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Antimicrobial resistance and new insights in the diagnosis of Carrión's Disease

Cláudia Sofia Paradela Gomes

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ANTIMICROBIAL RESISTANCE AND NEW INSIGHTS IN THE DIAGNOSIS OF CARRIÓN'S DISEASE

Dissertation submitted by **Cláudia Sofia Paradela Gomes** for the degree of Doctor in Medicine from the University of Barcelona, under the supervision of Dr. Joaquim Ruiz Blázquez.

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The director and tutor of this thesis, Dr. Joaquim Ruiz Blázquez and Dr. Jordi Vila Estapé respectively, from the Barcelona Global Health Institute (ISGlobal) certify that the PhD thesis entitled “Antimicrobial resistance and new insights in the diagnosis of Carrión’s disease” presented by Cláudia Sofia Paradela Gomes has been carried out under the supervision of Dr. Ruiz and fulfils the criteria for presentation as a compendium of articles in the Faculty of Medicine of the University of Barcelona.

Dr. Joaquim Ruiz	Dr. Jordi Vila	Cláudia Gomes
Director	Tutor	Author

Barcelona, September 2016

Aos meus pais,

ao meu irmão,

ao Nuno.

"É o tempo da travessia:

E, se não ousarmos fazê-la,

teremos ficado, para sempre,

à margen de nós mesmos."

Fernando Teixeira de Andrade

"I wouldn't change anything.

If you change one thing, that changes everything.

And some things are the way they should be."

Alan Ball and Nancy Oliver, Six feet under

ANTIMICROBIAL RESISTANCE AND NEW INSIGHTS IN THE DIAGNOSIS OF CARRIÓN'S DISEASE

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ATP	Adenosine triphosphate
CFU	Colony-forming unit
DBS	Dried blood spots
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
fla	Gene encoding flagellin
GC	Guanine-cytosine
gltA	Gene encoding the citrate synthase
gyrA	Gene encoding the DNA gyrase A subunit
gyrB	Gene encoding the DNA gyrase B subunit
IFA	Indirect fluorescence antibody assay
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
its	Hypervariable intergenic transcribed spacer *16S-23S rRNA*
MIC	Minimal inhibitory concentration
MLST	Multi-locus sequence typing
mRNA	Messenger ribonucleic acid
OMPs	Outer membrane proteins
parC	Gene encoding DNA topoisomerase IV A subunit
parE	Gene encoding DNA topoisomerase IV B subunit
PAβN	Phenylalanine-arginine-β-naphthylamide
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RND	Resistance-nodulation-cell division superfamily
rplD	Gene encoding the ribosomal protein L4
rplV	Gene encoding the ribosomal protein L22
rpoB	Gene encoding the β subunit of bacterial RNA polymerase
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SCS-α	Succinyl-CoA synthetase subunit α

SCS-β	Succinyl-CoA synthetase subunit β
VEGF	Vascular endothelial growth factor
23S rRNA	Gene encoding the 23S subunit of ribosomal RNA

Deadly, unknown and neglected tropical disease

7FACTS

ABOUT CARRION'S DISEASE

1

CARRION'S DISEASE

IS ENDEMIC

IN THE ANDEAN VALLEYS OF PERU, ECUADOR AND COLOMBIA, AND PARTICULARLY IN RURAL AREAS WITH FEW RESOURCES

Factors contributing to the spread of the disease to previously unaffected areas include climate change, the El Niño phenomenon and increased population mobility.

2

THE CAUSATIVE ORGANISM IS ***BARTONELLA BACILLIFORMIS***, **A BACTERIA THAT IS TRANSMITTED TO HUMANS THROUGH THE BITE OF A**

SANDFLY OF THE LUTZOMYIA GENUS.

As humans are the only known reservoir for the bacteria, this a potentially eradicable disease.

3

IN THE ACUTE PHASE,

KNOWN AS OROYA FEVER,

THE BACTERIA INFECT RED BLOOD CELLS, CAUSING FEVER, HEMOLYTIC ANEMIA AND IMMUNOSUPPRESSION.

Untreated, Carrion's disease is fatal in between 44% and 88% of cases → more deadly than the plague and as lethal as the Ebola virus.

4

SYMPTOMS ARE DIFFERENT IN INDIVIDUALS WHO HAVE BEEN EXPOSED TO THE DISEASE AND HAVE DEVELOPED SOME IMMUNITY.

THE CHRONIC PHASE:

In the chronic phase, the infection gives rise to lesions called "Peruvian warts".

5

ANTIBIOTIC TREATMENT CURES THE CLINICAL SYMPTOMS BUT DOES NOT

ALWAYS ERADICATE THE BACTERIA

In some endemic areas up to 40% of those infected are asymptomatic.

6

THE REAL NUMBER OF THOSE AFFECTED IS UNKNOWN OWING TO THE LACK OF RESOURCES FOR PROPER DIAGNOSIS IN RURAL AREAS

7

THE DISEASE WAS NAMED IN HONOUR OF

DANIEL ALCIDES CARRIÓN,

A MEDICAL STUDENT WHO ASKED TO BE INOCULATED WITH BLOOD FROM A "PERUVIAN WART".

Carrión developed Oroya fever, thereby demonstrating that the fever and the warts were different phases of the same disease.

The greatest impact of Carrión disease is on the most vulnerable and forgotten populations. It is truly a neglected disease.

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GRAPHIC DESIGN BY RACHEL JILL PAPERNICK

INTRODUCTION

1. HISTORY

1.1 THE PRE-INCA AND INCA PERIOD

It is difficult to know exactly when Carrión's disease first appeared in Peru. There are no written documents from the pre-Inca and Inca cultures and thus, there are no data regarding cases of bartonellosis during these periods. However, there are evidence that Carrión's disease was known to the pre-Columbian cultures [1]. Some pottery artwork pieces made by the indigenous Mochica and Chimú cultures in the pre-Columbian period (huacos) show images of people with warty lesions as do some stone figures from the Huaylas culture (Figure 1). Moreover, quechua, the language of the ancient Peruvians, has words to describe fever "*rupha*", anemia "*sirki*", and eruption "*kcepo*" and "*ticti*" [1].

Figure 1: Examples of huacos from the pre-Columbian period. Reproduced from [2].

In 1525, during the Inca period, the imperial city of Cusco suffered a devastating epidemic during which more than 200,000 lives were lost. Some authors attributed this deadly epidemic to Carrión's disease. At that time the Inca emperor was in Tumibamba (present day Ecuador), and he and his heirs and other family members were also victims of this epidemic. Some authors have suggested that he was infected by the messenger who delivered the reports of the epidemic [1, 3], but others believe that it was from the transfer of his mummy to Cusco after his death that the epidemic later arose [4]. Despite the description of the Cusco epidemic with fever, rash and a high mortality rate, it is not completely clear whether the cause was Carrión's disease because the warty phase was never described [1, 3]. In fact, the possible presence of the first American outbreak of smallpox or measles (also supported by the occurrence of rash) is highly probable, being either imported through the Spanish settlements in Panama [4] or from the Plate river settlements by

the expedition of the Portuguese explorer Aleixo Garcia to the Inca emporium in 1524 [5].

1.2 The Spanish Conquest

After the arrival of the Spanish conquerors to Coaque, Ecuador, in 1531 Pizarro lost a quarter of his men to an outbreak of a warty epidemic involving muscle and skeletal pain similar to what is described in Carrión's disease [1, 6]. Later, Juan Calvete described that during the invasion in 1547 the army departed from Panama to end the Gonzalo Pizarro rebellion, but many soldiers became sick with skin eruptions [7]. Accurate determination of the cause of these epidemics will probably never be possible and different opinions as to the possible causes have been given in the literature. For some authors, these epidemics were associated with bartonellosis [6, 8], but for others they were not. Some of the reasons to the contrary are that Coaque is a coastal area almost at sea level, and that the first report of Carrión's disease in Ecuador was not until the 1920s [9].

1.3 XVII and XVIII Centuries

The first written report of Carrión's disease in Peru is from the XVII century. In 1630, Gago de Vadillo described the first report of the presence of a warty disease in an area in Huaylas, in the Anchash department, and raised the hypothesis that the warts were the consequence of the drinking water [10].

The next landmark in the history of bartonellosis was in 1764 when Cosme Bueno, mentioned two important new facts: the disease is very bothersome and dangerous in the absence of warts, which indicate benignness, and the origin of the illness seems to be a small insect named uta [6, 11].

1.4 XIX Century – The tragic era

The event that first sparked medical attention to this disease was the appearance of a febrile disease that killed thousands of Chinese and Chilean men in 1870 while working on the railroad in Cocachacra, a village between Lima and Oroya city. The unexplained epidemic appeared suddenly. Fever and anemia were rampant as were the number of deaths which, according to some reports, accounted for more than 7,000 lives. Indeed, it was said that "every railroad tie has cost a life". The new disease became known as Oroya fever despite Oroya city being quite far away and no cases had been reported there [6].

The most remarkable contribution was made by Daniel Alcides Carrión, a young Peruvian medical student who inoculated himself with wart blood from a hospitalized child on 27 August 1885. The young researcher was doing his thesis and wanted to describe and understand the pre-eruption symptoms of the Peruvian wart because of the difficulty in diagnosing the disease before the appearance of the wart [11-13]. The results of this experiment were a huge surprise for everybody, including himself. Some expected the eruption of a normal wart with no associated dangers while others considered that the inoculation would not lead to anything. However, 21 days after inoculation the first symptoms appeared followed by rapid disease progression, being completely compatible with the clinical features of Oroya fever [1, 6, 11]. Despite his rapidly failing health Carrión had the wisdom to clearly understand what was happening and said to his medical student friends:

"Up to today, I thought I was only in the invasive stage of the verruga as a consequence of my inoculation, that is, in the period of anemia that precedes the eruption. But now I am deeply convinced that I am suffering from the fever that killed our friend, Orihuela. Therefore, this is the evident proof that Oroya fever and the verruga have the same origin, as Dr. Alarco once said" [12].

Unfortunately, this experiment ended tragically and Daniel Carrión died on the 5th of October, 39 days after inoculation. In his honor, the 5th of October was declared the National Medicine Day in Peru and the disease was named after him. Daniel Carrión became a hero in Peruvian medicine. His experiment of self-infection proved that the Oroya fever and Peruvian wart are 2 different phases of the same illness [1, 6, 8].

1.5 XX CENTURY AND THE MODERN TIMES

Following Daniel Carrión's experiment, new conclusions about Carrión's disease were accepted by the medical community in general. Moreover, in 1909 Alberto Barton announced the discovery of the causal agent of Carrión's disease. He also described that the bacilli multiplies in patients with Oroya fever and decreases until it practically disappears in the Peruvian wart phase of the illness [14].

Nonetheless, some researchers were not convinced. Richard Strong led the first Harvard Expedition to South America in 1913 and determined that Oroya fever and the Peruvian wart were 2 different illnesses. They were only able to find the bacillus in erythrocytes and other body tissues but not in Peruvian warts, thereby contradicting previous findings [3].

In the same period the entomologist Charles Townsend was in charge of finding the vector of the disease. He spent several months studying the possible role of ticks, acarus and diurnal bugs without success. Thereafter he decided to investigate the traditional native belief which considered a nocturnal sand fly called "*tititra*" as the vector. After several studies he confirmed the traditional knowledge and proposed that the vector was the "*titira*" a member of the *Phlebotomus* genus named *Phlebotomus verrucarum* (currently known as *Lutzomyia verrucarum*) [15].

Further doubts finally vanished in 1927 when a Japanese researcher, Hideyo Noguchi, published the isolation of the etiological agent from blood from patients with both Oroya fever and the Peruvian wart in his *Leptospira* media [16, 17]. Furthermore, the microorganisms isolated from the acute phase of the disease were inoculated into monkeys and produced both anemia and the characteristic eruptions on the skin [18]. Hideyo Noguchi had decisively proven the work of Daniel Carrión, and his results were later confirmed in 1937 by the second Harvard Expedition to Peru. The etiological agent of Carrión's disease was then named *Bartonella bacilliformis* in recognition of the discovery by Alberto Barton years previously [1, 8].

Meanwhile, along the way several other human inoculations were carried out with *B. bacilliformis*. In 1913, Richard Strong repeated the dangerous experiment by Daniel Carrión. Fortunately, however, the outcome was only the appearance of a Peruvian wart at the site of the inoculation. In 1928, Garcia Rosell suffered accidental inoculation when making a blood transfusion in a patient with Oroya fever. He developed moderate fever and some time later a Peruvian wart appeared on his skin. This was the inverse of Daniel Carrión's experiment; the formation of a Peruvian wart was also possible from the inoculation with blood from a patient with Oroya fever. The same happened to Oviedo Garcia. Nevertheless, Kuczynski-Godard went further and inoculated himself with *B. bacilliformis* in 1937 and presented the Oroya fever phase 19 days after inoculation. Fortunately, he did not die and the disease progressed to the Peruvian wart phase [6, 19].

Thereafter, a series of studies were undertaken to describe the endemic areas of the disease as well as the different aspects of the etiology of the vector, the characteristics of the pathogen and host responses.

Up to now, only the chapter on infectious diseases related to smallpox has been definitively closed. Poliomyelitis is being actively eliminated through intensive

vaccination, and yaws, measles, and several other infectious diseases have been included in the eradication agendas. However, a series of chapters on the history of Carrión's disease have been lost and several issues have apparently been forgotten with a large amount of information only being accessible in local publications or unpublished theses. This illness continues to be present in the poorest regions and affects the most disadvantaged populations, far from the western world and is consequently completely outside international focus. Indeed, the study of Carrión's disease shows that this is a truly neglected disease which only a few have attempted to describe and understand.

2. EPIDEMIOLOGY

2.1 PERU

Peru is the country most affected by Carrión's disease. In fact, this illness is considered to be among the diseases of special relevance in the country, with the reporting of new cases being mandatory. Information about the situation outside Peru is scarce and mostly outdated. Thus, the main data regarding the ecology and sociology of the disease is limited to Peru, although it can likely be extrapolated to the neighboring countries affected.

Peru has 1,285,216 Km^2. It is the 19th largest country in the world and the 3rd largest in South America [20]. Moreover, its peculiar orography favors the great diversity of climates and habitats. The south and center Pacific coastal areas are desert transited to humid areas of mangrove woodlands on the northern coasts. Parallel to the Pacific Ocean, the center of the country is fully occupied by the Andes Mountains, arriving to 6768 meters above sea level in the Huascaran peak. Meanwhile, at the center and north of the eastern side of the mountains there are humid woodlands that give place to a deep jungle in the border areas of the country (Figure 2).

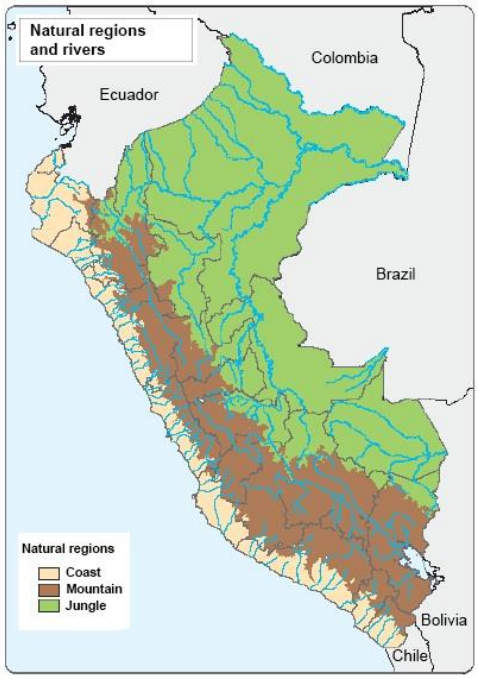

Figure 2: Peru geography. The coast, mountain and jungle cover 11.7, 27.9 and 60.3% of the national territory, respectively. Adapted from [20].

According to the World Bank classification, Peru is an upper middle income South American country [21], with a population of about 31 million, ranking 84th in the 188 positions of the human development index [22]. The high percentage of poverty in this country is of note, accounting for 22.7% of the population in 2014 and being extremely high in the rural mountain areas (50.4% of inhabitants) [23], as shown in figure 3.

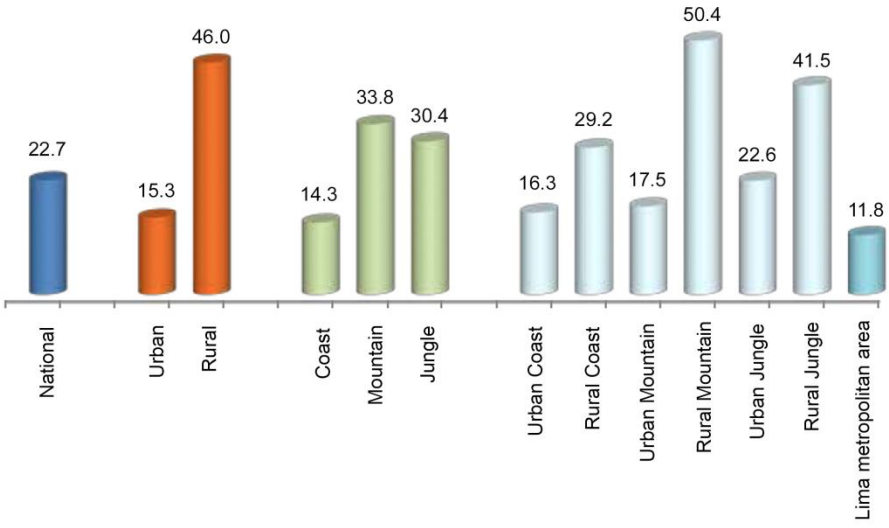

Figure 3: Total poverty rate by geographical areas of Peru in 2014. Adapted from [23].

The internal migratory phenomenon during the XX and XXI centuries resulted in an enormous population concentration in both the capital, the metropolitan area of Lima, as well as the coastal areas, with approximately 55% of the total country inhabitants [20] (Figure 4).

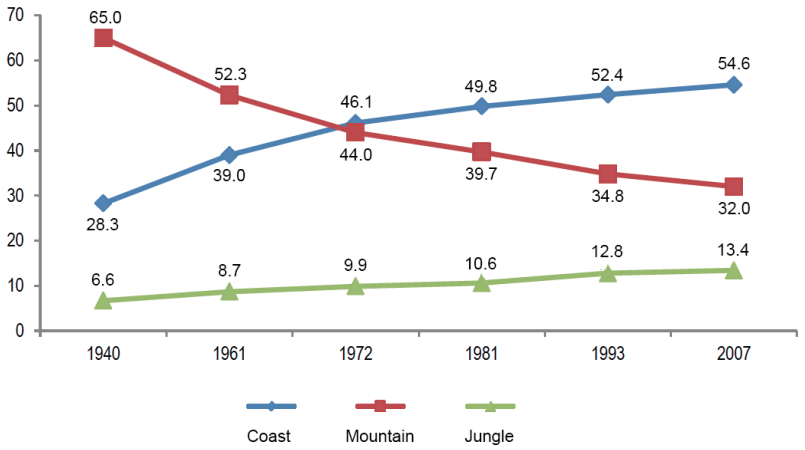

Figure 4: Percentage of censored population by natural regions of Peru between 1940 and 2007. Adapted from [23].

Peru is currently undergoing a demographic transition towards a general aging of the population. At present, the population under 15 years represents 28% of the total country inhabitants (Figure 5).

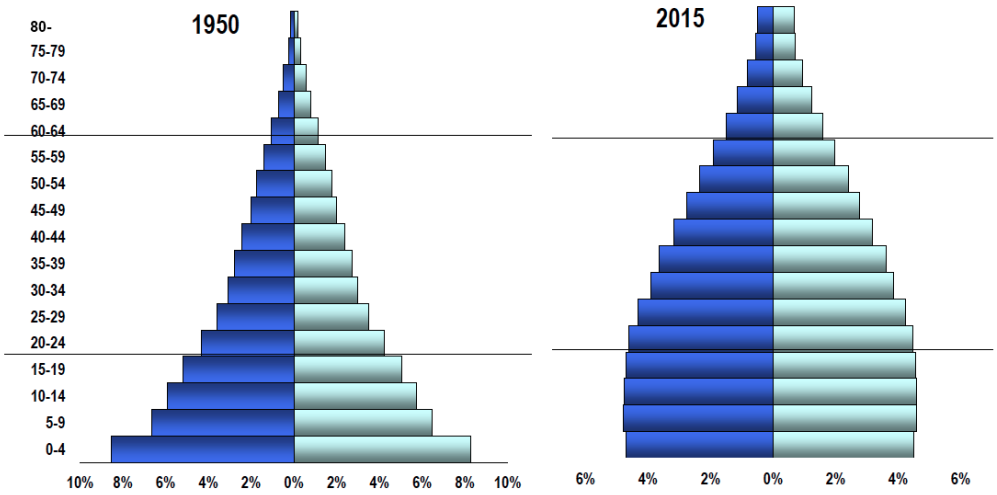

Figure 5: Population pyramid by age group in 1950 and 2015. Adapted from [20].

Economical advances have allowed the country to fulfill a series of challenges. Illiteracy has markedly decreased and now affects only 5.7% of the population, although it continues to affect up to 15.5% of the population in some rural areas [23].

Moreover, the rate of infant mortality has decreased from 56.6/1000 live births in 1990 to 12.9/1000 live births in 2013 [24]. Despite the high inequity between rural and urban areas, malnutrition has also decreased in the last 10 years. Overall, the level of chronic malnutrition in children under five is 18.1%, being 10.1% in urban areas and reaching 31.9 % in rural areas [25]. Access to health care facilities in rural areas remains one of the unsolved challenges for this country. The availability of hospitals and an effective national health system to the poorest populations is very low in Peru. Access to or the proximity to the closest health care center is often difficult leading to a lack of appropriate medical assistance in some areas (Figure 6). Another important issue is the uncontrolled and illegal selling of medications [26].

Figure 6: A) Example of the precarious access and isolated houses in rural areas in the north of Peru. B) Health centers in north Peru.

2.2 CHARACTERISTICS OF THE AFFECTED POPULATION

The persistence of Carrión's disease in endemic regions is mainly associated with poverty, warm weather, living conditions, low levels of education and the characteristics of the region that define the presence of the vector. In fact, most of these factors are completely interrelated. Andean areas have been largely unfunded during the XIX and XX century resulting in small communities which are difficult to reach and access to higher education requires migration to urban areas.

It has been shown that Carrión's disease usually affects both genders similarly, with a slightly higher prevalence among men [27-30]. Furthermore, Carrión's disease has been considered as an occupational disease by Gonzalez *et al*. [31] in light of vector exposure by seasonal workers, most of whom are men, in most of the affected areas (e.g.: coffee plantation workers and doctors) living outside and traveling to endemic areas. Moreover, the relationship between coffee plantations and the presence of *Lutzomyia* spp. has also been described [32]. Similarly, gender distribution of home workers might also affect vector exposure and the proportion of men/women infected.

Both children and pregnant women are two population segments that may be especially affected. Fetal deaths, miscarriages and premature births rank among the most serious complications affecting pregnant women in rural areas of Peru [33]. Regarding children, they are the most affected by the acute phase of the disease [29, 34]. Additionally, high levels of malnutrition enhance the severity of this pathogen [35].

Age and family members with bartonellosis are the best predictors of *B. bacilliformis* infection [36]. Patients are clustered in households and only 18% of cohort households account for 70% of the cases. This pattern of bartonellosis seems to follow a statistical pattern known as the "20/80" rule, implying that 20% of households or individuals in a susceptible population account for 80% of the disease. Thus, the risk of a patient's family member becoming infected is 2.6 times greater compared with a member of a disease-free household [36]. A study by Ellis *et al.* conducted during an outbreak also showed that cases were more likely to report bites than controls in houses near or far away [37].

2.3 CARRIÓN'S DISEASE IN PERU

Carrión's disease is an endemic illness affecting the inter-Andean valleys located between 500 and 3200 meters above sea level. Peru is endemic and is the country most affected by this disease. It is estimated that the endemic areas account for around 145,000 Km^2 [38], consisting in inter-Andean valleys positioned at right-angles to the prevailing wind [39]. More than 1.6 million inhabitants live in endemic areas [38] and a seroprevalence of greater than 60% has been described among the general population of these areas [6]. Although there are exceptions, the warty phase of the disease seems to prevail in endemic regions whereas severe Oroya fever appears to be more common in non-endemic areas [40]. Figure 7 shows the 14 endemic departments of Peru in 2014: Piura, Cajamarca, Amazonas, San Martin, La Libertad, Ancash, Lima, Huancavelica, Huánuco, Ica, Junín, Ayacucho, Madre de Dios and Cusco; with the department of Ancash, being the most significant endemic area.

Figure 7: Geographical distribution of Carrión's disease in Peru. Reproduced from [41].

Despite the trend towards a decrease in the number of cases over the last decade, 2004 showed a peak of Carrión's disease with 11,130 cases and a cumulative incidence of 40.4 per 100,000 inhabitants (Figure 8). From 2004 to 2010 about 134,000 sprayings with insecticides were done in order to control the disease [42]. However, an increase was observed in the number of cases in the Cutervo provinces of Cajamarca (2010 and 2011), Pataz - La Libertad (2011) and Huancabamba - Piura (2011 and 2013) [42] after discontinuation of this activity from 2010 onwards. In 2013/2014 an outbreak was also reported including 428 cases in the Piura department. More detailed information about this area and outbreaks is given in the

section on the study area because of its importance to this thesis. Thus, overall, 238 deaths occurred in Peru from 2002 to June 2012, with 83.2% of these deaths belonging to 4 departments (Cajamarca, Ancash, Amazonas and Piura) [42].

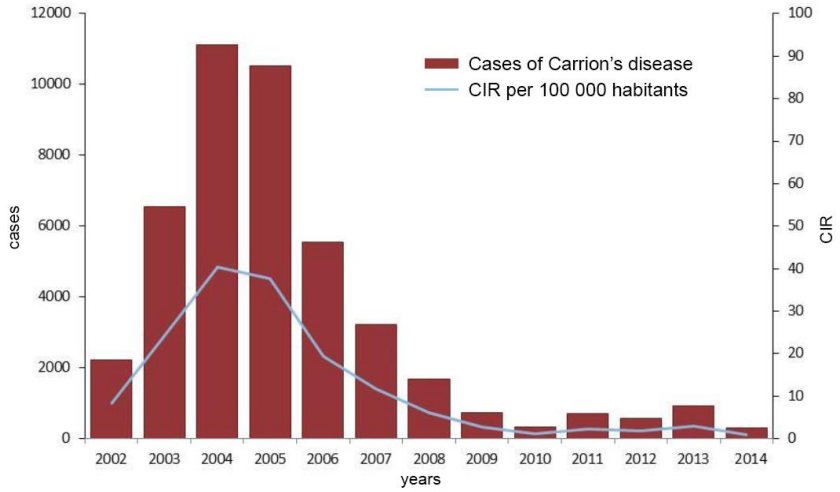

Figure 8: Evolution of number of cases of Carrión's disease between 2002 and week 35 of 2014. CIR: cumulative incidence rate. Adapted from [43].

After 2014 the number of cases returned to the lowest levels described, with only 64 acute and 52 chronic cases being reported in 2015 [44]. Until mid-February 2016, 6 cases of Oroya fever and 1 Peruvian wart were registered by the epidemiologic health system [45]. Although all these data provide a general picture of the situation of Carrión's disease in Peru, I believe that not all cases are reported or even diagnosed and thus, these numbers are underestimated and the real extension of the problem is largely unknown.

Human bartonellosis is considered an emerging disease which is expanding to new areas and is influenced by climate changes such as the El Niño [15, 46]. El Niño causes a warming in sea temperature every 5–7 years, which favorably affects the vector ecology [39], incrementing the number of cases in endemic regions as well as outbreaks in non-endemic areas [39, 47]. Elevation of the sea surface temperature seems to happen just before the appearance of an increase in the number of cases of bartonellosis [46]. Indeed, Chinga-Alayo *et al.* reported an almost 4-fold increase in monthly cases during an El Niño cycle [47]. Additionally, geographic expansion and the development of outbreaks should be taken into account in the epidemiology of the disease [40]. In the last years in depth studies of outbreaks have been described in the literature [27, 29, 37, 48-51]. Some examples are a study by Gray *et al*. [27]

describing a febrile outbreak in 1987 with a mortality rate of up to 88% in untreated cases. This outbreak was responsible for the death of 14 people and left 14 others seriously affected in Shumpillan, a small remote village in the Andes; Maguiña *et al*. reported the first outbreak in indigenous communities of the jungle in 1992, with 10-11% of lethality and several deaths [50]. Kosek *et al*. described an outbreak in the Amazonas department in 1996 with both acute and chronic cases, with a low case-mortality rate of 0.7% [51]. Ellis *et al.* reported a case-control study during an outbreak of acute bartonellosis in the Urubamba Valley (Cusco department) in 1998 [37], and more recently, in 2003, Sánchez Clemente *et al.* [29] reported an outbreak in the endemic area of Caraz (Ancash department).

2.4 CARRIÓN'S DISEASE OUTSIDE PERU

Some neighboring countries such as Ecuador and Colombia are also affected by Carrión's disease. There has been a cutaneous form of the disease in Ecuador possibly since pre-Columbian times [9]. However, it has only been since the 1930s that more reports of bartonellosis started to reappear in Ecuador [9, 52]. The distribution of the disease is widespread throughout the country [9], and a less aggressive form seems to be present with an unrecognized acute phase and a milder warty phase [53]. In this regard, the recent description of other *Bartonella* spp. able to produce Carrión's disease-like symptoms should be taken into account [54, 55]. Nonetheless, the illness seems to be underreported. In fact, one of the most relevant endemic Peruvian areas is on the Ecuadorian border [56], although only sporadic cases have been reported [57] since an outbreak in 1997 [58].

In Colombia the disease was apparently unknown until 1936 when an outbreak occurred [9, 59]. It was proposed that the disease had been imported from Peru by soldiers after the war between Colombia and Peru in 1932-1934 [9].

In addition, sporadic cases have been reported in Chile and Bolivia [27]. In distant geographical areas, such as Southeast Asia or Guatemala, compatible clinical symptoms have also been described [39]. Once again the possible role of other *Bartonella* species can not be ruled out, and the cosmopolitan distribution of *B. rochalimae* should particularly be considered [60].

Peru receives more than 700,000 tourists yearly [37]. Although the illness seems to be outside the most relevant tourist destinations, several imported *Bartonella*-like

cases have been described in travelers to and immigrants from endemic areas [55, 61, 62].

3. *BARTONELLACEAE*

The genus *Bartonella* was proposed after the description of *B. bacilliformis*. However, phylogenetic studies demonstrated a close phylogenetic and phenotypic relationship between *B. baciliformis* and *Rochalimaea quintana,* the latter of which at that time belonged to the genus *Rochalimaea* [63, 64]. Following these observations, the 2 genera were combined to become only *Bartonella* [65]. Later, the genus *Grahamella* was also added to the genus *Bartonella* [66]. Therefore, the genus *Bartonella* belongs to the *Bartonellaceae* family and combines all the species of the 3 genera *Bartonella*, *Rochalimaea* and *Grahamella,* which are members of alpha-2 subgroup of the alpha-proteobacteria [67], along with *Rickettsia* and *Brucella* [39]. *Bartonella* organisms are widely dispersed throughout nature [68]. Most of these bacteria are considered facultative intracellular pathogens, erythrocyte-adherent bacilli, fastidious, aerobic, short, pleomorfic Gram-negative coccobacillary or bacillary rods (0.6 µm x 1.0 µm) that take from 5 to 15 days and up to 45 days in primary culture to form visible colonies on enriched blood-containing media, as they are hemin dependent [67, 68].

Until 1993, the genus *Bartonella* contained only 1 species, *B. bacilliformis* [69]. Currently, 33 species of *Bartonella* have been identified and internationally recognized. In addition, 13 other species have been proposed and at least 8 are classified as *Candidatus* species [70]. New species of *Bartonella* continue to be discovered. Among the most recent species identified was *Bartonella rochalimae* in 2007 which was reported to have caused an Oroya-fever-like disease in an American traveler returning from Peru [55], and also identified in the blood of a Peruvian patient with Carrión's disease during a retrospective study [71]. *Bartonella ancashensis* spp. nov., was first reported in 2013 after isolation from the blood of 2 patients with Peruvian wart [54, 72]. Although few studies about these species have been made, *B. rochalimae* has been described to be present in fleas [73] and is widely disseminated [74-77]. On the other hand, *B. ancashensis* has only been described in the area of Ancash, and no information about its vectors or reservoirs is currently known.

The genus *Bartonella* can be separated into 4 phylogenetic lineages (Figure 9). Lineage 1 includes *B. bacilliformis* whereas lineage 2 contains ruminant-specific

species. Lineage 3 is composed of species infecting diverse mammals, and lineage 4 includes, for example, the cat-specific *Bartonella henselae* or human-specific *Bartonella quintana*, species. This distinct host adaptability in the different lineages of the genus *Bartonella* is associated with the different type IV secretion systems acquired by the different species [78-80]. The type IV secretion system is directly involved in adherence to erythrocytes facilitating host-specific adhesion [81]. The exception is lineage 1 in which the type IV secretion system is absent, and thus, other virulence proteins are implicated in erythrocyte adherence and invasion (detailed in section 5).

