics degut a que el mecanisme de resistència sigui innat en la soca bacteriana, o bé per l'habilitat de la soca d'adquirir mecanismes de resistència per diferents mitjans.

Des d'una perspectiva evolutiva, els bacteris han utilitzat dues estratègies per lluitar contra l'efecte dels antibiòtics: mutant gens associats amb el mecanisme d'acció de l'antibiòtic o adquirint ADN extern per transferència horitzontal de gens que contenen determinants de resistència antibiòtica.

Els principals antibiòtics utilitzats pel tractament d'infeccions degudes a *E. coli* són els aminoglicòsids (com la gentamicina o l'amicacina), els macròlids (inclouen l'azitromicina o l'eritromicina), les quinolones (principalment la ciprofloxacina), la rifaximina, el cotrimoxazol (antibacterià combinat de trimetoprim i sulfametoxazol) i els β-lactàmics (essent els més empleats la penicil·lina, l'ampicil·lina o les cefalosporines).

Els mecanismes de resistència que ha desenvolupat *E. coli* per tal d'evitar l'efecte d'aquests antibiòtics es basen en:

- L'adquisició de gens que codifiquen per enzims que causen canvis conformacionals o destrueixen l'antibiótic.
- Alteracions en la permeabilitat de la membrana bacteriana, evitant així l'entrada de l'antibiòtic al bacteri.

- Expressió de bombes d'expulsió activa per a expulsar l'antibiótic de la cèl·lula bacteriana.
- Mutacions en la diana bacteriana de l'antibiòtic, mantenint la funcionalitat d'aquesta però evitant que l'antibiòtic hi pugui actuar.

Els mecanismes de resistència més prevalents i importants trobats en les soques clíniques de bacteris Gram-negatius com *E. coli* són els que actuen enfront als antibiòtics β-lactàmics, mitjançant l'adquisició de gens que codifiquen per enzims que els destrueixen. Aquests enzims s'anomenen β-lactamases i es classifiquen segons la seqüència aminoacídica del gen que els codifica (classificació d'Ambler) o bé segons els substrats o perfils d'inhibició (classificació funcional de Bush i Jacoby). Les β-lactamases que provoquen més problemes de resistència (ja que solen presentar-se en soques resistents a múltiples antibiòtics) i, per tant, altes taxes de morbiditat i mortalitat en els pacients són les β-lactamases d'espectre extès (BLEEs). Els bacteris que expressen aquests enzims són capaços d'hidrolitzar les cefalosporines d'espectre extès com la ceftazidima, la cefotaxima o la ceftriaxona. La BLEE més representativa és la CTX-M-15, que presenta una gran capacitat d'hidròlisi de la cefotaxima i la ceftriaxona i està codificada pel gen $bla_{\text{CTX-M-15}}$. Aquest gen sol trobar-se ubicat en plasmidis estables i transferibles i en regions de resistència múltiple (que contenen altres gens de resistència a altres famílies d'antibiòtics). Aquestes regions s'estableixen en elements genètics que es transfereixen horitzontalment, com els transposons o les seqüències d'inserció (SIs), el que facilita la dispersió i disseminació del gen de resistència, amb el conseqüent augment de les taxes d'infecció per *E. coli* resistent a cefalosporines en la última dècada a nivell mundial.

Relació entre la virulència i la resistència antimicrobiana en aïllats clínics d'*E. coli*:

En els estudis realitzats a principis del segle XXI sobre la virulència de soques d'*E. coli* causants d'infeccions urinàries es va observar un fenomen característic que es repetia: aquelles soques que presentaven més GFVs solien ser susceptibles a les quinolones i viceversa. Els estudis es van ampliar a altres col·leccions d'*E. coli* patogèniques extraintestinals i fins i tot intestinals, i es va veure que la relació es donava pels GFVs que es localitzen en IAPs, illes que comparteixen característiques estructurals amb els bacteriòfags i que es poden escindir fàcil i

espontàniament del cromosoma bacterià. Paral·lelament, l'exposició a quinolones pot provocar un augment en la deleció i transposició de regions de DNA mitjançant l'activació del mecanisme de reparació SOS, que alhora escindeix pro-fags (genoma del bacteriòfag inserta al cromosoma bacterià) i per extensió també pot eliminar parcial o totalment les IAPs, causant una pèrdua de GFV en les soques que esdevenen resistents a quinolones.

8.1.4 L'epidemiologia d'*E. coli*

L'epidemiologia bacteriana és la metodologia utilitzada per a estudiar les relacions i distribucions de soques bacterianes segons el seu contingut genòmic i els seus perfils de virulència o resistència antibacteriana. Existeixen vàries estratègies de classificació dels aïllats d'*E. coli* basats en trets genotípics o fenotípics, que diferencien o ajunten les soques per tal de determinar si tenen un origen comú o per establir el potencial patogènic que posseeixen. Les metodologies moleculars actuals permeten l'associació de llinatges clonals amb el potencial de virulència o determinen els orígens patogènics de l'espècie, tot i l'alta recombinació genètica que pot donar-se en el genoma d'*E. coli*.

Desafortunadament, no existeix una única metodologia epidemiològica ràpida, precisa, barata i fiable que sigui capaç de diferenciar tots els grups patogènics d'*E. coli*, pel que cal fer combinacions de varis mètodes moleculars. En la present tesi doctoral, els mètodes de classificació epidemiològica principalment emprats foren els següents:

- **Grup filogenètic:**

Aquesta metodologia es basa en l'assignació dels aïllats d'*E. coli* en 7 grups principals (A, B1, C, E, D, F i B2) mitjançant les amplificacions d'uns fragments genòmics o gens per PCR (reacció en cadena de la polimerasa). La combinació de bandes amplificades determina el grup filogenètic de l'aïllat, i inicialment es va descriure un patró d'agrupació, en el qual les soques d'*E. coli* extraintestinal patogèniques pertanyien als grups B2 i D i les soques intestinals o comensals (menys virulentes, en general) eren assignades als grups A i B1. Amb l'ampliació d'estudis es veié que aquesta distribució no es podia generalitzar, i que per tant, aquesta

metodologia sola no era adequada per a predir el potencial patogènic dels aïllats d'*E. coli*. De fet, s'ha suggerit que la distribució de grups filogenètics pot estar més relacionada amb factors socioeconòmics o geogràfics.

La metodologia del grup filogenètic no és prou discriminativa i a no sempre és reproduïble a nivell global, però resulta una eina ràpida i barata per una primera revisió de la clonalitat genètica en col·leccions àmplies, i fins i tot per identificar brots en poblacions concretes.

- ***Multilocus sequence typing* (MLST)**

A finals del segle XX es desenvolupà aquesta tècnica epidemiològica en el model de *Neisseria meningitidis*, basada en la comparació de canvis nucleotídics en seqüències de múltiples gens, altament conservats en el genoma bacterià de l'espècie. Diversos investigadors van desenvolupar la tècnica per a *E. coli*, i fins a tres esquemes diferents es poden realitzar actualment per a la classificació de l'espècie. Tot i així, el més àmpliament utilitzat és l'esquema d'Achtman, que utilitza 7 gens, als quals assigna un número d'al·lel únic, i cada combinació numèrica d'al·lels forma un tipus de seqüència o *sequence type* (ST). Els aïllats que comparteixen com a mínim 6 dels 7 al·lels se'ls assigna al mateix complex clonal (CC) i se'ls anomena com el ST genotip ancestral. Els ST que no formen part de cap CC s'anomenen *singletons*. Les relacions entre STs i CCs es poden visualitzar gràficament amb l'algortime BURST, que utilitza un model simple d'evolució bacteriana basat en la diversificació de genotips ancestrals donant lloc a grups de genotips altament relacionats (CC) que descendeixen d'un mateix fundador. La primera implementació gràfica d'aquest algoritme utilitzada en aquesta tesi doctoral és l'eBURST.

Aquest mètode és car, lent i requereix un alt nivell d'experiència, però és molt adequat per estudis evolutius i per esbrinar possibles relacions filogenètiques en col·leccions d'aïllats.

- **Electroforesi en gel de camp pulsant**

Aquesta tècnica es la més usada per la classificació de nombroses espècies bacterianes, i es basa en tallar l'ADN total del bacteri immobilitzat en agarosa amb enzims de restricció de baixa freqüència, obtenint així un patró de bandes d'ADN particular per a aïllats relacionats epidemiològicament. El gel es corre en un camp elèctric de direccions i sentits variables que

permet separar bandes d'ADN grans i de mides similars. És una metodologia molt laboriosa que no dóna un valor numèric a les soques i que requereix personal tècnic especialitzat, però té un poder discriminatori molt alt i permet comparabilitat a nivell global.

8.2 Justificació del treball, hipòtesis i objectius

Donada la gran preocupació relacionada amb les infeccions causades pel bacteri *E. coli* a nivell mundial, l'emergent aparició d'aïllats resistents a les teràpies antibiòtiques actuals i la morbilitat i mortalitat que se li atribueixen, resulta important l'estudi d'aquest microorganisme de manera holística. Per això, aquesta tesi doctoral és un intent de proporcionar una aproximació integrada a la virulència, la resistència antimicrobiana i l'epidemiologia d' *E. coli*, considerant les següents hipòtesis:

- **Virulència:**

Varis FV confereixen a l'*E. coli* la capacitat de causar infeccions específiques. Aquests factors no han estat ben caracteritzats o bé s'han associat a patotips d'*E. coli* específics de manera errònia. La prevalença dels FV típics d'*E. coli* diarreogènics no s'ha estudiat en aïllats d'ECExP, però també hi podrien jugar un paper important en aquest tipus d'infeccions. Alguns GFV poden estar presents en llocs específics d'infecció, mentre altres ofereixen habilitats de colonització transversals.

- **Resistència antimicrobiana:**

Donat l'increment d'infeccions causades per aïllats resistents a antimicrobians a la família dels Enterobacteris, és important elucidar els principals mecanismes de resistència de les soques clíniques d'*E. coli*. Els mecanismes més prevalents són els enzimàtics, essent el més important el grup de les β-lactamases CTX-M, i més específicament l'enzim CTX-M-15, disseminat arreu del món.

Les guies terapèutiques disponibles pel tractament d'infeccions d'*E. coli* han d'estar actualitzades segons les corresponents taxes de resistència als diferents antibiòtics trobades en els aïllats causants de les diferents infeccions en cada àrea geogràfica. Per aquesta raó és important caracteritzar la prevalença de resistència dels aïllats d'*E. coli* causants cada infecció

a cada ciutat o districte, així com els percentatges de soques multiresistents, que també estan incrementant.

El potencial de virulència i la resistència antimicrobiana no són propietats aïllades del bacteri *E. coli*; podria existir una relació entre ambdós i una explicació biològica que la sustentés.

- **Epidemiologia**

La descripció de les relacions epidemiològiques entre els aïllats d'*E. coli* causants de diferents tipus d'infeccions proporcionarà informació sobre la capacitat de dispersió de clons específics per tal d'establir programes de vigilància si cal. Alguns grups filogenètics semblen més patogènics que altres o bé causen infeccions específiques. Per altra banda, els diferents patotips intestinals d'*E. coli* no poden estar relacionats filogenèticament a escala mundial, però altres factors importants s'haurien de tenir en compte.

Objectius:

Donades les hipòtesis descrites, els objectius de la present tesi doctoral són:

Objectiu general:

Estudiar el versàtil bacteri *E. coli* com un organisme amb implicacions clíniques en termes de virulència, resistència antimicrobiana i epidemiologia.

Objectius específics:

1. Determinar la prevalença de GFV típics d'ECEA en aïllats d'*E. coli* causants d'infeccions extraintestinals (*Articles 1* i *2*).
2. Determinar la prevalença i la potencial especialització ambiental de GFV específics en *E. coli* vaginals potencialment causants d'infeccions obstètriques (*Articles 3* i *4*).
3. Investigar la càrrega de FV en aïllats d'*E. coli* multiresistents a antibiòtics causants d'infeccions intestinals i extraintestinals (*Articles 4* i *7*).

4. Elucidar la possible relació entre virulència i resistència a agents antimicrobians específics en aïllats d'*E. coli* causants de diferents patologies extraintestinals (*Articles 1*, *3* i *4*).
5. Determinar la prevalença de resistència antimicrobiana en soques d'*E. coli* causants d'infeccions intestinals i extraintestinals (*Articles 4*, *5*, *6* i *Resultats Adicionals I*).
6. Estudiar l'evolució de la prevalença de resistència antimicrobiana en aïllats d'*E. coli* causants d'infeccions intestinals i extraintestinals per tal de determinar si les guies terapèutiques requereixen canvis (*Article 5* i *Resultats Adicionals I*).
7. Investigar les bases moleculars de la resistència als agents antimicrobians més freqüentment usats en el tractament clínic d'infeccions per *E. coli* (*Articles 5*, *6*, *7* i *Resultats Adicionals I*).
8. Determinar la prevalença i identificar els mecanismes enzimàtics més importants de resistència enfront a antibiòtics β-lactàmics en *E. coli* causants d'infeccions intestinals i extraintestinals (*Articles 4*, *5*, *6*, *7* i *Resultats Adicionals I*).
9. Establir relacions epidemiològiques entre els aïllats d'*E. coli* que comparteixen mecanismes de resistència i/o FV causants d'infeccions intestinals i extraintestinals (*Articles 1*, *2*, *3*, *6*, *7* i *Resultats Adicionals I*).
10. Determinar l'epidemiologia de soques d'*E. coli* causants de diarrea del viatger arreu del món per tal d'elucidar possibles disseminacions clonals (*Resultats Adicionals II*).

8.3. Resultats i discussió

Els resultats d'aquesta tesi han estat dividits en tres seccions depenent del tipus d'infecció causada pel bacteri *E. coli* i les principals propietats estudiades en cada col·lecció:

- SECCIÓ 1: Virulència en ECExP: Enterotoxines.
- SECCIÓ 2: Virulència i resistència antibiòtica en ECExP: Dones, nounats i infants.
- SECCIÓ 3: Resistència antibiòtica i epidemiologia de la diarrea del viatger.

SECCIÓ 1: Virulència en ECExP: Enterotoxines

Dos estudis varen ser realitzats per tal d'abordar els Objectius 1, 4 i 9:

(i) ARTICLE 1: Prevalença d'enterotoxines en aïllats d'*Escherichia coli* causants de bacterièmia.

Els gens codificants per les toxines ShET-1, ShET-2 i EAST-1 (*set1*, *sen* i *astA*) es van trobar en el 18%, 10% i 10% de soques d'*E. coli* causants de bacterièmia, respectivament. Altres gens codificants per enterotoxines, com el de la toxina Shiga *stx2*, s'han trobat en *E. coli* causants de bacterièmia, així com una soca d'ECEA fou la causant d'un cas de bacterièmia en un pacient alemany que tornava de les Filipines.

El gen *set1*, localitzat en una IAP, es va trobar més freqüentment en soques sensibles a àcid nalidíxic, corroborant la relació inversa entre la presència de GFV associats a IAP i la resistència a quinolones.

La caracterització per grup filogenètic de la col·lecció va categoritzar els aïllats en els 4 grups principals (A, B1, B2 i D), essent el D el grup més prevalent (53%). Tot i així, els aïllats que presentaven el gen codificant per l'enterotoxina ShET-1 pertanyien al grup filogenètic B2, mentre que els que tenien el gen de la toxina ShET-2 estaven més relacionats amb el grup D, establint una possible relació entre el GFV i el grup filogenètic.

