UNIVERSITAT DE BARCELONA

Caracterización de los mecanismos de resistencia a antomicrobianos en aislamientos clínicos de *Acinetobacter baumannii* (patógeno nosocomial) y *Shigella* spp. (patógeno comunitario)

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Caracterización de los mecanismos moleculares de resistencia a agentes antimicrobianos en aislamientos clínicos de *Acinetobacter baumannii* (patógeno nosocomial) y *Shigella* spp. (patógeno comunitario)

Tesis presentada por
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para optar al Grado de Doctor en Ciencias Biológicas

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CERTIFICAN

Que la memoria titulada " Caracterización de los mecanismos moleculares de resistencia a agentes antimicrobianos en aislamientos clínicos de *Acinetobacter baumannii* (patógeno nosocomial) y *Shigella* spp. (patógeno comunitario)", presentada por Margarita Maria Navia De Roux, ha sido realizada bajo su dirección y cumple los requisitos necesarios para ser leída delante del Tribunal correspondiente.

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Director

Joaquim Gascón Brustenga
Codirector

Barcelona, abril de 2004

A mis padres que me dieron la vida,

a Irene y Andres que la han continuado

y a John con quien la comparto

AGRADECIMIENTOS

Son muchas las personas que a lo largo de estos ocho años
han hecho posible la realización de este trabajo.
Son muchos también con quienes he compartido
buenos momentos durante este período
y otros tantos los que me han
enseñado, corregido, alentado y soportado!
A todos vosotros, muchísimas gracias!!

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1 INTRODUCCION

1.1 Breve historia de los agentes antimicrobianos

De manera inconsciente e indirecta, el ser humano ha utilizado sustancias con actividad antimicrobiana desde épocas prehistóricas. Buscando la sanación de heridas y enfermedades, se utilizaban emplastes e infusiones de preparados con plantas y elementos que tenían a su alcance, algunos de ellos con actividad antimicrobiana. El uso de las diferentes plantas con fines terapéuticos, por parte de nuestros antepasados, se hacía de manera empírica, basándose en la observación y utilizando un método de ensayo y error.

El primero en buscar activa y científicamente sustancias con actividad antibiótica, fue el médico alemán Paul Ehrlich, considerado el padre de la quimioterapia. Dedicó su vida a la química, específicamente a la búsqueda de colorantes con alguna actividad terapéutica. Sus grandes aportaciones fueron dos: 1) la primera modificación de un núcleo terapéutico mediante substituciones químicas (intentaba con ello eliminar la alta toxicidad del atoxil, derivado arsenical) obteniendo moléculas sintéticas capaces de unirse selectivamente al microorganismo patógeno y 2) el desarrollo de una experimentación científica en terapéutica, probando sus compuestos en animales previamente infectados.

Otro alemán seguidor de los trabajos de Ehrlich, el Dr. Gerhard Domagk, fue el descubridor en 1932 de la primera molécula con actividad antibacteriana probada: el protonsil (una sulfamida). En 1935 se introdujo en la clínica, iniciándose así la era de las sulfamidas y convirtiéndose en uno de los mayores avances de la medicina y del tratamiento de las infecciones (Domagk, 1935). Gerhard Domagk recibió el premio nobel de medicina en 1947 por su descubrimiento.

Previamente, Fleming había descubierto la penicilina (Fleming, 1929). Su hallazgo fue casual y solo un hombre metódico y de mente abierta como él supo ver la importancia en lo que otros habrían descartado como un " molesto accidente ". Tal vez por ello, Fleming no fue plenamente consciente de la magnitud de su hallazgo, y el estudio de su uso terapéutico se

retrasó hasta 1939 cuando H.W Florey encontró la manera de purificar grandes cantidades y empezó a dársela a sus pacientes

Fleming y Florey recibieron en 1945 el Premio Nobel de Medicina, por el descubrimiento y desarrollo clínico de la penicilina.

Un último premio Nobel fue dado por el descubrimiento y desarrollo de un antibiótico a Selman Waksman, por el descubrimiento en los años 40 de la estreptomicina y su aplicación como tratamiento de la tuberculosis. Desde entonces son muchas las moléculas que se han descubierto (en la Tabla 1 se hace un resumen de los grupos de antibióticos más relevantes).

Tabla 1.- Introducción en la práctica clínica de los grupos de antibióticos más relevantes. (Tomado de Ruiz, 1998)

Años	**Moléculas**
40	Estreptomicina
50	Primeros macrólidos, Tetraciclinas, Cloranfenicol
60	Cefalosporinas, aminoglicósidos, quinolonas
70	Glicopéptidos
80	Fluroquinolonas, Carbapenems

1.2 Problemática de la multiresistencia.

Desde la introducción en clínica de las sulfamidas en 1939, el uso de los antimicrobianos ha significado un gran avance en la medicina moderna, siendo una de las estrategias más importantes en el control de las enfermedades infecciosas, otrora la mayor causa de mortalidad y morbilidad en el mundo. Sin embargo, a lo que en un principio parecía ser una solución definitiva, pronto se le descubrió que tenía un talón de Aquiles. Al existir sustancias bacteriostáticas y bactericidas en la naturaleza (el mejor ejemplo siendo tal vez la penicilina), las bacterias poseían ya mecanismos mediante los cuales eran capaces de evadir o inhibir su efecto. En otras palabras, tenían mecanismos naturales de resistencia, que en presencia de nuevas sustancias antimicrobianas, han ido evolucionando hasta volverse eficaces frente a las nuevas moléculas que se han ido desarrollando o descubriendo. A su vez, el mal

uso y abuso de estas sustancias, ha ejercido una presión de selección que ha contribuido a que dicha evolución natural se desarrollase con mayor rapidez. Es así como, por ejemplo, tan solo cinco años después de la introducción de la penicilina en la práctica clínica, ya se observaba un 14% de resistencia entre aislamientos nosocomiales de *Staphylococcus aureus*, que en su introducción eran 99% sensibles (Towner, 1995). Hoy en día, más del 90% de los *S.aureus* son resistentes a la penicilina, como resultado de la producción de β-lactamasas (Sefton, 2002).

Aunque el problema de la resistencia es casi inherente al de las infecciones, lo cierto es que la dimensión del problema y la velocidad a la cual la resistencia es desarrollada varía de un microorganismo a otro, dependiendo también del nivel de transmisión en un área determinada y del antimicrobiano en cuestión. De la misma manera, los diferentes esfuerzos para intentar controlar y en última instancia disminuir la resistencia, no siempre han alcanzado su objetivo (Lipsitch, 2001). Los determinantes de la velocidad a la cual una población bacteriana responde a las reducciones en el uso a un antimicrobiano parecen estar directamente relacionados con el coste que para la supervivencia ("fitness") del microorganismo tiene el mantener los determinantes de resistencia. Así pues, si el ser resistente implica un coste sustancial en la transmisibilidad del organismo, lo esperable es que una vez desaparezca la presión de selección, la población resistente sea reemplazada por una susceptible. En caso contrario, si no supone un coste o si los determinantes de resistencia son adquiridos (en elementos transponibles) por bacterias que ya de por sí son altamente transmisibles, la reversión del problema será como mínimo más lenta y cada vez más difícil (Lenski, 1998)

1.3 Factores que favorecen el desarrollo de la multirresistencia

Aunque el desarrollo de resistencias es un mecanismo evolutivo natural, existen factores socio-económicos que favorecen su aparición y aceleran el proceso de selección natural de cepas multirresistentes. Probablemente las dos factores principales que contribuyen en el desarrollo de resistencias entre los microorganismos son el mal uso que se hace de los antimicrobianos y la venta de productos de mala calidad. El mal uso se da tanto en países desarrollados como en países de baja renta (PBR). En países desarrollados es común la venta

de antimicrobianos sin receta médica en farmacias que facilita el que los pacientes se "autoreceten" con fármacos y posologías inadecuados. También se da, en todo el mundo, su mal uso entre los mismos médicos, que los prescriben en casos innecesarios, como en el caso de infecciones virales (Sefton, 2002) o que prescriben antibióticos influenciados por sus hábitos y sin tener en cuenta los patrones de resistencia en la zona. En PBR, principalmente en zonas rurales, también se da el caso de su mal uso en manos de sanadores o personal con entrenamiento muy limitado. Es aquí también donde, debido a la falta de controles de calidad y a la especulación, es común que se dé el segundo factor: la circulación de productos de mala calidad. Estos pueden ser productos degradados, adulterados o con una concentración de principio activo inadecuada ("diluidos").

Existen otra serie de factores que favorecen ya no la aparición sino la diseminación de los microorganismos resistentes. Las más obvias son el hacinamiento y la falta de higiene, normalmente asociadas a la pobreza. Situaciones tales como los conflictos armados que no solo acaban con los sistemas de salud en una región sino que también propician los desplazamientos, las concentraciones humanas en campos de refugiados y que empobrecen aún más a poblaciones ya de por sí en un estado crítico, favorecen también la aparición de epidemias de muy difícil control (Okeke et al, 1999).

1.4 Mecanismos responsables de la adquisición de resistencia

La resistencia a los antimicrobianos puede ser natural o adquirida. La natural se da aún en la ausencia de presión selectiva de antibióticos e implica que no todas las bacterias son susceptibles a todos los antimicrobianos. Así por ejemplo, las Enterobacterias, tienen una resistencia intrínseca a la penicilina, puesto que la membrana externa de estos microorganismos impide el paso del antibiótico al interior celular. Otro ejemplo de resistencia intrínseca en las Enterobacterias es la que presentan algunas especies de *Morganella morganii*, varias especies de *Serratia* y algunas de *Proteus* a las polimixinas.

La resistencia adquirida es la más preocupante, pues a diferencia de la intrínseca, puede llegar a ser transferida de un microorganismo a otro, generando un fenómeno de

aumento de los niveles de resistencia por diseminación de los mecanismos que generan la misma.

A nivel bioquímico, los mecanismos pueden dividirse en cinco grupos principales, resumidos con ejemplos en la Tabla 2, aunque hay que tener en cuenta que dichos mecanismos pueden solaparse, es decir que la presencia de uno no es excluyente de los demás y de hecho es común encontrar varios de ellos presentes en una misma célula bacteriana.

Tabla 2.- Bases bioquímicas de los mecanismos de resistencia a los antimicrobianos

Tipo de mecanismo	**Ejemplos**
Producción de enzimas modificadoras	ß-lactamasas (que inhiben los ß-lactámicos) Enzimas modificadoras de los aminoglicósidos Acetil-cloranfenicol transferasa
Modificación de la diana	Modificación de las topoisomerasas (resistencia a las quinolonas)
Adquisición de un sistema de *bypass*	Resistencia al trimetroprim Resistencia a las sulfamida
Reducción de la permeabilidad de la célula	Resistencia al imipenem en *Pseudomonas aeruginosa*
Expulsión activa del antimicrobiano	Resistencia a la tetraciclina

1.4.1 Producción de enzimas modificadoras / inactivantes del antimicrobiano

La producción de enzimas modificadoras confiere resistencia a una cepa, como su nombre lo indica, por la modificación y consecuente inactivación del antimicrobiano en cuestión. Es un mecanismo específico, limitado por el rango de acción de la enzima. El ejemplo típico de adquisición de resistencia por la producción de enzimas modificadoras es la resistencia a los ß-lactámicos, mediada por las ß-lactamasas.

Tabla 3.- Ejemplos de Betalactamasas con especial relevancia en clínica.

Enzima	Produccción	Sustrato	Inhibición por clavulánico	Clasificación según Bush, Jacoby y Medeiros	Especies bacterianas
AmpC	Cromosómica, inducible. Mutaciones pueden conllevar a una sobreexpresión constitutive	Principalmente cefalosporinas, pero tambien penicilinas	NO	1	*Enterobacter spp* *M.morganii* *P.aeruginosa* *Serratia* y otras
TEM-1	Plasmídica	Penicilinas y cefalosporinas de primera generación	SI	2b	*E.coli* y otras Enterobacterias, *H.influenzae*, *N.gonorrhoaeae*
TEM-3...>100 (BLEAS)	Plasmídica	Penicilinas, cefalosporinas (incluidas cefotaxima, ceftazidima), aztreonam	SI	2be	*E.coli* *Klebsiella* y otras Enterobacterias
TEM-variantes (IRT)	Plasmídica	Penicilinas, cefalosporins (incluidas cefotaxima, ceftazidima), aztreonam.	NO	2br	*E.coli* *Klebsiella* y otras enterobacterias
SHV-1	Cromosómica	Penicilinas y cefalosporinas de primera generación. Monobactámicos	SI	2b	*K.pneumoniae*
SHV-2....>50 (ESBL)	Plasmídica	Penicilinas, cefalosporinas (incluidas cefotaxima, ceftazidima), aztreonam	SI	2be	*K.pneumoniae, E.coli*
CTX-M 1...>10 (BLEAS)	Plasmídica	Penicilinas, cefalosporinas (incluidas cefotaxima, ceftazidima), aztreonam	SI	2be	*E.coli* *Klebsiella* y otras Enterobacterias
OXA-1	Plasmídica	Penicilinas y cefalosporinas de primeras generaciones	NO	2d	*E.coli* y otras Enterobacterias
OXA-10...>30 (ESBL)	Plasmídica	Penicilinas, cefalosporinas (incluidas cefotaxima, ceftazidima), aztreonam	NO	2de	*P.aeruginosa*
K1 (ESBL)	Cromosómica; sobreexpresión en algunas cepas	Penicilinas cefalosporinas (en caso de sobreexpresión afecta cefuroxima y aztreonam, cefotaxima solo minimamente y no afecta ceftazidima)	SI	2be	*K.oxytoca*

Las ß-lactamasas son enzimas capaces de hidrolizar el enlace amida del anillo betalactámico de este grupo de antibióticos. Hasta la actualidad se han descrito alrededor de trescientas enzimas diferentes, siendo catalogados como el mayor éxito estratégico-evolutivo de los microorganismos en la lucha contra los antimicrobianos (Cantón et al, 1999). No existe en la actualidad ningún ß-lactámico que se escape a la acción de al menos una de estas enzimas.

La clasificación y nomenclatura de las ß-lactamasas ha cambiado varias veces desde su descubrimiento, habiendose propuesto varios esquemas para clasificar esta amplia familia de enzimas. El primer acercamiento fue el de dividir las ß-lactamasas en penicilinasas que hidrolizaban las penicilinas y cefalosporinasas que hidrolizaban las cefalosporinas. Más tarde se tuvieron en cuenta la posición de determinantes genéticos (si plasmídico o cromosómico), así como puntos isoeléctricos o información sobre la cinética de las diferentes enzimas. Sin embargo, en todas estas clasificaciones había anomalías, que con el desarrollo de técnicas de biología molecular – específicamente la secuenciación - han podido ser finalmente resueltas.

La nomenclatura y el sistema de clasificación que se utiliza actualmente es la propuesta por Bush, Jacoby y Medeiros en 1995 y que de alguna manera logra aunar criterios de clasificaciones anteriores. En él, se dividen las ß-lactamasas en cuatro grupos. En el grupo 1 están las betalactamasas de Gram negativos, que hidrolizan preferentemente las cefalosporinas y no son inhibidas por el ácido clavulánico. Incluye betalactamasas cromosómicas constitutivas (Amp C de *E.coli*), inducibles (de *Citrobacter, Serratia, P.aeruginosa, Enterobacter* y *Morganella*) así como otras de carácter plasmídico resultado de la incorporación de genes cromosómicos en plásmidos (MIR-1, FOX-1).

En el grupo 2, el más numeroso y heterogéneo, están las betalactamasas que presentan un residuo de serina en su centro activo. La mayoría de ellas son inhibidas por el ácido clavulánico. Incluyen las penicilinasas de Gram positivos, las betalactamasas plasmídicas clásicas de Enterobacterias (TEM-1, TEM-2, SHV-1), betalactamasas de espectro ampliado derivadas de las plasmídicas clásicas, oxacilinas clásicas (OXA-1, PSE-2etc) y de espectro ampliado (derivadas de las clásicas) entre otras. En el grupo tres están las metaloenzimas cromosómicas, que requieren zinc para ser funcionales, no son inhibidas por el ácido clavulánico y tienen capacidad de hidrolizar los carbapenems. Finalmente el grupo 4 reune a todas aquellas enzimas que no reunen características para estar en ninguno de los otros tres grupos y cuya caracterización está aún por terminar. En la Tabla 3 se muestra un resumen de las betalactamasas más relevantes desde el punto de vista clínico.

Otro ejemplo de enzimas modificadoras son las que confieren resistencia a los aminoglicósidos. Se trata del mecanismo más importante y de mayor trascendencia clínica en cepas resistentes a los aminoglicósidos, aunque dicha resistencia puede deberse también a otros mecanismos tales como a alteraciones de la permeabilidad o modificaciones de la diana (en este caso el ribosoma). Estas enzimas actúan sobre los grupos amino o hidroxilo de los aminoglucósidos, generando compuestos inactivos incapaces de cumplir su función a nivel del ribosoma. Se dividen en tres grupos dependiendo de su modo de acción (ver Tabla 4).

La cloranfenicol acetil transferasa, que inactiva el cloranfenicol por o-acetilación (Shaw et al., 1997) es también una enzima modificadora. En el caso del fenotipo resistente al cloranfenicol también se ha descrito una implicación de otros mecanismos, específicamente un aumento de la expulsión activa o la disminución de la permeabilidad (principalmente mediada por la sobreexpresión del gen *cmlA*) (Bissonette et al., 1991; Cohen et al., 1989). Sin embargo, al igual que con los aminoglucósidos o las betalactamasas, es la resistencia por enzimas modificadoras la más importante y relevante desde el punto de vista clínico.

Tabla 4. Enzimas modificantes de aminoglucósidos más frecuentes (tomado de Cantón et al., 1999)

Enzima modificante	**Perfil de modificación**	**Microorganismo**
Acetiltranferasas – acetilan grupos amino del aminoglucósido. Proceso acetil-CoA dependiente		
	Gm, Tb, Nt, Nm,Pm	*Providencia stuartii, Proteus*
AAC(2')	Gm, Tb, Nt, Nm, Km, Ak	*rettgeri*
AAC(6')		*Enterobacteriaceae,*
	Gm, Tb, Nt	*Pseudomonas*
AAC(3)-I	Gm, Km, Tb, Nt	*Staphylococcus, Enterococcus*
AAC(3)-II	Gm, Km, Tb, Nt, Nm	*faecium*
AAC(3)-III		*Enterobacteriaceae,*
		Pseudomonas
		Enterobacteriaceae
		Pseudomonas
Nucleotidiltransferasas (o adeniltransferasas) - transfieren un nucleótido monosfostato desde un nucleótido trifostato a un grupo hidroxilo del antibiótico. Proceso ATP dependiente		
ANT (3'')	Str	*1.4.1.1.1 Enterobacteriaceae,*
ANT (6)	Str	*Pseudomonas*
ANT (2'')	Gm, Km, Tb	*1.4.1.1.2 Staphylococcus,*
ANT (4) (4'')	Km, Nm, Pm, Tb, Ak	*Enterococcus faecium*
		Enterobacteriaceae,
		Pseudomonas
		1.4.1.1.3 Staphylococcus
Fosfotransfereasas - fosforilan grupos hidroxilo. Proceso ATP dependiente		
APH (3')	Km, Nm, Pm, (Ak)	*Enterobacteriaceae,*
		Pseudomonas,
APH(2'')-AAC(6')	Gm ,Km, Tb, Nt, (Ak)	*Staphylococcus, Enterococcus*
APH(3'')	Str	*faecalis*
		Staphylococcus, Enterococcus
APH(6)	Str	*1.4.1.1.4 Enterobacteriaceae,*
		Pseudomonas
		Staphylococcus, Enterococcus
		Pseudomonas

Gm:gentamicina, Tb:tobramicina, Nt:netimicina, Nm:neomicina, Km:kanamicina, Ak:amikacina, Str:estreptomicina, Pm:paramomicina ()aminoglucósido no siempre modificado

1.4.2 Modificación de la diana

La resistencia por modificación de la diana se da cuando, en la cepa resistente, se modifica el receptor para el antimicrobiano de manera que este último ya no puede ejercer su efecto. La resistencia a las quinolonas es un ejemplo de dicho mecanismo. Las quinolonas son antimicrobianos que inhiben la acción de la ADN girasa y la Topoisomeras II, enzimas esenciales involucradas en el control de la configuración helicoidal del ADN. La unión entre la quinolona y la ADN-girasa o la Topoisomerasa II se da con la ayuda de iones magnesio, en puntos muy específicos. Las cepas que han desarrollado resistencia, presentan mutaciones en uno o más de estos puntos de "fijación" de manera que el complejo Quinolona-Topoisomerasa no puede darse y por lo tanto la enzima no es inactivada. Otro ejemplo de resistencia por modificación de la diana es el de la resistencia a las penicilinas por modificación de las proteínas fijadoras de penicilina (*"Penicillin Binding Proteins"* o PBP).

1.4.3 Adquisición de un sistema de bypass

Si en vez de mutar la diana, el microorganismo obtiene una enzima que sustituya en funciones la que está siendo bloqueada por la acción del antimicrobiano y que a su vez sea resistente a la acción de este, logrará evadirse del efecto letal del antibiótico y sobrevivir. Los genes para estas "enzimas alternativas" suelen ir a menudo codificados en elementos transponibles, tipo plásmidos o transposones, de manera que se trata de un mecanismo de resistencia de dispersión fácil. Ejemplos de este tipo de mecanismo lo presentan los genes de dihidrofolato reductasas codificados en plásmidos, trasposones e integrones, que confieren a las bacterias que los reciben resistencia al trimetroprim.

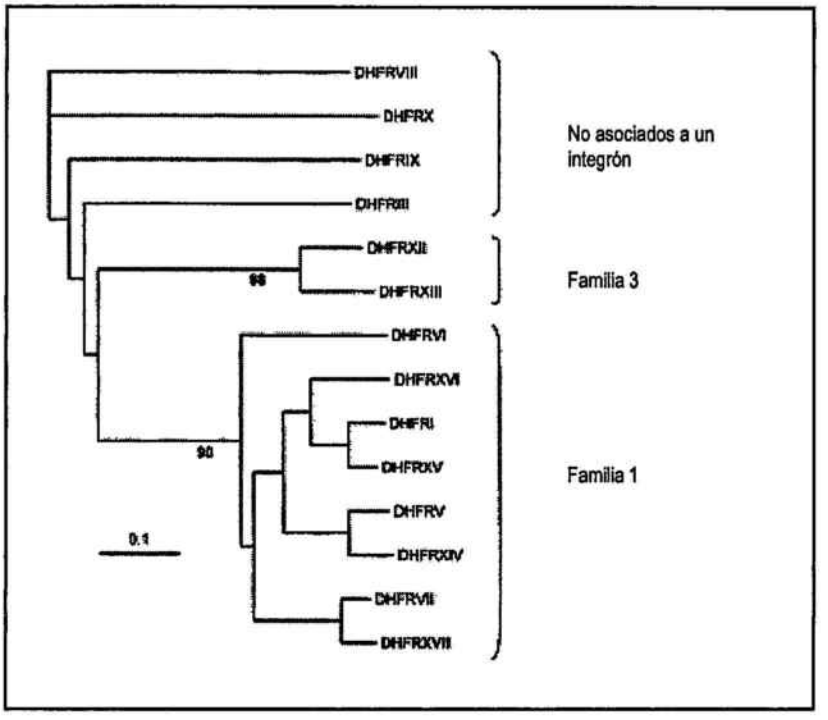

Figura 1.- Arbol filogenético consensuado que compara la secuencia de 14 enzimas DHFR . (Tomado de White et al., 2000.)

Hasta la fecha se han descrito al menos veinte clases diferentes de genes *dfr* (Amyes & Towner, 1990; Thomson, 1993). Las enzimas codificadas difieren entre sí y pueden ser distinguidas por su diferentes propiedades bioquímicas y biofísicas. White y colaboradores (2001) han construido un árbol filogenético basándose en la secuencia de 14 de los genes (Figura 1), identificando dos tipos de DHFRs: aquellos que se encuentran como *cassettes* de integrones (familias 1 y 3), y aquellos que no (familia 2).

1.4.4 Reducción de la permeabilidad de la célula

La impermeabilidad de una célula puede deberse bien a una estructura específica de la membrana o a un cambio de la misma. En la Figura 2 vemos la diferencia entre la membrana de un bacilo Gram positivo y otro Gram negativo. Los primeros están rodeados de una pared celular conformada por una sola capa de peptidoglicano. Aunque mecánicamente fuerte, esta membrana no provee mucha protección contra los antibióticos pues estos en su mayoría son hidrofóbicos y pueden difundirse a través de la misma (Nikaido 1994). Las bacterias Gram

negativas en cambio poseen una pared celular compuesta por varias capas de peptidoglicano, lipopolisacáridos y proteína. Poseen entonces una membrana exterior que provee una barrera efectiva contra moléculas pequeñas. Esta pared externa está compuesta por lipopolisacáridos (LPS) haciéndola menos fluida que la tradicional membrana de fosfolípidos.

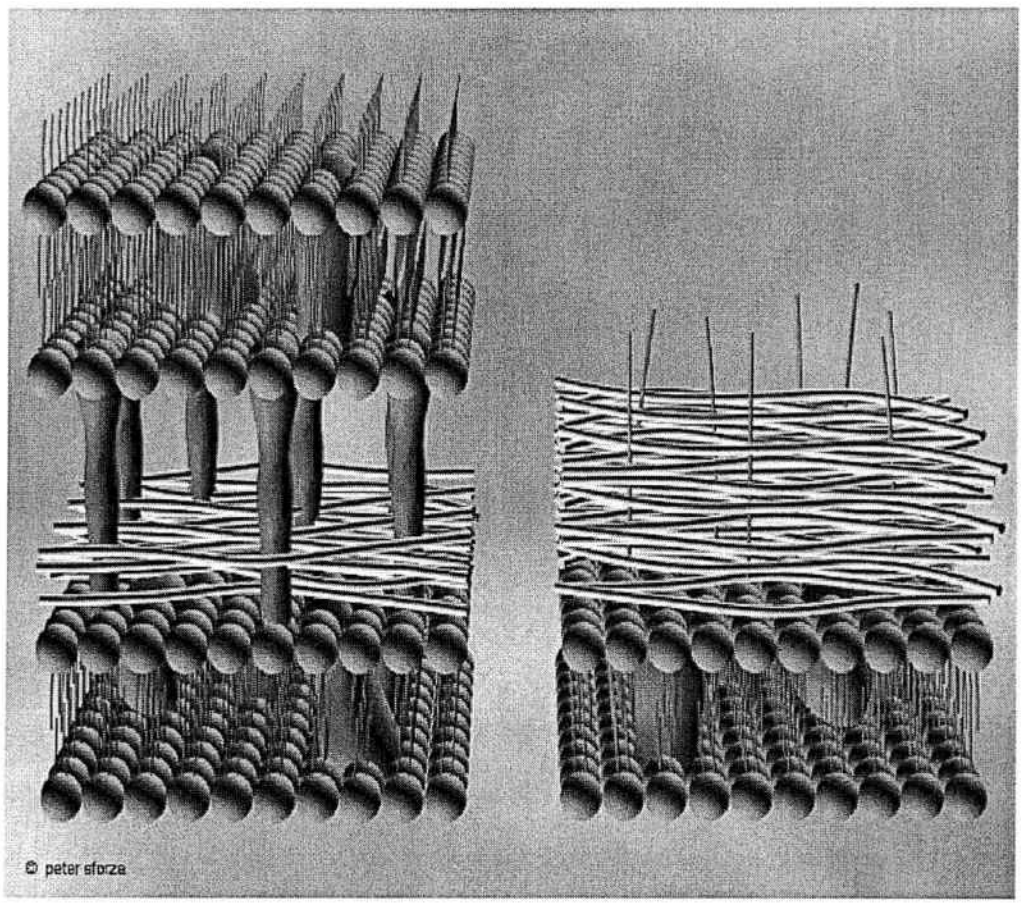

Figura 2.- Imagen comparativa de la estructura de la pared celular de una bacteria Gram negativa (izquierda) y una Gram positiva (derecha). Amarillo, peptidoglicano; violeta, proteína; verde, ácido teicóico; marrón, fosfolípido; naranja, lipopolisacárido.
Tomado de www.apsnet.org/education/K-12PlantPathways/TeachersGuide/Activities/DNA

La pared externa de los Gram negativos, posee porinas que permiten la entrada de nutrientes a la célula. Como resultado de la pérdida de porinas, la membrana celular externa suele tornarse aún más impermeable al paso de moléculas hidrofílicas, confiriendo así una mayor resistencia a la bacteria. Se trata por lo tanto de un mecanismo que suele ser relativamente inespecífico. Existen además especies bacterianas, como es el caso de

Acinetobacter baumannii, que tienen constitutivamente una membrana más impermeable, lo cual les hace intrínsecamente resistentes a muchos antimicrobianos.

1.4.5 Expulsión activa del antimicrobiano

A diferencia de la impermeabilidad de la membrana, que es un mecanismo pasivo, la expulsión del antimicrobiano es un proceso activo, es decir que requiere del consumo de energía por parte de la célula. En él están involucradas las llamadas bombas de expulsión. Estas estructuras están compuestas por tres o más unidades que, acopladas a la membrana celular, expulsan el antimicrobiano a medida que va entrando.

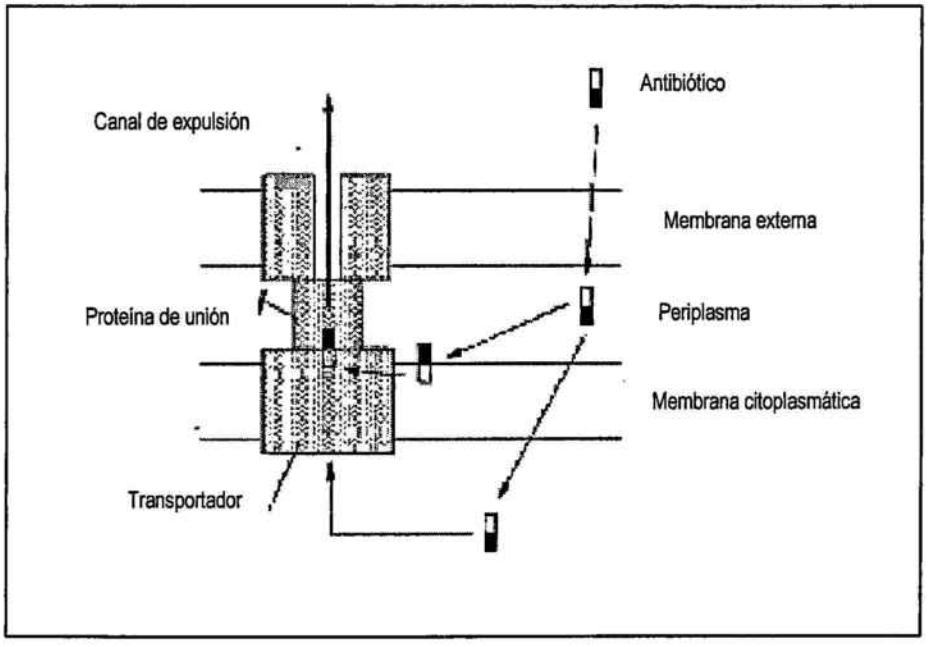

Figura 3.- Sistema de expulsión activa. Está formado por tres proteínas, un transportador situado en la membrana citoplasmática, una estructura similar a una porina situado en la membrana externa que actúa como canal de expulsión y una lipoproteína hidrofílica de unión entre las otras dos. (Modificado de Nikaido 1994)

El ejemplo más típico de resistencia por expulsión activa es la de la resistencia a la tetraciclina, aunque también se conocen bombas que expulsan antibióticos tan diferentes como el cloranfenicol, los betalactámicos o la eritromicina. La Tabla 5 muestra los sistemas de

expulsión más conocidos, los microorganismos en los que han sido descritos y los antibióticos afectados.

Tabla 5.- Principales sistemas de expulsión activa descritos hasta el momento.
(Tomado de Cantón et al., 1999.)

Sistema	**Transportador**	**Microorganismo**	**Fenotipo de resistencia**
MFS (major facilitator superfamily)	Nor A Qac A Tet L Bmr OtrB Erm B	*Staphylococcus aureus* *Staphylococcus aureus* *Bacillus subtilis* *Bacillus subtilis* *Streptomyces rimosus* *Escherichia coli*	Cloranfenicol, quinolonas, puromicina Amonios cuaternarios Tetraciclinas Cloranfenicol, quinolonas, puromicina, bromuro de etidio Oxitetraciclinas Acido nalidíxico
RND (resistance nodulation division)	Mex B Acr B	*Pseudomonas aeruginosa* *Escherichia coli*	Cloranfenicol, quinolonas, tetraciclinas, betalactámicos Novobiocina, eritromicina, ácido fusídico
Smr (Small multidrug resistance)	Qac E	*Klebsiella aerogenes*	Amonios cuaternarios
ABC (ATP-binding cassette)	Msr A	Estafilococos coagulasa (-)	Macrólidos

1.4.6 Transferencia horizontal de los genes.

La resistencia permanente puede surgir, bien por la mutación de un gen o por la adquisición de ADN del exterior. Existen tres formas mediante las cuales se puede dar el intercambio genético entre bacterias: conjugación transformación, y/o transducción.

La *conjugación* es el paso del material genético entre dos bacterias, una donante y otra receptora, por medio de un *pili* sexual que la primera genera (Cisterna Cancer, 1999). Fue inicialmente descrita en *E.coli* por Joshua Lederberg y Edward Tatum en 1946. De los tres mecanismos de transferencia de material genético, este es tal vez el más importante, al darse directamente entre dos células vivas, y ha sido el mayor responsable de la dispersión de plásmidos con genes de resistencia (Sefton, 2002).

La *transformación* observada por primera vez en 1928 en *Streptococcus pneumoniae* por Frederick Griffith, se da cuando la bacteria receptora adquiere ADN exógeno directamente del medio ambiente después de que otra bacteria lo ha liberado al exterior. Una vez dentro de la bacteria receptora, esta lo incorpora dentro de su genoma. Se ha demostrado el paso de genes de resistencia mediante transformación (ej. resistencia a la estreptomicina en *S.pneumoniae*). Sin embargo, no todas las bacterias son *competentes*, es decir, no todas tienen la capacidad de incorporar ADN exógeno mediante transformación.

Finalmente la *transducción* fue descubierta incialmente en *Salmonella typhimurium* en 1951 por Joshua Lederberg y Norton Zinder cuando intentaban reproducir el proceso de conjugación que se había descrito recientemente para *E.coli*. La transducción se da mediada por ciertos bacteriófagos que se ocupan de transferir el material genético entre dos bacterias compatibles. El proceso se da gracias a que de alguna manera, durante el ciclo lítico, los fagos se quedan con material genético de la bacteria que han lisado y posteriormente lo introducen dentro de la siguiente célula que infectan.

Existen dos tipos de elementos conjugativos que pueden intercambiarse entre bacterias no relacionadas (ej. Gram positivos y Gram negativos): se trata de los plásmidos y los transposones conjugativos:

- Los plásmidos son moléculas de ADN extra cromosómico, de doble cadena, helicoidal y circular. Suelen contener entre 5 y 100 genes y aunque no son esenciales para el crecimiento bacteriano, pueden aportar una vexntaja añadida a la bacteria. Es el caso de los plásmidos R, encontrados en bacterias Gram negativas, que codifican para un pilus conjugativo y multiresistencia a los antibióticos.

- Los transposones o "genes saltarines" se caracterizan por tener una serie de genes - entre ellos es común encontrar genes de resistencia – flanqueados a ambos lados por uno que codifica para una transposasa. La transposasa es

una enzima que cataliza el corte y sellamiento del ADN, permitiéndole al transposón moverse de un lugar a otro. Los transposones pueden encontrarse bien dentro del núcleo bacteriano o dentro de plásmidos.

Los plásmidos y transposones a menudo contienen un tercer elemento genético: el integrón. Se trata de una estructura que contiene uno o más genes – llamados *cassetes* - flanqueados por regiones conservadas. En los integrones de Clase 1, los más estudiados, en la region conservada 5' se codifica para un promotor que incrementa la expresión de los genes y una integrasa (*int*) responsable de la escisión e integración de los *cassetes* (Figura 4). De esta manera, los *cassetes* se convierten en elementos transponibles, moviéndose en bloque, a pesar de no codificar genes involucrados en su propia movilidad.

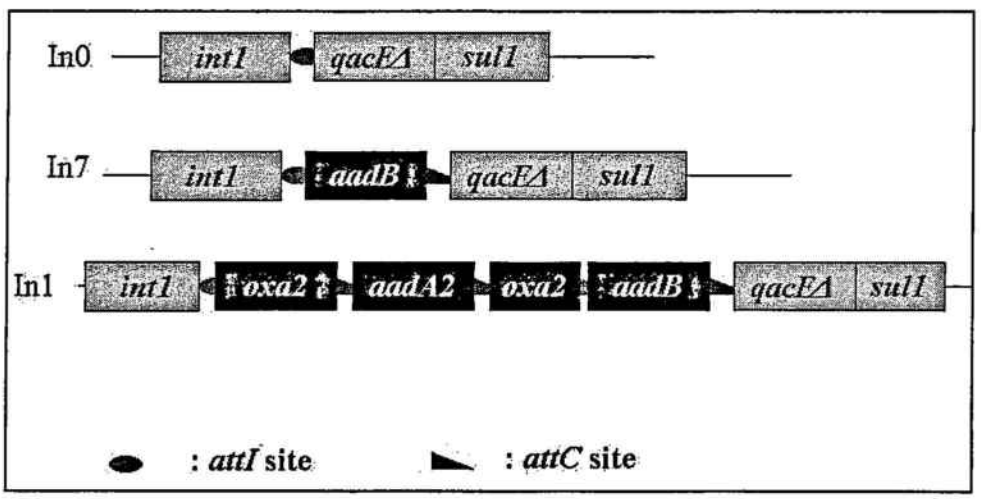

Figura 4.- Estructura y ejemplos de integrones de Clase 1. In0: Integrón base: en el extremo 5' de la región conservada se encuentran el promotor , la integrasa (*int 1)* y el sitio de recombinación *attI*, mientras que el extremo 3' alberga un gen de resistencia al bromuro de etidio (*qacEΔ*), otro de resistencia a la sulfamidas *sul1* así como un ORF de función desconocida. In7 e In1, dos ejemplos de integrones con *cassettes* correspondientes a genes de resistencia. Un *cassette* está compuesto por el gen sin promotor y un lugar de recombinación *attC*.

1.5 *Acinetobacter baumannii*

1.5.1 Características microbiológicas

El género Acinetobacter está compuesto por bacilos Gram negativos, no fermentadores de la glucosa, catalasa positivos, oxidasa negativos y aerobios estrictos. Inmóviles, a menudo se les encuentra dispuestos en parejas. Su historial taxonómico está lleno de cambios, habiéndoseles denominado con al menos quince nombres genéricos diferentes (Bergogne-Berenzin & Towner, 1996). La taxonomía actual se debe a Bouvet y Grimont (1986) dentro de la cual se distinguen hoy en día 20 genoespecies. De ellas, *Acinetobacter baumanii* es la más aislada y aquella con implicaciones clínicas más relevantes. Sin embargo, existen otras especies del género capaces de producir infección. De *Acinetobacter baumannii* se han definido 19 biotipos, de los cuales el 1,2,6 y el 9 son los más aislados en los hospitales (Bouvet 1987)

En el laboratorio crecen en la mayoría de los medios de cultivo. Sin embargo para su aislamiento a partir de muestras clínicas se recomienda el uso de medios selectivos. El medio LAM (Leeds Acinetobacter Médium) suele dar buenos resultados, puesto que permite diferenciar entre los tres tipos de microorganismos que crecen en él: *Acinetobacter, Pseudomonas* y *Stenotrophomonas* (Jawad et al.,1994). Por otro lado, para el crecimiento rutinario y el mantenimiento de las cepas en el laboratorio, el agar MacConkey es una buena opción.