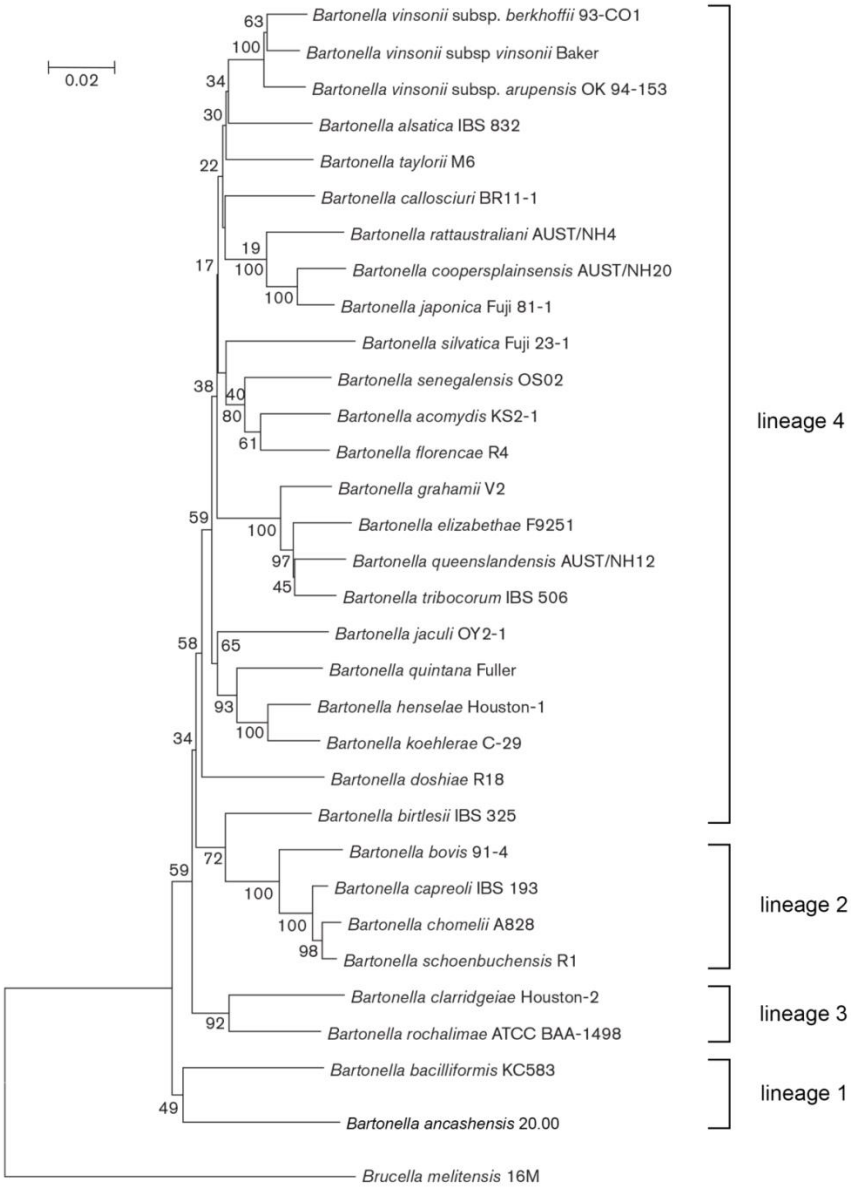

Figure 9: Phylogenetic relationships between 31 strains of species of the genus *Bartonella* with *Brucella melitensis* as the out group. In this MLST (multi-locus sequence typing) phylogeny a 3277 character fragment was considered consisting of concatenated gene fragments *rrs* (1352 characters), *rpoB* (825 characters), *gltA* (312 characters) and *ftsZ* (788 characters). Adapted from [72].

Several arthropod vectors such as fleas, ticks, flies and lice have been described for *Bartonella* spp. Coinfections by different *Bartonella* strains and species are common in mammals with a rate of co-infection of 90% in fleas from rats [82].

It is perhaps because *Bartonella* spp. can infect a wide variety of cells, including erythrocytes, endothelial cells, pericytes, dendritic cells, $CD34^+$ progenitor cells and various macrophage-type cells that the clinical and pathological manifestations of the infections appear to be very diverse in both animals and humans [83]. For example, *Bartonella vinsonii* subsp. *berkhoffii* has coevolved with cattle, *Candidatus Bartonella melophagi* has coevolved with sheep, and *Bartonella australis* has coevolved with kangaroos [83]. *B. rochalimae* has been described in raccoons, coyotes and red foxes [60]. Several species have been associated with different diseases and syndromes in humans: *B. bacilliformis*, *B. henselae*, *B. quintana*, *B. elizabethae*, *B. clarridgeae, B. rochalimae* and *B. ancashensis* [54, 55, 68, 72, 84]. Moreover, endocarditis has been associated with several *Bartonella* spp. in humans, supporting the important pathological role of this genus of bacteria in immunocompetent humans (Table 1) [83]. At least one mammalian reservoir host is known for each of the members of the genus *Bartonella* described [85]. Historically the term “bartonellosis” was attributed to infections with *B. bacilliformis* but at present this term has a wide definition and includes infections caused by any *Bartonella* spp. However, in this thesis ”bartonellosis” always means infection caused by *B. bacilliformis*.

Table 1: Species of *Bartonella* that are human pathogens, human diseases caused and primary hosts and vectors.

Microorganism	Disease in humans	Primary reservoir	Vector
B. alsatica	Endocarditis	Rabbit	Rabbit flea
B. ancashensis	Carrión's-like disease	Unknown	Unknown
B. bacilliformis	Carrión's disease	Human	Sand fly
B. clarridgeiae	CSD, endocarditis	Cat	Cat fleas
B. elizabethae	Endocarditis, neuroretinitis	Rat	Rat fleas
B. grahamii	Neuroretinitis	Wild mice	Rodent fleas
B. henselae	CSD, BA, endocarditis neuroretinitis, bacteremia	Cat	Cat fleas
B. quintana	Trench fever, BA, Endocarditis	Human	Body lice
B. rochalimae	Carrión's-like disease, bacteremia	Canids	Fleas
B. vinsonii arupensis	Endocarditis, bacteremia	Mouse	Unknown
B. vinsonii berkhoffi	Endocarditis	Dog	Unknown

BA: bacillary angiomatosis; CSD: cat scratch disease. Adapted from [83].

3.1 *BARTONELLA BACILLIFORMIS*

B. bacilliformis is responsible for Carrión's disease in humans. It is a Gram-negative, non-fermentative, aerobic, pleomorphic coccobacillus, with 0.2-0.5 µm per 1-2 µm, and with 2-16 flagella that confer high mobility. Conventional biochemical tests are not particularly helpful for presumptive identification of *Bartonella* species, but peptidase activity in L-proline and L-lysine is useful in presumptive identification of *B. bacilliformis*, being negative for proline and positive for lysine [38]. The optimum temperature is 28°C and the growth of this microorganism is slow in blood media [15, 16]. In fact, *B. bacilliformis* can remain viable at 4ºC for long periods of time [86, 87], similar to what has been described for other *Bartonella* spp. [88]. The *B. baciliformis* genome consists in a single circular deoxyribonucleic acid (DNA) molecule of about 1,600 kbp [89] and 39-40% GC content [63].

Besides *B. bacilliformis*, 2 other species of *Bartonella, B.quintana and B. henselae,* play an important role in human diseases and are associated with an increasing number of clinical manifestations.

3.2 *BARTONELLA HENSELAE*

B. henselae is the pathogen responsible for cat scratch disease, a zoonosis distributed worldwide and transmitted from infected cats to humans by bites or scratches [90]. Infections in other mammals have also been described. Johnson described a *B. henselae* infection causing the miscarriage of an equine fetus [91]. Cat scratch disease usually denotes a self-limiting illness characterized by fever and lymphadenopathy [83]. However, in immunocompromised patients the infection can result in vasculoproliferative disorders such as bacillary angiomatosis or peliosis hepatis [90]. Intraerythrocytic localization of *B. henselae* has been described in cats, but in a study done by Pitassi *et al.* it was shown that *B. henselae* can also infect human erythrocytes [92].

3.3 *BARTONELLA QUINTANA*

B. quintana was first isolated in 1960 by Vinson and it can invade erythrocytes and endothelial cells and cause trench fever, endocarditis and bacillary angiomatosis [83]. Bacillary angiomatosis is a chronic *B. quintana* disease that causes cutaneous nodular lesions and can develop in immunocompromised individuals [83]. Moreover, *B. quintana* has been demonstrated to also cause endocarditis, which is of particular medical concern in patients with human immunodeficiency virus infection and other immunocompromised individuals such as homeless people, who are at higher risk of human body lice exposure and persistent *B. quintana* bacteremia [83]. Humans are the reservoir and the bite of infected human body lice (*Pediculus humanus humanus*) is the principal vector [83]. Nonetheless, polymerase chain reaction (PCR) has been used to amplify *B. quintana* DNA from cat fleas [93], from the dental pulp collected from a domestic cat [94] and from blood of a dog with endocarditis [95], suggesting that other species may also be sporadically infected by this microorganism. Additionally, in one report culture and PCR techniques showed a naturally acquired *B. quintana* infection in a non-human primate [96].

3.4 GENETIC DIVERSITY

The phylogenetic relationships and genetic diversity among *B. bacilliformis* isolates have not been extensively studied, although several studies have addressed this aspect. Interestingly, in a study assessing the differentiation of *B. bacilliformis* isolates by the evaluation of *gltA* and 16S-23S ribosomal DNA intergenic spacer regions as well as an amplified fragment length polymorphism (AFLP), *B. bacilliformis* strains causing acute disease were also found to cause asymptomatic infection [40].

This finding demonstrates that infections with different severity may be caused by alterations in the expression of different genes related to host-pathogen interactions and are not only related to the presence of different virulence profiles. Moreover, the same study revealed that 3 different outbreaks were caused by 3 different unique genotypes of *B. bacilliformis* and not by the genotype most often encountered in the region of Peru where the disease is considered endemic [40]. Infrequent restriction site PCR and comparison of nucleotide sequences of *gltA* and *ialB* genes have demonstrated the presence of genotypic variation within populations of *B. bacilliformis* [97].

More recently, a multi-locus sequence typing (MLST) approach was developed consisting in the amplification of 7 different genetic loci (*ftsZ*, *flaA*, *ribC*, *rnpB*, *rpoB*, *bvrR* and *groEL*) [98]. To date, phylogenetic analyses have identified up to 12 distinct sequence types among *B. bacilliformis* clinical isolates [98, 99]. A comparative genomic approach revealed that the evolution of *B. bacilliformis* is shaped predominantly by mutations. Mutational divergence leads to sub-speciation but mutational convergence between clones of a sub-species shows evidence of common adaptative evolution [100].

4. CLINIC PRESENTATIONS OF CARRIÓN'S DISEASE

Once a person is bitten by an infected sand fly, an asymptomatic infection or a mild to severe disease is presented. The severity of Carrión's disease is probably determined by individual predisposition and the inherent virulence of the strain causing infection. According to Noguchi, strains isolated from people with very mild anemia or Peruvian warts are presumably less virulent [101], and in a study in which the disease manifestations were milder than the typical manifestations of Oroya fever, the authors proposed that a strain of *B. bacilliformis* with diminished virulence had caused the infection [51]. The average incubation time of Carrión's disease is 61 days (between 10 and 210 days) [68] and two syndromes, that occur independently or sequentially [38], have been described: an acute phase called Oroya fever and a chronic phase designated as Peruvian warts.

4.1 OROYA FEVER

In humans, the major clinical consequences of infection by *B. bacilliformis* result from invasion of the erythrocytes by the bacteria in the acute phase of Carrión's disease. *B. bacilliformis* infect the erythrocytes and subsequently these cells are destroyed in the spleen and liver leading to severe hemolytic anemia and transient immunosuppression. Hemolytic anemia involves the destruction of the infected erythrocytes by the reticuloendothelial system, with the erythrocytes remaining within the circulation less time than normal, thereby leading to the development of anemia [102]. However, it is interesting to note that not all the infected erythrocytes are removed from the circulation. The increased production of erythrocytes in response to the great destruction of these cells may be up to five-fold greater than normal. However, in Oroya fever there is a substantial reduction in the number of erythrocytes with a compensatory increase in plasma, so that the total blood volume is not severely affected [102]. On the other hand, a deregulation of the immune system causes a deterioration of cellular immunity leading to immunosuppression predisposing the patients to opportunistic infections [103, 104]. The amount of microorganisms in the blood is higher in patients with severe anemia than in patients with eruptions [101]. In fact, in a study by Maguiña *et al.* the mean percentage of infected erythrocytes in this phase of the disease was 61% (range 2-100%), and in 25% of patients >90% of the erythrocytes were infected at the time of admission [105].

The symptoms of Oroya fever are indistinguishable from the initial symptoms of other infectious diseases such as malaria, typhoid fever, dengue, tuberculosis or even viral hepatitis. The onset is usually gradual, with malaise, fever, headache and mild chills and can include pallor, hepatomegalia, abdominal pain and other unspecific symptoms [30, 34, 68, 106]. The acute phase seems to have a greater effect on children and young adults up to the age of 15 years [6, 30, 36, 46, 51]. Overall, patients in the acute phase are younger than those in the eruptive phase [105], suggesting the development of acquired immunity following exposure [36]. The mortality rate in the acute phase is of 40-85% in untreated patients, but with appropriate and timely treatment in reference centers this rate can be reduced to values of around 10% [6, 15, 68, 105, 107]. Complications or secondary opportunistic infections can dramatically worsen the clinical outcome and unfortunately they are quite common [30, 34, 68, 108, 109]. Non-infectious hematological, cardiovascular and neurological complications have been reported several times [30, 34, 106]. In regard to opportunistic infections, the most common pathogen involved is *Salmonella*

spp. being responsible for 90% of the deaths in Oroya fever [1]. Moreover, mortality is increased during outbreaks, mainly in new transmission areas because health personnel are unaware of the disease and laboratory staffs are not trained to diagnose the disease [6]. For example, in the late 1990's in La Convencion, a district of the Cusco Department, acute cases of Carrión's disease were systematically reported as viral hepatitis, with mortality rates of 39% in some hospitals [28]. The risk of mortality is high in pregnant women during acute bartonellosis, with the possible presentation of severe complications including fetal death [33, 68, 105].

4.2 PERUVIAN WART

The chronic phase of Carrión's disease, called Peruvian wart, is characterized by the development of dermal eruptions. This phase typically occurs weeks or months after the acute febrile syndrome and persists from one month to one year [1, 68]. Nonetheless, the chronic phase of the disease may present without any history of acute illness [110]. Lesions vary in size and number, mainly affecting the arms and legs, although other body areas can also be affected [110]. The microorganisms are observed within the warts as both intracellular inclusions and as free organisms within the extracellular matrix [110]. The lesions are classified as follows [105, 110] (Figure 10):

Miliary – These small, often numerous, reddish papules of less than 3 mm in diameter are situated at the papillary and medial dermis and may be pruriginous.

Mular – These erythematous nodular tumors are greater than 5 mm in diameter and tend to extend deeper into the hypodermis with the possible involvement of subcutaneous tissue and muscle.

Subdermic nodules – These diffuse subdermic nodules show no changes in the overlying skin and are larger and more prominent than the miliary and mular lesions.

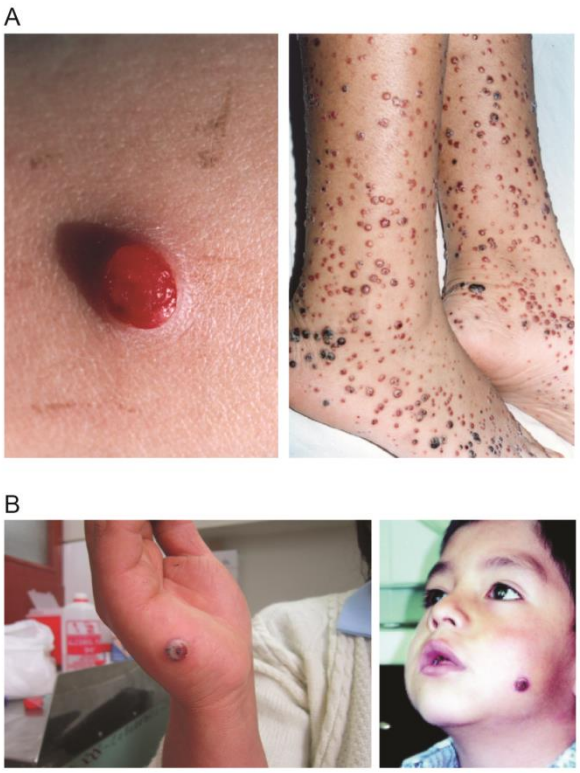

Figure 10: Different types of warty lesions. A) Miliary warts; B) Mular warts. Adapted from [6, 110].

The eruptions are most often miliary [110], although different types of warts may commonly be found in the same patient [6]. Miliary lesions are normally painless while the nodules of the mular lesions may be painful and two thirds of patients complain of bleeding warts.

The eruptive phase is a more common manifestation in inhabitants of endemic regions, and the risk of developing Peruvian wart increases with age [51]. This phase tends to heal spontaneously, and the mortality rate is insignificant. These eruptions are frequently accompanied by mild systemic symptoms such as fever, malaise, osteoarticular pain and headache [68, 110]. Notwithstanding, in more severe cases complications including bleeding may be observed in 66% and secondary infection in 12% of the patients, might leading occasionally to fatal outcomes in the absence of timely blood transfusions [6, 105].

Around 50% of the patients with Peruvian wart are bacteriemic [36, 111], with bacteremia being significantly correlated with the age of the lesions as well as low hemoglobin levels. On the other hand, the number and distribution of the lesions and the clinical symptoms are not associated with bacteremia [111].

4.3 ASYMPTOMATIC CARRIERS

In addition, it has long been known that these microorganisms can persist in blood for many years after the infection [112]. Indeed, *B. bacilliformis* was isolated from an individual who had visited Ecuador 3 years previously, demonstrating that these bacteria can persist in the blood for long periods of time [61]. Moreover, asymptomatic carriers have also been described in endemic areas [113, 114] and it is believed that these carriers perpetuate the disease and can introduce it to new areas.

5. PATHOGENESIS AND VIRULENCE FACTORS

Successful infection of a mammalian host by a bacterial pathogen typically involves a series of intimate host-pathogen interactions. *B. bacilliformis* can invade a variety of different human cell types *in vitro* [115], but during infection it is known that *B. bacilliformis* infects the erythrocytes and endothelial cells in Oroya fever and Peruvian wart, respectively. Bacteria belonging to the *Bartonella* genus are the only bacterial pathogen known to invade human erythrocytes. Erythrocytes have several properties including protection from the humoral immune system, absence of lysosomes and a reasonably long life [107]. Mature erythrocytes are non-endocytic and thus, erythrocyte involvement in the invasion process is necessarily passive [115], with several determinants and virulence factors being involved in the pathogenesis of *B. bacilliformis* including motility, erythrocyte deformation and invasive factors [116-120] (Figure 11).

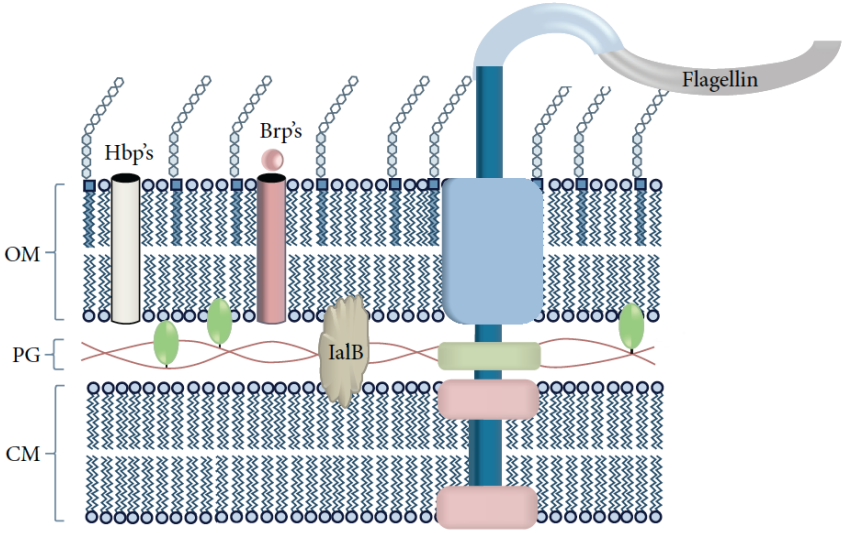

Figure 11: Various protein antigens and virulence factors of *B. bacilliformis* and their locations in the cell. Brp's: Bartonella repeat proteins; Hbp's: hemin-binding proteins; IalB: invasion-associated locus B protein; OM: outer membrane; PG: peptidoglycan; CM: cytosolic membrane. Adapted from [121].

In contrast, in the Peruvian wart, the endothelial cells actively participate in the uptake of *B. bacilliformis* by a process similar to phagocytosis [115]. The objectives of this complex adaptation to the human host are perhaps to achieve the persistence and maintenance of a reservoir state for vector-based transmission and immune evasion.

5.1 ERYTHROCYTES / OROYA FEVER

The first study reporting the ability of *B. bacilliformis* to penetrate erythrocytes was performed in 1969 [122]. Erythrocyte invasion is probably the most important step in the pathogenesis of Carrión's disease since it is related to the worst outcome of the illness. It is possible that competence for erythrocyte invasion must be acquired by an adaptation process in the primary niche [123]. Albeit controversial, some authors describe colonization of a primary niche after intravenous inoculation and before erythrocyte infection [38, 116, 123, 124]. This hypothesis is supported by some evidence. In a study by Schulein *et al.*, *B. tribocorum* was rapidly cleared from the circulating blood of infected rats, the blood of which remained sterile for 3-4 days thereafter, before erythrocyte infection [123]. Moreover, the incubation period (60 days on average) of Oroya fever after infection is long [124]. *In vivo* studies have attempted to understand the erythrocyte parasitism of *Bartonella* and have shown that *B. tribocorum* invade mature erythrocytes in rats followed by a period of intraerythrocytic replication. The cessation of bacterial replication could result from the deprivation of essential nutrients or growth factors or may indicate an active mechanism of growth control [123]. Erythrocytes lack the normal actin-based structures employed by most cells during endocytosis and therefore, as mentioned previously, *B. bacilliformis* play an active role during erythrocyte invasion [125, 126]. Infection of erythrocytes by *B. bacilliformis* involves at least 2 important steps; 1) the binding of the bacteria to the surface of the erythrocytes and; 2) deformation of the erythrocyte membrane, including deep invaginations and membrane fusion leading to the formation of intracellular vacuoles containing bacteria [127].

Although non-motile bacteria which have flagella can be bound to erythrocytes promoting surface contact interaction, the optimal binding of bacteria to the surface of erythrocytes requires *B. bacilliformis* energy, most likely proton motive force-dependent motility [107, 128]. Polar flagella (Figure 12) allow *B. bacilliformis* to be highly motile [129]. Flagella are composed of multiple 42 kDa flagellin subunits and provide the bacterium with a high degree of mobility during its search for erythrocytes [126, 129]. This bacterial motility seems to be essential for erythrocyte binding and

deformation by potentially providing mechanical force [124, 127]. A mutant lacking flagella results in a non-motile phenotype [130], and a significant decrease in the efficiency of invasion was described on pre-incubating *B. bacilliformis* with anti-flagella antibodies [129].

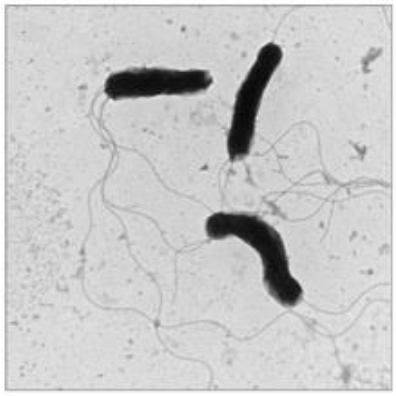

Figure 12: Transmission electron micrographs showing *B. bacilliformis* flagella. Adapted from [38].

Beyond flagella, the extracellular protein, deformin, plays a fundamental role in erythrocyte invasion. This protein can be isolated from the supernatant of cultures of *B. bacilliformis* and is able to deform human erythrocytes by producing deep invaginations in their membranes [120, 131]. Studies with purified deformin have shown its capacity to achieve these invaginations even in the absence of bacteria [120]. It is likely that flagellum-based motility and the deformation factor act together to facilitate the entry into the erythrocytes (Figure 13).

Figure 13: Deformation of erythrocytes as seen by scanning electron microscopy. Adapted from [127].

In addition, an invasion-associated locus which includes the *ialA* and *ialB* genes seems to facilitate erythrocyte invasion. The proteins encoded by these genes have shown the ability to invade human erythrocytes in the absence of the other *Bartonella* virulence factors [132]. Studies with *Escherichia coli* harboring a cloned *ialAB* locus have shown that both *ialA* and *ialB* are required for invasiveness *in vitro*, as indicated by a 6 to 39-fold increase in invasion when both genes are present. The *ialA* gene

reduces stress-induced dinucleotide levels during invasion, thereby enhancing pathogen survival, and *ialB* has shown to probably have a secreted protein with a direct role in human erythrocyte parasitism [118, 119, 132, 133]. It has also been shown that anti-IalB antibodies can block the interaction between IalB and erythrocytes [133]. A study by Coleman and Minnick showed that higher *ialB* mRNA (messenger ribonucleic acid) levels occur in acidic conditions (pH 5.0) or in bacteria grown at low temperatures (20ºC). By down-regulating *ialB* expression at 37ºC and near neutral pH, *B. bacilliformis* would presumably be less efficient at parasitizing circulating erythrocytes thus, emphasizing the chronic nature of Carrión's disease. Knowing that the insect midgut becomes acidified around 3 days post-ingestion and that female sand flies need to ingest a blood meal every 4-5 days, *ialB* expression is up-regulated leading to maximization of the *B. bacilliformis* erythrocytic invasion capacity. The authors also suggest that if for some reason sand fly feeding is interrupted, ingested *B. bacilliformis* would up-regulate the *ialB* expression and be primed for erythrocyte adherence and invasion following transfer to another human host when the insect next fed [134].

Erythrocyte adherence and invasion undoubtedly involves other proteins and factors, some of which have already been identified. For example, actin, α and β spectrin, band 3 protein, glycophorin A and glycophorin B are erythrocyte proteins implicated in the specific binding of *B. bacilliformis* to the human erythrocyte membrane [117, 131]. Moreover, the ability of *B. bacilliformis* to interact with actin and spectrin suggests that erythrocyte internalization may be related to alterations in cytoskeletal structure [117, 131]. *B. bacilliformis* repeat proteins also seem to play a role in the adherence of the bacteria to host cells by sharing common domains and structural characteristics with the trimeric autotransporter adhesion proteins of *B. henselae* and *B. quintana*: these proteins are known to be involved in adhesion to host cells and extracellular matrix proteins [121].

The severity of the hemolytic anemia characteristic of Oroya fever seems to be unique among *Bartonella* spp. and could be explained by massive infection of the erythrocytes [69]. Hemolytic activity is contact-dependent and is due to a *Bartonella* protein, not requiring direct involvement of the erythrocyte proteins. Lysis of erythrocytes results in the generation of intact ghost cells that have occasionally been observed to contain numerous highly motile *B. bacilliformis*. It is possible that *B. bacilliformis* uses these ghosts to evade the host immune system, providing the opportunity to invade microvasculature endothelial cells [135]. Moreover,

sequestration of parasitized erythrocytes by spleen cells, lymph nodes and the liver shortening their average life has also been described [102].

In vivo experiments in rats have shown a prolonged period of intraerythrocytic colonization, demonstrating for the first time, the persistence of *B. tribocorum* in erythrocytes [123]. In fact, the persistence of intracellular erythrocyte parasitism appears to be a central aspect of the pathogenesis of all *Bartonella* spp. In this way, bacteria can persist within an immunological environment and the possibility of transmission by blood-sucking arthropods increases [38, 123, 124] (Figure 14).

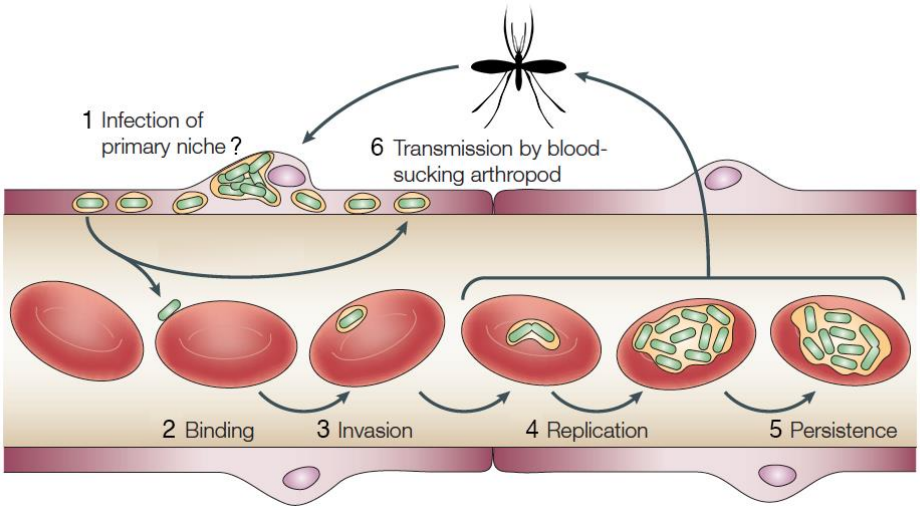

Figure 14: Model of the course of *B. tribocorum* infection in the mammalian reservoir host. 1) the primary niche of bacterial colonization is still poorly defined but is considered to include the vascular endothelium as a major constituent. Bacteria are released from the primary niche into the bloodstream, from where they can reinfect the primary niche to start another infection cycle, or 2) where they bind to erythrocytes, 3) invade, 4) replicate in an intracellular membrane-bound compartment, and 5) finally persist in a non-replicative intra-erythrocytic state. Adapted from [136].

5.2 ENDOTHELIAL CELLS / PERUVIAN WART

In the chronic phase of Carrión's disease, the Peruvian wart appears, with infection of the endothelial cells and their pronounced proliferation resulting in the characteristic skin eruptions. Epithelial or endothelial cells participate more actively in bacterial uptake than erythrocytes, being facilitated, in part, by the microfilament-dependent activity of these host cells [107, 115]. The existence of numerous capillaries within Peruvian warts suggests that infection of endothelial cells induces local angiogenic response, that is, the formation of new blood vessels. This fact was experimentally demonstrated by Garcia *et al*. who reported that *B. bacilliformis* possess *in vitro* activity that stimulates endothelial cell proliferation and the production of an angiogenic factor up to 3 times. This latter factor, the plasminogen activator t-PA

[137], may be responsible for the stimulation of capillary growth in Peruvian warts [107]. Additionally, *B. bacilliformis* extracts also stimulated the formation of new blood vessels in an *in vivo* model for angiogenesis [137]. Definite morphologic evidence of the presence of *B. bacilliformis* in Peruvian wart lesions was achieved by the finding of Rocha-Lima's inclusions by light microscopy. Moreover, the bacteria was also found in abundance in the extracellular spaces in the florid lesions and no organisms were present in the resolving nodules [138]. Further studies have been carried out in recent years to elucidate the pathogenesis of Peruvian wart. *In situ* hybridization has shown that high levels of expression of angiogenesis factors such as vascular endothelial growth factor (VEGF) receptors and angiopoietin-2 are observed in the endothelium of Peruvian wart. Cerimele and colleagues showed that VEGF was produced in the overlying epidermis thus, demonstrating cooperation between infected endothelium and the overlying epidermis to induce angiogenesis [139]. *In vitro* infection of human endothelial cells by *B. bacilliformis* resulted in activation of Rho-family GTPases (Rho, Rac and Cdc42 - key signaling proteins in pathways involving actin organization), and the cytoskeletal remodeling of endothelial cells are stimulated to form filopodia and lamellipodia that serve to introduce the bacteria into the cell, thereby showing that *B. bacilliformis* stimulates its own entry into endothelial cells [140-142]. A heat shock and highly immunogenic protein of *B. bacilliformis,* GroEL, also seems to play a key-role in the induction of vascular cell proliferation. GroEL is actively secreted by the supernatants of *B. bacilliformis* cultures and acts in a dose-dependent manner by increasing cell numbers by 6 to 20-fold. Cell proliferation is inhibited in the presence of anti-GroEL antibodies [143]. The levels of expression of this protein are regulated by different factors such as temperature or DNA supercoiling relaxation [144], which may be produced at different levels during the different infection phases. Furthermore, the increase in cell numbers could be caused by either increased cell division or reduced cell death. An anti-apoptotic mechanism has been reported in *Bartonella* accounting, in part, for its ability to induce vascular proliferation *in vivo* and enhancing the survival of the host cells, and therefore, itself [145]. Figure 15 illustrates a simple schematic model of *Bartonella*-triggered vasoproliferation.

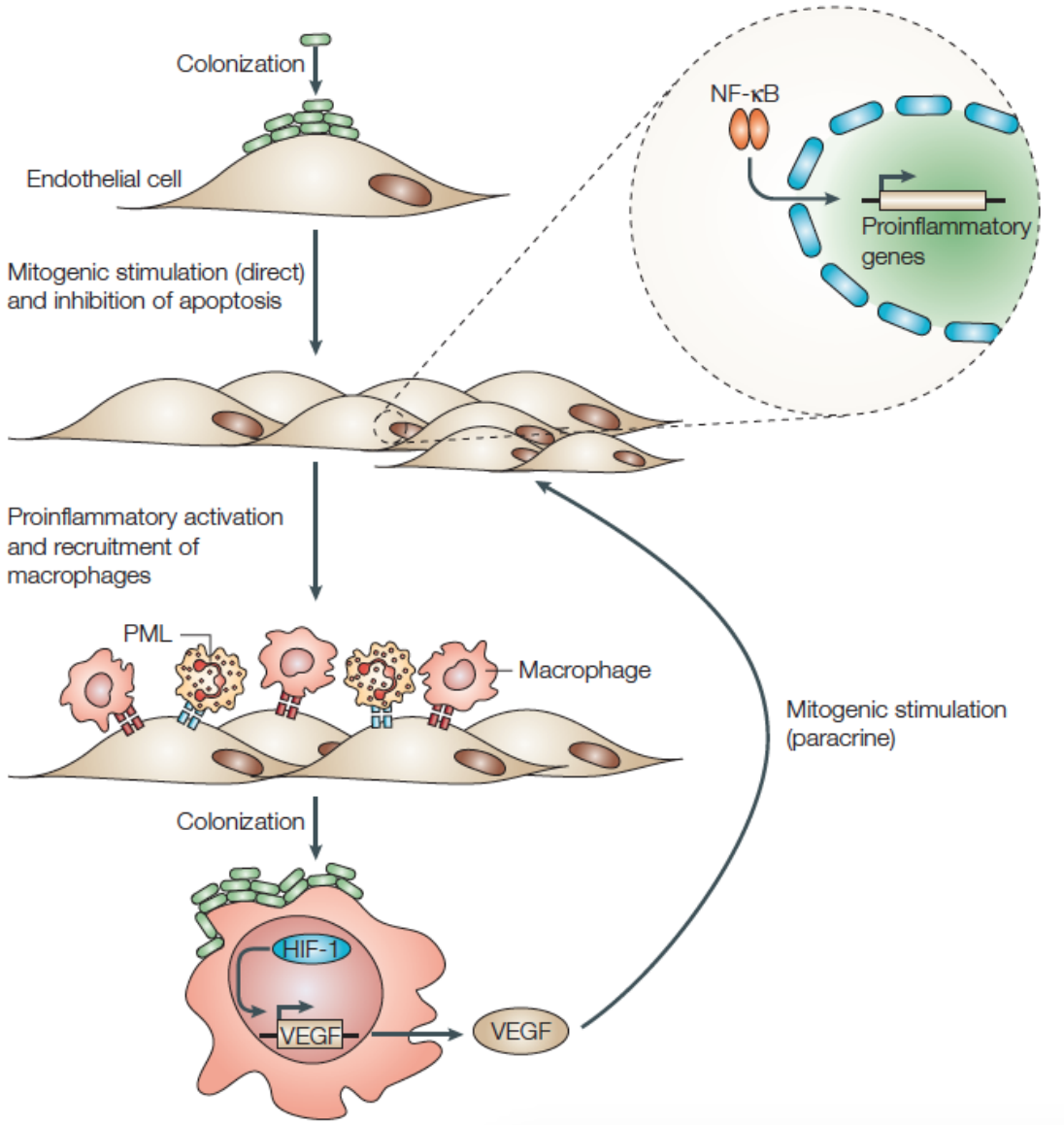

Figure 15: Model of *Bartonella*-triggered vascular tumour formation. PML: polymorphonuclear leukocyte; NF-κB: nuclear factor κB; HIF-1: hypoxia-inducible factor 1; VEGF: vascular endothelial growth factor. The vasoproliferative *bartonellae* adhere to and invade endothelial cells, which results in the direct stimulation of endothelial cell proliferation and inhibition of apoptosis. Bacteria also trigger NF-κB-dependent proinflammatory phenotype that leads to the recruitment of macrophages and other lymphocytes. Bacterial colonization of macrophages results in hypoxic conditions that lead to activation of HIF-1 and the subsequent upregulation of VEGF expression. The release of VEGF by macrophages results in paracrine stimulation of endothelial cell proliferation. Together, these events are thought to mediate vascular tumour formation. Reproduced from [136].