(ii) ARTICLE 2: Prevalença dels gens *set1B* i *astA* codificant per enterotoxines en aïllats clínics d'*Escherichia coli* uropatogènics.

Aquesta col·lecció d'*E. coli* uropatogènics presentava el gen *set1B* en el 16% dels aïllats i el gen *astA* en el 8% dels aïllats. Els dos gens han estat reportats en una altra col·lecció d'ECUP mexicana, en la qual el 31,4% i el 30,9% dels aïllats presentaven *set1B* i *astA*, respectivament, suggerint que les soques d'ECUP que adquireixen aquests gens poden esdevenir agents potencials de diarrea.

En la col·lecció d'aquest estudi, el grup filogenètic més prevalent fou el B2, com també ho fou en les soques positives pels gens d'enterotoxines, resultats en concordança amb la majoria d'estudis que classifiquen les soques més virulentes en aquest grup.

Tot i ser factors de virulència típics d'*E. coli* diarreogènics enteroagregatius, el fet de trobar els GFV que codifiquen per enterotoxines en soques extraintestinals emfatitzen la necessitat d'augmentar el coneixement en la potencial virulència extraintestinal de soques d'*E. coli* diarreogèniques o bé en el potencial diarreogènic de soques d'*E. coli* extraintestinals, així com de fer el seguiment de l'adquisició d'aquesta virulència.

SECCIÓ 2: Virulència i resistència antibiòtica en ECExP: Dones, nounats i infants

Aquesta secció inclou els següents 4 estudis, que participaven en l'assoliment dels Objectius 2 al 8:

(i) ARTICLE 3: Prevalença d'*Escherichia coli* en mostres recollides del tracte genital de dones embarassades i no embarassades: relació amb la virulència.

El bacteri *E. coli* es va aïllar en el 15% de dones embarassades i en el 12% de dones no embarassades d'aquesta col·lecció. Els GFV associats amb sistemes de reclutament de ferro *iroN*, *fyu* i *iutA* així com els gens *pap* d'adhesió van ser els més comunament amplificats, resultats en acord amb altres estudis realitzats amb *E. coli*s vaginals. Quan s'estudiaren els aïllats de dones embarassades i de no embarassades per separat, s'observà que els de dones embarassades tenien percentatges molt més alts de freqüència de GFV, essent els gens *hly*,

cnf1, *papC* i *iroN* significativament més freqüents en dones embarassades, els resultats i prevalences dels quals concorden amb altres estudis trobats a la literatura. Aquests gens, per tant, serien uns bons marcadors d'infeccions durant l'embaràs, incrementant llavors el risc de desenvolupar infeccions al fetus o nounat.

(ii) ARTICLE 4: Resistència antimicrobiana i caracterització de la virulència d'aïllats clínics d'*Escherichia coli* causants d'infeccions obstètriques severes en dones embarassades.

En aquest estudi es van caracteritzar soques clíniques d'*E. coli* causants de septicèmia d'origen obstètric i d'infecció intraamniòtica a nivell de resistència i de virulència.

Pel que fa la virulència, els GFV més prevalents en la col·lecció foren els gens *pap* i *fimA*. Els aïllats causants de septicèmia presentaren un major nombre de GFV que els causants d'infecció intraamniòtica, amb percentatges de prevalença significativament majors pels gens *hlyA*, *cnf1*, *papA*, *iha*, *fyuA*, i *papG*II, tots inclosos en IAP. Els dos primers gens han estat reportats en varis estudis per presentar altes prevalences en col·leccions d'ECUP i d'infeccions vaginals, mentre el gen *papGII* també fou trobat l'al·lel més prevalent en aïllats d'*E. coli* vaginals i causants de septicèmia. Els resultats de l'estudi demostraren doncs que els GFV *hly*, *cnf1* i *papGII* es troben més freqüentment en soques més patogèniques i que poden ser utilitzats com a marcadors de virulència en infeccions obstètriques d'*E. coli*. Aquest estudi deixa entreveure una possible especialització ambiental dels sistemes de reclutament de ferro, donat que el gen *iutA* es trobà significativament més freqüent en les soques causants d'infecció intraamniòtica, mentre la resta de GFV de sistemes de reclutament de ferro tenien prevalences més significatives en les soques causants de septicèmia d'origen obstètric, i també s'han observat diferents distribucions d'aquests factors en *E. coli* del tracte genital causants de diferents infeccions o en dones embarassades de diferents àrees geogràfiques.

A nivell de resistència, un 66% dels aïllats eren resistents a l'ampicil·lina, presentant una alta prevalença com la reportada en la literatura per soques d'ECExP, mentre que gairebé totes les soques eren sensibles a les cefalosporines de tercera generació, resultat positiu en comparació a l'emergència de soques resistents portadores de BLEEs en els últims anys

causants d'infeccions extraintestinals, suggerint doncs la correcta implementació d'aquests últims antimicrobians com a primera opció terapèutica per a infeccions obstètriques.

El vint-i-sis percent d'aquests aïllats eren resistents a més de tres famílies d'antibiòtics, presentant un fenotip de multiresistència a antibiòtics. El 70% d'aquests aïllats multiresistents eren causants de septicèmia i presentaven una distribució de GFV més prevalents molt diferent a la dels aïllats d'infecció intraamniòtica, suggerint també una especialització del perfil de virulència.

(iii) ARTICLE 5: Resistència antimicrobiana en soques d'*Escherichia coli* causants de septicèmia neonatal entre el 1998 i el 2008.

En aquest estudi s'observà una alta prevalença i una evolució en els percentatges de resistència a ampicil·lina i a gentamicina en les soques causants de septicèmia neonatal al nostre hospital, essent aquests dos dels tres antibiòtics utilitzats com a primera opció terapèutica per aquesta infecció, a més d'emprar-se com a tractament profilàctic durant el part. Donats aquests resultats, es recomanà un canvi en les guies terapèutiques, suggerint la cefalosporina cefotaxima com a primera opció terapèutica pel tractament de septicèmia neonatal. Altres estudis realitzats en hospitals de Barcelona mostraren també altes prevalences incrementades en el temps de resistència a aquests dos antibiòtics. Cal destacar però, que en aquest cas és important la vigilància a nivell local, ja que els percentatges poden ser més baixos en altres localitats i per tant l'ampicil·lina i la gentamicina poden ser essent efectives com a tractament empíric de la infecció. Posteriorment a la publicació d'aquest article, els neonatòlegs del nostre hospital varen començar a evitar l'administració d'ampicil·lina i gentamicina com a tractament empíric d'aquesta infecció quan s'havia donat profilaxis intrapart a la mare.

El mecanisme de resistència més comú entre les soques resistents a ampicil·lina fou el gen codificant per la β-lactamasa del grup TEM-1 amb una prevalença del 74%, mentre que les dues úniques soques resistents a cefalosporines presentaven la β-lactamasa CTX-M.

(iv) ARTICLE 6: Epidemiologia i caracterització molecular d'aïllats d'*Escherichia coli* resistents a múltiples antibiòtics portadors de la β-lactamasa d'espectre estès *bla*$_{CTX-M}$ del grup 1 causants de bacterièmia i infecció del tracte urinari a Manhiça, Moçambic.

La col·lecció de soques d'aquest article comprenia aïllats d'*E. coli* causants d'infecció urinària i bacterièmia en infants del districte de Manhiça, Moçambic. Aquestes infeccions estan associades a altes taxes de morbiditat i mortalitat en infants africans, pel que es va considerar important estudiar la càrrega del mecanisme de resistència a antibiòtics β-lactàmics més comú a nivell mundial. Sobre el total d'aïllats, un 11,3% eren productors de BLEEs, dels quals un 70,6% eren portadors del gen *bla*$_{CTX-M}$ del grup 1 (majoritàriament CTX-M-15 i només una CTX-M-37), a més de presentar un fenotip de multiresistència a antibiòtics. Aquests percentatges són força alts (i altres estudis del país encara reporten major prevalença) si tenim en compte que les cefalosporines de tercera generació no s'utilitzen com a tractament per aquestes infeccions a Moçambic, ja que són força difícils de disposar a més de cares. Per aquest fenomen podem suggerir dues possibles explicacions: (i) existeix una adquisició de resistència creuada, donat que aquests determinants de resistència es poden trobar en el mateix plasmidi que inclou altres gens de resistència a altres antibiòtics sota els quals sí que hi ha pressió selectiva, o bé (ii) es dóna un ús freqüent de cefalosporines pel tractament d'altres infeccions bacterianes altament prevalents en l'àrea geogràfica.

El gen *bla*$_{CTX-M}$ es va trobar majoritàriament en plasmidis conjugables pertanyents als grups d'incompatibilitat IncF i IncH, tot i que dues soques el presentaven inserit al cromosoma. Tots els aïllats presentaven el gen de resistència cadena avall de la seqüencia d'inserció ISEcp1, cosa que, juntament amb la localització plasmídica implica un alt potencial de disseminació d'aquest determinant de resistència.

Tots els aïllats causants d'infecció urinària pertanyien al grup filogenètic A, en concordança amb un estudi rus d'una col·lecció d'ECUP, però contraris a la majoria d'estudis filogenètics de soques uropatògenes. La majoria de soques amb BLEEs de la col·lecció no mantenien cap relació filogenètica ja que pertanyien a diferents STs, i només dos eren del mateix complex clonal, el CC10. A nivell global, els resultats de l'estudi indicaren que, mentre el mecanisme de

resistència a cefalosporines de tercera generació era el mateix , els aïllats productors de BLEEs mostraven un nivell baix de relació epidemiològica i que per tant no es tractava de cap disseminació clonal, però tampoc de la difusió d'un plasmidi concret.

SECCIÓ 3: Resistència antibiòtica i epidemiologia de la diarrea del viatger

Un Article i dos Resultats Addicionals es presenten per l'acompliment dels Objectius 3, 5, 6, 7, 8, 9 i 10 de la tesi doctoral:

(i) ARTICLE 7: *Escherichia coli* enteroagregatiu productor de CTX-M-15 com a causa de diarrea del viatger.

En aquest article s'observa com un 9,8% dels aïllats d'ECEA causant diarrea del viatger en pacients visitats a la Unitat de Medicina Tropical del nostre hospital durant la primera dècada dels anys 2000 són productors de la BLEE CTX-M-15 i tots provenen de l'Índia. Els 5 aïllats contenen la seqüència d'inserció IS*Ecp1* cadena amunt del gen codificant per la β-lactamasa i tres d'ells el presenten en plasmidis conjugables, mentre que els altres dos el tenen inserit al cromosoma. El GFV *aatA*, usat com un dels primers mètodes moleculars pel diagnòstic d'ECEA, es troba en tots els aïllats, demostrant que continua essent un bon marcador del patotip, tot i que encara cal identificar altres GFV per a tenir el 100% de correlació amb el marcador fenotípic d'adhesió a les cèl·lules HEp-2.

(ii) RESULTATS ADDICIONALS I: Sensibilitat antimicrobiana i mecanismes de resistència a quinolones i a antibiòtics β-lactàmics en *Escherichia coli* enteroagregatius i enterotoxigènics causants de diarrea del viatger.

Aquests resultats addicionals mostren com el percentatge de resistència antibiòtica en soques d'ECEA és major que el de soques d'ECET. En termes generals, s'observen altes taxes de resistència als antibiòtics més econòmics i usats en els països de renta mitjana o baixa com l'ampicil·lina, el cotrimoxazol o la tetraciclina. En estratificar els aïllats segons l'àrea geogràfica visitada pels pacients amb diarrea del viatger, s'observen prevalences més baixes en els que provenen de Llatinoamèrica respecte als del sud-est Asiàtic o l'Àfrica, tal i com ja s'havia

reportat en altres estudis. Els percentatges d'aïllats resistents a azitromicina i a rifaximina són baixos, demostrant que aquests antibiòtics encara són actius i recomanats com a teràpia enfront a ECEA i ECET causant diarrea del viatger.

Els aïllats de la col·lecció productors de BLEEs foren caracteritzats i gairebé tots eren portadors d'enzims CTX-M (CTX-M-15 i CTX-M-27). Ambdós enzims havien estat prèviament caracteritzats en soques d'ECEA, però cap estudi havia mostrat abans un ECET portador de CTX-M-27. Els grups d'incompatibilitat plasmídica descrits en aquests aïllats foren molt variables, i els principals STs descrits van ser el ST38 i el ST131.

(iii) RESULTATS ADDICIONALS II: Epidemiologia d'*Escherichia coli* enteroagregatius i enterotoxigènics causants de diarrea del viatger del sud-est Asiàtic, Llatinoamèrica i Àfrica.

Existeix molt poca bibliografia sobre l'epidemiologia d'ECEA i ECET causants de diarrea del viatger, que són alhora els principals agents etiològics de les infeccions intestinals que pateixen les poblacions de països de renta mitjana o baixa i provocant altes taxes de morbiditat i mortalitats sobretot en infants menors de 5 anys. Per aquesta raó s'ha analitzat a fons les possibles relacions epidemiològiques entre aquests aïllats, tot classificant el seu grup filogenètic i elaborant el MLST.

El grup filogenètic prevalent en aquesta col·lecció va ser el grup A, seguit del B i l'E. Cap dels aïllats va ser classificat dins del grup B2, suggerint que cap dels dos patotips estava associat a aquest llinatge. La classificació per MLST va demostrar que hi havia una alta diversitat clonal entre els aïllats causants de diarrea del viatger dels dos patotips, essent majoritaris el ST10 i el ST4 i distribuïts de manera diferencial per àrea geogràfica.

L'anàlisi de grups clonals de tots els STs representats en la col·lecció va agrupar correctament els STs que formen part del mateix complex clonal i també va agrupar força bé els STs pertanyents als grups filogenètics predominants, però en cap cas va separar els dos patotips, indicant que els aïllats d'ECEA i d'ECET són simplement qualsevol llinatge d'*E. coli* que adquireix, expressa i reté plasmidis que contenen factors de colonització i/o toxines, i que per tant, la classificació de patotips intestinals no està relacionada amb la filogènia d'*E. coli*. Tot i

així, es recomanen els dos mètodes de classificació epidemiològica per aquest tipus d'aïllats i s'encoratja el seu ús continuat i estès per a xarxes de vigilància.

8.4. Conclusions

1. Existeix una transferència de gens de factors de virulència (GFV) entre *E. coli* intestinals i extraintestinals, portant a trobar enterotoxines típiques de *Shigella* spp. i d'*E. coli* enteroagregatius en aïllats clínics causants de bacterièmia i infeccions del tracte urinari (ITUs).

2. Les soques d'*E. coli* aïllades de mostres vaginals i endocervicals de dones embarassades presenten més GFV que aquelles de dones no embarassades. Entre ells, l'hemolisina, el factor citotòxic necrotitzant i les fímbries P podrien jugar un paper important en el subseqüent desenvolupament de septicèmia neonatal.

3. Alguns GFV són més prevalents en aïllats d'*E. coli* causants d'infeccions extraintestinals específiques, i fins i tot poden mostrar una distribució depenent de l'ambient en relació als sistemes de reclutament del ferro en l'*E. coli* causant d'infeccions obstètriques. La presència d'aquests GFV normalment correspon al potencial de virulència del bacteri.

4. S'ha demostrat una relació entre els aïllats d'*E. coli* sensibles a quinolones i la presència de certs GFV com *set1* (en bacterièmia) i *hly*, *cnf* i els gens *pap* (en el tracte genital femení), possiblement a causa de la inducció de la pèrdua total o parcial d'illes associades a patogenicitat, on estan localitzats aquests gens.