La diferenciación entre la especie *A.baumannii* y las otras especies que conforman el llamado complejo *A.calcoaceticus- A.baumannii* (*A.calcoaceticus, A.baumannii* y las genoespecies 1 y 13) que comparten características bioquímicas similares, puede ser difícil y prestarse para confusiones en el laboratorio. Existen métodos comerciales, el más conocido siendo el API 20NE, pero no son totalmente confiables. Tal vez los métodos más sensibles para la diferenciación de las genoespecies son los moleculares, principalmente la hibridación DNA-DNA en sus diferentes variantes (para una revisión ver a Bergogne-Berenzin & Towner, 1996) o más recientemente una PCR-RFLP del gen 16S (Gerner-Smidt, 1992). *Acinetobacter baumannii* crece bien a 37ºC, pero se diferencia de la mayoría de las demás genoespecies (excepto de *A.calcoacéticus* y *A.lwoffii*), en que también es capaz de crecer a 44ºC. Recientemente se han

descrito también cepas de *Acinetobacter* genoespecie 3 capaces de crecer a esta temperatura (Ribera et al., 2004)

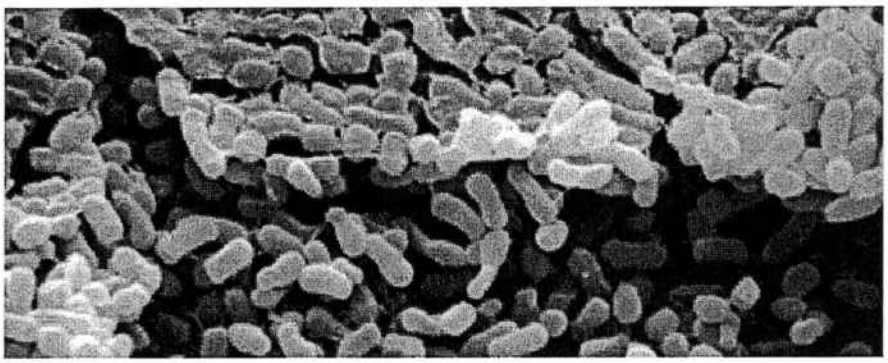

Figura 5.- .- Foto por microscopía de barrido (SEM – scanning electrón micrograph) de *Acinetobacter baumannii.* Tomado de http://mic.sgmjournals.org/content/vol149/issue12/cover.shtml

1.5.2 Patogenicidad

Se han descrito varios factores que predisponen o facilitan la infección por *A.baumannii.* Entre ellos los más relevantes son el haber sido sometido a una cirugía, el tener una enfermedad grave de base (cáncer, quemaduras, inmunosupresión) o edades extremas (se consideran poblaciones de riesgo los ancianos y los neonatos – especialmente los prematuros).

Dada su alta capacidad de supervivencia y su multiresistencia, es de agradecer que la virulencia de *A.baumannii* sea relativamente baja (Bergongne-Berenzin, 1997) Sin embargo, se han determinado ciertas características que incrementan la virulencia de aquellas cepas que las poseen (Bergongne-Berenzin & Towner, 1996):

1) La presencia de un polisacárido capsular. Dicho polisacárido hace que la superficie de la bacteria sea más hidrofílica.
2) La capacidad de adhesión a las células epiteliales humanas, gracias a la presencia de fimbrias o del mismo polisacárido capsular.
3) La producción de ciertas enzimas que dañan los lípidos tisulares.
4) El papel potencialmente tóxico del componente lipopolisacárido de la pared celular, así como la presencia del lípido A.

Adicionalmente se ha demostrado en modelos animales, que en infecciones mixtas con otras bacterias, *Acinetobacter* spp. se muestra más virulento que en infecciones de especies del género por sí solas (Obana,1986). Otro factor de virulencia del género Acinetobacter parece radicar en su capacidad para captar hierro de su huésped, específicamente por la producción de sideróforos y proteínas de membrana externa receptoras del hierro.

1.5.3 Epidemiología.

La información que se tiene sobre *Acinetobacter baumannii* no se remonta a más de dos décadas, debido a la complicada historia taxonómica de los Acinetobacter. Se trata de un género ampliamente distribuido en la naturaleza encontrándose en el suelo o en el ambiente. Diversas especies de *Acinetobacter* son además microbiota normal de la epidermis y mucosas humanas. Sin embargo, en un estudio con pacientes y controles llevado a cabo en un hospital alemán, (Seifert et at al) se encontró que si bien un 75% de los pacientes y un 42,5% de los controles estaban colonizados por *Acinetobacter* las especies prevalentes eran *A.lwoffii, A.johnsonii* y el grupo 3. mientras que *A.baumannii* solo se aisló en tres sujetos. Esta observación parece contradecir la creencia común de que la frecuencia de las infecciones nosocomiales por *A.baumannii* son debidas simplemente a que se trata de un microorganismo frecuente en el medio y habitante natural de la piel humana.

Aunque *A.baumannii* no se considera un habitante habitual del tracto digestivo de sujetos sanos (Grehn et al. 1978), en pacientes graves la microbiota intestinal puede verse modificada y bajo ciertas condiciones estar sujetos a la colonización de patógenos exógenos responsables de epidemias nosocomiales. En un estudio realizado en el Hospital de Bellvitge, Corbella y colaboradores (1976) encontraron que tras 30 días de ingreso en una UCI, la probabilidad de tener heces limpias de *A.baumannii* era menor del 25%. Contraria a la alta colonización intestinal de los pacientes, se halló una baja prevalencia de *A.baumannii* en cultivos ambientales, apuntando a los pacientes como el principal reservorio epidemiológico. Observaron también que la colonización de los pacientes se daba en etapas muy tempranas,

con un 25% de los pacientes con cultivo positivo tras 48 horas de ingreso y un 75% después de una semana. La relevancia de la contaminación cruzada con el personal médico como vehículo también fue demostrada con los cultivos positivos de los guantes de latex.

A. baumannii es un patógeno oportunista, y gracias a su capacidad para crecer en medios muy pobres, por ser capaz de utilizar una gran variedad de fuentes de carbono a través de vías metabólicas diferentes, se le puede encontrar creciendo en los más diversos medios, animados o inanimados. Así pues se ha aislado *A.baumannii* de objetos tan dispares como sábanas, ventiladores, colchones o pomos de puertas. Si a ello se le suma el hecho de que se trata de microorganismos con una habilidad asombrosa para desarrollar resistencias a los antimicrobianos más potentes (Towner,1997) no es de extrañar que se hayan convertido en una de las principales especies involucradas en epidemias nosocomiales. El uso extensivo de antimicrobianos de espectro ampliado en los hospitales, además, ha terminado por eliminar otras bacterias que competían por el mismo nicho ecológico, favoreciendo el establecimiento de los Acinetobacter multirresistentes. También ha contribuido a su establecimiento como patógeno nosocomial, y especialmente de UCIs, el uso de técnicas diagnósticas y terapéuticas cada vez más invasivas.

Se ha aislado *A.baumannii* de diversos tipos de muestras clínicas. En general, se puede decir que su distribución no varía de las de otras infecciones nosocomiales por bacterias Gram negativas (Towner,1997). El principal lugar de infección suelen ser las vías respiratorias bajas, especialmente como causante de neumonías asociadas al uso de ventiladores. También tienen una alta incidencia en las infecciones de vías urinarias. Otros patologías incluyen meningitis, infecciones de heridas o quemaduras, abscesos, septicemias, endocarditis y peritonitis. La transmisión de un paciente colonizado o infectado a otro suele darse fácilmente y por los medios más diversos, incluyendo la dispersión a través del personal sanitario o por uso de material (clínico o no) contaminado. Por su alta resistencia, se trata de bacterias capaces de sobrevivir en el ambiente durante muchos días sin apenas alimento.

En el hemisferio norte se ha observado una estacionalidad en las infecciones por *Acinetobacter*, siendo más comunes en los meses de verano (MacDonald et al. 1999). Se ha sugerido que este hecho puede deberse a que la humedad estacional favorece, no solo el crecimiento de las bacterias, sino su diseminación, que se ha probado puede darse, entre pacientes de un mismo cuarto vía aerosoles o incluso a través de sistemas de aire acondicionado de donde ya ha sido aislado (MacDonald et al. 1998).

La incidencia de infecciones nosocomiales por *Acinetobacter* spp. se ha calculado, según estudios recientes, en un 0,3% de las infecciones nosocomiales endémicas en pacientes graves y en un 1% del total de las bacteriemias hospitalarias (Struelens et al., 1993) Estas cifras, por supuesto, pueden ser mayores en centros en los que existen problemas serios de contaminación y de control de la trasmisión entre enfermos.

1.5.4 Tratamiento

La rápida aparición de resistencias entre las cepas clínicas de *Acinetobacter* ha hecho que su tratamiento sea complicado. La mayoría de las cepas son hoy en día altamente resistentes a los antimicrobianos más tradicionalmente utilizados en clínica, incluyendo las amino y ureidopenicilinas, las cefalosporinas (incluso las de espectro ampliado), la mayoría de los aminoglicósidos, el cloranfenicol y las tetraciclinas.

Las cefalosporinas de tercera generación, y las fluoroquinolonas aún muestran actividad. Heinemann y colobaradores hicieron un estudio comparativo de la acividad de siete fluoroquinolonas diferentes (seis nuevas vs. Ciprofloxacino) en cepas epidemiológicamente estudiadas de *A.baumannii* . Encontraron que las nuevas fluroquinolonas presentan una buena actividad, siendo la mayor la del clinafloxacino y la menor la de la ciprofloxacina. En el trabajo se encuentran niveles de resistencia a las fluroquinolonas mucho menores a los reportados por otros grupos y se concluye que probablemente se debe a que las cepas utilizadas eran en su mayoría epidemiológicamente no relacionadas y que en trabajos que analizan brotes, la inclusión de cepas epidémicas sobreestima los niveles de resistencia. Sin embargo, tratándose

de una bacteria que normalmente se presenta en forma de brotes multirresistentes, el que visto desde esta perspectiva parezca menos grave no es en sí ningún consuelo.

La mejor actividad contra *A.baumannii* la tienen los carbapenems. Sin embargo ya en 1985 se reportó un primer caso de infección por una cepa imipenem resistente aislado de un hemocultivo en el Royal Infirmary de Edinburgo (Bergogne-Berenzin, 1996). Desde entonces se han detectado otras cepas resistentes, aunque afortunadamente aún continúan siendo relativamente raras y por lo tanto el imipenem continúa siendo tratamiento de primera línea en la infección por *A.baumannii.* La resistencia al carbapenem puede deberse entre otras cosas a la presencia de una carbapenemasa. Puesto que se ha demostrado que dichas carbapenemasas están codificadas en plásmidos (Paton et al., 1993), tal vez sea solo cuestión de tiempo que la resistencia se propague. Se sabe, además, que las cepas imipenem R parecen presentar resistencia cruzada con las penicilinas, las cefalosporinas, los aminoglicósidos y las quinolonas (Afzal-Shah & Livermore, 1998).

Con el aumento de resistencias a moléculas específicas, el uso de terapias combinadas también ha dado buenos resultados en el tratamiento de infecciones multirresistentes. En un estudio piloto se evaluaron nueve combinaciones diferentes frente a 69 cepas nosocomiales de *A.baumannii,* (Marques et al., 1997) y se halló que en los casos en los que imipenem no puede utilizarse o está contraindicado, la mejor opción es la de ampicilina+sulbactam, bien sea solo o en combinación con aminoglicósidos. *Acinetobacter* es uno de los pocos patógenos frente a los cuales el inhibidor de ß-lactamasas, sulbactam, tiene actividad bactericida. Esto parece deberse a la actividad intrínseca del sulbactam, el cual no solo es inhibidor suicida (que inactiva la enzima modificandola covalentemente y en el proceso el inhibidor se autodestruye) sino que en el caso específico del complejo *A.calcoaceticus-A.baumannii* tiene la capacidad de unirse a sus PBP (Douboyas et al.,1994).

1.5.5 Mecanismos de resistencia a los agentes antimicrobianos

Si analizamos el caso de *A.baumannii* desde los diferentes factores que determinan la adquisición de la multirresistencia, hayamos que todos ellos, en mayor o menor medida, influyen.

- La resistencia intrínseca de la bacteria. *A.baumannii* es un microorganismo con altos niveles de resistencia intrínseca, por diferentes razones. Tanto en número como en tamaño, sus porinas son mucho menores que las de otras bacterias. Como resultado se ha observado por ejemplo, que para las penicilinas y cefalosporinas, la permeabilidad en este microorganismo es solo el 1% de la observada en *E.coli* e incluso un poco menor que para *P.aeruginosa* (J.Vila, 1998). Microorganismos con una altísima resistencia intrínseca se la deben en parte a la presencia de bombas de expulsión. Aunque en *A.baumannii* no se ha descrito aún ninguna, es probable que las tenga y que juegue un papel importante.

- La adquisición de elementos genéticos móviles que codifican para genes de resistencia. Tanto los plásmidos, como los transposones (y los integrones movilizados por los dos anteriores) han sido ampliamente descritos en *A.baumannii.* Se conoce además que es un microorganismo con facilidad para adquirir plásmidos por conjugación (aunque con dificultad para cederlos). Más aún, una de sus características es la de la transformación natural, es decir, la capacidad para adquirir material genético que se encuentra libre en el medio externo, sin necesidad de ser sometido a ningún tipo de estrés físico-químico.

- La capacidad de sobrevivir en reservorios humanos y ambienta les en los que los genes de resistencia se puedan transferir. El reservorio es el nicho ecológico en el que persiste un agente infeccioso, en el caso *A.baumannii* serían la piel, la cavidad oral, el tracto respiratorio y el tracto intestinal. Este último suele ser el reservorio

de las cepas más multirresistentes. En todos los nichos está compartiendo con otros microorganismos con quienes puede compartir material genético.

A nivel de mecanismos moleculares específicos para los diferentes antibióticos, es importante recalcar aquellos para los β-lactámicos, los aminoglucósidos y las quinolonas.

En la actualidad, *A.baumannii* es resistente a la mayoría de los betalactámicos y en especial a las penicilinas y las cefalosporinas (J.Vila et al.1999), habiéndose descrito en este microorganismo todos los mecanismos de resistencia hasta ahora conocidos para este grupo de antimicrobianos: producción de betalactamasas, modificación de las proteínas fijadoras de la penicilina y disminución de la penetración del antibiótico (Towner, 1997). Así pues, la resistencia a la ampicilina, las carboxipenicilinas y las uridopenicilinas normalmente están relacionadas con la presencia de betalactamasas tipo TEM-1, TEM-2 o del tipo OXA. Sin embargo, la resistencia no siempre es atribuible a la sola presencia de una de estas enzimas, describiéndose a menudo la presencia de más de una de ellas. En cuanto a la resistencia a imipenem, se han descrito diversos mecanismos:

- disminución de la permeabilidad de la membrana y por lo tanto menor acumulación del antibiótico intracelularmente
- alteración de una proteína fijadora de manera que disminuye la afinidad de esta por el imipenem
- producción de carbapenemasas

El fenotipo de resistencia a las cefalosporinas parece ser el resultado de la presencia de más de un mecanismo. En el caso de *A.calcoaceticus*, la resistencia a las cefalosporinas es intrínseca y atribuible a la presencia de un número reducido de porinas que además son más pequeñas de lo habitual. Aunque bien podría ser este el caso para *A.baumannii*, también es cierto que se ha observado la presencia de betalactamasas cromosómicas no inducibles (tipo ACE) con actividad cefalosporinasa. Aunque algunas tienen poca actividad contra las penicilinas,

ninguna la tiene contra el aztreonam o las cefalosporinas de tercera generación (Towner, 1997).

La resistencia a aminoglicósidos en bacterias se debe a uno de tres mecanismos: i) alteración de la diana ribosomal ii) reducción de la entrada del antibiótico y/o iii) modificación enzimática del antimicrobiano. La primera de ellas no tiene significancia en terapéutica, pues solo afecta la estreptomicina y la espectomicina. La segunda confiere resistencia a todos los aminoglicósidos y es común en *A.baumannii*. Sin embargo es la tercera la más importante para esta especie y la responsable de la mayoría de los fenotipos resistentes. Existen tres clases de enzimas modificadoras de aminoglicósidos: las O-fosfotransferasas (APH), las O-nucleotidiltransferasas (ANT) y las N-acetiltransferasas (AAC). Un número denota la posición en que se realiza la modificación del sustrato y existe una subclasificación de las enzimas dependiendo de los aminoglicósidos que modifiquen (Fluit et al. 2001). Hay una cierta especificidad en la distribución de estas enzimas entre bacterias Gram positivos y Gram negativos. Y aún dentro de una misma especie, parece haber diferencias geográficas que impiden extrapolar los resultados de un estudio a otras situaciones (Ploy et al., 1994; Seward et al., 1998; Tran Van Nieu, 1992). Este es el caso para *A.baumannii*, habiéndose realizado varios estudios con resultados contrastantes. Se puede decir que se han encontrado genes que codifican para enzimas de las tres clases, y que se suelen encontrar en diferentes combinaciones, aunque en términos generales es más común la fosforilación que la acetilación (Towner, 1997). Este tipo de resistencia puede estar mediada por plásmidos o transposones, los cuales juegan un papel esencial en su diseminación. Este es normalmente el caso dentro del género que nos ocupa, siendo más común en *Acinetobacter* la resistencia a aminoglicósidos mediada por plásmidos que la cromosómica.

Hasta hace menos de quince años las fluroquinolonas eran una excelente elección frente a Acinetobacter. Sin embargo, el aumento de resistencias ha sido rápido en los últimos años. El mecanismo responsable para dicho fenotipo es principalmente la mutación de los

genes diana: *gyr*A y *par*C, y *A.baumannii* no es una excepción (Vila et al., 1995 ; Vila et al., 1997).

1.6 *Shigella* spp.

1.6.1 Características microbiológicas

El género Shigella pertenece a la familia de Enterobacteriaceae. Se trata de bacilos Gram negativos, anaerobios facultativos, no móviles. Toman su nombre del científico japonés, llamado *Shiga*, que las descubrió hace poco más de un siglo. Están íntimamente relacionadas con *Escherichia coli* en lo que respecta a la patogenicidad, a la fisiología y la serología. De hecho, es motivo de discusión constante desde mediados del siglo pasado, el si ambos géneros deberían agruparse bajo uno solo (Rolland et al., 1998 Pupo et al., 2000, Pupo et al., 1997, Karaolis et al., 1994, Johnsons, 2000, Ochman et al. 1983). El género contiene cuatro serogrupos o especies, que a su vez tienen múltiples serotipos. *S.flexneri* (con quince serotipos, de entre los cuales el 2a es reconocido como el más virulento); S.*sonnei* (serotipo único); *S.dysenteriae* (12 serotipos) y *S.boydii* (18 serotipos).

Su aislamiento a partir de muestras biológicas se realiza sembrando estas en medio MacConkey. En el crecen como colonias lactosa negativas y se facilita el crecimiento de cepas que en medios más selectivos pueden ser inhibidas. Este mismo medio se utiliza para el mantenimiento de las cepas en el laboratorio. Para su identificación, pueden utlilizarse, bien métodos comerciales (tipo API) o una serie que incluya indol, glucosa, lactosa, urea, citrato, kliger y fenilalanina. El serogrupo se determina por medio de aglutinación con antisueros específicos.

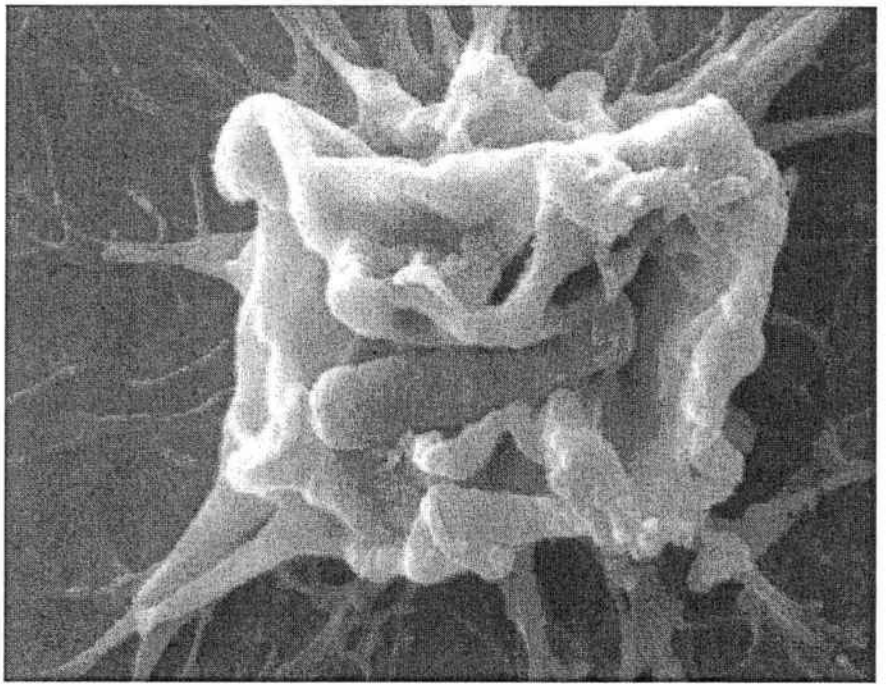

Figura 6.- Foto por microscopía de barrido (SEM – scanning electrón micrograph) que muestra una *Shigella flexneri* entrando en una célula HeLa. La bacteria interactúa con la superficie celular de la célula huésped, inyectando una invasina que actúa orquestrando un re-arreglo de la actina de la célula huésped que resulta en la formación de seudópodos que "engullen" la bacteria.
(Tomado de http://users.path.ox.ac.uk)

1.6.2 Patogenicidad

Las Shigellas son microorganismos altamente infectivos. Un inóculo de tan solo 10 células bacterianas es suficiente para causar infección (Sansoneti et al., 1999). Ello explica porqué el contagio puede darse no solo a través del consumo de aguas contaminadas con heces o comida lavada con dichas aguas sino de persona a persona. En general, pues, la ruta infectiva comienza en la boca (via fecal-oral). A diferencia de otros patógenos intestinales, las Shigellas suelen sobrevivir fácilmente al pH bajo del estómago, de manera que la mayor parte de las bacterias ingeridas llegan aún siendo viables hasta el intestino.

El proceso de invasión celular de las Shigellas ha sido ampliamente estudiado especialmente utilizando el modelo de las células HeLa (ver Figura 6). Las cepas virulentas de Shigella, más que realizar un proceso activo de "invasión" de las células huésped, desencadenan otro por medio del cual inducen su fagocitosis. Dicha fagocitosis inducida está

relacionada con una reorganización de la actina de la célula hospedadora, inducida por la bacteria al adherirse a esta, y que resulta en la formación de seudópodos que engullen la bacteria dentro de una vesícula fagocítica. Una vez dentro, la Shigella lisa la vacuola y es así liberada al citoplasma celular.

Una vez dentro del citoplasma, la bacteria utiliza diferentes proteínas del citoesqueleto de la célula huésped, principalmente la actina, para generar una cola. La continua polimerización de la actina da suficiente impulso a la bacteria para moverse a través del citoplasma celular y hasta las células vecinas (Goldberg, 2001). El paso de una célula a otra se realiza cuando la bacteria induce de nuevo la fagocitosis al empujar la membrana plasmática de su próxima huésped. A partir de este momento comienza de nuevo todo el proceso. La Figura 7 resume el proceso de invasión y dispersión celular de *Shigella*.

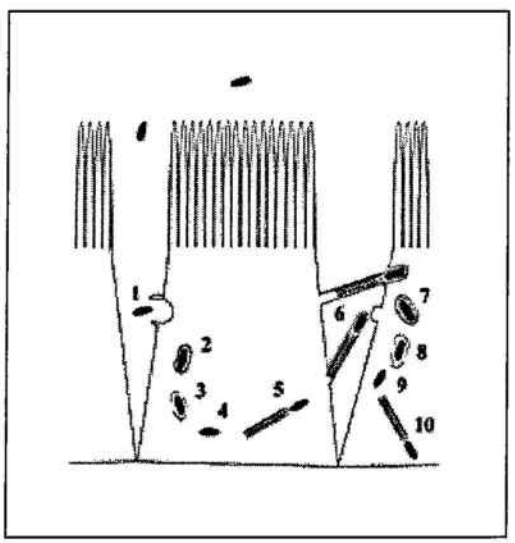

Figura 7.- Patogénesis de *Shigella* spp.
(1) La bacteria entra en la célula del mamífero por fagocitosis inducida. Una vez dentro (2), lisa la vacuola de fagocitosis (3) quedando libre en el citoplasma de la célula huésped (4)- La Shigella ensambla una cola de actina desde un polo (5). Este ensamblaje la impulsa a través del citoplasma y también le permite formar una protuberancia a partir de la superficie celular (6). Cuando dicha protuberancia hace contacto con la membrana de la célula adyacente, es incorporada junto con la bacteria (7). La bacteria está entonces en una vacuola de doble membrana, que procede a lisar (8) para quedar nuevamente libre en el citoplasma de la célula adyacente. El proceso comienza de nuevo al generar una nueva cola de actina que la ayude a moverse a través del mismo(10)
(Tomado de Goldberg, 2001)

La movilidad basada en la actina que presentan las Shigellas, son parte importante de su virulencia. Diferentes factores de virulencia, la mayoría de ellos codificados en plásmidos de gran tamaño, son los responsables de articular todo el proceso de adherencia e invasión de la célula huésped. Los más importantes están resumidos en la Tabla 6.

Son dos las enterotoxinas que parecen ser las responsables de la presentación líquida de la diarrea en infecciones por Shigella o algunos tipos de *E.coli.* Se trata de ShET1 y ShET2, ambas asociadas con la acumulación de líquido en el íleo de conejos. Mientras que ShET2 es plasmídica y se detecta en el 80% de las cuatro especies de Shigella así como en algunos tipos de *E.coli,* ShET1 está codificada a nivel cromosómico y se encuentra básicamente en *S.flexneri* 2a. Sin embargo, en un estudio sobre factores de virulencia de *E.coli* enteroagregativa (ECEA) realizado por Vila y colaboradores (2000), se observó la presencia del gen *set* (que codifica para ShET-1) tanto en cepas de casos como de controles, aunque su expresión solo se demostró en el grupo de los casos.

Tabla 6.- Principales factores de virulencia de *Shigella* spp. involucrados en la invasión celular

Factor de virulencia	Localización	Posible papel en la invasión
IpaD [1]	Plásmido	Adherencia
Ipa B, C	Plásmido	Invasión / lisis de la vesícula fagocítica
Proteínas Mxi [2]		Excreción de IpaA-D
Ics A [3]	Plásmido	Polimerización de la actina
Ics B	Plásmido	Lisis de membrana plasmática / dispersión intercelular
Olm		Movimiento a lo largo de los filamentos de actina
Vac C	Cromosoma	Regulador expresión *ipa*
Vac B	Cromosoma	Control post-transcripcional de *ipa* e *icsA*
KcpA	Cromosoma	Regulador expresión *icsA*

1.-Ipa : Invasion plasmid antigens 2.- Mxi :membrane expression of invasion 3.- Ics :intercellular spread

Una tercera toxina, expresada por *S.dysenteriae* serotipo 1, es la extremadamente potente Shiga-toxina. Esta citotoxina inhibe la expresión protéica de la célula huésped y experimentalmente se ha demostrado que tiene efecto enterotoxigénico. Su papel exacto en la virulencia de Shigella no está clarificado, pero se asocia su presencia con el síndrome urémico-hemolítico.

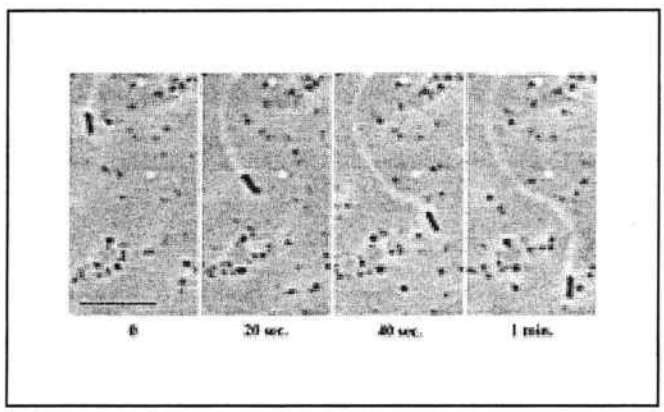

Figura 8. – Movimiento de una célula de Shigella a lo largo de un minuto, impulsada por la generación de una cola de actina.
(Tomado de Goldberg, 2001)

1.6.3 Cuadro clínico

El conjunto de síntomas causados por una shigellosis se conoce como disentería. Hasta mediados del siglo pasado, se atribuía solamente a la infección por especies de Shigella, pero desde entonces se sabe también que hay especies virulentas de *Escherichia coli* capaces de producir los mismos síntomas.

La infección por Shigella tiene dos presentaciones clínicas básicas: 1) bien una diarrea acuosa acompañada de vómitos que resultan en una deshidratación de leve a moderada o 2) una diarrea acompañada de sangre y moco, causante de dolores abdominales y tenesmo. La presencia de fiebre en los primeros días de sintomatología no es infrecuente, mientras que en un 25% de los pacientes menores de 4 años, se reportan convulsiones.

En general, se puede afirmar que la shigelosis es una infección aguda, y que la sintomatología suele aparecer entre 24 y 48 horas después del contagio. Aparentemente, el tamaño del inóculo está directamente relacionado con el tiempo que tardan en aparecer los primeros síntoma. La síntomatología suele tener una duración, en adultos sanos no tratados, de alrededor de 7 días, aunque puede variar entre 1 – 30, pudiéndose aislar bacterias de las heces de pacientes hasta 3 – 4 semanas post infección (Carroll et al., 2000) En niños o adultos malnutridos o inmunocomprometidos, la infección puede llegar a ser fatal. En un porcentaje que varía entre un 3%-50% (dependiendo de la virulencia de la cepa infectante y del estado nutricional e inmune del paciente), la infección inicial puede evolucionar en bacteremias, complicaciones neurológicas o afectación renal (síndrome urémico-hemolítico).

Tabla 3.- Características clínicas de infección por *Shigella* spp. Basado en datos del hospital de Dacca, Bangladesh [1]

Síntoma	Porcentaje aproximado de pacientes		
	S.sonnei	*S.flexneri*	*S.dysenteriae*
Diarrea acuosa	75	30	30
Heces mucosas	50	75	95
Heces sanguinolentas	10	50	80
Dolor abdominal	50	70	85
Vomito	60	30	40
Fiebre	5	10	10

[1]Tomado del capítulo sobre *Shigella* spp. por Tomas L. Hale y Gerald T.Keusch en http://gsbs.utmb.edu/microbook/ch022.htm

1.6.4 Epidemiología.

Los humanos son el principal reservorio de las Shigellas, aunque algunos primates en cautiverio pueden también llegar a infectarse. La shigellosis es endémica en los PBR, en los cuales las condiciones higiénicas y sanitarias son inadecuadas, facilitando el mantenimiento y la dispersión de estos bacilos. *S.flexneri* es la especie más prevalente en países en áreas endémicas, en donde se le atribuyen alrededor del 60% de los aislamientos positivos (Kottlof et

al.). *S.sonnei* (serotipo único) es la especie más comúnmente aislada en países desarrollados (aprox. 77% de los aislamientos) (Kottlof, 1999) y suele infectar sobretodo a lactantes y niños en guarderías o ancianos viviendo en residencias. *S.dysenteriae* es la especie más comúnmente encontrada en áreas con sobrepoblación y bajos niveles de higiene. El serotipo 1 (aprox. 30% de los aislamientos de *S.dysenteriae*) suele ser el responsable de grandes brotes epidémicos de disentería, muy virulentos y multirresistentes, con altos índices de mortalidad. Finalmente, *S.boydii,* es el serogrupo más infrecuente de los cuatro. *S.flexneri* y *S.dysenteriae* son las dos especies de Shigella más virulentas.

Se calcula que anualmente se dan unos 164.7 millones de episodios de Shigellosis, de los cuales 163.2 millones ocurrren en PBR. Un 69% de todos los episodios de Shigella y un 61% de todas las muertes (estimadas en aproximadamente 1,1 millones al año) son en niños menores de cinco años (Kotloff et al., 1999).

El aumento de viajes internacionales, y el que cada vez más frecuentemente se escojan como destino lugares exóticos en países tropicales, ha contribuido también a que la infección por Shigella se convierta en una causa relativamente común de la diarrea del viajero (DV), que a su vez suele afectar aproximadamente a un 50% de los turistas a PBR (RE.Black,1990). Aunque no se trata del agente etiológico por excelencia en dicha patología, si es el que produce síntomas más agudos e invalidantes. En un estudio que se realizó en el Hospital Clínico hace diez años, se aisló un patógeno en el 39% de los viajeros que consultaron por diarreas, y entre estos se aisló *Shigella* spp. en el 9% de los afectados (Gascón et al., 1993).

Más recientemente, desde el inicio de la epidemia del VIH/SIDA, se ha identificado un nuevo grupo de riesgo: los pacientes seropositivos. La intersección entre la infección por Shigella en pacientes ya infectados con VIH ha tenido consecuencias graves. Se ha demostrado que el estar infectado por VIH es un factor de riesgo importante para la shigelosis (Baer et al., 1999). Por otro lado, en pacientes seropositivos, la intensidad de los síntomas es mayor. Adicionalmente, es común que estos pacientes desarrollen una diarrea persistente o recurrente

por Shigella, aún estando bajo tratamiento antimicrobiano adecuado, y están expuestos a un mayor riesgo de bacteriemia por Shigella que puede ser recurrente, grave o incluso mortal (Dougherty et al. 1996 ; 1988, Clerinx et al. 1995, Hueber et al., 1993).

En un estudio reciente se ha demostrado que el 75% de los contactos asintomáticos de pacientes con una Shigella, daban positivos en una prueba por PCR, evidenciando que la inmunidad del huésped juega un papel importante en la determinación de cuales de las personas expuestas realmente desarrollarán una infección clínica (Gaudio et al, 1997). Esto explicaría en parte la mayor susceptibilidad de personas inmunocomprometidas por VIH.

1.6.5 Tratamiento

El tratamiento está indicado en todos los casos confirmados o sospechosos de shigelosis infantil así como en pacientes con diarrea invasiva, severa o prolongada, así como en aquellos que tienen alto riesgo de complicaciones (personas mayores, diabéticos, cirróticos, imnunocomprometidos) (Olldfield & Wallace, 2001). En adultos sanos se recomienda generalmente para disminuir el tiempo y la severidad de los síntomas.

El tratamiento tradicional infantil ha sido bien con ampicilina o con cotrimoxazol (trimetoprima-sulfametoxazol). Sin embargo, los altos niveles de resistencia desarrollados en las últimas décadas a ambos antimicrobianos han hecho que se haya limitado su empleo en la actualidad. El ácido nalidíxico (55mg/kg/día) ha pasado a ser el tratamiento de primera línea en PBR. A pesar de su probada toxicidad sobre el cartílago en modelos animales (Burkhardt et al., 1997), cuando se ha administrado quinolonas como uso compasivo en niños, los efectos adversos observados no son superirores a los de los adultos, siendo además la mayoría reversibles (A. Black et al., 1990). Otra opción bastante extendida en PBR, específicamente Africa, es el uso de cloranfenicol. Así por ejemplo, en Ifakara, el cloranfenicol es la cuarta droga más utilizada mientras que suele ser el antibiótico de primera o segunda línea para tratar la mayoría de infecciones y la primera opción para diarreas en niños (Dr. David Schellemberg,

comunicación personal). En adultos, el tratamiento se basa en el uso de las fluoroquinolonas (normalmente norfloxacino o ciprofloxacino).

Un grupo de antimicrobianos bastante prometedor es el de las cefalosporinas de tercera generación, actualmente utilizadas en pediatría (en países desarrollados) y para tratar cepas de Shigella multiresistentes. Su mayor problema es el de un elevado precio, que hace que no puedan ser considerados como alternativa para el tratamiento de infecciones en PBR, donde el impacto en cuanto a mortalidad y morbilidad es mayor. Es por lo tanto necesario encontrar nuevos tratamientos, eficaces pero también asequibles a las poblaciones más necesitadas.

Actualmente no existe vacuna alguna que proteja de la infección por Shigella, aunque se están realizando pruebas de seguridad y eficacia de algunas que parecen ser prometedoras (Coster et al., 1999; Noriega et al., 1996; Cohen et al., 1997). La principal limitación en el desarrollo de una vacuna contra *Shigella* spp. es la variedad no solo de serogrupos, sino de serotipos, que en total suman 47 presentaciones antigénicas diferentes! Según Kotloff y colaboradores, en un análisis detallado de la epidemiología de Shigella con miras a desarrollar una estrategia vacunal, el producto idóneo tendría que proteger contra *S.sonnei* (serotipo único y el más prevalente en países desarrollados), contra el serotipo 1 de *S.dysenteriae* (extremadamente virulento y causante de pandemias) y el 100% de *S.flexneri*. En este último caso se ha demostrado que existe una reactividad serológica cruzada en humanos frente a los 15 serotipos de la especie *flexneri*, de manera que no seria descabellado pensar el poder desarrollar un vacuna polivalente con estas características utilizando tecnologías punta (Kotloff et al., 1999). Por el momento, sin embargo, la mejor opción de control continúa siendo la prevención, tanto mediante la mejora de las medidas de higiene como mediante la educación de la población en riesgo.

1.6.6 Mecanismos moleculares de resistencia a los agentes antimicrobianos

Trabajando con cepas de Shigella multiresistentes, ya en 1959 Ochiai y colaboradores encontraron que dicha multiresistencia podía transferirse a otras cepas susceptibles, incluso de *E.coli*, y que durante la transferencia no había intercambio de ADN cromosómico ni segregación de las resistencias. Dos años más tarde, Watanabe y Fukusawa hacían la primera descripción de los "factores de resistencia" o R-factors, aunque pasarían más de 20 años antes de que se demostrara que la transferencia de estos factores R era mediada por plásmidos (Tanaka y col. 1983).

El género *Shigella* es ampliamente reconocido por su habilidad para ganar y transferir plásmidos. Suelen albergar poblaciones heterogéneas de plásmidos, habiéndose descrito hasta 10 diferentes en una misma cepa (Jamieson y col., 1979). Dado que un gran número de determinantes de resistencia se encuentran codificados en elementos transponibles como los plásmidos, no es de extrañar que las Shigellas hayan adquirido rápidamente resistencia a prácticamente todos los antibióticos utilizados en los PBR. En un estudio realizado en 1994 en el Hospital Clínico con cepas ailsladas del viajeros internacionales con diarrea (Vila y col. 1994), se observaron niveles de multiresistencia del 72% para *S.sonnei* y el 63% para *S.flexneri*. Los primeros eran especialmente resistentes frente al cotrimoxazol y la tetraciclina mientras que los segundos lo eran frente a ampicilina y cloranfenicol. La resistencia a las quinolonas empieza a aparecer en países donde su uso es extensivo, como en el caso de India, pero aún es incipiente.

Frente a tres de estos cinco antibióticos (ampicilina, tetraciclina y trimetroprim-sulfametoxazol) el fenotipo resistente se explica por la presencia de genes normalmente encontrados en elmentos transponibles tales como plásmidos, integrones o transposones. Así pues, la resistencia a la ampicilina ha estado tradicionalmente mediada en Shigella por genes tipo TEM (Schumacher y col.,1992) aunque más recientemente la presencia de genes tipo OXA comienza a ser comúnmente reportada (Siu y col., 2000). Por supuesto, también pueden estar

involucrados otros mecanismos, tales como la modificación de las PBP, pero no son los más frecuentes.

La forma más común de resistencia al trimetroprim es la mediada por la adquisición de variantes de la enzima dihidrofolato reductasa (DHFR) codificada en un plásmido, y el género Shigella no es la excepción. Al igual que en otras Enterobacterias, es el gen *dfr*Ia, que codifica para la variante DHFR I, generalmente asociada al transposon Tn7, es el que se identifica con mayor frecuencia (Heikkila y col., 1990).

La resistencia a la tetraciclina, muy común hoy en día entre microorganismos Gram negativos, fue descrita por primera vez en Shigella, donde también fue por primera vez relacionada con la presencia de plásmidos-R y demostrada su transferencia a otras especies por conjugación (Roberts, 1996). Se conoce que varios genes que codifican proteinas de membrana involucrados en la expulsión activa de la tetraciclina se encuentran en plásmidos y transposones. Sin embargo, el fenómeno de la resistencia a tetraciclina en Shigella ha sido poco estudiado.

La resistencia a las quinolonas entre cepas de Shigella aún es incipiente a nivel global, pero comienza a ser notoria en países en los que el uso de este grupo de antibióticos está extendido, como en el caso de la India. Al igual que en otras Enterobacterias, el fenotipo resistente en Shigella es debido a la mutación de los genes diana, principalmente *gyr*A y *par*C (Ruiz, J., 2003). Aunque se trata de una resistencia mediada a nivel cromosómico, se ha observado en cepas de *S.dysenteriae* que la presencia de algunos plásmidos en concreto, en los que se codifican ciertas moléculas involucradas en el sistema de reparación del ADN, tal y como el sistema SOS de *E.coli*, puede contribuír a la resistencia a quinolonas aumentando la tasa de mutación espontánea (Ambler et al. 1993, Ashraf et al., 1991).