B. bacilliformis is a paradigmatic example of strong and specific host adaptation. This microorganism has the ability to manipulate the host cell with a refined elegance with the purpose of bacterial entry. Indeed, despite its lethality in the acute phase, it has developed the capacity to modify its virulence characteristics during infection allowing it to survive for a long time in intimate interplay with the host immune system.

6. IMMUNOLOGY

Little is known about the immunology of Carrión's disease and more studies in this field are definitely warranted in the future. In fact, most of the present knowledge is inferred from what has been obtained by analyzing other members of the genus. Current knowledge strongly suggests that the stimulation of interleukin (IL)-10 secretion is an important part of the immune modulation by members of the *Bartonella* genus [124]. This cytokine is a key regulator of immunity and might be involved in the control of the inflammatory response induced by bacterial products [124, 146]. It has a moderating effect on immune responses by suppressing several inflammatory mediators including T helper cells, monocytes/macrophages and dendritic cells, thus interfering both with innate immunity and with the establishment of an adaptative immune response [124, 147]. Accordingly, the few studies developed on Carrión's disease have shown a significant elevation of IL-10 and interferon (INF)-γ in patients in the acute phase, especially in systemic inflammatory response, which would explain the severe course developed by some patients [148]. Elevation of IL-10 levels was also noticed in 2 cases of acute phase of Carrión's disease during pregnancy, being more prominent in the patient with a more serious clinical outcome [149]. Finally, a case of neuro-bartonellosis with 98% parasitemia showed the highest IL-10 levels among 13 patients diagnosed with acute disease. The patient showed an initial elevation of INF-γ and significantly low $CD4^+$ and $CD8^+$ T-lymphocyte counts which resolved after appropriate treatment [150]. The effects of elevated IL-10 secretion greatly favor asymptomatic infection and the state of immune peripheral tolerance needed to establish persistent infection [124, 150, 151]. Similarities have been observed between Carrión's disease and other *Bartonella* spp. infections. Circulating IL-10 levels have been described to be significantly higher in patients with cat scratch disease (*B. henselae*) and in asymptomatic homeless people with *B. quintana* bacteremia. Moreover, this specific immune profile and an attenuation of inflammatory response may also account for the chronic persistence of *B. quintana* in homeless people [152, 153]. *In vivo* studies showed IL-10 production in culture supernatants of spleen cells from mice inoculated with *B. henselae* [154]. The lack of bacteremia in IL-10 knock-out mice infected with *B. birtlesii* confirms the key role of IL-10 in the establishment of *Bartonella* infection [155].

High rates of seroprevalence are observed in endemic regions and native populations seem to be less susceptible to infection and advanced hemolytic disease than

foreigners, and it is believed that antibodies may provide long-term protective immunity [156]. Besides, in endemic regions an opposite correlation between patient age and disease incidence or severity suggests that humoral immunity confers partial immunological protection related to the patient's immune status [36, 156]. In the first four weeks of the acute phase of bartonellosis IgM antibody levels rise, while IgG and IgA levels remain normal. IgG levels increase after approximately the fourth week. In the eruptive phase there is a significant albeit not very marked increase of IgA, IgG and IgM levels [46, 157]. The transient depression of the cellular immunity that occurs during the acute hematic phase is of note [158], with patients presenting lymphopenia with a decrease in $CD4^+$ and a slight increase of $CD8^+$ T-lymphocytes. A resemblance between this immunosuppression and AIDS was recently established [104]. Immunosuppression develops in both diseases causing a deterioration of cellular immunity [104] which could thereby explain the predisposition to opportunistic infections [6, 104, 106]. Treatment may induce immune reconstitution and favor the manifestation of atypical symptoms. The intensity of this inflammatory response probably depends on previous exposure to *Bartonella* and on individual genetic predisposition [104].

Data of immune response in the eruptive phase is very scarce. Leukocyte levels remain normal with a slight tendency to present lymphocytosis [6, 106]. Among 21 patients in the chronic phase of the disease, significantly elevated levels of INF-γ and IL-4 were reported [150].

The development of a vaccine inducing both humoral and cellular immune response is perhaps the most valuable strategy to advance towards disease eradication and overall, to improve the life of people living side by side with Carrión's disease as well as to improve public health [38, 121, 133]. Several proteins involved in *B. bacilliformis*' interactions with the human host, such as flagellin, FtsZ or IalB, have been proposed as good candidates for the development of a vaccine [121, 133]. However, the only study regarding vaccines in Carrión's disease was carried out in 1943 by Howe and Hertig and consisted in the active immunization of 22 military guards posted to zones with warty disease. The vaccine consisted in a suspension of 4 inactivated strains of *B. bacilliformis* and 86% of guards developed high titers of agglutinins for *B. bacilliformis* as a result of one or more inoculations. Although active immunization did not prevent the development of infection and positive cultures in 55% of guards, only one guard required hospitalization. These results showed improvement in the course of potentially severe Carrión's disease on comparison with

the controls. During one month of exposure 75% of non-immune individuals developed Peruvian wart and in the other group with 4 months of exposure approximately 90% of the guards became infected, with two-thirds becoming incapacitated by severe illness [159]. On the other hand, the sensitization of rabbits with viable cells of *B. bacilliformis* increased their susceptibility to the lethality of subsequently administered *Bartonella* metabolites, indicating that hyper-reactive states are dependent on the degree of sensitization [160].

7. TRANSMISSION

Up to now different ways of transmission of *Bartonella* have been described or proposed. Nonetheless, Carrión's disease is a vector-borne illness, being this the most common and relevant way of disease transmission.

7.1 VECTORS

Townsend identified *P. verrucarum* as the vector of Carrión's disease by observing its habits and characteristics, the conditions of endemic zones as well as the distribution of the disease and the insect [15, 161]. Nevertheless, the role of *L. verrucarum* as the vector of Carrión's disease was not firmly supported until the studies of Noguchi in the late1920s [162], and was definitively corroborated by Hertig in 1942 [163]. Currently, *Lutzomyia* and *Phlebotomus* are two different genera within the *Phlebotominae* subfamily, belonging to the Psychodidae family. While *Phlebotomus* members live in temperate regions of the Old World, *Lutzomyia* genus is present in tropical and subtropical areas of America, from Argentina and Chile to the USA [164].

According to the Theodor classification, the *Lutzomyia* genus is composed by at least 26 subgenera accounting overall for approximately 400 species [165]. According to both morphological and molecular criteria a sub-classification has been proposed in which *L. verrucarum*, and at least 40 related species including *Lutzomyia maranonensis* or *Lutzomyia columbiana*, have been placed in the so-called verrucarum group [166, 167] (Figure 16). The *Psychodidae* family has a complex taxonomy which is under constant revision, and the most recently proposed taxonomy by Galati has resulted in new genera and in the re-classification of different species, including *L. verrucarum,* (classified within the genus *Pintomyia*, *Pafinomyia* subgenus and verrucarum series) [168]. The Theodor classification (genus *Lutzomyia* and *L. verrucarum* species) are referred in the present thesis.

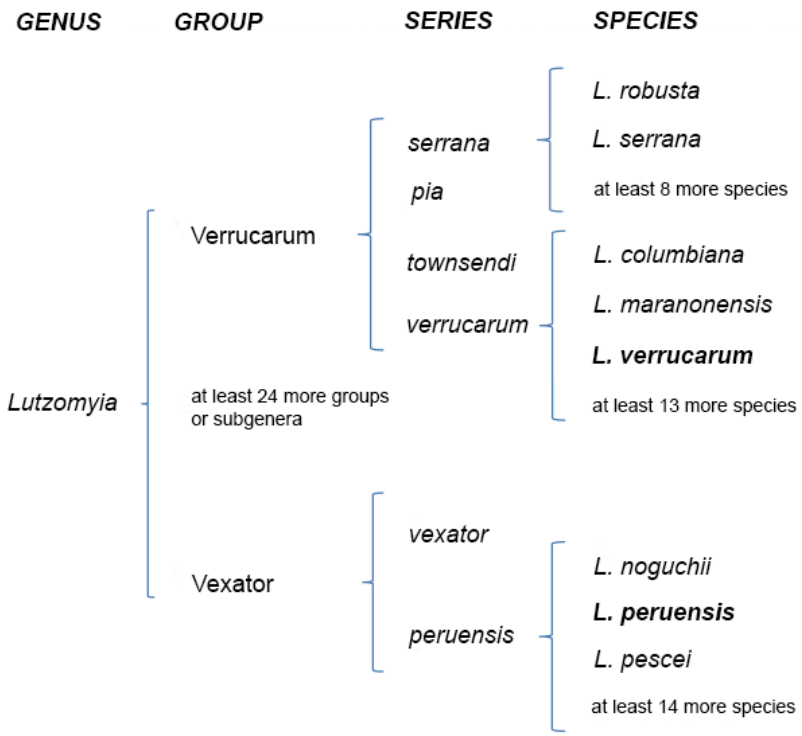

Figure 16: Classification of suspected and confirmed (in bold) vectors of Carrión's disease following the scheme of Theodor 1965.

Although *L. verrucarum* lives in relatively high arid zones, the presence of humidity and organic material facilitates the establishment of *Lutzomyia* [32]. These findings, together with the presence of sugar sources have been related to the association between traditional coffee plantations and the presence of *Lutzomyia* spp. [32]. Both *Lutzomyia* males and females feed on honey dew from aphids or vegetable sugars, but females (except for some autogenous species) also have blood requirements for egg development [164]. Thus, *L. verrucarum* females are responsible for the transmission of *B. bacilliformis* to humans. The mechanism of this transmission is still unclear [38].

L. verrucarum has classically been distributed at altitudes of 500 to 3200 meters above sea level in inter-Andean valleys and slopes of the center and northern Peruvian Andes [38, 68]. *L. verrucarum* and other *Lutzomyia* species have a unimodal annual distribution pattern, with the highest population densities occurring prior to the onset of the summer rainy season [38]. Sand fly population densities are directly correlated with the average minimum ambient temperatures and relative humidity. Sand flies are usually found inside houses [36, 37, 169], feeding at dusk and during the evening when the ambient temperatures drop and the relative humidity rises [15, 38, 106]. In fact, unusually high numbers of sand flies were collected after El Niño affected Peru [36, 170].

The distribution of Carrión's disease and the presence of *L. verrucarum* do not always seem to match, raising the possibility of the involvement of other *Lutzomyia* species in disease transmission [169, 171]. Several studies have been carried out to determine the *Lutzomyia* species that might play a role in the transmission of the disease. In endemic zones of the Cajamarca and Amazonas departments, *L. maranonensis* and *Lutzomyia robusta* were considered the probable vectors [169, 172]. On the other hand, in the jungle of the Huanuco department the authors found that *Lutzomyia serrana* is the most probable vector [173]. The presence of *L. verrucarum* in Ecuador and Colombia were never reported [9]. As indicated in chapter 2.4, Colombia was Carrión's disease free prior to 1936. Indeed, the first outbreak reported more than 6000 deaths [59], highlighting the possible adaptation of another arthropod to the vector-role of Carrión's disease and the latent risk of illness expansion. Moreover, it has been postulated that the closely related *L. verrucarum* species, *L. columbiana,* is implicated in disease transmission. These studies suggest that there may be another vector for Carrión's disease other than *L. verrucarum,* although the findings are not conclusive. The results were based on the collection of insects in endemic areas using traps followed by the identification of the species collected. However, *B. bacilliformis* was not identified in any of the insects.

In the brilliant study by Noguchi, several insect species were collected, including three species of *Lutzomyia* (*L. verrucarum* and two species which have been named *Lutzomyia noguchii* and *Lutzomyia peruensis*), from districts of Peru in which the disease prevails. The presence of *B. bacilliformis* in the insects was established by infecting *Macacus rhesus* with the extract of the crushed insects and making cultures of the monkeys' blood at different time points after the inoculation. To corroborate the results, the blood from the animals was cultured *in vitro,* yielding cultures of *B. bacilliformis* which produced typical verrucous lesions on inoculation into other monkeys. Moreover, the monkeys which had recovered from the infection with the *Lutzomyia* strains showed resistance to a human strain of *B. bacilliformis* that was later inoculated. The results of this work show that *L. noguchii* very likely carries *B. bacilliformis*, and that *L. verrucarum* is also a probable vector. However, there continue to be reservations with respect to *L. peruensis* [162]. Similar studies in which the monkeys were directly bitten by *L. verrucarum* wild sand flies were later performed by Hertig resulting in infection of 5 out of 8 monkeys [163]. The presence of *B. bacilliformis* in *L. verrucarum* was later confirmed by PCR and Real-Time PCR (RT-PCR) [174]. In a posterior study done in Cusco using PCR, 1% of the *L.*

peruensis collected were infected with *B. bacilliformis,* and this vector was implicated in the transmission of bartonellosis to humans [37] (Table 2).

Table 2: *Lutzomyia* species confirmed or proposed as Carrión's disease vectors according to the Theodor 1965 classification.

Specie	**Group**	**Serie**	**Status**	**Distribution**
L. verrucarum	verrucarum	verrucarum	Confirmed	PE
L. peruensis	vexator	peruensis	Confirmed	BO, PE
L. noguchii	vexator	peruensis	Highly probable	PE
L. columbiana	verrucarum	verrucarum	Potential	CO
L. maranonensis	verrucarum	verrucarum	Potential	EC, PE
L. pescei	vexator	peruensis	Potential	PE
L. robusta	verrucarum	serrana	Potential	EC, PE
L. serrana	verrucarum	serrana	Potential	BO, BR, BZ, CO, CR, EC, GF, GT, HN, MX, NI, PA, PE, VE

BO: Bolivia; BR: Brazil; BZ: Belize; CO: Colombia; CR: Costa Rica; EC: Ecuador; GF: French Guyana; GT: Guatemala; HN: Honduras; MX: Mexico; NI: Nicaragua; PA: Panama; PE: Peru; VE: Venezuela.

The role of the vector in the maintenance of the microorganism by vertical transmission was assessed by Ponce and colleagues. Similar to what happens in some viruses, it was hypothesized that the insect would be responsible for maintaining the bacteria and thus, no other animal reservoir was needed. *L. verrucarum* from an area with a high prevalence of the insect and the illness were fed from two patients' diagnosed with Oroya fever, with a positive blood smear and bacteremia of 3 and 80%. After oviposition and the death of the insects, PCR was performed to detect the microorganism. The results showed that insects fed with a higher bacteremia had a shorter life and consequently a decrease in oviposition, supporting the hypothesis of a higher mortality of *L. verrucarum* due to a higher bacteremia and the absence of vertical transmission of *B. bacilliformis* in *L. verrucarum* [175]. A recent report compared *B. bacilliformis* colonization in both a competent and non-competent vector, *L. verrucarum* and *L. longipalpis*, respectively. Initially, colonization of the two fly species was indistinguishable. However, at day 3 the bacteria remained in the abdominal midgut of the non-competent vector and was progressively digested until no viable bacteria remained at day 7. In *L. verrucarum*, *B. bacilliformis* colonizes the lumen of the digestive tract and persists for more than 14 days. These results suggest that *L. longipalpis* eliminates *B. bacilliformis* while bacteria in *L. verrucarum* survive on blood meal digestion and colonize the sand fly's

entire tract [176].

Other kinds of vectors can not be excluded. One report described the transmission of *B. bacilliformis* from an infected to a normal *rhesus* monkey by the bite of the tick *Dermacentor andersoni.* The infection was mild and the bacteria were recovered from the lymph nodes and blood from the animals [177].

7.2 ARTICLE "Carrión's disease: more than a sand fly-vectored illness"

Besides the vectorial transmission of Carrión's disease, other methods of transmission are possible as mentioned and described in depth in the following manuscript published in the PLOS Pathogens Journal.

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PEARLS

Carrion's Disease: More Than a Sand Fly–Vectored Illness

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What Is Carrion's Disease?: The Forgotten

Carrion's disease is a biphasic illness (S1 Fig) caused by an infection of *Bartonella bacilliformis*, a bacterium that is transmitted through bites of certain phlebotomine sand flies in the Andean valleys of Peru and in some areas of Ecuador and southern Colombia [1,2]. The acute phase, called Oroya fever, is a serious, life-threating illness that mainly affects immunologically naïve populations, such as children. It is also of special concern in pregnant women, because high mortality rates have been described as well as miscarriages, preterm births, and fetal deaths [3]. In this acute phase, the absence or delay of antibiotic treatment may lead to fatal outcomes. In fact, it is considered that, in the pre-antibiotic era, the lethality of this illness ranked between 40% and 88% [1,2]. In the chronic phase, classically considered to occur in previously exposed inhabitants, *B. bacilliformis* induce endothelial cell proliferation, producing skin lesions called Peruvian warts. In this phase, the lethality is very low [1]. Additionally, the presence of asymptomatic carriers is frequent, although the real numbers remain uncertain because of the difficulty in detecting these subjects (Table 1) [4].

Until now, no reservoir other than humans has ever been described, and, thus, due to the geographically defined area and the lack of animal reservoirs, this disease could be potentially eradicated.

The role of undescribed *Bartonella* spp. as a cause of Carrion's-disease–like symptoms cannot be ruled out. Indeed, other *Bartonella* spp. have correlated to Carrion's-disease–like presentations. Thus, *Bartonella rochalimae*, which is disseminated worldwide [5], was associated with a mild Oroya-fever–like episode in a tourist after a trip to Peru, whereas *Bartonella ancashensis* has been isolated from Peruvian warts of children living in an endemic Peruvian area [1].

Despite vector transmission being by far the most relevant route of transmission, other possible routes should be highlighted. Due to the nature of the illness, all direct inoculation or contact with infected human blood may result in its acquisition; thus, blood transfusions as well as accidental contact with infected blood in laboratories or during medical practices need to be considered. Additionally, vertical transmission and contact with other human fluids should also be considered (Fig 1).

Vector Transmission of *B. bacilliformis*: The Story Always Told

Native inhabitants traditionally considered sand flies as causing Carrion's disease, and, in the middle of the 18th century, Cosme Bueno proposed their role as vectors of Carrion's disease and *Leishmania* [7]. Nonetheless, the vector role of *Lutzomyia verrucarum* in the illness was

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not confirmed until 1913, more than 40 years after the devastating Carrion's disease episode in 1870–1871 that occurred during the construction of the Lima-La Oroya railway, causing several thousands of deaths [1,8].

B. bacilliformis is naturally transmitted by sand flies belonging to the *Lutzomyia* genus, mainly *L. verrucarum*, but also *Lutzomyia peruensis* [1,2]. However, the illness is also present in areas where these vectors are absent, suggesting the presence of undescribed vectors, or the undetected established vectors of *B. bacilliformis* [1]. In fact, the vector role of *L. peruensis* was described after an outbreak in an *L. verrucarum*-free area [1].

Vector sand flies are present in the inter-Andean valleys between 400 and 3,200 m [1,2]. Nonetheless, since the late 1990s, a continuous expansion of the illness to previously considered free areas, including coastal and high jungle areas, has been evident. It has been associated with the current situation of climate change, which, together with human activities, is presumably affecting the vector distribution, leading to vector expansion [1]. Additionally, the El Niño phenomenon results in increasing humidity levels, favoring sand fly reproduction and facilitating the development of Carrion's disease outbreaks [1]. Moreover, the potential of Lutzomyia spp. living in non-endemic areas to become adapted as vectors of B. bacilliformis should not be ruled out.

Blood Transfusion: The Hidden Risk

Similar to other *Bartonella* species [9], *B. bacilliformis* is able to survive a long time in infected blood at 4°C [10,11]. In 1926, Noguchi reported that *B. bacilliformis* can survive 152 days in experimentally infected monkey blood samples stored at 4°C [10]. More recently, viable *B. bacilliformis* was recovered from Oroya fever patients' blood stored at 4°C for as long as 30 months [11]. It has been considered that infections with *B. bacilliformis* occur in populations

Table 1. Main facts on Carrion's disease.

Facts[1]	Oroya fever	Asymptomatic carrier	Peruvian wart
Symptoms and Signs	Abdominal pain		Arthralgia
	Anorexia/Hiporexia		Bone pain
	Arthralgia		Fever
	↑ Bilirubin		Headache
	Chills 1		Joint pain
	Diarrhea		Lymphadenopathy
	Dyspnea		Malaise
	Fever (up to 99%) [2]		Myalgia
	Headache		**Skin lesions**
	↓ **Hematocrit (up to >80%)**		
	Hemolytic Anemia (up to >90%) [3]		
	Hepatomegaly		
	Hypothermia		
	Jaundice		
	Lymphadenopathy		
	Malaise		
	Myalgia		
	Nausea/Vomiting		
	Pallor		
	Pollakiuria		
	↑ Protein C Reactive		
	Splenomegaly		
	Sweats		
	Systolic murmur		
	Tachycardia		

(*Continued*)

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Table 1. (*Continued*)

Facts[1]	Oroya fever	Asymptomatic carrier	Peruvian wart
Complications	**Immunosuppression**		Bleeding
	Co-infections[4]		Dermal infection
	bloodstream *Salmonella*		Necrosis
	bloodstream *S. aureus*		
	Leptospirosis		
	Latent infections[4]		
	Histoplasmosis		
	Toxoplasmosis		
	Tuberculosis		
	Cardiovascular		
	Anasarca		
	Cardiovascular shock		
	Congestive heart failure		
	Myocarditis		
	Pericardial effusion		
	Pericardial tamponade		
	Gastrointestinal		
	Digestive hemorrhage		
	Gyneco-obstetrics		
	Fetal death		
	Miscarriages		
	Pre-term births		
	Hepatical		
	Acute cholecystitis		
	Hepatocellular necrosis.		
	Neurological		
	Altered Mental Status		
	Increased intracranial pressure		
	Coma		
	Convulsion		
	Respiratory		
	Acute Pulmonary Edema		
	Other		
	Purpura		
	Renal insufficiency		
Treatment	Blood Transfusions	Amoxicillin plus clavulanic acid[5]	Erythromycin, Azithromycin
	Amoxicillin plus clavulanic acid[5]	Ciprofloxacin	Rifampicin
	Chloramphenicol ± other antibiotics		
	Ciprofloxacin ± Cephalosporin		
Outcome	Development of partial immunity[6]	Perpetuators of the illness	Development of partial immunity
	Lack of bacterial clearance[6]	Potential infected blood/organ donations	Lack of bacterial clearance
	Vertical transmission risk	Vertical transmission risk	Vertical transmission risk
	Death[7]		

In bold, the more frequent and/or relevant signs and symptoms, as well as complications. Orange shaded, complications that more often result in a fatal outcome.

[1] Nonexhaustive list.

[2] Moderate (usually less than 39˚C) and intermittent.

[3] With negative Coombs test.

[4] Listed some of the most commonly detected.

[5] Mainly in pregnant women.

[6] Uncertain number due to the low blood bacterial burden and the lack of sensitive diagnostic tools. It has been reported that 45% of inhabitants from an endemic area have antibodies against *B. bacilliformis* [2].

[7] If correctly treated, case fatality rates ranks from 0.5%–1% in peripheral health centers and 8%–10% in reference centers (because of the reception of complicated cases); when not treated, case fatality ranks between 40% and 88%. In any case, it is especially relevant among children and pregnant women.

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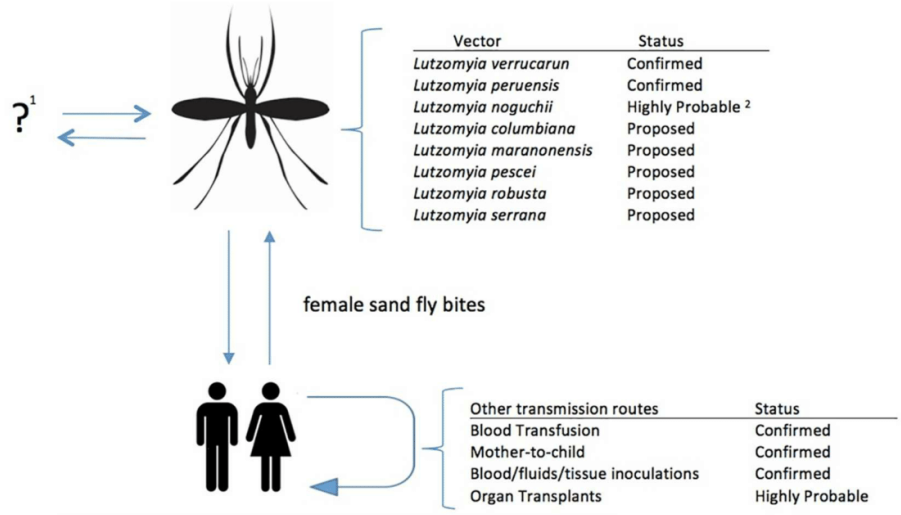

Fig 1. *B. bacilliformis* transmission routes. [1] Humans are the only known reservoir. [2] Despite a report by Noguchi et al [6], no confirmatory result has been published.

doi:10.1371/journal.ppat.1005863.g001

unlikely to be qualified as blood donors [12]. Nonetheless, the slow bacterial growth, the inability of definitive diagnostic approaches to consistently detect carriers [4], and the undefined duration of the asymptomatic carrier status, which may be up three years [13], are clear risks that may result in posttransfusion infections. Moreover, this risk, which is not limited to endemic regions, may extend to other areas due to the migration phenomena from rural to urban areas and low- or middle- to high-income countries.

In endemic zones, blood banks perform blood smears to detect the presence of *B. bacilliformis*, while in neighboring regions, blood donations of persons living in endemic areas are not accepted [14]. In blood banks from other regions or countries, no specific measures are taken to determine the presence of *B. bacilliformis*. Nonetheless, confirmed or suspected cases of posttransfusion Carrion's disease are scarce. This may be due to the low-income nature of the main regions affected, which results in a low number of cases being reported, as well as a high number of previously exposed inhabitants, of up to 45% [2], who have developed partial immunity that may prevent or minimize the effect of transfusion-mediated transmission. In fact, earlier 20th century reports considered that almost all local inhabitants had partial immunity [8]. Moreover, most data on Carrion's disease are only reported at a local level, thereby contributing to lack of disease visibility at an international level.

From the few reports present in the literature, two are strongly suggestive of transfusion transmission. In 1972, a newborn died from Oroya fever after a blood transfusion in a *B. bacilliformis*-endemic area. In this case, vertical transmission was not considered, as the mother did not have a previous *Bartonella* infection [15]. More recently, the acquisition of Oroya fever has been described in a chronic myeloid leukemia patient receiving multiple blood and platelet transfusions, one of which was contaminated with *B. bacilliformis* [14].

The possibility of *B. bacilliformis* transmission during organ transplantation is also plausible. Indeed, posttransplant infection with a Bartonellaceae (*Bartonella henselae*) has been proposed [16].

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Vertical Transmission: The Nightmare

Vertical transmission was first proposed in 1858 by Tomas de Salazar [15]. Similarly, in 1913, Strong et al stated "We saw cases in young nursing children, and Campodonico and Monge state it occurs in newly born infants," again suggesting the presence of vertical transmission [8]. Subsequently, both Malpartida and Colareta proposed the same in the mid-1930s [15].

Nonetheless, unequivocal reports in the recent literature are scarce. In addition to the reasons for the low number of reports of transfusion transmission, the serious consequences of the acute phase of Carrion's disease during pregnancy should also be considered, especially when the infection occurs in the first months of pregnancy, affecting both the mother with serious complications and high lethality for the fetus, including preterm births, miscarriages, or fetal deaths, among others [3,15]. Additionally, social attitudes and traditional practices may lead to delays or nonuse of health centers during pregnancy or after child birth [17], resulting in underestimation or misdiagnosis of mother-to-child *B. bacilliformis* transmissions, which thus may be higher than considered.

In 2003, it was reported that blood samples collected from a preterm child, 90 minutes after birth, of a mother with verrucous lesions, resulted in *B. bacilliformis*-positive culture [15]. In 1994, a neonate (19 days) was reported with Oroya fever, in whom 30% of the red blood cells were infected, thereby implicating the mother with general malaise, and diagnosed with a positive blood smear [18]. Finally, in 2015, another case was reported in which a 22-day-old child of a mother with a Peruvian wart was admitted with Oroya fever [19].

Intentional or Accidental Inoculation with Fluids or Blood: Heroes, Crazies, and Unfortunates

Inoculation with contaminated body fluids is a direct way to acquire Carrion's disease. Although currently direct human inoculations with infected human fluids are limited to accidents, in the last years of the 19th and in the early 20th centuries, experiments inoculating volunteers with either infected blood or wart exudates were performed. The most classical example is the self-inoculation of Daniel Alcides Carrión in 1885. He inoculated himself with a wart exudate and developed Oroya fever with a fatal outcome [20]. Subsequently, in 1928, Garcia Rosell received an accidental inoculation from the contaminated blood of a patient with Oroya fever and developed a febrile illness that was cured, followed by eruption of Peruvian warts [20].

Conclusion: Risks and Opportunities

Although Carrion's disease is currently restricted to specific geographical zones, increasing tourism to endemic regions together with continuous human migratory processes may lead to both imported cases and presence of asymptomatic carriers outside of traditional areas, driving towards establishment of non-vectorial *B. bacilliformis* mother-to-child transmission or through blood transfusions. Those, along with the incessant and growing movement of goods, may also facilitate the accidental introduction of vectors into atypical habitats. These findings highlight the risk of *B. bacilliformis* transmission beyond traditionally affected regions and reinforce the need to develop a Carrion's disease eradication agenda.

Supporting Information

S1 Fig. *B. bacilliformis* may infect three different populations: healthy never exposed, healthy pre-exposed, or asymptomatic carriers (the infection of persons with either active Oroya fever or Peruvian wart is not considered, because these people should be under

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treatment). The illness evolution may vary leading to Oroya fever, Peruvian wart, or asymptomatic infection with different easiness. Moreover, although no data are available, the natural bacteria clearance may not be ruled out. Although not to scale, the arrows' size represents the probability of infection evolution.? [1] No data about.
(DOCX)

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7.3 CONTROL AND PREVENTION

The vector is the primary target for the control and prevention of Carrión's disease. The use of dichlorodiphenyltrichloroethane (DDT) sprayings was efficiently used to control sand flies after World War II and have continued to be applied in endemic areas [1]. This use of DDT in Peru was successful in reducing the sand fly population to negligible numbers as well as virtually ceasing the appearance of new cases of bartonellosis, and this effect persisted for at least 1 year and a half [178]. In a study done in Peru between 1945 and 1947, DDT spraying of stone walls and houses reduced the sand fly population to an extremely low level [178, 179], as did DDT spraying of school buildings in a zone with warty disease [112] and the spraying of village dwellings during an Oroya fever epidemic [27]. However, it is not clear if DDT sprayings continue at present, and if so, which areas are covered.

Other preventive measures have been taken by health authorities. Nonetheless, although they are not always directed against Carrión's disease, these measures also play a role in the control of the sand fly population and are useful in the fight to stop this illness. These measures include regular maintenance of irrigation canals or gullies together with campaigns aimed at making the population aware of the risks of stagnant water in the transmission of other vector-borne diseases like dengue (Figure 17). Moreover, albeit not frequent, the use of mosquito nets in windows or beds has also been described in endemic areas [180]. However, a great deal of work is still necessary in order to make the population truly aware of a problem which can be prevented and treated [180].

Figure 17: Prevention posters used in endemic zones of Carrión's disease. Kindly provided by Dr Isabel Sandoval.

8. RESERVOIRS OF *B. BACILLIFORMIS*

Determination of the reservoirs of *B. bacilliformis* is of special relevance. Data regarding the characteristics, habitats and geographical location, together with their direct or indirect interaction with humans would allow better understanding of the risk of developing the illness, the presentation of outbreaks and geographical spread as well as the feasibility of eradicating this disease.

Various candidates have been suggested as the reservoir of Carrión's disease. Euphorb plants were initially proposed as reservoirs because of the geographical correlation between this kind of vegetation and the warty zones as well as the seasonal incidence of the disease and the period of greatest plant growth. Herrer tried to recover *B. bacilliformis* from Euphorb plants in endemic zones and infect these plants experimentally with cultures of this microorganism. Thereafter he determined whether latex from plants allowed the development of *B. bacilliformis* in culture. The results of all of the above experiments were negative: *B. bacilliformis* were not recovered from the Euphorbiaceous plants, no infection was produced in the plants and the conditions for the *in vitro* development of the bacteria in latex were adverse. Altogether, these results demonstrated that Euphorb plants do not act as a reservoir of Carrión's disease [181].

Several studies have been performed in animals in the search for other potential reservoirs. Noguchi successfully demonstrated that *macacus rhesus* is susceptible to infection by *B. bacilliformis*. The microorganisms were found in the red blood cells of the animals and recovered in *in vitro* cultures. The clinical manifestations in monkeys are less severe than those of human disease and the nodules which developed resembled those of the Peruvian wart [18]. Similarly, in 1942, Hertig was able to isolate *B. bacilliformis* from blood of rhesus macaques experimentally exposed to the bite of wild *L. verrucarum* [163]. More recently, it has been shown that Owl Monkeys (*Aotus nancymaae*), which are present in the south American jungles including those of northern Peru, may be experimentally infected. In 4 out of 6 monkeys the presence of the microorganism was observed within the erythrocytes 21 days after either intradermal (in order to simulate the bites of the sand fly), or intravenous inoculation. Moreover, a more than 4-fold increase in IgM levels was observed in the 3 intradermally and 1 intravenously inoculated animals. Similar to the previous study, the symptoms were less evident and no positive PCR or successful culture was

obtained [182].