5. La prevalença de resistència antimicrobiana en soques d'*E. coli* causants d'infeccions obstètriques va ser similar que la trobada en altres *E. coli* extraintestinals patogèniques (ECExP), excepte taxes més baixes de resistència a cefalosporines de tercera generació. Aquest fet demostra que el tractament empíric amb ceftriaxona o ampicil·lina-cefoxitina al nostre hospital és adequat.

6. S'han observat alts percentatges de resistència a ampicil·lina i gentamicina en aïllats d'*E. coli* causants de septicèmia neonatal, fent necessari un canvi en la teràpia empírica de nounats infectats al nostre hospital. La baixa prevalença de resistència a cefalosporines en aquests aïllats suggereix que aquests agents antimicrobians podrien ser inclosos com a teràpia de primera elecció per aquesta infecció.

7. Donat que l'increment de resistència a ampicil·lina en els aïllats d'*E. coli* causants de septicèmia neonatal ha estat associat a l'ús d'aquest antibiòtic per la profilaxis intrapart, el tractament empíric actual d'aquesta infecció al nostre hospital ha estat revisat i ara té en compte l'administració prèvia de la profilaxis intrapart a la mare.

8. Es trobà un percentatge significant d'aïllats resistents a múltiples antibiòtics portadors de β-lactamases d'espectre estès (BLEEs) en *E. coli* causants de bacterièmies i ITUs en infants de Moçambic, tot i que les cefalosporines no s'utilitzin per aquestes infeccions en aquest indret. Aquest fet pot ser atribuït a fenòmens d'adquisició de resistència creuada o bé a l'ús d'aquesta família d'antimicrobians per a tractar altres infeccions bacterianes altament prevalents en aquesta àrea geogràfica.

9. La resistència a quinolones i a cefalosporines de tercera generació ha augmentat significativament durant l'última dècada en soques d'*E. coli* enteroagregatiu (ECEA) i enterotoxigènic (ECET) causants de diarrea del viatger, majoritàriament en pacients que viatgen a l'Índia o a l'Àfrica sub-Sahariana. Per aquesta raó, les fluoroquinolones (actual teràpia) no deurien ser considerades com l'antibiòtic d'elecció pels viatgers en aquestes àrees d'alt risc.

10. La baixa prevalença d'aïllats d'ECEA i ETEC resistents a azitromicina i rifaximina obtinguda demostra que aquests agents antimicrobians continuen sent adequats pel tractament de diarrea del viatger, essent l'azitromicina recomanada per infants i pacients que viatgen a àrees endèmiques de *Campylobacter* spp. com el sud-est Asiàtic.

11. El principal mecanisme molecular de resistència a antibiòtics β-lactàmics en aïllats d'*E. coli* clínicament rellevants és la BLEE CTX-M. El gen codificant d'aquest enzim es troba majoritàriament en plasmidis conjugables i cadena avall de la seqüència d'inserció IS*Ecp1*, permetent doncs una ràpida i fàcil disseminació d'aquest determinant de resistència.

12. El grup d'incompatibilitat plasmídica més prevalent en els aïllats d'*E. coli* productors de BLEEs de les coleccions estudiades fou l'IncF.

13. Tot i que els grups filogenètics B2 i D són els descrits com a més virulents en els aïllats d'ECExP, els grups A i B1 també van ser descrits en els aïllats clínicament rellevants d'*E. coli* estudiats.

14. Tot i que varis aïllats clínics d'*E. coli* portadors del gen $bla_{CTX\text{-}M}$ del grup 1 de les col·leccions estudiades pertanyien als complexes clonals CC20 i CC38 , la resta d'aïllats no estaven epidemiològicament relacionats.

15. Els grups clonals ST38 i ST131 portadors dels gens $bla_{CTX\text{-}M\text{-}15}$ i $bla_{CTX\text{-}M\text{-}27}$ respectivament, són altament prevalents en els aïllats d'ECEA i ETEC productors de BLEEs causants de diarrea del viatger.

16. Els aïllats d'ECEA i ECET causants de diarrea del viatger arreu del món són epidemiològicament molt heterogenis, pertanyent majoritàriament al complex clonal CC10 i essent distribuïts de manera variable en les diferents àrees geogràfiques.

17. Donades les poques dades disponibles sobre virulència, resistència antimicrobiana i prevalença clonal de l'*E. coli* causant de les infeccions compilades en la present tesi doctoral, és important establir o mantenir xarxes de vigilància específiques per aquests tipus d'infeccions per tal d'adaptar les guies terapèutiques quan sigui necessari.

IX. ANNEX

IX.ANNEX

- E. Sáez-López, E. Guiral, SM. Soto. "*Neonatal Sepsis by Bacteria: A Big Problem for Children"*. Clinical Microbiology Open Acess 2013.

Clinical Microbiology: Open Access

Saez-Lopez et al., Clin Microbial 2013, 2:6
http://dx.doi.org/10.4172/2327-5073.1000125

Short Communication Open Access

Neonatal Sepsis by Bacteria: A Big Problem for Children

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Introduction

Neonatal sepsis is an important but underestimated problem around the world. It is defined as disease affecting newborns ≤ 1 month of age with clinical symptoms and positive blood cultures. Infection is an important cause of morbidity and mortality during the neonatal period, despite the great improvements in intensive neonatal care and the use of extended spectrum antimicrobial agents. The incidence of this disease in developed countries is 1/1,000 in normal term neonates and 4/1,000 in preterm neonates. These values increase in low-weight preterm neonates [1]. In developing countries, this incidence increases to 2.2-8.6/1,000 live births [2]. Neonatal sepsis can be subdivided into early-onset neonatal sepsis and late-onset neonatal sepsis.

Early Onset Neonatal Sepsis

EONS can be acquired vertically from the pregnant woman before or during delivery. In this case, microorganisms present in the genital tract of the mothers are of great importance [3]. The symptoms appear within the 72 hours of life. EONS is a serious problem among very-low-birth-weight (VLBW) neonates and is associated with at least a three-fold increased risk of mortality [4]. The estimated incidence in this group is about 15-19/1000 live births [5].

Among the risk factors associated with EONS, the duration of gestation at delivery and the presence of maternal genital tract infection are the most common. In the case of early neonatal sepsis caused by bacteria, these microorganisms could arise from a prematurely ruptured amniotic membrane which becomes infected generally affecting the amniotic fluid or preterm delivery in a mother colonized by such bacteria and who may have a much higher risk of infecting their offspring due to the immaturity of their immune system [6,7]. The intraamniotic infection can affect maternal tissues such as decidua and myometrium, and also fetal tissues such as amniotic and chorionic membranes (chorioamnionitis), amniotic liquid (amnionitis), umbilical cord (funisitis) and placenta (vilitis) [8]. The microorganisms can arrive to the amniotic cavity through the blood system of the placenta, by invasive procedures during gestation (amniocentesis, etc) and by an ascending pathway [9].

Ascending infection from the genital tract of the mother to the fetus requires the following steps [9]:

I- Presence of bacteria in the vagina/cervix.

II- Bacteria residing in the decidua.

III- The bacteria might colonize the amnion or the chorion, going through the fetal vessels or crossing the amnion, reaching the amniotic cavity.

IV- The bacteria enter the fetus through contact with the infected amniotic liquid and reach the blood, causing sepsis.

Intrauterine or congenital transmission through the placenta affecting the fetus during pregnancy should be differentiated from perinatal transmission, which takes place at delivery and is caused by contact with the microbiota of the birth canal and perineal area.

The main vertically transmitted microorganisms causing EONS are *Streptococcus agalactiae* (or group B *Streptoccocus* GBS) and *Escherichia coli*, followed by Coagulase-negative *Staphylococcus* (CONS), *Haemophylus influenza* and *Listeria monocytogenes* [3]. These microorganisms are an important source of problems for the health of neonates worldwide.

Reference	Geographical area	Period	*GBS*	*E.coli*
Brzarro et aL (2005) [12]	Yale (USA)	1989-2003	49%	24%
Coben-Wolanarez et al. (2009) [13]	USA	1996-2007	1.01/1,000	0.65/1,000
Van den hoggen et al. (2010)[14]	Netherlands	2003-2006	0.7%	0.2%
Vergnano et al. (2011)[15]	England	2006-2008	50%	18%
Sgro et al. (2011) [16]	Canada	2006-2008	163%	264%
Stoll et al. (2011)[17]	USA	2006-2009	43%	29%
Weston et al. (2011) [18]	USA	2005-2008	38%	24%

Table 1: Frequencies of GBS and *E. coli* described in several studies on EONS

Several studies have corroborated this etiological data. Stoll et al. [10] found that 44% and 10.7% of EONS were caused by *E. coli* and SGB, respectively. Among EONS cases Vergnano et al. [11] reported that 50% were caused by SGB and 18% by *E. coli,* this last microorganism being more frequent among very low birth-weight (VLBW) neonates. Other studies are compiled in Table 1 [12-18].

Dagnew et al. [19] carried out a review of the studies about the frequency of GBS causing EONS in developing countries. The incidence rate of 0-2.06 per 1,000 live births and the prevalence of other microorganisms causing EONS varied within and between geographic regions. In Arabic countries, Gram-negative microorganisms are more frequently found as cause of EONS than Gram-positive microorganisms [20]. Finally, *Klebsiella* spp. (from blood samples) and *Staphylococcus aureus* (from pus swabs samples) were the bacteria more frequently involved in EONS in Tanzania [21].

Late Onset Neonatal Sepsis

LONS occurs at 4-90 days of life and is acquired from the care giving environment [22]. The incidence ranges from 1.87 to 5.42 per 1,000 live births [11]. The microorganisms most frequently found to cause LONS are CONS, *Staphylococcus aureus*, *E. coli*, *Klebsiella*,

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Received July 26, 2013; **Accepted** August 14, 2013; **Published** August 16, 2013

Citation: Saez-Lopez E, Guiral E, Soto SM (2013) Neonatal Sepsis by Bacteria: A Big Problem for Children. Clin Microbial 2: 125. doi: 10.4172/2327-5073.1000125

Reference	Geographical area	Period	GBS	E.coli	S. aureus	CONS
Bizzaro et al. [12]	Yale (USA)	1989-2003	4%	10%	7%	43%
Cohen-Wolkowiez et al. [15]	USA	1996-2007	0.24/1,000	0.6/1,000	1.01/1,000	1.22/1,000
Vergnano at al. [14]	England	2006-2008	8%	13%	18%	54%
Waters et al. (23]	Low & middle income countries	1980-2010	2.4%	12.2%	14.9%	
Hammoud et al. (26]	Kuwait	2005-2009	0.3%	5.8%	1.7%	35.5%
Didier C et al. [25]	Alasce (France)	2007	7%	56%	12.7%	13.6%
Downie et al. [27]	Developing countries	1993-2009	6%	8%	25%	2%

Table 2: Studies about etiology of LONS

Pseudomonas, *Enterobacter, Candida*, GBS, *Serratia*, *Acinetobacter* and Anaerobes [11, 20, 23]. The main risk factors associated with LONS are prematurity, central venous catheterization (duration > 10 days), nasal canula, gastrointestinal tract pathology, exposure to antibiotics, and prolonged hospitalization [7, 24]. Didier et al. [25] found three major types of late onset neonatal infections (LONI): *E. coli*-induced urinary tract infection, CONS septicemia affecting preterm infants and severe GBS infections. Other studies are compiled in Table 2.

In spite of the decrease of early-onset GBS sepsis due to the implementation of universal screening and intrapartum prophylaxis, late-onset GBS sepsis remains unchanged, being an important public health problem and associated with a high mortality and morbidity in preterm newborns [25]. This observation is in accordance with the hypothesis that LONS is usually acquired from the environment.

Several studies have related antenatal antibiotic treatment to the increase of antibiotic-resistant cases of LONS, mainly due to *E. coli* [25-28].

To prevent nosocomial infections, it is important that good the hand hygiene that has been promoted by several global programs is carried out. Intravascular catheters and the fragile skin of the neonates are important points of entrance for intrahospital microorganisms with the consequent risk of neonatal sepsis [29].

Symptoms and Diagnosis

The clinical symptoms manifested by neonates with EONS and LONS are non-specific and usually include temperature instability, respiratory problems, apnea, feeding intolerance, etc. [7]. Generally, the diagnosis of neonatal sepsis diagnosis is carried out by blood, CSF and urine cultures. Nowadays, other diagnostic tools such as complete blood cell count, C-reactive protein, procalcitonin, mannose binding lectin, cytokine profile, etc. are being studied. In the case of LONS, acute phase reactants, chemokines and cytokines, and cell-surface antigens are non-specific biomarkers that have been studied for diagnosis and management [30]. More recently, the use of genomics and proteomics are being analyzed for detecting neonatal sepsis.

The diagnosis of well-defined neonatal sepsis is difficult due to the high number of negative cultures. For this reason, the term of clinical sepsis has been created based on the symptoms and clinical characteristics presented by the neonate [31].

Neonatal Sepsis Treatment

Antimicrobial treatment of neonates with suspected sepsis must start immediately after birth and without delay. The isolation and antimicrobial susceptibility tests are not immediately available and results are not obtained in 24 hours. For these reasons, antimicrobial treatment is usually empirical using antibiotics effective against the most likely pathogens [32]. The empirical treatment of EONS consists of ampicillim (Am) and gentamicin (Gm), which cover common pathogens such as GBS, Gram-negative bacteria and *Listeria* and have synergic action. The combination of ampicillim - cefotaxime is only given in the case of meningitis determined by CSF positivity or by clinical suspicion. In the case of LONS, the therapy must be of extended spectrum antibiotics in order to cover Gram-negative and Gram-positive microorganisms. The duration of antibiotic therapy is of 10 days in EONS without meningitis, 10-14 days in LONS without meningitis, and 14-22 days in the cases of neonatal meningitis. However, an increase in the percentage of Gram-negative bacteria resistant to Am and Gm has been observed [24, 33, 34]. Several studies found that the 75-78% of *E. coli* strains causing EONS were ampicillin-resistant and 19-53% were gentamicin-resistant [24, 33]. In the case of *E. coli* isolates from LONS, between 19-50% were ampicillin-resistant and 9-16% were gentamicin-resistant [24, 33]. This trend has also been observed in developing countries [35]. For these reasons, although the current guidelines for empirical therapy in neonates seem to be appropriate [32], it is necessary to carry out studies about the susceptibility of bacteria causing neonatal sepsis in order to avoid an emergence and/or an increase in resistance levels. After empirical treatment, the choice of the antibiotics depends on the microorganism isolated, their antimicrobial susceptibility and the mechanisms of resistance used by the microorganism.