2 JUSTIFICACIÓN Y OBJETIVOS

La adquisición de resistencias a diferentes antimicrobianos, bien sea mediante mutaciones o por transferencia de material genético, se ve facilitada por la presión de selección que ejercen los mismos antibióticos. Debido a diferencias en el uso y manejo de los antimicrobianos, la presión de selección y por lo tanto el nivel de resistencia difiere entre los países desarrollados y los PBR. Sin embargo, se puede decir que en ambos casos existen tres ambientes en los que se promueve o al menos se facilita dicha adquisición y que son los siguientes:

1.- **El mundo de la Medicina Veterinaria**, en el que se usan los antibióticos no solo como tratamiento o profilaxis sino como promotores de crecimiento. No solo se induce a la selección de bacterias resistentes, sino que quedan residuos de antibióticos en la comida que posteriormente son ingeridos por los humanos en concentraciones subinhibitorias que favorecen la selección de cepas resistentes a nivel de tracto intestinal.

2.- **El mundo hospitalario.** Aproximadamente entre 20-40% de los pacientes ingresados en un hospital español reciben tratamiento con antimicrobianos (Barrio et al., 1999). Sumado a esta altísima presión de selección, está la ejercida por la utilización de numerosos desinfectantes y medidas para reducir la dispersión de bacterias resistentes, que elimina una buena parte de la microbiota bacteriana dejando el nicho libre para las más virulentas y resistentes.

En los PBR suele darse la situación de que, bien por falta de medicamentos y/o por falta de apoyo del laboratorio microbiológico, se utilizan como profilaxis antibióticos para los cuales existen altos niveles de resistencia. Por otro lado suele darse un mal uso de los antibióticos en manos de personal no cualificado que suple las necesidades de la población ante la falta de sistemas de salud bien constituidos.

3.- **La comunidad.** La libre venta de productos antimicrobianos en farmacias, resulta en un mal uso y abuso de estos mismos que ejerce a su vez una presión de selección. Es un

ambiente en donde, bajo condiciones de higiene deficientes, puede darse fácilmente la dispersión de cepas entre individuos, incluso a través de alimentos o agua contaminada. Otro factor a tener en cuenta es el de la importación de bacterias resistentes de otros países, traídas por viajeros a lugares exóticos.

En el caso de los PBR, sumado al hecho de que son lugares en los que por las condiciones higiénicas se dan a menudo y se propagan con facilidad cepas epidémicas, se conjugan además una serie de factores de alto riesgo para la adquisición y diseminación de factores de resistencia. Además del mal uso que se da en general a los antimicrobianos a nivel mundial, es común que en estos países debido a una falta de control, se consuman medicamentos de mala calidad o caducados, y por lo tanto con cantidades subinhibitorias de producto

Dentro de los principales patologías ***comunitarias,*** causadas por patógenos que presentan altos niveles de resistencia, está el del síndrome diarréico. Se trata de una problema especialmente grave en ***países de baja renta,*** en los cuales la infección por algunos microorganismos suele ser endémica. Uno de los patógenos responsables es el género Shigella, que se caracteriza entre otras cosas por tener una gran facilidad para ganar plásmidos y con ellos, adquirir nuevas resistencias.

En cuanto al ***ambiente hospitalario*** y primordialmente en los ***países desarrollados***, la persistencia de cepas multirresistentes de *Acinetobacter* es una preocupación constante. Se trata de un microorganimo con una elevada resistencia intrínseca, capaz de crecer bajo condiciones extremas, con lo cual ocupa nichos en los cuales tiene poca competencia. Suele ser responsables de brotes de difícil manejo entre pacientes delicados.

Teniendo en cuenta el escenario anterior, nos planteamos el ver en estos dos microorganismos con epidemiología y microhabitat muy diferente (casi opuesto), con resistencias intrínsecas no comparables pero ambos con altos niveles de multiresistencia:

a.- Como se da la adquisición de la multiresistencia

b.- Como se realiza su diseminación clonal

Para responder a estas preguntas, se delinearon los siguientes objetivos:

2.1 Objetivo general

Estudiar el patrón de resistencias y conocer los mecanismos moleculares implicados en el fenotipo resistente en dos patógenos con epidemiología y microhábitat muy diferente: *Shigella* spp. patógeno comunitario y *A. baumannii*, patógeno nosocomial.

2.2 Objetivos específicos

1.- Analizar la epidemiología de un patógeno nosocomial (*A.baumanni*) y de un patógeno comunitario (*Shigella* spp.)

2.- Estudiar los niveles de resistencia encontrados en cepas de *Shigella* spp. aisladas en una zona endémica o provenientes de pacientes con DV y por lo tanto con diversos orígenes geográficos.

3.- Estudiar los perfiles de resistencia de cepas clínicas de *A.baumannii* y analizar los mecanismos de resistencia implicados en los fenotipos resistentes.

4.- Analizar los diferentes mecanismos de resistencia de las cepas de *Shigella* spp. multirresistentes.

5.- Estudiar la prevalencia y composición de los integrones en las cepas de *Shigella* spp y en las cepas de *A.baumannii*.

6.- Buscar tratamientos alternativos para la Shigellosis.

3 RESULTADOS

3.1 *Acinetobacter baumanii*

3.1.1 Artículo 1.- "Spread of Amikacin resistance in *Acinetobacter baumannii* strains isolated in Spain due to an Epidemic Strain."

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Se estudiaron 16 cepas de *A.baumannii* provenientes de nueve hospitals españoles, con el fin de determinar si la alta incidencia de resistencia a la amikacina entre esto microorganismos se debía a la dispersión de un solo clon (cepa epidémica), así como determinar cual era el mecanismo de resistencia responsable de dicho fenotipo. La relación epidemiológica se determinó mediante dos métodos diferentes: REP-PCR y análisis del ADN genómico mediante digestión con enzimas de baja frecuencia de corte y electroforesis de campo pulsante (PFGE). Se hallaron cinco patrones diferentes por REP-PCR, uno de los cuales agrupaba 9 de las 16 cepas, provenientes de 7 hospitales diferentes. Por PFGE se repitieron los resultados, excepto que una cepa proveniente de un octavo hospital, y que por REP-PCR presentaba un patrón único y diferente, por PFGE presentaba el mismo bandeo que el clon epidémico. Todas las cepas presentaban una CMI a la amikacina >32 µg/ml. Al buscar el mecanismo de resistencia responsable mediante PCR con cebadores específicos para los genes *aph*(3')–*VIa*, *aac*(6')–*Ib* y *aac*(6')-*Ih*, se observó que las 16 cepas daban el amplicón esperado de 234pb con los cebadores para *aph*(3')–*VIa* pero no amplificaban con los otros dos.

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Spread of Amikacin Resistance in *Acinetobacter baumannii* Strains Isolated in Spain Due to an Epidemic Strain

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Sixteen amikacin-resistant clinical *Acinetobacter baumannii* isolates from nine different hospitals in Spain were investigated to determine whether the high incidence of amikacin-resistant *A. baumannii* was due to the dissemination of an amikacin-resistant strain or to the spread of an amikacin resistance gene. The epidemiological relationship studied by repetitive extragenic palindromic PCR and low-frequency restriction analysis of chromosomal DNA showed that the same clone was isolated in eight of nine hospitals, although other clones were also found. The strains were studied for the presence of the *aph(3')-VIa* and *aac(6')-I* genes, which encode enzymes which inactivate amikacin, by PCR. All 16 clinical isolates had positive PCRs with primers specific for the amplification of the *aph(3')-VIa* gene, whereas none had a positive reaction for the amplification of the *aac(6')-I* gene. Therefore, the high incidence of amikacin resistance among clinical *A. baumannii* isolates in Spain was mainly due to an epidemic strain, although the spread of the *aph(3')-VI* gene cannot be ruled out.

Several outbreaks of nosocomial infections caused by amikacin-resistant *Acinetobacter baumannii* have been documented (3, 4, 11, 20). The most frequent cause of resistance to aminoglycosides in *A. baumannii* is the modification of hydroxyl or amino groups of the antibiotic by aminoglycoside-modifying enzymes (1, 5–7, 14, 15, 21), although other mechanisms such as diminished permeability or alteration of the binding sites have been suggested (21). Until recently, amikacin remained the most active aminoglycoside in the treatment of infections caused by *Acinetobacter* spp. The 6′-aminoglycoside-acetylating enzyme found in *Acinetobacter* spp. inactivates amikacin (13); however, the most frequently found amikacin-modifying enzyme in *A. baumannii* is aminoglycoside-3′-phosphotransferase VI [APH(3′)-VI], a type of 3′-*O*-phosphotransferase which also inactivates amikacin (8). Buisson et al. (3) found a significant correlation between amikacin consumption and the emergence of amikacin resistance mediated by APH(3′)-VI in *Acinetobacter* species. The main purpose of this study was to develop a PCR method for the detection of the *aph(3′)-VIa* gene and to determine if the spread of amikacin resistance in *A. baumannii* strains isolated in nine Spanish hospitals was due to an epidemic strain carrying the *aph(3′)-VIa* or *aac(6′)-I* gene.

MATERIALS AND METHODS

Bacterial strains. Sixteen amikacin-resistant clinical isolates of *A. baumannii* were collected from nine Spanish hospitals (Hospital Clinic, Barcelona; Hospital Valle Hebrón, Barcelona; Hospital de Sant Joan de Reus, Reus, Tarragona; Hospital Virgen del Rocio, Seville; Hospital Clinico, Seville; Hospital de La Princesa, Madrid; Hospital 12 de Octubre, Madrid; Hospital Ramón y Cajal, Madrid; and Hospital Nuestra Señora [Ntra. Sra.] del Pino, Las Palmas, Grand Canary Islands). The identification of *A. baumannii* was performed by standard biochemical reactions by following the criteria of Bouvet and Grimont (2). Moreover, all the strains analyzed in this study were causing pneumonia in patients in the intensive care units of each hospital.

Susceptibility testing. Susceptibility testing was performed by using an agar dilution method in accordance with the guidelines established by the National Committee for Clinical Laboratory Standards (16). Approximately 10^4 CFU of each isolate was inoculated onto freshly prepared media containing serial dilutions of amikacin (Bristol-Myers Laboratories, Hounslow, United Kingdom) with a multipoint replicator.

REP-PCR and low-frequency restriction analysis of chromosomal DNA (PFGE). The repetitive extragenic palindromic PCR (REP-PCR) was performed with the primers and under the conditions described previously (22). Samples (5 μl) of each PCR end product were analyzed by polyacrylamide gel electrophoresis with Genephor precast 12.5% polyacrylamide gels run at 600 V and 25 mA. After that, the gel was stained with silver. The analysis of chromosomal DNA by digestion with low-frequency-of-cleavage restriction enzymes and separation of the fragments by pulsed-field gel electrophoresis (PFGE) was performed with the *ApaI* enzyme under the conditions mentioned elsewhere (12).

PCR amplification of the *aph(3′)-VIa* and *aac(6′)-I* genes. Two oligonucleotide primers were designed on the basis of the nucleotide sequence of the *aph(3′)-VIa* gene in comparison with the sequences of different phosphotransferases in an attempt to find specific sequences of this gene which do not anneal with the other genes. These primers were 5′-ATACAGAGACCACCATACAG T-3′ (from nucleotides 140 to 159) and 5′-GGACAATCAATAATAGCAAT-3′ (from nucleotides 355 to 374) (Genosys Biotechnologies, Cambridge, United Kingdom). The PCR was performed as follows. One colony grown on MacConkey agar was resuspended in 25 μl of sterile distilled water and boiled for 10 min. After a centrifugation step at 15,000 × *g* for 1 min, 25 μl of the reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM potassium chloride, 3.0 mM magnesium chloride, 0.1% (wt/vol) gelatin, 400 μM deoxynucleoside triphosphates, and 1 μM (each) primer was added, together with 2.5 U of *Taq* polymerase (BRL, Life Technologies Inc., Gaithersburg, Md.). Each reaction mixture was overlaid with mineral oil and amplified with the following temperature profiles: 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplification was performed in a DNA thermal cycler (model 480; Perkin-Elmer Cetus). The amplified DNA products were resolved by electrophoresis in 1.5% NuSieve and 1% agarose gels. In order to confirm that the amplified product

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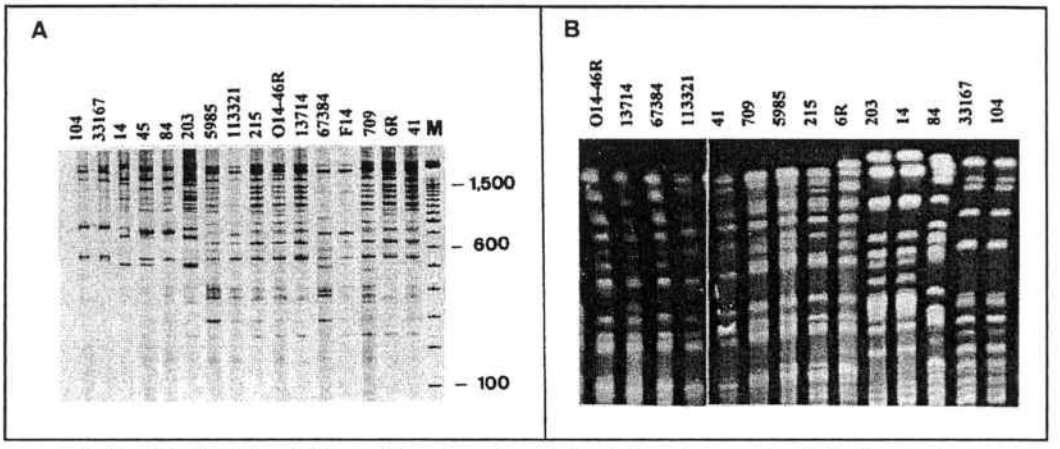

FIG. 1. Patterns obtained by REP-PCR (A) and PFGE (B). The numbers above the lanes indicate the strains. Lane M, DNA molecular size marker (100-bp DNA Ladder; Life Technologies Inc.).

belongs to the *aph(3')-VIa* gene, the fragment was recovered from the reaction mixture with the QIAquick Spin PCR purification kit (Qiagen, Chatsworth, Calif.) and processed with a DNA sequencing kit (Taq DyeDeoxy Terminator Cycle Sequencing Kit; Applied Biosystems, Foster City, Calif.) and analyzed in an automatic DNA sequencer (model 373A; Applied Biosystems).

Eight clinical isolates carrying the genes for other aminoglycoside phosphotransferases were used as controls; they were *Escherichia coli* carrying the gene for APH(3')-I, *E. coli* carrying the gene for APH(3')-II, *Enterobacter cloacae* carrying the gene for APH(3')-II, *Staphylococcus epidermidis* carrying the gene for APH(3')-II, *E. cloacae* carrying the gene for APH(3')-III, *Staphylococcus aureus* carrying the gene for APH(3')-IV, and *Enterococcus faecalis* carrying the gene for APH(3')-IV.

Detection of the *aac(6')-Ib* and *aac(6')-Ih* genes was performed by PCR by following the procedure described by Ploy et al. (17).

RESULTS

The 16 amikacin-resistant clinical *A. baumannii* isolates were epidemiologically analyzed by REP-PCR and by low-frequency-of-cleavage restriction enzyme analysis of chromosomal DNA and PFGE. The analysis by REP-PCR allowed the distribution of the strains into five different groups with DNA bands ranging from 200 to 2,000 bp (Fig. 1A). Nine of the 16 strains belonged to the same type (type 1) and came from seven different hospitals, whereas two strains each were of types 2, 3, and 4 and one strain was of type 5. The strains belonging to type 2 were isolated in a Madrid hospital and in another hospital in Seville, whereas strains belonging to type 4 were isolated in two different hospitals in Seville (Table 1). The analysis of chromosomal DNA by PFGE confirmed the results obtained by REP-PCR (Table 1 and Fig. 1B) with one exception. The PFGE pattern of the strain determined to be of type 5 by REP-PCR had the same PFGE pattern as the strains determined to be of type 1 by REP-PCR; therefore, all these strains must be considered the same type (type A by PFGE). The results were analyzed by following the criteria described by Tenover et al. (19).

For all amikacin-resistant clinical *A. baumannii* isolates tested in this study, the amikacin MIC was >32 μg/ml (Table 1). To investigate whether the resistance was due to the synthesis of the aminoglycoside-modifying enzyme APH(3')-VIa, a PCR for the amplification of the *aph(3')-VIa* gene was used. A DNA fragment of the expected size of 234 bp, from nucleotides 140 to 374 of the *aph(3')-VIa* gene, was obtained (Fig. 2A). Its nucleotide sequence showed 100% homology with that described by Martin et al. (13). This DNA fragment was not obtained from the other strains which carried the genes corresponding to four different phosphotransferases (Fig. 2A), showing that the primers were specific for *aph(3')-VIa*. All the strains analyzed in our study were positive for the *aph(3')-VIa* gene by PCR (Fig. 2B), whereas when primers specific for the amplification of the *aac(6')-Ib* and *aac(6')-Ih* genes were used, the genes were not amplified from any of the strains.

The number of beds in the hospitals from which the strains included in this study were obtained ranged from 274 to 1,150. All hospitals but one (Hospital San Joan de Reus, Reus, Tarragona, Spain) have both oncology and transplant patients. The distribution of the *A. baumannii* isolates by clinical source was quite similar in all hospitals, with respiratory specimens being the most common clinical specimens containing *A. baumannii*, followed by wound and urine specimens.

DISCUSSION

We studied whether the high incidence of amikacin resistance in *A. baumannii* strains isolated in Spain was due to the dissemination of the amikacin resistance gene *aph(3')-VIa* or

TABLE 1. Epidemiological characteristics of amikacin-resistant *A. baumannii* strains

Strain no.	Hospital[a]	PFGE pattern	REP-PCR pattern	AK[b] MIC (μg/ml)	PCR result for *aph(3')-VIa*
F14	VH, Barcelona	A	1	256	+
O14-46R	VH, Barcelona	A	1	256	+
5985	HC, Barcelona	A	1	64	+
41	HC, Barcelona	A	1	64	+
113321	HNSP, Canarias	A	1	128	+
6R	12O, Madrid	A	1	128	+
67384	RC, Madrid	A	5	256	+
13714	VR, Seville	A	1	64	+
215	HLP, Madrid	A	1	64	+
709	Re, Tarragona	A	1	64	+
203	HLP, Madrid	B	2	32	+
84	HLP, Madrid	C	3	32	+
45	HLP, Madrid	C	3	64	+
14	HCP, Seville	B	2	256	+
104	HCP, Seville	D	4	128	+
33167	VR, Seville	D	4	64	+

[a] VR, Hospital Virgen del Rocio; RC, Hospital Ramón y Cajal; HNSP, Hospital Ntra. Sra. del Pino; VH, Hospital del Valle Hebrón; Re, Hospital San Joan de Reus; HC, Hospital Clinic; HLP, Hospital La Princesa; HCP, Hospital Clinico Provincial; 12O, Hospital 12 de Octubre.

[b] AK, amikacin.

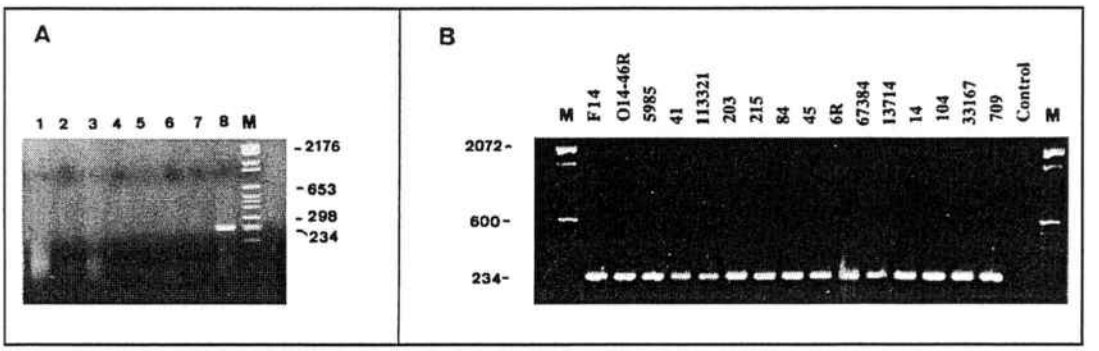

FIG. 2. (A) Analysis of PCR-amplified DNA with primers specific for amplification of *aph(3')-VIa*. Lanes: 1, *E. coli* carrying the gene for APH(3')-I; 2, *E. coli* carrying the gene for APH(3')-II; 3, *E. cloacae* carrying the gene for APH(3')-II; 4, *S. epidermidis* carrying the gene for APH(3')-II; 5, *E. cloacae* carrying the gene for APH(3')-III; 6, *S. aureus* carrying the gene for APH(3')-IV; 7, *E. faecalis* carrying the gene for APH(3')-IV; 8, *A. baumannii* carrying the gene for APH(3')-VIa; and M, DNA molecular mass marker VI (Boehringer Mannheim, Mannheim, Germany). (B) PCR analysis of the *aph(3')-VIa* gene of the amikacin-resistant clinical *A. baumannii* isolates studied. Control indicates an amikacin-susceptible *A. baumannii* strain used as a negative control. Lane M, DNA molecular size marker (100-bp DNA Ladder; Life Technologies Inc.).

aac(6')-I or to the spread of an amikacin-resistant strain of *A. baumannii*. The epidemiological relationship among the 16 selected clinical isolates of *A. baumannii* studied by REP-PCR and PFGE showed that clone A (as determined by PFGE) was isolated from patients in eight of nine hospitals. Therefore, the same clone has spread throughout Spain, even to the Canary Islands, which are far from the Spanish peninsula. Moreover, one clone (clone D4) spread between two hospitals in Seville. Another clone (clone B2) spread from a hospital in Seville to a hospital in Madrid or vice versa. Overall, four different clones of *A. baumannii* were identified.

Of the aminoglycoside-modifying enzymes detected in *A. baumannii*, so far, only APH(3')-VI and AAC(6')-I confer resistance to amikacin. APH(3')-VI is primarily associated with *Acinetobacter* spp. and is less frequently observed in other gram-negative bacteria (18). The *aph(3')-VIa* gene, which encodes APH(3')-VI, has been cloned from *A. baumannii* (13). APH(3')-VI production is characterized by resistance to kanamycin, neomycin, paromomycin, ribostamycin, butirosin, and gentamicin B, as well as to amikacin and isepamicin (9). AAC(6')-I production confers resistance to amikacin, netilmicin, sisomicin, and tobramycin. Ten genes, named *aac(6')-Ia* to *aac(6')-Ij*, encoding AAC(6')-I enzymes have been described (18). Of these genes, *aac(6')-Ib* and *Aac(6')-Ih* have frequently been found in *A. baumannii* (17). The resistance determinant of AAC(6') was apparently chromosomally located (15), although recently it has been described to be plasmid mediated (10), and it has been suggested that the *aph(3')-VIa* gene could reside on a transposon (9). These genes can therefore be easily transferred among strains carried on these genetic elements, contributing to the spread of amikacin resistance. In this study, we have developed a PCR for the specific detection of the *aph(3')-VIa* gene, and we have detected this gene in 100% of the amikacin-resistant clinical *A. baumannii* isolates from different hospitals in different geographic areas in Spain, whereas these strains did not have a positive PCR result when primers specific for the *aac(6')-Ib* and *aac(6')-Ih* genes were used. Our data are consistent with the results of previous studies in which dot blot hybridization was used (9, 18). In those studies 82.7 and 95% of amikacin-resistant *Acinetobacter* strains hybridized with an *aph(3')-VIa* probe (9, 18). In part, our results agree with those of Lambert et al. (9), who showed that in France the dissemination of amikacin resistance in *Acinetobacter* spp. was due to a gene, although the dissemination of an amikacin-resistant clone is also true in Spain.

PCR for the detection of aminoglycoside-modifying enzymes may play a major role in delineating the types of genes involved in epidemics, may help define their modes of transmission, and may make large studies of the movement of specific resistance determinants during outbreaks of nosocomial and community-acquired infections possible. In the outbreaks caused by *A. baumannii*, the use of antibiotics can contribute to the persistence and spread of the outbreak. Sometimes the patients are only colonized with this microorganism and do not require antimicrobial therapy; therefore, it is important to differentiate between colonization and infection before therapy is begun. In conclusion, our study has shown the contribution of the *aph(3')-VIa* gene to the incidence of amikacin resistance in *A. baumannii* studied by PCR. This, together with the molecular epidemiological analysis of strains isolated from different hospitals in Spain, has shown that the dissemination of amikacin resistance was due to an epidemic strain carrying the *aph(3')-VIa* gene and demonstrates how easily this microorganism is spread from hospital to hospital.

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3.1.2 Artículo 2.- "Distribution of ß-lactamases in *Acinetobacter baumannii* clinical isolates and the effect of Syn 2190 (AmpC inhibitor) on the MIC of different ß-lactam antibiotics"

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Se estudiaron 20 cepas de A.baumannii aisladas de diferentes hospitals españoles y con resistencia a ß-lactámicos, con el fin de analizar su distribución de ß-lactamasas así como la actividad del Syn 2190, un inhibidor de la betalactamasa cromosómica AmpC.

Todas las cepas eran resistentes a la ampicilina, once de ellas con una CMI >256 µg/ml. En nueve de las once, se pudo correlacionar el valor de la CMI con la presencia y expresión de una OXA y/o una TEM. En las once cepas restantes (con CMIs entre 32 y 256µg/ml) se observó una disminución de la CMI a ampicilina en presencia del Syn 2190. 90% de las cepas eran resistentes a la cefotaxima, 60% al cefepime, 55% a la ceftazidima , 35% a la piperacilina y 15% al imipenem. La presencia del Syn 2190 no modificó la CMI a este último, mientras que frente a la ceftazidima se observó una disminución de la CMI en el 82% de las cepas resistentes. Frente a la piperacilina, la afectación del inhibidor fue diferente incluso entre cepas que presentaban las mismas ß-lactamasas.

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Distribution of β-lactamases in *Acinetobacter baumannii* clinical isolates and the effect of Syn 2190 (AmpC inhibitor) on the MICs of different β-lactam antibiotics

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The distribution of β-lactamases in a group of 20 epidemiologically well defined *Acinetobacter baumannii* clinical isolates and the *in vitro* activity of Syn 2190, a novel β-lactamase AmpC inhibitor, were determined. Twenty-five per cent of the strains carried and expressed a TEM-type β-lactamase, whereas 35% had an OXA-type β-lactamase. In nine out of 11 (82%) ceftazidime-resistant and four out of 13 (30.7%) cefepime-resistant strains, the MIC of these β-lactam antibiotics decreased when determined in the presence of Syn 2190. Thus, our results suggest that in a high percentage of *A. baumannii* clinical isolates the increased production of AmpC, in combination or not with other resistance mechanisms, contributes to the resistance pattern in *A. baumannii* to β-lactams.

Introduction

Acinetobacter baumannii is an emergent opportunistic nosocomial pathogen, which very often causes nosocomial outbreaks that are difficult to control due to the ease with which this microorganism disseminates and its persistence in the hospital environment. Successive surveys have reported increased resistance in *A. baumannii* clinical isolates.[1]

A. baumannii is currently resistant to most β-lactam antibiotics, particularly penicillins and cephalosporins, and lately to carbapenems.[1,2] Ceftazidime, piperacillin and imipenem are among the most active β-lactam antibiotics against *A. baumannii*. Several authors have studied the presence of β-lactamases as a mechanism of resistance to these antibiotics.[1–3] Some of these reports were performed using *Acinetobacter calcoaceticus*, since they were carried out before the studies that defined 13 genospecies, including one species for *A. calcoaceticus* and another for *A. baumannii*.[1] This study aimed to investigate the distribution of β-lactamases in a group of epidemiologically well defined *A. baumannii* clinical isolates. Moreover, the *in vitro* activity of Syn 2190, a novel AmpC inhibitor,[4,5] in combination with various penicillins and cephalosporins was also determined.

Materials and methods

Bacterial strains

The 20 *A. baumannii* clinical isolates analysed during this study are part of a collection from the Hospital Clinic, and were isolated in Spanish hospitals during a 10 year period.[6,7]

Susceptibility testing

All susceptibility testing was by Etest (AB Biobisk, Sölna, Sweden) following the manufacturer's recommendations. The MICs of ampicillin, piperacillin, ceftazidime, imipenem and cefepime were determined in both the presence and absence of 4 mg/L Syn 2190[4,5] (Naeja Pharmaceutical Inc., Edmonton, Canada). In addition, the MICs of ticarcillin, cefotaxime and cefoxitin were determined. The MIC of imipenem was also determined in the presence of 25 mg/L reserpine.

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Staphylococcus aureus ATCC 29213 and *Escherichia coli* ATCC 25922 were used as quality controls.

Characterization of β-lactamase

Determination of isoelectric points was performed as described previously.[8] The presence of *tem*, *oxa 2*-like, *oxa 3*-like and *shv*-type genes was determined by PCR with primers and conditions described previously.[3,8] Determination of the presence of *oxa 20* and *oxa 37* genes was carried out under the same conditions as the other *oxa* genes, but using the following primers: 5′-CACATCGGTTTATAATGAAT-3′ and 5′-TTGGGTGGCAAAGCATTGACG-3′.

Results and discussion

Twenty *A. baumannii* clinical isolates were studied. These isolates were identified as *A. baumannii* based on biochemical criteria and by amplified ribosomal DNA restriction analysis. Previous studies have shown that, with the exception of strains 6R and 67384, the isolates were epidemiologically unrelated.[6,7] Susceptibility to different β-lactam antibiotics is shown in Table 1. All strains were resistant to ampicillin. Eleven out of 20 *A. baumannii* strains had an MIC of ampicillin >256 mg/L, and in nine, this high level of resistance was correlated with the presence of an OXA and/or TEM β-lactamase (Table 2). Twenty-five per cent of the strains carried and expressed a TEM-type β-lactamase, whereas 35% of the strains had an OXA-type β-lactamase (Table 2). Plasmid-mediated TEM-1- and TEM-2-type β-lactamases have been described previously.[1,9] In a previous study, Joly-Guillou *et al.*[9] established a prevalence of TEM-1-type β-lactamase in *Acinetobacter* spp. of *c.* 34%. Several OXA-type β-lactamases have been described in *A. baumannii*, with most being located in an integron.[2,3] The remaining 11 strains not presenting an OXA- or TEM-type β-lactamase had a MIC range of ampicillin between 32 and >256 mg/L, and a decrease in the MIC of ampicillin was observed for all when it was determined in the presence of 4 mg/L Syn 2190, an AmpC inhibitor. These results suggest that at least 50% of the strains present an overexpressed chromosomal AmpC β-lactamase, with different levels of expression depending on the strain. Syn 2190 is an inhibitor of AmpC β-lactamases but does not affect other types of these enzymes.[4,5] Although a CARB-type β-lactamase has been found in an *A. calcoaceticus* subsp. *anitratus* strain,[1] in our study this type of β-lactamase was not detected. Susceptibility to piperacillin was also low, with 35% (seven out of 20) of the strains being resistant and 45% (nine out of 20) being intermediately resistant. Strains carrying the same set of β-lactamases, for instance strains 6R, 661 and 704R producing TEM and OXA β-lactamases, or 67384, 875 and 74I producing an OXA β-lactamase, showed different susceptibility to the piperacillin plus Syn 2190 combination. In strains 6R, 67384 and 875, the MIC of piperacillin was

Table 1. MICs of different β-lactam antibiotics, some plus Syn 2190, for 20 *A. baumannii* clinical isolates

	MIC (mg/L)													
Strain	AMP	AMP+I	TIC	PIP	PIP+I	CTX	CAZ	CAZ+I	FOX	FEP	FEP+I	IPM	IPM+I	IPM+R
6R	>256	>256	>256	>256	>256	>32	>256	32	>256	>32	>32	>32	>32	>32
67384	>256	>256	>256	>256	>256	>32	>256	32	>256	>32	>32	>32	>32	>32
875	>256	>256	>256	>256	>256	>32	>256	16	>256	>32	12	>32	>32	>32
46Y	>256	>256	>256	>256	>256	>32	>256	>256	>256	>32	>32	4	4	4
36	>256	24	>256	64	16	32	16	3	>256	4	1.5	0.25	0.25	0.25
65	128	64	>256	64	48	>32	>256	16	>256	>32	>32	0.25	0.25	0.25
661	>256	>256	>256	>256	32	32	32	24	>256	>32	>32	1	1	0.19
F14	96	64	96	24	24	>32	>256	16	>256	>32	>32	0.25	0.25	0.25
198	48	24	64	48	24	>32	8	8	>256	24	6	0.12	0.12	0.25
92	48	8	24	24	12	>32	8	16	96	4	6	1	1	0.38
77	>256	16	12	48	12	>32	8	8	>256	>32	>32	0.5	0.5	0.5
78	>256	8	12	24	8	>32	>256	16	>256	>32	>32	0.25	0.25	0.25
31	>256	>256	256	64	64	8	4	4	>256	>32	>32	0.25	0.25	0.25
83	32	4	8	8	3	>32	>256	16	48	1.5	1	0.12	0.12	0.12
183	32	4	8	4	4	8	8	8	24	1	0.5	0.06	0.06	0.06
61	32	6	16	12	6	>32	4	2	>256	2	1.5	0.19	0.12	0.12
86	48	8	24	16	12	>32	4	3	>256	4	3	0.094	0.12	0.12
60	64	8	32	32	16	>32	8	8	>256	4	3	0.12	0.12	0.12
74I	>256	>256	>256	>256	64	>32	>256	32	>256	>32	16	1	1	0.19
704R	>256	>256	>256	>256	32	>32	>256	64	>256	>32	32	1	1	0.19

I, Syn 2190; AMP, ampicillin; TIC, ticarcillin; PIP, piperacillin; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; FEP, cefepime; IPM, imipenem; R, reserpine.

β-Lactamases in *Acinetobacter baumannii*

Table 2. Distribution of β-lactamases in the 20 *A. baumannii* clinical isolates

Strain	Isoelectric focusing (pI)	PCR		Resistance phenotype							
		bla_{TEM}	bla_{OXA}[a]	AMP[b]	PIP	CTX	CAZ	FEP	IPM	TIC	FOX
31	5.4	+	–	R	I	S	S	R	S	R	R
36	>8, 5.4	+	–	R	I	R	I	S	S	R	R
6R	>8, 7.0–7.5, 5.4	+	+(21-like)	R	R	R	R	R	R	R	R
661	>8, 7.0–7.5, 5.4	+	+(21-like)	R	R	R	R	R	S	R	R
704R	>8, 7.0–7.5, 5.4	+	+(21-like)	R	R	R	R	R	S	R	R
67384	>8, 7.0–7.5	–	+(21-like)	R	R	R	R	R	R	R	R
875	>8, 7.0–7.5	–	+(21-like)	R	R	R	R	R	R	R	R
46Y	>8, 7.0–7.5	–	+(21-like)	R	R	R	R	R	S	R	R
74I	>8, 7.0–7.5	–	+(37)	R	R	R	R	R	S	R	R
65	>8	–	–	R	I	R	R	R	S	R	R
F14	>8	–	–	R	I	R	R	R	S	I	R
198	>8	–	–	R	I	R	S	I	S	I	R
92	>8	–	–	R	I	R	S	S	S	I	R
77	>8	–	–	R	I	R	S	R	S	S	R
78	>8	–	–	R	I	R	R	R	S	S	R
83	>8	–	–	R	S	R	R	S	S	S	R
183	>8	–	–	R	S	S	S	S	S	S	I
61	>8	–	–	R	S	R	S	S	S	S	R
60	>8	–	–	R	I	R	S	S	S	I	R
86	>8	–	–	R	S	R	S	S	S	I	R

pI, isoelectric point; S, susceptible; I, intermediate; R, resistant.
[a]The type of *oxa* gene is given in parentheses.
[b]Breakpoints for the different β-lactams: AMP ≤8 S, ≥32 R; TIC ≤16 S, ≥128 R; PIP ≤16 S, ≥128 R; CAZ ≤8 S, ≥32 R; CTX ≤8 S, ≥32 R; FOX ≤8 S, ≥32 R; FEP ≤8 S, ≥32 R; IPM ≤4 S, ≥16 R.

>256 mg/L, in both the presence and absence of Syn 2190, whereas strains 661, 704R and 74I showed a decrease in the MIC of piperacillin from >256 to 32–64 mg/L when it was determined in the presence of the AmpC inhibitor. Three possible explanations could be posed for this phenomenon: (i) there may be a decrease in Syn 2190 penetration in strains 6R, 67384 or 875 so that it would not inhibit AmpC; (ii) a complementary mechanism of resistance to piperacillin besides the expression of β-lactamases in these strains, such as a decrease in permeability, increased efflux or modifications in the penicillin-binding proteins; or (iii) various chromosomal cephalosporinases with different levels of inhibition by Syn 2190 could be synthesized by each strain. In fact, several cephalosporinases, which are very heterogeneous from a functional point of view, have been described.[1,10]

The proportion of cefotaxime-resistant strains (90%) was higher than that of ceftazidime-resistant strains (55%), whereas in general cefepime showed low activity, with 60% of the strains being resistant. For nine out of 11 (82%) ceftazidime-resistant strains, the MIC of this β-lactam antibiotic decreased at least four-fold when determined in the presence of Syn 2190, whereas this was the case for only three of the 12 (25%) cefepime-resistant or intermediate strains. Recently, Bou & Martinez-Beltrán[10] cloned an AmpC β-lactamase from *A. baumannii* with a typical cephalosporinase profile that slightly affected cefepime. However, in *Pseudomonas aeruginosa*, a total de-repression of the AmpC enzyme compromises cefepime.[11] This could be the case in the above-mentioned strains, whereas in those strains showing no change in the MIC of cefepime (>32 mg/L) in the presence of Syn 2190, the resistance could be related to decreased permeability or increased efflux. This is further supported by the fact that strains such as 77 or 31 are highly resistant to cefepime but susceptible to ceftazidime, whereas neither MIC is affected by Syn 2190.

Three out of 20 (15%) *A. baumannii* strains showed a MIC of imipenem >32 mg/L, indicating the presence of a carbapenemase. Several β-lactamases affecting imipenem have been described,[1,2] some of which have been located in a plasmid that can be transferred, such as ARI-1, which is now OXA-23.[1] The combination of imipenem plus Syn 2190 did not modify the activity of this carbapenem, suggesting that AmpC has no effect on this antimicrobial agent. A set of oxacillinases with activity against carbapenems has recently been described.[2] The imipenem-susceptible strains presented a range of imipenem MICs between 0.06 and 4 mg/L. All four

strains with a MIC of imipenem of 1 mg/L had an MIC of 0.19 (three strains) and 0.38 mg/L (one strain) in the presence of reserpine (25 mg/L). Reserpine is an efflux pump inhibitor, in both Gram-positive and -negative bacteria. These results suggest that an efflux pump, inhibited by reserpine, could be involved in the moderate increase in resistance to imipenem.

In summary, despite the fact that other mechanisms of resistance to β-lactam antibiotics, such as decreased permeability or increased efflux, may contribute to the final MIC, our results indicate that a high percentage of *A. baumannii* clinical isolates show increased production of AmpC, and that other mechanisms, such as OXA- and TEM-type β-lactamases, play an important role in resistance to β-lactam antibiotics.

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3.1.3 Artículo 3.- "Cloning and Nucleotide Sequence Analysis of a Gene encoding an OXA-derived ß-lactamase in *Acinetobacter baumannii*"

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La cepa de *A.baumannii*, Ab41, fué aislada durante un brote epidémico en una unidad de cuidados intensivos del Hospital Clinico de Barcelona. Presentaba resistencia a todos los ß-lactámicos testados, excepto ceftazidima, ceftriaxone, ceftizoxima e imipenem. Así, presentaba una MIC >256µg/ml para ampicilina, amoxicilina, y ticarcilina; 256µg/ml para piperacilina y ticarcilina más ácido clavulánico, 64µg/ml para aztreonam; 16µg/ml para cefotaxima; 16/8µg/ml para amoxicilina más ácido clavulánico; 8µg/ml para ceftazidima, ceftizoxima, ceftriaxone y ampicilina más sulbactam y 0,5µg/ml frente a imipenem. Utilizando técnicas moleculares se estudiaron los mecanismos responsables para dicho fenotipo multirresistente. Mediante isoelectroenfoque se observó la presencia de tres ß-lactamasas con pI de >8.0 (ß-lactamasa cromosómica), 5.4 (tipo TEM) y 7.0. Esta última resultó ser una ß-lactamasa tipo OXA muy similar a la OXA-3 de la cual difería a nivel de secuencia de ADN en tres nucleótidos, dos de los cuales eran silenciosos. El tercero, sin embargo, generaba una sustitución en la Ile-217 a Met, a cuatro aminoácidos de la triada conservada. La nueva enzima fue designada como OXA-21

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Cloning and Nucleotide Sequence Analysis of a Gene Encoding an OXA-Derived β-Lactamase in *Acinetobacter baumannii*

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A clinical strain of *Acinetobacter baumannii* (strain Ab41) that was resistant to all β-lactam antibiotics tested except ceftazidime, ceftriaxone, ceftizoxime, and imipenem produced three β-lactamases: a presumptive chromosomal cephalosporinase, a TEM-1-like β-lactamase (pI 5.4), and a novel OXA-derived β-lactamase named OXA-21 (pI 7.0). The gene encoding OXA-21 was located in an integron. The nucleotide sequence showed three mutations compared with the sequence of OXA-3, with two being silent; the nonsilent mutation generated a substitution of Ile-217 to Met.