Cooper *et al.* proposed that human bartonellosis is a zoonosis in which wild animals, most probably rodents, are the natural reservoir [58, 183]. However, this proposal was based on case-control studies using questionnaires. No animal was tested for *B. bacilliformis* and the results were never confirmed in later studies. In 1942 the isolation of *B. bacilliformis* was reported from the blood of a single rodent, belonging to the genus *Phyllotis*, from an endemic area in Central Peru (Rimac valley) but no attempts to infect other rodents were successful [178]. To my knowledge no other isolation of *B. bacilliformis* from blood samples of an animal has been reported, and possible misidentification cannot be ruled out [6]. It is known that inhabitants of endemic zones usually have pets which are susceptible to infection of *Bartonella* spp. and these animals can develop lesions similar to those observed on the skin of patients with Peruvian wart. However, the presence of *B. bacilliformis* in warts from these animals has never been microbiologically demonstrated [6]. A survey in which the blood of 50 animals from 11 homes of families with children who had recently had bartonellosis was collected was unable to identify an animal reservoir for *B. bacilliformis* [184]. Moreover, in a study done by Solano and colleagues blood smears and blood cultures were obtained from dogs, cobayos guinea pigs and wild mice as well as healthy individuals from endemic areas of Peru. No positive results were obtained for blood smears, and no positive cultures were obtained from the wild animals. On the other hand, 12% of human samples were found to be positive for *B. bacilliformis* [185]. In studies by Chamberlin and Lescano 47% and 54%, respectively, of patients with warty disease were bacteriemic suggesting that chronic patients are the most likely reservoir [36, 111].

Currently humans are the only established reservoir for Carrión's disease, although the absence of other vegetal or animal reservoirs for *B. bacilliformis* has never been convincingly demonstrated. Most reports have been done with techniques with a low sensitivity, and thus, studies using techniques able to detect low bacteremia, serological techniques and/or RT-PCR are needed.

9. DIAGNOSIS OF CARRIÓN'S DISEASE

Carrión's disease is a restricted disease that affects the poorest populations living in remote isolated rural areas, which are badly communicated or have precarious access and poorly equipped laboratories [39]. The current methods employed for the diagnosis of bartonellosis have significant limitations, and the diagnosis of Carrión's disease remains an unsolved problem. The initial febrile stage is often misdiagnosed because its symptomatology is similar to that of other illnesses [37-39, 41, 105]. Although the warty phase is easier to diagnose by the clinic manifestations, incorrect diagnosis can be made, especially in regions where the disease is not present and in cases imported from endemic areas. One example of this was reported by Maguiña *et al.* in which erroneous initial diagnoses were made in 19% of patients including skin tumor in 7 patients, hemangioma in 5 patients, polyarthralgias in 2 patients, and systemic lupus erythematosis in 1 patient [105]. Moreover, without the diagnosis of the asymptomatic carriers perpetuation of the disease will never end.

9.1 ROUTINE DIAGNOSTIC TECHNIQUES

In endemic rural areas the diagnosis of the acute phase of Carrión's disease is mainly based on clinical symptoms and peripheral blood smears using Giemsa staining due to low cost and ease of use of this technique. Nonetheless, this method is expertise-dependent and despite the high specificity of 96%, a very low sensitivity (24-36%) has been reported, especially in mild cases of disease and in the subclinical and chronic phases of illness [37, 39, 186]. Moreover, the unspecific initial symptoms of Carrión's disease should be taken into account, since they are common to several pathologies present in these areas, such as dengue and other arboviral diseases, including malaria or tuberculosis, thereby making diagnosis based only on clinical symptoms difficult [15]. In the chronic stage of the disease clinical diagnosis is made by the presence of cutaneous angiomatous skin lesions. The sensitivity of microscopic techniques decreases from the 36% described in the acute phase to less than 10% in the Peruvian wart [106], highlighting the lower blood bacterial carriage. Histopathologic diagnosis is possible and when sectioned and stained with Warthin-Starry silver or Giemsa stain the histopathology of the skin eruptions reveals bacteria, albeit with difficulty [38].

A more reliable method is culture in blood agar. *B. bacilliformis* grows on solid or semisolid leptospira medium containing blood of the rabbit, sheep, horse or man [16].

The colonies are typically small, round, and lenticular and range from translucent to opaque [38]. Nevertheless, bacterial culture is not clinically useful for diagnosis due to the culture requirements and the slow bacterial growth rate (1 to 6 weeks). Moreover, it is cumbersome, time-consuming and contaminations have been described in 7-20% of the cultures [15, 105]. Additionally, the sensitivity of this method is extremely low. In one study a considerable portion of Peruvian wart cases yielded negative blood cultures [112], and another study showed that only 13% of the patients with Peruvian wart had a positive culture or blood film [105]. Moreover, a report in which cultures were performed over a period of 6 months from persons known to be infected with *B. bacillifomis* supported the idea that the proportion of positive cultures is much higher in the first months of infection than later [112]. A novel, chemically modified, insect-based liquid culture medium has been described, suggesting that the detection and isolation of *Bartonella* species can be enhanced. Although *B. bacilliformis* were not included in the study, the authors suggested that this new medium could be an alternative method to isolate these fastidious microorganisms from patient samples [187].

9.2 MOLECULAR TECHNIQUES

Several PCR approaches have been described in the literature in the last years. However, these studies do not generally involve a large number of samples and additionally, as occurs with the remaining diagnostic tools, they are hampered by the lack of a standard case definition. Beyond detection, genetic targets that provide sufficient sequence diversity to allow identification at the species levels are required to identify strains associated with human illness [188]. It has been shown that PCR approaches are more effective than optical microscopic and culture techniques [41], being able to diagnose Carrión's disease patients in the acute phase previously classified as negative by thin blood smear. Nonetheless, a critical issue is the detection limit of these techniques, raising doubts about their usefulness in the detection of low-bacteremia or asymptomatic carriers. Moreover, it should be kept in mind that molecular techniques are very difficult to be implemented in routine practice in remote endemic rural areas [39]. In fact, molecular techniques are only implemented in reference centers, mainly located in Lima. This fact also leads to another problem that is the need to transfer samples in a rapid consistent manner and the return of diagnostic results from reference centers to local health facilities.

PCR can be done directly in affected tissues and/or blood or in enrichment cultures [38]. Enrichment of the sample before conventional PCR has been proposed to

increase the positivity by 55% when compared with the original blood samples [189]. However, this enrichment technique results in a 14-day delay in sample processing, thereby making it unaffordable for diagnostic purposes. Dried blood spots (DBS) may be an easy solution to both the transportation of samples from endemic areas to reference laboratories and for small blood volume collection, especially in the pediatric setting.

Several PCR assays for the detection and identification of *Bartonella* species have been reported in the literature. A nested PCR using the cell division gene *ftsZ* to differentiate among *B. bacilliformis*, *B. quintana* and *B. henselae* was described by Kelly *et al.* [190]. Additional approaches include several PCR-restriction fragment length polymorphism (RFLP) methods: RNA polymerase beta-subunit (*rpoB*) gene analysis [191]; the citrate synthase (*gltA*) gene [192, 193]; the *ftsZ* gene [194] and the *16S* ribosomal ribonucleic acid (rRNA) gene [195]. Molecular analyses based on only one-step PCRs have also been described: the riboflavin synthesis genes, such as *ribC, ribD* and *ribE* genes, are well suited for either the detection of bacterial DNA in human specimens or for the differentiation of *Bartonella* species due to their presence in bacterial species and their absence in humans [196]. The *ialB* gene has been proposed for the diagnosis of *B. bacilliformis* on demonstrating high sensitivity and specificity in 10 blood samples from acute patients with confirmed thin blood smear [197]. Nonetheless, further studies showed its presence in other members of the genus, such as *B. henselae* and *B. birtlesii*, being thus a useful tool for *Bartonella* spp. infection [133], but not discriminatory for *B. bacilliformis*. The amplification of *16S rRNA* has also been proposed for the diagnosis of Carrión's disease in Peru [41]. However, several studies have warned about for the utility of the *16S rRNA* gene as a means of differentiating *Bartonella* species due to the high conservation in this gene [198, 199]. Hypervariable intergenic transcribed spacer *16S-23S rRNA* (*its*) amplification allows differentiation between *B. bacilliformis* and *B. ancashensis* from the main pathogenic *Bartonella* spp. [200]. In fact, the *its* region has been used in different studies of *Bartonella* spp. [76, 201]. Another PCR approach is multiplex PCR based on *its* gene amplification combined with reverse line blotting which allows distinguishing among 20 different *Bartonella* species [202].

Finally, recent studies have demonstrated the utility of more sensitive molecular techniques such as quantitative PCR in which 24.6% of 65 children studied were positive whereas only 3% were blood-culture positive [203]. In another study with animal blood samples, the sequences amplified by RT-PCR of a region of the *ssrA*

gene were able to discriminate *Bartonella* species [188]. A two-step protocol consisting of a genus-specific RT-PCR with the *gltA* gene as the target followed by pyrosequencing of RT-PCR positive specimens directed against a segment of the *rpoB* gene was also described for differentiation of at least 11 medically relevant *Bartonella*. Despite being a two-step protocol, it can be done in 5 hours, and it is an alternative to the time consuming sequencing of a PCR product [204]. On the other hand, a whole-genome scanning technique to identify the NADH dehydrogenase gamma subunit (*nuoG*) was found to be sensitive and specific enough to detect a diverse number of *Bartonella* species in a wide range of environmental samples while maintaining minimal cross-reactivity to mammalian host and arthropod vector organisms [205]. Angkasekwinai *et al*. [206] described a loop-mediated isothermal amplification with *pap31* as the target gene. This is a simple method to detect the presence of *B. bacilliformis* DNA within 1 hour and requires less specialized equipment. This method achieved good results on analyzing *Lutzomyia* samples, with a detection limit between 1 and 10 copies/µL depending on whether bacterial genomic DNA is used alone or in the presence of human DNA, respectively. Nonetheless, its usefulness remains to be validated in human clinical samples.

9.3 SEROLOGICAL TECHNIQUES

It is clear that serological tests are useful diagnostic tools for Carrión's disease, especially when combined with other techniques such as PCR and blood culture. However, immune diagnostic methods for this disease are relatively underdeveloped [38], and as mentioned above, the most studied approaches, such as indirect fluorescence antibody assay (IFA) or Enzyme-linked immunosorbent assay (ELISA) have the disadvantage of the limited technical resources in endemic areas. In this context, the characterization of the antigens expressed during *B. bacilliformis* infection is not only fundamental to elucidate the pathogenesis of this disease but may also be useful for the development of a rapid diagnostic tool [39, 207]. A rapid diagnostic test which does not need experienced personnel or high technology machinery could be perfectly introduced in low-income areas after basic training. Moreover, the results can be quickly obtained thereby facilitating early initiation of treatment.

Studies of *B. bacilliformis* antigens in the literature are scarce compared with reports of other pathogens. To my knowledge, the first study identifying *B. bacilliformis* antigens was published by Knobloch in 1988 [208] who described 24 protein antigens including two main antigens with 65 and 75 kDa. BB65 is a heat shock protein

posteriorly identifed as GroEL, and the 75 kDa antigen corresponds to the cell division protein FtsZ of *B. bacilliformis* [208-211]. Some studies have found that GroEL does not bind to *Bartonella* Ig (immunoglobulin) M but does bind to IgG antibodies after the first two weeks, thereby demonstrating its utility to detect persisting IgG from the first to the third year after *B. bacilliformis* infection. However, only 60% of sera from Peruvian wart patients react with GroEL [209]. The presence of GroEL is correlated with mitogenicity activity against human vascular endothelial cells leading to the development of the verrucous lesions. This mitogenic activity is inhibited *in vitro* by the presence of specific anti-GroEL antibodies, suggesting the protective role of specific IgG in asymptomatic carriers [143].

The first study which attempted to identify the outer membrane proteins (OMPs) of *B. bacilliformis* led to the identification of 14 OMPs ranging from 11.2 to 75.3 kDa. The most prominent immunoprecipitants with rabbit anti-*Bartonella* hyperimmune serum were the proteins of 31.5, 42, and 45 kDa which may represent the immunodominant OMPs of the pathogen [125].

Padmalayam *et al*. described an immunogenic 43 kDa lipoprotein as an antigen by screening a genomic DNA lambda library with serum from a patient in the chronic wart phase of bartonellosis [207]. An ELISA assay was described using the 43 kDa lipoprotein in its recombinant form. The sensitivity of IgG and IgM ELISA as 70.4 and 85.2%, respectively, and the specificity for both IgG and IgM was 90%. Despite the good sensitivity and specificity three sera from chronic patients had high titers of IgM indicating that the IgM ELISA does not discriminate between the two phases of the disease [212].

Another ELISA assay using the recombinant flagellin gene (*fla*) of *B. bacilliformis* was assessed against the sera from patients with bartonellosis and healthy controls. The sensitivity of this method was 58% and when sera from people with other pathologies were tested a specificity of 69% was obtained. Moreover, 15% of sera from healthy people also gave a positive result [213]. One possible explanation for the worse results on comparison with the 43 kDa lipoprotein ELISA may be that the flagellin of *B. baciliformis* is very similar to other flagellin and may share epitopes with other bacteria [212]. More recently, Pap31 (or hemin-binding protein A) was described to be immunologically dominant and highly expressed in *B. bacillifomis* cultures and was considered a good candidate for use in ELISA [214]. Furthermore, hemin-binding proteins seem to be good candidates for the development of serodiagnostic tools for

Bartonella infections, as proposed for *B. quintana* infections [215].

B. bacilliformis immunoblot sonication of whole organisms is remarkably sensitive to *B. bacilliformis* antibodies present in the sera of patients with chronic disease (94%) and also yields reasonable results in acute disease (70%). However, some cross reactions with sera with antigenically similar bacteria such as *Brucella* and *Chlamydia psittaci* were reported in 34% and 5% of the cases, respectively [186]. Indeed, an intense cross-reactivity between *B. bacilliformis* and *C. psittaci* was also previously reported due to common surface epitopes [216].

Chamberlin *et al*. described an IFA with irradiated *B. bacilliformis* whole cell antigen preparation cocultivated with Vero cells. This assay showed that 81% of acute confirmed cases presented a positive IFA test. On the other hand, 74% of the volunteers from endemic areas with a positive IFA test had had bartonellosis within the last year. This percentage decreased to 39% when a more distant or a non-bartonellosis episode was reported, showing that in general 45% of the volunteers from an area of *Bartonella* endemicity were seropositive for *B. bacilliformis* by the IFA test [113].

10. TREATMENT OF CARRIÓN'S DISEASE

The treatment differs depending on which phase of the disease is presented, including blood transfusions and antimicrobial agents. With regard to antimicrobial therapy, different antibacterial agents have been tested since the beginning of the antibiotic era [217]. In fact, the discovery and production of antibiotics in the first half of the last century have been among of the greatest achievements in medicine. The use of antimicrobial agents has reduced the morbidity and mortality of infectious disease and has substantially contributed to the increase of human life expectancy. Although several studies were developed in the XIX century [218, 219], it is largely considered that the first antimicrobial discovered was penicillin by Alexander Fleming in 1929 [220]. Shortly thereafter, and coinciding with the World War II, this antimicrobial was introduced in clinical practice and its use rapidly spread. Many other antimicrobial agents were discovered in the following years and today the different classes of antimicrobial agents known are classified according to their mechanism of action (Table 3).

Table 3: Decade of introduction in clinical practice of the main antibiotic families and their respective targets.

Decade[1]	Main Antibiotic Families	Target
1930s	Sulphamides	Folic acid synthesis
1940s	β-lactams (Penicillin)	Cell wall synthesis
	Aminoglycosides	Protein synthesis
	Nitrofurans	DNA, RNA and protein synthesis
	Polymyxins	Outer membrane disruption
1950s	Amphenicols	Protein synthesis
	Tetracyclines	Protein synthesis
	Glicopeptids	Cell wall synthesis
	Macrolides	Protein synthesis
1960s	Cephalosporins (β-lactams)	Cell wall synthesis
	Quinolones	DNA replication
	Streptogramins	Protein synthesis
	Lincosamides	Protein synthesis
	Rifamycins	Protein synthesis
1970	β-lactamase inhibitors	β-lactamases
	Phosphonic acids	Cell wall synthesis
1980	Carbapenems (β-lactams)	Cell wall synthesis
	Mupirocin	Protein synthesis
	Lipopeptids	Outer membrane disruption
2000	Oxazolidones	Protein synthesis

[1] Introduction into clinical practice.

The current clinical practice relies mostly on personal experience, expert opinion, and data of microbiological susceptibility [221]. In any case, no clinical assays have been developed to define the real usefulness of the different antibiotic schedules proposed to treat Oroya fever, and only one clinical assay has been developed for the chronic phase [222].

10.1 TREATMENT OF OROYA FEVER

It is often considered that *B. bacilliformis* was the most lethal bacteria in the pre-antibiotic era. Left untreated, Oroya fever is frequently referred to as having one of the highest death rates of all infectious diseases, being from 40 to 85% [107] and achieving 88% in a study by Gray and colleagues [27]. Fortunately, treatment is available, and the mortality rates have decreased.

Blood transfusions are perhaps the most classical treatment and are recommended to treat the severe anemia typical in the acute phase of the illness [34, 68, 110, 223]. On the other side, the first treatments with penicillin were reported in 1944 [217, 224]. Since 2003 and according to the Ministry of Health of Peru recommendations and guidelines, ciprofloxacin is the drug of choice for adults in the acute phase of Carrión's disease (400 mg intravenously twice a day during 3 days and then 200 mg orally twice a day until 14 days of treatment). In severe cases, ceftriaxone (2 g every 24 hours during 7 to 10 days) should be added to the treatment [225]. However, as mentioned previously, *B. bacilliformis* is intrinsically resistant to nalidixic acid [226]. Moreover, there are *in vitro* evidence suggesting that ciprofloxacin is not adequate for treatment of the acute phase and should be removed from the current guidelines [39, 227]. Nonetheless, the issue of the most adequate antimicrobial remains controversial. On one hand successful treatment has been reported, as in the case of a patient with a massive erythrocyte infection (more than 95%) who received a 10-day course of ciprofloxacin and ceftriaxone with full recovery [228], while on the other hand, therapeutic failure and persistent bacteremia of up to 22.6% have been described in cases after having completed treatment schedules [229].

Prior to the inclusion of ciprofloxacin in the guidelines, some studies reported the use of chloramphenicol with good results [6, 105, 230]. In fact, chloramphenicol was considered the treatment of choice in acute cases after a medical consensus in 1998, and prior to its replacement by ciprofloxacin in 2003 due to the lack of clinical response in some patients [231-233]. In a previously mentioned study by Gray and colleagues none of 10 patients treated with chloramphenicol died compared with 88% mortality among non-treated patients [27]. In another study, 19 patients received chloramphenicol for 5 days and similar results were obtained; the temperature usually returned to normal within 24 hours and the erythrocyte count and size were reestablished. Relapse was infrequent and when it occurred, further administration of chloramphenicol resulted in recovery [234]. Moreover, in a retrospective report with 215 patients clinical cure was obtained with chloramphenicol in 89% of the cases [232]. The use of chloramphenicol and another antibiotic also showed good response to therapy in 23 patients [105].

It is known that a co-infection should be suspected if there is no improvement within the first 72 hours of treatment [6]. The acute illness is often complicated by other infections, usually *Salmonella* spp., which greatly intensify the clinical condition of the patients [34, 108]. A treatment scheme based on chloramphenicol has the advantage

of being a low cost, broad-spectrum antibiotic which also covers potential co-infections [38]. However, it is interesting to note that successful treatment with chloramphenicol does not seem to eliminate the risk of subsequently developing eruptive-phase bartonellosis [37, 105]. Chloramphenicol is effective in the majority, but not all, patients with acute bartonellosis [105]. Thus, despite the effectiveness of chloramphenicol in some patients, therapeutic failures and persistent bacteremia, leading to asymptomatic carrier status, have been reported in other patients receiving this drug. In a study by Maguiña *et al.* 3 out of 42 patients did not respond well to chloramphenicol therapy, being of note that these 3 patients had an initial organism burden > 80% [105]. Another study reported 50% of persistent bacteremia after chloramphenicol treatment in 66 patients, 28.8% of whom presented posterior Peruvian warts, and the remaining presented positive blood cultures or PCR results [235]. Moreover, in Europe as well as other countries, the use of chloramphenicol in humans is restricted, while its use in livestock production is strictly forbidden because of its potential to produce side effects in the bone marrow [236].

10.2 Treatment of Peruvian wart

From 1969 until 1975 streptomycin was the drug of choice for the treatment of the chronic phase of Carrión's disease, and in the mid-1970s rifampin was introduced and became the first line drug, showing better results compared with streptomycin [105, 237]. In a study in 260 chronic patients receiving rifampin clinical cure was observed in 93.1% [232]. Nonetheless, treatment failure with rifampin for Peruvian wart has also been reported [230, 238, 239] and alternatives have been used in the treatment of chronic patients. Good response was obtained in an eruptive case treated with chloramphenicol [62], and in another case, a 12-year child, was treated with sultamicillin and deflazacort for 10 days, presenting rapid improvement of overall symptoms and complete remission of skin lesions at 21 days [240]. More recently, the Ministry of Health of Peru proposed the use of azithromycin (500 mg orally per day during 7 days) [225] for the eruptive phase of the disease after successful use of this antimicrobial [230].

In this thesis 4 antimicrobial agents were studied, azithromycin, chloramphenicol, ciprofloxacin and rifampicin which we mention in more detail below.

10.3 AZITHROMYCIN

Azithromycin was first synthetized in 1980 [241] and belongs to the macrolide family. It is the prototype of a new class of macrolides, the azalides, characterized by inserting a methyl-substituted nitrogen in C-9 of the erythromycin molecule (Figure 18). This structural change improved the acid stability of the molecule [242]. Furthermore, compared to erythromycin, azithromycin has fewer side effects and a longer half-life. Macrolide compounds inhibit protein synthesis by binding to domains II and IV of *23S rRNA* [243].

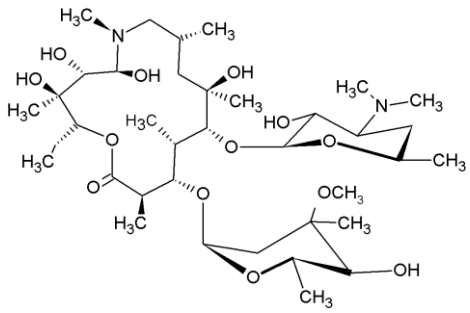

Figure 18: Chemical structure of azithromycin.

Like other macrolides, azithromycin has been primarily used to treat infections caused by Gram-positive microorganisms [244]. However, it has been shown to have good activity against Gram-negative microorganisms, being more active than erythromycin [245-247]. Currently, azithromycin is the antibiotic of choice to treat the warty phase of Carrión's disease [225].

10.4 CHLORAMPHENICOL

Chloramphenicol is an early antimicrobial agent isolated in 1947 from a culture of *Streptomyces venezuelae* [248] (Figure 19). This drug acts as an inhibitor of protein synthesis, preventing peptide chain elongation by binding to the 50S ribosomal center [249]. For decades it was used to treat the acute phase of Carrión's disease, but at present, it is no longer included in the treatment guidelines.

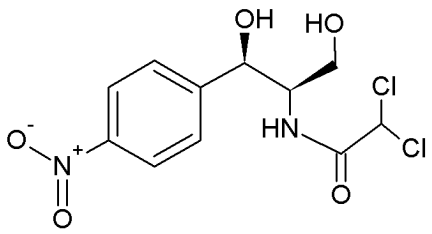

Figure 19: Chemical structure of chloramphenicol.

10.5 CIPROFLOXACIN

Ciprofloxacin belongs to the quinolone family of antibiotics which was introduced into clinical practice in 1967 [250] and characterized by a broad spectrum of action (Figure 20). Since their introduction, quinolone antibiotics have become one of the antibiotics most commonly used worldwide [251] and are the treatment of choice for respiratory diseases, gastrointestinal infections, skin related diseases, tuberculosis, urinary tract infections and soft tissue infections. The mechanism of action consists in the inhibition of the proteins involved in the replication and transcription of DNA, particularly DNA Gyrase and Topoisomerase IV by forming a quinolone-enzyme-DNA complex which blocks the enzyme. This causes inhibition of bacterial DNA synthesis, and thus, eventually causes cell death [252, 253]. DNA Gyrase and Topoisomerase IV, two enzymes that have a high homology between them, are tetramers (A_2B_2). The two subunits of the DNA gyrase are GyrA and GyrB, encoded by the *gyrA* and *gyrB* genes, and ParC and ParE are the two subunits of topoisomerase IV encoded by the *parC* and *parE* genes [251, 252]. Ciprofloxacin belongs to this group of antibiotics and is currently the treatment of choice for the acute phase of Carrión's disease in the current guidelines of the Ministry of Health of Peru.

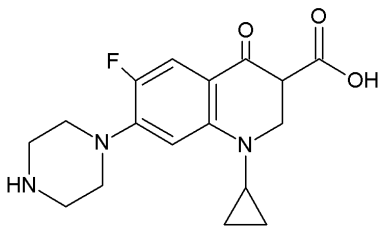

Figure 20: Chemical structure of ciprofloxacin.

10.6 RIFAMPICIN

Rifampicin is a member of rifamycin family, discovered in 1957 and used to treat several bacterial infections, namely tuberculosis (Figure 21). The mechanism of action consists in the inhibition of the replication of the genetic material by inhibiting the bacterial DNA-dependent RNA polymerase (encoded by the *rpoB* gene) [254]. Rifampicin is included in the current guidelines as a second line treatment for the warty phase of Carrión's disease.

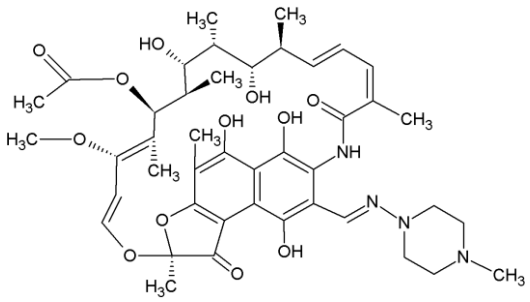

Figure 21: Chemical structure of rifampicin.

11. MECHANISMS OF ANTIMICROBIAL RESISTANCE

Antimicrobial resistance is considered to be one of the most important health problems worldwide. The emergence of resistant bacteria is due to the occurrence of *de novo* mutations that may be transfered by vertical gene transfer, changes in expression of certain genes and/or the ability of resistance genes to transfer between strains and species by horizontal gene transfer [255, 256]. Horizontal gene transfer is considered a major factor in the development of antimicrobial resistance since it contributes to the spread of genes that confer resistance to antimicrobial agents. The main processes leading to horizontal transfer of genetic material between bacteria are conjugation, transformation and transduction [257]. However, for intracellular bacteria like *B. bacilliformis*, antibiotic resistance is mainly due to mutations or intrinsic mutations in target genes. In other words, vertical gene transfer is more common and horizontal gene transfer has never been described for *B. bacilliformis*. Nonetheless only a few studies have determined the presence of plasmids in members of *Bartonella* genus, being the first described in 2003 in *Bartonella grahamii* [258]. In this thesis we only broach vertical gene transfer since only antimicrobial resistance in mutant strains was studied.

In general antibiotic resistance mechanisms can be grouped into: a) target modifications, b) inactivation of the drug or c) permeability changes (Figure 22). This thesis focuses on target modifications and changes in permeability.

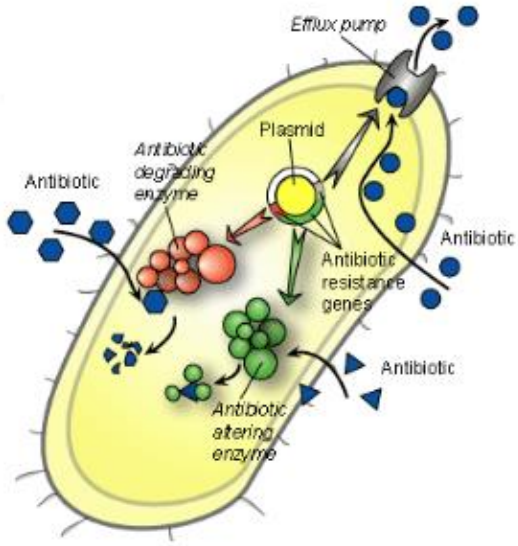

Figure 22: General antibiotic resistance mechanisms: plasmids, efflux pumps, enzymes that modify the antibiotic in a way that it loses its activity, and enzymes that degrade the antibiotic thereby inactivate it. Reproduced from [259].

Fortunately, *Bartonella* spp. continues to be highly susceptible to antibiotics [260]. Although not extensive, *in vitro* assays have shown general high levels of antimicrobial susceptibility. Nonetheless, constitutive nalidixic acid resistance and related diminished fluoroquinolone susceptibility have been reported [226, 261]. Additionally, relatively high minimal inhibitory concentration (MIC) levels of clindamycin and colistin [262], sporadic isolates presenting resistance to ciprofloxacin or even chloramphenicol, and a trend towards diminished susceptibility to aminoglycosides have been observed in clinical isolates [233, 263]. However, antimicrobial resistance levels have been established in a few *B. bacilliformis* clinical or collection isolates [226, 233, 262-264], and it should be taken into account that *in vitro* susceptibility does not consistently correlate with the *in vivo* outcomes of patients [239]. Moreover, treatment guidelines have changed over time due to the high percentage of clinical cure without microbiological elimination being described in more than 22% ciprofloxacin and up to 50% of chloramphenicol treatments [38, 229]. The lack of studies with clinical isolates as well as clinical assays emphasizes the need to obtain *in vitro* information about the ease with which this microorganism develops resistance and which antibiotic resistance mechanisms are selected. On the other hand, the slow bacterial growth and special culture requirements as well as the aforementioned high levels of antibiotic susceptibility hinder the development of studies of *in vitro B. bacilliformis* antibiotic-resistant mutants. To my knowledge only three *in vitro* studies have been developed with *B. bacilliformis*, selecting *in vitro* resistance to different antimicrobial agents, including coumermycin, ciprofloxacin,

rifampicin and erythromycin [238, 265, 266]. However, these studies were developed using either the KC583 or KC584 strain alone and were limited to the analysis of point mutations. In fact, Minnick *et al.* [266] showed that the frequency of mutation of *B. bacilliformis* KC583 is 10-fold higher than that of *E. coli*, and Biswas *et al.* [238] only needed 5 passages to select high ciprofloxacin-resistant isolates from strain KC584. Regarding rifampicin and azithromycin, full resistance was obtained after 3 and 4 passages, respectively [238].

Figure 23 shows the resistance mechanisms of the four antibiotics studied in this thesis followed by a brief explanation of the main resistance mechanisms for each antibiotic.

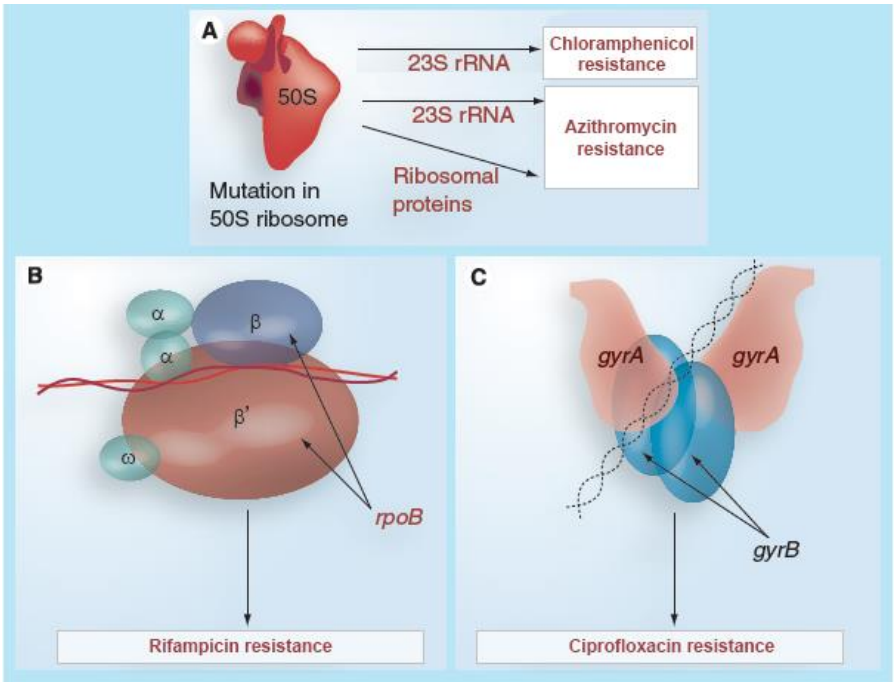

Figure 23: Main molecular mechanisms of resistance to azithromycin, chloramphenicol, ciprofloxacin and rifampicin in *Bartonella* spp. (A) Mechanism of azithromycin and chloramphenicol resistance due to changes in the 50S ribosomal subunit; (B) Mechanism of rifampicin resistance due to changes in the *rpoB* gene in RNA polymerase; (C) Mechanism of ciprofloxacin resistance due to changes in the *gyrA* and *gyrB* genes in the DNA Gyrase. Adapted from [239].

11.1 Resistance mechanisms to Azithromycin

Resistance to erythromycin appeared shortly after its introduction, with the first strain of erythromycin-resistant *staphylococci* being reported in the United Kingdom in 1959. The use of macrolides has considerably increased over the last 20 years as has the description of different resistance mechanisms in both Gram-positive and Gram-negative microorganisms. Changes in ribosomal proteins L4 and L22, encoded

by genes *rplD* and *rplV* respectively, as well as mutations in domain V of the *23S rRNA* gene can confer resistance by altering the conformation of the antibiotic union site [267, 268]. However, it should be taken into account that multiple copies of the *23S rRNA* gene vary according to the microorganism. Regarding *Bartonellaceae*, and following the *B. bacilliformis* numbering throughout the text, the mutations at L4 (Gly-70→Arg and Gly-70→Arg + His-74→Tyr) have been previously described in *in vitro* obtained macrolide-resistant mutants of *B. henselae* [269]. In *23S rRNA* the mutations A2058G, A2058C, A2059G and C2611T have also been described in macrolide-resistant mutants of *B. henselae* [269, 270].

11.2 RESISTANCE MECHANISMS TO CHLORAMPHENICOL

Despite largely being used in clinical practice, there is practically no information regarding the mechanisms of resistance to chloramphenicol in *B. bacilliformis*. The most relevant mechanisms of resistance in Gram-negative microorganisms are transferible, including antibiotic modification mediated by chloramphenicol acetyl transferases and the expulsion of the antibiotic mediated by specific transferable efflux pumps, such as CmlA or FloR [249]. The chloramphenicol susceptibility usually described in *B. bacilliformis* as well as the high levels of resistance conferred by these transferable mechanisms do not support the presence of these mechanisms of resistance in clinical isolates. Regarding chromosomal mechanisms, the *23S rRNA* methylation confers high level of resistance to chloramphenicol although it is not very frequent in Gram-negative microorganisms. Alterations in the ribosome, such as the presence of mutations of the *23S rRNA,* have been described, conferring chloramphenicol resistance to other microorganisms [256].