CDC Prophylaxis Guidelines

Streptococcus agalactiae or group B *Streptococcus* (GBS) has been the main etiologic agent of early neonatal sepsis in developed countries. In developing countries, this remains to be confirmed, although the few reports available point out that GBS is also a highly prevalent cause of neonatal infections. This microorganism belongs to the gastrointestinal tract microbiota from where it can colonize the vagina. Colonization of a pregnant woman's vagina is very important, as it implies an enhanced risk of GBS being transmitted vertically to the child before or at birth, and subsequently causing infection in the newborn. In Spain, it has been reported that 10-18.5% of pregnant women are colonized by GBS [36] ; 22.76% in Tehran [37] ; 6% in Iran [38]; 19% in Poland [39]; and 20% in Taiwan [40]. To avoid this enhanced risk of vertical transmission, several diagnostic and prophylactic protocols have been proposed. In 1996, the Center of Disease Control (CDC) recommended taking vaginal and rectal samples from pregnant women in their last antenatal visit and administering a prophylactic antibiotic such as penicillin G or ampicillin during pregnancy or at the time of delivery in women found to be colonized by GBS in antenatal screenings. If the pregnant woman is allergic to betalactamics, erythromycin or clindamycin must be used [41]. When implemented, the use of these prophylactic measures resulted in a decrease in the incidence of infection by GBS. A good example of this success was a study carried out in 10 hospitals of Barcelona (Spain) in which it was found that the incidence of GBS as cause of neonatal sepsis was reduced from 1.92/1.000 newborns in 1994 to 0.26/1.000 newborns in 2001 ($p < 0,001$) [42]. Another study revealed a decrease in the incidence of GBS vertically transmitted

Reference	Before IAP		After IAP	
	GBS	*E. coli*	*G B S*	*E. coli*
Levine et al. [56]	1.7/1,000	0.29/1,000	0	1.3/1,000
Stoll et al. [10]	5.9/1,000	3.2/1,000	1.7/1,000	6.8/1,000
Dairy et al. [57]	1.43/1,000	0.32/1,000	0.25/1,000	no change
Lopez-Sastre et al. [43]	1.10/1,000	0.17/1,000	0.7/1,000	0.38/1,000
Schrag et al. [1]	1.7/1,000	3.2/1,000	0.34/1,000	6.8/1,000
van den Hoo chen et al. [14]	1.8%	1%	0.7%	0.3%
Lin et al. [55]	45.4%	40.9%	20%	70%

Table 3. Studies on the effect of intrapartum prophylaxis and the percentage of GBS and *E. coli*.

neonatal sepsis from 65.4% to 36.4% due to the CDC prophylaxis [43]. Data reported by the CDC showed that after implementation of the guidelines, the incidence of EONS by GBS reduced from 1.7/1000 live births in 1993 to 0.34/1000 live births in 2004 [44].

With intrapartum prophylaxis, the proportion of women exposed to intrapartum antibiotics has doubled [45]. In addition, the incidence of bacterial species causing EONS has changed. Several studies have associated this change in the etiology of EONS with the implementation of GBS prophylaxis. Thus, EONS by GBS has decreased but an increase in the rates of other microorganisms has been reported, mainly *E. coli* [1, 46, 47] especially in low-birth weight infants [43].

Nonetheless, not only has a change in the etiology of EONS been observed but an increase in Am-resistant bacteria causing EONS has also been described [24,33,34]. In the last years, GBS presenting reduced penicillin susceptibility (PRGBS) has been reported [48,49]. The increase in the levels of penicillin resistance has been attributed to amino acid substitutions in the penicillin-binding protein 2X. These isolates also presented fluoroquinolone and/or macrolide resistance [50,51]. In addition, it is estimated that about the 12.45-48% of GBS isolates from EONS were erythromycin-resistant and about the 11.8-28% were clindamycin-resistant [41, 52,53] being a serious problem for empirical prophylaxis.

Several studies have found a relationship between the increase of the administration of intrapartum prophylaxis and the increase of EONS by non-group B streptococcal microorganisms that are resistant to ampicillin [10,43, 54]. Friedman et al. [24] found an association between the emergence of resistant *E. coli* and PROM, high temperature and intrapartum prophylaxis. However, other studies did not find a significant change in the incidence of ampicillin-resistant non-group B streptococcal microorganisms causing EONS after implementation of GBS screening and intrapartum prophylaxis. Lin et al. [37] described an incidence of ampicillin-resistant *E. coli* of about 88.9% in 2004 and 92.9% in 2008. Schrag et al [1] determined that exposure to intrapartum antibiotic therapy did not increase early-onset *E. coli* infection but it was only effective in preventing *E. coli* infection among term neonates (Table 3) [55-57].

Conclusion

Neonatal sepsis remains an important but underestimated problem around the world. In spite of intrapartum prophylaxis, epidemiological surveillance of other pathogens causing early-onset neonatal sepsis is needed. The development of pathogen-specific strategies to prevent this infection could be an important diagnostic tool to reduce the cases of early-onset neonatal sepsis. In addition, studies on antimicrobial resistance of the microorganisms causing neonatal sepsis are needed in order to improve empirical treatment and avoid the emergence of resistances.

References

1. Schrag SJ, Stoll BJ (2006) Early-onset neonatal sepsis in the era of widespread intrapartum chemoprophylaxis. Pediatr Infect Dis J 25: 939-940.
2. Vergnano S, Sharland M, Kazembe P, Mwansambo C, Heath PT (2005) Neonatal sepsis: an international perspective. Arch Dis Child Fetal Neonatal Ed 90: F220-224.
3. Sgro M, Shah PS, Campbell D, Tenuta A, Shivananda S, et al. (2011) Early-onset neonatal sepsis: rate and organism pattern between 2003 and 2008. J Perinatol 31: 794-798.
4. Klinger G, Levy I, Sirota L, Boyko V, Reichman B, et al. (2009) Epidemiology and risk factors for early onset sepsis among very-low-birthweight infants. Am J Obstet Gynecol 201: 38.
5. Stoll BJ, Hansen NI, Higgins RD, Fanaroff AA, et al. (2005). Very low birth weight preterm infants with early onset neonatal sepsis: the predominance of gram-negative infections continues in the National Institute of Child Health and Human Development Neonatal Research Network, 2002-2003. Pediatr Infect Dis J. 24: 635-639.
6. Raymond J, Lopez E, Bonacorsi S, Poyart C, Moriette G, et al. (2008) Evidence for transmission of Escherichia coli from mother to child in late-onset neonatal infection. Pediatr Infect Dis J 27: 186-188.
7. Camacho-Gonzalez A, Spearman PW, Stoll BJ (2013) Neonatal infectious diseases: evaluation of neonatal sepsis. Pediatr Clin North Am 60: 367-389.
8. Henneke P, Berner R (2006) SIRS and group-B streptococcal sepsis in newborns: pathogenesis and perspectives in adjunctive therapy. Semin Fetal Neonatal Med 11: 333-342.
9. Cornette L, Miall L (2006) Development of regional guidelines: the way forward for neonatal networks? Arch Dis Child Fetal Neonatal Ed 91: F318-319.
10. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, et al. (2002) Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. N Engl J Med 347: 240-247.
11. Vergnano S, Menson E, Kennea N, Embleton N, Russell AB, et al. (2011) Neonatal infections in England: the NeonIN surveillance network. Arch Dis Child Fetal Neonatal Ed 96: F9-9F14.
12. Bizzarro MJ, Raskind C, Baltimore RS, Gallagher PG (2005) Seventy-five years of neonatal sepsis at Yale: 1928-2003. Pediatrics 116: 595-602.
13. Cohen-Wolkowiez M, Moran C, Benjamin DK, Cotten CM, Clark RH, et al. (2009) Early and late onset sepsis in late preterm infants. Pediatr Infect Dis J 28: 1052-1056.
14. van den Hoogen A, Gerards LJ, Verboon-Maciolek MA, Fleer A, Krediet TG (2010) Long-term trends in the epidemiology of neonatal sepsis and antibiotic susceptibility of causative agents. Neonatology 97: 22-28.
15. Vergnano S, Menson E, Kennea N, Embleton N, Russell AB, et al. (2011) Neonatal infections in England: the NeonIN surveillance network. Arch Dis Child Fetal Neonatal Ed 96: F9-9F14.
16. Sgro M, Shah PS, Campbell D, Tenuta A, Shivananda S, et al. (2011) Early-onset neonatal sepsis: rate and organism pattern between 2003 and 2008. J Perinatol 31: 794-798.
17. Stoll BJ, Hansen NI, Sánchez PJ, Faix RG, Poindexter BB, et al. (2011) Early onset neonatal sepsis: the burden of group B Streptococcal and E. coli disease continues. Pediatrics 127: 817-826.
18. Weston EJ, Pondo T, Lewis MM, Martell-Cleary P, Morin C, et al. (2011) The burden of invasive early-onset neonatal sepsis in the United States, 2005-2008. Pediatr Infect Dis J 30: 937-941.
19. Dagnew AF, Cunnington MC, Dube Q, Edwards MS, French N, et al. (2012) Variation in reported neonatal group B streptococcal disease incidence in developing countries. Clin Infect Dis 55: 91-102.
20. Tosson AM, Speer CP (2011) Microbial pathogens causative of neonatal sepsis in Arabic countries. J Matern Fetal Neonatal Med 24: 990-994.
21. Mhada TV, Fredrick F, Matee MI, Massawe A (2012) Neonatal sepsis at Muhimbili National Hospital, Dar es Salaam, Tanzania; aetiology, antimicrobial sensitivity pattern and clinical outcome. BMC Public Health. 12: 904-910.
22. Bizzarro MJ, Raskind C, Baltimore RS, Gallagher PG (2005) Seventy-five years of neonatal sepsis at Yale: 1928-2003. Pediatrics 116: 595-602.

23. Waters D, Jawad I, Ahmad A, LukÅ¡iÄ‡ I, Nair H, et al. (2011) Aetiology of community-acquired neonatal sepsis in low and middle income countries. J Glob Health 1: 154-170.

24. Friedman S, Shah V, Ohlsson A, Matlow AG (2000) Neonatal escherichia coli infections: concerns regarding resistance to current therapy. Acta Paediatr 89: 686-689.

25. Didier C, Streicher MP, Chognot D, Campagni R, Schnebelen A, et al. (2012) Late-onset neonatal infections: incidences and pathogens in the era of antenatal antibiotics. Eur J Pediatr 171: 681-687.

26. Hammoud MS, Al-Taiar A, Thalib L, Al-Sweih N, Pathan S, et al. (2012) Incidence, aetiology and resistance of late-onset neonatal sepsis: a five-year prospective study. J Paediatr Child Health 48: 604-609.

27. Downie L, Armiento R, Subhi R, Kelly J, Clifford V, et al. (2013) Community-acquired neonatal and infant sepsis in developing countries: efficacy of WHO's currently recommended antibiotics--systematic review and meta-analysis. Arch Dis Child 98: 146-154.

28. Terrone DA, Rinehart BK, Einstein MH, Britt LB, Martin JN Jr, et al. (1999) Neonatal sepsis and death caused by resistant Escherichia coli: possible consequences of extended maternal ampicillin administration. Am J Obstet Gynecol 180: 1345-1348.

29. Coronell W, Perez C, Guerrero C, Bustamante H (2009) Sepsis neonatal. Rev. Enferm. Infec. Pedr. 23: 57-68.

30. Ng PC, Lam HS (2010) Biomarkers for late-onset neonatal sepsis: cytokines and beyond. Clin Perinatol 37: 599-610.

31. Ng PC (2004) Diagnostic markers of infection in neonates. Arch Dis Child Fetal Neonatal Ed 89: F229-235.

32. Muller-Pebody B, Johnson AP, Heath PT, Gilbert RE, Henderson KL, et al. (2011) Empirical treatment of neonatal sepsis: are the current guidelines adequate? Arch Dis Child Fetal Neonatal Ed 96: F4-8.

33. Guiral E, Bosch J, Vila J, Soto SM (2012) Antimicrobial resistance of Escherichia coli strains causing neonatal sepsis between 1998 and 2008. Chemotherapy 58: 123-128.

34. Maayan-Metzger A, Barzilai A, Keller N, Kuint J (2009) Are the "good old" antibiotics still appropriate for early-onset neonatal sepsis? A 10 year survey. Isr Med Assoc J 11: 138-142.

35. Thaver D, Ali SA, Zaidi AK (2009) Antimicrobial resistance among neonatal pathogens in developing countries. Pediatr Infect Dis J 28: S19-21.

36. Ramos JM, Milla A, López-García P, Gutiérrez F (2009) [Colonization by Streptococcus agalactiae in foreign and Spanish gestating women in the area of Elche (Spain)]. Enferm Infecc Microbiol Clin 27: 249-251.

37. Javanmanesh F, Eshraghi N (2013) Prevalence of positive recto-vaginal culture for Group B streptococcus in pregnant women at 35-37 weeks of gestation. Med J Islam Repub Iran 27: 7-11.

38. Hamedi A, Akhlaghi F, Seyedi SJ, Kharazmi A (2012) Evaluation of group B Streptococci colonization rate in pregnant women and their newborn. Acta Med Iran 50: 805-808.

39. Brzychczy-Wloch M, Wojkowska-Mach J, Helwich E, Heczko PB (2013) Incidence of maternal GBS colonization and neonatal GBS disease among very low birth weight Polish neonates. Med Sci Monit 19: 34-39.

40. Yu HW, Lin HC, Yang PH, Hsu CH, Hsieh WS, et al. (2011) Group B streptococcal infection in Taiwan: maternal colonization and neonatal infection. Pediatr Neonatol 52: 190-195.

41. Verani JR, McGee L, Schrag SJ; Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC) (2010) Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recomm Rep 59: 1-36.

42. Andreu A, Sanfeliu I, Viñas L, Barranco M, Bosch J, et al. (2003) [Decreasing incidence of perinatal group B streptococcal disease (Barcelona 1994-2002). Relation with hospital prevention policies]. Enferm Infecc Microbiol Clin 21: 174-179.

43. Lopez Sastre JB, Fernandez Colomer B, Coto Cotallo GD, Ramos Aparicio A; Grupo de Hospitales Castrillo (2005) Trends in the epidemiology of neonatal sepsis of vertical transmission in the era of group B streptococcal prevention. Acta Paediatr. 94: 451-457.

44. Centers for Disease Control and Prevention (CDC) (2005) Disparities in universal prenatal screening for group B streptococcus--North Carolina, 2002-2003. MMWR Morb Mortal Wkly Rep 54: 700-703.

45. Schrag S, Gorwitz R, Fultz-Butts K, Schuchat A (2002) Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. MMWR Recomm Rep 51: 1-22.

46. Edwards RK, Jamie WE, Sterner D, Gentry S, Counts K, et al. (2003) Intrapartum antibiotic prophylaxis and early-onset neonatal sepsis patterns. Infect Dis Obstet Gynecol 11: 221-226.

47. Laugel V, Kuhn P, Beladdale J, Donato L, Escande B, et al. (2003) Effects of antenatal antibiotics on the incidence and bacteriological profile of early-onset neonatal sepsis. A retrospective study over five years. Biol Neonate 84: 24-30.

48. Kimura K, Suzuki S, Wachino J, Kurokawa H, Yamane K, et al. (2008) First molecular characterization of group B streptococci with reduced penicillin susceptibility. Antimicrob Agents Chemother 52: 2890-2897.

49. Kimura K, Matsubara K, Yamamoto G, Shibayama K, Arakawa Y (2013) Active screening of group B streptococci with reduced penicillin susceptibility and altered serotype distribution isolated from pregnant women in Kobe, Japan. Jpn J Infect Dis 66: 158-160.

50. Nagano N, Nagano Y, Kimura K, Tamai K, Yanagisawa H, et al. (2008) Genetic heterogeneity in pbp genes among clinically isolated group B Streptococci with reduced penicillin susceptibility. Antimicrob. Agents Chemother. 52: 4258-4267.

51. Kimura K, Nagano N, Nagano Y, Suzuki S, Wachino J, et al. (2013) High frequency of fluoroquinolone- and macrolide-resistant streptococci among clinically isolated group B streptococci with reduced penicillin susceptibility. J. Antimicrob. Chemother. 68: 539-542.

52. Capanna F, Emonet SP, Cherkaoui A, Irion O, Schrenzel J, et al. (2013) Antibiotic resistance patterns among group B Streptococcus isolates: implications for antibiotic prophylaxis for early-onset neonatal sepsis. Swiss Med. Wkly. 143: w13778.