Acinetobacter baumannii is recognized as an important opportunistic pathogen which mainly causes pneumonia, bacteremia, and meningitis in immunocompromised patients (2, 13, 14). Currently, it is resistant to a wide variety of antibiotics, and this complicates the treatment of serious infections (11, 12, 15, 17). The low level of susceptibility of this microorganism to β-lactam antibiotics is linked to either an intrinsic or an acquired resistance. Sato and Nakae (10) showed that the outer membrane permeability of *Acinetobacter* to β-lactam antibiotics was 1 to 3% of that observed in *Escherichia coli*, suggesting that one of the causes for the high level of antibiotic resistance of *Acinetobacter calcoaceticus* is attributable to the presence of a small number of small porins. However, the most common mechanism of resistance to β-lactam antibiotics is due to the inactivation of these antibacterial agents by β-lactamases encoded either by the chromosome or by plasmids (3, 17). The plasmid-encoded β-lactamases TEM-1 and CARB-5 are the β-lactamases most frequently found in *Acinetobacter* (3, 17). Chromosomally encoded enzymes in *Acinetobacter* have also been extensively studied (3). In a previous study (17), β-lactamase detection was performed with 54 epidemiologically unrelated clinical isolates of *A. baumannii*, yielding a TEM-type β-lactamase in 16% of the clinical isolates analyzed and an unknown β-lactamase with a pI of 7.0 in 11% of the clinical isolates analyzed. The main purpose of the present work was to clone and sequence the gene encoding this unknown β-lactamase.

A strain of *A. baumannii* (strain Ab41) that was isolated during an outbreak in an intensive care unit in our hospital (16) and that has been epidemiologically and biochemically analyzed (16, 19) was studied. The susceptibility testing of this strain was performed by an agar dilution method in accordance with the guidelines established by the National Committee for Clinical Laboratory Standards (8). Approximately 10^4 CFU of the isolate was inoculated onto freshly prepared medium containing serial dilutions of the following antimicrobial agents: ampicillin (Antibioticos, S.A., León, Spain); amoxicillin, amoxicillin plus clavulanic acid, ticarcillin, and ticarcillin plus clavulanic acid (Beecham Laboratories, Brentford, United Kingdom); ceftazidime (Glaxo Wellcome, Greenford, United Kingdom); cefotaxime (Hoechst, Frankfurt, Germany); ceftriaxone (Roche, Basel, Switzerland); piperacillin (Lederle Laboratories, Pearl River, N.Y.); imipenem (Merck Research Laboratories, Rahway, N.J.); ampicillin plus sulbactam (Pfizer, Inc., New York, N.Y.); ceftizoxime (Smith Kline & French, Philadelphia, Pa.); and aztreonam (Squibb, Princeton, N.J.). Ampicillin plus clavulanic acid and ampicillin plus sulbactam were tested at ratios of 2:1, whereas ticarcillin plus clavulanic acid was tested at 2 μg of clavulanic acid per ml.

A. baumannii Ab41 was resistant to all β-lactam antibiotics except ceftazidime, ceftriaxone, ceftizoxime, and imipenem. The MICs were >256 μg/ml for ampicillin, amoxicillin and ticarcillin; 256 μg/ml for piperacillin and ticarcillin plus clavulanic acid; 64 μg/ml for aztreonam; 16 μg/ml for cefotaxime; 16/8 μg/ml for amoxicillin plus clavulanic acid; 8 μg/ml for ceftazidime, ceftizoxime, ceftriaxone, and ampicillin plus sulbactam; 0.5 μg/ml for imipenem; and 4 μg/ml for sulbactam.

β-Lactamases were analyzed by isoelectric focusing as described by Matthew et al. (7). The pIs were determined by comparison with those of enzymes with known pIs. The extract of the strain contained three β-lactamases: one gave a band with a pI above 8.0, likely corresponding to a chromosomal cephalosporinase, the other focused at pI 5.4 (TEM-1 type), and the third focused at pI 7.0. By using PCR, the presence of an integron was detected in this strain. PCR was carried out with a 50-μl volume containing 25 μl of a suspension of the strain which was prepared as described previously (18) and 25 μl of a reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM potassium chloride, 3.0 mM magnesium chloride, 0.1% (wt/vol) gelatin, 400 μM deoxynucleoside triphosphates, 1 μM primers, and 2.5 U of *Taq* polymerase (GIBCO-BRL). The primers 5′-AAGCAGACTTGACCTGA3′ (upper primer) and 5′GGCATCCAAGCAGCAAG3′ (lower primer) were used (6). The reaction mixture was overlaid with sterile mineral oil and was submitted to the following program of amplification: 1 min at 94°C, 1 min at 55°C, and 5 min at 72°C, with a final extension of 16 min at 72°C. The amplified DNA product was resolved by electrophoresis in a 1% (wt/vol) agarose gel containing ethidium bromide. This strain yielded a PCR product of approximately 1.5 kb, and this product was extracted from the agarose gel by using the Gene-Clean kit (Bio 101, Inc., La Jolla, Calif.) and was cloned by using the TA cloning kit (Invitrogen BV, Leek, The Netherlands). Sequencing was done with the *Taq* DyeDeoxiTerminator Cycle Sequencing Kit, and the sequence was analyzed in an automatic

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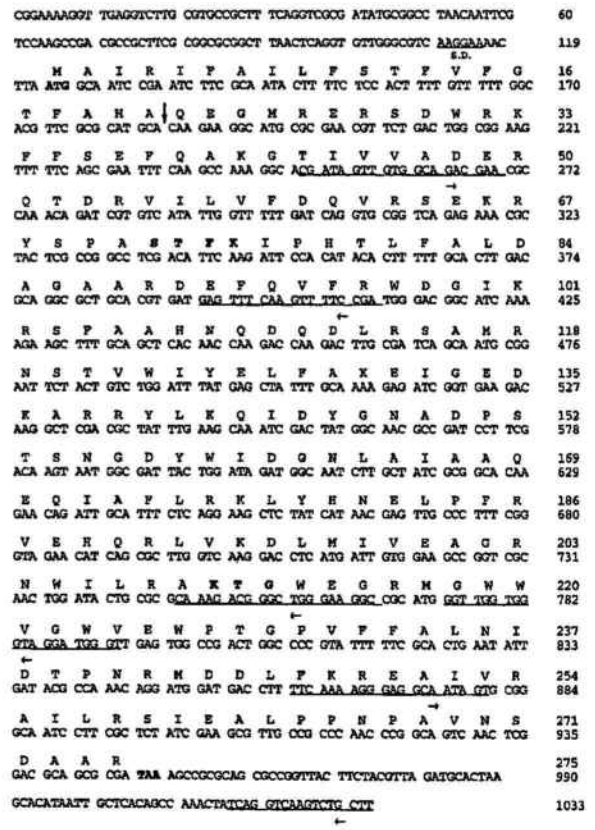

FIG. 1. Nucleotide and amino acid sequences of the OXA-21 gene and the OXA-21 protein from *A. baumannii* Ab41. The locations of the primers used to sequence the gene are underlined, and the direction of the sequence is indicated with an arrow. S.D., a putative Shine-Dalgarno ribosomal recognition site. The boldface ATG and TAA represent the initiation and termination codons, respectively. The β-lactamase-active site S-T-F-K and the conserved triad K-T-G are presented in boldface. A proposed cleavage site generating a possible signal sequence is indicated with a vertical arrow.

DNA sequencer (373A; Applied Biosystems). The entire sequence of the gene was determined twice in order to prove the accuracy. The DNA sequence of this integron in both directions was determined by using both PCR primers. A 665-bp fragment was sequenced, with the lower primer showing a high level of homology with the *aadB* gene, whereas the upper primer yielded a 505-bp fragment, which was sequenced and which showed homology with a type OXA β-lactamase. The complete nucleotide sequence obtained by the sequencing strategy described above is presented in Fig. 1. An ATG codon initiated an 825-bp open reading frame, which ended with a TAA codon. The initiation codon was preceded by a Shine-Dalgarno ribosome-binding sequence, AAGGAA. The nucleotide sequence differed from that of OXA-3 β-lactamase by three point mutations, which represented 99.5% similarity. However, two of these three mutations were silent. The gene was flanked at the 3′ end by a short recombination element. At the 5′ end another antibiotic resistance gene, *aadB*, was found, and this gene was also flanked by another recombination element, indicating that the two genes are inserted independently in the integron. Therefore, this *oxa* gene is integrated as a cassette in an integron, similar to other oxacillinases. The deduced amino acid sequence is presented in Fig. 1. The enzyme was 275 amino acids long and began with about 21 hydrophobic residues suitable for a signal peptide. The Ser-Thr-Phe-Lys (positions 72 to 74) and Lys-Thr-Gly (positions 210 to 212) conserved regions corresponding to the enzyme active site and the conserved triad, similar to that found in class A β-lactamases, were observed. The amino acid sequence homology between this β-lactamase and the OXA-3 β-lactamase was 99.6%. In comparison with the OXA-3 sequence (9), the nonsilent mutation generated a substitution of Ile-217 to Met, which is located four amino acids from the conserved triad (Table 1).

TABLE 1. Nucleotide and amino acid changes between OXA-3 and OXA-21

Nucleotide position	Nucleotide change (OXA-3→OXA-21)	Amino acid change (OXA-3→OXA-21)
18	T→C	No change
651	T→G	I→M
696	C→T	No change

The oxacillinases are often plasmid-mediated enzymes which belong to the molecular class D β-lactamases (1) and are included in group 2d of the recent functional classification by Bush et al. (4). They share a number of unusual characteristics and show hydrolytic activity for isoxazolyl penicillins such as oxacillin and cloxacillin and other penicillins such as methicillin. Eighteen oxacillinases have been characterized so far. Phylogenetic data have revealed five groups of class D β-lactamases, with large evolutionary distances between each group (9). Group II includes OXA-2, OXA-3, and OXA-15. The OXA-3 β-lactamase shows properties similar to those of OXA-2 and shares a significant degree of immunological cross-reactivity with OXA-2 (5). Comparison of the amino acid sequence of the β-lactamase investigated in this study with those of OXA-2 and OXA-15 shows similarities of 91 and 90.5%, respectively. Met-217 was found in both OXA-2 and OXA-15 but was not found in OXA-3. The high degree of similarity among this group of class D β-lactamases indicates a common ancestor.

In summary, the present study has described a new OXA-derived β-lactamase in *A. baumannii*. It is the first of its class described in this microorganism. We suggest the designation OXA-21 for the enzyme.

Nucleotide sequence accession number. The nucleotide sequence of the OXA-21 gene has been given EMBL database accession no. Y10693.

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3.1.4 Artículo 4.- "Characterization of an integron carrying a new class D β-lactamase (OXA-37) in Acinetobacter *baumannii*"

Molecular Drug Resistance, 2002; 8(4): 261-265

Se ha analizado una cepa de *Acinetobacter baumannii* altamente resistente a ß-lactámicos, con excepción de amoxi-clavulánico (para la que era intermedia), imipenem y ampi-sulbactam. Por isoelectroenfoque, la cepa presentaba dos ß-lactamasas: una con punto isoeléctrico >8.0 y probablemente correspondiente a una ß-lactamasa cromosómica, y otra con pI de aproximadamente 7,4. Al buscar la presencia integrones de tipo I, se amplificaron dos bandas diferentes: una de 550 pb y otra de 2.2Kb. El integrón más pequeño contenía un solo cassette, con un gen *aac*CI. El más grande, en cambio, presentaba tres cassettes diferentes: un gen *aac*A4 seguido por un marco de lectura abierto (ORF) desconocido y finalmente por una OXA. La caracterización de la oxa mostró que se trataba de un gen muy similar a la oxa-20 descrita en *P.aeuruginosa*, con una mutación en la posición 69 que resulta en la sustitución de un glutámico por un aspártico. La clonación de dicha oxacilinasa y su transformación en una *E.coli* susceptible demostró que parecía tener una buena actividad especialmente sobre la ceftazidima, cefotaxima, ticarcillina y la ampicilina+sulbactam. La nueva oxacilinasa ha recibido el nombre de OXA-37.

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Characterization of an Integron Carrying a New Class D β-Lactamase (OXA-37) in *Acinetobacter baumannii*

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ABSTRACT

Integrons from a clinical strain of *Acinetobacter baumannii* highly resistant to β-lactams have been analyzed. The largest (2.2 kb) contained three gene cassettes: an *aacA*4, an open reading frame of 417 nucleotides, and an OXA-type encoding gene. The *oxa* gene nucleotide sequence differed from that of the *oxa*-20 in 2 bp, one of the mutations being silent. The nonsilent mutation generated a substitution of glutamic acid for aspartic acid. The new OXA has been named OXA-37.

INTRODUCTION

ACINETOBACTER BAUMANNII is a nosocomial pathogen, recognized as being responsible for a wide spectrum of infections including bacteremia, secondary meningitis, pneumonia, and urinary tract infections.[2] It is often resistant to a wide variety of antimicrobials, including cotrimoxazole, quinolones, β-lactam antibiotics, or aminoglycosides. In fact, isolates of *A. baumannii* which are only susceptible to colistin, have been recently described.[12]

Partly responsible for such levels of resistance is the presence of integrons, genetic elements frequently found in Gram-negative bacteria, among which *A. baumannii* is not an exception.[17,19,20] By definition, integrons are flanked by two conserved regions between which gene cassettes are integrated by recombination and can be later rearranged or deleted.[8] Currently, over 59 gene cassettes have been described[7] with most of them encoding proteins involved in antibiotic resistance. At least six unknown open reading frames (ORFs) have also been reported.[9]

Along with plasmids and transposons, integrons are responsible for the movement of resistance genes, among others, both intra- and interspecies. Not all genes conferring antibiotic resistance have been found in integrons. Actually some of them, such as those for chloramphenicol acetyl transferases (*cat* genes), tetracycline resistance (*tet* genes), or certain types of β-lactamase-encoding genes (TEM- or SHV-type genes) are usually encountered in genetic environments different from integrons, such as plasmids. However, a wide variety have already been described to be integron borne, including those responsible for resistance to aminoglycoside (*aad* and *aac* genes), trimethoprim (*dfr*), chloramphenicol (*cml* A), or β-lactam antibiotics.[7]

At least 15 different β-lactamase gene cassettes have been found within integrons.[7] To date, this family of antimicrobial drugs is classified into four major groups, with the OXA-type being included in the Ambler class D,[15] or group 2d from Bush's classification.[6] These are enzymes that hydrolyze cloxacillin faster than benzylpenicillin, have a pI ranging between 5.5 and 9.0, and may have an incomplete inhibition by clavulanic acid.[5,15] OXA-type β-lactamases have been described in different gram-negative microorganisms such as *Pseudomonas aeruginosa, Salmonella typhimurium, Klebsiella pneumoniae,* or *Escherichia coli* (for a review, see ref. 15). As for *A. baumannii*, in 1989 an oxacillin-hydrolizing β-lactamase with a pI of 6.3 was reported to be responsible for the resistance to imipenem in a clinical strain isolated in a French hospital.[10] Recently, a Spanish clinical isolate was described to carry an integron-borne OXA-3-related β-lactamase, named OXA-21,[20] while more recently, an integron-borneOXA-20 has been found in a French strain.[17] Finally, genes of new OXA-type β-lactamases (OXA-23, OXA-24, OXA-25, OXA-26, and OXA-27) presenting carbapenemase activity have been lately found in different strains of *A. baumannii* from various geographical regions.[1,4,16]

While studying a series of clinical strains of *A. baumannii* isolated from different hospitals around Spain, one was found to be highly resistant to β-lactams. The aim of this study was to characterize the integrons from such isolates to determine, if any, their contribution to β-lactams resistance.

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MATERIALS AND METHODS

Microorganism

The *A. baumannii* Spanish clinical strain 74-I included in this study was isolated and identified using conventional methods.[21]

Susceptibility testing

Susceptibility testing of the isolate was performed by E-test to the following β-lactamases: ampicillin, amoxicillin + clavulanic, ampicillin + sulbactam, ceftazidime, piperacillin, cefotaxime, cefoxitin, cefepime, ticarcillin, piperacillin + tazobactam, imipenem, and aztreonam, following manufacturer's indications. Appropriate American Type Culture Collection (ATCC) strains were used as controls. An additional antibiogram was done by disk diffusion in which the following non-β-lactam antimicrobial drugs were included: tetracycline, chloramphenicol, cotrimoxazole, spectinomycin, netilmicin, amikacin, nalidixic acid, and ciprofloxacin.

Isoelectric point determination

β-Lactamase crude extracts were obtained as previously described[20] and analyzed by isoelectric focusing. The exact pI values were determined by comparison with enzymes of known pI values.

Integron amplification and sequencing

The presence of integrons was determined by PCR, using primers and conditions previously described.[11] Amplified products were resolved in a 1.5% agarose gel and stained with 50 μg/ml ethidium bromide. Selected bands were cut from the gel and DNA was recovered using the Gene-Clean kit (Bio 101 Inc., La Jolla, CA). It was then cloned into a pCRII vector and transformed into a competent *Escherichia coli* strain (Invitrogen B.V., Leek, The Netherlands). Plasmid DNA was extracted using the Quantum Prep Plasmid Miniprep Kit (BioRad Laboratories, Richmond, CA). Sequencing reactions were carried out with a Thermosequenase Dye Terminator Sequencing Kit (Amersham, Amersham Place, England) and run in an automatic DNA sequencer (377, Applied Biosystems). The entire integron sequence was determined twice in both senses to prove its accuracy. The sequence's GeneBank accession number is AY007784.

Amplification of the OXA-37 gene and modification of the initiation codon

The OXA-37 gene was amplified by PCR using the following specific primers: upperTTG-*Nco*I, 5′-CCA TGG TTG ATA ATC CGA TTT CTA-3′, and lower-*Pst*I, 5′-CTG CAG CTA GTT GGG TGG C-3′. To change the initiation codon from a TTG to an ATG, a new upper primer was designed and used (upperATG-*Nco*I, 5′-CCA TGG ATG ATA ATC CGA TTT CTA-3′). The PCR program used for both reactions was of 30 cycles with denaturation (94°C), annealling (55°C), and extension (72°C) times of 1 min each.

Cloning of the OXA-37 and the ATG-amplicon

The PCR amplification products (813 bp) were run in a 1.5% agarose gel; the bands were cut and recovered using the Concert Rapid Gel Extraction System kit (Life Technologies, Gaithersburg, MD). The vector used to clone both amplicons was pAlter (Promega, Madison, WI), which confers resistance to chloramphenicol and tetracycline. The cloning procedure was as follows: both plasmid and amplicons were digested with *Pst*I and *Nco*I at 37°C for 2 hr, after which the enzymes were inactivated at 80°C for 20 min. The digestion products were each run in an agarose gel, and the bands corresponding to the open plasmid and the *oxa* genes with sticky ends were cut and recovered. This extra step was done to avoid repairing of the small cut fragments. After an overnight ligation reaction, competent *E. coli* strains (Invitrogen B.V., Leek, The Netherlands) were transformed with one of three plasmids (one containing the OXA-37 (pAOxa 37), a second one with the oxa-37 precursor gene with a modified initiation codon (pAOxa ATG), and finally a pAlter plasmid without an insert to be used as a control). The transformed bacteria were plated onto MH agar supplemented with tetracycline and ampicillin (only in the case of the plasmids containing inserts). In the case of the strain transformed with the pAlter-OXA ATG plasmid, one colony was isolated and plasmid extracted, and sequencing was carried out as previously described, to ensure that the TTG codon had been really changed for an ATG. Susceptibility testing of the transformed *E. coli* was also done using the E-test and determined for the same β-lactams as for the parental strain.

RESULTS

The susceptibility pattern to β-lactams of strain 74I is shown on Table 1. It was highly resistant to most of those tested, with the exception of amoxicillin + clavulanic, to which it was intermediate (16 μg/ml), and ampicillin + sulbactam (3 μg/ml) and imipenem (1 μg/ml), to which it was susceptible. As for the non-β-lactam antimicrobials, the strain showed resistance in front of all of those tested, except for amikacin and spectinomycin, for which it was intermediate.

The extract from strain 74-I contained two β-lactamases: one with a pI above 8.0, probably corresponding to a chromosomal cephalosporinase, and a second one with a pI of about 7.4.

PCR with integron primers resulted in the amplification of two bands: one of about 550 bp and another of about 2.2 kb. Sequencing revealed that the 550-bp amplicon contained an integron with an *aac*CI gene. On the other hand, the largest amplicon was found to contain three gene cassettes. (Fig. 1 shows the complete nucleotide and amino acid sequence of the integron as well as the sequencing strategy followed to obtain it.) On the 5′ end, starting with a GTG codon and ending with a TAA, was an *aac*A4 gene with 552 bp. On the second place, starting with an ATG and ending with a TAG was an unknown open reading frame of 417 bp. Finally, on the 3′ end, there was a cassette with an OXA-type β-lactamase gene of 798 bp. The *oxa* gene here described has a 99.7% nucleotide sequence identity and a 99.6% amino acid identity with that of the OXA-20, first described in *P. aeruginosa*[14] and recently in *A. baumannii*,[17] thus differing in two base pairs and one amino acid, respectively. The mutation that brings about the amino acid change is on nucleotide position 69 and results in a glutamic acid being replaced by an aspartic acid (see Table 2). Both amino acids are negatively charged and could be considered as

TABLE 1. SUSCEPTIBILITY PATTERNS OF STRAIN 74-I AND THE CLONED OXA-37, WITH AND WITHOUT MODIFICATION OF THE INITIATION CODON

Antibiotic	*Strain 74-I MIC (μg/ml)*	*E. coli + pAlter MIC (μg/ml)*	*E. coli + pAoxa37 MIC (μg/ml)*	*E. coli + pAoxaATG MIC (μg/ml)*
Ampicillin	>256	0.25	4 (16×)	6
Ceftazidime	>256	0.023	0.75 (32×)	0.75
Piperacillin	>256	0.25	4 (16×)	3
Cefotaxime	>256	0.004	0.19 (48×)	0.19
Cefoxitin	>256	1	16 (16×)	16
Ticarcillin	>256	0.5	12 (24×)	12
Aztreonam	>256	<0.016	0.19 (>5×)	0.25
Cefepime	>32	<0.002	<0.002	<0.002
Amoxicillin + Clavulanic	16	1	6 (6×)	6
Ampicillin + Sulbactam	3	0.032	3 (93×)	3
Imipenem	1	0.125	0.125	0.125

In parentheses the fold-increase in MIC of the *E. coli* + pAoxa37 when compared to the *E. coli* + pAlter.

practically equivalent. As with the *oxa* 20 gene, this one starts with a low transcription codon, TTG.

To determine how this gene contributes to the resistance of the *A. baumannii* strain, it was amplified, cloned, and transformed into a susceptible *E. coli* strain. The results obtained (Table 1) show that the OXA-37 seems to have a good activity, especially over ceftazidime, cefotaxime, ticarcillin, and ampicillin + sulbactam, for which the MIC was increased in 32, 48, 24, and 93-fold, respectively, when comparing the susceptibilities of the *E. coli* strain with and without the pAlter-Oxa37. This activity did not increase when the low transcription codon TTG was changed to an ATG.

DISCUSSION

Analysis of the integrons in a highly β-lactam resistant isolate of *A. baumannii* was performed. Results showed the presence of two integrons, one containing an *aac*C1 and a second one of about 2,200 bp with three gene cassettes: an *aac*A4, an unknown open reading frame, and an *oxa* gene. All three cassettes were flanked on the 3′-end by recombination hot spot sequences (GTTRRRY), indicating that each of them was inserted into the integron independently.

Two similar integrons have been described recently in literature, in both cases found in French clinical strains. The first one was described in an isolate of *P. aeruginosa*,[14] while the second one was found in an *A. baumannii*.[17] Although the three integrons present a strong similarity, they possess some important internal differences. In the *P. aeruginosa* OXA-20-containing integron, the β-lactamase gene cassette is also preceded by an *aac*A4 gene cassette, which differs from the one here described in base pair 305 (C → T), which brings about one amino acid change (S → T). However, there is no ORF between the two genes, as is the case here. In terms of selection, the loss of the ORF would mean an increase in the expression of the oxacillinase, which would pass from a third to a second place from the integron's promoter. Of course, this would be true if the unknown protein did not in itself pose an advantage. It could be argued, however, that because the OXA has a low transcription initiation codon, and an apparently very small activity against most common β-lactams, bringing it near to the promoter would not be so significant in the end result. In any case, it is considered in general that the probability of losing a cassette from an integron is greater than that of gaining one.[3] On the other hand, Naas *et al.*[14] have analyzed the GC content of the OXA-20 and arrived at the conclusion that with 45% (it is practically the same for OXA-37), it probably had an Enterobacterial origin. However, taking into account the fact that the GC content for *Acinetobacter* is between 38% and 47%,[13] an *Acinetobacter* origin should not be discarded. In fact, we analyzed as well the GC content of the ORF and found it had a 46.43%, which still leaves it within the range described for *A. baumannii*.

When cloned into a susceptible *E. coli* strain, OXA-37 increased the MIC in different levels to all β-lactams tested except for cefepime and imipenem. Although the final MIC attained by the *E. coli* still could be considered as that of a susceptible strain, the increase in certain cases, such as ampicillin + sulbactam, cefotaxime, or ceftazidime was considerable (94×, 48×, and 32×, respectively). If we take into account that *A. baumannii* has to start with a low membrane permeability, which makes it intrinsically more resistant than *E. coli*, the contribution of this *oxa* gene to the acquisition of resistance in this microorganism could be relevant. In a previous study, in which the effect of the *oxa*28 genes in *Pseudomonas* and *E. coli* was compared, Poirel *et al.*[18] observed the same effect.

Thus, the high levels of resistance to β-lactams presented by this strain must be explained by the sum of different mechanisms. It can be partly explained by the presence of the *oxa* gene. However, the intrinsic resistance of *A. baumannii* due to a low membrane permeability plays, as it is well known, a definite role. Besides, the presence of a chromosomal β-lactamase (pI about 8.0) should also be taken into account. As part of an-

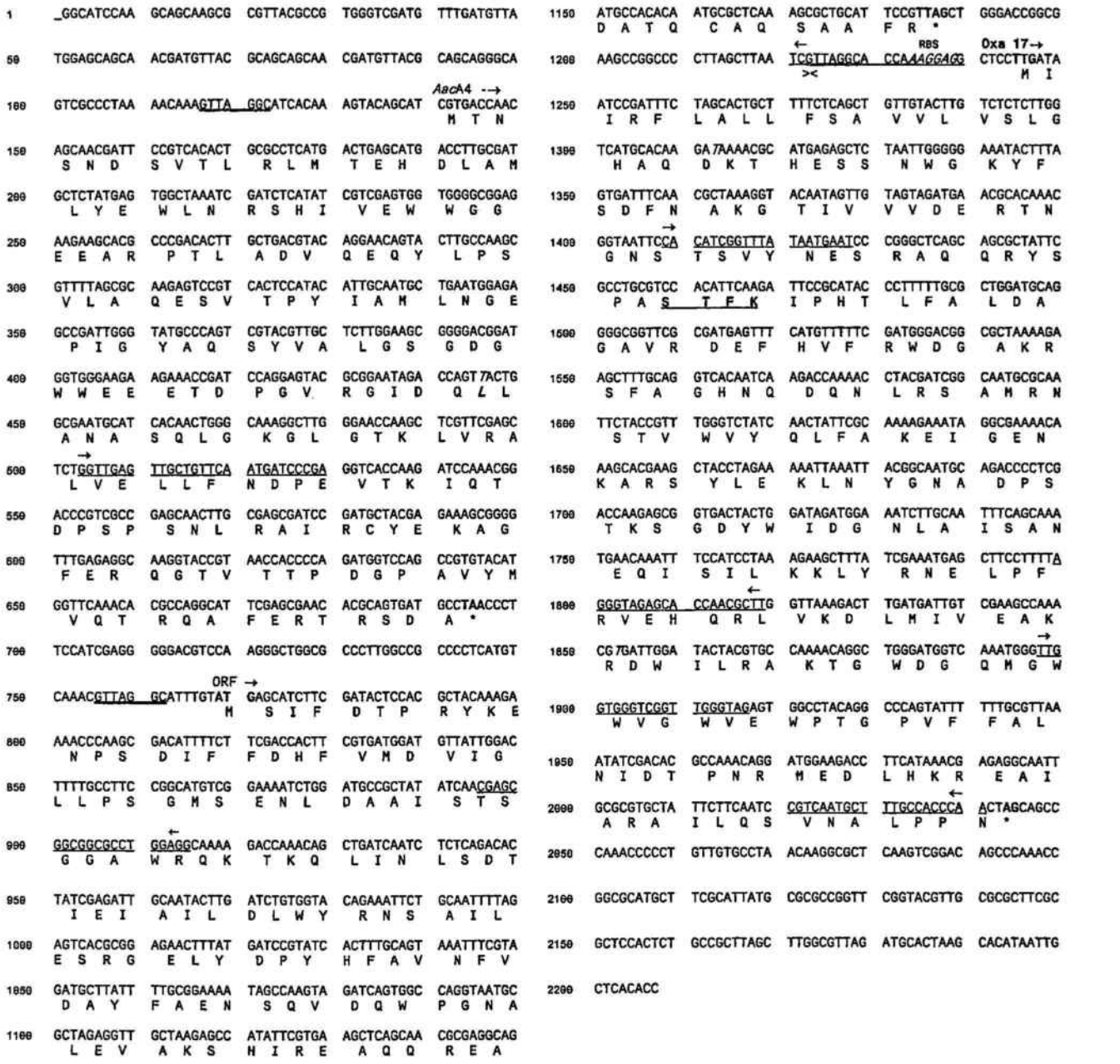

FIG. 1. Nucleotide sequence of the integron and amino acid sequences of its three gene cassettes. The sequences of the primers used to sequence the integron are underlined and the direction is indicated with an arrow. The recombinational hot-spot sequences or composite core sites (GTTRRRY) are double underlined whereas facing arrows indicate the putative integrase cleavage site. The initiation and termination codons of each of the three gene cassettes are shown in boldface. The β-lactamase active sites S-T-F-K and the conserved triad K-T-G are in boldface as well. In the *aac*A4 and *oxa*-37 gene cassettes, the nucleotide changes with respect to those in the *oxa*-20 containing integron sequence are shown in italics, while the amino acid changes are in boldface. RBS stands for the ribosome binding site for *oxa*-37. GenBank accession number is AY007784.

TABLE 2. NUCLEOTIDE AND AMINO ACID CHANGES BETWEEN OXA-20 AND OXA-37

Nucleotide position	*Nucleotide change (OXA 20 → OXA 37)*	*Amino acid change (OXA 20 → OXA 37)*
69	A → T	E → D
609	C → T	No change

other study (Danes *et al.*, unpublished results) the MIC to β-lactams of strain 74I were analyzed in the presence of Syn 2190, an AmpC inhibitor. In the presence of such product, the MIC to piperacillin decreased from >256 to 64, that of ceftazidime was brought down from >256 to 32, and the one to cefepime decreased from >32 to 16. Still, other mechanisms such as the presence of efflux pumps or changes in the penicillin binding proteins (PBPs) cannot be completely discarded. For example,

in this same study, the MIC to imipenem of strain 74I decreased from 1 μg/ml to 0.19 μg/ml in the presence of reserpine, an efflux pump inhibitor.

The putative protein encoded by the ORF here described would have 140 amino acids. The integron described by Ploy *et al.*[17] does contain an ORF (sequence AJ251519) between the acetylase and oxacillinase gene cassettes. However, it has a termination codon 30 bp before the one presented here, thus being 10 amino acids shorter.

The description of three closely related integrons with internal differences shows once again the great plasticity of these elements—the relative facility with which they gain and/or lose gene cassettes. These facts, shown in previous studies,[3] acquire special relevance when considering that an amino acid substitution could in some cases result in an increased or decreased fitness of the microorganism in a particular environment. The ability of integrons to gain or lose gene cassettes and the possibility of them being spread horizontally can also be considered as traits that could contribute to evolution.

In summary, an integron similar to those found first in *P. aeruginosa* and recently in *A. baumannii*, has been encountered in a Spanish clinical strain of the latter species. Once again, the importance of this genetic element in the dissemination of antimicrobial resistance genes both within and between species is being shown. Within the integron, a novel *oxa* gene cassette has been found. Following the international accepted nomenclature, we suggest the designation of OXA-37 for this enzyme.

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3.1.5 Artículo 5.- "Integron Mediated Antibiotic Multiresistance in *Acinetobacter baumannii* Clinical Isolates From Spain."

Clinical Microbiology Infection 2003; 9(9): 907-911

Con el fin de determinar la implicación de los integrones tipo I en los niveles de resistencia a antimicrobianos en cepas clínicas de *A.baumannii*, se analizó la población de un conjunto de siete cepas multirresistentes, con origen geográfico variado. Se encontraron cuatro integrones diferentes, con un total de seis cassettes correspondientes a los genes *aacC1, aacA4, aadA1, aadB, oxa-21* and *oxa-37* más un marco de lectura abierto (ORF). Se observó que el mismo integrón estaba presente en cepas no relacionadas, mientras que cepas epidemiológicamente relacionadas podían contener integrones diferentes

ORIGINAL ARTICLE

Integron-mediated antibiotic multiresistance in *Acinetobacter baumannii* clinical isolates from Spain

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Objective To determine whether non-epidemiologically related, antibiotic-resistant isolates of *Acinetobacter baumannii* from different geographical origins posses common type 1 integrons.

Methods The epidemiologic relationships between seven *A. baumannii* strains recovered from different Spanish hospitals were established by pulsed-field gel electrophoresis, the presence of integrons being determined by PCR and DNA sequencing.

Results Integron analysis showed the presence of four different integrons, containing six different known genes (*aacC1, aacA4, aadA1, aadB, oxa21* and *oxa37*) plus an ORF. It was found that the same integron was present in different unrelated strains and that related strains could have different integrons.

Conclusion These results show the potential risk of integron dissemination among different strains of *A. baumannii*.

Keywords Integron, *Acinetobacter baumannii*, resistance

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INTRODUCTION

An important factor that influences the development of multiresistance is the acquisition of mobile genetic elements. Thus, plasmids and transposons carrying resistance genes have been widely described in the scientific literature [1–7]. Integrons have been identified on these mobile elements.

To our knowledge nine different types of integrons have been described up to date, those included in class 1 being by far the most extensively analyzed [8–10].

Class 1 Integrons are composed of three different elements. Two conserved regions: an integrase encoding gene in the 5′ segment (5′CS) and the genes *qacE1*, *sulI* and the *orf5* in the 3′ segment (3′CS). Between these conserved regions a variable region is found in which gene cassettes are inserted [2]. Up to three different gene cassettes are commonly found inserted in one integron. However, exceptionally, a higher number of genes can be found [8]. Furthermore, composed integrons possessing a second 3′CS have also been described. Such structures posses a common ORF (*orf513*) after the first sulfonamide-resistance gene, carrying different resistance genes between this ORF and the second 3′CS [11,12].

Acinetobacter baumannii is the most frequent and clinically important species of the genus *Acinetobacter* [1], usually presenting multiple antibiotic resistance [1,3,13–17]. Different reports identifying integrons as responsible for the presence and acquisition of antibiotic resistance in *A. baumannii* have been published [6,10,18–22]. The presence of type 1 integrons carrying aminoglycoside-resistance genes (*aadB, aacA4, aacC1*) or β-lactamases encoding genes as *oxa21, oxa24* or *oxa37* in *A. baumannii* from Spain have also been previously reported [10,18,22].

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The aim of this study was to investigate the role of type 1 integrons in mediating antibiotic resistance in *A. baumannii*, analyzing whether non-related isolates from different geographical origins possessed common integrons.

MATERIALS AND METHODS

Microorganisms

Seven strains of *A. baumannii* obtained from six different Spanish hospitals (Table 1) were randomly chosen to perform this study. All the strains were characterized as *A. baumannii* according to the criteria of Bouvet and Grimont [23].

Antimicrobial Susceptiblity Testing

Susceptibility to ampicillin, ceftazidime, imipenem, amoxicillin plus clavulanic acid, cotrimoxazole, tetracycline, chloramphenicol, spectinomycin, netilmicin, amikacin, nalidixic acid and ciprofloxacin was determined by the disk diffussion method in accordance with the NCCLS guidelines [24].

Epidemiologic relationships

The genetic relationship of these clinical isolates was established with low-frequency restriction analysis of chromosomal DNA using *Apa*I. Plugs were prepared following the procedure of Gautom [25], and the DNA fragments were separated in a 1% agarose pulsed field gel electrophoresis (PFGE). The electrophoresis conditions were 200 V, 20 h, with pulse times ranging from 5 to 8 seconds. Banding patterns were digitized and stored as TIFF files. Patterns were analyzed using the Lane Manager software (TDI, Madrid, Spain) to calculate Dice coefficients of correlation and to generate a dendrogram by the unweighted pair group method using arithmetic averages (UPGMA) clustering.

Amplification of integrons

PCR amplification of type 1 integrons was done with the set of primers described by Levesque and Roy [27], following previously described conditions and procedures [18]. The amplified products were resolved in 2% agarose gel and stained with ethidium bromide, 0.5 mg/L. The bands were recovered from the gel using the Gene-Clean kit (Bio101, Inc., La Jolla, CA, USA), cloned in a pCRII vector, and transformed into *Escherichia coli* competent cells (Invitrogen BV, Leek, The Netherlands).

DNA Sequencing procedures

Plasmid DNA, with the cloned integrons, was extracted and directly sequenced with the Thermosequenase Dye Terminator Sequencing kit (Amersham, Cleveland, OH, USA) in an automatic DNA sequencer (377; Applied Biosystems,

Table 1 Source and epidemiologic relationships between the strains

Strain/Year	Hospital/City	Drug Resistances	PFGE type	Integron type
87/1988	HCP/Barcelona	Cm, Amp, Caz, A/C	B	1700/*aadB, oxa-21*
74I/1997	HLP/Madrid	Tc, Cm, Sxt, Spt, Net, Ak, Amp, Caz, A/C, Nal, Cip	D	550/*aacC1* 2100/*aacA4, ORF, oxa37*
875/1995	HVR/Sevilla	Tc, Cm, Sxt, Spt, Net, Ak, Amp, Caz, Imp, A/C, Nal, Cip	C	550/*aacC1* 750/*aadB*
6R/1997	HDO/Madrid	Tc, Cm, Sxt, Spt, Net, Ak, Amp, Caz, Imp, A/C, Nal, Cip	A	550/*aacC1* 750/*aadB* 1700/*aadB, oxa21*
F_{14}/1990	HVH/Barcelona	Tc, Cm, Sxt, Spt, Ak, Amp, Caz, A/C, Nal, Cip	A	>2Kb/*aacA4, oxa21, aadA1*
709R/1997	HSJ/Reus	Tc, Cm, Sxt, Spt, Net, Ak, Amp, Caz, A/C, Nal, Cip	A	550/*aacC1* 750/*aadB*
203/1997	H.LP/Madrid	Cm, Spt, Net, Ak, Amp, Caz, A/C, Nal, Cip	B	750/*aadB*

Tc, Tetracycline; Cm, Chloramphenicol; Sxt, Cotrimoxazole; Spt, Spectinomycin; Net, Netilmycin; Ak, Amikacin; Amp, Ampicillin.
Caz, Ceftazidime; Imp, Imipenem; A/C, Amoxicillin plus clavulanic acid; Nal, Nalidixic acid, Cip, Ciprofloxacin.
HCP, Hospital Clínic i Provincial; HDO, Hospital Doce de Octubre; HLP, Hospital La Princesa; HSJ, Hospital Sant Joan; HVH, Hospital Vall d'Hebró; HVR, Hospital Virgen del Rocio.

Foster City, CA, USA). The sequencing strategy included an initial sequencing with the primers originally used to amplify the integrons and the posterior designing of novel primers to move downstream into the more central gene cassettes.