11.3 RESISTANCE MECHANISMS TO CIPROFLOXACIN

It has been described that several amino acid changes in the target proteins (GyrA, GyrB, ParC and ParE) are involved in the acquisition of resistance to quinolones [271, 272]. To date, only constitutive nalidixic acid resistance and related diminished fluoroquinolone susceptibility have been reported in association with the presence of an Ala as wild type amino acids at positions 91 and 85 of GyrA and ParC, respectively [226, 261]. These characteristic GyrA and ParC sequences are extended to other members of the genus *Bartonella* [261]. Changes at positions Asp-90 (ParC) and Asp-95 (GyrA) have previously been described in studies analyzing quinolone-resistant mutants of *B. bacilliformis*, *B. henselae* and *B. quintana* [238, 266, 273]. In addition, a mutation at amino acid codon 91 of GyrA leading to replacement of Ala to

Val has previously been described in *B. henselae* mutants [270].

11.4 Resistance mechanisms to rifampicin

Resistance to rifampicin has been linked to different mutations in the *rpoB* gene. These mutations are clustered in three highly conserved regions in the mid-portion of the *rpoB* gene. In *E. coli,* cluster I includes codons 505–537, cluster II codons 563–575 and cluster III codons 684–690 [274, 275]. The amino acid change Ser-545-Phe has previously been observed in *B. bacilliformis* and *B. quintana* in *in vitro* rifampicin-resistant mutants [238]. Amino acid substitutions at equivalent amino acid position have been observed as being involved in the development of rifamycin resistance in other microorganisms including *Mycobacterium tuberculosis* [276] or *E. coli* [277].

11.5 Efflux pumps

Efflux pumps are proteins involved in the expulsion of toxic substances from the bacteria, including for example, antimicrobials. Efflux pumps can be specific to a single substrate or can carry a variety of compounds, including antibiotics from different families. This is important for the development of multiresistance, since only one type of pump can confer simultaneous resistance to a wide range of antimicrobial agents [278]. It is of note that the efflux pumps mechanism contributes to an intrinsic resistance to antibiotics. Bacterial efflux transporters are classified into five major superfamilies: the multi-antimicrobial extrusion protein family, the major facilitator superfamily, the small multidrug resistance family, the resistance-nodulation-cell division superfamily (RND) (including AcrAB-TolC) and the adenosine triphosphate (ATP)-binding cassette superfamily ABC. All of these systems use eject protons as an energy source, except for the ABC superfamily, which uses ATP hydrolysis to promote the export of substrates (Figure 24). Thus, the effect of efflux pumps on resistance should be considered, as well as the role of their inhibitors to evaluate potential drug combinations for new treatments [279, 280].

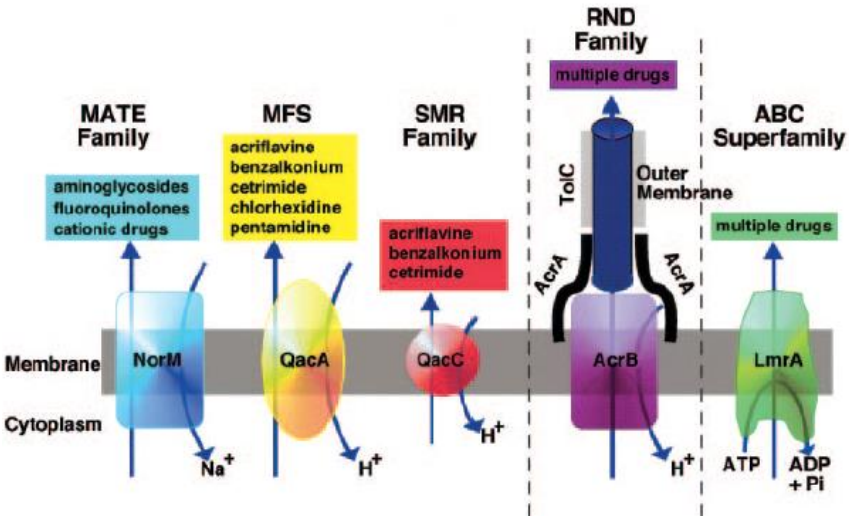

Figure 24: Diagrammatic comparison of the five families of bacterial efflux pumps. MATE: multi-antimicrobial extrusion protein family; MFS: major facilitator superfamily; SMR: small multidrug resistance family; RND: resistance-nodulation-cell division superfamily; ABC: ATP-binding cassette superfamily. Reproduced from [280].

Although no efflux pump inhibitor has been introduced in clinical practice with this purpose, different efflux pump inhibitors have been studied for the future design of combination treatments [281]. It is believed that the use of such strategies could facilitate the reintroduction of antimicrobials that are not effective today, avoiding the continuous growth of resistant strains [282]. Various efflux pump inhibitors have been studied, such as phenylalanine-arginine-β-naphthylamide (PAβN) which is one of the most frequently studied *in vitro* inhibitor [283-285], and artesunate that also inhibits the AcrAB-TolC system. To date, PAβN has only been used *in vitro* and so far has not been authorized for use in humans [283]. On the other hand, artesunate is currently used in the treatment of malaria. This inhibitor has been shown to increase the sensitivity of certain bacteria, such as *E. coli*, increasing the accumulation of β-lactam antibiotics inside the bacteria by inhibition of the multidrug efflux pump system AcrAB-TolC [286]. Nonetheless, despite its clinical safety, up to now no direct clinical application as an efflux pump inhibitor has been approved.

12. ERADICATION OF CARRIÓN'S DISEASE

During the last two centuries the fight against infectious diseases has been enormously enhanced with its burden having dramatically decreased, especially in developed countries. A series of milestones has marked these advances. The introduction of vaccines and antimicrobial agents in the XIX and XX centuries, respectively, may be considered the most relevant. However, social developments, access to health care and potable water have also played an invaluable role. The

eradication of smallpox and advances towards that of poliomyelitis are probably the main goals that have been achieved by vaccination campaigns. Indeed, at present, the eradication of illnesses such as malaria, elephantiasis, teniasis, measles, mumps, rubella or yaws has been proposed [287-289].

Eradication is defined as *"Permanent reduction to zero of the worldwide incidence of infection caused by a specific agent as a result of deliberate efforts; intervention measures are no longer needed"*. There are four principles that make infectious disease eradication feasible: sufficient funding, an adequate public health infrastructure, sustained socio-political commitment and biological feasibility [290]. Although eradication strategies need strong social and political commitment, consume major human and financial resources and require enormous effort to be implemented, a successful eradication program can provide a high benefit-cost ratio. A sustainable improvement in health should be a reality for all those having to live side by side with neglected illnesses, such as Carrión's disease. Futhermore, it cannot be forgotten that the reality of those outside this scenario could change with climatic phenomenon and the possible consequences of vector expansion.

In 1997 Dahlem's criteria on the feasibility of eradicating an illness were described [291]. Three specific points were considered as the most relevant: the effectiveness of an intervention; the sensitivity of diagnostic tools and humans being the only vertebrate reservoir. Carrión's disease fulfils all these points. Therefore, research efforts need to be strengthened to design and validate vaccine candidates. Additionally, the high antibiotic susceptibility of *B. bacilliformis* suggests that mass treatment could lead to the elimination of the illness in some areas and to a significant decrease of carriers in others. In both cases, a geographically well-delimited affected area would be favorable, facilitating the actions of eradication. The development of an efficient diagnosis tool is extremely relevant for the detection of carriers after mass treatment or vaccinations. Economic and social factors should also be considered, as proposed in the Dhalem conference. Among these, two points are of special concern: the need for consensus regarding the actions to be taken, including international support and the perception of the illness as a relevant public health problem. Obviously, an illness restricted to small isolated rural areas far from the tourist routes of middle-income countries and mainly affecting the poorest population may not be perceived as relevant and may not generate sufficient interest to gain consent for its eradication. At present, the window within which Carrión's disease could potentially be eradicable might be about to close, due to the

aforementioned expansion of the illness to nearby areas and the risk of accidental introduction of vectors into remote areas resulting in expansion of the illness to far away areas, similar to what has occurred with Chikungunya in southern Europe [292]. In summary, despite being potentially eradicable, this insidious and slowly expanding disease continues to scourge the Andean valleys, remaining outside the main scientific interest and hidden from international agendas.

HYPOTHESES AND OBJECTIVES

HYPOTHESIS

1. To date, excluding the intrinsic resistance to quinolones, antibiotic-resistant isolates of *B. bacilliformis* have not been reported. However, antibiotic-resistant clones may emerge in the future. We hypothesize that it is possible to develop *B. bacilliformis* mutants highly resistant *in vitro* to the common antibiotics used for treatment. Moreover, several different mechanisms of resistance will be selected. These mechanisms affect bacterial fitness, thereby explaining the apparent lack of antibiotic resistance among clinical isolates.

2. The way to diagnose Carrión's disease is far from ideal, resulting in misdiagnosis. We hypothesize that the current methodological approaches are not sensitive and specific enough to correctly diagnose Carrión's disease, and especially to detect asymptomatic carriers. The identification of antigenic candidates could be the first step towards more sensitive approaches able to efficiently diagnose this disease.

OBJECTIVES

1. Develop and characterize *in vitro B. bacilliformis* mutants resistant to the main antibiotics used to treat Carrión's disease.

1.1. Determine the ability of azithromycin, chloramphenicol, ciprofloxacin and rifampicin to select highly resistant *B. bacilliformis* mutants.

1.2. Establish the mechanisms of resistance selected as well as the role of the efflux pumps in the acquisition of resistance.

1.3 Evaluate the stability of the resistance acquired.

2. To provide new insights into the diagnosis of Carrión's disease. Compare several techniques as well as explore new antigenic candidates that could be useful in a future diagnostic test to be implemented in endemic rural areas.

2.1. Characterize a possible *B. bacilliformis* outbreak in a non endemic area.

2.2. Establish the detection limit and compare different PCR approaches to detect *B. bacilliformis* in blood samples.

2.3. Characterize a population living in endemic and post-outbreak areas with several diagnostic techniques.

2.4. Identify *B. bacilliformis* antigenic candidates.

THESIS OUTLINE

Carrión's disease is a neglected disease restricted to the poorest areas of the Andean regions, and only a few groups work in this field worldwide. Indeed, this disease is outside the focus of international research and, thus, *B. bacilliformis* remains an understudied pathogen. Until recently, it was thought that Carrión's disease fulfilled the most relevant criteria for eradication, however, in the last years a reemergence of the disease and progressive extension to new areas has clouded this option. Although this goal still remains possible, a handful of issues should first be addressed.

One of the issues is the lack of well defined efficient treatment schedules. Both *in vitro* antimicrobial resistance studies as well as clinical assays are needed to determine the best antibiotic treatment approaches. Despite the use of antibiotics, *B. bacilliformis* remains susceptible to almost all of these drugs. While the administration of antimicrobial agents usually improves the patient's health status and clinical cure is achieved, the latter is not always followed by bacterial clearance. This is of special concern because humans are the only bacterial reservoir known and asymptomatic carriers are those who perpetuate and spread the disease. Studies focused on the antimicrobial resistance mechanisms in mutant strains will contribute to fill in this knowledge gap and will be the first step to guide the design of clinical assays and new therapeutic strategies. On the other hand, the most imperative need is perhaps an easy way to perform correct diagnosis since adequate treatment is crucial to save lives. New insights in diagnostic and immunological studies will help to improve the diagnosis of Carrión's disease and will permit to identify new molecules with diagnostic potential.

The design of antibiotic schedules which minimize the selection of antibiotic resistance during treatment, together with the development of diagnostic techniques which can be implemented in isolated rural areas and are sensitive enough to detect asymptomatic carriers are essential for the control, elimination and eradication of Carrión's disease, which, in turn, will have an impact on the both lives of the people living in these areas as well as on public health.

The results of this thesis have been separated into 2 different important aspects related to Carrión's disease; on one hand, antimicrobial resistance (Chapter I) and, on other hand, the diagnosis and characterization of clinical samples (Chapter II). Chapter I describes a study regarding the resistance mechanisms developed in the presence of the 4 most common antibiotics used in the treatment of Carrión's

disease. Chapter 2 discusses several aspects of Carrión's disease which are addressed in 3 studies. These studies were performed in close collaboration with Dr. Juana del Valle from the Universidad Peruana de Ciencias Aplicadas and Instituto de Investigación Nutricional in Lima, Peru. As a result of this collaboration we were given access to DNA samples from an outbreak in the Amazonas department, and we also had the opportunity to visit some endemic zones of Carrión's disease and enthusiastically participate in field-work doing sample collection. Nonetheless, most of the results of this chapter were achieved largely due to collaboration with Dr. Mayumi Matsuoka from the National Institute of Infectious Diseases of Tokyo, Japan.

STUDY AREA

The studies in this thesis were done with both collection strains acquired from the Institute Pasteur (Paris, France) (articles 1, 3 and 4), and blood/serum samples collected in different areas of northern Peru (articles 2 and 4). A brief description of the areas where the samples were collected is provided below:

Amazonas Department, province of Rodriguez de Mendoza: an area in which Carrión's disease has never been described, but in which an outbreak was reported in March 2013. Blood samples were collected by our collaborators in Peru, and the DNAs were sent to Barcelona in order to perform molecular studies (article 2).

Piura Department, province of Huancabamba: we actively collected blood and serum samples in this area to carry out the studies reported in article 4 of this thesis. The main characteristics of this area as well as the field work performed are described below.

Despite the fact that Piura is on the Pacific coast, on the western side of the Andean mountains, the province of Huancabamba is mostly located on the eastern slope of the Andes, belonging to the Amazonas headwaters. It is close to the equatorial line and at around 970 km from Lima (Figure 25). The climate is warm and humid being Cerro Negro the biggest peak with approximately 3,950 meters above sea level. This province covers 4,254 Km^2, and in 2015 had a semirural population of 126,683 inhabitants (6.87% of Piura inhabitants) with a slight trend to a fall in population [293]. Administratively Piura is divided into 8 provinces, including Huancabamba, which in turn, is subdivided into 8 districts: Canchaque, El Carmen de la Frontera, Huancabamba, Huarmaca, Lalaquiz, San Miguel de El Faique, Sondor and Sondorillo.

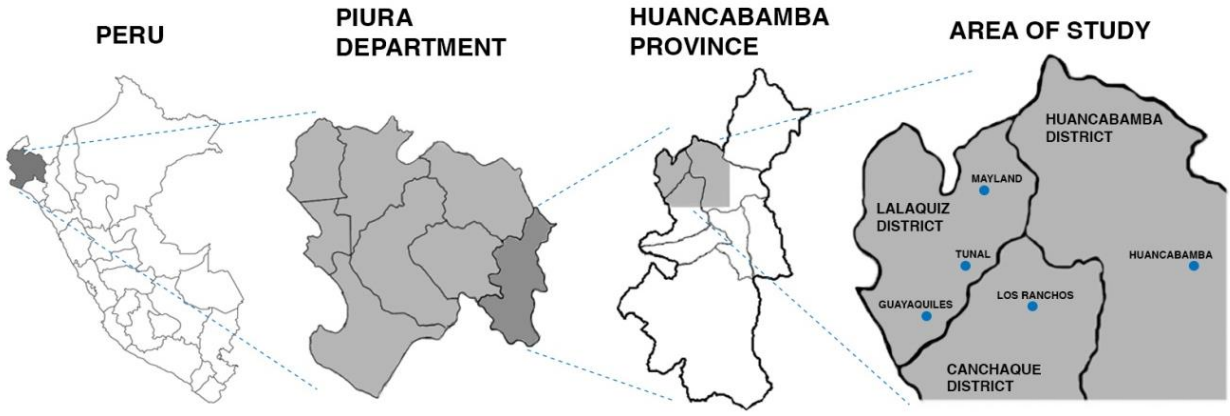

Figure 25: Geographical localization of the study area.

The health system in this area is clearly deficient, with only 1 hospital, 3 health centers, and 55 small centers able to perform very basic health surveillance throughout the Huancabamba province [294].

Several studies have described the presence of Carrión's disease in the Huancabamba province but have only included the Sondor, Sondorillo, Carmen de la Frontera, Huarmaca and Huancabamba districts [30, 264, 294]. It should be taken into account that these districts are located in an inter-Andean valley on the eastern Andean slope, with natural access to Jaen province. Moreover, the neighboring San Ignacio province and the direct route to this district should also be considered. Both Jaen and San Ignacio, belong to the Cajamarca department and are two of the most relevant endemic areas for *B. bacilliformis*.

In this thesis 5 villages were included in the sampling in March 2014. Four (Tunal, Guayaquiles, Los Ranchos and Mayland) were selected because a confirmed outbreak of *B. bacilliformis* had been reported several months previously [43], while the remaining village, Huancabamba, is a well established endemic area of *B. bacilliformis*.

In relation to post-outbreak areas, samples were collected mostly in three villages in the district of Lalaquiz (Tunal, Guayaquiles and Mayland) and Los Ranchos in the Canchaque district, near the border with Lalaquiz (Figure 25). These areas remained free of Carrión's disease until 2011, when the first 5 cases were described [295, 296]: 2 acquired the disease in the Cajamarca department, 1 was probably imported from Huancabamba, and the remaining 2 cases were considered the first autochthonous cases in the area [296].

Before our team arrived to the north of Peru to collect the samples in the four post-outbreak villages, the health center of each village was notified to recruit all individuals that were diagnosed with Carrión's disease during the outbreak (with positive thin blood smear and/or clinical symptoms). These individuals were invited by the personnel of the health center of each village to participate in the study and donate a blood sample on a specific day (the day that we went to collect the samples). All the participants were informed of what day they should go to the corresponding health center to donate the sample. Over four days (one day per village) our team collected all the samples.

At the time of sample collection, all the participants were apparently healthy, but a series of mild symptoms were reported when a questionnaire was given.

The endemic Huancabamba village is located within the homonymous district. Carrión's disease has largely been described here. In 2001 a total of 44 acute cases were reported leading to 3 deaths [30]. In Huancabamba, the participants were randomly recruited by house-to-house visits during which we explained the study and asked the people to participate. Everyone who agreed to participate donated a blood sample.

Blood and serum samples were processed in the field. Afterwards all the samples were stored in refrigerated conditions and sent by air to Lima and Tokyo. Figure 26 illustrates the field work carried out.

Figure 26: Pictures taken in the Piura disctrict during blood sample collection.

RESULTS

CHAPTER I – ANTIMICROBIAL RESISTANCE

Development and characterisation of highly antibiotic resistant *Bartonella bacilliformis* mutants

Gomes C, Martínez-Puchol S, Ruiz-Roldán L, Pons MJ, del Valle Mendoza J, Ruiz J.

Scientific Reports, 2016; 6:33584
Impact factor (2015): 5.228; Quartile: 1 (Multidisciplinary)

CHAPTER II – DIAGNOSIS

An unidentified cluster of infection in the Peruvian Amazon region

Cornejo A, Gomes C, Suarez L, Martínez-Puchol S, Bustamante P, Pons MJ, Ruiz J, del Valle Mendoza J.

The Journal of Infection in Developing Countries, 2015; 9: 524-529
Impact factor (2015): 1.139; Quartile: 4 (Infectious Diseases)

Evaluation of PCR approaches for detection of *Bartonella bacilliformis* in blood samples

Gomes C, Martínez-Puchol S, Pons MJ, Bazán J, Tinco C, del Valle J, Ruiz J.

PLoS Neglected Tropical Diseases, 2016; 10(3): e0004529
Impact factor (2015): 3.948; Quartile: 1 (Tropical Medicine)

Succinyl-CoA synthetase: new antigen candidate of *Bartonella bacilliformis*

Gomes C, Palma N, Pons MJ, Magallón-Tejada A, Sandoval I, Tinco-Valdez C, Gutarra C, del Valle Mendoza J, Ruiz J, Matsuoka M.

PLoS Neglected Tropical Diseases, 2016; 10(9): e0004989
Impact factor (2015): 3.948; Quartile: 1 (Tropical Medicine)

CHAPTER I – ANTIMICROBIAL RESISTANCE

ARTICLE 1

Development and characterisation of highly antibiotic resistant *Bartonella bacilliformis* mutants

Data on antimicrobial resistance in clinical isolates of *B. bacilliformis* is currently scarce and is mainly focused on constitutive quinolone resistance. Additionally, to date, there are only 3 reports in the literature characterizing *B. bacilliformis* mutants. Moreover, no study has selected chloramphenicol-resistant mutants, or has determined the stability of the acquired resistance or the role of efflux pumps in the selection of resistance.

The aim of this paper was to obtain information about the development of *B. bacilliformis* antibiotic resistance *in vitro* for the 4 antibiotics most commonly used in clinical practice in order to answer the following questions: When does antibiotic resistance appear? What is the best approach to treat Carrión's disease? What mechanisms of resistance are the most easily selected? Is resistance stable over time or does it disappears when antibiotic pressure is removed? Do efflux pumps play a role in the acquisition of resistance in *B. bacilliformis*?

To address our objective we developed 12 (3 for each antibiotic) *B. bacilliformis* mutants highly resistant to the most common antibiotics used to treat Carrión's disease in clinical practice (azithromycin, chloramphenicol, ciprofloxacin and rifampicin). The strains were grown for 35 or 40 consecutive passages in the presence of antibiotic pressure. The stability of the resistance acquired was assessed by culture the mutants obtained in the absence of antibiotic pressure, and the possible presence of mutations in the target genes was determined by PCR and sequencing. Additionally, two efflux pumps inhibitors, PAβN and artesunate, were used to evaluate the role of efflux pumps in the development of antibiotic resistance.

In all cases except one chloramphenicol mutant, the final MICs achieved for azithromycin, chloramphenicol and rifampicin were > 256 mg/L and > 32 mg/L for ciprofloxacin. Table 4 shows the number of passages required for the mutants to achieve confluent growth (except for one mutant) in the presence of the respective antibiotic disc.

Table 4: Number of cultures needed to obtain confluent growth.

	Number of cultures			
	Strains			
Antibiotic	**57.18**	**57.19**	**57.20**	**Mean**
Azithromycin	10	12	20	14
Chloramphenicol	> 40	32	40	>37.3
Ciprofloxacin	6	11	15	10.6
Rifampicin	20	4	25	16.3

Overall, resistant mutants were most easily selected by ciprofloxacin. On the other hand, in our study, resistance to chloramphenicol was the most difficult to obtain and the resistance mechanisms selected reverted, probably due to the high biological cost of the development of chloramphenicol resistance. Only mutants selected with rifampicin consistently showed resistance stability. Antibiotic resistance was mainly related to mutations found in the target genes of each antibiotic (Table 5), being found in 11 of the 12 mutants.

Table 5: Mechanisms of resistance obtained for the mutants in study.

Ciprofloxacin		Azithromycin		Chloramphenicol	Rifampicin
gyrA	*gyrB*	*rplD*	*rplV*	*23S rRNA*	*rpoB*
Val91	Lys475	Δ62-65	83::VSEAHVGKS	A2372	Arg527
Gly95		Arg70			Tyr540
		Lys66			Phe545
		Tyr74			Tyr588

In addition, *B. bacilliformis* efflux pumps play a role in the development of the resistance. We observed the effect of PAβN and/or artesunate in the MIC of 1 azithromycin, 1 chloramphenicol and 1 rifampicin mutants.

The present study highlights the ability of *B. bacilliformis* to become resistant to the main antibiotics used to treat Carrión's disease, by both the development of antibiotic-target alterations as well as mediation by efflux pump overexpression. In general, a high biological cost seems to occur when resistance is selected because of the total or partial reversion of the antibiotic resistance levels acquired. This finding was especially of note in the case of chloramphenicol in which resistance selection was difficult and always returned to parental levels in the absence of antibiotic pressure. Although mutant studies obtained *in vitro* can not be directly translated to the community, chloramphenicol seems to be the best treatment option.

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Development and characterisation of highly antibiotic resistant *Bartonella bacilliformis* mutants

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The objective was to develop and characterise *in vitro Bartonella bacilliformis* antibiotic resistant mutants. Three *B. bacilliformis* strains were plated 35 or 40 times with azithromycin, chloramphenicol, ciprofloxacin or rifampicin discs. Resistance-stability was assessed performing 5 serial passages without antibiotic pressure. MICs were determined with/without Phe-Arg-β-Napthylamide and artesunate. Target alterations were screened in the *23S rRNA*, *rplD*, *rplV*, *gyrA*, *gyrB*, *parC*, *parE* and *rpoB* genes. Chloramphenicol and ciprofloxacin resistance were the most difficult and easiest (>37.3 and 10.6 passages) to be selected, respectively. All mutants but one selected with chloramphenicol achieved high resistance levels. All rifampicin, one azithromycin and one ciprofloxacin mutants did not totally revert when cultured without antibiotic pressure. Azithromycin resistance was related to L4 substitutions Gln-66 → Lys or Gly-70 → Arg; L4 deletion Δ_{62-65} (Lys-Met-Tyr-Lys) or L22 insertion 83::Val-Ser-Glu-Ala-His-Val-Gly-Lys-Ser; in two chloramphenicol-resistant mutants the *23S rRNA* mutation G2372A was detected. GyrA Ala-91 → Val and Asp-95 → Gly and GyrB Glu474 → Lys were detected in ciprofloxacin-resistant mutants. RpoB substitutions Gln-527 → Arg, His-540 → Tyr and Ser-545 → Phe plus Ser-588 → Tyr were detected in rifampicin-resistant mutants. In 5 mutants the effect of efflux pumps on resistance was observed. Antibiotic resistance was mainly related to target mutations and overexpression of efflux pumps, which might underlie microbiological failures during treatments.

Bartonella bacilliformis is the causative agent of Carrion's disease, a biphasic endemic illness of the Andean valleys. In the acute stage (the so-called Oroya fever) severe haemolytic anaemia is present, resulting in 40–85% of deaths in untreated people and decreases to around 10% if correctly treated[1–3]. In this stage the presence of concomitant infections such as bloodstream *Salmonella* infections, among others[1,4–6], are frequent due to the temporal immunosuppression induced by *B. bacilliformis*[7].

Peruvian wart is the chronic non life-threatening phase, characterised by cutaneous proliferative vascular lesions, occurring some weeks or months after the acute infection[3].

Additionally, the number of asymptomatic carriers is uncertain, although some studies have shown that 45% of the inhabitants of endemic areas present evidence of previous contact with the pathogen[8].

The usual treatments are chloramphenicol (CHL) or ciprofloxacin (CIP), alone or combined with cephalosporins or aminoglycosides in for the acute stage. Rifampicin (RIF) or azithromycin (AZM) are used in the chronic stage[6,9]. Despite the reported ~10% of deaths among patients receiving adequate treatment[3], it is widely considered that this microorganism has good clinical response to the above mentioned treatments.

The use of antibacterial agents in the treatment of Carrion's disease has been profuse in recent decades[10]. Moreover, in asymptomatic carriers the microorganisms are also under antibiotic pressure related to the treatment of other infections. However, descriptions of antibiotic-resistant *B. bacilliformis* clinical isolates are scarce[11,12]. To date, only constitutive nalidixic acid resistance and related diminished fluoroquinolone susceptibility have been reported in association with the presence of an Ala as WT amino acids at positions 91 and 85 of GyrA and ParC, respectively[13,14]. These characteristics are extended to other members of the *Bartonella* genus[13].

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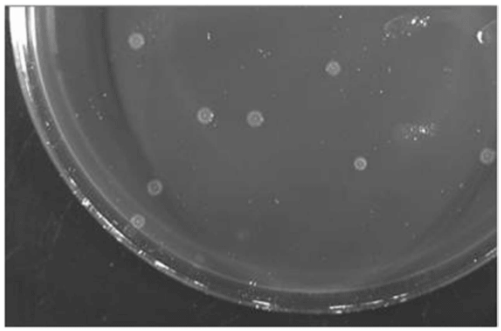

Figure 1. Colony morphology. The photograph shows *B. bacilliformis* presenting T1 colony morphology[20]. The colony is characterised by a small, translucent round morphology, with a regular edge and a small halo. The colonies present a "bubble" in the center of the colony. The morphology was unstable and disappeared after reculture.

	Number of passages			
	Strains			
Antibiotic	57.18	57.19	57.20	Mean
Azithromycin	10	12	20	14
Chloramphenicol	>40*	32	40	>37.3
Ciprofloxacin	6	11	15	10.6
Rifampicin	20	4	25	16.3

Table 1. Number of passages needed to obtain confluent growth. *At the end of 40 passages, in presence of a chloramphenicol disc, a halo of 18 mm was observed.

Additionally, relatively high Minimal Inhibitory Concentration (MIC) levels of clindamycin and colistin[12], have been observed as well as sporadic isolates presenting resistance to CHL or CIP and a trend towards diminished susceptibility to aminoglycosides[11,16]. Moreover, *in vitro* resistance to different antimicrobial agents, including coumermycin, CIP, RIF and erythromycin, has been described[17–19]. However, these studies were developed using either the KC583 or KC584 strain alone, being limited to the analysis of point mutations, and to date, no study has determined the role of efflux pump overexpression or the stability of the antibiotic resistance selected.

Analysis of *in vitro* obtained mutants may provide information in order to better understand antibiotic-resistance acquisition and evolution. The aim of this study was to develop and characterise a series of *in vitro B. bacilliformis* antibiotic resistant mutants and determine the presence of target mutations, the role of efflux pumps as well as the stability of selected resistance.

Results

Development of antibiotic-resistant mutants. The time required for bacterial lyophilised reactivation varied from 5 weeks (strains 57.19 and 57.20) to 9 weeks (strain 57.18). Interestingly, strain 57.18 showed an initially different morphology, coinciding with the previously described T1 morphology[20], although this reverted in the next passage (Fig. 1).

The development of the antibiotic-resistant mutants required approximately 18 months, therefore, 4 antibiotic-resistant mutants were obtained from each parental strain, one for each antibiotic included in the study.

The first antibiotic to generate inhibitory halo 0 mm was RIF, with only 4 passages to obtain confluent growth (strain 57.19). However, overall, the antibiotic requiring the least number of passages to generate confluent growth was CIP with a mean of 10.6 passages. On the other hand, CHL required >37.3 passages (Table 1). Thin growth was observed inside the halo recorded during the process of mutant selection of 57.20_{Azm}. Thus, after the initial 35 passages, most antibiotic-resistant mutants showed confluent growth in the presence of the antibiotic disc (inhibitory halo 0 mm) except for two out of three mutants selected with CHL, which presented inhibitory halos of 18 mm ($57.18_{Chl\text{-}35}$) and 32 mm ($57.20_{Chl\text{-}35}$). After 5 additional serial passages (total: 40 passages), $57.20_{Chl\text{-}40}$ achieved a halo of 0 mm, while $57.18_{Chl\text{-}40}$ remained with an inhibitory halo of 18 mm (Fig. 2).

Analysis of MIC levels and stability of resistance. A maximum of 1 dilution difference between the MICs at 7 and 14 days was observed. Thus, those obtained at 7 days were used throughout the manuscript.

Analysis of the MIC levels showed that all but 1 mutant achieved MICs up to the E-test detection limit >256 mg/L (AZM, RIF and CHL) and >32 mg/L (CIP). The exception was the above mentioned $57.18_{Chl\text{-}40}$ mutant that reached a CHL MIC level of 4 mg/L (Table 2).

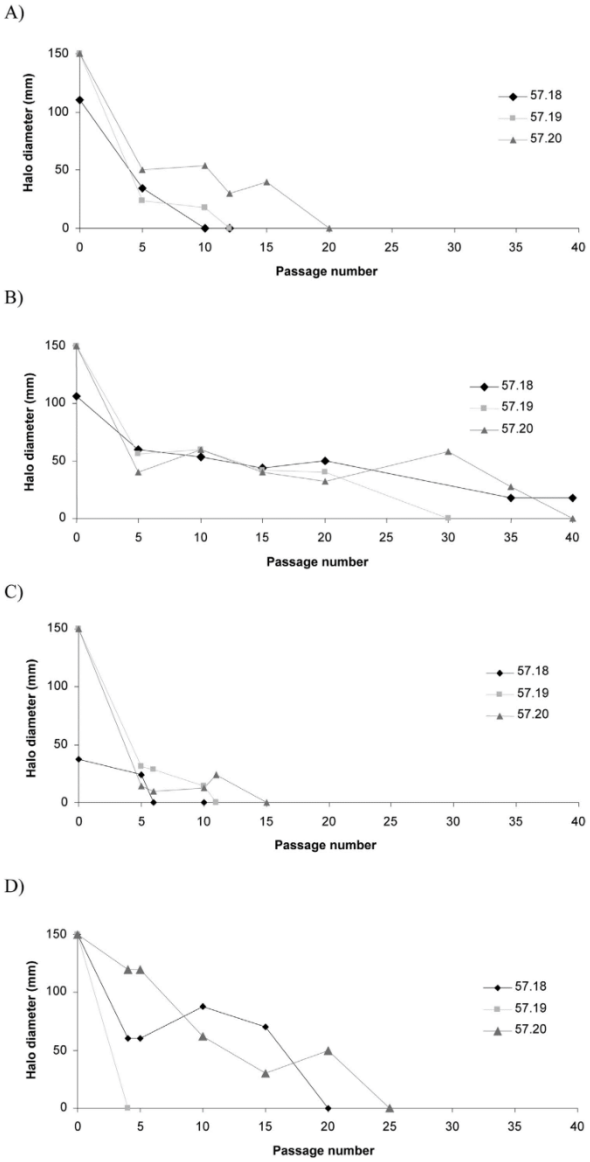

Figure 2. Evolution of disc diameter halo during serial passages. (**A**) Azithromycin, (**B**) Chloramphenicol, (**C**) Ciprofloxacin, (**D**) Rifampicin. This figure demonstrates the ease with each mutant are selected for each antibiotic. The halo diameters (measured in mm) are reported every 5 passages or at the passage in which halo zero was obtained. In (**B**) is clearly visualised the difficulty with which resistance to chloramphenicol (CHL) is developed.

Interestingly, analysis of the stability of the resistance obtained showed that all RIF selected mutants were stable, as were $57.18_{Azm\text{-}5St}$ and $57.18_{Cip\text{-}5St}$. Two strains ($57.18_{Chl\text{-}5St}$ and $57.19_{Chl\text{-}5St}$) returned to the parental MIC levels, while the remaining isolates showed intermediate MICs between parental and final mutant values. In the case of mutants $57.19_{Azm\text{-}5St}$ and $57.20_{Cip\text{-}5St}$ at 14 days the presence of colonies within the inhibitory halo were observed. Thus, $57.19_{Azm\text{-}5St\text{-}WH}$ showed a MIC of AZM of 32 mg/L while $57.20_{Cip\text{-}5St\text{-}WH}$ showed a MIC > 32 mg/L for CIP (Table 2).

Antibiotic cross resistance. When the mutants were selected with AZM the MICs to other antibiotics generally increased, while the selection with CHL usually resulted in decreases in other MICs, and in those selected with CIP and RIF the results were more variable (Tables 2 and 3).

Target mutations. None of the genes of the parental strains analysed presented differences with respect those of the KC583 strain except the amino acid codon change Thr-13 → Ala at L4 protein and Arg-9 → Cys at L22 protein.