53. Gonzalez JJ, Andreu A, Grupo de Estudio de Infección Perinatal, Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (2004) Susceptibility of vertically transmitted Group B streptococci to antimicrobial agents. Multicenter study. Enferm. Infecc. Microbiol. Clin. 22: 286-291.

54. Towers CV, Carr MH, Padilla G, Asrat T (1998) Potential consequences of widespread antepartal use of ampicillin. Am J Obstet Gynecol 179: 879-883.

55. Lin CY, Hsu CH, Huang FY, Chang JH, Hung HY, et al. (2011) The changing face of early-onset neonatal sepsis after the implementation of a maternal group B Streptococcus screening and intrapartum prophylaxis policy--a study in one medical center. Pediatr. Neonatol. 52: 78-84.

56. Levine EM, Ghai V, Barton JJ, Strom CM (1999) Intrapartum antibiotic prophylaxis increases the incidence of gram-negative neonatal sepsis. Infect Dis Obstet Gynecol 7: 210-213.

57. Daley AJ, Isaacs D, Australasian Study Group for Neonatal Infections (2004) Ten-year study on the effect of intrapartum antibiotic prophylaxis on early onset group B streptococcal and Escherichia coli neonatal sepsis in Australasia. Pediatr. Infect. Dis. J. 23: 630-634.

Citation: Saez-Lopez E, Guiral E, Soto SM (2013) Neonatal Sepsis by Bacteria: A Big Problem for Children. Clin Microbial 2: 125. doi: 10.4172/2327-5073.1000125

X. REFERENCES

X. References

1. Bentley R, Meganathan R. Biosynthesis of vitamin K (menaquinone) in bacteria. Microbiol Rev. 1982;46(3):241–80.
2. Blount ZD. The unexhausted potential of *E. coli*. Elife. 2015;4:1–12.
3. Savageau MA. *Escherichia coli* habitats, cell types, and molecular mechanisms of gene control. Am Nat. 1983;122(6):732–44.
4. Russo TA, Johnson JR. Medical and economic impact of extraintestinal infections due to *Escherichia coli*: Focus on an increasingly important endemic problem. Microbes Infect. 2003;5(5):449–56.
5. O'Neill J. Tackling drug-resistant infections globally: final report and recommendations. Review on Antimicrobial Resistance. 2016.
6. Study.com: Growth requirements of *E. coli* and auxotrophs. Available from: http://study.com/academy/lesson/growth-requirements-of-e-coli-and-auxotrophs.html
7. Bright Hub: Theodor Escherich and the Discovery of *E. coli*. 2009. Available from: http://www.brighthub.com/science/genetics/articles.
8. Escherich TH. The intestinal bacteria of the neonate and breast-fed infant. Rev Infect Dis. 1989;11(2):352–6.
9. Backhed F. Host-bacterial mutualism in the human intestine. Science. 2005;307(5717):1915–20.
10. Chang D-E, Smalley DJ, Tucker DL, Leatham MP, Norris WE, Stevenson SJ, et al. Carbon nutrition of *Escherichia coli* in the mouse intestine. Proc Natl Acad Sci U S A. 2004;101(19):7427–32.
11. van Elsas JD, Semenov A V, Costa R, Trevors JT. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. ISME J. 2011;5(2):173–83.
12. Russo TA, Johnson JR. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. J Infect Dis. 2000 May;181(5):1753–4.
13. Lawrence JG, Ochman H. Molecular archaeology of the *Escherichia coli* genome. Proc Natl Acad Sci U S A. 1998;95(16):9413–7.

14. Dobrindt U. (Patho-)Genomics of *Escherichia coli*. Int J Med Microbiol. 2005;295(6–7):357–71.
15. Aminov RI. A brief history of the antibiotic era: Lessons learned and challenges for the future. Front Microbiol. 2010 Dec 8;1:134.
16. Levy SB. The antibiotic paradox: How miracle drugs are destroying the miracle. N Engl J Med. 1993:1792.
17. One Health Initiative. Available from: http://www.onehealthinitiative.com/
18. European Centre for Disease Prevention and Control. Available from: https://ecdc.europa.eu/en
19. World Health Organization [Internet]. Available from: http://www.who.int
20. Gomes TAT, Elias WP, Scaletsky ICA, Guth BEC, Rodrigues JF, Piazza RMF, et al. Diarrheagenic *Escherichia coli*. Brazilian J Microbiol. 2016;47:3–30.
21. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev. 1998;11(1):142–201.
22. Weintraub A. Enteroaggregative *Escherichia coli*: epidemiology, virulence and detection. J Med Microbiol. 2007;56(Pt 1):4–8.
23. Nataro JP, Yikang D, Yingkang D, Walker K. AggR, a transcriptional activator of aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli*. J Bacteriol. 1994;176(15):4691–9.
24. Dreyfus LA, Jaso-Friedmann L, Robertson DC. Characterization of the mechanism of action of *Escherichia coli* heat-stable enterotoxin. Infect Immun. 1984;44(2):493–501.
25. Fasano A, Noriega FR, Liao FM, Wang W, Levine MM. Effect of *Shigella* enterotoxin 1 (ShET1) on rabbit intestine in vitro and in vivo. Gut. 1997;40(4):505–11.
26. Al-Hasani K, Adler B, Rajakumar K, Sakellaris H. Distribution and structural variation of the she pathogenicity island in enteric bacterial pathogens. J Med Microbiol. 2001;50(9):780–6.
27. Fasano A, Noriega FR, Maneval DR, Chanasongcram S, Russell R, Guandalini S, et al. *Shigella* enterotoxin 1: an enterotoxin of *Shigella flexneri* 2a active in rabbit small intestine in vivo and in vitro. J Clin Invest. 1995;95(6):2853–61.
28. Flores J, Okhuysen PC. Enteroaggregative *Escherichia coli* infection. Curr Opin

Gastroenterol. 2009;25(1):8–11.

29. Henderson IR, Nataro JP. Virulence functions of autotransporter proteins. Infect Immun. 2001;69(3):1231–43.
30. Jensen BH, Olsen KEP, Struve C, Krogfelt KA, Petersen AM. Epidemiology and clinical manifestations of enteroaggregative *Escherichia coli*. Clin Microbiol Rev. 2014;27(3):614–30.
31. Fang GD, Lima AA, Martins CV, Nataro JP GR. Etiology and epidemiology of persistent diarrhea in northeastern Brazil: a hospital-based, prospective, case–control study. J Pediatr Gastroenterol Nutr. 1995;21:137–44.
32. Opintan JA, Newman MJ, Ayeh-Kumi PF, Affrim R, Gepi-Attee R, Sevilleja JEAD, et al. Pediatric diarrhea in Southern Ghana: Etiology and association with intestinal inflammation and malnutrition. Am J Trop Med Hyg. 2010;83(4):936–43.
33. Gaastra W, Svennerholm AM. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). Trends Microbiol. 1996;4(11):444–52.
34. Nada RA, Shaheen HI, Khalil SB, Mansour A, El-Sayed N, Touni I, et al. Discovery and phylogenetic analysis of novel members of class b enterotoxigenic *Escherichia coli* adhesive fimbriae. J Clin Microbiol. 2011;49(4):1403–10.
35. von Mentzer A, Connor TR, Wieler LH, Semmler T, Iguchi A, Thomson NR, et al. Identification of enterotoxigenic *Escherichia coli* (ETEC) clades with long-term global distribution. Nat Genet. 2014;46(12):1321–6.
36. Qadri F, Svennerholm A-M, Faruque ASG, Sack RB. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev. 2005;18(3):465–83.
37. Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. Nat Rev Microbiol. 2004;2(2):123-40.
38. Levine MM. *Escherichia coli* that cause diarrhea : Enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J Infect Dis. 2016;155(3):377–89.
39. Silva RM, Toledo MR, Trabulsi LR. Biochemical and cultural characteristics of invasive *Escherichia coli*. J Clin Microbiol. 1980;11(5):441–4.

40. Ewing WH, Gravatti JL. *Shigella* types encountered in the Mediterranean area. J Bacteriol. 1947;53(2):191–5.
41. Harris JR, Mariano J, Wells JG, Payne BJ, Donnell HD, Cohen ML. Person-to-person transmission in an outbreak of enteroinvasive *Escherichia coli*. Am J Epidemiol. 1985;122(2):245–52.
42. Tulloch EF. Invasive Enteropathic *Escherichia coli* dysentery. Ann Intern Med. 1973;79(1):13.
43. Vila J, Oliveira I, Zboromyrska Y, Gascon J. Diarrea del viajero. Enferm Infecc Microbiol Clin. 2016;34(9):579–84.
44. Al-abri SS, Beeching NJ, Nye FJ. Traveller's diarrhoea. 2005;5(June):349–60.
45. Bettelheim KA. *Escherichia coli* in the normal flora of humans and animals. In: *Escherichia coli*: Mechanisms of Virulence [book]. Cambridge University Press; 1997.
46. Sáez-López E, Guiral E, Fernández-Orth D, Villanueva S, Goncé A, López M, et al. Vaginal versus obstetric infection *Escherichia coli* isolates among pregnant women: antimicrobial resistance and genetic virulence profile. PLoS One. 2016;11(1):e0146531.
47. Obata-Yasuoka M, Ba-Thein W, Tsukamoto T, Yoshikawa H, Hayashi H. Vaginal *Escherichia coli* share common virulence factor profiles, serotypes and phylogeny with other extraintestinal *E. coli*. Microbiology. 2002;148:2745–52.
48. Johnson JR, Russo TA. Extraintestinal pathogenic *Escherichia coli*: "The other bad E coli." J Lab Clin Med. 2002;139(3):155–62.
49. Russo TA, Johnson JR. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. J Infect Dis. 2000;181(5):1753-4.
50. Johnson JR, Russo TA. Extraintestinal pathogenic *Escherichia coli*: "The other bad *E coli*". J Lab Clin Med. 2002 Mar;139(3):155–62.
51. Rasko DA, Rosovitz MJ, Myers GSA, Mongodin EF, Fricke WF, Gajer P, et al. The pangenome structure of *Escherichia coli*: Comparative genomic analysis of *E. coli* commensal and pathogenic isolates. J Bacteriol. 2008;190(20):6881–93.
52. Johnson JR, Stell a L. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis. 2000;181(1):261–72.

53. Fratamico PM, DebRoy C, Liu Y, Needleman DS, Baranzoni GM, Feng P. Advances in molecular serotyping and subtyping of *Escherichia coli*. Front Microbiol. 2016;3(7):644.

54. Picard B, Garcia JS, Gouriou S, Duriez P, Brahimi N, Bingen E, et al. The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection? Infect Immun. 1999;67(2):546–53.

55. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. 1992 J;101(6):1644–55.

56. Minino A, Smith B. Deaths: Preliminary data for 2000. National Vital Statistics Reports. 2001.

57. Smith JL, Fratamico PM, Gunther NW. Extraintestinal pathogenic *Escherichia coli*. Foodborne Pathog Dis. 2007;4(2):134–63.

58. Pitout JDD. Extraintestinal pathogenic *Escherichia coli*: A combination of virulence with antibiotic resistance. Front Microbiol. 2012;3(January):9.

59. Soares Tibo LH, Wildgrube Bertol J, Bernedo-Navarro RA, Yano T. Cytotoxic factor secreted by *Escherichia coli* associated with sepsis facilitates transcytosis through human umbilical vein endothelial cell monolayers. Braz J Infect Dis. 2016;20 (3):298–302.

60. Williams JM, Keijzers G, Macdonald SPJ, Shetty A, Fraser JF. Review article: Sepsis in the emergency department – Part 3: Treatment. EMA - Emerg Med Australas. 2018;30(2):144–51.

61. Rhodes A, Evans LE, Alhazzani W, Levy MM, Antonelli M, Ferrer R, et al. Surviving sepsis campaign: International guidelines for management of sepsis and septic shock: 2016. Vol. 43, Intensive Care Medicine [book]. Springer Berlin Heidelberg; 2017:304-377.

62. Foxman B. The epidemiology of urinary tract infection. Nat Rev Urol. 2010;7(12):653–60.

63. Wiles TJ, Kulesus RR, Mulvey M a. Origins and virulence mechanisms of uropathogenic *Escherichia coli*. NIH Public Access. 2008;85(1):11–9.

64. Terlizzi ME, Gribaudo G, Maffei ME. Uropathogenic *Escherichia coli* (UPEC) infections:

virulence factors, bladder responses, antibiotic, and non-antibiotic antimicrobial strategies. Front Microbiol. 2017;8(1566).

65. Bien J, Sokolova O, Bozko P. Role of uropathogenic *Escherichia coli* virulence factors in development of urinary tract infection and kidney damage. Int J Nephrol. 2012;2012 :681473.
66. Tantillo E, Colistra A, Vannini E, Cerri C, Pancrazi L, Baroncelli L, et al. Bacterial toxins and targeted brain therapy : New insights from cytotoxic necrotizing factor 1 (CNF1). Int J Mol Sci. 2018 May 31;19(6). pii: E1632
67. de Cueto M, Aliaga L, Alós JI, Canut A, Los-Arcos I, Martínez JA, et al. Resumen ejecutivo del diagnóstico y tratamiento de las infecciones del tracto urinario. Guía de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Enferm Infecc Microbiol Clin. 2017;35(5):314–20.
68. Wait RB. Urinary tract infection during pregnancy. Asymptomatic bacteriuria, acute cystitis, and acute pyelonephritis. Postgrad Med. 1984;75(8):153–7, 161.
69. Newton ER. Chorioamnionitis and intraamniotic infection. Clin Obstet Gynecol. 1993;36(4):795–808.
70. Berg CJ, Callaghan WM, Syverson C, Henderson Z. Pregnancy-related mortality in the United States, 1998 to 2005. Obstet Gynecol. 2010;116(6):1302–9.
71. Krohn MA, Thwin SS, Rabe LK, Brown Z, Hillier SL. Vaginal colonization by *Escherichia coli* as a risk factor for very low birth weight delivery and other perinatal complications. J Infect Dis. 1997;175(3):606–10.
72. Guiral E, Bosch J, Vila J, Soto SM. Prevalence of *Escherichia coli* among samples collected from the genital tract in pregnant and nonpregnant women: relationship with virulence. FEMS Microbiol Lett. 2011;314(2):170–3.
73. Carey JC, Klebanoff MA. Is a change in the vaginal flora associated with an increased risk of preterm birth? Am J Obstet Gynecol. 2005;192(4):1341–7.
74. Newton ER. Preterm Labor, preterm premature rupture of membranes, and chorioamnionitis. Clin Perinatol. 2005;32(3):571–600.
75. Gibbs RS, Duff P. Progress in pathogenesis and management of clinical intraamniotic infection. Am J Obstet Gynecol. 1991;164:1317–26.