RESULTS

All seven *A. baumannii* isolates were resistant to chloramphenicol, ampicillin, amikacin, ceftazidime and amoxicillin plus clavulanic acid. Only isolate 87 showed susceptibility to nalidixic acid, ciprofloxacin, tetracycline, cotrimoxazole, spectinomycin, Finally isolates 87 and F14 both showed susceptibility to netilmicin (Table 1).

The application of the Dice coefficient to the results obtained by low-frequency restriction analysis of chromosomal DNA and PFGE showed the presence of four different profiles, which were arbitrarily named A to D. The criteria used to define clonal relations among strains was based on a difference of less than three bands, corresponding in the dendrogram to 85% similarity (Figure 1). Group A was composed of three strains (6R, F_{14} and 709R) group B included two strains (87 and 203) while the remaining groups were all of single isolates.

PCR amplification with integron-specific primers (Figure 2) resulted in the amplification of two common bands of circa 550 bp and 750 bp respectively in 5 out of 7 strains. Upon sequencing, the smallest band was found to be an integron carrying an *aacC1* gene, while the larger one carried a single *aadB* gene. Two unrelated strains (87 and 6R) carried an integron with a molecular size of 1700 bp containing two genes: an *aadB* and an *oxa21*. The two largest integrons, with a 2.1 kb and >2.1 kb were found in isolates 74I and F_{14} respectively. The 2.1 kb integron contained an *aacA4* and an *oxa37*, plus a putative unknown protein encoding region with a longer sequence than AJ251519. The largest one with >2.1 kb contained three genes.an *oxa21*, *aadA1* and an *aacA4* (Table 1).

DISCUSSION

Many different genes responsible for antibiotic resistance have been found in integrons, among which those coding for different aminoglycoside-modifying enzymes, β-lactamases or dihydrofolate reductases can be found [2,8,18,19,27]. Moreover, some different ORF with unknown functions have been described [8,20,27,28]. In this study, five different integrons have been found, all of them presented at least one gene encoding an aminoglycoside-modifying enzyme. The *aacC1* and the *aadA1* genes were only found in one type of integron each, whereas *aadB* and *aacA4* genes

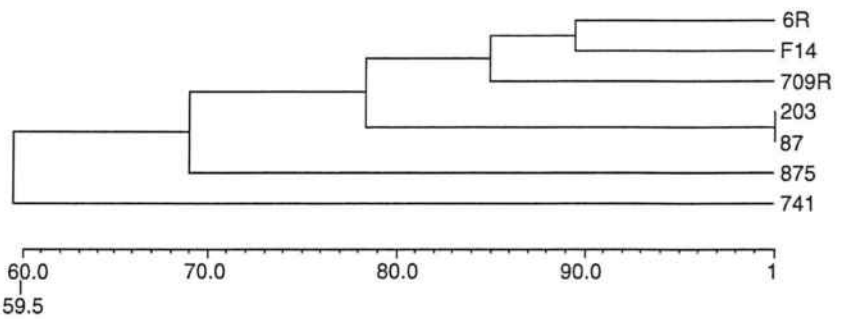

Figure 1 Dendrogram of the clonal relationship of the selected strains.

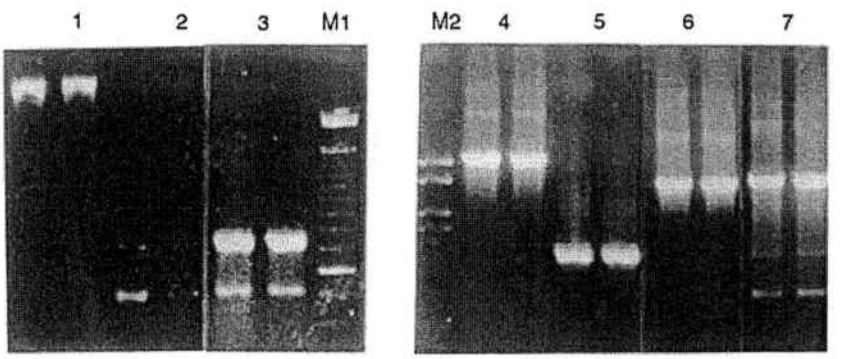

Figure 2 PCR amplification of integrons. PCR amplification of integrons. Lane M Molecular weight marker (100 bp ladder, GIBCO BRL, Gaithersburg, MD), lane 1, strain 875, lane 2, strain 6R; lane 3, strain 87; lane 4, strain 709; lane 5, strain 74I; lane 6, strain 203; lane 7, strain F_{14}.

were each located in two different sized integrons. As for the β-lactamases, an *oxa21* gene was found in two different integrons, while an *oxa37* gene was located in one. This high prevalence of aminoglycoside-modifying enzymes in *A. baumannii* integrons is in accordance with Bissonnette and Roy [8], who described *aadA1* and *aadA2* genes as being the most frequently found cassettes in multiresistance integrons. Furthermore, these genes have been previously described in *A. baumannii* clinical isolates from other European countries, suggesting wide dissemination [19,20]. In a similar way, the most frequently encoded β-lactamase genes in these genetic elements include the OXA type [7,8,18,19]. Once more these results are in agreement with what has been published regarding *A. baumannii* integrons, among which a high prevalence of both aminoglycoside-modifying enzymes and β-lactamases have been found [10,18–20].

Our results point out the possibility that unrelated strains, with a different geographical origin, acquire the same integron, as shown with the common integrons of circa 550 bp (present in strains belonging to all the different PFGE-types) and 750 bp (from strains belonging to the PFGE-types A, B, and C) as well as the integron carrying an *aadB* and an *oxa21* of circa 1700 bp present in strains of type A and B. Interestingly, these results also show that related strains may possess unrelated integrons. Thus, the two isolates belonging to type B (strains 87 and 203) had a different integron profiles. Similarly, isolate F_{14} had an integron >2 kb that was absent from the other isolates of type A.

The integrons found in our strains carried genes that were identical or closely related to others that have been previously found in integrons from other organisms such as *Pseudomonas aeruginosa* [5,20,27,29]. Both *A. baumannii* and *P. aeruginosa* are important nosocomial pathogens living in environments with high antibiotic pressure such as intensive care units, and thus frequently showing multiresistance. The close similarity between the integrons present in *P. aeruginosa* and *A. baumannii*, as for example that found in isolate 74I, suggest the transfer of genetic elements between these two microorganisms, which could be plasmid-mediated as has previously been observed in *Salmonella* [4]. Further studies are required to elucidate the process by which microorganisms evolve towards multirresistance.

In conclusion, our results show that integrons can play an important role in the acquisition of multiresistance in *A. baumannii*, especially resistance to β-lactam and aminoglycoside antibiotics, and suggest the potential transfer of genetic material between *A. baumannii* and *P. aeruginosa*.

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3.2 *Shigella* spp.

3.2.1 Artículo 6.- "Typing and Characterization of the Mechanisms of Resistance of *Shigella* spp. isolated from faeces of children under 5 years of age in Ifakara, Tanzania."

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Ochenta y seis cepas de *Shigella* spp.(78 *S.flexneri*, 4 *S.dysenteriae*, 4 *S.sonnei*) aisladas durante la época seca en niños menores de 5 años con diarrea en Ifakara, Tanzania, fueron analizadas para determinar su relación epidemiológica, sus niveles de resistencia así como los mecanismos implicados en los fenotipos multirresistentes. La relación epidemiológica se determinó mediante REP-PCR y análisis de ADN cromosómico por digestión con enzimas de restricción de baja frecuencia de corte seguida por electroforesis en campo pulsante. Los resultados obtenidos con ambas metodologías fueron iguales. Se halló un grupo mayoritario compuesto por 51 cepas de *S.flexneri,* considerado como la cepa epidémica. Las restantes cepas de *S.flexneri* se encontraron agrupadas dentro de otros 3 grupos, mientras que las cepas de *S.sonnei* y las de *S.dysenteriae* estaban agrupadas todas dentro de un grupo cada especie. Se realizó un tercer análisis del perfil plasmídico de las cepas. Entre las cepas de *S.flexneri* se encontraron 8 perfiles diferentes, que diferían entre sí por tres a seis bandas. Los niveles de resistencia observados fueron altos, especialmente entre las cepas de *S.flexneri* que presentaron un 92% de resistencia a la ampicilina y al cloranfenicol, un 99% a la tetraciclina, y un 91% al cotrimoxazol. Las cepas de *S.dysenteriae* eran 100% resistentes a los cuatro antimicrobianos, mientras que las cepas de *S.sonnei* eran todas susceptibles al cloranfenicol, solo una de ellas resistente a la ampicilina aunque todas resistentes a la tetraciclina y al cotrimoxazol. Al analizar los mecanismos de resistencia a la ampicilina, se observó que en el caso de *S.flexneri* se explicaba por la presencia de una ß-lactamasa tipo OXA-1 (p.I.7.0) en el 75% de los casos, y por una TEM en el 25% restante. Las cepas de *S.dysenteriae* también presentaban una OXA-1 mientras que la única cepa de *S.sonnei* resistente a ampicilina presentaba una TEM. Todas las cepas resistentes al cloranfenicol poseían actividad cloranfenicol

acetil transferasa, mientras que la resistencia al cotrimoxazol en la mayoría de las cepas se explicó por la presencia de una *dfr*A1

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Typing and Characterization of Mechanisms of Resistance of *Shigella* spp. Isolated from Feces of Children under 5 Years of Age from Ifakara, Tanzania

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Eighty-six strains of *Shigella* spp. were isolated during the dry season from stool samples of children under 5 years of age in Ifakara, Tanzania. The epidemiological relationship as well as the antimicrobial susceptibility and mechanisms of resistance to ampicillin, chloramphenicol, and co-trimoxazole were investigated. Four different epidemiological tools, pulsed-field gel electrophoresis (PFGE), repetitive extragenic palindromic (REP)-PCR, plasmid analysis, and antibiogram, were compared for typing *Shigella* strains. Seventy-eight (90%) strains were *Shigella flexneri* and were distributed into four groups, by either PFGE or REP-PCR, with 51, 17, 7, and 3 strains. The four strains of *Shigella dysenteriae* belonged to the same group, and the four strains of *Shigella sonnei* were distributed in two groups with three and one strain each. Plasmid analysis showed a high level of heterogeneity among strains belonging to the same PFGE group, while the antibiogram was less discriminative. REP-PCR provided an alternative, rapid, powerful genotyping method for *Shigella* spp. Overall, antimicrobial susceptibility testing showed a high level of resistance to ampicillin (81.8%), chloramphenicol (72.7%), tetracycline (96.9%), and co-trimoxazole (87.9%). Ampicillin resistance was related to an integron-borne OXA-1-type β-lactamase in 85.1% of the cases and to a TEM-1-type β-lactamase in the remaining 14.8%. Resistance to co-trimoxazole was due to the presence of a *dhfr Ia* gene in all groups except one of *S. flexneri*, where a *dhfr VII* gene was found within an integron. Chloramphenicol resistance was associated in every case with positive chloramphenicol acetyltransferase activity. All strains were susceptible to nalidixic acid, ciprofloxacin, ceftazidime, cefotaxime, and cefoxitin. Therefore, these antimicrobial agents may be good alternatives for the treatment of diarrhea caused by *Shigella* in Tanzania.

Acute infectious diarrheal disease is one of the most frequent causes of childhood deaths in the developing world. Diarrheal disease accounts for approximately 25% of all deaths in children younger than 5 years of age in these areas (21). Infections caused by *Shigella* species are an important cause of diarrheal disease, in both developing and developed countries. Worldwide, it is estimated that shigellosis causes around 600,000 deaths per year, two-thirds of the deceased being children under 10 years of age. *Shigella dysenteriae* and *Shigella flexneri* are the predominant species in the tropics, while *Shigella sonnei* is the predominant species in industrialized countries (18).

Shigellosis is one of the acute diarrheal diseases for which antimicrobial therapy is effective. However, today it also presents a pressing challenge, as *Shigella* spp. have progressively become resistant over the past decades to most of the widely used and inexpensive antimicrobials (21). Thus, the history of the genus suggests that resistance will emerge to any antimicrobial agent used intensively (25). Antimicrobial resistance in enteric pathogens is of the greatest importance in the developing world, where the rate of diarrheal diseases is highest and indiscriminate use of antimicrobial agents is a fact.

The comparative analysis of different epidemiological markers is important in order to know which is the best for tracing the source of infection during an outbreak. Several conventional typing methods and newly introduced molecular biology typing techniques have been described (3, 5, 11, 13). On the other hand, the study of the mechanisms of resistance of the resistant pathogenic bacteria may provide insight into the means by which multiple resistance is spreading among the bacterial population.

The aim of this article is to characterize *Shigella* strains isolated from children under 5 years of age in Ifakara, Tanzania. The work includes comparative epidemiological typing with various epidemiological tools, as well as a determination of antimicrobial susceptibility and the molecular characterization of the mechanisms of resistance to ampicillin, chloramphenicol, and co-trimoxazole.

MATERIALS AND METHODS

Bacterial strains. Eighty-six strains of *Shigella* spp. were isolated from stool samples of children under 5 years of age during the dry period (July to September) of 1997 in Ifakara, Tanzania. The children included in the study were seen at Saint Francis Designated District Hospital. *Shigella* spp. were identified by conventional methods (16) and by serotyping. All the strains with different plasmid patterns or antibiograms were investigated in detail to determine their mechanisms of resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole.

Antimicrobial susceptibility testing. Susceptibility testing was performed by an agar diffusion disk method as recommended by the National Committee for Clinical Laboratory Standards (17). Mueller-Hinton agar was obtained from Becton Dickinson (Cockeysville, Md.), and antimicrobial disks were obtained

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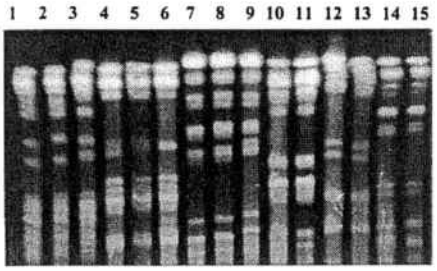

FIG. 1. PFGE. Lanes 1, 2, and 3, *S. flexneri* strains belonging to group F-I; lanes 4, 5, and 6, *S. flexneri* strains belonging to group F-II; lanes 7, 8, and 9, *S. flexneri* strains belonging to group F-III; lanes 10 and 11, *S. flexneri* strains belonging to group F-IV; lanes 12 and 13, *S. dysenteriae* strains; lanes 14 and 15, *S. sonnei* strains.

from BBL Microbiology Systems (Cockeysville, Md.). *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms and tested weekly. Each time a new batch of Mueller-Hinton agar was introduced, *Enterococcus faecalis* ATCC 29212 was tested to detect the presence of inhibitors of trimethoprim-sulfamethoxazole. The MICs of ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, tetracycline, cefoxitin, cefotaxime, ceftazidime, nalidixic acid, and ciprofloxacin for the selected strains were determined by E-test strips (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar plates, following the manufacturer's instructions. *E. coli* ATCC 25922 was used as a reference strain for quality control.

Low-frequency restriction analysis of chromosomal DNA by PFGE. Genomic DNA was prepared as described previously (15), digested with *Xba*I, and separated in 1% agarose gels with a contour-clamped homogeneous-field apparatus (CHEF-DR2; Bio-Rad). It was run under 200 V, with the pulse time increasing from 5 to 8 for 20 h. Pulsed-field gel electrophoresis (PFGE) patterns were interpreted by using the criteria established by Tenover et al. (26).

REP-PCR. Repetitive extragenic palindromic (REP)-PCR was carried out following the method previously described by Gallardo et al. (6), with some modifications. Briefly, the primer 5' GCG CCG ICA TGC GGC ATT 3' was used under the following conditions: 30 cycles of 1 min at 94°C, 1 min at 40°C, and 1 min at 65°C, with a final extension of 16 min at 65°C. The reaction was prepared with 5 μl of boiled bacterial suspension, 1 μl of 5 mM primer, and PCR beads (Pharmacia A.B., Uppsala, Sweden). Fifteen microliters of the PCR products was separated in a 12.5% precast polyacrylamide gel with a Genephor apparatus (Pharmacia) and silver stained.

Plasmid analysis. Plasmid DNA was extracted from overnight bacterial cultures with the commercial kit Wizard Plus SV Minipreps DNA purification system (Promega, Madison, Wis.) according to the manufacturer's instructions. The plasmids obtained were visualized and analyzed by 0.8% agarose gel electrophoresis.

β-Lactamase detection. β-Lactamase analysis was performed by the following methods.

(i) Isoelectrofocusing. Isoelectrofocusing was performed as described elsewhere (6). Gels were run in a PhastSystem apparatus (Pharmacia A.B.) and developed with nitrocefin, and the isoelectric points were determined. Several strains carrying β-lactamases of known pI were used as controls and focused in parallel with the extracts.

(ii) PCR. All PCR amplifications of the different β-lactamase genes were carried out in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Emeryville, Calif.), using the primers previously described (6) and under the following conditions: 30 cycles of denaturation at 94°C, annealing at 55°C, and extension at 72°C, plus a final extension of 7 min at 72°C. The PCR product was run and visualized in 0.7% agarose gels stained with ethidium bromide.

Chloramphenicol acetyltransferase detection. The chloramphenicol acetyltransferase activity assay was performed as described elsewhere (2), with slight modifications (6). Briefly, the strains were grown overnight on MacConkey agar. A heavy suspension of bacteria in 0.2 ml of 1 M NaCl, 0.01 M EDTA, and 0.05% sodium dodecyl sulfate (pH 8) was incubated in an Eppendorf tube at 37°C for 60 min. After a short centrifugation in a microcentrifuge, 50 μl was transferred to a microtitration plate. Duplicate wells were prepared with each strain, and 50 μl of a solution containing two parts 0.2 M Tris-HCl (pH 8), 2 mM acetyl coenzyme A, and one part 10 mM 5,5-dithio-bis-(2-nitrobenzoic acid) in 0.1 M Tris-HCl, pH 8, was added to each well. A 50-μl amount of 5 mM sterile chloramphenicol (dissolved in water) was added to one well (test reaction), and an equivalent amount of water was added to the duplicate well (control). The plate was reincubated at 37°C for 5 min. The reaction was stopped by adding 1 N H_2SO_4 and read spectrophotometrically.

Detection of trimethoprim resistance genes. Both *dhfr Ia* and *dhfr VII* genes were amplified under the same conditions used for β-lactamases and with the following primers: *dhfr Ia* upper (5' GTG AAA CTA TCA CTA ATG G 3') and lower (5' TTA ACC CTT TTG CCA GAT TT 3') and *dhfr VII* upper (5' TTG AAA ATT TCA TTG ATT G 3') and lower (5' TTA GCC TTT TTT CCA AAT CT 3'). The sizes of the PCR products for both genes were the same, 474 bp, and included the entire gene.

Integron amplification and cloning. Reaction mixtures for integron amplification were prepared in the same way as those for β-lactamase PCR but with the following primers: upper (5' GGC ATC CAA GCA GCA AG 3') and lower (5' AAG CAG ACT TGA CCT GA 3') (10). The conditions for amplification were as follows: 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 8 min, plus a final extension of 72°C for 16 min. Twenty-five microliters of the amplified products was run in a 1.5% agarose gel and stained with ethidium bromide. The bands were excised from the gel, and the DNA was recovered with a GeneClean kit (Bio 101, Inc., La Jolla, Calif.) and cloned into pCRII vector (Invitrogen BV, Leek, The Netherlands).

DNA sequencing. Plasmid extraction was performed as described above. The sequencing of the plasmids with the cloned inserts was done with a Thermosequenase dye terminator sequencing kit in an automatic DNA sequencer (model 377; Applied Biosystems, Perkin-Elmer, Emeryville, Calif.) following the manufacturer's instructions.

RESULTS

The eighty-six strains of *Shigella* spp. that were isolated were distributed as follows: 78 (90%) were *S. flexneri*, 4 (4.6%) were *S. dysenteriae*, and 4 (4.6%) were *S. sonnei*. No *Shigella boydii* strains were isolated. The 78 *S. flexneri* strains were grouped into four epidemiological groups by PFGE or REP-PCR (Fig. 1 and 2). The distribution of strains according to these epidemiological markers was as follows: 51 strains in group F-I, 17 strains in group F-II, 7 strains in group F-III, and 3 strains in group F-IV. However, the four major *S. flexneri* groups were subdivided into nine different subgroups based on antibiogram and plasmid analysis (Table 1). Eight different plasmid patterns were obtained among *S. flexneri* strains (Fig. 3). These patterns contained from three to six different plasmids each, although in some cases the difference between two patterns was due to the gain or loss of only one plasmid.

On the basis of antibiotic susceptibility, six phenotypes were defined: phenotype I (Amp^r Cm^r Tet^r Sxt^r), phenotype II (Amp^s Cm^s Tet^s Sxt^s), phenotype III (Amp^s Cm^s Tet^r Sxt^s), phenotype IV (Amp^s Cm^s Tet^r Sxt^r), phenotype V (Amp^r Cm^s Tet^r Sxt^r), and phenotype VI (Amp^r Cm^r Tet^r Sxt^s). In spite of belonging to the same clone by PFGE, REP-PCR, or plasmid analysis (Fig. 1 to 3), the four strains of *S. sonnei* were distrib-

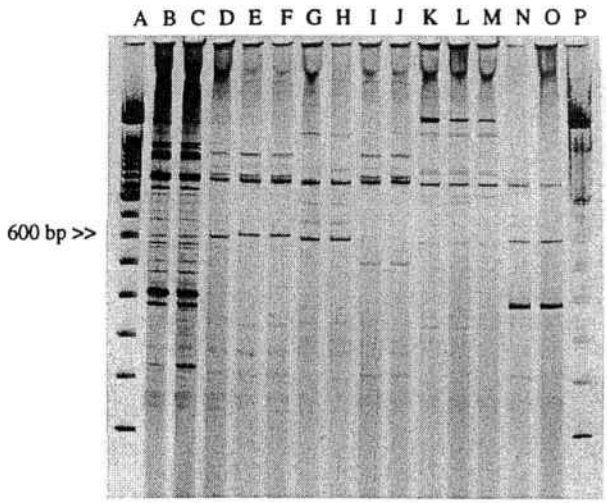

FIG. 2. REP-PCR. Lanes A and P, molecular size markers; lanes B and C, *S. flexneri* strains belonging to group F-II; lanes D, E, and F, *S. flexneri* strains belonging to group F-I; lanes G and H, *S. flexneri* strains belonging to group F-III; lanes I and J, *S. flexneri* strains belonging to group F-IV; lanes K, L, and M, *S. dysenteriae* strains; lanes N and O, *S. sonnei* strains.

TABLE 1. Characterization of *Shigella* spp. by four different epidemiological markers

Species	Group[a] (no.)	Subgroup[b] (no.)	PFGE	Antibiogram[c]	Plasmid profile	REP-PCR group
S. flexneri	F-I (51)	F_1 (46)	A	I	b	1
		F_2 (4)	A	I	a	1
		F_3 (1)	A	II	c	1
	F-II (17)	F_4 (14)	B	I	d	2
		F_5 (3)	B	VI	e	2
	F-III (7)	F_6 (5)	C	I	f	3
		F_7 (2)	C	III	g	3
	F-IV (3)	F_8 (1)	D	IV	h	4
		F_9 (2)	D	III	h	4
S. sonnei	S-I (4)	S_1 (3)	F	IV	j	5
		S_2 (1)	F	V	j	5
S. dysenteriae	D-I (4)	D_1 (4)	E	I	i	6

[a] Distribution of strains based on PFGE and REP-PCR.
[b] Distribution of groups according to antibiogram and plasmid analyses.
[c] See text for phenotypes.

uted in two groups based on the antibiogram. Three strains showed phenotype IV (group S1), and one strain showed phenotype V (group S2). The four strains of *S. dysenteriae* were all the same clone (Table 1).

Fourteen *S. flexneri* strains, three *S. sonnei* strains, and three *S. dysenteriae* strains were used for detailed investigations of the mechanisms of resistance to ampicillin, chloramphenicol, and co-trimoxazole. The MICs of ampicillin, chloramphenicol, tetracycline, co-trimoxazole, nalidixic acid, ciprofloxacin, ceftazidime, cefotaxime, and cefoxitin for these strains are shown in Table 2. For all the strains resistant to ampicillin, chloramphenicol, and tetracycline, the MICs of the drugs were >256 μg/ml, and for those resistant to co-trimoxazole, the MIC was >32 μg/ml. Overall, 92% of *S. flexneri* strains were resistant to ampicillin and chloramphenicol, 99% were resistant to tetracycline, and 91% were resistant to co-trimoxazole. *S. dysenteriae* strains were 100% resistant to ampicillin, chloramphenicol, tetracycline, and co-trimoxazole. While *S. sonnei* strains were all susceptible to chloramphenicol, only one of four strains was resistant to ampicillin and all showed resistance to tetracycline and co-trimoxazole. All *Shigella* sp. strains tested were susceptible to nalidixic acid, ciprofloxacin, cefotaxime, ceftazidime, and cefoxitin (Table 2).

Isoelectric focusing was used first to detect the production of β-lactamase, and PCR with specific primers was used to corroborate the results, which are shown in Table 3. The ampicillin resistance of *S. flexneri* was explained in 75% of the cases by the presence of an OXA-1-type β-lactamase (pI 7.0), whereas the remaining 25% had a TEM-1-type β-lactamase (pI 5.4). The *S. dysenteriae* clone also carried an OXA-1-type β-lactamase, whereas the ampicillin-resistant *S. sonnei* strain had a TEM-1-type β-lactamase. In all cases, the OXA-1-type β-lactamase was located in an integron (data not shown).

All strains resistant to chloramphenicol showed chloramphenicol acetyltransferase activity (Table 3), and also, all co-trimoxazole-resistant strains presented genes encoding dihydrofolate reductases (Table 3). Four of five co-trimoxazole-resistant *S. flexneri* epidemiological groups showed the *dhfr Ia* gene, and the fifth group showed a *dhfr VII* gene, while co-trimoxazole-resistant *S. sonnei* and *S. dysenteriae* strains also had the *dhfr Ia* gene.

DISCUSSION

The predominant species of *Shigella* during the studied period of time was *S. flexneri*, which is usually the predominant species in areas of endemicity, accounting for 50% of culture-positive disease (25). *S. sonnei* and *S. dysenteriae* were found in the same proportions. The most common typing procedures currently used with *Shigella* spp. are plasmid analysis and PFGE (7, 8, 12, 13, 24). *Shigella* species usually harbor a heterogenous population of plasmids, ranging in number from 2 to as many as 10 (9). Plasmid analysis has proven to be a useful typing technique (7, 8). Moreover, it is inexpensive and quick to perform, but it can be limiting if we take into account the fact that many plasmids are unstable and may be easily gained and/or lost. PFGE has a high discriminatory power, although it is cumbersome and expensive. However, it has been widely used for typing *Shigella* spp. (13, 24). Taking PFGE as a reference epidemiological tool, strains belonging to the same PFGE group but having different plasmid profiles and different antibiograms were observed (for instance, subgroups F_4 and F_5). Therefore, the mechanisms of resistance are probably carried in the missing plasmid. The contrary is also true; two strains belonging to the same PFGE group with the same plasmid profile showed different antibiograms (for instance, subgroups F_8 and F_9). This is probably due to an integron or transposon carrying the resistance gene integrated in the chromosome.

Recently, Liu et al. (13) compared plasmid profiles, PFGE, and enterobacterial repetitive intergenic consensus PCR for typing 20 clinical isolates of *S. sonnei*. PCR-based techniques have the advantages of being quick and easy to perform, and in this case they proved to be as good at discriminating epidemiologically related strains as PFGE. We found something similar with REP-PCR, another PCR-based technique, in which the amplification of the regions between REP sequences gives a good fingerprinting pattern valid for epidemiological typing. As long as the protocol is strictly followed and conditions are kept constant, this technique provides a degree of discrimination equivalent to that of PFGE with the advantages of speed, simplicity, and economy. To our knowledge, this is the first time that such a technique has been used in comparison with PFGE and plasmid profiles to type different species of *Shigella*.

Antimicrobial susceptibility testing showed a high degree of resistance to antibiotics most commonly used in the area (tetracycline, ampicillin, co-trimoxazole, and chloramphenicol). No resistance to quinolones and cephalosporins was observed,

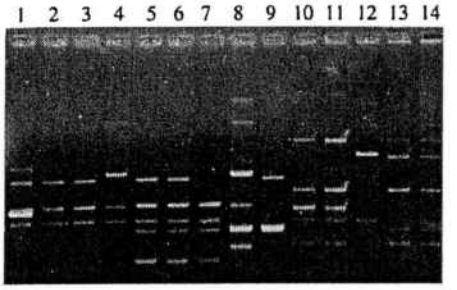

FIG. 3. Plasmid patterns. Lane 1, *S. flexneri* strain belonging to subgroup F_2; lanes 2 and 3, *S. flexneri* strains belonging to subgroup F_1; lane 4, *S. flexneri* strain belonging to subgroup F_3; lanes 5 and 6, *S. flexneri* strains belonging to subgroup F_4; lane 7, *S. flexneri* strain belonging to subgroup F_5; lane 8, *S. flexneri* strain belonging to subgroup F_6; lane 9, *S. flexneri* strain belonging to subgroup F_7; lanes 10 and 11, *S. flexneri* strains belonging to subgroups F_8 and F_9; lane 12, *S. dysenteriae*; lanes 13 and 14, *S. sonnei* strains belonging to subgroups S_1 and S_2.

TABLE 2. Antimicrobial susceptibility of *Shigella* spp.

Antimicrobial agent	MIC (μg/ml)											
	S. flexneri									*S. sonnei*		*S. dysenteriae*
	F_1[a]	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	S_1[b]	S_2	D_1[c]
Ampicillin	>256	>256	1	>256	>256	>256	1.5	1.5	0.5	2	>256	>256
Tetracycline	>256	>256	1	>256	>256	>256	>256	>256	>256	>256	>256	>256
Chloramphenicol	>256	>256	1	>256	>256	>256	2	1	0.75	1.5	3	>256
Co-trimoxazole	>32	>32	0.032	>32	0.38	>32	0.125	>32	0.032	>32	>32	>32
Nalidixic acid	2	4	1.5	1.5	1.5	4	3	2	2	3	4	3
Ciprofloxacin	0.008	0.016	0.006	0.006	0.006	0.008	0.008	0.016	0.016	0.012	0.008	0.012
Ceftazidime	0.094	0.19	0.094	0.094	0.094	0.094	0.064	0.064	0.064	0.094	0.064	0.094
Cefotaxime	0.064	0.125	0.064	0.064	0.064	<0.016	0.094	0.064	0.064	0.064	0.023	0.047
Cefoxitin	2	3	2	2	2	2	2	3	2	1	1	1

[a] F_1 to F_9, subgroups of *S. flexneri* based on antibiogram and plasmid analysis.
[b] S_1 and S_2, subgroups of *S. sonnei* based on antibiogram and plasmid analysis.
[c] D_1, group of *S. dysenteriae*.

which can be explained by the fact that they are not used as alternative therapies in this area due to their high cost and lack of availability. However, a trend to quinolone resistance has been observed by Ries et al. (20) in *S. dysenteriae* strains isolated in Burundi. *S. dysenteriae* is considered the most resistant of the *Shigella* spp. (21). However, in our study *S. flexneri* showed the same level of resistance as *S. dysenteriae*. This pattern of resistance and susceptibility is commonly seen in developing countries, in contrast with strains from developed countries, which are less resistant to these antimicrobial agents (4, 27). In this study, the antimicrobial resistance pattern is not a useful epidemiological marker, due to the lack of variability in susceptibility patterns (i.e., the high level of resistance shown by most isolates). Resistance to ampicillin in *S. flexneri* groups F_1 and F_2 and *S. dysenteriae* (group D) is explained by the presence of an OXA-1-type β-lactamase within an integron. Group F_3 *S. flexneri* and the one ampicillin-resistant *S. sonnei* (group S_2) isolate had a TEM-1-type β-lactamase. Both genes have been previously described in *Shigella* strains isolated in Denmark and Greece (14, 22). Therefore, this is the most frequent mechanism of ampicillin resistance found in *Shigella*.

Besides ampicillin, the drug of choice for treating shigellosis is co-trimoxazole. Eighty-eight percent of the strains studied showed resistance to this drug, and in most cases this resistance could be explained by the presence of a *dhfr Ia* gene previously described in *Shigella* and considered the most common dihydrofolate reductase gene in the genus. In one group of *S. flexneri*, however, the *dhfr* gene found was *dhfr VII*, first described in *E. coli* (1). These genes were found inserted in an integron. Both genes were detected with specific primers to amplify the entire gene, which was further sequenced, showing in both cases 100% homology with the *dhfr Ia* and *dhfr VII* genes previously described (19, 23). Chloramphenicol resistance was explained in every case by a positive chloramphenicol acetyltransferase activity generating a high level of resistance. The use of this antibiotic has rapidly declined in many countries. However, due to the fact that it is inexpensive and presents a broad-spectrum activity it is extensively employed in developing countries, thereby ensuring strong selection pressure for the maintenance of chloramphenicol resistance.

In this study, we suggest that antibiotic resistance determinants are carried by plasmids, as well as in integrons which contain resistance genes, such as bla_{OXA} or *dhfr* genes. The spread of multiresistant *Shigella* strains among a population in which diarrheal disease is one of the major causes of child morbidity and mortality requires greater attention to the appropriate use of antibiotics, the establishment of hygienic measures to prevent or decrease transmission, and the development of new effective drugs that can be safely used with children. Moreover, the guidelines for the treatment of shigellosis in developing countries should be updated, since in this study co-trimoxazole, one of the recommended antimicrobial agents for the treatment of shigellosis, has been shown to have little activity against *Shigella* spp.

TABLE 3. Characteristics of *Shigella* sp. clinical isolates[a]

Shigella sp.	Subgroup[b]	β-Lactamase profile			CAT	DHFR
		pI	TEM	OXA		
S. flexneri	F_1 (4)	Ca. 7	−	+	+	Ia
	F_2 (1)	Ca. 7	−	+	+	Ia
	F_3 (1)	ND	ND	ND	ND	ND
	F_4 (2)	Ca. 7	−	+	+	Ia
	F_5 (1)	Ca. 7	−	+	+	ND
	F_6 (2)	Ca. 5.4	+	−	+	VII
	F_7 (1)	ND	ND	ND	ND	ND
	F_8 (1)	ND	ND	ND	ND	Ia
	F_9 (1)	ND	ND	ND	ND	ND
S. sonnei	S_1 (2)	ND	ND	ND	ND	Ia
	S_2 (1)	Ca. 5.4	+	−	ND	Ia
S. dysenteriae	D_1 (3)	Ca. 7	−	+	+	Ia

[a] CAT, chloramphenicol acetyltransferase; DHFR, dihydrofolate reductase; ND, not determined; −, absent; +, present.
[b] Number of strains analyzed in each subgroup is shown in parentheses.

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3.2.2 Artículo 7.- "Prevalence of the *sat* gene among clinical isolates of *Shigella* spp. causing travelers' diarrhea: geographical and specific differences."

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Se determinó la prevalencia del gen *sat,* que codifica para un factor de urovirulencia en *Escherichia coli,* entre 79 cepas de *Shigella* spp (36 *S.flexneri* y 43 *S.sonnei*) aisladas de pacientes con DV. La determinación se realizó mediante amplificación por PCR utilizando cebadores específicos para el gen. Cuarenta y cuatro (55.69%) del total de las cepas fueron positivas: 11 *S.sonnei* (25,58%) y 23 *S.flexneri* (63.89%). La mayor prevalencia en ambas especies se encontró entre cepas aisladas de viajeros provenientes de América Latina ($p<0.001$). Del total de *S.flexneri,* 19 (48.72%) pertenecían al serotipo 2a. De estos, 15 (78,95%) eran positivos para el gen *sat,* mientras que solo 4 (20%) de los serotipos restantes de *S.flexneri* lo fueron

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Letters to the Editor

Prevalence of the *sat* Gene among Clinical Isolates of *Shigella* spp. Causing Travelers' Diarrhea: Geographical and Specific Differences

In the last several years, developing countries have become popular tourist destinations. The large number of travelers to these areas has brought an increase in the so-called travelers' diseases (5). These diseases are due both to the sanitary conditions encountered in some of these places and to the irresponsible attitudes of the travelers themselves. In fact, travelers' diseases may be seen as a mirror of the reality of the sanitary conditions of the tourist destinations influenced by the conduct of the travelers. Thus, analysis of the prevalence and characteristics of travelers' diseases may be considered an indirect source of sanitary information for the visited areas. Among the aforementioned diseases, traveler's diarrhea is one of the most common pathologies found among those returning from developing countries (5).

Diarrhea associated with *Shigella* infections results in around 1,100,000 deaths each year, especially among children in developing countries (3). To study in depth the virulence determinants of this microorganism is a public health priority. Sequences homologous to Sat, a urovirulence factor of *Escherichia coli*, have been described for *Shigella* spp. (2). However, no extensive studies on the prevalence of this virulence factor among *Shigella* spp. have been performed.

The prevalence of the *sat* gene in 36 *Shigella sonnei* and 43 *Shigella flexneri* isolates causing traveler's diarrhea collected during the period of 1995 to 2000 has been established in this study.

The presence of the *sat* gene was determined by PCR amplification of 387 bp using the primers 5′-ACTGGCGGACTC ATGCTGT-3′ and 5′-AACCCTGTAAGAAGACTGAGC-3′ under previously reported conditions (4). Some of the PCR products were sequenced, and all presented 100% homology to those described previously for *E. coli*.

TABLE 1. Geographical distribution of the *Shigella* isolates

Area (total no. of isolates)	No. of isolates showing presence or absence of *sat*			
	S. sonnei		*S. flexneri*	
	sat negative	*sat* positive	*sat* negative	*sat* positive
Africa (32)	12	2	8	10
West Africa	5	1	4	8
East Africa	2	0	1	1
North Africa	5	1	2	1
South Africa	0	0	1	0
Latin America (28)	9	5	4	10
Central America	9	5	3	5
South America	0	0	1	5
Asia (14)	9	3	1	1
India	6	3	0	1
Middle East	3	0	0	0
Southeast Asia	0	0	1	0
ND[a] (5)	2	1	0	2
Total (79)	32	11	13	23

[a] ND, not determined.

TABLE 2. Geographical prevalence of the *sat* gene

Continent	Prevalence of *sat* in *Shigella* isolates (%)	
	S. sonnei	*S. flexneri*
Africa	14.28	55.55
Latin America	35.71	71.43
Asia	25.00	50.00

Overall, 44 isolates (55.69% of the total) were positive; 11 were *S. sonnei* (25.58% of the total number of *S. sonnei* isolates analyzed), while 23 were *S. flexneri* (63.89% of the *S. flexneri* isolates analyzed). Independent of its exact geographical origin, the prevalence of the *sat* gene has always been found to be 30 to 40% higher in *S. flexneri* than in *S. sonnei*. In both species, the higher prevalence of *sat* (35.71 and 71.43% in *S. sonnei* and *S. flexneri*, respectively) was found in isolates obtained from travelers returning from Latin America (Tables 1 and 2). Using a chi-square test, such a proportion was found to be significant ($P < 0.001$). The results of the Mantel and Haensel stratified analysis by continent were still significant ($P = 0.002$).

Nineteen (48.72%) out of the 39 *S. flexneri* strains belonged to serotype 2a. This high prevalence of *S. flexneri* serotype 2a might be explained by the particularly enhanced virulence of this serotype (1). Furthermore, 15 (78.95%) out of these 19 *S. flexneri* 2a strains were positive for the *sat* gene. In contrast, among the remaining 20 *S. flexneri* strains belonging to other serotypes, only 4 (20%) contained it.

In summary, our results show a high incidence of the *sat* gene among clinical isolates of *Shigella* spp. of different intercontinental origins. Further studies are necessary to establish, with greater accuracy, the geographical distribution of this toxin, as well as its relevance as a diarrheagenic factor of *Shigella* spp.

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3.2.3 Artículo 8.- "Genetic diversity of *Shigella* species from different intercontinental sources"

Enviado a : *Genetics Infection and Immunity*

De un total de 7023 pacientes que consultaron el Servicio de Medicina Tropical del Hospital Clínico entre 1995 y 2000, aproximadamente un 18% lo hizo por presentar una DV. De ellos se recolectaron muestras fecales y de aproximadamente un 9,75% se aisló una Shigella. De las ciento veinticuatro cepas de Shigella spp., 58 fueron identificadas como S.flexneri, 54 como S.sonnei, 2 como S.dysenteriae, 1 como S.boydii y ocho identificadas solamente hasta género.