Strain	MIC[1] (mg/L)				Mechanisms of Resistance									
					Fluoroquinolones				Macrolides		Macr + Amph[2]	Rifamycins	EPIs[3]	
	AZM	CHL	CIP	RIF	GyrA	GyrB	ParC	ParE	L4	L22	*23S rRNA*	RpoB	PAβN[4]	ART[5]
57.18	0.19	0.38	0.5	<0.016	wt	wt	Wt	wt	wt	wt	wt	wt	—	—
57.18_{Azm-35}	**>256**	**2**	0.75	<0.016	—	—	—	—	Δ_{62-65}	wt	wt	—	Y	Y
$57.18_{Azm-5St}$	>256	—	—	—	—	—	—	—	Δ_{62-65}	wt	wt	—	—	—
57.18_{Chl-40}	<0.016	**4**	0.38	<0.016	—	—	—	—	—	—	wt	—	Y	Y
$57.18_{Chl-5St}$	—	0.125	—	—	—	—	—	—	—	—	wt	—	—	—
57.18_{Cip-35}	<0.016	0125	**>32**	<0.016	Val91	wt	Wt	wt	—	—	—	—	N	N
$57.18_{Cip-5St}$	—	—	>32	—	Val91	wt	Wt	wt	—	—	—	—	—	—
57.18_{Rif-35}	<0.016	0.75	0.38	**>256**	—				—	—	—	Arg527	N	N
$57.18_{Rif-5St}$	—	—	—	>256	—				—	—	—	Arg527	—	—
57.19	<0.016	0.125	0.38	0.016	wt	wt	Wt	wt	wt	wt	wt	wt	—	—
57.19_{Azm-35}	**>256**	**0.5**	0.125	<0.016	—	—	—	—	Arg70	wt	wt	—	N	N
$57.19_{Azm-5St}$	0.5	—	—	—	—	—	—	—	Arg70	wt	wt	—	—	—
$57.19_{Azm-5St-WH}$	32	—	—	—	---	—	—	—	Arg70+Tyr74	wt	wt	—		
57.19_{Chl-35}	<0.016	**>256**	0.125	<0.016	—	—	—	—	—	—	A2372	—	N	N
$57.19_{Chl-5St}$	—	0.094	—	—	—	—	—	—	—	—	wt	—	—	—
57.19_{Cip-35}	**0.064**	**0.5**	**>32**	<0.016	wt	Lys475	Wt	wt	—	—	—	—	N	N
$57.19_{Cip-5St}$	—	—	1.5	—	wt	Lys475	Wt	wt	—	—	—	—	—	—
57.19_{Rif-35}	<0.016	**0.75**	**2**	**>256**	—	—	—	—	—	—	—	Tyr540	N	Y
$57.19_{Rif-5St}$	—	—	—	>256	—	—	—	—	—	—	—	Tyr540	—	—
57.20	0.064	0.25	0.38	<0.016	wt	wt	Wt	wt	wt	wt	wt	wt	—	—
57.20_{Azm-35}	**>256**	0.38	**1**	<0.016	—	—	—	—	Lys66	83::VSEAHVGKS	—	—	N	Y
$57.20_{Azm-5St}$	2	—	—	—	—	—	—	—	Lys66*	83::VSEAHVGKS	—	—	—	—
57.20_{Chl-40}	<0.016	**>256**	0.25	<0.016	—	—	—	—	—	—	A2372*	—	N	N
$57.20_{Chl-5St}$	—	0.5	—	—	—	—	—	—	—	—	wt	—	—	—
57.20_{Cip-35}	0.094	0.25	**>32**	<0.016	Gly95	wt	Wt	wt	—	—	—	—	N	Y
$57.20_{Cip-5St}$	—	—	0.38	—	Gly95*	wt	Wt	wt	—	—	—	—	—	—
$57.20_{Cip-5St-WH}$	—	—	>32	—	Gly95	wt	Wt	wt	—	—	—	—		
57.20_{Rif-35}	0.094	**1**	0.047	**>256**	—	—	—	—	—	—	—	Phe545+ Tyr588	N	Y
$57.20_{Rif-5St}$	—	—	—	>256	—	—	—	—	—	—	—	Phe545 + Tyr588	—	—

Table 2. MICs and mechanisms of resistance. AZM: azithromycin; CHL: Chloramphenicol; CIP: Ciprofloxacin; RIF: Rifampicin. The asterisks indicate the presence of double peaks, suggesting either the presence of a double bacterial population or the presence of mutations in one of the two copies of the *B. bacilliformis* gene. Mutants in which cross-resistance to other antibiotics were observed are in underlined font. The final MIC of the antibiotic used in the mutant selection is shown in bold. [1]Minimal Inhibitory Concentration. [2]Macrolides and Amphenicols. [3]Efflux Pumps Inhibitors. [4]Phe-Arg-β-Naphtylamide. [5]Artesunate.

AZM resistance was related to the presence of alterations in the *rplD* and *rplV* genes, encoding the L4 and L22 proteins, respectively. No mutation was observed in the *23S rRNA* gene. Thus 57.18_{Azm} and $57.18_{Azm-5st}$ showed a predicted 4 amino acid deletion (Lys-Met-Tyr-Lys) at L4 protein from codons 62 to 65 (Δ_{62-65}). Nonetheless, close analysis of the spherogram showed the presence of a double sequence from the deletion onwards under the majority peaks with in the reading-frame together with a non-deleting *rplD* (Supplementary Figure 1). Meanwhile, in mutants 57.19_{Azm-35} and $57.19_{Azm-5st}$ we observed the presence of the L4 amino acid codon change Gly-70 → Arg, with a change His-74 → Tyr also being observed in $57.19_{Azm-5st-WH}$. The mutant 57.20_{Azm} showed the presence of alterations at both the *rplD* and *rplV* genes. Thus, the presence of an amino acid codon change Gln-66 → Lys was observed in the *rplD* gene, while a 27 base-pair insertion (leading to the 9 amino acid Val-Ser-Glu-Ala-His-Val-Gly-Lys-Ser) was observed at one of the two *rplD B. bacilliformis* genes after position 83 (Supplementary Figure 1). Interestingly the $57.20_{Azm-5St}$ *rplD* gene showed a double peak which presented a mixed population with a partial reversion of the change Gln-66 → Lys.

Regarding quinolone resistance, no mutation was observed in the Topoisomerase IV encoding genes (*parC* and *parE*). A GyrA amino acid substitution was observed in 2 out of 3 selected mutants, 57.18_{Cip-35} and 57.20_{Cip-35} (Ala-91 → Val and Asp-95 → Gly, respectively), and a GyrB amino acid substitution (Glu-475 → Lys) was observed in 57.19_{Cip-35}. The mutations present in 57.18_{Cip-35} and 57.19_{Cip-35} were stable and were also observed in the mutants $57.18_{Cip-5St}$, and $57.19_{Cip-5St}$. In the case of 57.20_{Cip-35} the analysis of the sequence spherogram showed the presence of a double peak with two bacterial populations, one with the WT *gyrA* gene sequence and other possessing the aforementioned amino acid codon substitution. This was confirmed by analysing $57.20_{Cip-5St}$ (possessing a WT *gyrA* gene sequence) and $57.20_{Cip-5St-WH}$ (which maintained the amino acid change Asp-95 → Gly).

	MIC [1]/ (fold)[2]											
	Azithromycin (AZM)[3]			Chloramphenicol (CHL)[3]			Ciprofloxacin (CIP)[3]			Rifampicin (RIF)[3]		
Ab[4]	57.18_{Azm-35}	57.19_{Azm-35}	57.20_{Azm-35}	57.18_{Chl-40}	57.19_{Chl-35}	57.20_{Chl-40}	57.18_{Cip-35}	57.19_{Cip-35}	57.20_{Cip-35}	57.18_{Rif-35}	57.19_{Rif-35}	57.20_{Rif-35}
AZM	—	—	—	**≤0.016 (≤0.08)**	<0.016 (ND)	**≤0.016 (≤0.25)**	**≤0.016 (≤0.08)**	**0.064 (>4.00)**	0.094 (1.47)	**≤0.016 (≤0.08)**	<0.016 (ND)	0.094 (1.47)
CHL	**2 (5.26)**	**0.5 (4.00)**	0.38 (1.52)	—	—	—	0.125 (0.33)	**0.5 (4.00)**	0.25 (1.00)	0.75 (1.97)	**0.75 (6.00)**	**1 (4.00)**
CIP	0.75 (1.50)	**0.125 (0.33)**	**1 (2.63)**	0.38 (0.76)	**0.125 (0.33)**	0.25 (0.66)	—	—	—	0.38 (0.76)	**2 (5.26)**	**0.047 (0.12)**
RIF	<0.016 (ND)	<0.016 (<1.00)	<0.016 (ND)	<0.016 (ND)	<0.016 (<1.00)	<0.016 (ND)	<0.016 (ND)	<0.016 (<1.00)	<0.016 (ND)	—	—	—

Table 3. Cross resistance levels for the mutants obtained. The cases in which the MIC of the mutant decreased with respect to the parental isolate are highlighted in underlined font while those in which the MIC increased, (expressed in mg/L) are shown in bold. ND: not-determined. [1]Minimal Inhibitory Concentration in mg/L. [2]Fold: MIC fold increase/decrease compared to parental isolate. When the quotient ranks between 0.5 and 2 no effect was considered. Values ≥ 2 represent a co-selection of resistance, while values < 0.5 indicate that the mutant strain increased its susceptibility levels to the antimicrobial agent analysed. [3]Antibiotic used in the selection of resistant mutants. [4]Antibiotic tested.

The *in silico* determination of the hydrophobicity pattern showed an alteration in the presence of GyrA Val-91, while the presence of GyrA Asn-95 altered both the charge and slightly affected the hydrophobicity pattern (Fig. 3).

In the 57.19_{Chl-35} mutant an alteration in the *23S rRNA* gene (G2372A) was detected, which reverted after 5 passages without antibiotic pressure. The same mutation was also observed in the 57.20_{Chl-40} mutant, but a double peak was observed showing the presence of a double bacterial population, or, more probably, that the mutation was only present in one of the two *23S rRNA* genes of the *B. bacilliformis* genome (Supplementary Figure 1).

Finally, the presence of mutations in the *rpoB* gene was observed in all RIF-selected mutants which were also present after the 5 passages without antibiotic pressure, leading to the amino acid changes Gln-527 → Arg (57.18_{Rif-35}, $57.18_{Rif-5St}$), His-540 → Tyr (57.19_{Rif-35}, $57.19_{Rif-5St}$) and Ser-545 → Phe plus Ser-588 → Tyr (57.20_{Rif-35} and $57.20_{Rif-5St}$). Interestingly, when the *rpoB* gene in the 57.20 RIF-derived mutant at passage 13 (57.20_{Rif-13}), was sequenced, only the presence of the amino acid codon change Ser-588 → Tyr was observed.

Effect of Efflux Pumps Inhibitors (EPIs). The study of the effect of Phe-Arg-β-Napthylamide (PAβN) and artesunate (ART) on normal bacterial growth showed that high PAβN concentrations (7.5, 10, 20 mg/L) allowed bacterial, albeit non normal, growth. Thus, a concentration of 5 mg/L was used in the assays. No effect of ART was observed on bacterial growth with 20 mg/L. Additionally, we confirmed the lack of effect on bacterial growth with ethanol at the concentration and required volume used.

Both PAβN and ART had a visible effect on the CHL MIC of 57.18_{Chl-40}, and this effect was also confirmed by the increased disc inhibitory halo in the presence of both EPIs. Both EPIs also affected the AZM susceptibility levels of the 57.18_{Azm-35} mutant. Additionally, ART was found to affect the RIF MIC of 57.19_{Rif-35}, and also enhanced the activity of RIF and CIP in 57.20_{Cip-35} and 57.20_{Rif-35} mutants respectively (Tables 2 and 4).

Discussion

Currently antimicrobial resistance levels have only been established in a few *B. bacilliformis* clinical or collection isolates[11,12,14–16]. Information about the mechanisms of antibiotic resistance exhibited by these isolates is very scarce and strictly focused on constitutive quinolone resistance[13–15]. This lack of data, together with the high percentage of clinical cure without microbiological elimination described in more than 22% of CIP and up to 50% of CHL treatments as well as the high-lethality related to the acute illness phase in the absence or delay of treatment[2,3,21], highlight the need for *in vitro* information about the ease with which this microorganism develops resistance and which antibiotic resistance mechanisms are selected. To the best of our knowledge only three *in vitro* studies has been developed to date[17–19], and none has determined the stability of the antibiotic resistance selected or the role of efflux pump overexpression.

Although the 57.19 strain only needed 4 consecutive passages to develop full resistance to RIF, overall, the antibiotic which most easily selected resistant mutants was CIP. This is in accordance with the possession of Ala-91 and Ala-85 of GyrA and ParC respectively which affect the hydrophobicity pattern of these proteins, impairing the interaction of quinolones and acting as a factor favouring the selection of resistance to fluoroquinolones[14,22]. In fact, it has been shown that the frequency of mutation of *B. bacilliformis* KC583 was 10-fold higher than that of *Escherichia coli*, while only 5 passages were needed to select high CIP-resistant mutants from strain KC584[18,19]. Regarding RIF and AZM, full resistance was obtained after 3 and 4 passages respectively[18]. There are no previous reports on the selection of *Bartonella* spp. CHL-resistant mutants. In our study, resistance to CHL was consistently the most difficult to obtain. Although this finding is in apparent disagreement with the observed 50% of persistent bacteraemia after CHL treatments[21], it might be related to changes at the fitness level as has been observed in the presence of specific *23S rRNA* gene point mutations in other microorganisms[23–25].

The MIC of the mutants selected was higher than that of the parental isolates, achieving high MICs levels with all the antibacterial agents tested. This finding shows a worrisome scenario: the feasibility of selecting high antibiotic resistant mutants with the 4 main antibiotic families used in the treatment of Carrion's disease. However, only those mutants selected with RIF showed consistent resistance stability, while only 1 out of 3 of CIP or

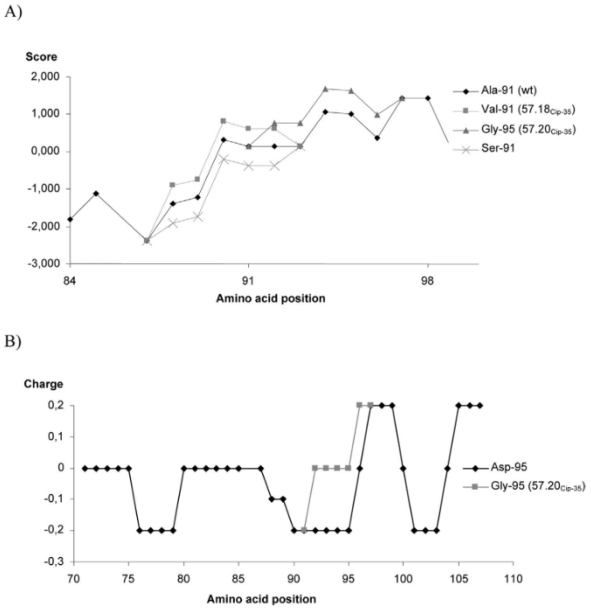

Figure 3. Hydrophobic and charge patterns associated with GyrA amino acid substitutions. (**A**) Alterations in the hydrophobic pattern. Additionally to the amino acid substitutions detected (Ala-91 → Val and Asp-95 → Gly) the theoretical effect of the presence of Ser-91 also shows the gradual effect on the hydrophobic pattern related to the presence of Ser, Ala or Val at position 91. (**B**) Effect on the charge pattern. This graph only shows the effect of Asp-95 and Gly-95 since the presence of Val-91 does not result in charge pattern alterations. The (**A**) comprises the amino acid sequence from amino acids 84 to 99, while in (**B**) the amino acid sequences analysed are from amino acids 70 to 107.

		Mutant[1]		Susceptibility level in presence of EPIs			
				PAβN		ART	
Mutant	Antibiotic	disc[2]	MIC[3]	disc	MIC	disc	MIC
57.18_{Azm-35}	AZM	0	>256	**24**	>256	**20**	**48**
57.18_{Chl-40}	CHL	18	4	**40**	**1**	**48**	**1.5**
57.18_{Cip-35}	CIP	0	>32	0	>32	0	>32
57.18_{Rif-35}	RIF	0	>256	0	>256	0	>256
57.19_{Azm-35}	AZM	0	>256	0	>256	0	>256
57.19_{Chl-35}	CHL	0	>256	0	>256	0	>256
57.19_{Cip-35}	CIP	0	>32	0	>32	0	>32
57.19_{Rif-35}	RIF	0	>256	0	>256	0	**96**
57.20_{Azm-35}	AZM	0	>256	0	>256	**20**	>256
57.20_{Chl-40}	CHL	0	>256	0	>256	0	>256
57.20_{Cip-35}	CIP	0	>32	0	>32	**20**	>32
57.20_{Rif-35}	RIF	0	>256	0	>256	**26**	>256

Table 4. Effect of efflux pump inhibitors (EPIs) on the antibiotic susceptibility levels. ART: Artesunate; AZM: azithromycin; CHL: Chloramphenicol; CIP: Ciprofloxacin; RIF: Rifampicin. The samples in which the effect of EPIs was visible highlighted in bold. [1]Antibiotic susceptibility of mutant isolates at last selection passage. [2]Disc halo diameter measured in mm. [3]Minimal inhibitory concentration by E.-test in mg/L.

AZM-resistant mutants did not revert either totally or partially. Regarding CHL, all the resistant mutants selected reverted, supporting the idea of a high biological cost of the development of CHL resistance. In the same line, the presence of double sequences when analysing some of antibiotic target gene sequences may be interpreted in three different ways: The survival of original susceptible parental strains, the partial reversion of the mutants obtained when antibiotic pressure disappears or the presence of mutations in only one of the gene copies when there is more than one. The first possibility is unlikely because of the high susceptibility levels of the parental strains to all the antibacterial agents tested (halo diameter >100 mm) which prevent the survival of the microorganisms when the disc was placed on the center of the plate (the maximum possible halo diameter is 50 mm). Meanwhile

the other two possibilities may be considered, and, moreover, are not mutually exclusive. They might underlie the high number of microbiological failures, isolation of viable microorganisms from blood, after successful clinical treatments[21]. Moreover, the reversion observed in the 57.20_{Azm-35} mutant may be related to the slower growth described in association with L4 and L22 alterations[26]. Along this line, the reversion of resistance, the presence of double bacteria populations in at least two mutants (one selected with CIP and other with AZM) as well as the additional time needed by some of the resistant strains ($57.19_{Azm-5St-WH}$, $57.20_{Cip-5st-WH}$) to grow, support the idea that the development of antibiotic resistance has a high biological cost in *B. bacilliformis*, resulting in the dilution of strains carrying specific antibiotic-related mutations within a general antibiotic susceptible bacterial population. Similarly, both the loss of biological efficiency and the decrement of virulence after the development of antimicrobial resistance have been observed in other microorganisms[27,28]. However, compensatory mutations might result in the development and spread of resistant isolates[27,29].

Although non-uniform, cross effects, either increases or decreases, in the MIC levels were observed. These alterations might be explained by collateral antibiotic resistance effects that could interfere with normal bacteria biochemical processes. Three of antibacterial agents tested act at a ribosome level, while the remaining agent acts at the proteins involved in DNA decatenation and duplication. Ribosomal level alterations may differentially affect the expression of genes or may have a direct effect on the activity of other agents. In other microorganisms, specific points placed in *23S rRNA* and in the ribosomal proteins may result in concomitant resistance between CHL and macrolides[30,31]. Among these, it has been suggested that L4 deletions Δ_{65-66} (Trp-Arg) and Δ_{68-69} (Lys-Gly) leading to macrolide-resistance in *Streptococcus pneumoniae* also confres low levels of CHL resistance. Thus, *S. pneumoniae* L4 Δ_{65-66} has been associated with an increase of the CHL MIC of up to 4-fold when cloned in a pan-susceptible *S. pneumoniae* strain[31]. Amino acid codons 65 and 66 of *S. pneumoniae* are equivalent to positions 64 and 65 of *B. bacilliformis*, both included within the Δ_{62-65} of 57.18_{Azm-35} mutant. Thus, the role of L4 deletions in the acquisition of the low levels of CHL resistance observed in this mutant (MIC 2 mg/L, 5.26-fold increases) is plausible, either directly due to alterations in the ribosome conformation affecting CHL binding or indirectly due to the possible induction of efflux pump overexpression as discussed below. This fact highlights the risk of co-selection of antibiotic resistance during *B. bacilliformis* treatment.

On the other hand, selection of CHL seems to negatively affect the ability of bacteria to survive in the presence of other antibiotic agents. This fact may also be related to the above mentioned possible effect on fitness, being probably underlain by alterations in the expression or regulation of different bacterial genetic factors. Additionally, selection of hypersusceptibility to unrelated antibiotics, such as macrolides, has also been observed in relation to specific *23S rRNA* mutations[32].

Two differences leading to the substitutions Thr-13 → Ala at L4 and Arg-9 → Cys at L22, were observed between the parental and KC583 strains. Nonetheless, both substitutions were present in other *B. bacilliformis* genomes. This finding, together with the high antibiotic susceptibility of the parental isolates, suggests the presence of DNA polymorphisms.

Regarding AZM resistance, in all the cases the presence of alterations at L4 and L22 was found, while no mutation at *23S rRNA* was observed. This latter finding differs from that described in microorganisms with a low number of *23S rRNA* gene copies[33] and observed in previous studies developed in *B. bacilliformis* and *Bartonella henselae*[18,34,35].

Amino acid changes at L4 and L22, including insertions and deletions, have been described in a series of unrelated microorganisms, such as *E. coli*[26,36,37]. Regarding *Bartonellaceae*, the mutations at L4 (Gly-70 → Arg and Gly-70 → Arg + His-74 → Tyr) have been previously described in *in vitro* obtained macrolide-resistant mutants of *B. henselae*[35]. In *E. coli*, a series of amino acid changes involving the L4 residues Gln-62 and Gly-66 equivalent to *B. bacilliformis* Gln-66 and Gly-70 have been reported as being involved in the development of AZM resistance[26,36]. Interestingly, in *E. coli* substitutions at position Ser-70 (equivalent to *B. bacilliformis* His-74) have been described[36], but never alone, as reported by Biswas *et al.*[34], suggesting a minor role of this substitution in the development of macrolide resistance.

This is the first report describing insertions and deletions in the L4 and L22 proteins of *B. bacilliformis*. The predicted L4 deletion is located close to the L4 "hot spot" region involved in macrolide resistance in which a deletion of the 2 equivalent later amino acids in macrolide-resistant *S. pneumoniae* has been reported[31], while to the best of our knowledge no insertion has been described in equivalent positions in other microorganisms.

Two out of three CHL-resistant mutants (57.19_{Chl-35} and 57.20_{Chl-40}) presented a G2372A mutation in the *23S rRNA* gene. The equivalent mutation has previously been involved in CHL resistance in yeasts and *E. coli*[25,38]. Although in the case of the 57.20_{Chl-40} mutant double peaks were obtained suggesting either the presence of a double bacterial population, or, more probably, the presence of a mutation affecting only one of the two *23S rRNA* genes. This fact, together with the rapid reversion of this mutation and the slow bacterial growth, which might be longer than 2 months in clinical samples cultures[10,39], might explain the presence of microbiological failures after CHL treatments, contributing to the lack of reports of CHL resistance in clinical isolates. Supporting the biological cost of the development of CHL resistance, the equivalent G2447A mutation in *E. coli* results in both resistance to CHL and in retarded growth rates[25].

The amino acid alterations observed in two out of three GyrA were located in the classical quinolone-DNA Gyrase interaction points, while resistance in the third isolate was related to the presence of mutations in the *gyrB* gene. Up to now, no amino acid change at GyrA position 91 has been described in *B. bacilliformis*. However, alterations at equivalent GyrA position have been extensively described in other quinolone-resistant microorganisms[22], including the presence of Val-91, detected both in *B. henselae* and unrelated microorganisms[22,34,40]. The presence of an Ala in position 83 of the GyrA of *E. coli* (equivalent to 91 of *B. bacilliformis)* results in a decreased CIP MIC, and intermediate or low-level of nalidixic resistance[40–42], while the presence of Val results in full fluoroquinolone-resistance as well as in high levels of resistance of nalidixic acid[40]. These findings may be directly related to the different effect on the GyrA hydrophobicity pattern and the subsequent involvement of the

ability of quinolones to bind with their targets in relation to the presence of Ser (WT amino acid in *E. coli* and most other microorganisms) Ala or Val residues, as shown in Fig. 3.

Changes at position Asp-95, including Asp-95 → Gly, have been previously described in studies analysing quinolone-resistant mutants of *B. bacilliformis*, *B. henselae* and *Bartonella quintana*[18,19,43]. Changes at equivalent position have frequently been reported in other microorganisms[22,40]. The effect of different mutations is additive[22], thus, in the absence of the effect on bacterial fitness, it would be more successful to add a change in a new position than to vary the amino acid at position 91. The presence of Gly at position 95 slightly affects the hydrophobicity pattern, but mainly affects the pattern of charges at this area, avoiding the interaction with radical 7 of the quinolones[44].

This is the first description of a GyrB amino acid substitution Glu-475 → Lys in *B. bacilliformis*. The same amino acid change at the equivalent position of *S. pneumoniae* (Glu-474) has been associated with quinolone resistance[45]. Moreover, in *S. pneumoniae* a series of amino acid changes including Glu-476 → Ala and Arg-477 → His has also been described in the same region, showing the relevance of the GyrB region in the development of quinolone-resistance[46,47]. The $57.19_{Cip\text{-}5St}$ mutant showed a MIC of 1.5 mg/L (3.95-fold higher than its parental isolate). Thus, by itself the Glu-475 → Lys alteration results in a slight increase in the CIP MIC. Moreover, this finding, together with the lack of PAβN and ART effect on the CIP MIC, highlight the presence of unstable mechanism/s of resistance in the $57.19_{Cip\text{-}35}$ mutant.

Rifamycin-resistant *rpoB* gene mutations are usually located within 3 highly conserved regions in the mid portion of the gene. In *E. coli* these regions include codons 505–537 (cluster I), 563–575 (cluster II) and 684–690 (cluster III)[48,49]. In the present study, the amino acid codon changes Gln-527 → Arg; His-540 → Tyr; Ser-545 → Phe are located within the *B. bacilliformis* equivalent cluster I, while Ser-588 → Tyr is located within the equivalent cluster II. Of these, the amino acid change Ser-545-Phe has been previously observed in *B. bacilliformis* and *B. quintana in vitro* RIF-resistant mutants[18]. However, substitutions at equivalent amino acid positions have been observed as being involved in the development of rifamycin resistance in other microorganisms including *Mycobacterium tuberculosis*[50]. Thus, in *E. coli* RpoB substitutions of Gln-513, His-526, Ser-531 or Ser-574 (equivalents to Gln-527, His-540, Ser-545 and Ser-588 of *B. bacilliformis* respectively) have been described; moreover, in some cases the same amino acid change (e.g. Ser-531 → Phe or His-526 → Tyr) has also been reported[43,51,52]. In one case ($57.20_{Rif\text{-}35}$) a double mutation (Ser-545 → Phe plus Ser-588 → Tyr) was observed. However, when the presence of an RpoB alteration was sought in 57.20_{Rif} at passage 13, only the presence of the amino acid change Ser-588 → Tyr was observed. This fact shows the sequential selection of RIF resistance related to continuous antibiotic pressure. In *E. coli* the substitution at Ser-574 (equivalent to Ser-588) results in low-levels of RIF resistance, being its effect strongly diminished in the presence of PAβN[51]. Thus, the selection of a mutation conferring low RIF resistance levels is followed by that of another additive mechanism of resistance, leading to an increase in the RIF resistance levels. Similarly, although substitutions at the equivalent Ser-545 position seem to result in high RIF MIC levels in *E. coli*[49], in other microorganisms such as *Rhodococcus equi* only low levels of RIF resistance are conferred[53].

This is the first determination of the role of efflux pumps in the development of antibiotic resistance in *B. bacilliformis*. The two EPI tested act differently, while PAβN competes with other efflux pump substrates to be extruded from bacteria, ART diminishes the expression levels of some efflux pump encoding genes[54,55]. These differences may underlie the disparity of results obtained in the present study with these inhibitors.

Both PAβN and ART affected the susceptibility levels of $57.18_{Azm\text{-}35}$ and $57.18_{Chl\text{-}40}$ mutants. In the first case the mutant presented L4 Δ_{62-65}. Although related to deletions in L22, a similar scenario has been observed in *E. coli* strains carrying 3 amino acid deletions in L22 in which the inactivation of different efflux pump components results in a strong decrease in the erythromycin MIC levels[56]. Thus, it has been proposed that deletions at L22 alter the translation of specific proteins, possibly via changes in programmed stalling, modifying the cell envelope and resulting in efflux pump overexpression and lowering steady-state macrolide levels[56]. It is of interest to note that in $57.18_{Azm\text{-}35}$, the use of PAβN was only visible in the disc diffusion analysis. The most probable scenario for this is that the MIC of this strain is much higher than 256 mg/L and thus, the effect on the MIC levels, albeit present, was not observed. Regarding the $57.18_{Chl\text{-}40}$ mutant both the effect of PAβN and ART decreased the CHL MIC levels to only slightly higher than the parental isolate, showing that efflux pumps may play a role in the development of low levels of CHL resistance in *B. bacilliformis*. No effect was observed with any inhibitor tested in either $57.19_{Chl\text{-}35}$ or $57.20_{Chl\text{-}40}$. This finding, together with the effect of ART on the antimicrobial susceptibility levels of all remaining 57.20 derivative mutants, highlight two facts: (a) the high levels of resistance to CHL derived from the presence of *23S rRNA* mutations which when present mask the effect of efflux pumps, and (b) the presence of efflux pumps able to extrude the 4 antimicrobial families tested in *B. bacilliformis*. Thus, the different level of antimicrobial resistance of each selected mechanism may allow or not visualization of the overexpression of efflux pumps and might underlie the differences in the effect of ART on the RIF-selected mutants. ART also affects the susceptibility to RIF in $57.19_{Rif\text{-}35}$ and $57.20_{Rif\text{-}35}$ mutants, showing that efflux pumps may also affect this antibiotic in *B. bacilliformis* and reinforcing the possibility that the selection of a double RpoB substitution in the $57.20_{Rif\text{-}35}$ mutant was due to the low level of RIF resistance conferred. In the case of the $57.19_{Rif\text{-}35}$ mutant the effect was only observed on the MIC levels, while in the case of $57.20_{Rif\text{-}35}$ the effect was observed in the disc diameter halo. Further studies are needed to determine the mechanisms of efflux pump overexpression (alterations in signal patterns, punctual mutations in promoter regions, among others).

In summary, the present data highlight the ability of *B. bacilliformis* to become antibiotic resistant both by the development of antibiotic-target alterations, but also mediated by efflux pump overexpression. However, regarding AZM, CHL and CIP, the instability of resistance detected in different mutants, either related to reversions and/or to dilution within a higher fitness susceptible population, suggests a high biological cost, which may underlie the rarity of antibiotic-resistant *B. bacilliformis* clinical isolates. Further studies analysing antibiotic target sequences

Strain			E.D.			Clonal		
CIP	NCTC[1]	Original[1]	Year[1]	Place	Source[1]	AFLP[2]	ISR[2]	Ref.
57.18	12134	267	1949	Lima	Blood	B	2	57
57.19	12135	529	1941	NK	NK	B	2	57
57.20	12136	CPX	1957	NK	Blood	B	2	58

Table 5. Parental isolates characteristics. E.D.: Epidemiological data; Original: Name of the original isolate. AFLP: Amplified Fragment Length Polymorphism; ISR: Intergenic Spacer Regions; Ref: Older reference including these strains that we found. NK: Not known. [1]Information present in http://www.phe-culturecollections.org.uk/. [2]In Birtles *et al.*[58].

from a relevant number of *B. bacilliformis* clinical isolates prior, during and after antibiotic treatment are necessary to better understand the natural history of antibiotic resistance in this microorganism.

Methods

Microorganisms. Three *B. bacilliformis* strains from the Institute Pasteur (Paris, France) collection were used (Table 5). The strains were reactivated following the instructions of the Institute Pasteur. Briefly, after opening, 0.2 ml of LB was added to the vial to resuspend the bacteria. Thereafter, the suspension was spread onto Columbia blood agar (Ref: 254005, BD, Heidelberg, Germany) and incubated at 28 °C. The plates were inspected weekly until bacterial growth was observed. Prior to the development of the antibiotic resistant mutants, the bacterial identity of all three isolates was confirmed by amplification and sequencing of the *16S rRNA* gene (Table 6)[59].

In vitro development of resistant mutants. The antibiotic resistant mutants were selected by plating the *B. bacilliformis* on blood agar with a disc of AZM (15 μg), CIP (5 μg), CHL (30 μg) or RIF (30 μg) (BD, Franklin Lakes, USA) which was initially put in the corner of each plate, and subsequently in the center of the plate according to halo diameter[18]. The confluent growth outside the zone of inhibition was recovered with a pre-sterilized plastic inoculation loop and subcultured 35 consecutive times. In those cases in which no halo zero was obtained, the isolates were grown in the presence of antibiotic for 5 additional passages.

At the beginning of the study, the passages with the selected antibiotic discs were performed every other week because of the difficulty to visualise the bacterial growth in the first weeks due the large halo sizes. Then, we changed to a weekly basis, according to the evolution of each strain.

Throughout the text, the mutants obtained are referred to by indicating the name of the parental isolate, the antibiotic used in its selection and the passage number (e.g.: $57.18_{Azm\text{-}35}$ is the AZM resistant mutant derived from strain 57.18 at passage 35).

Stability of resistance. Resistance stability was assessed by doing 5 final additional passages on Columbia blood agar, on a weekly basis, in the absence of antibiotic pressure. These are referred to in the text following the above mentioned nomenclature but including "5st" (5 serial passages to determine stability instead of passage number). When a mixed bacterial population was obtained (e.g.: growth of isolated colonies inside the inhibitory halo), isolates from inside of halo were additionally marked as "WH" (within halo).

Antibiotic susceptibility levels. The MICs of AZM, CIP, CHL and RIF of the parental isolates, the mutants at the final passage and after the 5 additional passages without antibiotic were determined by E-test (Liofilchem, IZASA, Barcelona, Spain). The MICs were read at 7 and 14 days. To determine the effect on the MIC levels the quotient MIC_F (final MIC)/MIC_I (initial MIC) was used. In all cases MIC differences >2-fold were considered as significant.

Antibiotic cross resistance. In the final passage in the presence of antibiotic discs, antibiotic cross resistance was determined by testing the MIC of each mutant with all the other antibiotics under study.

DNA extraction. *B. bacilliformis* were collected by adding sterile PBS into the plates and suspending the colonies with a one-use loop. This bacterial suspension was transferred to a sterile vial and the DNA was extruded by boiling at 100 °C for 10 minutes and stored at −20 °C until use.