76. Romero R, Quintero R, Oyarzun E, Wu YK, Sabo V, Mazor M, et al. Intraamniotic infection and the onset of labor in preterm premature rupture of the membranes. Am J Obstet Gynecol. 1988;159(3):661–6.
77. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine Infection and Preterm Delivery. N Engl J Med. 2000;342(20):1500–7.
78. Burke C. Perinatal sepsis. J Perinat Neonatal Nurs. 2009;23(1):42–51.
79. Guiral E, Sáez-López E, Bosch J, Goncé A, López M, Sanz S, et al. Antimicrobial resistance and virulence characterization among *Escherichia coli* clinical isolates causing severe obstetric infections in pregnant women.
80. Tamelienė R, Barčaitė E, Stonienė D, Buinauskienė J, Markūnienė E, Kudrevičienė A, et al. *Escherichia coli* colonization in neonates: prevalence, perinatal transmission, antimicrobial susceptibility, and risk factors. Medicina (Kaunas). 2012 Jan;48(2):71–6.
81. Morbidities I. Infection and Inflammation. J Nutr 2003 May;133(5 Suppl 2)1656S-1660S.
82. Mercer BM, Arheart KL. Antimicrobial therapy in expectant management of preterm premature rupture of the membranes. Lancet. 1995;346(8985):1271–9.
83. Watt S, Lanotte P, Mereghetti L, Moulin-Schouleur M, Picard B, Quentin R. *Escherichia coli* strains from pregnant women and neonates: intraspecies genetic distribution and prevalence of virulence factors. J Clin Microbiol. 2003;41(5):1929–35.
84. Soto SM, Bosch J, Jimenez de Anta MT, Vila J. Comparative study of virulence traits of *Escherichia coli* clinical isolates causing early and late neonatal sepsis. J Clin Microbiol. 2008;46(3):1123–5.
85. Bonacorsi S, Bingen E. Molecular epidemiology of *Escherichia coli* causing neonatal meningitis. Int J Med Microbiol. 2005;295(6–7):373–81.
86. Vila J, Sáez-López E, Johnson JR, Römling U, Dobrindt U, Cantón R, et al. *Escherichia coli*: an old friend with new tidings. FEMS Microbiol Rev. 2016;005(40):437–63.
87. Santos RP, Tristram D. A practical guide to the diagnosis, treatment, and prevention of neonatal infections. Pediatr Clin North Am. 2015;62(2):491–508.
88. Hooper DC, Jacoby GA. Mechanisms of drug resistance: quinolone resistance. Ann N Y Acad Sci. 2015;1354(1):12–31.
89. Frye JG, Jackson CR. Genetic mechanisms of antimicrobial resistance identified in

Salmonella enterica, *Escherichia coli*, and *Enteroccocus* spp. isolated from U.S. food animals. Front Microbiol. 2013;4(May):135.

90. Da Silva GJ, Domingues S. Insights on the horizontal gene transfer of carbapenemase determinants in the opportunistic pathogen *Acinetobacter baumannii*. Microorganisms. 2016 23;4(3).
91. Munita JM, Arias CA, Unit AR, Santiago A De. Mechanisms of antibiotic resistance. Microbiol Spectr. 2016;4(2):1–37.
92. Manson JM, Hancock LE, Gilmore MS. Mechanism of chromosomal transfer of *Enterococcus faecalis* pathogenicity island, capsule, antimicrobial resistance, and other traits. Proc Natl Acad Sci U S A. 2010;107(27):12269–74.
93. Mahillon J, Chandler M. Insertion sequences. Microbiol Mol Biol Rev. 1998 Sep [cited 2018;62(3):725–74.
94. Bennett PM. Plasmid encoded antibiotic resistance: Acquisition and transfer of antibiotic resistance genes in bacteria. Br J Pharmacol. 2008;153(SUPPL. 1):347–57.
95. Brolund A. Overview of ESBL-producing Enterobacteriaceae from a Nordic perspective. Infect Ecol Epidemiol. 2014;4(1).
96. Novick RP. Plasmid incompatibility. Microbiol Rev. 1987;51(4):381–95.
97. Couturier M, Bex F, Bergquist PL, Maas WK. Identification and classification of bacterial plasmids. Microbiol Rev. 1988;52(3):375–95.
98. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods. 2005;63(3):219–28.
99. Johnson TJ, Wannemuehler YM, Johnson SJ, Logue CM, White DG, Doetkott C, et al. Plasmid replicon typing of commensal and pathogenic *Escherichia coli* isolates. Appl Environ Microbiol. 2007;73(6):1976–83.
100. Mazel D. Integrons: Agents of bacterial evolution. Nat Rev Microbiol. 2006;4(8):608–20.
101. Sosa A de J, Byarugaba DK, Amábile-Cuevas CF, Hsueh P-R, Kariuki S, Okeke IN. Antimicrobial resistance in developing countries [book]. New York, NY: Springer New York; 2010.
102. Simonsen KA, Anderson-Berry AL, Delair SF, Dele Davies H. Early-onset neonatal sepsis.

Clin Microbiol Rev. 2014;27(1):21–47.

103. Garneau-Tsodikova S, Labby KJ. Mechanisms of resistance to aminoglycoside antibiotics: Overview and perspectives. Medchemcomm. 2016;7(1):11-27.
104. Ramirez MS, Nikolaidis N, Tolmasky ME. Rise and dissemination of aminoglycoside resistance: the *aac(6')-Ib* paradigm. Front Microbiol. 2013;4:121.
105. Aires JR, Nikaido H. Aminoglycosides are captured from both periplasm and cytoplasm by the AcrD multidrug efflux transporter of *Escherichia coli*. J Bacteriol. 2005;187(6):1923–9.
106. Pediatric Allergy: Principles and Practice [book]. Elsevier; 2016.
107. Kaneko T, Dougherty TJ, Magee TV. Macrolide Antibiotics. Comprehensive Medicinal Chemistry II [book]. Elsevier; 2007:519–66.
108. Phuc Nguyen MC, Woerther P-L, Bouvet M, Andremont A, Leclercq R, Canu A. *Escherichia coli* as reservoir for macrolide resistance genes. Emerg Infect Dis. 2009;15(10):1648–50.
109. Dasgupta A. Advances in antibiotic measurement. Adv Clin Chem. 2012;56:75–104.
110. Ojo KK, Ulep C, Van Kirk N, Luis H, Bernardo M, Leitao J, et al. The *mef(A)* gene predominates among seven macrolide resistance genes identified in gram-negative strains representing 13 genera, isolated from healthy Portuguese children. Antimicrob Agents Chemother. 2004;48(9):3451–6.
111. Walker RC. The fluoroquinolones. Mayo Clin Proc. 1999;74(10):1030–7.
112. Basu S, Mukherjee SK, Hazra A, Mukherjee M. Molecular characterization of uropathogenic *Escherichia coli*: nalidixic acid and ciprofloxacin resistance, virulent factors and phylogenetic background. J Clin Diagn Res. 2013;7(12):2727–31.
113. Vila J, Vargas M, Ruiz J, Espasa M, Pujol M, Corachán M, et al. Susceptibility patterns of enteroaggregative *Escherichia coli* associated with traveller's diarrhoea: emergence of quinolone resistance. J Med Microbiol. 2001;50(11):996–1000.
114. Aldred KJ, Kerns RJ, Osheroff N. Mechanism of quinolone action and resistance. Biochemistry. 2014;53(10):1565–74.
115. Fàbrega A, Madurga S, Giralt E, Vila J. Mechanism of action of and resistance to quinolones. Microb Biotechnol. 2009;2(1):40–61.

116. Andres P, Lucero C, Soler-Bistué A, Guerriero L, Albornoz E, Tran T, et al. Genes in clinical enterobacteria with unusual phenotypes of quinolone susceptibility from Argentina. Antimicrob Agents Chemother. 2013;57(6):2467–75.
117. Pons MJ, Mensa L, Gascón J, Ruiz J. Fitness and molecular mechanisms of resistance to rifaximin in in vitro selected *Escherichia coli* mutants. Microb Drug Resist. 2012 Aug
118. Hopkins KL, Mushtaq S, Richardson JF, Doumith M, de Pinna E, Cheasty T, et al. *In vitro* activity of rifaximin against clinical isolates of *Escherichia coli* and other enteropathogenic bacteria isolated from travellers returning to the UK. Int J Antimicrob Agents. 2014;43(5):431–7.
119. Gomes C, Ruiz L, Pons MJ, Ochoa TJ, Ruiz J. Relevant role of efflux pumps in high levels of rifaximin resistance in *Escherichia coli* clinical isolates. Trans R Soc Trop Med Hyg. 2013;107(9):545–9.
120. Huovinen P, Sundstrom L, Swedberg G, Skold O. Trimethoprim and sulfonamide resistance. Antimicrob Agents Chemother. 1995;39(2):279–89.
121. Eliopoulos GM, Huovinen P. Resistance to trimethoprim-sulfamethoxazole. Clin Infect Dis. 2001;32(11):1608–14.
122. Marín M. Antibióticos betalactámicos. Enferm Infecc Microbiol Clin 2003;21(1):42-55.
123. Mechanisms in Medicine. ß-Lactams: Mechanisms of Action and Resistance - YouTube [Internet]. 2011.
124. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ V. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol. 2015;13(1):42–51.
125. Bush K, Jacoby G a. Updated functional classification of beta-lactamases. Antimicrob Agents Chemother. 2010;54(3):969–76.
126. Eun H-M, Eun H-M. Marker/reporter enzymes. Enzymology Primer for Recombinant DNA Technology [book]. Elsevier, 1996:567–645.
127. Ambler RP. The structure of beta-lactamases. Philos Trans R Soc Lond B Biol Sci. 1980 May;289(1036):321–31.
128. Bush K. Characterization of beta-lactamases. Antimicrob Agents Chemother. 1989;33(3):259–63.
129. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for beta-lactamases

and its correlation with molecular structure . Antimicrob Agents Chemother. 1995;39(6):1211-33.

130. Ghafourian S, Sadeghifard N, Soheili S, Sekawi Z. Extended-spectrum beta-lactamases: Definition, classification and epidemiology. Curr Issues Mol Biol. 2014;17(1):11–22.
131. Bradford P. Extended-spectrum beta-lactamase in the 21 century: characterization, epidemiology, and detection of this important resistant threat. Clin Microbiol Rev. 2001;14(4):933–51.
132. Livermore DM, Woodford N. The beta-lactamase threat in Enterobacteriaceae, *Pseudomonas* and *Acinetobacter*. Trends Microbiol. 2006;14(9):413–20.
133. Pitout JD. Infections with extended-spectrum beta-lactamase-producing enterobacteriaceae: changing epidemiology and drug treatment choices. Drugs. 2010;70(3):313–33.
134. Cantón R, Coque TM. The CTX-M beta-lactamase pandemic. Curr Opin Microbiol. 2006;9(5):466–75.
135. Bonnet R. Growing group of extended spectrum: the CTX-M enzymes. Antimicrob Agent Chemother. 2004;48(1):1–14.
136. Zhao W-H, Hu Z-Q. Epidemiology and genetics of CTX-M extended-spectrum β-lactamases in Gram-negative bacteria. Crit Rev Microbiol. 2013;39(April 2012):79–101.
137. Humeniuk C, Arlet G, Gautier V, Grimont P, Labia R, Philippon A. Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob Agents Chemother. 2002;46(9):3045–9.
138. He D, Partridge SR, Shen J, Zeng Z, Liu L, Rao L, et al. CTX-M-123, a novel hybrid of the CTX-M-1 and CTX-M-9 group β-lactamases recovered from *Escherichia coli* isolates in China. Antimicrob Agents Chemother. 2013;57(8):4068–71.
139. Coque TM, Novais Â, Carattoli A, Poirel L, Pitout J, Peixe L, et al. Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. 2008;14(2):195–200.
140. Karim A, Poirel L, Nagarajan S, Nordmann P. Plasmid-mediated extended-spectrum beta-lactamase (CTX-M-3) from India and gene association with insertion sequence IS*Ecp1*. FEMS Microbiol Lett. 2001;201:237–41.

141. Peirano G, Pitout JDD. Molecular epidemiology of *Escherichia coli* producing CTX-M beta-lactamases: the worldwide emergence of clone ST131 O25:H4. Int J Antimicrob Agents. 2010;35(4):316–21.

142. Eckert C, Gautier V, Hidri N, Verdet C, Barnaud G, Delisle F, et al. Dissemination of CTX-M-type β-lactamases among clinical isolates of *Enterobacteriaceae* in Paris, France. Antimicrob Agents Chemother. 2004;48(4):1249–55.

143. Velasco M, Horcajada JP, Moreno-martinez A, Vila J, Martinez A, Ruiz J, et al. Decreased invasive capacity of quinolone-resistant *Escherichia coli* in patients with urinary tract infections. Clin Infect Dis. 2001;33:1682–6.

144. Vila J, Simon K, Ruiz J, Horcajada JP, Velasco M, Barranco M, et al. Are quinolone-resistant uropathogenic *Escherichia coli* less virulent? J Infect Dis [Internet]. 2002 Oct 1;186(7):1039–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12232848

145. Johnson JR, Kuskowski M a, Gajewski A, Soto S, Horcajada JP, Jimenez de Anta MT, et al. Extended virulence genotypes and phylogenetic background of *Escherichia coli* isolates from patients with cystitis, pyelonephritis, or prostatitis. J Infect Dis. 2005;191(1):46–50.

146. Horcajada JP, Soto S, Gajewski A, Smithson A, Jime MT, Anta D, et al. Quinolone-resistant uropathogenic *Escherichia coli* strains from phylogenetic group B2 have fewer virulence factors than their susceptible counterparts. J Clin Microbiol. 2005;43(6):2962–4.

147. Soto SM, Zúñiga S, Ulleryd P, Vila J. Acquisition of a pathogenicity island in an *Escherichia coli* clinical isolate causing febrile urinary tract infection. Eur J Clin Microbiol Infect Dis. 2011;30(12):1543–50.

148. Soto SM, Bosch J, Jimenez de Anta MT, Vila J. Comparative study of virulence traits of *Escherichia coli* clinical isolates causing early and late neonatal sepsis. J Clin Microbiol. 2008;46(3):1123–5.

149. Kawamura-Sato K, Yoshida R, Shibayama K, Ohta M. Virulence genes, quinolone and fluoroquinolone resistance, and phylogenetic background of uropathogenic *Escherichia coli* strains isolated in Japan. Jpn J Infect Dis. 2010;63(2):113–5.

150. Martínez-Martínez L, Fernández F, Perea EJ. Relationship between haemolysis

production and resistance to fluoroquinolones among clinical isolates of *Escherichia coli*. J Antimicrob Chemother. 1999;43(2):277–9.

151. Liu X, Liu H, Li Y, Hao C. Association between virulence profile and fluoroquinolone resistance in *Escherichia coli* isolated from dogs and cats in China. J Infect Dev Ctries. 2017;11(4):306–13.
152. Telli M, Guiral E, Martínez J a, Almela M, Bosch J, Vila J, et al. Prevalence of enterotoxins among *Escherichia coli* isolates causing bacteraemia. FEMS Microbiol Lett. 2010;306(2):117–21.
153. Soto SM, Anta MTJ De, Vila J. Quinolones induce partial or total loss of pathogenicity islands in uropathogenic *Escherichia coli* by SOS-dependent or -independent pathways, respectively. Antimicrob Agents Chemother. 2006;50(2):649–53.
154. Hacker J, Blum-Oehler G, Mühldorfer I, Tschäpe H. Pathogenicity islands of virulent bacteria: Structure, function and impact on microbial evolution. Mol Microbiol. 1997;23(6):1089–97.
155. Vila J, Simon K, Ruiz J, Horcajada JP, Velasco M, Barranco M, et al. Are quinolone-resistant uropathogenic *Escherichia coli* less virulent? J Infect Dis. 2002;186(7):1039–42.
156. CDC. What is Epidemiology? Available from: https://www.cdc.gov/careerpaths/k12teacherroadmap/epidemiology.html
157. Gautom RK. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157 : H7 and other gram-negative organisms in 1 Day. 1997;35(11):2977–80.
158. Chaudhuri RR, Henderson IR. The evolution of the *Escherichia coli* phylogeny. Infect Genet Evol. 2012;12(2):214–26.
159. Llovet T, Coll P, March F, Montserrat I, Atela I, Mirelis B, et al. Comparison of macrorestriction analysis of genomic DNA by pulsed-field gel electrophoresis and ribotyping with conventional methods for differentiation of *Escherichia coli* 0124 isolates. Clin Microbiol Infect. 1995;1(2):127–33.
160. Crandalll K a, Posada D. Phylogenetic approaches to molecular epidemiology. Epidemiology. 2002;25–40.
161. Köhler C-D, Dobrindt U. What defines extraintestinal pathogenic *Escherichia coli*? Int J

Med Microbiol. 2011;301(8):642–7.