Del total de aislamientos de Shigella, se analizaron 82 de ellas (43 *S.sonnei*, 37 *S.flexneri*, 2 *S.dysenteriae*) mediante digestión del ADN cromosómico con *XbaI* y posterior electroforesis en campo pulsante, con el fin de determinar su relación epidemiológica. Los resultados muestran un alto nivel de heterogeneidad, tanto entre las cepas de *S.flexneri* como entre las de *S.sonnei*.

Al analizar el número de aislamientos de una cierta especie proveniente de un área geográfica en concreto, se observa que en ciertos lugares parecería existir a través de los años predominancia de una sola especie, bien de *S.flexneri* o de *S.sonnei*.

Genetic diversity of *Shigella* species from different intercontinental sources

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ABSTRACT

One hundred and twenty four strains of *Shigella* spp. (mostly *S.sonnei* and *S.flexneri*) were isolated between 1995 and 2000 from patients suffering from traveler's diarrhea. Seventy nine of them have been typed by digestion of their chromosomal DNA with *Xba* I and pulsed field gel electrophoresis. Results show a high degree of heterogeneity in both *S.sonnei* and *S.flexneri* isolates. This is the first time that such a high number of geographically unrelated isolates of *Shigella sp.* are analyzed from a genetic point of view.

Key words: *Shigella* sp. Molecular typing, Genetic variability, Epidemiology

INTRODUCTION

Diarrhea caused by *Shigella* spp, also known as dysentery, is characterized by bloody stools containing mucus. A very small inoculum (10-100 bacterial cells) is enough to cause disease (Sansonetti, 1999). It is usually acquired by drinking contaminated water or by eating uncooked food, previously washed or watered with it.

Shigella causes around 165 million cases and over one million deaths each year, with approximately 91% of the episodes occurring in developing countries (Kotloff et al., 1999), where it especially affects children. Although in industrialized countries the incidence of *Shigella* infections is not alarming, it is still present within certain groups of risk such as children or elder people in day care centers. A third susceptible group is that of travelers, among whom a diarrhea caused by *Shigella* tends, to be more invalidating than that caused by enterotoxigenic *Escherichia coli*, the main infectious agent responsible for traveler's diarrhea (TD).

Lately, the choice of exotic countries as tourist destinations has been rising. According to the World Tourism Organization, 664 million people traveled abroad during 1999 and this number increased by 7.4% during 2000. Moreover, it is estimated that around 40% of world tourism is for developing countries (Vargas, 2001) places that from a sanitary point of view are considered as being of high risk. Thus, it is not surprising that a large amount of TD cases are reported each year, nor that it is in fact one of the most common diseases reported amongst travelers. At our hospital, around 27% of the outpatient visits were due to this pathology ten years ago (Gascon et al., 1993).

Because of its low infectious dose, Shigellosis is a highly contagious disease. Thus, outbreaks are common and in most cases due to the clonal spread of one or few strains. As most of the works published for *Shigella* study the epidemiology of local outbreaks, little is known about the variability of the different species of the genus on a worldwide basis.

In this work we have done a description of *Shigella* TD cases between 1995 and 2000 in our hospital and have analyzed over 67% of the isolates recovered in order to establish their genotypic relationship.

MATERIALS AND METHODS

Bacterial strains

Shigella strains were isolated between 1995 and 2000 from stool samples of patients who visited the Tropical Medicine Unit at the Hospital Clinic in Barcelona, because of a persistent diarrhea acquired during a trip abroad. All strains were identified to a genus level by conventional methods (Murray et al., 1995) while agglutination with specific antiserum (Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France) was used to identify the species.

Low frequency restriction analysis of chromosomal DNA and PFGE.

Genomic DNA was prepared as previously described (Matushek et al., 1996), digested with *Xba* I (Chiou et al., 2001) and separated in 1% agarose gels using a contour-clamped homogenous-field apparatus (CHEF-DR2; Bio-Rad Lab., Hercules, CA). Gels were run under 200V with pulse times increasing from 5 to 20 for 20 hours, after which they were stained with an ethidium bromide solution (0.5 μg/ml) for 30 min. The sizing ladder ProMega-Markers Lambda Ladders (Promega, Madison, WI), with a range between 50 kb and 1,000 kb, was ran in both ends and the middle lane of every gel. Certain strains were included in more than one gel as an internal control.

PFGE pattern analysis

PFGE patterns were analysed using the Diversity Database package (BioRad Lab., Hercules, CA). Bands were aligned to the molecular weight standard present in both extremes and in the middle row of each gel. Those bands that were too faint (in all repeats of the same isolate) or which were larger or smaller than the largest and smallest molecular weights respectively, were not included in the analysis. Dice coefficients of similarity were calculated and dendograms constructed by the unweighed pair group method with arithmetic averages (UPGMA). PFGE patterns differing from each other in 3 or less bands (or with a Dice coefficient ≥77) were considered as one type, as their differences can be explained from the appearance of one single genetic event (Tenover et al., 1995). Those with four or more band differences were considered to be genetically unrelated (Chiou et al., 2001).

RESULTS

One hundred and twenty three strains of *Shigella* sp. were isolated from patients visiting the Tropical Medicine Unit at the Hospital Clínic in Barcelona presenting diarrhea after a trip abroad between 1995 and 2000. A diarrhea starting during the trip or within the first week after returning, was diagnosed as a TD.

From a total of 7023 patients attending the Tropical Medicine Unit at the Hospital Clinic, around 19% presented a TD. Stools samples were collected and a Shigella was isolated in approximately 9% of the cases (Table 1). Out of a total of 124 *Shigella* spp. isolates, 58 were identified as *S.sonnei*, 54 as *S.flexneri*, 2 were *S.dysenteriae* and 1 *S.boydii* while 8 were identified only to a genus level. Strains were recovered from travelers visiting nine different geographical zones. Their geographical origin was varied, as shown in Table 2. The predominance of a certain species in some of the zones can be observed. Thus, for example, all strains but one coming from South America were *S.flexneri*, while most of the ones isolated from travelers to the Indian subcontinent were *S.sonnei*.

Molecular typing by restriction analysis of the chromosomal DNA with *Xba* I of 79 of the strains (43 *S.sonnei*, 34 *S.flexneri*, 2 *S.dysenteriae*) yielded a great variety of profiles with an average of 20 to 22 bands. Construction of the dendograms confirmed that isolates from both *S.flexneri* and *S.sonnei*. were genetically diverse. With the exception of two *S.flexneri* strains (No. 31658 and 31498), isolated from the same patient after a trip to Mali, the remaining strains that appeared to be genetically related did not have a common geographical origin. However, and with only one exception, they did have in common the year in which they were isolated.

DISCUSSION

According to the World Traveler's Organization, about 40% of the long distance trips each year are to developing countries. In the year 2000, this meant that around 279 millions people chose as a destination, some exotic developing country. If we take into account that up to 50% of all traveler's end up with a diarrhea (Consensus Development Conference Panel, 1986) and that on a worldwide basis approximately 1% of all TD is due to *Shigella* (Black, 1990), we can estimate that, during the year 2000, 1.3 million persons suffered from TD caused by these microorganisms. If we instead used the 9% incidence for *Shigella* found in our hospital among patients with TD, the number would increase to 12.55 million.

Despite its relative epidemiological importance as a diarrheal disease causing pathogen, little is known about its genotypic variability on a worldwide basis. Previous epidemiological studies have mainly focused on studying either apparent outbreaks or geographically localized populations over a certain period of time. Works based on the study of a set of *Shigella* isolates with such a varied origin as those presented in this work are the exception. (Karakaolis et al., 1994)

When looking at the geographical distribution of the *S.sonnei* and *S.flexneri* isolates in this study, the first thing that comes to one's attention is the fact that there are certain areas in which one species predominates among the isolates. Such is the case of South America or Western Africa (for S.*flexneri*), or the Indian Subcontinent or Northern Africa (for *S.sonnei*). The predominance of certain species of *Shigella* in certain geographical regions is a well known phenomenon.(Kotloff et al., 1999)

One of the main objectives of this work was to study the genetic diversity of the Shigella strains isolated as a cause of diarrhea in travelers to different geographical areas. When it comes to techniques used for molecular epidemiology and typing, PFGE has been shown to be a method with an excellent discriminatory power. This is also true in the case of *Shigella* typing. In a previous study, *Shigella* strains from an outbreak were typed using different techniques, finding this one to be, along with REP PCR, the best choice (Navia et al., 1999). In this work, vast number of PFGE patterns (almost as many as number of strains) among geographically independent strains of *S.sonnei* and *S.flexneri* have been traduced into

dendograms which show that the populations of *Shigella sonnei* and *Shigella flexneri* from around the world appear to be highly genetically diverse.

Up till now, a great deal of the epidemiological work done with *Shigella* at this level has concentrated on strains from a determined geographical area (Talukder et al., 2003) and, furthermore, in many cases they were isolates from an outbreak (Litwin et al., 1997; Navia et al., 1999). This could give the misleading idea of a certain degree of clonality within the different species of *Shigella*. Bacterial populations reproduce by binary fission and are thus made up of clones which eventually acquire variability by genetic recombination, lateral gene transfer or mutations. Thus, when studying a population of pathogens, the definition of "clonality" must be carefully stated, specially when such pathogens tend to appear in localized outbreaks. The virulence of the outbreak causing strain will also play an important role in the maintenance and wide spreading of the outbreak.

The degree of clonality of *Shigella* spp. has been previously discussed based on studies in which sequencing of housekeeping genes or ribotyping have been performed (Karakaolis et al., 1994; Rolland et al.,1998; Pupo et al., 2000). When reading such works, however, it must not be forgotten that housekeeping genes are subject to a stabilizing selection and thus, although very useful when studying evolutionary relationships between species, are not the optimum to study the genetic divergence of pathogenic species.

Our results, when analyzing by PFGE, strains isolated from very diverse geographical origins, show a high degree of genetic variability among, both *Shigella sonnei* and *Shigella flexneri*.

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Table 1.- Description of patients visiting the Tropical Medicine Unit at the Hospital Clinic

Year	No.Patients	No. TD	No.Shigellas
2000	1498	282 (18,8%)	18 (6,3%)
1999	1393	284 (20,38%)	19 (6,69%)
1998	1298	262 (20,18%)	31 (11,83%)
1997	1085	190 (17,5%)	20 (10,52%)
1996	1081	200 (18,5%)	16 (8,0%)
1995	848	149 (17,6%)	19 (12,75%)
TOTAL	**7203**	**1367 (18,97%)**	**123 (8,99%)**

Table 2.- Geographical distribution of the *Shigella* strains isolated between 1995 and 2000

		Number of strains per species			
Geographical origin	**Number of TD cases per zone**	***S.sonnei***	***S.flexneri***	**Other**	**Total Shigella/area (% of total TD cases)**
North Africa	169	13	6	1 dysent.	20 (11,83%)
South Africa	13	-	2	-	2 (15,38)
Eastern Africa	60	3	3	2 *spp.*	8 (13,33%)
Western Africa	204	8	18	1 *dysent.* 1 *boydii*	28 (13,72%)
Central Africa	11	-	-	-	- (0%)
Middle East	42	4	1	-	5 (11,90%)
S.E. Asia	63	-	1	-	1 (1,45%)
Indian subcontinent	285	12	3	2 *spp.*	17 (5,96)
South America	142	1	11	2 *spp.*	14 (9,86%)
C.America/Caribbean	378	17	9	2 *spp.*	28 (7,41%)
Total	**1367**	**58**	**54**	**11**	**123 (8,99%)**

LEGENDS TO FIGURES

Figure 1.- UPGMA dendogram based on Dice coefficients of similarity for *S.sonnei* strains, collected between 1995 and 2000 from patients with traveler's diarrhea. Strain numbers, from top to bottom: 61, 175, 27691, 33124, 36498, 260, 106, 10681, 192, 35037, 73897, 94, 154, 92645, 32818, 33570, 98, 85153, 150, 21054, 130, 220, 175, 86865, 13051, 1059, 73, 35129, 33047, 85253, 14249, 85832, 132, 31498, 13156, 5499, 314, 85833, 26781, 11512, 14655, 13558, 3805. On the right hand side, a representation of the PFGE pattern for each strain.

Figure 2.- UPGMA dendogram based on Dice coefficients of similarity for *S.flexneri* strains, collected between 1995 and 2000 from patients with traveler's diarrhea. Strain numbers, from top to bottom: 16742, 76, 93085, 174, 13844, 16092, 37758, 31658, 31498, 297, 33385, 86414, 88193, 13040, 2129, 67429, 196, 85128, 34990, 86551, 20393, 20124, 15746, 15681, 305, 86415, 123, 1333, 119, 138, 17, 35078, 113, 179. On the right hand side, a representation of the PFGE pattern for each strain.

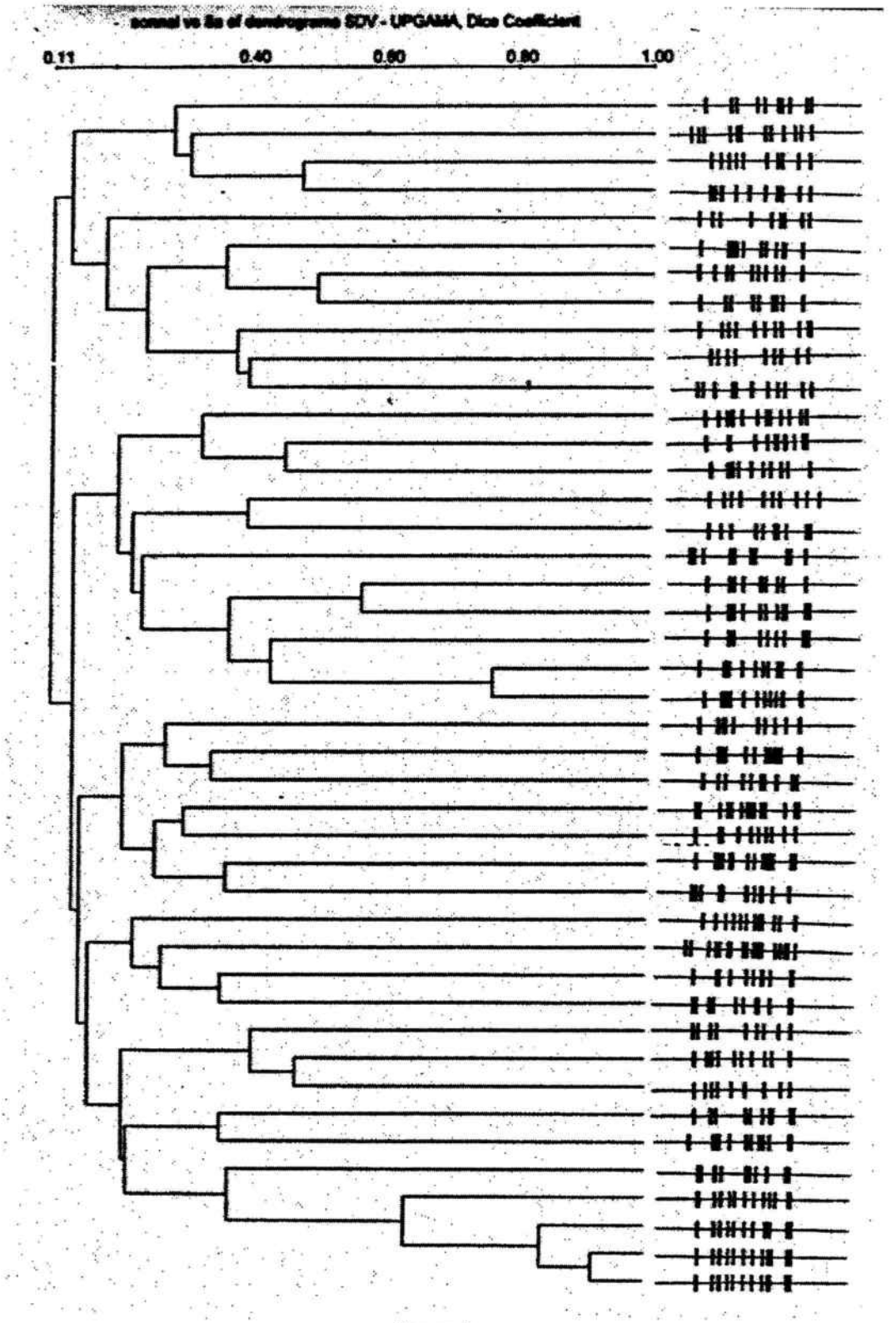

Figure 1.

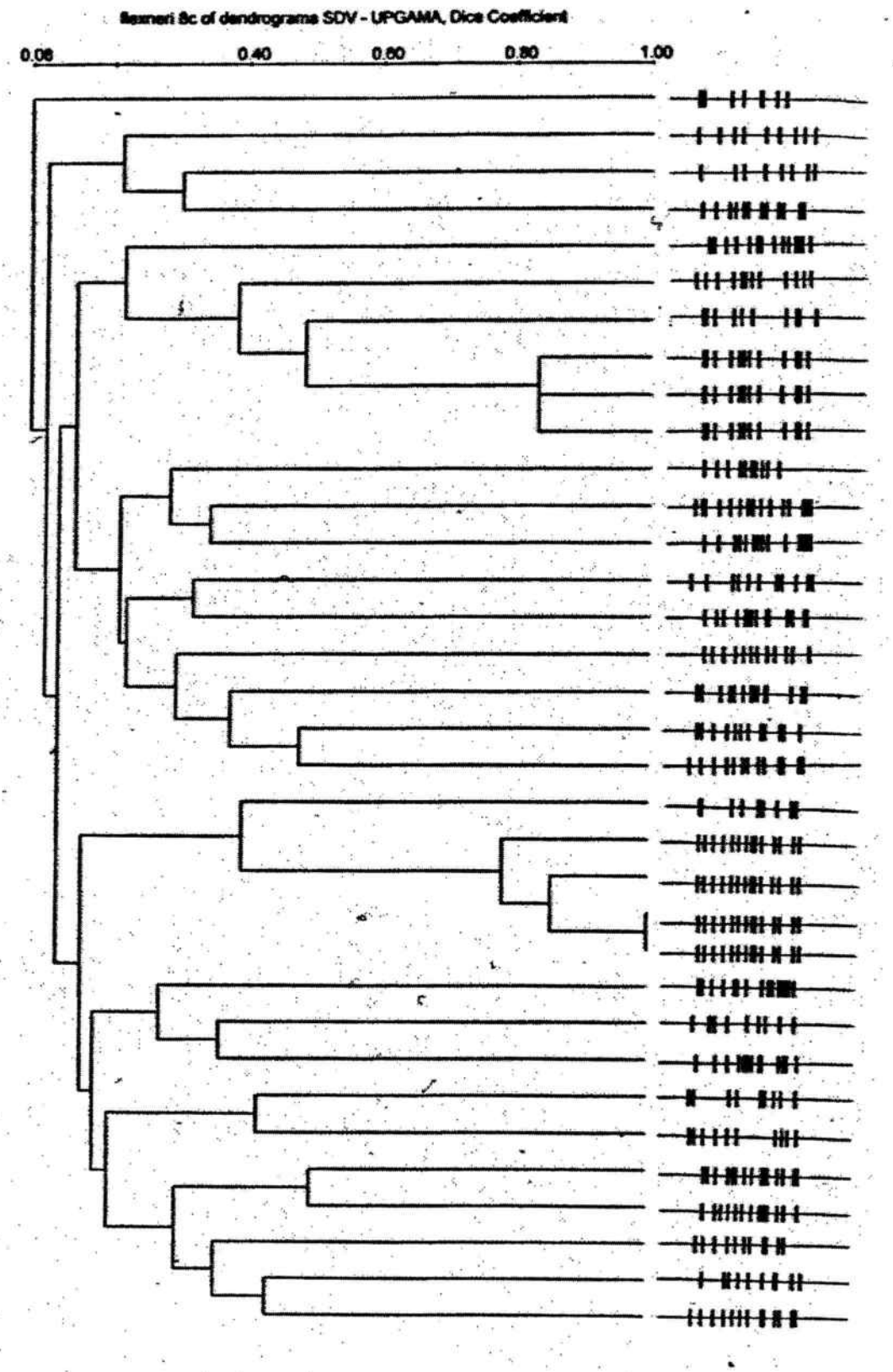
Resmeri 8c of dendrograms SDV - UPGAMA, Dice Coefficient
0.08
0.40
0.60
0.80
1.00

Figure 2

3.2.4 Artículo 9.- "Analysis of the mechanisms of resistance of *Shigella* spp. causing traveler's diarrhea"

Enviado a: *Journal of Antimicrobial Chemotherapy*

Se han estudiado ochenta y tres cepas de *Shigella* spp. (43 *S.sonnei*, 37 *S.flexneri*, 2 *S.dysenteriae* and 1 *S.boydii*) aisladas de pacientes con diarrea del viajero (DV). Se determinó la CMI a ampicilina, tetraciclina, cloranfenicol, trimetroprim y ácido nalidíxico, encontrándose altos niveles de resistencia, especialmente entre las cepas de *S.flexneri*. En general las cepas eran altamente resistentes a la tetraciclina (78%), al trimetroprim (75%) y a la ampicilina (65%). Al analizar los mecanismos de resistencia, se encontró una alta prevalencia (65.6%) de genes *dfr*A1 entre las cepas con fenotipo trimetroprim, mientras que se observó una predominancia de genes *oxa* entre las cepas ampicilina resistentes (en el 88,5% de las cepas $Ampi^R$). Se encontró que en las cepas resistentes a cloranfenicol, todas ellas *S.flexneri*, la resistencia era debida a la producción de la cloranfenicol acetil transferasa. Las cuatro cepas resistentes al ácido nalidíxico presentaban una mutación en la región QRDR del *gyr*A pero no en *par*C.

Se observaron en general altos niveles de resistencia, poniendo una vez más en manifiesto que el problema de la multiresistencia es mundial y que puede tener graves implicaciones a nivel de la salud pública.

Analysis of the Mechanisms of Resistance to Several Antimicrobial Agents in Shigella Species Causing Traveler's Diarrhea

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Running title: Mechanisms of resistance in *Shigella* species.

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ABSTRACT

Eighty three strains of *Shigella* species (43 *S.sonnei*, 37 *S.flexneri*, 2 *S.dysenteriae* and 1 *S.boydii*) isolated from patients with travelers' diarrhea have been studied. Their susceptibility to ampicillin, tetracycline, chloramphenicol, trimethoprim and nalidixic acid has been determined. High levels of resistance have been found, specially among the *S.flexneri* strains. Overall, strains were highly resistant to tetracycline (78%), trimethoprim (75%) and ampicillin (65%). When analyzing the mechanisms of resistance, *dfr*A1 genes were found to be prevalent among the trimethoprim resistant phenotype (65,6% of the resistant isolates), while *oxa* genes were the predominant among the ampicillin resistant strains (88,5% of the resistant isolates). Chloramphenicol resistance, found only in *S.flexneri*, was due to chloramphenicol acetyltransferase production. Four strains were resistant to nalidixic acid, presenting a mutation in the QRDR region of the *gyr*A gene but no mutation in the *par*C gene. High levels of resistance were found, putting once more into evidence that the multiresistance issue is a worldwide problem with public health implications.

INTRODUCTION

Acute infectious diarrheal disease continues to be one of the major causes of childhood death in the developing world. Since the introduction of rehydration therapy as part of diarrhea management three decades ago, mortality due to diarrhea has decreased considerably. (9). However, this innovation has been of little advantage in front of diarrheas produced by invasive organisms such as *Shigella* spp., so that antimicrobial treatment is still necessary in such cases. With the increase of intercontinental travels to exotic destinations, infection by *Shigella* has become an important cause of travelers' diarrhea (TD). Moreover, *Shigella* causes much more severe and invalidating symptoms than those from infection with enterotoxigenic *Escherichia coli,* TD's main etiologic agent.

The *Shigella* genus contains four species: *Shigella boydii* – the less common and seldom isolated. *Shigella flexneri,* which is the most prevalent in endemic areas (up to 50% of positive cultures) (24). *Shigella dysenteriae,* the pathogen usually causing diarrhea in overcrowded areas and often the agent in large outbreaks. And finally, *Shigella sonnei,* the most prevalent *Shigella* species in developed countries, posing a problem in day care centers and elderly homes.

Over the past decades, *Shigella* species has progressively become resistant to most antibiotics used for their treatment due in part to the facility they have to acquire resistance genes within plasmids or transposons. (18) Thus, sulphonamides, tetracycline, ampicillin, ampicillin plus sulbactam , trimethoprim, trimethoprim-sulphamethoxazole and more recently nalidixic acid, have all been first line treatment options. Some of them, as is the case with sulphonamides, tetracycline or even ampicillin, have already been discarded due to the very high levels of resistance encountered. Others, although still under use, show increasing levels of therapy failure because of a steady state decrease in the susceptibility of the shigellae. Such is the case for nalidixic acid, the current first line treatment in many countries despite its suspected cartilage toxicity in young children. A recent study has detected 59,6% of resistant *Shigella flexneri* isolates in Hong Kong (5). Resistant strains in India, where it is also widely used, are also increasingly reported (6, 8, 30).

A series of socioeconomic and behavioral factors, such as the misuse of antibiotics by health practitioners and patients, poor quality of the antimicrobial drugs or the prevalence of unhygienic conditions lead to the acquisition of bacterial resistance to antibiotics in developing countries (15). In addition, the facility for international travels nowadays means that the appearance of multiresistant pathogenic strains anywhere in the world might end up becoming a public health problem somewhere else around the globe, as they may be repeatedly dispersed by travelers. Thus, the treatment decision for Shigellosis in developed countries is now commonly influenced by the patient's history of a recent trip abroad (24, 27)

In this work we have studied the susceptibility patterns and the mechanisms of resistance in strains of *Shigella* species with a wide geographical origin, isolated from patients with TD.

MATERIALS AND METHODS

Bacterial strains

Shigella strains were isolated between 1995 and 2000 from stool samples of patients, presenting traveler's diarrhea, who visited the Tropical Medicine Unit at the Hospital Clinic in Barcelona, Spain. Strains were identified to a genus and species level by conventional biochemical methods (12) and agglutination with specific antisera (Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France).

Antimicrobial susceptibility testing

The determination of the minimum inhibitory concentration (MIC) of ampicillin, tetracycline, chloramphenicol, trimethoprim, nalidixic acid and ciprofloxacin was performed using E-test strips (AB BioDisk, Sölna, Sweden) following the manufacturer's recommendations. ATCC control strains were used in every case.

β-lactamase detection.

Presence of β-lactamases was studied using two different methods: Isoelectric focusing (IEF) and PCR using specific primers for TEM, CARB, SHV type genes as previously described (7,13). A series of primers to amplify different *oxa* genes were also used: OXA 1-4 (capable of amplifying *oxa* 1, *oxa* 31, *oxa* 4 and *oxa* 30) and OXA 35 primers were used as previously described (7,13). Primers to amplify *oxa* 2-3 like genes (capable of amplifying *oxa* 2, *oxa* 3, *oxa* 15, *oxa* 21, *oxa* 32, *oxa* 34) and *oxa* 5-7 like genes (*oxa* 5, *oxa* 7, *oxa* 10, *oxa* 11, *oxa* 13, *oxa* 14, *oxa* 16, *oxa* 17, *oxa* 19, *oxa* 28) were also used. The sequences of such primers, given from 5´to 3´, are as follows: *oxa 2-3U*: CGA TAG TTG TTG TGG CAG ACG AA; *oxa 2-3L*: CCA CTC AAC CCA TCC TAC CC; *oxa 5-7U*: TAT ATT CCA GCA TCA ACA TT and *oxa 5-7L*: ATG ATG CCC TCA CTT GCC AT.

Chloramphenicol acetyltransferase detection.

The presence of chloramphenicol acetyltransferase activity was determined with a modification of the method described by Azemun et al (4) and as described elsewhere (13).

Trimethoprim resistance genes.

The presence and type of *dfr* genes was determined using a PCR-RFLP methodology described elsewhere (14).

Mutations in the *gyr*A and *par*C genes.

PCR amplification and sequencing of the QRDR regions of the *gyr*A and *par*C genes of those strains presenting resistance to nalidixic acid, was done using the primers and following the conditions described elsewhere (28,29).

RESULTS

Eighty three strains of *Shigella* spp. (37 *S.flexneri*, 43 *S.sonnei*, 2 *S.dysenteriae* and 1 *S.boydii*) from various geographical origins have been studied. In a previous study (unpublished results) the strains were typed using pulsed field gel electrophoresis. They were shown to be epidemiologically unrelated according to Tenover's criteria (25) except for two strains isolated from the same patient on a different date which proved to be the same clone and a group of 5 strains that were considered as closely related (3 or less band differences).

A high percentage of multiresistance was observed. The highest level of resistance was in front of tetracycline with a 78%, followed by trimethoprim (76%), ampicillin (65%) and chloramphenicol (26,5%). (Table 1). Table 2 shows the different resistance phenotypes encountered for the strains. Differences in the susceptibility levels were observed between the two most prevalent species: *S.flexneri* and *S.sonnei* with *S.flexneri* being in general more resistant, except in front of trimethoprim. Interestingly, the Tet^R Tm^R Amp^R Clm^R Nal^S phenotype is only observed for *S.flexneri* mainly because no *S.sonnei* was found to be resistant to chloramphenicol

The mechanisms of resistance underlying the ampicillin and trimethoprim phenotypes are shown in Tables 3 and 4 respectively. Briefly, with one exception, the resistance phenotype in front of ampicillin was explained in every case by the presence of a β-lactamase gene, among which the most common where those encoding the OXA-type β-lactamases. Only in six strains was such resistance not explained (at least in part) by the presence of an *oxa* type gene. In all of these, resistance was due to the presence of a *tem*-type gene. The *oxa* 1 type genes were specially abundant among *S.flexneri* strains, while among *S.sonnei* there were many *oxa* 2 and *oxa* 5 type genes. The genes encoding CARB and SHV-type β-lactamases were rare but also found: the first one in two *S.sonnei* strains and the latter in one *S.flexneri*

In the case of trimethoprim resistance strains, dihydrofolate reductase genes were found in most of them, with *dfr*A1 being the most prevalent type

Chloramphenicol resistant strains had a positive chloramphenicol acetyl transferase activity, while some of the susceptible strains, used as controls, did not. Only three strains were resistant to nalidixic acid (with MICs ranging between 64-128μg/ml) but susceptible for ciprofloxacin (<1 μg/ml). Two of the strains were *S.sonnei*, originally from India and the remaining one was a *S.flexneri* from Mali. When analyzing the mechanism of resistance responsible for this phenotype, we found that all three strains presented a mutation in the *gyr*A gene (Ser_{83} to Leu in the *S.flexneri* and one of the *S.sonnei* strains and Asp_{87} to Tyr in the remaining *S.sonnei*) but not in *par*C, explaining their susceptibility to ciprofloxacin.

The two clonal strains, isolated from the same patient on different dates, showed exactly the same antibiotic susceptibility profile and shared the same mechanisms of resistance. As for the closely related strains, their susceptibility profiles were similar with the exception of that to chloramphenicol, as two of the strains were resistant, one was intermediate and the fourth was susceptible. The mechanisms of resistance were the same for trimethoprim, and all had an OXA-1 type ß-lactamase, but two of them had a TEM-type β-lactamase as well.

DISCUSSION

Increasing antibiotic resistance levels have been continuously reported for *Shigella* species since the 1940's, when the first sulphonamides-resistant strains were found in Japan (32). Since then, new first line therapies have been established and eventually abandoned because of treatment failure. We have analyzed the antimicrobial resistance profiles of eighty three strains of Shigella , mainly *S.flexneri* and *S.sonnei*, isolated from patients with TD.

Strains were highly resistant to tetracycline, trimethoprim and ampicillin. This is in accordance with what has been previously reported on strains from developing countries. In a previous study carried out by our group with *Shigella* strains (13) isolated from children in Ifakara, Tanzania, we found percentages of resistance between 96,9% to tetracycline and 81,8% to ampicillin. Other authors have reported similar findings with percentages of resistance all over 50% for these antibiotics (3, 11, 12).

In general terms, and in accordance with what has been previously reported (5) *S.flexneri* strains showed higher levels of resistance than the *S.sonnei*. It is especially interesting in the case of chloramphenicol, where 56% of the *S.flexneri* strains were resistant, compared to none in the *S.sonnei* group. This higher resistance in front of chloramphenicol has been previously reported (5, 11, 13, 31). Furthermore, retrospective studies in which resistance trends of *Shigella* strains from a specific site are evaluated over time, show that there has been a very important decrease in the levels of resistance to chloramphenicol in *S.sonnei*, while for *S.flexneri* the resistant levels may have even increased. (10). This result could suggest that *S.sonnei* is naturally more susceptible in front of chloramphenicol and/or that the mechanism of resistance to chloramphenicol in *S.sonnei* somehow differ from those of *S.flexneri*, being either more easily lost or less easily acquired

The only exception of *S.flexneri* overall greater resistance was observed for trimethoprim, in front of which *S.sonnei* showed higher levels of resistance. If results are looked at by region, differences are certainly observed in the percentage of resistance to certain antibiotics for different

geographical areas. However, it is difficult to assess such results because of the variable and sometimes very small number of strains collected from certain regions. A more detailed study, with a larger and more representative sample size for the different regions would be required to draw conclusions. Yet, it would be expected that the antibiotic consumption trends of each region would influence the resistance profiles of the isolates

This could be the case with the nalidixic acid resistant strains hereby found. India, and to a lesser extent, Mali are known for the wide use and sometimes abuse of nalidixic acid, and nalidixic acid resistant strains of different Enterobacteria have been previously reported to be isolated in these regions (6, 8, 30). Although the three strains were susceptible to ciprofloxacin, because of the sequential mutations that lead to different quinolone resistance phenotypes, it is only a question of time that ciprofloxacin resistance will start to appear and become common. The mutations found have been both previously described in Shigella. The Ser_{83} to Leu substitution has been found in *S.sonnei*, *S.flexneri* and *S.dysenteriae* (16, 17, 26) while the Asp_{87} to Tyr substitution was described in five strains of *S.sonnei* (26). Our findings are in accordance with these previous descriptions.

Analysis of the mechanisms of resistance to trimethoprim were very much in line with what has been previously described, with the *dfr*A1 gene being the most prevalent among both *S.flexneri* and *S.sonnei*. (23) Quite an important number of *dfr*A14 genes were also found. The DHFR Ib enzyme (encoded by the *dfr*A14 gene) has very similar biochemical properties, to those of the DHFR I , to which it is thought to be related. To our knowledge, it is the first time that *dfr*A14 genes are described in *Shigella*, although it has previously been shown that this genes are quite common among Enterobacteriaceae, specially in certain areas (1,2) . Other less common types of dihydrofolate reductases were also found (*dfr*A5, *dfr*A15, *dfr*A12, *dfr*A7), all of them as cassettes within type I integrons (unpublished data) along with a couple of the *dfr*A1 genes.

As for the mechanisms of resistance to ampicillin, previous studies have described *oxa* genes as being specially prevalent in *S.flexneri* while *S.sonnei* usually harbour TEM type β-lactamases.(19,

20, 22). In this study, we have found *oxa* genes as being by far the most prevalent in both species. This is in accordance with a previous study carried out with strains from Tanzania (13). Furthermore, it is interesting to note that there seems to be a species preference for certain genes, as is the case with the *oxa* 1 type for *S.flexneri* or the *oxa* 2 like and *oxa* 5 like types for *S.sonnei* . The clonal spread of Shigella species carrying these specific β-lactamase genes is, in this case, overruled. The results could thus suggest that these genes are within incompatibility group R- plasmids. In a previous study, Siu et al. (22) screened 91 ampicillin resistant *S.flexneri*, finding out that 21% harbored a TEM-1 while the majority, 79% contained an *oxa*-1 like gene, later identified as encoding for a novel OXA-30 β-lactamase. In this study, we have also found a high prevalence of *oxa*-1 like genes in *S.flexneri*. The primers we used would amplify any *oxa*-1 like genes, among which *oxa*-30 is included although confirmation would require sequencing. No specific correlation between geographical area and molecular mechanism of resistance to β-lactams or trimethoprim is observed.

The history of the treatment of Shigellosis is a nightmare of continuous failures. The levels of resistance to the current first line treatments in developing countries is not encouraging. Nalidixic acid has become the first line treatment in children from endemic, developing areas, despite its proven toxicity, and chloramphenicol still continues to be used in many African rural areas (Dr.David Schellemberg, personal communication). Yet resistance levels are high and rising. Better alternatives, such as cephalosporines are good options in the treatment of TD but not as first line treatment in developing, endemic areas because of high price and lack of availability. The need for other cheaper options (21) is a time pressing issue. Moreover, the number of travelers to developing countries is steadily rising. Multiresistant bacteria carried within their intestinal tracts may be incorporated into the ecosystem of their hometowns and countries, with further spreading of the multiresistant microorganisms and/or the horizontal transfer of the genetic elements carrying resistance determinants, aggravating antimicrobial resistance in the zone. Therefore, surveillance studies are necessary to determine not only the level of local resistance at a certain point in time, but as well that found in imported strains.

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Table 2.- Resistance phenotypes of *Shigella* species causing travelers' diarrhea. For each species, the number of strains with each phenotype is shown. In brackets, the percentage.

Phenotype	*S.flexneri* (n=37)	*S.sonnei* (n=43)
Tet^R Tm^R Amp^R Clm^R Nal^S	14 (37,8%)	0
Tet^R Tm^R Amp^R Clm^S Nal^S	7 (19%)	13 (30,2%)
Tet^R Tm^R Amp^S Clm^S Nal^S	2 (5,4%)	13 (30,2%)
Tet^R Tm^S Amp^R Clm^R Nal^S	3 (8,1%)	0
Tet^R Tm^R Amp^R Clm^S Nal^R	0	2 (4,6%)
Tet^S Tm^S Amp^R Clm^S Nal^S	0	7 (16,2%)
Tet^S Tm^R Amp^S Clm^S Nal^S	0	3 (7%)
Tet^S Tm^S Amp^S Clm^S Nal^S	2 (5,4%)	1 (2,3%)
Others	9 (24,3%)	4 (9,3%)

Table 3.- Distribution of β-lactamases among the *Shigella* species isolates.

β-lactamase	S.sonnei n= 25	S.flexneri n=27
TEM-type (pI circa 5.2)	7 (28%)	9 (33,3%)
SHV-type (pI circa 7.6)	0	1 (3,7%)
OXA 1-2 type	1 (4%)	20 (74%)
OXA 2-3 type	10 (40%)	0
OXA 5-7 type	19 (76%)	3 (11%)
CARB-type (pI circa 5.6)	2 (8%)	0
Undetermined	1 (4%)	0

Table 4.- Distribution of dihydrofolate reductases among the *Shigella* species isolates.

Dihydrofolate reductase gene	***S.sonnei*** **n= 36**	***S.flexneri*** **n=25**
*dfr*A1	27 (75%)	13 (52%)
*dfr*A14	14	6
*dfr*A5	0	1
*dfr*A15	1 (2,7%)	0
*dfr*A12	0	1
*dfr*A7	0	1
undetermined	4 (11,1%)	6 (24%)

Table 1.- Resistance profiles in *Shigella* species isolated from patients with TD.