Target mutation detection. The presence of mutations in the *rplV* and *rplD* genes (AZM) as well as in the *23S rRNA* (AZM and CHL), quinolone resistance-determining regions (QRDR) of the *gyrA*, *gyrB*, *parC* and *parE* genes (CIP) and the *rpoB* gene (RIF) were determined by PCR using the primers and conditions listed in Table 6. The PCR products were purified using a commercial kit according to the manufacturer's instructions (Gel Extraction Kit from Omega Bio-tek, Georgia, USA) and thereafter sequenced (Beckman Coulter, Takeley, United Kingdom). The sequences obtained were compared with those of their respective parental strains and with that of the type strain KC583 (http://www.bacterio.net/bartonella.html). In cases in which the presence of non-silent mutations was observed, the deduced amino acid sequence was compared against *B. bacilliformis* amino acid sequences present in GenBank. Regarding GyrA, hydrophobicity was determined using the method of Kyte and Doolittle as defined in the ProtScale software[60,61], while the polarity pattern was determined using the charge software (http://www.bioinformatics.nl/cgi-bin/emboss/charge).

Throughout the text, both the DNA and amino acid numeration is referred to as that of *B. bacilliformis* KC583. When DNA or amino acid positions of other microorganisms are used for comparison purposes this is explicitly indicated.

Primer	Target	Sequence (5′-3′)	Ann (°C)	Size (bp)	Ref.
Resistance to quinolones					
gyrA-F	*gyrA*	CAT GCG ATG AAT GAA ATG GGA CTT TTG	55	233	19
gyrA-R		AAA CGA CAT TCC GTG TAA CGC ATC GC			
gyrB-F	*gyrB*	CTG AAG TCC GTC CAA TTG TT*	48	634	TS
gyrB-R		TCT TCA AAT GCT GCT TCA TT*			
parE-F	*parE*	CAA TAC GTG ATC CTT TCG AT*	47	564	TS
parE-R		TTC CTC CTT GTG ATA TTC TG*			
parC-F	*parC*	TCT TAT GCT AAG TGT GCA CGG A	55	349	14
parC-R		TAC CAA CAG CAA TCC CTG AAG AA			
Resistance to macrolides					
rplD_F	*rplD*	AGA AGT CTC TGT AGC TGA GGG	49	688	TS
rplD_R		ACT GGA CTG ACA ATT ACA TCA			
rplV_F	*rplV*	CTG GAC TGA CAA TTA CAT CAT	49	702	TS
rplV_R		GGC GAC TCC AAT AGC AGA AG			
Resistance to macrolides and amphenicols					
23S_rRNA_F	*23S rRNA*	AGT GAA ATT GAA TTC CCC	46	780	TS
23S_rRNA_R		GGA ATA CTC GTT TTC AGG T			
Resistance to rifamycins					
rpoB-F	*rpoB*	GAT GAT ATC GAC AAT CTT GGT A**	49	818	TS
rpoB-R		GCA GCA CCT GAA TCA CGA GCC			
Bacterial identification					
16SBartonella-F	*16S rRNA*	CCT TCA GTT MGG CTG GAT C	55	438	59
16SBartonella-R		GCC YCC TTG CGG TTA GCA CA			

Table 6. Primers used in the study. TS. This Study. *These primers are modifications of those described by Angelakis *et al.*[43]. **This primer is a modification of the BarpoBF primer designed by Biswas *et al.*[18].

Role of Efflux Pumps. The role of efflux pumps in the development of antibiotic resistance was established by determining the antibiotic susceptibility levels by disc diffusion and E-test in commercial blood agar media supplemented *in house* with PAβN, or ART. In order to determine the EPI concentrations to be used, the effect of different concentrations of these products (from 2.5 mg/L to 20 mg/L) on normal bacterial growth was tested. Additionally, the effect of 100% ethanol (ART solvent) was also independently assessed.

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Author Contributions
J.R., C.G. and J.d.V.M. design the experiment; C.G., S.M.-P., L.R.-R. and J.R. developed the mutant strains, and perform the antibiotic susceptibility studies; C.G. and M.J.P. perform the molecular studies. C.G. and J.R. analyzed the data; J.R. and C.G. wrote the manuscript draft. All authors read the manuscript critically, provide suggestions and approved the final version.

Additional Information
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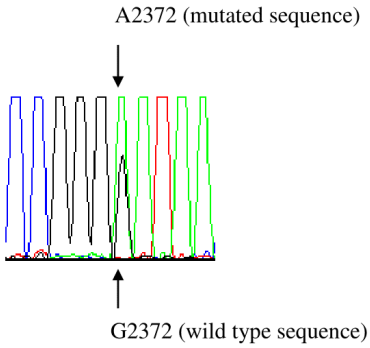
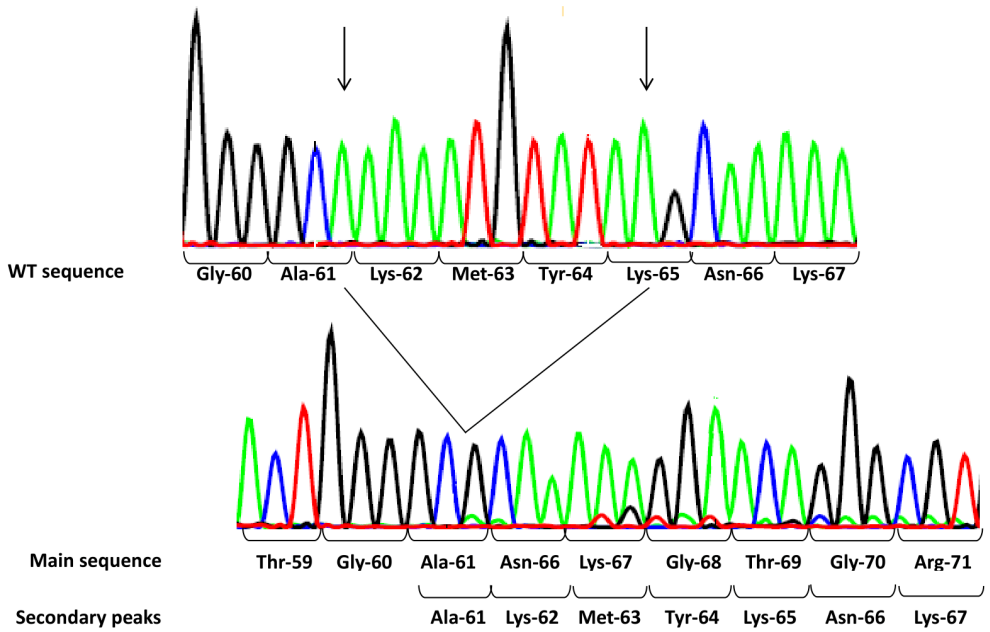
Supplementary Figure 1:

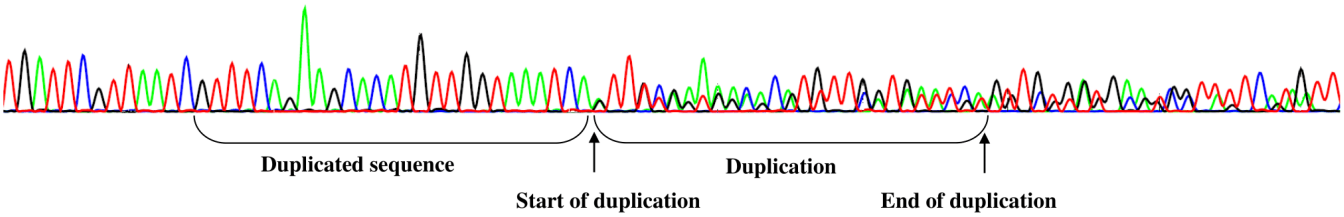
A) Example of double peak showing the presence of a WT and mutated 23S rRNA sequences (strain 57.20$_{Chl-40}$).



B) Delection of 4 amino acids between position 62-65 of L4 (strain 57.18$_{Azm-35}$). In the upper side the DNA sequences belonging to the 57.18 parental isolate, with arrows marking first and the last delected base in the mutant. In the lower side the sequence of the 57.18$_{Azm-35}$. In both cases the derived protein sequence is annotated. In addition, the secondary sequence observed under the main sequence is also marked.



C) Duplication of 9 amino acids at position 83 of L22 (strain 57.20$_{Azm-35}$).



CHAPTER II – DIAGNOSIS

ARTICLE 2

An unidentified cluster of infection in the Peruvian Amazon region

In March 2013 an outbreak of Oroya fever was reported in a non-endemic rural area of northeast Peru. The diagnosis was made on the basis of clinical suspicion as well as microscopy techniques. In this article the objective was to characterize the outbreak using molecular techniques. Fifty-three blood samples recovered from people diagnosed with Oroya fever were included in the study. Revision of the clinical data showed that the most common symptoms were headache and general discomfort (50.9%) followed by chills (32.1%) and fever (24.5%). To address our objective we performed PCR of both universal *16S rRNA* and *Bartonella*-specific *16S RNA*. Additionally, all blood samples were cultured according to *B. bacilliformis* conditions but no growth of *B. bacilliformis* was observed. PCR did not obtain any amplified products for the *16S rRNA* specific for *Bartonella* spp. However, with universal *16S rRNA* primers, all the samples were amplified, and the presence of *Sphingomonas faeni* was identified by sequencing.

Nonetheless the question as to the origin of the infection remained. Knowing that *S. faeni* is an environmental microorganism and that 17% of patients reported aquatic activities, we also analyzed domestic water samples and water-well samples. The results showed the presence of aquatic microorganisms belonging to the *Sphingomonas* genus.

We proposed that this outbreak was mistakenly attributed to *B. bacilliformis* and the results strongly suggested *S. faeni* as the causative agent. To my knowledge, this is the first report of a community-acquired outbreak caused by *Sphingomonas* spp.

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Brief Original Article

An unidentified cluster of infection in the Peruvian Amazon region

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Abstract

Introduction: *Bartonella bacilliformis* is the etiological agent of Carrion's disease, which is a neglected disease linked to people in low-socioeconomic populations in Andean valleys. An outbreak of *B. bacilliformis* was reported in a rural area of the Peruvian Amazon region. The aim of this study was to characterize this outbreak using molecular techniques.

Methodology: Fifty-three blood samples from patients diagnosed with Carrion's disease were analyzed by molecular tools, using both a *Bartonella*-specific polymerase chain reaction (PCR) and an universal PCR, both based on 16S rRNA gene amplification. Additional water samples from the area were also analyzed.

Results: Unexpectedly, the samples were positive only when the universal PCR was used. Although environmental contamination cannot be ruled out, the results showed that *Sphingomonas faeni* was the possible causative agent of this outbreak, and that water was the most feasible infection source.

Conclusions: Diagnosis by clinical criteria or microscopy may lead to misdiagnosis. There is a need to include molecular tools in the routine diagnosis of febrile syndromes, including Carrion's disease.

Key words: *Bartonella* spp.; *Sphingomonas*; diagnosis; outbreak; Peru.

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Introduction

In northern Peru, the presence of different etiologic causes of febrile syndromes, including dengue, oropuche, mayaro, *Plasmodium* spp., or *Bartonella bacilliformis*, is well established [1-4]. Their correct diagnosis is necessary to enable administration of correct treatment. Nonetheless, in rural areas of low- and middle-income countries, correct diagnosis is a challenge, due to the presence of a series of common vague and undefined symptoms, especially in the early disease phases, and to the local diagnostic facilities.

Bartonella bacilliformis is the etiological agent of Carrion's disease, which is a neglected disease linked to people in low-socioeconomic populations in Andean valleys. This bacterium is transmitted by the bite of a sandfly, a member of the genus *Lutzomyia*. No reservoir other than humans has been identified [2]. This illness has two well-differentiated phases. The first is the acute so-called Oroya fever phase, characterized by fever and severe anemia (related to the fact that *B. bacilliformis* invade the red blood cells) in addition to a transitional immunosuppression [2]. Additionally, several symptoms and signs, including headache and jaundice, have also been described [1,5]. Regardless of its being considered the most lethal bacterial infection in the pre-antibiotic era, this illness may be successfully treated with a series of antimicrobial agents, including quinolones and macrolides, among others. However, it should be noted that the clinical cure has no direct correlation with microbiological eradication; asymptomatic carriers are frequent in endemic areas. The second phase of the disease, affecting semi-immune people, is characterized by the presence of endothelial proliferation resulting in warts (the so-called Peruvian warts) [2].

Since this illness mainly affects people in rural areas, one of the main problems is the lack of adequate technical and human resources for definitive diagnosis. Although some molecular diagnostic tools have been proposed [1,2,5], the diagnosis of Carrion's disease in rural endemic areas is mostly restricted to clinical criteria or optical microscopy and it is frequently misdiagnosed by both [1,2].

In this context, the presence of an active outbreak of Oroya fever was reported on March 2013 in Rodríguez de Mendoza, a rural area in the northeast of Peru [6]. Diagnosis was primarily based on the sudden presence of ill people, mostly with fever and chills, and was confirmed by positive microscopy results. This outbreak was of special concern because Carrion's disease had never been reported in this area, but had been reported in some endemic areas are relatively near the affected population; it has been reported that current climatic alterations underlie an expansion of this illness to new areas [2,7].

The aim of this study was to characterize this outbreak using molecular techniques.

Methodology

Patients and sampling

Fifty-three blood samples recovered in Rodríguez de Mendoza (Amazonas, Peru) were sent to the Instituto de Investigación Nutricional/Universidad Peruana de Ciencias Aplicadas to be included in the study. In all cases, patients were previously diagnosed with Carrion's disease following clinical or microscopy criteria (Figure 1). Clinical and demographic data were recorded in a questionnaire.

Additionally, domestic water samples and water-well samples were also recovered and processed.

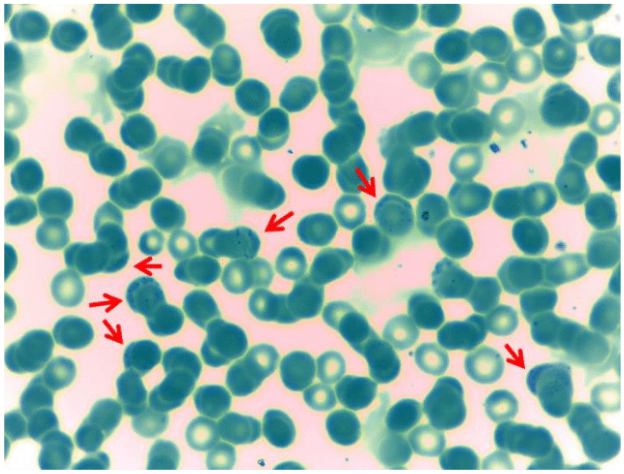
Bacterial culture conditions

Microorganisms were cultured as previously described [1]. Briefly, 2 mL of the blood samples were grown in Columbia agar medium supplemented with 10% sheep blood and incubated at 28°C under anaerobic conditions. The plates were visually inspected at 24, 48, and 72 hours to detect contamination, and then every seven days for bacterial growth.

DNA extraction

The DNA was extracted from 200 µL of blood/water samples using a commercial extraction kit (High Pure Kit Preparation template, Roche Applied Science, Mannheim, Germany). Bacterial DNA obtained after extraction was diluted in 100 µL of nuclease-free water and then processed or stored at -20°C until use.

Figure 1. Giemsa-stained red blood cells. Arrows mark red blood cells with internal bodies, which leads to misidentification.



PCR procedures

Two different 16S rRNA PCR approaches were used: one using specific primers for *Bartonella* genus, and the other using universal primers. In both cases, primers and conditions have been previously described [1]. All the products obtained were recovered and sequenced (Macrogen, Seoul, Korea).

Results

A demographic analysis showed that 52.8% of the subjects included in the study were men and ranged in age from 4 months to 85 years; the subjects were mostly adults (60.4%), and 22 (41.5%) reported engaging in agrarian activities. Additionally, 11 patients (20.7%) were not residents of the area, having reported a visit of less than one month in length (median, 2.4 days; range, 1-7 days) to the area. Nine (17%) patients, mostly outsiders (8/9) with a median stay in the area of 1.6 days (range, 1 to 3), reported engaging in aquatic activities, including in the river and thermal baths (Table 1).

The review of the clinical data showed that the most frequent symptoms were headache and general discomfort (50.9%), followed by chills (32.1%) and fever (24.5%) (Table 2). Regarding treatment, 28 (52.8%) were treated with ciprofloxacin, while 12 did not receive antibiotic treatment; the remaining 8 patients were treated with amoxicillin plus clavulanic acid (4 patients), cotrimoxazole (2 patients), and sulfamethoxazole and metronidazole (1 patient each).

Table 1. Epidemiological characteristics

	No.	Frequency (%)
Age		
< 5 years	5	9.4 %
6–18 years	16	30.2 %
> 18–55 years	24	45.3 %
> 56 years	8	15.1 %
Gender		
Male	28	52.8 %
Female	25	47.2 %
Ocupation[1]		
Farmer	22	41.5 %
Housewife	6	11.3%
Not indicated[2]	7	13.2 %
Living in the area		
Resident[3]	28	52.8 %
Non-resident	11	20.7 %
No data	14	26.4 %
Other		
Water activities	9	17 %

[1]Only adults or minor developing economical activities; [2]Four were not residents of the area; [3]Patients reporting more than one month in the area

Table 2. Clinical presentation

Clinical presentation	Number of cases n (%)
Headache General discomfort	27 (50.9)
Chills	17 (32.1)
Fever	13 (24.5)
Joint pain	10 (18.9)
Vomiting	9 (17)
Abdominal pain	7 (13.2)
Dizziness	5 (9.4)
Myalgia	4 (7.5)
Pallor Cough Diarrhea Hyporexia	3 (5.7)
Low back pain Epigastralgy	2 (3.8)
Jaundice Expectoration Itching Ecchymosis	1 (1.9)

All samples were analyzed by microscopy, microbiological and molecular techniques. Despite 29 (54.7%) cases being initially reported as positive by microscopy techniques, no amplified products were obtained with the 16S rRNA *Bartonella*-specific primers, showing that microscopy leads to a misidentification of *Bartonella* infections. Meanwhile, correct amplification of 1,503 bp was obtained in all the cases using the 16S rRNA universal primers. Twenty-six (49%) of these amplified products were randomly recovered and sequenced. Unexpectedly, the sequencing showed the presence of *Sphingomonas faeni*, suggesting that this microorganism was the causative microorganism of this outbreak.

To determine the presence of *B*. *bacilliformis*, all samples were cultured under non-aerobic conditions. Bacterial growth was only detected in three samples. The DNA was extracted from the three growing microorganisms, and the different 16S rRNA PCR approaches were performed. Amplified product was obtained only with 16S rRNA universal primers. Analysis of the sequences showed the presence of *Staphylococcus epidermidis*, which was considered a contaminant.

Domestic water samples and water-well samples were collected. DNA extraction followed by PCR amplification using the 16S rRNA universal primers was performed; the results showed the presence of different microorganisms, including previous uncultured aquatic microorganisms related to GenBank access numbers gb|GU758935.1 and GU758935.1 from water well, and HM238175 from both domestic and well samples, the last one belonging to a member of the *Sphingomonas* genus.

Discussion

The present report shows the presence of a misdiagnosed B. bacilliformis outbreak. The results showed the presence of S. faeni, an environmental microorganism that to the best of our knowledge, has never been described as a causative agent of an infectious outbreak. Although it cannot be ruled out, environmental PCR contamination was unlikely because PCR amplifications and DNA extractions were performed more than once, at different times, in two different settings. Moreover, negative controls were used, and no other Sphingomonas spp. was detected in any of the other laboratory samples analyzed prior, during, or after this study.

In a previous report, the symptoms significantly associated with Oroya fever were chills, joint pain, cough, loss of appetite, pollakiuria and jaundice [1]. With the exception of chills, these symptoms were absent or had a minimal presence, being then non-suggestive of Carrion's disease. The fact that this outbreak was first associated with *B*. *bacilliformis* was probably due to the unexpected amount of cases, the proximity of endemic areas and, especially, due to the presence of positive blood thin smears (Figure 1).

It has been shown that *Sphingomonas* spp. possess virulence factors showing a degree of identity with *Brucella* intracellular survival factors [8]. Moreover, *Sphingomonas* spp. possess the ability to penetrate within epithelial cells *in vitro* [9], and some reports have shown the presence of intracellular Gram-negative microorganisms during *Sphingomonas* spp. infections [10]. However, to the best of our knowledge, no data about the ability of *S*. *faeni* or other *Sphingomonas* spp. to invade erythrocytes exists in the literature; the possible concomitant presence of other pathogens able to invade erythrocytes cannot be ruled out. A possibility is the presence of hemoplasma, which are wall-less erythrocytic bacteria unable to be cultured *in vitro*, classified within the genus *Mycoplasma*, which have been described in human and animal infections [11]. However, an *in silico* analysis showed that the 16S rRNA universal primers used were able to amplify a fragment of 1,433 bp of the 16S rRNA gene of hemoplasma species (i.e., *Candidatus Mycoplasma haemominutum*, GeneBank access NC_021007.1). Similarly, the primers used were also able to detect other well-known intraerythrocytic bacteria such as *Anaplasma* spp.

Bacterial growth was only detected in three samples, but the growing microorganism was *S*. *epidermidis*, which was considered to be a sample contamination. These low positivity rates may have been found because the samples were directly plate-cultured but no hemoculture was done, and because of the specific growth conditions cultured in order to detect the presence of *B*. *bacilliformis* [1]. *Sphingomonas* is a strictly aerobic microorganism commonly distributed both in hospitals and the natural environment in soil and water [12-14]. Several *Sphingomonas* spp. infections in humans have been reported, mostly limited to sporadic case reports or intra-hospital outbreaks, and mostly related to *Sphingomonas paucimobilis* [13,15]. To our knowledge, this is the first infectious *Sphingomonas faeni* outbreak described, and the first *Sphingomonas* spp. outbreak described in a non-hospital environment.

Although *S*. *faeni* has low clinical virulence, it has a close relationship with *S*. *paucimobilis*, which is able to infect healthy non-compromised patients [16]. This

fact, together with the possible acquisition of virulence factors, as has been proposed for other member of the genus [8], may allow this microorganism to infect healthy people, causing an outbreak. Moreover, the specific socio-sanitary conditions of the area should be considered, including nutritional status, which may enhance the possibility of infections by low-virulence microorganisms [17]. In this sense, malnutrition and anemia among the infant population of the Amazonas region is about 32.8% and 56.7%, respectively [18].

Regarding the focus of infection, all probes seemed to be from the water. *Sphingomonas* is an environmental microorganism, and in this outbreak, almost 17% of the patients were reported to have engaged in aquatic activities; moreover, another *Sphingomonas* spp. (coincident with that recorded as GeneBank HM238175.1) was also detected in both domestic and well water sources.

Unfortunately, we were not able to recover water from the thermal baths. Nevertheless, the environmental nature of *S. faeni* together with the presence of other *Sphingomonas* spp. in the potable water suggest an association between the water consumed and participation in aquatic activities with this outbreak.

Conclusions

Though sample contamination or the presence of a non-detect microorganisms cannot be ruled out, our findings strongly suggests the emergence of *S. faeni* as the causative agent of a community-acquired outbreak, probably associated with water. To our knowledge, this is the first report of a *Sphingomonas* spp. extra-hospital outbreak, as well as the first description of *S. faeni* as a causative infectious agent.

This outbreak was mistakenly attributed to *B. bacilliformis*, demonstrating that diagnosis of febrile syndromes by clinical criteria or microscopy may lead to misdiagnosis. Training of health personnel and the development of new diagnostic tools able to be implemented in endemic rural areas are urgently needed to overcome erroneous diagnoses and to avoid inappropriate treatments.

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Authors' contributions

CG, JR, and JdV designed the study. AC, CG, SMP, and MJP performed the experiments. LS and PB gathered clinical and epidemiological data. JR and JdV analyzed the data. CG, JR, and JdV wrote the manuscript. All the authors read and approved the final manuscript.

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ARTICLE 3

Evaluation of PCR approaches for detection of *Bartonella bacilliformis* in blood samples

Despite not being applicable in most endemic rural areas, in reference centers the PCR technique is a rapid method to diagnose *B. bacilliformis*. Nonetheless, the detection limit of this technique and its usefulness for the diagnosis and detection of asymptomatic people or low-bacteremia carriers have never been addressed.

In this article, we present a comparative study of 3 PCR approaches designed to detect *B. bacilliformis*. We determined the detection limit of the PCR approaches both from artificially infected blood and DBS. We verified the specificity and discussed the different possibilities of the use of PCR in the clinical setting. The PCR approaches chosen were fragments of *Bartonella*-specific *16S rRNA*, *fla* as well as the *its*. The results obtained are summarized in table 6.

Table 6: Detection limit for the 3 PCR approaches studied in both blood samples and dried blood spots.

Blood samples (CFU/µL)			Dried blood spots (CFU/µL)		
16S rRNA	***fla***	***its***	***16S rRNA***	***fla***	***its***
5	5	500	5	500	500

CFU: colony-forming unit

The *16S rRNA* PCR presented the lowest detection limit, 5 colony-forming unit (CFU)/µL and all *Bartonella* spp. could be amplified. Nonetheless, this PCR approach also showed a positive result for *Brucella melitensis*. PCR amplification of the *its* gene increased the detection limit. However, this approach is specific for *Bartonella* spp. and the amplified products have different sizes which allow differentiation of the 3 species causing Carrión's disease-like syndromes (*B. bacilliformis*, *B. rochalimae* and *B. ancashensis*). Regarding the *fla* gene, we also noted the specificity to *Bartonella* spp., and with this approach we were able to distinguish the different species of *Bartonella* causing Carrión's disease from the remaining *Bartonella* causing human infection, with the exception of *B. clarridgeiae*. In all cases we observed that the sensitivity of the PCR decreases in DBS.

It seems that the sensitivity of these techniques might allow the diagnosis of acute cases of Carrión's disease, but their applicability to detect healthy or low-bacteremia carriers' remains unclear. We propose concomitant use of these PCR approaches in the clinical setting.

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RESEARCH ARTICLE

Evaluation of PCR Approaches for Detection of *Bartonella bacilliformis* in Blood Samples

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Abstract

Background

The lack of an effective diagnostic tool for Carrion's disease leads to misdiagnosis, wrong treatments and perpetuation of asymptomatic carriers living in endemic areas. Conventional PCR approaches have been reported as a diagnostic technique. However, the detection limit of these techniques is not clear as well as if its usefulness in low bacteriemia cases. The aim of this study was to evaluate the detection limit of 3 PCR approaches.

Methodology/Principal Findings

We determined the detection limit of 3 different PCR approaches: *Bartonella*-specific *16S rRNA*, *fla* and *its* genes. We also evaluated the viability of dry blood spots to be used as a sample transport system. Our results show that *16S rRNA* PCR is the approach with a lowest detection limit, 5 CFU/μL, and thus, the best diagnostic PCR tool studied. Dry blood spots diminish the sensitivity of the assay.

Conclusions/Significance

From the tested PCRs, the *16S rRNA* PCR-approach is the best to be used in the direct blood detection of acute cases of Carrion's disease. However its use in samples from dry blood spots results in easier management of transport samples in rural areas, a slight decrease in the sensitivity was observed. The usefulness to detect by PCR the presence of low-bacteriemic or asymptomatic carriers is doubtful, showing the need to search for new more sensible techniques.

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Author Summary

Carrion's disease is an endemic illness in the Andean valleys of Peru that achieves high mortality rates in the absence of antibiotic treatment. There are three clinical manifestations, febrile acute patients, chronic patients as well as asymptomatic carriers. No effective diagnostic tool exists nowadays leading to misdiagnosis and the perpetuation of the illness. The objective of this study was to determine the detection limit of three PCR approaches both from blood samples as well as from filter papers. Furthermore, the specificity was also accessed. We found that the best PCR approach studied was the amplification of the *16S rRNA* from blood samples with a detection limit of 5 CFU/μL, the same when using dry blood in filter paper, although the obtained bands were not so evident. Present results highlight the need to develop more sensitive techniques able to be used both in rural areas and in the detection of asymptomatic carriers.

Introduction

Bartonella bacilliformis is the etiological agent of Carrion's disease, an overlooked illness with a lethal febrile stage and a warty phase. Its endemicity is restricted to Peru, Ecuador and Colombia, with some cases having been described in Bolivia and Chile. The transmission is by a sandfly of the genera *Luyzomyia*, mostly *Lutzomyia verrucarum* [1]. The human is the only reservoir known, and in endemic areas about 40% of asymptomatic carriers have been described [2]. In addition, Carrion's disease-like syndromes have been related to two other *Bartonella* species: *Bartonella rochalimae* and *Bartonella ancashensis* [3–5]. Although its relevance remains uncertain, these species may be an explanation for the Carrion's disease cases sporadically reported in distant areas such as Guatemala or Thailand [1]. In fact, *B. rochalimae* has been isolated worldwide [6,7].

Although the warty phase is easy to diagnose by the clinic manifestations, the initial febrile stage as well as asymptomatic carriers, are often misdiagnosed or non-diagnosed leading to perpetuation of the illness. Correct diagnosis of both acute and asymptomatic carriers is extremely important and adequate treatment is imperative to save lives. In endemic areas the diagnosis is usually made by thin blood smear and/or by clinical data. Despite having a specificity of microscopy of 96%, a low sensitivity of 36% has been described [8]. Moreover, other diseases such as malaria, dengue or tuberculosis that are also present, should be taken into account, since the first symptoms are common and may lead to misdiagnosis and erroneous treatments. All these factors are of enormous relevance since the mortality rates of Carrion's disease are of 40–85% without treatment [9]. Furthermore, even despite receiving correct treatment the mortality rate is of 10% [10]. A more reliable method is blood culture but this is cumbersome, time-consuming and contaminations have been described in the 7–20% of the cultures [11]. Serologic tests have also been described and show a higher specificity of about 85% for both IgM ELISA and indirect fluorescence antibody test, but are difficult for routine practice [1]. Molecular diagnosis by PCR is probably the easiest way to achieve a more accurate diagnosis in endemic areas, as the equipment required is not as sophisticated or expensive, may be installed in different Health Regional Centers which may provide diagnosis to more peripheral patients, and the personnel may be easily trained in technique management. Several PCR approaches have been described in the literature in the last years [12,13]. However, these studies do not generally involve a large number of samples and additionally, as occurs with the remaining diagnostic tools, they are hampered by the lack of a standard case definition. In any case, PCR approaches have been showed as more effective that optical microscopic [12], being

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able to diagnostic Carrion's disease patients in acute phase previously classified as negatives by thin blood smear. Nonetheless, a critical issue is the detection limit of these techniques, raising doubts about its usefulness in the detection of low-bacteraemia carriers.

Dried blood spot (DBS) is used for the diagnosis of several infectious diseases [14,15], and has been proposed for use as easy method to transfer blood samples from endemic areas to reference centers in order to carry out molecular techniques for the diagnosis of Carrion's disease [13]. Therefore, since this illness principally affects children in rural areas, DBS may be an easy solution to both the transportation of samples and for small blood volume collection in the pediatric setting.

The aim of this study was to evaluate the detection limit of three PCR approaches designed to detect *B. bacilliformis*, both in blood and filter papers to test their potential use for transferring samples from endemic areas to reference centers.

Materials and Methods

Bacterial strain

We used a collection strain of *B. bacilliformis* from the Institute Pasteur, CIP 57.20 (NCTC 12136). The strain was grown on blood agar (BD, Germany) at 28°C and 5% CO_2 until confluent growth.

Blood samples

To accurately quantify the amount of *B. bacilliformis* we used flow cytometry from the Citomics core facility of the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). For this, one grown agar plate was diluted in appropriate buffer and Perfect-count microspheres were used. Serial dilutions (10^6 CFU/mL—10 CFU/mL) were made in whole blood provided by the blood bank of the Hospital Clinic.

Dry blood spots

One-hundred μL of the above mentioned bacterial serial dilutions were transfered to Whatmann 903 filter papers and let dry at least one week at room temperature to mimic the sample transfer conditions in a real scenario.

DNA extraction

DNA extraction was done from 100 μL blood and from dry blood spots with the Qiamp DNA Mini Kit (Qiagen, Germany), according to the manufacturer's instructions except that the final elution volume was 100 μL.

PCR amplification

Fragments of *Bartonella*-specific *16S rRNA*, flagellin (*fla*) genes as well as the variable-intergenic region (*its*) were amplified. The primers used were 5'-CCTTCAGTTMGGCTGGATC-3' and 5'-GCCYCCTTGCGGTTAGCACA-3' for *16S rRNA* [16], 5'-ATAGAAAGAGCCTGAA TACC-3' and 5`-TGATGAAGCATGACAGTAACAC-3' for flagellin and 5'-AGATGAT GATCCCAAGCCTTCTGG-3' [17], and 5'-CTTCTCTTCACAATTTCAAT-3' [18] for the amplification of variable-intergenic region. The PCRs were performed in a 25-μL total reaction volume with 500 nM forward primer, 500 nM reverse primer, 9,75 μL H_2O and 5 μL of DNA following the conditions: 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 72°C for 30 cycles. A 2% agarose gel stained with Sybr Safe was performed, and the results were

visualized with an ImageQuant LAS4000 transiluminator (GE Healthcare Europe GmbH, Barcelona, Spain).

Detection limit

The detection limit was considered as the lowest dilution at which a positive result was obtained and considering the number of copies of each gene in the *B. bacilliformis* genome. All the above mentioned experiments were done in duplicate intra-assay and at two different times.

Specificity

The specificity was tested by doing the same PCR approaches to other member of the *Bartonella* genus both *in vitro*: *Bartonella elizabethae* (strain 30455), *Bartonella grahamii* (strain 50771), *Bartonella henselae*, *Bartonella koehlerae* (strain 30773), *Bartonella tamiae* (Strain Th307), and *Bartonella vinsonii* subsp. vinsonii (strain 30453), and *in silico* for the remaining 25 recognized species plus *B. ancashensis*. In addition other plate-grown bacteremia microorganisms such as *Escherichia coli*, *Pseudomonas* spp., *Shigella* spp., *Klebsiella* spp., *Haemophilus* spp., *Staphylococcus aureus* and *Streptococcus* spp., as well as an intracellular microorganism such as *Ricketsia* spp. and *Brucella melitensis* were also tested.

Results

When DNA was directly extracted from the blood, the detection limit was 5 CFU/μL for both the *Bartonella*-specific *16S rRNA* and the *fla* genes. Meanwhile, a limit of 500 CFU/μL was obtained on amplification of the *its* region. In the case of DBS, the *Bartonella*-specific *16S rRNA* PCR approach showed the lowest detection limit, which was also of 5 CFU/μL. Concerning dry blood, despite the detection limit being the same for *16S rRNA* and *its*, the sensitivity decreased for *fla* when the detection limit dropped to 500 CFU/μL compared with 5 CFU/μL obtained directly from blood (Table 1). It was of note that fainter bands were always obtained with DBS.