162. Herzer PJ, Inouye S, Inouye M, Whittam TS. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. J Bacteriol. 1990;172(11):6175–81.

163. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol. 2000;66(10):4555–8.

164. Gordon DM, Clermont O, Tolley H, Denamur E. Assigning *Escherichia coli* strains to phylogenetic groups: Multi-locus sequence typing versus the PCR triplex method. Environ Microbiol. 2008;10:2484–96.

165. Picard B, Picard-Pasquier N, Krishnamoorthy R, Goullet P. Characterization of highly virulent *Escherichia coli* strains by ribosomal DNA restriction fragment length polymorphism. FEMS Microbiol Lett. 1991;66(2):183–8.

166. Bingen E, Picard B, Brahimi N, Mathy S, Desjardins P, Elion J, et al. Phylogenetic analysis of *Escherichia coli* strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains. J Infect Dis. 1998;177(3):642–50.

167. Clermont O, Christenson JK, Denamur E, Gordon DM. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep. 2013;5(1):58–65.

168. Munkhdelger Y, Gunregjav N, Dorjpurev A, Juniichiro N, Sarantuya J. Detection of virulence genes, phylogenetic group and antibiotic resistance of uropathogenic *Escherichia coli* in Mongolia. J Infect Dev Ctries. 2017;11(1):51–7.

169. Wijetunge DSS, Gongati S, DebRoy C, Kim KS, Couraud PO, Romero IA, et al. Characterizing the pathotype of neonatal meningitis causing *Escherichia coli* (NMEC). BMC Microbiol. 2015 Oct 14;15(1):211.

170. Usein C-R, Papagheorghe R, Oprea M, Condei M, Strãuţ M. Molecular characterization of bacteremic *Escherichia coli* isolates in Romania. Folia Microbiol. 2016;61(3):221–6.

171. Chakraborty A, Adhikari P, Shenoy S, Saralaya V. Genomic analysis and clinical importance of *Escherichia coli* isolate from patients with sepsis. Indian J Pathol Microbiol. 2015;58(1):22.

172. Imuta N, Ooka T, Seto K, Kawahara R, Koriyama T, Kojyo T, et al. Phylogenetic analysis of enteroaggregative *Escherichia coli* (EAEC) isolates from Japan reveals emergence of CTX-M-14-producing EAEC O25:H4 clones related to sequence type 131. J Clin Microbiol. 2016;54(8):2128–34.

173. Salmani H, Azarnezhad A, Fayazi MR, Hosseini A. Pathotypic and phylogenetic study of diarrheagenic *Escherichia coli* and uropathogenic *E. coli* using multiplex polymerase chain reaction. 2016;9(2):e28331

174. Duriez P, Clermont O, Bonacorsi S, Bingen E, Chaventré A, Elion J, et al. Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. Microbiology. 2001;147(6):1671–6.

175. Tenaillon O, Skurnik D, Picard B, Denamur E. The population genetics of commensal *Escherichia coli*. Nat Rev Microbiol. 2010;8(3):207–17.

176. Maiden MCJ, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci. 1998;95(6):3140–5.

177. Selander RK, Caugant DA, Ochman H, Musser JM, Gilmour MN, Whittam TS. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environ Microbiol. 1986;51(5):873–84.

178. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol. 2006;60(5):1136–51.

179. Clermont O, Gordon D, Denamur E. Guide to the various phylogenetic classification schemes for *Escherichia coli* and the correspondence among schemes. Microbiol. 2015;161(2015):980–8.

180. eBURST. Available from: http://eburst.mlst.net/default_v2.asp

181. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: Inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004;186(5):1518–30.

182. Riley LW. Pandemic lineages of extraintestinal pathogenic *Escherichia coli*. Clin Microbiol Infect. 2014;20(5):380–90.

183. Durmaz R, Otlu B, Koksal F, Hosoglu S, Ozturk R, Ersoy Y, et al. The optimization of a

rapid pulsed-field gel electrophoresis protocol for the typing of *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella* spp. Jpn J Infect Dis. 2009 Sep;62(5):372–7.

184. Foley SL, Lynne AM, Nayak R. Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. Infect Genet Evol. 2009;9(4):430–40.
185. Sáez-López E, Bosch J, Salvia MD, Fernández-Orth D, Cepas V, Ferrer-Navarro M, et al. Outbreak caused by *Escherichia coli* O18: K1: H7 sequence type 95 in a neonatal intensive care unit in Barcelona, Spain. Pediatr Infect Dis J. 2017;36(11):1079–86.
186. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog Dis. 2006;3(1):59–67.
187. Kauffmann F. The serology of the coli group. J Immunol. 1947;57(1):71–100.
188. Ørskov F, Ørskov I. *Escherichia coli* serotyping and disease in man and animals. Can J Microbiol. 1992;38(7):699–704.
189. Joensen KG, Tetzschner AMM, Iguchi A, Aarestrup FM, Scheutz F. Rapid and easy *in silico* serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. J Clin Microbiol. 2015;53(8):2410–26.
190. Lacher DW, Gangiredla J, Jackson SA, Elkins CA, Feng PCH. Novel microarray design for molecular serotyping of Shiga toxin-producing *Escherichia coli* strains isolated from fresh produce. Appl Environ Microbiol. 2014;80(15):4677–82.
191. Poolman JT, Wacker M. Extraintestinal pathogenic *Escherichia coli*, a common human pathogen: challenges for vaccine development and progress in the field. J Infect Dis. 2016;213(1):6–13.
192. Woods CR, Versalovic J, Koeuth T, Lupski JR. Whole-cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. J Clin Microbiol. 1993;31(7):1927–31.
193. Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 2017;19(24):6823–31.

194. Bae IK, Kim J, Sun JYH, Jeong SH, Kim Y-R, Wang K-K, et al. Comparison of pulsed-field gel electrophoresis & repetitive sequence-based PCR methods for molecular epidemiological studies of *Escherichia coli* clinical isolates. Indian J Med Res. 2014;140(5):679–85.

195. Herbold NM, Clotilde LM, Anderson KM, Kase J, Hartman GL, Himathongkham S, et al. Clustering of clinical and environmental *Escherichia coli* O104 isolates using the DiversiLab™ Repetitive Sequence-Based PCR System. Curr Microbiol. 2015;70(3):436–40.

196. Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev. 2011;35:736–55.

197. Mathers AJ, Peirano G, Pitout DD. The role of epidemic resistance plasmids and international high- risk clones in the spread of multidrug-resistant *Enterobacteriaceae*. 2015;28(3):565–91.

198. Wasey A, Salen P. *Escherichia coli* (*E Coli* 0157 H7). StatPearls. StatPearls Publishing; 2018.

199. Olesen B, Scheutz F, Andersen RL, Menard M, Boisen N, Johnston B, et al. Enteroaggregative *Escherichia coli* O78:H10, the cause of an outbreak of urinary tract infection. J Clin Microbiol. 2012;50(11):3703–11.

200. Itoh Y, Nagano I, Kunishima M, Ezaki T. Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable : H10 associated with a massive outbreak of gastrointestinal illness. 1997;35(10):2546–50.

201. Bielaszewska M, Mellmann A, Zhang W, Köck R, Fruth A, Bauwens A, et al. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. Lancet Infect Dis. 2011 Sep;11(9):671–6.

202. Wester AL, Brandal LT, Dahle UR. Extraintestinal pathogenic *Escherichia coli* carrying the Shiga toxin gene *stx2*. J Clin Microbiol. 2013;51(12):4279–80.

203. Herzog K, Engeler Dusel J, Hugentobler M, Beutin L, Sägesser G, Stephan R, et al. Diarrheagenic enteroaggregative *Escherichia coli* causing urinary tract infection and

bacteremia leading to sepsis. Infection. 2014;42(2):441–4.

204. Vargas M, Gascon J, Jimenez De Anta MT, Vila J. Prevalence of *Shigella* enterotoxins 1 and 2 among *Shigella* strains isolated from patients with traveler's diarrhea. J Clin Microbiol. 1999;37(11):3608–11.

205. Vila J, Vargas M, Henderson IR, Gascon J, Nataro JP. Enteroaggregative *Escherichia coli* virulence factors in traveler's diarrhea strains. J Infect Dis. 2000;182(6):1780–3.

206. Paniagua-Contreras GL, Hernández-Jaimes T, Monroy-Pérez E, Vaca-Paniagua F, Díaz-Velásquez C, Uribe-García A, et al. Comprehensive expression analysis of pathogenicity genes in uropathogenic *Escherichia coli* strains. Microb Pathog. 2017;103:1–7.

207. Mendez-Arancibia E, Vargas M, Soto S, Ruiz J, Kahigwa E, Schellenberg D, et al. Prevalence of different virulence factors and biofilm production in enteroaggregative *Escherichia coli* isolates causing diarrhea in children in Ifakara (Tanzania). Am J Trop Med Hyg. 2008;78(6):985–9.

208. Paiva de Sousa C, Dubreuil JD. Distribution and expression of the *astA* gene (EAST1 toxin) in *Escherichia coli* and *Salmonella*. Int J Med Microbiol. 2001;291(1):15–20.

209. Abe CM, Salvador FA, Falsetti IN, Vieira MAM, Blanco J, Blanco JE, et al. Uropathogenic *Escherichia coli* (UPEC) strains may carry virulence properties of diarrhoeagenic *E. coli*. FEMS Immunol Med Microbiol. 2008;52(3):397–406.

210. Chattaway MA, Jenkins C, Ciesielczuk H, Day M, Donascimento V, Day M, et al. Evidence of evolving extraintestinal enteroaggregative *Escherichia coli* ST38 clone. Emerg Infect Dis. 2014;20(11):1935–7.

211. Nunes KO, Santos ACP, Bando SY, Silva RM, Gomes TAT, Elias WP. Enteroaggregative *Escherichia coli* with uropathogenic characteristics are present in feces of diarrheic and healthy children. Pathog Dis. 2017;75(8).

212. López-Banda DA, Carrillo-Casas EM, Leyva-Leyva M, Orozco-Hoyuela G, Manjarrez-Hernández ÁH, Arroyo-Escalante S, et al. Identification of virulence factors genes in *Escherichia coli* isolates from women with urinary tract infection in Mexico. Biomed Res Int. 2014;2014:959206.

213. Pompilio A, Crocetta V, Savini V, Petrelli D, Nicola M Di, Bucco S, et al. Phylogenetic relationships, biofilm formation, motility, antibiotic resistance and extended virulence

genotypes among *Escherichia coli* strains from women with community-onset primitive acute pyelonephritis. PLoS ONE 13(5): e0196260.

214. Kaczmarek A, Skowron K, Budzy A, Gospodarek-Komkowska E. Virulence-associated genes and antibiotic susceptibility among vaginal and rectal *Escherichia coli* isolates from healthy pregnant women in Poland. Folia Microbiol (Praha). 2018;63(5):637-64.
215. Cook SW, Hammill HA, Hull RA. Virulence factors of *Escherichia coli* isolated from female reproductive tract infections and neonatal sepsis. Infect Dis Obstet Gynecol. 2001;9(4):203–7.
216. Birošová E, Siegfried L, Kmeťová M, Makara A, Ostró A, Grešová A, et al. Detection of virulence factors in α-haemolytic *Escherichia coli* strains isolated from various clinical materials. 2004;10: 569–573
217. Padilla C, Padilla A, Lobos O. Virulence genes and antimicrobial susceptibility of *Escherichia coli* taken from women with vaginitis in Talca, Chile. J Infect Dev Ctries. 2014;8(3):265–70.
218. Morin MD, Hopkins WJ. Identification of virulence genes in uropathogenic *Escherichia coli* by multiplex polymerase chain reaction and their association with infectivity in mice. Urology. 2002;60(3):537–41.
219. Al-Mayahie SMG. Vaginal colonization by *papG* allele II+ *Escherichia coli* isolates from pregnant and nonpregnant women as predisposing factor to pyelonephritis. Infect Dis Obstet Gynecol. 2013;2013:860402.
220. Jauréguy F, Carbonnelle E, Bonacorsi S, Clec'h C, Casassus P, Bingen E, et al. Host and bacterial determinants of initial severity and outcome of *Escherichia coli* sepsis. Clin Microbiol Infect. 2007;13(9):854–62.
221. Sáez-López E, Cossa A, Benmessaoud R, Madrid L, Moraleda C, Villanueva S, et al. Characterization of vaginal *Escherichia coli* isolated from pregnant women in two different African sites. PLoS One. 2016;11(7):1–10.
222. Beceiro A, Tomás M, Bou G. Antimicrobial resistance and virulence: A successful or deleterious association in the bacterial world? Clin Microbiol Rev. 2013;26(2):185–230.
223. Sawma-Aouad G, Hashwa F, Tokajian S. Antimicrobial resistance in relation to virulence determinants and phylogenetic background among uropathogenic *Escherichia coli* in

Lebanon. J Chemother. 2009;21(2):153–8.

224. Harwalkar A, Gupta S, Rao A, Srinivasa H. Lower prevalence of *hlyD*, *papC* and *cnf-1* genes in ciprofloxacin-resistant uropathogenic *Escherichia coli* than their susceptible counterparts isolated from southern India. J Infect Public Health. 2014;7(5):413–9.
225. Zhang L, Levy K, Trueba G, Cevallos W, Trostle J, Eisenberg JNS, et al. Effects of selection pressure and genetic association on the relationship between antibiotic resistance and virulence in *Escherichia coli*. Antimicrob Agents Chemother. 2015;59(11):6733–40.
226. Calhau V, Domingues S, Ribeiro G, Mendonça N, Da Silva GJ. Interplay between pathogenicity island carriage, resistance profile and plasmid acquisition in uropathogenic *Escherichia coli*. J Med Microbiol. 2015;64(8):828–35.
227. Guiral E, Bosch J, Vila J, Soto SM. Antimicrobial resistance of *Escherichia coli* strains causing neonatal sepsis between 1998 and 2008. Chemotherapy. 2012;58:123–8.
228. Bryce A, Hay AD, Lane IF, Thornton H V, Wootton M, Costelloe C. Global prevalence of antibiotic resistance in paediatric urinary tract infections caused by *Escherichia coli* and association with routine use of antibiotics in primary care: systematic review and meta-analysis. BMJ. 2016;352:i939.
229. García-Hernández AM, García-Vázquez E, Hernández-Torres A, Ruiz J, Yagüe G, Herrero JA, et al. Bacteriemias por *Escherichia coli* productor de betalactamasas de espectro extendido (BLEE): Significación clínica y perspectivas actuales. Rev Esp Quimioter. 2011;24(2):57–66.
230. Mazzariol A, Bazaj A, Cornaglia G. Multi-drug-resistant Gram-negative bacteria causing urinary tract infections: a review. J Chemother. 2017;29:2–9.
231. Thaver D, Ali SA, Zaidi AKM. Antimicrobial resistance among neonatal pathogens in developing countries. Pediatr Infect Dis J. 2009;28(Supplement):S19–21.
232. Amaya E, Caceres M, Fanc H, Torres Ramirez A, Palmgren A-C, Nord CE, et al. Antibiotic resistance patterns in gram-negative and gram-positive bacteria causing septicemia in newborns in León, Nicaragua: correlation with environmental samples. J Chemother. 2010;22(1):25–9.
233. Heideking M, Lander F, Hufnagel M, Pfeifer Y, Wicker E, Krause G, et al. Antibiotic susceptibility profiles of neonatal invasive isolates of *Escherichia coli* from a 2-year

nationwide surveillance study in Germany, 2009-2010. Eur J Clin Microbiol Infect Dis. 2013;32(9):1221–3.