Area	Tetracycline			Chloramphenicol			Ampicillin			Trimethoprim		
	S.flexneri	*S.sonnei*	TOTAL	*S.flexneri*	*S.sonnei*	TOTAL	*S.flexneri*	*S.sonnei*	TOTAL	*S.flexneri*	*S.sonnei*	TOTAL
West Africa	12/13	5/6	17/19 (89,5%)	6/13	0/6	6/19 (31,5%)	7/13	3/6	10/19 (53%)	6/13	6/6	12/19 (63%)
Eastern Africa	2/2	1/2	3/4 (75%)	2/2	0/2	2/4 (50%)	2/2	2/2	4/4 (100%)	2/2	1/2	3/4 (75%)
South Africa	1/1	n	1/1 (100%)	1/1	n	1/1 (100%)	1/1	n	1/1 (100%)	1/1	n	1/1 (100%)
North Africa	1/3	3/7	4/10 (40%)	0/3	0/7	0/10 (0%)	1/3	3/7	4/10 (40%)	2/3	5/7	7/10 (70%)
C.Amer. / Caribbean	6/8	10/14	16/22 (73%)	4/8	0/14	4/22 (18%)	7/8	10/14	17/22 (77%)	5/8	11/14	16/22 (73%)
South America	5/6	n	5/6 (83%)	4/6	n	4/6 (66%)	5/6	n	5/6 (83%)	5/6	n	5/6 (83%)
South East Asia	1/1	n	1/1 (100%)	1/1	n	1/1 (100%)	1/1	n	1/1 (100%)	1/1	n	1/1 (100%)
Middle East	n	2/3	2/3 (66%)	n	0/3	0/3 (0%)	N	1/3	1/3 (33.33%)	n	2/3	2/3 (66%)
India / Nepal	1/1	8/9	9/10 (90%)	1/1	0/9	1/10 (10%)	1/1	4/9	5/10 (50%)	1/1	8/9	9/10 (90%)
Undetermined	2/2	2/2	4/4 (100%)	1/2	0/2	1/4 (25%)	2/2	2/2	4/4 (100%)	2/2	2/2	4/4 (100%)
Total/ specie *(%)*	31/37 *84%*	31/43 *72%*		20/37 *54%*	0/43 *0%*		27/37 *73%*	25/43 *58%*		25/37 *65%*	35/43 *81%*	

3.2.5 Artículo 10.- "Molecular characterization of the integron population in *Shigella* strains isolated from patients with traveller's diarrhea"

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Se analizó la población de integrones de tipo I en cepas de *Shigella* spp. epidemiológicamente no relacionadas, aisladas en un período de cinco años de pacientes con DV. Se halló una prevalencia del 13,25% (11/83). Entre las once cepas que presentaban uno, se observaron nueve tipos diferentes de integrones. Al secuenciar, se encontraron 10 cassettes diferentes, con una altísima prevalencia de genes de dihidrofolato reductasas. También se hallaron algunos que codificaban para ß-lactamasas tipo OXA así como para enzimas modificantes de los aminoglucósidos

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Molecular characterization of the integrons in *Shigella* strains isolated from patients with traveler's diarrhea

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Abstract

The prevalence and characterization of Class 1 integrons has been performed in eighty three strains of *Shigella spp.*, isolated between 1995 and 2000 from patients with traveler's diarrhea. A low prevalence (13.25%) was recorded. Nine different integrons were found among 11 multiresistant strains, with a total of 10 different gene cassettes encoding for resistance to trimethoprim (*dfr*A1, *dfr*A5, *dfr*A7, *dfr*A12, and *dfr*A15), aminoglycosides (*aad*A1a and *aad*A2), β-lactam antibiotics (*oxa2*) or ORF with unknown function (orfD and orfF). A high prevalence of *dfr* and *aad* gene cassettes was observed. The low incidence of Class 1 integrons observed in this study is in contrast with the known facility that the *Shigella* genus has to gain and transfer plasmids.

Keywords: Genetic elements; Antibiotic resistance; Gene cassettes

1. Introduction

Diarrheas continue to be an important cause of disease with high morbidity and mortality rates, especially in developing countries (DCs). They are ranked as the fourth cause of death and the second cause of years of productive life lost due to premature mortality or disability (Kottlof et al., 1999). Infection by *Shigella* spp. is characterized by a watery diarrhea that may progress to mucoid bloody diarrhea, also known as dysentery. It has been estimated that each year over 163.2 million episodes of endemic shigellosis occur in DCs while only 1.5 million occur in developed ones (Kottlof et al., 1999). The increasing number of travelers to DCs, which have become attractive tourist destinations, has also increased the number of diarrhea episodes among the millions of persons who travel each year from industrialized countries to DCs (Virk, 2001).

As is the case with most enteropathogens, there has been an alarming increase in the development of resistance to different antimicrobial agents and especially to those most commonly used in DCs (ampicillin, trimethoprim/sulfamethoxazole, chloramphenicol or tetracycline). In a previous study from our group, when analyzing strains isolated from Tanzanian children, percentages of resistance as high as 96.9% to tetracycline or 87.9% to cotrimoxazol were found, with *S. flexneri* strains being in general more resistant than those of *S. sonnei* (Navia et al., 1999). These results were in agreement with those found in another study (Vila et al., 1994) developed on strains from traveler's diarrhea (TD).

The fast spread of resistance determinants among *Shigella* spp. can be partly explained by the facility this genus has to acquire plasmids (Kottlof et al., 1999). The horizontal transfer of resistance genes is a successful mechanism to disseminate multiple drug resistance among bacteria. These genes are mobilized from one microorganism to another in genetic transposable elements such as plasmids or transposons. More recently, the existence of integrons within these mobile elements has been described. Integrons are DNA elements with three essential components: 1) an *Int* I integrase gene encoding for a site-specific recombinase; 2) an insertion *att*I "receptor" site recognized by the integrase and into which; 3) gene cassettes, often encoding for antibiotic resistance mechanisms, can be inserted. From the various integron classes described to date, Class 1 is by far the most commonly found in clinical isolates and also that which has been most extensively studied (Martinez-Freijo et al., 1998; Carattoli, 2001).

Since their discovery, Class 1 integrons have been

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looked for in most *Enterobacteriaceae* (Martinez-Freijo et al., 1998; Leverstein-van Hall et al., 2002; White et al., 2001). However, little has been done for *Shigella* spp. a genus which is well known for easily gaining and transferring plasmids, which in turn are a common vehicle for integrons (McIver et al., 2002; Navia et al., 1999). In this study, we have analyzed the Class 1 integron population in strains of *Shigella* isolated between 1995 and 2000 from patients with TD.

2. Materials and methods

2.1. Bacterial strains

Eighty-three *Shigella* strains out of the 123 isolated between 1995 and 2000 were recovered from the frozen stock. These 123 strains were originally isolated from stool samples of patients visiting the Tropical Medicine Unit at the Hospital Clinic in Barcelona with a persistent diarrhea acquired during a trip abroad and appearing either during the trip or up to one week upon returning. Strains were identified to a genus and species level by conventional biochemical methods (Murray et al., 1995) and agglutination with specific antiserum (Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France).

2.2. Susceptibility testing

Susceptibility testing was performed using E-test (AB BioDisk, Sölna, Sweden) strips according to manufacturer's instructions. Susceptibility levels to tetracycline, chloramphenicol, ampicillin, trimethoprim, cotrimoxazole, and nalidixic acid were assessed. Strains ATCC29213 of *Staphylococcus aureus* and ATCC25922 of *Escherichia coli* were used as control microorganisms.

2.3. Integron amplification

Polymerase chain reaction (PCR) amplification of Class 1 integrons was performed using the Levesque and Roy primers 5'-GGCATCCAAGCAGCAAG- 3' and 5'-AAGCAGACTTGACCTGA-3' (Levesque and Roy, 1993), following previously described conditions and procedures (Vila et al., 1997). Alternatively, to increase specificity, some PCRs were ran using an annealing temperature of 59°C. The amplified products were resolved in 2% agarose gels and stained with ethidium bromide (0.5 mg/L). Bands were recovered from the gel using the Gene-Clean kit (Bio 101, Inc., La Jolla, CA, USA) and sequenced.

2.4. DNA sequencing

Recovered DNA was directly sequenced using the Thermosequenase Dye Terminator Sequencing Kit (Amersham, Cleveland, OH, USA) in an automatic DNA sequencer (model 377; Applied Biosystems, Foster City, CA, USA). In most cases the use of the Levesque and Roy primers was enough to cross the complete integron. Where necessary, novel primers were designed over the obtained sequence to move forward in the internal sequence of the integrons. These primers were: dfrA1upper 5' GTGAAACTATCACTAATGG 3' to sequence the whole integron of strains 76, 98 and 66170, primer drfA12 5' GGTGAGCAGAAGATTTTTCGC 3' to sequence the integron of strain 196; primer oxa2 5' CGATAGTTGTGGCAGACGAA 3' to sequence the integron of strains 14249 and 35037 and primer dfrA1lower 5' TTAACCCTTTTGCCAGATTT 3' to sequence the integron of strain 314. Sequences obtained were compared with those in GenBank, in order to identify the exact genes.

3. Results

A group of 83 *Shigella* spp. was studied. Of these, 43 were *S. sonnei*, 37 *S. flexneri*, 2 *S. dysenteriae*, and 1 *S. boydii*. Upon amplification with Class 1 integron specific primers, 11 (13.25%) of these isolates presented at least one band (Table 1). None of these 11 strains was, when analyzed by PFGE, related to any of the remaining 83 strains (Navia, unpublished results).

The susceptibility patterns for the integron containing strains is shown in Table 1. All except one in each case (91%) presented high levels of resistance to trimethoprim, cotrimoxazole and ampicillin. Resistance to tetracycline was next with a 63.3% prevalence, while only 27% of the isolates were resistant to chloramphenicol. From the total of 62 trimethoprim-resistant Shigella strains 9 (14.5%) had an integron carrying a *dfr* gene, 1 (1.6%) had an integron non associated with resistance to trimethoprim, and the remaining 52 (83.9%) did not have an integron. In the same way, 2 out of 52 ampicillin-resistant isolates (3.8%) had an integron carrying a β-lactamasa, while 8 (15,4%) had an integron not related with resistance to ampicillin, and the remaining 42 (80.8%) did not have an integron.

Nine different class 1 integrons were found among the eleven strains. The amplified bands ranged in size between 650 bp and 1800 bp and contained a minimum of one (bands ranging from 650, to 1000 bp) and a maximum of three gene cassettes (bands ranging from 1300 to 1800 bp). Upon sequencing the entire integrons, 10 different gene cassettes were found, including genes encoding for resistance to trimethoprim (*dfrA1*, *dfrA5*, *dfrA7*, *dfrA12*, *dfrA15*), β-lactam antibiotics (*oxa2*) and aminoglycosides (*aadA1a*, *aadA2*) as well as two different ORFs (D and F) without known function. Despite the diversity of integrons, only isolate 76 carries more than one (Table 1).

Although only 11 strains contained a Class 1 integron, when PCR reactions were first carried out, almost all strains presented an amplification product that had one of two patterns: one specific for *S. sonnei* and another found only

Table 1
Characteristics of the integron-containing *Shigella* clinical isolates

Strain	Species	Year of isolation	Country of travel	Integron size	Minimal Inhibitory Concentration						Gene cassettes
					TC	CL	Nal	Amp	Sxt	Tm	
76	*S. flexneri*	1995	México	1500 1000	1	4	1	>256	>32	>32	dfrA1/aadA1a aadA1a
196	*S. flexneri* (+)	1995	Venezuela	1800	0.5	8	2	32	>32	>32	dfrA12/orfF/aadA2
98	*S. sonnei*	1995	Nicaragua	1600	1	8	1	>256	>32	>32	dfrA1/aadA1a
314	*S. sonnei*	1996	Turkey	1400	1	4	2	1	>32	>32	aadA2/dfrA1
1385	*S. dysenteriae*	1997	Tunesia	650	>256	128	2	>256	>32	>32	dfrA1
119	*S. flexneri*	1997	Nicaragua	650	>256	128	2	>256	>32	>32	dfrA1
66170	*S. sonnei*	1998	East Africa	1500	0.5	4	2	>256	>32	>32	dfrA15/aadA1a
14249	*S. sonnei*	1998	Guatemala	1300	>256	8	2	>256	0.38	0.5	oxa2/orfD
35037	*S. sonnei*	1998	Guatemala	1300	>256	4	1	128	>32	>32	oxa2/orfD
86551	*S. flexneri* (+)	1999	Kenya	700	128	128	1	>256	>32	>32	dfrA7
88193	*S. flexneri*	2000	Egypt	720	0.5	1	2	>256	>32	>32	dfrA5

(+) Shigella flexneri type 2a strains.
TC = tetracycline; CL = chloramphenicol, Nal = Nalidixic Acid, Amp = Ampicillin, Tm = Trimethoprim, Sxt = cotrimoxazole.

among *S. flexneri*. Sequencing of the bands revealed they corresponded to segments of chromosomal genes unrelated to Class 1 integrons. A more detailed analysis, matching the primers with the complete sequence of each of these genes, revealed that there was a non-specific annealing of the primers in each case This explained the species specific pattern for *S. sonnei* and *S. flexneri*. When the PCR was performed with an annealing temperature of 59°C the non-specificity disappeared.

4. Discussion

Infection by *Shigellae* is of special burden within the developing world. However, the increasing number of tourists traveling to exotic destinations has also given shigellosis an important place among the causes of TD. The study of strains from different geographical regions provides a wider picture than that obtained from strains isolated in a determined area. We have analyzed strains from different parts of Africa, South and Central America, the Middle East, and the Indian subcontinent.

Since their first description in 1989 (Stokes and Hall, 1989) much has been found out about integrons. They have been shown to be present in a variety of clinical strains, and widely studied in different Enterobacteriaceae. Curiously, little is known about their presence in the *Shigella* genus, despite the fact that it is well known for easily acquiring and keeping large numbers of plasmids, one of the vehicles used by integrons for horizontal dissemination.

From the 83 strains analyzed, only 11 had a Class 1 integron. It is a smaller proportion than what could be initially expected. This low prevalence of Class 1 integrons found in both *S. sonnei* and *S. flexneri* in the present study is in agreement with previous studies carried out in *S. sonnei* (McIver et al., 2002; Oh et al., 2003), in which a higher prevalence of Class 2 integrons than Class 1 integrons was observed. Despite this low prevalence, a high diversity was observed. Thus, 9 different Class 1 integrons were detected, all previously described in other microorganisms (Huovinen et al., 1995; Adrian et al., 1998; White et al., 2001; Fluit and Schmitz, 1999), indicating the high potential of these structures to be transferred within microorganisms because of their transportation within plasmids or conjugative transposons.

The strains being studied were mostly multiresistant to those antibiotics tested, specially trimethoprim, cotrimoxazole, and ampicillin. These antibiotics are widely used in DCs and resistance toward them has grown alarmingly in the past decades (Vila et al., 1994; Sack et al., 1997). With one exception (strain 35037), trimethoprim resistance might be explained by the presence of integron-borne dihydropholate reductase (*dfr*) genes. A high prevalence of these genes was found, as cassettes, within the sequenced integrons. Only two out of the nine different integrons did not have a *dfr* gene present as a cassette. In contrast, and despite the fact that *oxa* type genes are commonly found as cassettes within integrons (Naas and Nordman, 1999), in this study there was only one *oxa* gene cassette found in one of the integrons present in two unrelated strains, which interestingly came from the same geographical area (Guatemala). Furthermore, taking into account the high number of isolates resistant to trimethoprim and ampicillin that did not carry a Class 1 integron, it can be assumed that the high levels of resistance to these antibiotics in these strains are probably due to genes encoded either within plasmids or in another type of integron.

A probable explanation for the high prevalence of *dfr* genes as cassettes could be that trimethoprim continues to be the therapy of choice in many DCs, where the options for alternative treatments are not obvious. A relatively high prevalence of streptomycin/spectinomycin resistance encoding genes (*aad*A1 and *aad*A2) was also found. Streptomycin has long been excluded from the treatment of shig-

ellosis and the same fate is being followed in many regions by cotrimoxazole, due to the widespread dissemination of cotrimoxazole resistance encoding genes, usually harbored within plasmids and in the past decade very often encountered within integrons. It is now quite accepted that even after an antimicrobial drug is withdrawn from use, their resistance determinants continue to be present in the environment for many decades (Chiew et al., 1998). It is interesting to note the levels of resistance to chloramphenicol. Despite its supposed withdrawal from the market years ago, chloramphenicol continues to be used in many DCs, where it is an available and working treatment for infections such as dysentery.

An initial screening for integrons among all the available strains, resulted in the amplification of the aforementioned Class 1 integrons in 11 isolates as well as in the amplification of a constant pattern of bands in the remaining 69 isolates of *S. flexneri* and *S. sonnei*, apparently species specific. Posterior sequencing of the bands from at least 30% of those strains, revealed that in every case the amplicons corresponded to the same fragments of chromosomal genes without any relation to Class 1 integrons. The theoretical matching of the primers' sequences with the complete sequence of each of these genes, revealed that there was an non-specific annealing of the primers in each case. To our knowledge, it is the first time that similar results are described with the primers from Levesque and Roy (Levesque and Roy, 1993), which have been classically and extensively used to amplify and study Class 1 integrons (Da Silva et al., 2002; Gombac et al., 2002; Kwon et al., 2002; Leversteing van-Hall et al., 2002; Martinez-Freijo et al., 1998). It is of interest to note that RFLP with *Hinf I* has been used to characterize integrons (Seward et al., 1999; Van Belkum et al., 2001). Attending to our results, this methodology without a posterior sequencing has the risk to consider as an integron a non-specific amplification.

Increasing the annealing temperature by 4°C was enough to avoid the non-specific annealing. However, in certain cases, it could also impair the amplification of certain integrons otherwise visible, so that it was not an optimal solution. Thus, to optimize results, alternative primers are required for *Shigella* spp.

The widespread dissemination of resistant determinants in integrons has been well documented in the past decade. In this study we have found a low incidence of Class 1 integrons within the *Shigella* strains, belonging to different species, a result that contrasts with the known facility this genus has to gain and transfer plasmids. However, this is by no means encouraging, taking into account the high levels of multiresistance found among the strains.

Acknowledgments

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3.2.6 Artículo 11.- "*In vitro* activity of rifaximin against bacterial enteropathogens causing diarrhea in children under 5 years of age in Ifakara, Tanzania"

Journal of Antimicrobial Chemotherapy 2001; 47:893-905

La CMI a rifaximina, un agente antibacteriano de baja absorción a nivel intestinal, fué probada en cepas de *E.coli* y *Shigella* spp. causantes de diarrea en niños menores de cinco años de Ifakara, Tanzania. En estudios anteriores se habían determinado los niveles de resistencia de estas cepas a los diferentes antimicrobianos utilizados normalmente como tratamiento, encontrándose altos niveles de resistencia. Se encontró que todas las cepas presentaban CMIs muy inferiores a las concentraciones de rifaximina que normalmente se alcanzan en la luz intestinal, haciendo de la rifaximina una alternativa interesante para el tratamiento de diarreas por patógenos multirresistentes

Journal of Antimicrobial Chemotherapy (2001) **47**, 893–905

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Correspondence

In vitro activity of rifaximin against bacterial enteropathogens causing diarrhoea in children under 5 years of age in Ifakara, Tanzania

J Antimicrob Chemother 2001; **47:** 904–905

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Sir,

Among the bacterial pathogens associated with the development of diarrhoea in children in developing areas, some pathogenic strains of *Escherichia coli* and *Shigella* spp. are especially important, because of both the frequency with which they are isolated and the severity of the symptoms they produce.[1,2] In developing countries it is common to treat infections empirically. Therefore, knowledge of the local bacterial resistance pattern is a valuable weapon for clinicians in these areas. In Ifakara, as well as in other developing areas, the usual therapy for severe diarrhoea in children is oral rehydration plus an antibiotic, currently co-trimoxazole.

In previous studies carried out in Ifakara, in which the resistance levels of 130 pathogenic *E. coli* and 86 *Shigella* spp. were analysed, high rates of resistance, amounting to 100% in some cases, to ampicillin, chloramphenicol, tetracycline and co-trimoxazole were found in the microorganisms studied.[1,2] Only quinolones and cephalosporins retained a good activity.[1,2] However, neither is a viable alternative because of their high price. Furthermore, quinolones are not recommended for paediatric use.

Rifaximin is a non-absorbable antibacterial agent, reaching high concentrations in the intestinal tract.[3,4] Some studies have shown its potential as a treatment for diarrhoea,[3] but its activity against clinical isolates from developing areas remains unknown.

Table. Activity of rifaximin against *E. coli* and *Shigella* spp.

		MIC (mg/L)		
Microorganism	*n*	range	MIC_{50}	MIC_{90}
EaggEC	65	4–32	8	16
ETEC	44	1–32	8	16
EPEC	21	4–16	8	16
S. flexneri	78	2–16	4	8
S. sonnei	4	8–16	4	16
S. dysenteriae	4	4–8	4	8

EaggEC, enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*.

The MIC of rifaximin (Alfa Wassermann, Italy) for these previously studied microorganisms was determined by an agar dilution method following the guidelines of the NCCLS.[5] *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were used as quality controls.

The MIC_{50} and MIC_{90} of the three types of diarrhoeagenic *E. coli* analysed were 8 and 16 mg/L, respectively (Table). Moreover, the highest MIC was 32 mg/L (four enteroaggregative *E. coli* and one enterotoxigenic *E. coli*), while the MIC_{50} of all *Shigella* spp. tested was 4 mg/L, and the MIC_{90} was 8 mg/L for *Shigella flexneri* and *Shigella dysenteriae*, and 16 mg/L for *Shigella sonnei* (Table). No clinical isolate of *Shigella* showed an MIC > 16 mg/L.

The rates of resistance to rifaximin should not be determined based on the criteria valid for absorbable antibiotics. Blood concentrations achieved after a usual oral dose of rifaximin (400 mg) are always negligible, usually <5 ng/mL (ranging from undetectable values to <25 ng/mL),[3,6] whereas the concentrations in the intestinal lumen are much higher. In a study to evaluate its concentration in faeces of travellers after therapy for 3 days with 400 mg of rifaximin every 12 h, it was found that, by the end of the therapy, concentrations of 8000 ± 830 μg/g of rifaximin were achieved in faeces, and 3 days later the concentration was still *c.* 3000 ± 422 μg/g.[5] As the concentration achieved in the intestinal tract remains above the MIC for the strains, rifaximin can be considered as a potential treatment for children with diarrhoea. In fact, in previous studies in which children between 3 and 12 years of age with diarrhoea were treated with rifaximin, satisfactory results were obtained.[3]

In summary, co-trimoxazole, ampicillin and tetracycline

have lost their activity against pathogenic *E. coli* and *Shigella* spp. from Ifakara, Tanzania, making it necessary to change the first-line treatment. Rifaximin may be an alternative, although studies evaluating its *in vivo* activity for the treatment of diarrhoea in children in developing countries should be performed.

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.Artículo 12.- "Detection of dihydrofolate reductase genes by PCR and RFLP"

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Se ha diseñado una metodología utilizando amplificación por PCR seguido de un análisis por RFLP para determinar la presencia de genes de dihidrofolato reductasas, responsables del fenotipo trimetroprim resistente. Utilizando un juego de 5 primers y 5 enzimas de restricción, se puede diferenciar entre 15 genes de dfr diferentes. Se trata de una alternativa más rápida y menos laboriosa que la hasta ahora existente que implicaba hibridación con sondas específicas.

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Detection of dihydrofolate reductase genes by PCR and RFLP

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Abstract

The presence of plasmid-encoded trimethoprim resistant *dfr* genes is the most common mechanism responsible for the acquisition of trimethoprim resistance. The usual method to detect the presence of these genes is hybridization with specific probes. We describe an alternative, faster and easier method, based on PCR amplification and RFLP analysis, to discriminate up to sixteen different *dfr* genes.

Trimethoprim is a synthetic broad-spectrum, antimicrobial agent. Although there are no naturally occurring enzymes to inactivate it, resistant bacterial strains rapidly developed after its introduction in 1962, partly due to its extensive use in both human and veterinary medicine. (Amyes & Towner, 1990; Huovinen et al., 1995)

Resistance to trimethoprim may be due to the presence of one or more of the following mechanisms: auxotrophy in thymine/thymidine, impaired permeability, efflux pumps, alterations in the chromosomal dihydrofolate reductase (DHFR) and/or presence of a plasmid-encoded trimethoprim resistant DHFR. Of these mechanisms, the latter is by far the most frequent (Thomson, 1993; Huovinen, 2001). To date, at least twenty *dfr* genes have been reported (Amyes & Towner, 1990; Thomson, 1993). The corresponding enzymes differ, and thus can be distinguished by biochemical and biophysical properties.

Usually, the detection of DHFRs within a bacterial population involves hybridization using specific DNA probes for the different *dfr* genes (Adrian et al, 1995; Amyes & Towner, 1990). However, this technique has important limitations, being relatively cumbersome, time consuming and expensive. Moreover, in cases where the genes differ in a few base pairs, the discriminatory power of this technique will be limited to the identification of the cluster. The aim of this study is to propose a new, easy and cheap alternative method for the identification of *dfr* genes.

The strains used in this study are listed in Table 1. To design the different primers the *dfr* gene sequences found in GeneBank were downloaded, compared and grouped by sequence similarity. Five different pairs of primers (named Ia, Ib, II, VII and XII) were selected (Table 2). To perform the PCR reactions one colony of the strain to be tested was boiled in 25 μL of sterile distilled water for 10 min and 25 μL of a mixture containing 1.0 mM of each primer (Table 2), 0.4 mM dNTPs, 2× PCR buffer with Mg^+ and 2.5 units of *Taq* polymerase were added. The mixture was overlaid with mineral oil and the reaction carried out in a Perkin Elmer Cetus Thermocycler. The program consisted in 30 cycles: 1 min denaturation (94°C), 1 min annealing and 1 min elongation (72°C), plus a final extension of 10 min at 72°C. Annealing temperatures varied for the different primers and are shown in Table 2, along with the expected size of the amplicons. The reaction products were ran in 1.5% agarose gels with 0.5 μg/ml of ethidium bromide, cut from the gel and recovered using a Concert Rapid PCR Purification System kit (Life Technologies, Gaithersburg). This analysis, when performed on the selected strains (Table 1), gave as result PCR amplicons with the predicted

Table 1
Strains used in the study

dfr gene	Strain	References
dfrA1	*Shigella sonnei*, strain 10681	This article
dfrA14	*Shigella sonnei*, strain 35037	This article
dfrB1	*Escherichia coli*, strain SC3	S.G.B. Amyes
dfrA5	*Shigella sonnei*, strain 66170	This article
dfrA6	*Escherichia coli*, strain SC9	S.G.B. Amyes
dfrA7	*Shigella flexneri*, strain 831	Navia et al. (1999)
dfrA12	*Shigella flexneri*, strain 196DV	This article
dfrA15	*Shigella flexneri*, strain 88193c	This article

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Table 2
Sequence of the primers for amplification of the *dfr* genes

	Primers (Name and sequence)	Amplified *dfr* genes	Amplicon size	Anealling temperature
Ia	up 5′ GTG AAA CTA TCA CTA ATG G 3′ low 5′ TTA ACC CTT TTG CCA GAT TT 3′	*dfrA1, dfrA5, dfrA15, dfrA15b, dfrA16, dfrA16b**	474 bp	55°C
Ib(+)	up 5′ GAG CAG CTI CTI TTI AAA GC 3′ low 5′ TTA GCC CTT TII CCA ATT TT 3′	*dfrA14, dfrA6*	393 bp	60°C
VII	up 5′ TTG AAA ATT TCA TTG ATT G 3′ low 5′ TTA GCC TTT TTT CCA AAT CT 3′	*dfrA7, dfrA17,*	474 bp	55°C
II	up 5′ GAT CAC GTG CGC AAG AAA TC 3′ low 5′ AAG CGC AGC CAC AGG ATA AAT 3′	*dfrB1, dfrB2, dfrB3*	141 bp	60°C
XII	up 5′ GGT GSG CAG AAG ATT TTT CGC 3′ low 5′ TGG GAA GAA GGC GTC ACC CTC 3′	*dfrA12, dfrA13*	319 bp	60°C

(+) also amplifies *dfrA1, dfrA5, dfrA15, dfrA16*. Sequence analysis showed that the Ib upper primer could in theory anneal with the *dfrA3*, although a specific lower primer would be required for amplification.

* Two different genes *dfrA16* are found in Gene Bank. Thus, one was named *dfrA16b*.

sizes (Table 2, Figure 1A). To confirm that the amplicons corresponded to the expected genes, sequencing of the recovered DNA was carried out with a Thermosequenase Dye Terminator Sequencing Kit (Amersham, Amersham Place, England) and analyzed in an automatic DNA sequencer 377A (Perkin Elmer, Emeryville, CA). In every case it was confirmed that the amplicon corresponded to the expected *dfr* gene.

The appropriate restriction enzymes to perform RFLP of each group were selected performing theoretical digestions of the amplicons with various enzymes. Those chosen and used are shown in Table 3. The theoretical study was performed for both the *dfr* genes present in the available resistant strains (*dfrA1, dfrA5, dfrA6, dfrA14* and *dfrA15*) and for those that could be amplified with the designed primers, even though no practical experiment could be carried out. As shown in Table 3, the predicted RFLP resulted in distinguishable patterns for each *dfr* gene. Digestions were performed following each manufacturer's indications and digested DNA was resolved in 2% agarose gels and stained with ethidium bromide (0.5 μg/ml). The RFLP of the PCR products resulted in different patterns in agreement with the theoretical predictions (Table 3, Figure 1B and C).

Recent studies have been conducted in which PCR has been used to detect specific *dfr* genes (Guerra et al., 2000; Navia et al., 1999). However, since over twenty *dfr* genes have been described (White et al., 2000), this approach is inadequate for the routinary detection of *dfr* genes, making it necessary to perform at least twenty different PCRs, one for each known *dfr* gene. Alternatively, DNA sequencing can be carried out on the PCR products obtained with specific primers for each cluster of enzymes. However, this technique is also time consuming and expensive.

In this study we propose an easy and rapid technique to

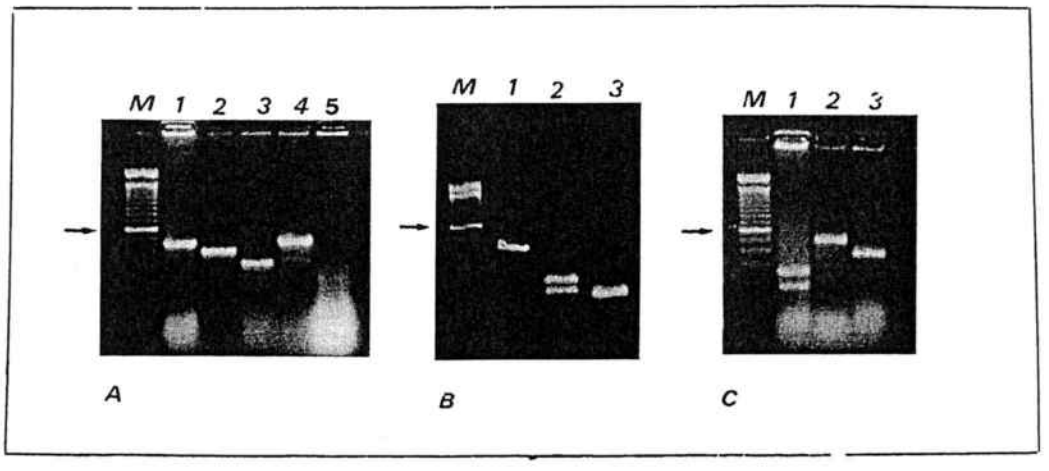

Fig. 1. Amplification of *dfr* genes and RFLP analysis.Group1A. RFLP pattern. Lane M.- Molecular weight marker (Gibco BRL 100bp ladder). Lane 1.- *dfrA1*. Lane 2, *dfrA14*. Lane 3.- *dfrA12*. Lane 4.- *dfrA7*. Lane 5.- *dfrB1*.Group 1B. RFLP pattern. Lane M, Molecular weight marker (Gibco BRL 100bp ladder). Lane 1, *dfrA14* amplicon prior to digestion. Lane 2, *dfrA14* amplicon after digestion with *Alu* I. Lane 3, *dfrA6* amplicon after digestion with *Alu* I.Group 1C. RFLP pattern. Lane M, Molecular weight marker (Gibco BRL 100bp ladder). Lane 1, *dfrA1* amplicon after digestion *Eco*RI*. Lane 2, *dfrA5* amplicon after digestion with *Eco*RI*. Lane 3, *dfrA15* amplicon after digestion with *Eco*RI*. The arrows in all cases show the 600bp.

Table 3
Theoretical fragment sizes from RFLP of the PCR amplicons of *dfr* genes

Primer	Enzyme	Expected band sizes (bp)	*dfr* gene	Accession No.
Ia	*Eco*RI* (also known as *Tsp*509 I)	**236, 163, 42, 33**	*dfrA1*	X00926
		455, 19	*dfrA5*	X12868
		356, 62, 33, 23	*dfr15*	Z83311
			dfr15b	AF156486
		356, 62, 33, 23	*dfrA16*	AF077008
		219, 101, 94, 41, 19	*dfrA16b*	AF174129
		219, 101, 94, 41, 19		
Ia	*Mae* III[1]	183, 265, 26	*dfrA15*	Z83311
		448, 26	*dfrA15b*	AF156486
Ia	*Mse* I[2]	250, 190, 31, 3	*dfrA16*	AF077008
		190, 155, 95, 31, 3	*dfrA16b*	AF174129
Ib	*Alu* I (only *dfrA14* and *dfrA6*)	**212, 175, 6**	*dfrA14*	Z50804
		166, 161, 60, 6	*dfrA6*	Z86002
VII	*Alu* I	268, 206	*dfrA7*	AF139109
		216, 206, 52	*dfrA17*	AF169041
II	*Eco*RI* (also known as *Tsp*509 I)	63, 78	*dfrB1*	U36279
		119, 22	*dfrB2*	J01773
		No cut (i.e 141 bp)	*dfrB3*	X72585
XII	*Taq* I	286, 33	*dfrA12*	Z21672
		187, 132	*dfrA13*	Z50802

In bold, the results shown in Fig. 1B and 1C
[1] To distinguish between *dfrA15* and *dfrA15b*
[2] To distinguish between *dfrA16* and *dfrA16b*

identify the *dfr* genes present in trimethoprim resistant strains. RFLP of PCR products has proven to be a reliable technique, with which we have obtained a 100% concordance between the theoretical and the practical results, confirmed by sequencing analysis. This technique has been previously used to identify point mutations (Ruiz et al., 1995), different gene types involved in acquired resistance (Fluit et al., 2001) and in epidemiologic studies (Tudó et al., 2001). However, its real advantage is its flexibility. It may be modified, by selecting alternative restriction enzymes or by the designing of new primers. As novel *dfr* genes are discovered, the technique can be adapted to include them. With the 5 pairs primers and the 5 enzymes used in this study, sixteen *dfr* genes can be identified using only two PCR conditions. Such genes happen to belong, according to the phylogenetic tree built by White et al. (2000) and with the exception of the *dfrB* group which was not included in the analysis, to the two families of enzymes encoded by gene cassettes. Moreover, if two or more *dfr* genes were to be present in the same strain and happen to amplify with one of the proposed sets of primers, RFLP analysis will result in a combined band pattern that should allow identification of the *dfr* genes present.

The limitation of this work lies in the small number of strains used. However, the results obtained are consistent and there are no reasons to suppose that the technique will not work with those *dfr* genes that were not experimentally tested. To summarize, an alternative method based on the PCR amplification of the *dfr* genes followed by RFLP analysis is presented. It is an easy, rapid and non-cumbersome technique capable of differentiating among closely related *dfr* genes.

Acknowledgments

We thank Dr. S.G.B. Amyes for kindly providing some of the strains. This work was partially supported by grant FIS00/0997 from the Fondo de Investigaciones Sanitarias (Spain). JSC has a grant associated to project PETRI no. PTR1995-0430-OP

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3.2.7 Artículo 13.- " Dispersión intercontinental de una cepa de *Shigella flexneri* resistente a trimetoprima"

Enfermedades Infecciosas y Microbiología Clínica, 2003; 21(8):401-3.

Se buscaba caracterizar una cepa de *Shigella flexneri* resistente a trimetoprima aislada de las heces de un viajero a su retorno de Kenia, y analizar su relación epidemiológica con un conjunto de cepas de similares características aisladas en Tanzania. Los estudios epidemiológicos mostraron que el total de seis cepas estudiadas pertenecían a un mismo clon. Asimismo, todas ellas poseían el mismo gen codificante para resistencia a trimetoprima (*dfr*A7), el cual estaba localizado en un integrón situado a nivel cromosomal. Es preciso mantener una vigilancia epidemiológica para controlar la difusión de microorganismos patógenos, o de genes de resistencia, entre distantes áreas geográficas

ORIGINALES

Dispersión intercontinental de una cepa de *Shigella flexneri* resistente a trimetoprima

Margarita M. Navia, Joaquim Ruiz y Jordi Vila

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INTRODUCCIÓN. Caracterizar una cepa de *Shigella flexneri* resistente a trimetoprima, aislada de las heces de un viajero a su retorno de Kenia, y analizar su relación epidemiológica con un conjunto de cepas de similares características aisladas en Tanzania.
MÉTODOS. Las relaciones clonales se estudiaron mediante análisis del perfil plasmídico, REP-PCR y electroforesis en geles de campos pulsantes (PFGE). Se estudió la presencia de integrones tipo 1 mediante reacción en cadena de la polimerasa (PCR) y secuenciación. Mediante técnicas de conjugación y PCR se analizó la localización y transferabilidad del integrón detectado.
RESULTADOS. Los estudios epidemiológicos mostraron que el total de seis cepas estudiadas pertenecían a un mismo clon. Asimismo, todas ellas poseían el mismo gen codificante para resistencia a trimetoprima (*dfrA7*), el cual estaba localizado en un integrón situado a nivel cromosómico.
CONCLUSIONES. Es preciso mantener una vigilancia epidemiológica para controlar la difusión de microorganismos patógenos, o de genes de resistencia, entre áreas geográficas distantes.

***Palabras clave:* Trimetoprima. Resistencia. *dfrA7*. Viajeros. Diseminación clonal.**

Intercontinental spread of a trimethoprim-resistant strain of *Shigella Flexneri*

INTRODUCTION. In this study we characterize a trimethoprim-resistant strain of *Shigella flexneri* recovered from stool samples of an international traveler after a trip to Kenya, and analyze its epidemiological relationship with a set of strains having similar characteristics from Tanzania.
METHODS. Clonal relationships were studied by three techniques - plasmid profile, repetitive-element (REP)-PCR and pulse-field gel electrophoresis (PFGE). The presence of type 1 integrons was studied by PCR and sequencing. The location and transferability of the detected integron was analyzed by conjugation and PCR.
RESULTS. The epidemiological studies showed that all six strains studied belonged to the same clone. Furthermore, all of them carried the same gene encoding for trimethoprim resistance (*dfrA7*), which was located in an integron within a chromosome.
CONCLUSION. Continuous epidemiological surveillance is required to control the spread of pathogenic microorganisms and the dissemination of resistance-encoding genes among geographical areas.

***Key words:* Trimethoprim. Resistance. *dfr*A7. Travelers. Clonal spread.**

Introducción

Se calcula que alrededor de 3 millones de seres humanos, niños en su mayoría, mueren anualmente durante el curso de afecciones diarreicas[1]. Así, una de cada 4 muertes de niños menores de 5 años acontecida en países en vías de desarrollo se debe a un proceso diarreico[1,2]. Un tercio de estas muertes se deben a infecciones causadas por *Shigella* spp.[3].

Esta situación sociosanitaria repercute, asimismo, en los viajeros provenientes de países desarrollados. Se calcula que aproximadamente entre el 20 y el 60% de los viajeros a estas áreas, durante o después del viaje, sufren procesos diarreicos, la denominada diarrea del viajero[4]. Si se tiene en cuenta que, en la actualidad, el conjunto de estos países son uno de los principales destinos turísticos a nivel mundial, recibiendo millones de turistas cada año, la importancia de esta enfermedad queda fuera de toda duda.

El tratamiento de elección para las infecciones asociadas a *Shigella* spp. ha sido, durante largo tiempo, el cotrimoxazol. No obstante, el amplio uso de la combinación de trimetoprima con sulfonamidas, uso que incluye, además de la terapéutica humana, aplicaciones veterinarias, ha facilitado la aparición de aislamientos de *Shigella* spp. resistentes al cotrimoxazol[5-8].

En la actualidad, se conocen diferentes mecanismos capaces de conferir resistencia a trimetoprima, siendo el más difundido la presencia de dihidrofolato reductasas (DHFR) resistentes a trimetoprima[9]. Así, hasta la actualidad, se han descrito más de 20 enzimas diferentes[9,10]. La mayoría de ellas se haya codificada en genes que se localizan en integrones[7,10], con un mayor o menor grado de diseminación. Entre estas últimas, se halla la DHFRVII. La primera cita de esta enzima es del año 1989, año en el que se describió en un plásmido aislado en cepas de *Escherichia coli* causantes de un brote de diarrea porcina[11]. En años subsiguientes, se describió en cepas de *E. coli* pro-

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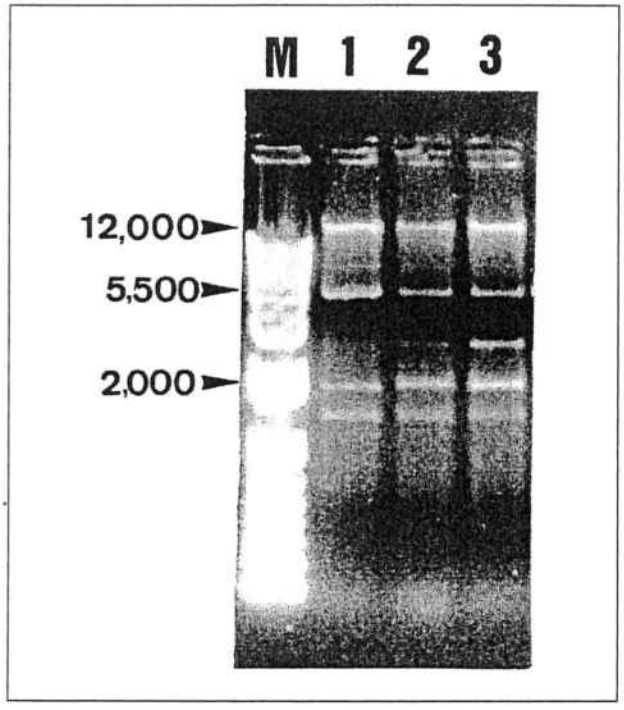

Figura 1. Perfil plasmídico de las cepas. 1, Cepa de *S. flexneri* procedente del viajero a Kenia. 2 y 3, Cepas de *S. flexneri* aisladas de niños tanzanos. M: marcador de pesos.