234. Shakir S, Goldbeck J, Robison D, Eckerd A, Chavez-Bueno S. Genotypic and phenotypic characterization of invasive neonatal *Escherichia coli* clinical isolates. Am J Perinatol. 2014;31(11):975–82.
235. García-Basteiro A, Ribeiro R, Brew J, Sacoor C, Valencia S, Bulo H, et al. Tuberculosis on the rise in southern Mozambique (1997-2012). Eur Respir J. 2017;49:1601683.
236. Maltha J, Guiraud I, Kaboré B, Lompo P, Ley B, Bottieau E, et al. Frequency of severe malaria and invasive bacterial infections among children admitted to a rural hospital in Burkina Faso. PLoS One. 2014;9(2):e89103.
237. González R, Munguambe K, Aponte JJ, Bavo C, Nhalungo D, Macete E, et al. High HIV prevalence in a southern semi-rural area of Mozambique: A community-based survey. HIV Med. 2012;13(10):581–8.
238. van der Meeren BT, Chhaganlal KD, Pfeiffer A, Gomez E, Ferro JJ, Hilbink M, et al. Extremely high prevalence of multi-resistance among uropathogens from hospitalised children in Beira, Mozambique. South African Med J. 2013;103(6):382–6.
239. Aslani MM, Alikhani MY, Zavari A, Yousefi R, Zamani AR. Characterization of enteroaggregative *Escherichia coli* (EAEC) clinical isolates and their antibiotic resistance pattern. Int J Infect Dis. 2011;15(2):e136-9.
240. Ali MMM, Ahmed SF, Klena JD, Mohamed ZK, Moussa TAA, Ghenghesh KS. Enteroaggregative *Escherichia coli* in diarrheic children in Egypt: molecular characterization and antimicrobial susceptibility. J Infect Dev Ctries. 2014;8(5):589–96.
241. Guerra JA, Romero-Herazo YC, Arzuza O, Gómez-Duarte OG. Phenotypic and genotypic characterization of enterotoxigenic *Escherichia coli* clinical isolates from northern Colombia, South America. Biomed Res Int. 2014;2014:1–11.
242. Nguyen T V., Le Van P, Le Huy C, Gia KN, Weintraub A. Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam. J Clin Microbiol. 2005 Feb;43(2):755–60.
243. Medina AM, Rivera FP, Pons MJ, Riveros M, Gomes C, Bernal M, et al. Comparative analysis of antimicrobial resistance in enterotoxigenic *Escherichia coli* isolates from two

paediatric cohort studies in Lima, Peru. Trans R Soc Trop Med Hyg. 2015;109(8):493–502.

244. Begum YA, Talukder KA, Azmi IJ, Shahnaij M, Sheikh A, Sharmin S, et al. Resistance pattern and molecular characterization of enterotoxigenic *Escherichia coli* (ETEC) strains isolated in Bangladesh. Ahmed N, editor. PLoS One. 2016 Jul;11(7):e0157415.
245. Odetoyin BW, Hofmann J, Aboderin AO, Okeke IN. Diarrhoeagenic *Escherichia coli* in mother-child Pairs in Ile-Ife, South Western Nigeria. BMC Infect Dis. 2015 Dec;16(1):28.
246. Cordero L, Sananes M, Ayers LW. Bloodstream infections in a neonatal intensive-care unit: 12 years' experience with an antibiotic control program. Infect Control Hosp Epidemiol. 1999;20(4):242–6.
247. Amstey MS. Group B streptococcal prophylaxis. Am J Obstet Gynecol. 1994;171(6):1675.
248. Mendoza-Palomar N, Balasch-Carulla M, González-Di Lauro S, Céspedes MC, Andreu A, Frick MA, et al. *Escherichia coli* early-onset sepsis: trends over two decades. Eur J Pediatr. 2017;176(9):1227–34.
249. Weissman SJ, Hansen NI, Zaterka-Baxter K, Higgins RD, Stoll BJ. Emergence of antibiotic resistance-associated clones among *Escherichia coli* recovered from newborns with early-onset sepsis and meningitis in the United States, 2008-2009. J Pediatric Infect Dis Soc. 2016;5(3):269–76.
250. Mendez Arancibia E, Pitart C, Ruiz J, Marco F, Gascón J, Vila J. Evolution of antimicrobial resistance in enteroaggregative *Escherichia coli* and enterotoxigenic *Escherichia coli* causing traveller's diarrhoea. J Antimicrob Chemother. 2009;64(2):343–7.
251. Kong FYS, Rupasinghe TW, Simpson JA, Vodstrcil LA, Fairley CK, McConville MJ, et al. Pharmacokinetics of a single 1g dose of azithromycin in rectal tissue in men. PLoS One. 2017;12(3):e0174372.
252. Sierra JM, Ruiz J, Navia MM, Vargas M, Vila J. In vitro activity of rifaximin against enteropathogens producing traveler's diarrhea. Antimicrob Agents Chemother. 2001;45(2):643-4.
253. Shakil S, Akram M, Ali SM, Khan AU. Acquisition of extended-spectrum β-lactamase producing *Escherichia coli* strains in male and female infants admitted to a neonatal

intensive care unit: molecular epidemiology and analysis of risk factors. J Med Microbiol. 2010;59(8):948–54.

254. Anandan S, Thomas N, Veeraraghavan B, Jana AK. Prevalence of extended- spectrum beta-lactamase producing *Escherichia coli* and *Klebsiella* spp in a neonatal intensive care unit. Indian Pediatr. 2009;46(12):1106–7.
255. Jain A, Roy I, Gupta MK, Kumar M, Agarwal SK. Prevalence of extended-spectrum beta-lactamase-producing Gram-negative bacteria in septicaemic neonates in a tertiary care hospital. J Med Microbiol. 2003;52:421–5.
256. Sáez-López E, Guiral E, López Y, Montero I, Bosch J, Vila J, et al. Characterization of CTX-M-14 and CTX-M-15 producing *Escherichia coli* strains causing neonatal sepsis. Microb Drug Resist. 2014;20(4):281–4.
257. Pitout JDD, Laupland KB. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis. 2008;8(3):159–66.
258. Mshana SE, Imirzalioglu C, Hossain H, Hain T, Domann E, Chakraborty T. Conjugative IncFI plasmids carrying CTX-M-15 among *Escherichia coli*ESBL producing isolates at a University hospital in Germany. BMC Infect Dis. 2009 Dec;9(1):97.
259. Berkley JA, Lowe BS, Mwangi I, Williams T, Bauni E, Mwarumba S, et al. Bacteremia among children admitted to a rural hospital in Kenya. N Engl J Med. 2005;352(1):39–47.
260. Sigaúque B, Roca A, Mandomando I, Morais L, Quintó L, Sacarlal J, et al. Community-acquired bacteremia among children admitted to a rural hospital in Mozambique. Pediatr Infect Dis J. 2009;28(2):108–13.
261. Mandomando I, Sigaúque B, Morais L, Espasa M, Vallès X, Sacarlal J, et al. Antimicrobial drug resistance trends of bacteremia isolates in a rural hospital in southern Mozambique. Am J Trop Med Hyg. 2010;83(1):152–7.
262. Kim H, Kim YA, Park YS, Choi MH, Lee GI, Lee K. Risk Factors and molecular features of sequence type (ST) 131 extended-spectrum β-Lactamase-producing *Escherichia coli* in community-onset bacteremia. Sci Rep. 2017;7(1):14640.
263. Musicha P, Feasey NA, Cain AK, Kallonen T, Chaguza C, Peno C, et al. Genomic landscape of extended-spectrum β-lactamase resistance in *Escherichia coli* from an urban African

setting. J Antimicrob Chemother. 2017;72(6):1602–9.

264. Carattoli A. Plasmids and the spread of resistance. Int J Med Microbiol. 2013;303(6–7):298–304.

265. Bagus Wasito E, Shigemura K, Osawa K, Fardah A, Kanaida A, Raharjo D, et al. Antibiotic susceptibilities and genetic characteristics of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates from stools of pediatric diarrhea patients in Surabaya, Indonesia. Jpn J Infect Dis. 2017;70(4):378–82.

266. Herrera-León S, Llorente MT, Sánchez S. Plasmid-mediated quinolone resistance in different diarrheagenic *Escherichia coli* pathotypes responsible for complicated, noncomplicated, and traveler's diarrhea cases. Antimicrob Agents Chemother. 2015;60(3):1950–1.

267. Alizade H, Fallah F, Ghanbarpour R, Aflatoonian MR, Goudarzi H, Sharifi H. Phylogenetic groups, extended-spectrum beta-lactamases and metallo-beta-lactamase in *Escherichia coli* isolated from fecal samples of patients with diarrhea in Iran. Gastroenterol Hepatol from Bed to Bench. 2015;8(3):207–14.

268. Bai L, Wang L, Yang X, Wang J, Gan X, Wang W, et al. Prevalence and molecular characteristics of extended-spectrum β-lactamase genes in *Escherichia coli* isolated from diarrheic patients in China. Front Microbiol. 2017 Feb;8:144.

269. Franz E, Veenman C, van Hoek AHAM, Husman A de R, Blaak H. Pathogenic *Escherichia coli* producing extended-spectrum β-lactamases isolated from surface water and wastewater. Sci Rep. 2015 Nov;5(1):14372.

270. Smet A, Vaes R, Praud K, Doublet B, Daminet S, Cloeckaert A, et al. New broad-spectrum β-lactamases emerging among Enterobacteriaceae from healthy cats and dogs: a public health concern? Int J Antimicrob Agents. 2014;44(1):81–2.

271. Ortega A, Oteo J, Aranzamendi-Zaldumbide M, Bartolomé RM, Bou G, Cercenado E, et al. Spanish multicenter study of the epidemiology and mechanisms of amoxicillin-clavulanate resistance in *Escherichia coli*. Antimicrob Agents Chemother. 2012;56(7):3576–81.

272. Ribeiro TG, Novais Â, Peixe L, Machado E. Atypical epidemiology of CTX-M-15 among Enterobacteriaceae from a high diversity of non-clinical niches in Angola. J Antimicrob

Chemother. 2016;71(5):1169–73.

273. Zurfluh K, Glier M, Hächler H, Stephan R. Replicon typing of plasmids carrying *bla*$_{\text{CTX-M-15}}$ among *Enterobacteriaceae* isolated at the environment, livestock and human interface. Sci Total Environ. 2015;521–522:75–8.

274. de Angelis G, Fiori B, Menchinelli G, D'Inzeo T, Liotti FM, Morandotti GA, et al. Incidence and antimicrobial resistance trends in bloodstream infections caused by ESKAPE and *Escherichia coli* at a large teaching hospital in Rome, a 9-year analysis (2007–2015). Eur J Clin Microbiol Infect Dis. 2018;1–10.

275. Gottesman B, Shitrit P, Katzir M, Chowers M. Antibiotic exposure in the community and resistance patterns of *Escherichia coli* community-acquired bloodstream infection. Isr Med Assoc J. 2018;20:382–4.

276. Scheuerman O, Schechner V, Carmeli Y, Gutiérrez-Gutiérrez B, Calbo E, Almirante B, et al. Comparison of predictors and mortality between bloodstream infections caused by ESBL-producing *Escherichia coli* and ESBL-producing *Klebsiella pneumoniae*. Infect Control Hosp Epidemiol. 2018;39(6):660–7.

277. Lee JH, Subhadra B, Son Y-J, Kim DH, Park HS, Kim JM, et al. Phylogenetic group distributions, virulence factors and antimicrobial resistance properties of uropathogenic *Escherichia coli* strains isolated from patients with urinary tract infections in South Korea. Lett Appl Microbiol. 2016;62(1):84–90.

278. Grude N, Potaturkina-Nesterova NI, Jenkins A, Strand L, Nowrouzian FL, Nyhus J, et al. A comparison of phylogenetic group, virulence factors and antibiotic resistance in Russian and Norwegian isolates of *Escherichia coli* from urinary tract infection. Clin Microbiol Infect. 2007;13(2):208–11.

279. Kõljalg S, Truusalu K, Stsepetova J, Pai K, Vainumäe I, Sepp E, et al. The *Escherichia coli* phylogenetic group B2 with integrons prevails in childhood recurrent urinary tract infections. APMIS. 2014;122(5):452–8.

280. Ejrnæs K. Bacterial characteristics of importance for recurrent urinary tract infections caused by *Escherichia coli*. Dan Med Bull. 2011 Apr;58(4):B4187.

281. Suzuki S, Shibata N, Yamane K, Wachino J, Ito K, Arakawa Y. Change in the prevalence of extended-spectrum-beta-lactamase-producing *Escherichia coli* in Japan by clonal

spread. J Antimicrob Chemother. 2009;63(1):72–9.

282. Okeke IN, Wallace-Gadsden F, Simons HR, Matthews N, Labar AS, Hwang J, et al. Multi-locus sequence typing of enteroaggregative *Escherichia coli* isolates from nigerian children uncovers multiple lineages. PLoS One. 2010;5(11):e14093.

283. Brisse S, Diancourt L, Laouénan C, Vigan M, Caro V, Arlet G, et al. Phylogenetic distribution of CTX-M- and non-extended-spectrum-β-lactamase-producing *Escherichia coli* isolates: Group B2 isolates, except clone ST131, rarely produce CTX-M enzymes. J Clin Microbiol. 2012;50(9):2974–81.

284. Chattaway MA, Jenkins C, Rajendram D, Cravioto A, Talukder KA, Dallman T, et al. Enteroaggregative *Escherichia coli* have evolved independently as distinct complexes within the *E. coli* population with varying ability to cause disease. PLoS One. 2014;9(11):e112967.

285. Matsumura Y, Pitout JDD, Gomi R, Matsuda T, Noguchi T, Yamamoto M, et al. Global *Escherichia coli* sequence type 131 clade with $bla_{\text{CTX-M-27}}$ gene. Emerg Infect Dis. 2016 Nov;22(11):1900–7.

286. Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P, et al. Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. PLoS Genet. 2009;5(1):e1000344.

287. Escobar-Paramo P, Grenet K, Le Menac'h A, Rode L, Salgado E, Amorin C, et al. Large-scale population structure of human commensal *Escherichia coli* isolates. Appl Environ Microbiol. 2004;70(9):5698–700.

288. Nature newsblog. Available from: http://blogs.nature.com/news/2011/06/europes_e_coli_outbreak_time_f.html.

289. Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol. 2015;13(5):269–84.

290. Romero R, Mazor M. Infection and preterm labor. Clin Obstet Gynecol. 1988 Sep;31(3):553–84.

291. European Centre for Disease Prevention and Control. ECDC Surveillance Atlas - Antimicrobial resistance. https://ecdc.europa.eu/en/antimicrobial-resistance.

292. CDC. The PFGE Process | PulseNet Methods. https://www.cdc.gov/pulsenet/pathogens/protocol-images.html.