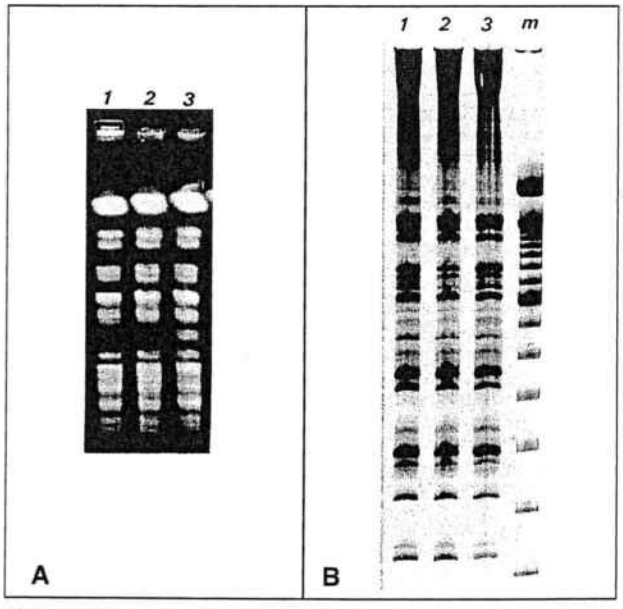

Figura 2. Técnicas de epidemiología molecular:
A) Electroforesis en campo pulsante: 1 y 2, cepas de *S. flexneri* aisladas de niños tanzanos, 3 cepa de *S. flexneri* aislada de las heces del viajero a Kenia.
B) REP-PCR: 1 y 2, cepas de *S. flexneri* aisladas de niños tanzanos; 3, cepa de *S. flexneri* aislada de las heces del viajero a Kenia. M: marcador de pesos (100 bp ladder; GIBCO-BRL, Gaithersburg, USA).

venientes de Suecia, Sri Lanka, Finlandia, Nigeria o Sudáfrica, así como en otros microorganismos como *Salmonella typhi* de la India[12-14].

Más recientemente se ha descrito esta enzima en un clon de *S. flexneri* aislado de heces de niños con diarrea en Tanzania[7]. En este trabajo se analiza la dispersión de dicho clon entre Kenia y Tanzania, así como su introducción en Europa, a través de viajeros internacionales.

Métodos

En un trabajo previo desarrollado en 1997 sobre epidemiología y niveles de resistencia a antimicrobianos en cepas de *Shigella* spp. causantes de diarrea en niños menores de 5 años en Tanzania, se comunicó la presencia de cinco cepas de *S. flexneri* que poseían un integrón que contenía el gen *dfrA7*[7]. Posteriormente, en 1999, se aisló una cepa de *S. flexneri* de las heces de un viajero procedente de Kenia (previamente se verificó que únicamente se hubiese desplazado a este país).

El patrón de sensibilidad antibiótica se obtuvo mediante tiras de E-test (AB Biodisk, Sölna, Suecia) en placas de Müeller-Hinton, siguiendo las instrucciones del fabricante. En concreto se analizó la sensibilidad a ampicilina, cloranfenicol, tetraciclina, ciprofloxacino y cotrimoxazol.

La relación epidemiológica entre las cepas se determinó mediante tres técnicas diferentes: REP-PCR, digestión con enzimas de baja frecuencia de corte y posterior electroforesis en geles de campos pulsantes (PFGE) y análisis plasmídico. La REP-PCR y el PFGE se realizaron siguiendo metodologías descritas previamente[7], mientras que los plásmidos se extrajeron de cultivos bacterianos usando el kit Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, EEUU), que posteriormente fue visualizado en geles de agarosa al 0,7% tras ser teñidos con bromuro de etidio.

El estudio de la presencia y contenido de integrones se realizó mediante reacción en cadena de la polimerasa (PCR) y secuenciación según la metodología previamente descrita[7]. Para determinar la posible transferencia mediante conjugación de la resistencia a trimetoprima se realizaron conjugaciones, tanto en medio líquido como en medio sólido sobre filtro, usando como bacteria receptora la cepa J53 de *E. coli* (F-, pro, Tm^S, Amp^S, Rif^R, Lac^+), gentilmente cedida por el Dr. Gómez-Lus (Universidad de Zaragoza). En total se efectuaron tres ensayos por cepa y método.

La ubicación del gen *dfrA7* en plásmido o cromosoma se determinó mediante PCR con cebadores específicos, tanto para la *dfrA7* como para integrones[7] sobre ambos productos previamente purificados. Como control, para detectar la presencia de ADN cromosómico, se utilizaron cebadores específicos para *gyrA*[15].

Resultados

Tanto las cepas aisladas en Tanzania, como aquella proveniente de Kenia, presentaban un patrón de resistencia a ampicilina, cotrimoxazol, cloranfenicol y tetraciclina, siendo sensibles al ciprofloxacino (concentración inhibitoria mínima [CIM] de 0,006 μg/ml) y al ácido nalidíxico (CIM de 1,5 μg/ml).

Las tres técnicas epidemiológicas utilizadas mostraron la existencia de relaciones clonales entre las cepas de Tanzania y la procedente de Kenia (figs. 1 y 2). En la cepa originaria de Kenia se encontraron 5 plásmidos (> 12 kb, 7,5 kb, 5 kb, 2 kb, 1,5 kb), que con excepción de aquel de 7,5kb también se encontraron en las cepas de Tanzania (fig. 1).

Al utilizar los cebadores para amplificar integrones de tipo 1 se detectó, en todos los casos, una banda de aproximadamente 750 pb, que al ser secuenciada mostró la presencia de un único gen *(dfrA7)* y una homología del 100% con la secuencia presente en el banco de genes.

Para localizar la ubicación de este integrón, se efectuaron estudios de conjugación y de PCR. Los estudios de conjugación mostraron que el gen no era transferible. Por su lado, los estudios de PCR mostraron la presencia en el cromosoma del integrón de 750 pb, así como más específicamente del gen *dfrA7*; los intentos de amplificación a partir de ADN plasmídico fueron negativos.

Discusión

En este estudio se demuestra la diseminación clonal de una cepa de *S. flexneri* multirresistente entre Tanzania y Kenia, así como su llegada a Europa en un viajero con diarrea del viajero.

La localización en un integrón del gen *dfrA7* es algo que ya había sido previamente descrito[7,10], aún cuando las únicas cepas de *Shigella* spp. en las que ha sido hallado hasta la fecha son las presentes en este estudio. Asimismo, se ha descrito con frecuencia en diferentes microorganismos en zonas relativamente próximas a Kenia y Tanzania, como sería el caso de Sudáfrica[12]. Este hecho, junto con la escasa información a nivel microbiológico y molecular, de los microorganismos presentes en esta zona geográfica, y la amplia movilidad de buena parte de la población que la habita, hacen presuponer una posible amplia diseminación de este gen en la zona.

Es interesante notar que en todas las cepas incluidas en este estudio, el gen *dfrA7* estaba codificado a nivel cromosómico, hecho frecuente, a tenor de las observaciones de Adrian[12]. En un estudio realizado por su grupo, se detalla que entre todos los genes que codifican para DHFR hallados en cepas comensales de *E. coli*, *Klebsiella* spp. y *Enterobacter* spp., aisladas de heces de voluntarios sanos en Sudáfrica, es el gen *dfrA7* el que con más frecuencia se hallaba codificado en el cromosoma, no siendo transferible por conjugación en 62 de 67 cepas (92,54%) que lo presentaban. Estos resultados contrastaban con lo que se encontraba para el resto de genes *dfr* incluidos en este estudio. La segunda DHFR, que presentaba peores niveles de transferencia por conjugación, fue la DHFR Ia, conjugando en 11 de las 52 cepas (21,15%) que la presentaban. Este dato pone de manifiesto las dificultades que presenta este gen para propagarse de manera horizontal. No obstante, en este estudio de Adrian et al[12] no se analizaron las relaciones epidemiológicas presentes entre las cepas, por lo que quedó sin establecer si las cepas que presentaban una codificación cromosómica del gen *dfrA7* pertenecían a un mismo clon o a varios. En nuestro estudio se constata, mediante tres técnicas diferentes, la estrecha relación epidemiológica existente entre las cepas incluidas en el mismo, demostrando que poseen un mismo origen clonal. Se ha de tener en cuenta que numerosos integrones se hallan en un transposón, lo que permitiría explicar su localización tanto plasmídica como cromosómica. Otra posibilidad teórica que debe tenerse en cuenta sería la posible integración, total o parcial, en el cromosoma, de un plásmido portador del citado gen.

El hecho de que una cepa bacteriana se disemine lentamente entre zonas limítrofes, como sería el caso de Kenia y Tanzania, debido a lo que se podría dar en llamar movimientos naturales de sus huéspedes, o por simples medios mecánicos, como pueden ser corrientes de agua, es algo connatural a todo ser vivo. No obstante, el que una cepa bacteriana específica pueda desplazarse miles de kilómetros en cuestión de horas, es algo que hasta la aparición de los actuales medios de transporte no podía tener lugar. Esto pone de manifiesto la necesidad de iniciar estudios epidemiológicos para controlar, en la medida de lo posible, la difusión de microorganismos, o genes de resistencia, entre diferentes y lejanas áreas geográficas.

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4 DISCUSION

A comienzos del siglo pasado, las infecciones bacterianas (principalmente la tuberculosis, la pneumonía y las diarreas) eran la primera causa de mortalidad y morbilidad en todo el mundo (Cohen, 1997). El desarrollo de vacunas, el descubrimiento de los antibióticos y cambios en la sociedad incluyendo mejoras en la higiene y sanidad, provocaron una disminución gradual de las infecciones. Sin embargo, a comienzos del siglo XXI, volvemos a vernos enfrentados al resurgimiento de infecciones que creíamos controladas debido, entre otras causas, al rapidísimo aumento de resistencias.

La prevalencia de las resistencias bacterianas a los agentes antimicrobianos, depende tanto de la adquisición de la misma, como de su posterior dispersión. Ambos procesos están directamente influenciados por la presión selectiva de los antimicrobianos. Se pueden diferenciar tres ambientes en los cuales, habiendo un uso de antimicrobianos, se promueve o al menos se facilitan dichos procesos: el ambiente veterinario, el hospitalario y la comunidad.

En este proyecto, se buscaba analizar como se adquiere la multiresistencia y como se disemina la misma en los dos ambientes con mayor uso de antimicrobianos: el hospitalario y la comunidad. Para ello, se estudiaron un patógeno nosocomial, *Acinetobater baumannii*, y uno comunitario: *Shigella* spp. El primero es uno de los bacilos Gram negativos con mayor morbi-mortalidad en las infecciones nosocomiales (Sefton,2002), siendo además altamente multiresistente. El género Shigella es por su parte, uno de los que causa síntomas más invalidantes entre los diferentes agentes etiológicos de la diarrea. Se trata además de un patógeno comunitario que normalmente responde bien al tratamiento, pero que en las últimas décadas ha mostrado un aumento preocupante de resistencia a los agentes antimicrobianos (Ashkenazi et al., 2003). Adicionalmente, estos dos microorganismos resultan ser buenos ejemplos de un patógeno problema en el mundo desarrollado (*A.baumannii*) y uno epidémico en PBR (*Shigella* spp.).

4.1 *Acinetobacter baumannii*

A.baumannii es una bacteria con una resistencia intrínseca elevada, que además al estar expuesta a una alta presión de selección dentro del ambiente hospitalario, ha desarrollado

altos niveles de resistencia. Al estudiar cepas multirresistentes aisladas de diferentes hospitales españoles, observamos la dispersión de los mismos clones en diferentes hospitales de la Península e incluso Canarias (Artículo 1). Estudios realizados para clarificar epidemias dentro de hospitales han demostrado que son a menudo debidas a la dispersión de cepas locales (Corbella et al., 2000). El que estos microorganismos tengan una gran capacidad para sobrevivir en superficies inanimadas (equipos, pomos de puertas, colchones) y sobre la piel, facilita y aumenta las probabilidades de dispersión y por lo tanto de epidemias. Lo que además hemos hallado es que la dispersión inter-hospitalaria de cepas multirresistentes de *A.baumannii*, al menos en España, también está ocurriendo.

Los aminoglucósidos y los betalactámicos han sido utilizados como primera línea de tratamiento para infecciones por *A.baumannii*. Sin embargo, los niveles de resistencia han ido aumentando hasta el punto de que los primeros ya prácticamente no se utilizan y en el caso de los segundos, grupos como las penicilinas o las cefalosporinas tampoco son efectivos. Intentando contestar a la pregunta sobre cuales eran los mecanismos implicados en los fenotipos multirresistentes de cepas clínicas de *A.baumannii*, se estudiaron cepas resistentes a amikacina (Artículo 1) y a betalactámicos (Artículo 2) provenientes de diferentes hospitales españoles.

La resistencia a amikacina era debida en un 100% de los casos a la presencia del gen *aph*(3')-VI, siendo consistente con lo que se ha encontrado en estudios previos (Lambert et al., 1990; Shaw et al. 1993) que describen a este gen como el principal mecanismo responsable de la resistencia a amikacina en *A.baumannii*. Se trata de un gen transponible (Lambert et al., 1990) lo que facilita su dispersión. En este caso, sin embargo, se ha demostrado que la dispersión de la resistencia no solo está relacionada con la del gen, sino con la de una cepa resistente.

Actualmente, *A.baumannii* es resistente frente a la mayoría de antibióticos ß-lactámicos, y particularmente las penicilinas y las cefalosporinas (Vila, 1998). Más recientemente, dicha

resistencia se ha observado también frente a los carbapenems (Bou et al., 2000; Corbella et al., 2000). Con el fin de esclarecer la distribución y el papel de las ß-lactamasas en *A.baumannii* resistentes a los ß-lactámicos, se estudiaron una serie de cepas clínicas epidemiológicamente bien definidas (Artículo 2). El 100% de las cepas eran resistentes a la ampicilina y encontramos que en el 60% de las cepas esto se debía bien sea una ß-lactamasa plasmídica, tipo TEM (25%) o tipo OXA (35%). Ambos tipos de ß-lactamasas han sido descritas anteriormente en *A.baumannii* y parecen jugar un papel protagonista en la resistencia a las penicilinas (Vila, 1998). Sin embargo, en las cepas restantes (también resistentes a la ampicilina), se observó una disminución de la CMI a ampicilina cuando se determinaba en presencia del Syn2190, un inhibidor de la ß-lactamasa cromosómica AmpC. Nuestros resultados sugieren que al menos en el 50% de las existe una ß-lactamasa cromosómica del tipo AmpC sobrexpresada que es inhibida por el Syn2190. El nivel de expresión varía entre cepa y cepa, viéndose reflejado en la CMI a ampicilina. La susceptibilidad a la piperacilina era también baja, con 35% de las cepas resistentes y 45% con resistencia intermedia. Curiosamente, cepas con las mismas ß-lactamasas, presentaban diferentes CMIs a la piperacilina en presencia de Syn 2190. Mientras que en un grupo de ellas no se veía afectada la CMI en presencia del inhibidor de AmpC, en otras disminuía. Pueden argumentarse tres posibles explicaciones:

1.- Que en aquellas células que no muestran una disminución de la CMI haya una entrada disminuída del Syn 2190

2.- Que en estas mismas exista un mecanismo de resistencia a piperacilina complementario tal como una disminución de la permeabilidad, un aumento de expulsión o modificación de las PBP

3.- La presencia de varias cefalosporinasas cromosómicas con diferentes niveles de inhibición frente al Syn 2190

Un 15% de las cepas eran resistentes al imipenem, indicando la presencia de una carbapenemasa. Hasta el momento se han descrito varias en *A.baumannii* localizadas en plásmidos y por lo tanto con facilidad de ser transferidas, como es el caso de la OXA-23 (primero descrita como ARI-1). En presencia de la reserpina, un inhibidor de bombas de expulsión, se observó una disminución de la CMI de imipenem de 1mg/L a 0,25mg/L, sugiriendo que una bomba de expulsión podría de esta disminución a la sensibilidad a imipenem en estas cepas.

En general, no hay que menospreciar la contribución que otros mecanismos tales como la disminución de la permeabilidad por alteración en las porinas, el aumento de expulsión activa o la alteración de las PBP tienen sobre la CMI final de una cepa resistente.

Así, por ejemplo, en un trabajo publicado en el 2002, Fernández-Cuenca y colaboradores estudiaron los mecanismos de resistencia a los carbapenems, encontrando que el fenotipo resistente se debía a la presencia de multiples mecanismos entre los cuales los más comunes eran la producción de oxacilinasas y la ausencia de la PBP2. En algunas cepas resistentes parecía también tener relación con dicho fenotipo la ausencia de una OMP específico.

En este caso, sin embargo, podemos decir que en las cepas analizadas de *A.baumannii*, la resistencia a ß-lactámicos se explica en un alto porcentaje por la producción de ß-lactamasas, bien sea de las transferibles (tipo TEM u OXA) o por sobreexpresión de las cromosómicas (tipo AmpC).

Analizando la resistencia por ß-lactamasas en *A.baumannii*, hayamos dos del tipo OXA que anteriormente no habían sido descritas. La primera, una OXA-21 (Artículo 3) se deriva de la OXA-3, diferenciándose de esta por tres mutaciones puntuales (dos silentes y una que resultaba en el cambio de la Ile-217 en una metionina. Este aminoácido está localizado a cuatro de la

triada conservada. Este gen se halló en al menos 6 de las cepas estudiadas (Artículo 2), y estaba localizado en un integrón, con un gen de *aadB* en el extremo 3'.

También en un integrón, se hayó una OXA-37 (Artículo 4). Esta ß-lactamasa se deriva de la OXA-20, previamente descrita en *Pseudomonas aeruginosa* (Naas et al., 1998) y en *A.baumannii* (Ploy et al., 1994), diferenciándose en dos mutaciones puntuales, una de las cuales resulta en el cambio del acido glutámico 69 por un ácido aspártico. Al tratarse ambos de aminoácidos de carga negativa, se trata de un cambio que estructural y funcionalmente no comporta importancia alguna. Con el fin de entender la contribución de esta ß-lactamasa a la resistencia de la cepa de la que fue aislada, se clonó el gen de la *oxa* 37 y se transformó con él una cepa de *E.coli* sensible. Hallamos que la mayor actividad de la ß-lactamasa era frente a la ticarcilina, ceftazidima, cefotaxima y ampicilina+sulbactam.

El estudio del integrón dentro del que se encontró la OXA-37, permitió hacer un análisis de su posible origen. Al igual que aquel descrito por Naas et al., el primer cassette dentro del integrón codificaba para una N-acetiltransfereasa (específicamente una *aac* A4) y que previamente ya ha sido descrita en *A.baumannii*. Como segundo cassette y antes de la OXA-37 hayamos una ORF que no estaba presente en el integrón de *P.aeruginosa* y que en un trabajo posterior al de Naas se describió, aunque más corto, en *A.baumannii* (Ploy et al., 2000). Podría argumentarse entonces que en términos de selección, el perder la ORF tiene ventajas pues sitúa a la OXA en segundo en vez de tercer lugar puesto que se sabe que en un integrón, entre más alejado esté el cassette del promotor, menor será su nivel de transcripción. Por supuesto, esto sería cierto siempre y cuando la transcripción del ORF en sí no tuviese alguna ventaja que desconocemos. Además, se sabe que la probabilidad de perder un cassette de un integrón es en general, mayor que aquella de ganarlo (Bissonnette et al. 1992). Finalmente, tras el análisis de contenido de GC de la OXA-20, Naas y colaboradores llegaron a la conclusión de que con un 45% (prácticamente igual que para la OXA-37) debía ser originariamente de una Enterobacteriaceae. El contenido de GC en genes originarios de *Acinetobacter* suele estar entre el 38% y el 47% (Maslow et al. 1993). Analizando el contenido de GC de la ORF encontramos

que era del 46.43% con lo cual aún está dentro del rango. Creemos entonces que hay evidencia suficiente para no descartar la posibilidad de que el integrón y la OXA hallados en un principio en *P.aeruginosa* sean de hecho originarios de *A.baumannii,* más aún si se tiene en cuenta que se trata de dos microorganismos que comparten nicho ecológico, hecho que en sí facilita el que se pueda dar la transferencia de material genético entre las dos especies. La descripción de integrones estrechamente relacionados muestra de nuevo la gran plasticidad de estos elementos y la relativa facilidad con que ganan o pierden genes

El anterior es solo un ejemplo de la importancia de este elemento genético en la diseminación de genes de resistencia a antimicrobianos tanto inter como intraespecie. La aportación que estos tienen en la multirresistencia presentada por *A.baumannii* ha sido valorada en diferentes ocasiones. Tras encontrar en cepas de *A.baumannii* integrones de Clase 1 con genes de resistencia a aminoglicósidos (Artículos 1,3 y 4) y a ß-lactámicos (Artículos 3 y 4), se decidió establecer el papel de estos elementos en el fenotipo multirresistente de cepas clínicas de la especie. Se hallaron cinco diferentes integrones en las cepas analizadas. Todos tenían al menos una enzima modificadora de aminoglucósidos, principalmente del tipo *aadB* y *aacA4.* Aunque ninguno de los dos genes se ha descrito como de los más comunes en *A.baumannii,* en España parecen estar ampliamente distribuídos en esta especie. En un trabajo realizado por nuestro grupo y en el que se estudiaron 69 cepas de *A.baumannii* procedentes de diferentes hospitales españoles (Ribera et al., 2004), se encontró que en el 95% de las cepas con integrones Clase 1 (18/19) tenían *aadB* o *aacA4* como cassette. Más aún, la gran mayoría (15/19) tenían un integrón de 700pb cuyo único cassette era el *aadB.* Las cepas no estaban ni epidemiológicamente ni geográficamente relacionadas y sin embargo presentaban en su mayoría el mismo integrón, planteando la pregunta de si *A.baumannii* tiene una clara afinidad por ciertos tipos específicos de integrón.

La alta resistencia de la especie a los aminoglicósidos, que es principalmente debida a la presencia de enzimas modificadoras mediadas por plásmidos y transposones, es ampliamente reconocida para el género. El predominio del fenotipo resistente se debe en gran

parte a su capacidad para adquirir genes de resistencia (Towner, 1997), con los integrones, en este sentido jugando un papel importante.

El otro tipo de gen encontrado como *cassette* fue el de ß-lactamasas tipo OXA, específicamente *oxa*-21 y *oxa*-37, ambas descritas anteriormente por nosotros en *A.baumannii* (Artículos 3 y 4).

Nuestros resultados mostraban que cepas de diferente origen geográfico y epidemiologicamente no relacionadas podían presentar el mismo integrón, mientras que otras epidemiológicamente relacionadas presentaban elementos con *cassettes* muy diferentes. En todos los casos se trataba de integrones anteriormente descritos, sino en *A.baumannii*, en especies tales como *P.aeruginosa*, también patógeno nosocomial y expuesta a la misma presión de selección antibiótica

4.2 *Shigella* spp.

La epidemiología de la infección por las diferentes especies de Shigella siempre se suele asociar con la dispersión de clones epidémicos. En el estudio realizado con cepas aisladas en niños tanzanos (Artículo 6) se observó que 90% de las cepas aisladas eran *S.flexneri* (la especie más común en PBR) y de estas un 65% (51/78) correspondían a un solo clon, que además era multirresistente. Al igual que en este estudio, gran parte de lo que a nivel epidemiológico se ha publicado hasta el momento sobre Shigella, se ha centrado en cepas de un área geográfica determinada y en un gran número de casos con cepas provenientes de un brote epidémico. Esto ha dado la idea errónea de que hay un alto grado de clonalidad entre las cepas de una misma especie de Shigella. Las poblaciones bacterianas se reproducen por fisión binaria, y por lo tanto están compuestas de clones que eventualmente adquieren variabilidad genética por recombinación, transferencia lateral de genes o mutaciones. Por lo tanto, al estudiar una población de patógenos, la definición de "clonalidad" debe ser asumida con cautela, especialmente cuando dicho patógeno, como en este caso, tiende a aparecer en brotes localizados. Al analizar un grupo de cepas de Shigella aisladas de regiones geográficas muy

diversas, provenientes de pacientes con DV (Artículo 8), hallamos un alto grado de variablidad genética tanto en *S.sonnei* como en *S.flexneri.* En otras palabras las especies de Shigella tienen un alto grado de variabilidad genética pero tienden a generar brotes epidémicos que, por definición, se deben principalmente a la dispersión de una o pocas cepas clónicas en zonas geográficas delimitadas.

El incremiento de viajes internacionales, y el aumento de los viajeros entre PBR y países desarrollados, está sin embargo abriendo fronteras y haciendo que estas zonas inicialmente tan delimitadas dejen de serlo. En Estados Unidos se ha asociado la Shigellosis multiresistente con los viajes internacionales (Tauxe et al., 1990). Esto concuerda con lo hallado en nuestro estudio con cepas de DV. Un 9% de las cepas aisladas de pacientes que consultaron por diarrea eran Shigellas importadas de un país extranjero tras un viaje. Este porcentaje es el mismo encontrado hace diez años en otro estudio similar realizado en el hospital (Gascón et al., 1993). La dispersión de una cepa multirresistente quedó patente en el estudio de un clon de *S.flexneri* inicialmente aislado de niños tanzanos y posteriormente identificado en un paciente con DV que regresaba a Barcelona después de un viaje a Kenya, país limítrofe con Tanzania y entre los cuales hay una gran actividad comercial. Este hecho pone de manifiesto la necesidad de iniciar estudios epidemiológicos para controlar, en la medida de lo posible, la difusión de microorganismos, o genes de resistencia, entre diferentes y lejanas áreas geográficas.

Uno de los factores que juegan un papel importante dentro del alcance y mantenimiento de un brote epidémico es la virulencia de la cepa involucrada. Se han identificado genes cuya presencia está directamente relacionada con el grado de virulencia de una cepa: los determinantes de virulencia. La toxina SAT (secreted autotransporter toxin) fue inicalmente descrita como un determinante de virulencia en cepas de *E.coli* uropatógenas (Guyer et al., 2000), siendo la responsable del daño glomerular durante la infección. Posteriormente ha sido descrita en otros patotipos diarreogénicos de *E.coli* incluídas las EPEC, EHEC y DAEC (Guyer et al., 2000; Taddei et al., 2003) Además, la toxina Sat está estrechamente relacionada con la toxina Pet, encontrada en cepas diarreagénicas de esta

misma especie. El gen *sat* se encuentra localizado dentro de una isla de patogenicidad, lo cual hace que su movilidad sea mucho mayor y su transferencia a otras especies altamente posible. Pertenece a la familia de toxinas autotransportables de Enterobacterias, proteínas con funciones específicas y bien definidas que, dentro de una sola molécula, tienen todos los requerimientos para ser secretadas a través de la membrana externa y sin necesidades energéticas asociadas que solo se ha identificado en bacterias patogénicas (Henderson y Nataro, 2001).

Al estudiar la prevalencia del gen *sat* entre las cepas aisladas de pacientes con DV (Artículo 7), observamos entre un 30-40% más de presencia de este gen en *S.flexneri* que en *S.sonnei*. Estos resultados concuerdan con el hecho de que prácticamente la mitad de las *S.flexneri* eran del tipo 2a, de reconocida virulencia, y que dentro de este grupo solo 4/19 no presentaban el gen. Una mayor prevalencia se observó también para las cepas provenientes de América Latina, independientemente de la especie. Sin embargo, se trata de datos preliminares y es necesario realizar más estudios para determinar si realmente existe una distribución geográfica de la toxina y si esta tiene alguna relevancia como factor diarreagénico).

Al estudiar los niveles de resistencia, tanto de las cepas tanzanas, como las de DV, observamos que eran altos para los antibióticos normalmente encontrados en países de baja renta (PBR) (ampicilina, cotrimoxazol, cloranfenicol) y utilizados en ellos como primera línea de tratamiento según disponibilidad. Esto es concordante con lo que se ha descrito anteriormente para PBR. Los niveles de resistencia más elevados en ambos grupos de cepas fueron frente a la tetraciclina. Así, la primera descripción de una bacteria tetraciclina resistente fue el de una *S.dysenteriae* en 1953, y la primera Shigella multirresistente (tetraciclina^R, estreptomicina^R, cloranfenicol^R) fue aislada en 1955. Cinco años más tarde,un 10% de las cepas aisladas en Japón eran multirresistentes. Posteriormente se ha demostrado que las Shigellas son capaces de transferir determinantes de resistencia a través de plásmidos R conjugativos (Roberts, 1999).

En general, los niveles de resistencia fueron mayores en las cepas tanzanas que en las de DV, resultados que se explican por el hecho de tratarse en gran medida de una cepa epidémica multirresistente. En ambos casos se observó una mayor resistencia en las cepas de *S.flexneri* que en las de *S.sonnei,* siendo especialmente notorio el caso del cloranfenicol, pues no se halló ninguna cepa de *S.sonnei* resistente. Aunque todas las cepas estudiadas fueron susceptibles a la ciprofloxacina, se detectaron ya cepas resistentes al ácido nalidíxico, provenientes de zonas con un alto uso de quinolonas como es el caso de la India.

Los mecanismos moleculares responsables del fenotipo resistente frente a los diferentes agentes antimicrobianos testados, fueron semejantes en ambos grupos de cepas. La resistencia al trimetroprim se explicó en la mayoría de las cepas por la presencia de genes codificantes para dihidrofolato reductasas, siendo el más prevalente el *dfr*A1. Este resultado está de acuerdo con lo descrito anteriormente. Sin embargo, también se hallaron otras DHFR y por primera vez se describió en *Shigella* la presencia de una *dfr*A7 (Artículo 6) y de la *dfr*A14 (Artículo 9), aunque en este último caso se ha descrito como bastante común en otro tipo de Enterobacterias, especialmente en ciertas áreas geográficas (Adrian et al., 1995)

Estudios anteriores han descrito la presencia de genes *oxa* como el mecanismo prevalente de resistencia a ampicilina en cepas de *S.flexneri* , mientras en el caso de *S.sonnei* describían una mayor prevalencia de betalactamasas tipo TEM (Siu et al., Sirot et al., Schumacher et al). Aunque este bien podría ser el caso en las cepas tanzanas (Artículo 6), el número de *S.sonnei* era demasiado pequeño como para sacar conclusiones. En cambio, en el caso de las cepas de DV (Artículo 9), se observó que los genes *oxa* eran, con ventaja, los más prevalentes para ambas especies. Más aún, parecía existir una especificidad de ciertos genes por una especie o por otra, de manera que los genes tipo *oxa* 1 estaban principalmente en cepas de *S.flexneri* mientras que los de tipo *oxa* 2 y *oxa* 5 eran más prevalentes en cepas de *S.sonnei* . Al ser cepas epidemiológicamente no relacionadas, la dispersión de un clon está descartada. Estos resultados podrían sugerir que los genes están en plásmidos - R de grupo de incompatibilidad.

La transferencia horizontal de genes es un mecanismo exitoso para la diseminación de multirresistencia entre bacterias. Los genes son movilizados de un microorganismo a otro en elementos transponibles, como plásmidos o transposones, dentro de los cuales pueden encontrarse como cassettes de un integrón. El genero Shigella es ampliamente reconocido por su facilidad para adquirir plásmidos (Kottlof et al., 1999) pero curiosamente poco se ha estudiado sobre las poblaciones de integrones en este género. Al utilizar cebadores específicos para amplificar por PCR integrones de Clase 1 en las cepas de DV, se halló una baja prevalencia (13,25%) comparado con el 50% que se ha descrito anteriormente para otras Enterobacterias (Martinez-Freijo et al., 1988; White et al., 2001). Sin embargo, lo que se encontró fue una altísima variedad, hallándose nueve integrones diferentes en tan solo once cepas (Artículo 10). La baja prevalencia podría tener su explicación en resultados obtenidos por McIver y colaboradores (2001), quienes observaron en *Shigella* una alta prevalencia de integrones Clase 2 y al igual que en nuestro estudio, muy pocos de Clase 1. La secuenciación de los diferentes cassettes reveló que predominan los genes codificantes para dihidratofolato reductasas, así como los responsables de resistencia a aminoglucósidos. En contraste y a pesar de tratarse de cepas muy resistentes a la ampicilina presuntamente por la presencia de oxacilinasas (Artículo 9), que además han sido descritas como genes comúnmente encontrados en *casettes,* (Naas & Nordman, 1999) solo un tipo de integrón contenía una oxa tipo 2. Una posibilidad es que dichas oxacilinasas se encuentren codificadas dentro de plásmidos, y por su distribución, como se comentó antes, podrían ser de grupos de incompatibilidad.

La creciente multiresistencia de las especies de Shigella complica la consecución de un tratamiento de primera línea efectivo. La mejor alternativa en el momento actual es la de las fluroquinolonas. Sin embargo se trata de una opción no recomendable en pediatría y por lo tanto limitada. La búsqueda de nuevas alternativas comienza a ser imperiosa. La rifaximina es un antibiótico que no se absorbe a nivel intestinal, y por lo tanto alcanza muy altas concentraciones en este que además resulta ser la "puerta de entrada" tanto de *Shigella* spp. como de *E.coli*, otro gran agente causal de diarrea. Por ello, y puesto que un estudio anterior

había planteado su potencial como tratamiento para diarreas (Gillis et al., 1995) se decidió determinar los niveles de resistencia de las cepas de Shigella aisladas de niños con diarrea en Tanzania y previamente descritas en el artículo 6. Se observó que efectivamente, la CMI de las cepas a la rifaximina era de 200 - 2000 veces menor que las concentraciones que se sabe se alcanzan en la luz intestinal tras tres días de terapia con rifaximina. DuPont y colaboradores (2001) han probado su seguridad y eficacia *in vivo.* En un ensayo clínico, randomizado, doble ciego, se comparó la seguridad y efectividad de la rifaximina vs. ciprofloxacina en 187 voluntarios con DV. No observaron diferencias significativas entre los dos grupos en lo que respecta a mejoría clínica durante las primeras 24 horas, la respuesta al tratamiento o la cura microbiológica. Posteriormente realizaron un segundo ensayo (Sack,SR 2003), esta vez comparando dos dosis de rifaximina (600 y 1200 mg/dia) en 380 voluntarios obteniendo los mismos resultados positivos y determinando como segura y eficaz la dosis de 600mg día. Los resultados obtenidos por nuestro grupo en el laboratorio (Artículo 11 y Sierra et al., 2001), así como lo observado por otros grupos tanto *in vitro* como *in* vivo (DuPont et al.,2001; Sack,SR 2003) parecen sugerir que la rifaximina es activa frente a infecciones entéricas y causantes de la diarrea del viajero. Se podría plantear entonces como una posible alternativa a los tratamientos actuales que empiezan a mostrar altos niveles de fallo terapéutico debido a los crecientes niveles de resistencia actuales. En cuanto a su posible uso como tratamiento de diarreas en PBR sería necesario realizar estudios en el campo, aunque es probable que funcione adecuadamente.

El estudio aquí realizado, caracteriza niveles y mecanismos de resistencia a antimicrobianos de dos microorganismos tipo, uno representante de lo que sería el medio hospitalario, y otro propio de infecciones comunitarias, observándose en ambos casos tanto unos elevados niveles de resistencia, como una diversidad de mecanismos, que demuestran la facilidad que poseen para adquirir, o desarrollar, resistencia frente a antibióticos.

Si bien, inicialmente, el problema de la multirresistencia se detectó como tal en infecciones nosocomiales, como aquellas producidas por *A.baumannii*, debido a la ingente

presión antibiótica que se genera en este ambiente, hoy en día debido al uso inapropiado, abusivo y a menudo incontrolado, que de los antimicrobianos se hace en la comunidad, así como a la extensiva practica de incorporarlos como aditivos a los piensos, para facilitar el engorde de animales de granja, este problema se ha extendido a los patógenos causantes de infecciones comunitarias, como sería el caso de *Shigellas* spp. Más aún, debido a esta presión continua, los microorganismos comensales o simbiontes que habitan en el tracto intestinal, fundamentalmente, han devenido un reservorio de genes implicados en el desarrollo de resistencia a antimicrobianos. La relevancia de este problema es indudable, de hecho, ya se han aislado cepas de *A.baumannii* resistentes a todos los antimicrobianos comercializados, y las alternativas, incluyendo combinaciones de farmacos, para tratar estas y otras infecciones, son cada vez más limitadas. La via más lógica para intentar controlar esta situación es iniciar una política restrictiva de uso de antimicrobianos, que limite al máximo las ventajas ecológicas de que disponen las cepas que poseen genes de resistencia, lo que redundaría en una ventaja selectiva de aquellas cepas que no los poseen, facilitando así, a largo plazo, un cambio en la tendencia actual de incremento de resistencia.

5 CONCLUSIONES

5.1 *Acinetobacter baumannii*

- La aparición de cepas *A.baumannii* multirresistentes en diferentes hospitales españoles, en ocasiones específicas, se ha debido a la diseminación intrahospitalaria de un clon epidémico multirresistente.

- La alta incidencia de resistencia a la amikacina en cepas de *A.baumannii* en diferentes hospitales españoles era debida a la dispersión de una cepa epidémica portadora del gen *aph*(3')–VI

- Los integrones de Clase 1 juegan un papel importante en la multirresistencia de *A.baumannii.* Son además claves en la dispersión de genes de resistencia, siendo traspasados horizontalmente tanto intra como interespecie

- *A.baumannii* y *P.aeruginosa*, además de compartir nicho ecológico, comparten también integrones y por lo tanto determinantes de multirresistencia.

- *A.baumannii* es reconocido por sus altos niveles de resistencia a los aminoglucósidos. Dicha resistencia se debe en gran medida a una alta prevalencia de enzimas modificadoras de aminoglicósidos, encontrándose en cepas españolas una alta prevalencia de genes *aadB* y *aacA4*, codificados en integrones.

- La resistencia a los ß-lactámicos se explica en gran parte a la presencia ß-lactamasas plasmídicas, sobretodo las de tipo TEM y OXA, así como a la sobreexpresión de ß-lactamasas cromosómicas de tipo AmpC.

5.2 *Shigella* spp

- Las cepas de *Shigella* spp. provenientes de PBR, presentan un alto nivel de resistencia a los antibióticos más utilizados en estas zonas: la tetraciclina, el cloranfenicol, el cotrimoxazol y la ampicilina. Se comienzan además a aislar cepas resistentes a las quinolonas.

- La resistencia al trimetroprim se asocia con la presencia de dihidrofolato reductasas, principalmente pero no exclusivamente *dfr*1A, normalmente presentes como *cassettes* en integrones

- La PCR-RFLP para la detección de genes de dihidrofolato reductasas (*dfr*) es una técnica rápida, fácil y muy flexible en cuanto permite hacer un tamizaje para los principales genes *dfr* actualmente conocidos y puede ser adaptada a medida que nuevos genes sean descritos.

- El principal mecanismo de resistencia al cloranfenicol en *Shigella* spp.es la producción de cloranfenicol acetil transferasa.

- La resistencia a ampicilina se explica en un gran número de casos con la presencia de una ß-lactamasa tipo OXA, seguida en prevalencia por las de tipo TEM.

- Los integrones de Clase 1, aunque presentes, no son tan comunes en Shigella como en otras Enterobacterias. En los integrones circulantes hay una gran diversidad de cassettes, aunque podría ser un reflejo de la gran diversidad geográfica de las cepas. Aún así, hay una claro dominio de las betalactamasas como *cassettes* dentro de los integrones hallados en las cepas de *Shigella* spp analizadas.

- *S.sonnei* y *S.flexneri* son especies con un alto grado de variabilidad genética, aunque pueden generar brotes epidémicos por cepas clónicas en zonas geográficas delimitadas.

- Hay una gran facilidad y rapidez de dispersión de clones multirresistente de Shigella entre zonas lejanas, que sugieren la importancia cada vez mayor de un seguimiento epidemiológico de microorganismos patógenos, tanto a nivel de centros nacionales como de patógenos importados.

- La rifaximina se plantea como una posible alternativa terapéutica para la infección por Shigella.

- Hay una alta incidencia de genes *sat* entre la población de Shigella estudiada, siendo especialmente significativa entre cepas de viajeros provenientes de América Latina y *S.flexneri* tipo 2a.

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