Regarding specificity, the *16S rRNA* gene amplifies for all *Bartonella* species (either *in vivo* or *in silico*) but a positive result was also obtained when tested *B. melitensis*. The *its* amplification assay was specific for *Bartonella* spp., and no other of the tested microorganisms had a positive PCR. Moreover, the *its* scheme might allow to distinguish between different *Bartonella* spp. by the different amplified size. The *fla* gene amplification was also specific for *Bartonella* species and differentiates between *Bartonella* spp. causing Carrion's disease (*B. bacilliformis*, *B. rochalimae* and *B. ancashensis)* and the remaining *Bartonella* causing human disease (Table 2) once no amplification was obtained or predicted for the last ones.

Discussion

Carrion's disease is an overlooked and restricted disease that affects the poorest populations living in remote rural areas, which badly communicated, without equipped laboratories, and with many other illnesses with a common symptomatology [1]. Thus, correct diagnosis of

Table 1. Detection limit for the 3 PCR approaches studied both for blood samples and dried blood spots.

Blood samples (CFU/μL)			Dried blood spots (CFU/μL)		
16S rRNA	***fla***	***its***	***16S rRNA***	***fla***	***its***
5	5	500	5	500	500

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Table 2. Amplification sizes in different *Bartonella* spp. for each of the three PCR approaches in study.

		PCR approaches (bp)		
Microorganism[1]	Illness[2]	*16S rRNA*	*its*	*fla*
Carrion's disease involved *Bartonella* spp.				
B. bacilliformis	**Carrion's disease**	**438**	**545**	**937**
B. rochalimae	Carrion's disease [3]	438	696	974
B. ancashensis	Carrion's disease [4]	438	590	940
Main *Bartonella* spp. causing human illness				
B. alsatica	Endocarditis	438	654	NA
B. clarridgeiae	Cat scratch disease	438	692	997
B. elizabethae	**Endocarditis**	**438**	**777**	**NA**
B. henselae	**Cat scratch disease**	**438**	**719**	**NA**
B. grahamii	**Retinitis**	**438**	**715**	**NA**
B. quintana	Trench fever, Cat scratch disease	438	619	NA
B. vinsonii subsp. berkhoffi	Endocarditis	438	727	NA
B. vinsonii subsp. arupensis	Bacteremia	438	742	NA
Other *Bartonella* spp.[5]				
B. bovis		438	525	NA
B. capreoli		438	564[6]	NA
B. coopersplainsensis		438	701	NA
B. koehlerae		**438**	**677**	**NA**
B. pachyuromydis		438	545	NA
B. queenslandensis		438	715	NA
B. schoenbuchensis		438	527	1008
B. silvatica		438	690	NA
B. taylorii		438	689	NA

[1].- The 32 currently recognized *Bartonella* species (including the three *B. vinsonii* subsp.) plus *B. tamiae* were considered.

[2].- Only the more relevant pathologies have been referenced here.

[3].- Described as one case of Oroya fever-like in a tourist returning from Peru [4].

[4].- Described as a cause of Peruvian Wart in children living in an endemic area [3,5].

[5].- Most of them isolated from animals, and some sporadically reported from human infections. Indicated are only those microorganisms that have the *fla* gene or that the amplified *its* product differing 20 bp or less respecting any of *Bartonella* spp. involved in Carrion's disease.

[6].- Uncertain amplification (two gaps close to primer 3' terminal).

NA: non amplified or non-predicted amplification.

Highlighted in bold when experimental amplification of the three PCR approaches in study were performed.

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Carrion's disease is essential, particularly since misdiagnosis is frequent [12, 19]. PCR techniques rank among the most rapid techniques to diagnose *B. bacilliformis*. For this reason, the determination of the detection limit of these techniques is extremely important. For this study we have chosen three approaches, the amplification of *16S rRNA*, the hypervariable intergenic transcribed spacer 16S-23S rRNA and the *fla* gene which codes for the flagelin protein of *B. bacilliformis*. The amplification of *16S rRNA* has been proposed for Carrion's disease diagnostic in Peru [12]. All tested *Bartonella* had an amplified product of 438 bp. Moreover, the *in silico* analysis showed that these primers are able to amplify all *Bartonella* spp. Then, this PCR approach may be also useful in other environments to detect and identify other *Bartonella* spp. either combining with sequencing or RFLP.

The *its* amplification permits to differentiate between *B. bacilliformis* and *B. ancashensis* from the main pathogenic *Bartonella* spp [20]. In fact the *its* region has been used in different studies of *Bartonella* spp [7].

Regarding *fla*, this gene permit to distinguish between the three *Bartonella* causing Carrion's disease: *B. bacilliformis* (940 pb), *B. rochalimae* (974 bp) and *B. ancashensis* (937 bp) from the remaining *Bartonella* spp. with clinical interest. However, one exception is *B. clarridgeiae* (997 bp). Additionally, and *in silico* analysis showed that *B. schoenbuchensis* will also results in a positive fragment of 1008 bp.

Our results show that the *Bartonella*-specific *16S rRNA* PCR seems to be the best of the techniques analyzed to detect the presence of *B. bacilliformis* in blood samples (5 CFU/μL) since the lowest detection limit was achieved on comparison with *fla* and *its* PCRs. These results are in accordance with Angkasekwinai *et al.* [21], who reported a detection limit of 1 and 10 copies/μL in a loop-mediated isothermal amplification when the detection limit was determined using bacterial genomic DNA alone or in the presence of human plasma respectively. This sensitivity might allow diagnosing the acute cases of Carrion's disease, in which the mean percentage of infected RBCs is 61% (ranging from 2 to 100%) [22]. Nonetheless, the concomitant use of these PCR approaches will provide information about other *Bartonella* spp. infections.

Filter paper may be an alternative for easy transportation of samples from endemic areas to reference laboratories but the decreasing sensitivity of the results must been taken into account which may lead to the non-detection of cases with a low bacteremia. Although the same detection limit was obtained for *16S rRNA* PCR both directly from blood and filter papers, the bands were fainter in the latter. It is true that 1 week delay in the sample processing could affect the PCR by increasing the detection limit. Nonetheless, in rural settings the transfer of samples to reference centers is associated with bad communications ways, resulting in some days from sample collection to molecular determinations.

None of the non-*Bartonella* microorganisms included in the study were positive when *its* or *fla* PCRs were performed. Nonetheless, when *Brucella* spp. was tested, amplification was obtained to *16S rRNA* PCR. Although this is a limitation, it is need to take into account that a diagnostic should to be performed both in the adequate clinic context and in parallel with other diagnostic tools such as differential PCR for *Brucella* diagnostic when needed [23].

The prevalence of asymptomatic people in endemic areas has already been described by PCR being 0.5% [1]. However, the number of inhabitants previous exposed increases to around 40% when serologic techniques like ELISA or IFA are performed [1]. It is need to take into account that *B. bacilliformis* possess tropism for both erythrocytes and endothelial cells, being then present a non-blood circulating bacterial. In the chronic illness stage (verrucuous patients) the sensitivity of the microscopical techniques decreases from the 36% described in the acute phase to less than 10% [24], highlighting the lower blood bacterial carriage and a possible transient bacteremia. Those facts might results in false PCR-negative when the technique is applied in the detection of both verrucous patients and asymptomatic carriers.

It is important to remark that in the last years 2 more sensitive PCR techniques have been described in the literature: qPCR [13] in which 24.6% of DBS samples are positive, as well as a loop-mediated isothermal amplification [21] that achieves good results on analysing *Lutzomyia* samples. However, qPCR requires the expertise of trained personnel and is more expensive and difficult to be implemented. Meanwhile the usefulness of loop-mediated isothermal amplification remains to be validated to detect the presence of *B. bacilliformis* in human clinical samples. Enrichment of the sample before conventional PCR has been proposed to increase the positivity by 55% when compared with the original blood samples [25]. However, this enrichment

technique results in a 14-days delay in sample processing thereby making it unaffordable for diagnostic purposes.

To conclude, here we show that *16S rRNA* PCR have low cfu detection limit and should be used with special attention to test samples from individuals with clinical suspicion of Carrion's disease since the applicability to detect healthy carriers is not clear. The use of DBS could facilitate the transfer of samples from rural endemic areas to health facilities, despite the possibility of a small decrease in positivity. It is critical to develop rapid, sensitive and specific techniques which may be applied in endemic rural areas to avoid misdiagnosis and to facilitate the detection of asymptomatic carriers and thereby the decrease the number of *B. bacilliformis* cases.

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Author Contributions

Conceived and designed the experiments: CG JR. Performed the experiments: CG CT SM MJP. Analyzed the data: CG SM JB. Wrote the paper: CG JdV JR.

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ARTICLE 4

Succinyl-CoA synthetase: new antigen candidate of *Bartonella bacilliformis*

Accurate diagnosis of both patients and asymptomatic carriers of Carrión's disease in endemic areas remains one of the unsolved issues in this field. There are few reports in the literature about *B. bacilliformis* antigens, and a sufficiently sensitive rapid diagnostic tool has yet to be developed. The aim of this study was to characterize a collection of sera and blood samples from people living in exposed areas, and identify and characterize *B. bacilliformis* antigenic candidates.

To address this objective, 198 blood and 177 serum samples were collected from volunteers diagnosed with Carrión's disease during an outbreak that had occurred a few months earlier in 4 non-endemic localities in Peru. One endemic area was also included in the study. The samples were characterized by RT-PCR, IFA, and IgM / IgG ELISA with sonicated *B. bacilliformis* whole cell as the antigen.

To identify immunogenic proteins, sonicated agar-grown *B. bacilliformis* was separated by SDS-PAGE and Western blot was performed for each serum for both IgG and IgM. Taking into account the antibody levels by whole cell ELISA for each serum, immunogenic candidate proteins were chosen and N-terminal amino acid sequencing was done.

The immunogenic candidate proteins identified were cloned, expressed and purified. To determine and characterize the response of the immunogenic candidates to the sera collected, ELISA was performed for both IgM and IgG using the immunogenic proteins as antigens.

As shown in table 7, approximately half of the volunteers in the endemic area and about 40% of individuals in the post-outbreak areas had a positive result by RT-PCR, demonstrating the high number of asymptomatic carriers in these zones. Although re-infections cannot be ruled out, the presence of microbiological failures seems evident as all volunteers from post-outbreak areas were treated with ciprofloxacin during the outbreak. In all cases, the positivity observed in the RT-PCR was close to the detection limit of the technique, showing that the presence of bacteria in the blood may have been very low. Regarding IFA, our rate of positivity was lower than expected; being 26.8% in the post-outbreak areas compared to the 74% previously described in the literature. Meanwhile, IgG anti-*B. bacilliformis* antibody levels

obtained by ELISA for people from the endemic area were significantly higher than those found in the volunteers from the post-outbreak areas where the disease had never been described prior to this outbreak. This highlights the presence of a high percentage of people with high levels of antibodies against *B. bacilliformis* living in endemic areas. Moreover, we found that IgM / IgG levels were age-dependent, suggesting the development of partial immunity along life. The differences between the antibody levels and the blood carriage of *B. bacilliformis* may support the bacterial evasion of the host immune system by differential expression of bacterial epitopes and the facility to invade different host tissues.

Table 7: Positive results for each technique in the endemic and post-outbreak areas.

		Post-outbreak areas n (%)	Endemic area n (%)	*P*	Total n/N (%)
RT-PCR		64/173 (37)	13/25 (52)	0.1883	77/198 (38.9)
IFA		41/153 (26.8)	3/24 (12.5)	0.0575	45/177 (25.4)
ELISA	**IgM**	50/153 (32.7)	10/24 (41.7)	0.4869	60/177 (33.9)
	IgG	32/153 (20.9)	16/24 (66.7)	0.0001	48/177 (27.1)
Positive for at Least one technique*		119/153 (77.8)	22/24 (91.7)	0.1715	141/177 (79.7)

*Only individuals with both blood and serum samples were considered (n=177).
The Fisher exact test was used in all the cases.

Four immunogenic proteins of *B. bacilliformis* were identified by Western blot: 2 with a secondary antibody anti-human IgM, Pap31 and Succinyl-CoA synthetase subunit-α (SCS-α) and another 2 with anti-human IgG as a secondary antibody, GroEL and Succinyl-CoA synthetase subunit-β (SCS-β) (Figure 27).

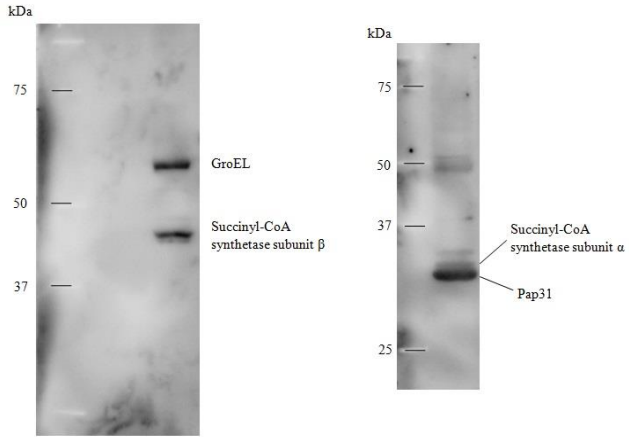

Figure 27: Antigenic candidates of *B. bacilliformis* identified. Example of a Western blot performed with a positive serum by whole cell ELISA. The left panel corresponds to IgG and the right panel to IgM.

As far as I know, this is the first description of SCS-α and SCS-β as immunogenic proteins of *B. bacilliformis*. These new antigenic candidates are implicated in the tricarboxylic acid cycle and described as an immunogenic protein of *B. melitensis* (SCS-α) and involved in the pathogenesis of *B. henselae* infection (SCS-β).

In all the cases, the positivity for IgM was greater than 77% according to the ELISAs of the immunogenic proteins while for IgG the values ranged from 35% for SCS-α to 60% for SCS-β. It is of note that in the case of IgM, we observed nearly complete concordance between the ELISAs for antigenic candidates and that for whole cell *B. bacilliformis*, suggesting that in a future rapid diagnostic test the antigens can be used indistinctly.

Studies identifying and characterizing *B. bacilliformis* antigens are essential to advance towards the development of a rapid diagnostic tool that can be implemented in rural areas where this disease is endemic.

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RESEARCH ARTICLE

Succinyl-CoA Synthetase: New Antigen Candidate of *Bartonella bacilliformis*

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Abstract

Background

Bartonella bacilliformis is the causative agent of Carrion's disease, a neglected illness with mortality rates of 40–85% in the absence of treatment. The lack of a diagnostic technique to overcome misdiagnosis and treat asymptomatic carriers is of note. This study aimed to identify new *B. bacilliformis* antigenic candidates that could lead to a new diagnostic tool able to be implemented in endemic rural areas.

Methodology/Principal Findings

Blood (n = 198) and serum (n = 177) samples were collected in northern Peru. Clinical data were recorded. Specific *16S rRNA* amplification by RT-PCR, IFA and ELISA for IgM/IgG with whole cells as antigens was done. Western blot analysis and N-terminal amino acid sequencing detected seroreactive proteins. ELISAs for IgM/IgG for the antigenic candidates were performed. Of the population 33.3% reported at least one symptom compatible with Carrion's disease; 25.4% (IFA), 27.1% (ELISA-IgG), 33.9% (ELISA-IgM) and 38.9% (RT-PCR) of samples were positive. Four proteins were considered potential antigenic candidates, including two new antigenic candidates, succinyl-CoA synthetase subunit α (SCS-α) and succinyl-CoA synthetase subunit β (SCS-β). On Western blot both Pap31 and SCS-α interacted with IgM, while GroEL and SCS-β interacted with IgG. The presence of specific antibodies against the antigenic candidates varied from 34.5% (IgG against SCS-α) to 97.2% (IgM against Pap31).

Conclusions/Significance

RT-PCR and the high levels of positivity for specific ELISAs demonstrate high levels of *B. bacilliformis* exposure and asymptomatic carriers among inhabitants. The new antigens identified might be used as a new rapid diagnostic tool to diagnose acute Carrion's disease and identify asymptomatic carriers.

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Author Summary

B. bacilliformis is a neglected pathogen causing Carrion's disease, a febrile illness with two distinct phases, the acute so-called Oroya fever that can be life-threatening, and the chronic so-called Peruvian wart. This illness is currently limited to poor inhabitants of Andean valleys of Ecuador, Colombia and Peru and for this reason is understudied. One of the most significant limitations is the lack of an adequate diagnostic tool able to be implemented in rural areas. It is imperative to unequivocally detect cases of Carrion's disease as well as identify asymptomatic carriers who perpetuate the illness. The present study describes the identification of 4 antigenic candidates potentially useful in the future development of a rapid diagnostic test. Moreover, 2 of these candidates have not been described in the literature. Additionally, four post-outbreak and one endemic community were studied and characterized. The identification of new antigens is essential for the development of a cheap, sensitive diagnostic tool, able to be implemented in low-income areas.

Introduction

Bartonella bacilliformis is the etiological agent of Carrion's disease, a neglected endemic illness in Peru which has also been reported in Ecuador and Colombia [1]. Two well-established phases have been described in this infection. In the acute phase, also called Oroya Fever, *B. bacilliformis* infects the red blood cells which may result in severe anemia and transient immunosuppression [2,3]. The absence of treatment leads to high levels of mortality (40% to 85%) [4]. The chronic phase, *'Verruga Peruana'* (Peruvian wart), is characterized by the development of nodular dermal eruptions. This phase typically occurs in survivors weeks or months after the acute febrile syndrome [5].

Clinical cure does not necessarily result in bacterial clearance. In fact, viable *B. bacilliformis* have been cultured from blood samples of treated patients [6,7]. This lack of clearance together with the development of partial immunity and the presence of continuous *B. bacilliformis* exposure, means that endemic areas have a high number of individuals who are asymptomatic carriers. Indeed, it has been described that 45% of inhabitants of endemic areas are *B. bacilliformis* seropositive when antibodies are tested by Indirect Fluorescence Antibody (IFA) assay [8].

Studies of *B. bacilliformis* antigens are scarce in the literature compared with reports of other pathogens, and a rapid diagnostic method to detect acute and/or chronic infections has yet to be developed. To our knowledge, the first report identifying *B. bacilliformis* antigens was described in 1988 by Knobloch [9]. Twenty-four protein antigens were found, including one main antigen with 65 kDa (BB65; a heat shock protein posteriorly identified as GroEL) [9–11]. Nonetheless, BB65 never bound to *Bartonella* IgM but did bind to IgG antibodies after the first two weeks, thereby demonstrating its utility to detect persisting IgG from the first to the third year after a *B. bacilliformis* infection. However, only 60% of sera from Verruga Peruana patients react with BB65 [10]. Padmalayam *et al.* described an immunogenic 43 kDa lipoprotein as an antigen by screening a genomic DNA lambda library with serum from a patient in the chronic wart phase of bartonellosis [12]. More recently, Pap31 was described to be immunologically dominant and a good candidate for use in ELISA, but once again only one serum was used for identification [13]. Unfortunately, none of the antigens described above have resulted in a rapid diagnostic tool.

The objective of this study was to identify and characterize *B. bacilliformis* antigenic candidates and take a step towards a rapid diagnostic tool able to be implemented in rural areas.

Materials and Methods

Bacterial strains

The microorganisms used in this study are listed in Table 1. *B. bacilliformis* was cultured at 28°C on Columbia agar with 5% sheep blood for 7 days.

Study population

Blood (n = 198) and serum samples (n = 177) were collected in March 2014 in five different villages of Piura, in the north of Peru: Tunal, Guayaquiles, Los Ranchos, Mayland and Huancabamba. Serum samples were collected in sodium citrate and gel SSTII advance vacutainers (BD, Heidelberg, Germany). A *B. bacilliformis* outbreak occurred in 4 of these villages: between May and November 2013 in Tunal, Guayaquiles and Mayland, and between November 2013 and March 2014 in Los Ranchos [14]. According to the national guidelines [15], all participants had received ciprofloxacin treatment during 14 days following diagnosis during the outbreak. All people living in the aforementioned four villages who were diagnosed (clinical symptoms and/or thin blood smear) with acute Carrion's disease during the previous outbreak were invited to participate in the study. Those who agree to participate were notified by the local health center and all samples from that village were collected on the same day. On the other hand, Huancabamba is a long established endemic area for Carrion's disease and the volunteers were randomly recruited by house-to-house visits. Clinical and demographic data were recorded in all cases. After collection the samples were kept in the appropriate refrigeration conditions: 4°C for blood samples and sera samples were frozen. All samples were transferred to Lima (Peru) and sera samples were also sent to Tokyo (Japan) to perform immunological assays. In all cases transportation was performed under frozen conditions.

Ethics statement

In all the cases, signed informed consent was obtained and clinical data were anonymized. In the case of children a parent or their guardian provided informed consent on their behalf. The study was approved by both the Ethic Committees of the Universidad Peruana de Ciencias Aplicadas (Lima, Peru) and the Hospital Clinic (Barcelona, Spain).

Table 1. Bacterial strains and plasmids.

	Description	Source or reference
Strains		
B. bacilliformis	CIP 57.20 –NCTC12136	Institute Pasteur
E. coli TOP10	Host strain for cloning	Invitrogen
E. coli BL21Star (DE3)	Host strain for gene expression	Invitrogen
Plasmids		
pCR4-TOPO	TA-cloning vector	Invitrogen
pET100D/TOPO	Expression vector	Invitrogen
pGroEL	pET100D/TOPO containing GroEL	This study
pPap31	pET100D/TOPO containing Pap31	This study
pSCS-α	pET100D/TOPO containing SCS-α	This study
pSCS-β	pET100D/TOPO containing SCS-β	This study

SCS-α: succinyl-CoA synthetase subunit α

SCS-β: succinyl-CoA synthetase subunit β.

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Real-Time (RT) PCR

DNA extraction was done from 200 μL blood samples with the Qiamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions except that the final elution volume was 100 μL. A RT-PCR was performed based on Smit *et al.* [16], using a BHQ quencher probe at 125 nM and 250 nM of primers in final volume of 25 μL. RT qPCR conditions were 95°C for 15 minutes, 60 cycles of 15 seconds at 95°C and 45 seconds at 60°C and the procedure was performed in an ABI Prism 7500 RT system and data was analyzed in the 7500 System SDS software (Applied Biosystems, Warrington, UK). The primers and the probe used are shown in Table 2.

IFA assay

The Chamberlin method was used with slight modifications [8]. Briefly, Vero cells were cultured in sterile plates maintaining the same MOI and concentrations throughout the experiment. At harvest, the Vero cell monolayer was removed with trypsin in a final volume of 10 mL. The slides were mounted with 65 μL of cells suspension per well and 20 μL 1:100 of Fluorescein (FITC)-conjugated goat anti-human IgG (heavy plus light chains) (Jackson IR, Baltimore, PA). Slides were read using a 20X objective, 10X oculars and an Olympus IX51 microscope. We used the same cut off established by Chamberlin *et al.*, considering 1/256 serum dilution end point as a positive IFA test.

ELISA

IgM and IgG antibody levels were measured by ELISA as described by Matsuoka *et al.* [17] using sonicated *B. bacilliformis* whole cells as antigens. Total protein concentration was quantified by the Pierce assay (Thermo Scientific, Rockford, USA). IgM were detected with rabbit anti-human IgM (1:1000) conjugated with peroxidase and using o-Phenylenediamine (Dako, Glostrup, Denmark) as substrate (Sigma, St. Louis, MO). IgG were detected with rabbit anti-human IgG (1:1000) conjugated with alkaline phosphatase and using Sigma P104 phosphatase substrate. Optical densities were measured as absorbance at 450 nm and 405/655 nm for IgM and IgG, respectively. Each sample was analyzed in triplicate intra-plate and results were reported as three independent ELISA experiments. The negative control, included a pool of sera from 30 healthy donors (X0939, Dako, Glostrup, Denmark). In the absence of an

Table 2. Primers used in this study.

	Sequence (5' → 3')	Ref
16S rRNA F	TTGATAAGCGTGAGGTCGGAGG	[16]
16S rRNA R	GCAACCACACATAGTAAGCCTAA	[16]
16S rRNA probe	ATGTCTGCCTAGAAATCAATCATAGGCC	[16]
GroEL F	*CACCATGGCTGCTAAAGAAGTAAAATTT	This study
GroEL R	TTAGAAATCCATTCCGCCCATTCCGCC	This study
Pap31 F	*CACCATGAATATAAAATGTTTAGTGACA	This study
Pap31 R	TCAGAATTTGTAAGCAACACCAACGCG	This study
SCS α F	*CACCATGTCAATTCTTATC	This study
SCS α R	CTAACCCTTCAAGACTGAAACC	This study
SCS β F	*CACCATGAATATCCATGAAT	This study
SCS β R	TTAAGCTCCTTTTACGGCTGC	This study

* CACC at the 5' end is a sequence added to allow pET directional cloning.

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established cut off, evidence of infection was considered in the samples above the Finite Mixture Model (FMM) cut off that provides a statistical framework for the analysis of immunoglobulin values [18].

Western blotting

Agar-grown *B. bacilliformis* were lysed by sonication, separated on a 10 to 20% SDS-PAGE gel and electrotransferred onto a PVDF membrane (GE Healthcare, Buckinghamshire, UK). The different lanes were cut and immunoblotted with each serum for both IgG/IgM. The negative control used in ELISA was used here.

Amino acid sequencing

Immunogenic candidate proteins were chosen according to the antibody levels obtained by whole cell ELISA results. The membranes were stained with Coomasie brilliant blue staining solution. Candidate proteins were directly cut out, and N-terminal amino acid sequencing was done in APROScience (Naruto, Japan).

Amplification and cloning of antigenic candidates

Genes coding for the candidate proteins were amplified by PCR using the primers in Table 2. Amplified products were purified and cloned in Champion pET Directional 100/D-TOPO vector (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions in order to generate Xpress-tagged-full-length versions of the candidate antigenic proteins. The DNA sequences were verified. For the detection, *E. coli* lysates suspended in lysis buffer were separated on a 12.5% SDS-PAGE and immunoblotted with anti-Xpress antibody. The His_6-tagged proteins were purified using His-Bind kits (GE Healthcare, Buckinghamshire, UK).

Candidate antigens ELISA

Purified candidate proteins were used to perform ELISA and determine the reaction with sera in study, following the same protocol mentioned previously. Here, the cut off was established considering 3 times the standard deviation for the X0939 negative control (Dako).

Statistical analysis

Statistical analysis was performed using the Graphpad package. Significance was considered with $P<0.05$. *P*-values were calculated using the Fisher and Mann-Whitney tests.

Results

A Carrion's disease outbreak occurred in the Lalaquiz district (S1 Fig) between May and November 2013. Sixty-five villages were affected, with Tunal, Guayaquiles and Mayland being among the 10 most affected with 53.8, 19.3 and 2.56% of the cases, respectively. Meanwhile, in Los Ranchos (in the neighboring Canchaque district) 21 cases were reported between November 2013 and March 2014. This outbreak was the first description of this illness in these villages. During the outbreak, a total of 428 cases were reported, diagnosed by clinical symptoms and/or thin blood smear. The mean age of the persons affected during the outbreak was 34 years; 52.3% of the cases were females, and 18.5% of the cases involved children up to 10 years of age. Headache (74.7%), malaise (66%), fever (39.2%), arthralgia (31.6%), myalgia (24.4%) and pallor (13%) were the most common symptoms.

We were able to collect 198 blood samples from volunteers in March 2014 and Table 3 shows the study population data. All samples collected in the 4 post-outbreak villages were

Table 3. General data about the study population (n = 198).

	Tunal (n = 94)	Guayaquiles (n = 25)	Los Ranchos (n = 44)	Mayland (n = 10)	Huancabamba (n = 25)	Total
n (%)	94 (47.5)	25 (12.6)	44 (22.2)	10 (5.1)	25 (12.6)	198 (100)
Mean age (min–max)	39.3 (1–94)	34.2 (6–67)	38.6 (5–77)	30.2 (6–73)	30.2 (9–68)	36.9 (1–94)
Male	31 (33)	8 (32)	17 (38.6)	4 (40)	16 (64)	76 (38.4)
Female	63 (67)	17 (68)	27 (61.4)	6 (60)	9 (36)	122 (61.6)
CD symptoms*	23 (24.5)	17 (68)	14 (31.8)	3 (30)	9 (36)	66 (33.3)

*The presence of at least one symptom compatible with Carrion's disease, including fever, joint pain, headache, malaise, pallor, myalgia and warts.

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from people who had been diagnosed with acute Carrion's disease a few months earlier when an outbreak had occurred. Despite the fact that all volunteers appeared healthy, mild symptoms were reported. The most common symptom described in our study population was headache in 27.3% of the population, followed by 4% of people reporting fever, myalgia and malaise. Joint pain and pallor were present in 5 and 2.5%. With regard to the endemic village, 1 (0.5%) person from the endemic area (Huancabamba) presented Verruga Peruana at the time of sample collection. RT-PCR results showed amplification for 77 (38.9%) individuals, but it should be taken into account that all positive samples presented very low bacteremia in blood. Significant differences were found between the positivity results of Tunal and Guayaquiles ($P = 0.0189$), Guayaquiles and Los Ranchos ($P = 0.0370$), Tunal and Mayland ($P = 0.0006$), Los Ranchos and Mayland ($P = 0.0001$) and Los Ranchos and Huancabamba ($P = 0.0178$). Additionally, the results between Mayland and Huancabamba were almost statistically significant ($P = 0.0548$) (Table 4).

Serum samples were taken from 177 (89.4%) of the individuals. Of these 45 (25.4%) had an IFA titer $\geq$ 256 being considered as positive by the IFA test (Figs 1 and 2, Table 4). The IFA results were almost significantly lower in the endemic area than in the 4 post-outbreak villages taken together ($P = 0.0575$).

For the whole cell *B. bacilliformis* IgM ELISA, the 3 standard deviation cut off method resulted in 95.5% of volunteers presenting levels of IgM equivalent to evidence of infection, while with the use of FMM, the cut off was established at 0.35 (IgM) and 0.53 (IgG), with approximately 34 and 27% of volunteers showing evidence of infection by IgM and IgG values, respectively. Thus, in order to differentiate the most positive samples we applied the FMM cut off.

Differences in the antibody levels between genders were not found. However, it was observed that higher IgM levels tended to be more frequent in females ($P = 0.0526$) while IgG values were significantly higher in men ($P = 0.0224$). On analyzing the antibody levels by localities, we saw that IgG levels were significantly higher ($P<0.0001$) in Huancabamba compared to post-outbreak areas where the disease had never been described before this outbreak. This shows the presence of a high percentage of healthy people with high levels of antibodies against *B. bacilliformis*. Additionally, for IgG levels statistically significant differences (P values ranging from <0.0001 to 0.0229) were obtained on comparing each of the post-outbreak areas with the endemic area (Huancabamba) (Table 4). When the IgM levels were analyzed taking into consideration the different age groups, we observed that young people ($\leq$25 years) had significantly higher IgM levels ($P<0.0001$) than adults $\geq$26 years, while IgG levels only showed a tendency to be higher in people $\geq$26 years ($P = 0.0753$). On comparing the IgG levels of the younger population ($\leq$10 years) with each of the other age groups, we found statistically

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Table 4. Positive results for each technique in the different study sites.

		Tunal	Guayaquiles	Los Ranchos	Mayland	Post-outbreak areas	Endemic area (Huancabamba)	P^{1}	Total
		n/N (%)	n/N (%)	n/N (%)	n/N (%)	n/N (%)	n/N (%)		n/N (%)
RT-PCR		30/94 (31.9)[a,c]	15/25 (60)[a,b]	10/44 (22.7)[b,d,e]	9/10 (90)[c,d]	64/173 (37)	13/25 (52)[e]	0.1883	77/198 (38.9)
IFA		21/75 (28)	8/25 (32)	8/43 (18.6)	4/10 (40)	41/153 (26.8)	3/24 (12.5)	0.0575‡	45/177 (25.4)
ELISA	**IgM**	19/75 (25.3)	9/25 (36)	17/43 (39.5)	5/10 (50)	50/153 (32.7)	10/24 (41.7)	0.4869	60/177 (33.9)
	IgG	14/75 (18.7)[f]	6/25 (24)[g]	10/43 (23.3)[h]	2/10 (20)[i]	32/153 (20.9)	16/24 (66.7)[f,g,h,i]	0.0001**	48/177 (27.1)
Positive for at least one technique†		55/75 (73.3)	23/25(92)	31/43 (72.1)	10/10 (100)	119/153 (77.8)	22/24 (91.7)	0.1715	141/177 (79.7)

†Only individuals with both blood and serum samples were considered (n = 177).

[1]Statistical significance between the positivity in the 4 post-outbreak villages taken together and the positivity in the endemic area (Huancabamba).

‡IFA results were almost significantly lower in the endemic area than in the 4 post-outbreak villages taken together ($P = 0.0575$).

**ELISA IgG values were significantly higher in Huancabamba than in the 4 post-outbreak villages taken together ($P = 0.0001$).

The superscript letters represent the statistically significant differences between the positivity in 2 specific areas

[a] $P = 0.0189$

[b] $P = 0.0370$

[c] $P = 0.0006$

[d] $P = 0.0001$

[e] $P = 0.0178$

[f] $P<0.0001$

[g] $P = 0.0041$

[h] $P = 0.0007$

[i] $P = 0.0229$.

The RT-PCR values between Mayland and Huancabamba were almost statistically significant ($P = 0.0548$). In all cases the Fisher exact test was used.

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significant differences with all groups except people ≥70 years (between ≥11 - ≤25 $P = 0.0281$; ≥26 - ≤55 $P = 0.0074$; ≥56 - ≤70 $P = 0.0246$) (Fig 3).

No concordance was observed between clinical data and the positivity obtained with the diagnostic tools used. It is interesting to note that the case of Peruvian wart was only detected by ELISA IgG (Table 5).

Table 6 shows the concordance between each pair of techniques used. Six samples had a positive result for at least 3 out of the 4 techniques used. This comparative analysis showed a certain degree of dispersion and a lack of concordance (Table 6).

By doing the Western blots with the sera in the study and against whole sonicated *B. bacilliformis* we chose four proteins as possible antigenic candidates, two identified with anti-human IgM and two with anti-human IgG (Fig 4). Amino acid sequencing revealed that the IgM candidates identified were Pap31 and succinyl-CoA synthetase subunit α (SCS-α) whereas for IgG we identified GroEL and succinyl-CoA synthetase subunit β (SCS-β) (Table 7).

After cloning, expression and purification of the four antigenic candidates, we performed ELISA for each IgM and IgG testing.

The IgM results showed a high prevalence of reactivity, from 77.4% for SCS-α to 97.2% for Pap31. Meanwhile, the IgG reactivity in the ELISAs done for the antigens ranged from 34.5 to SCS-α and 59.9% to SCS-β IgG (Table 8). The person with the Verruga Peruana was positive

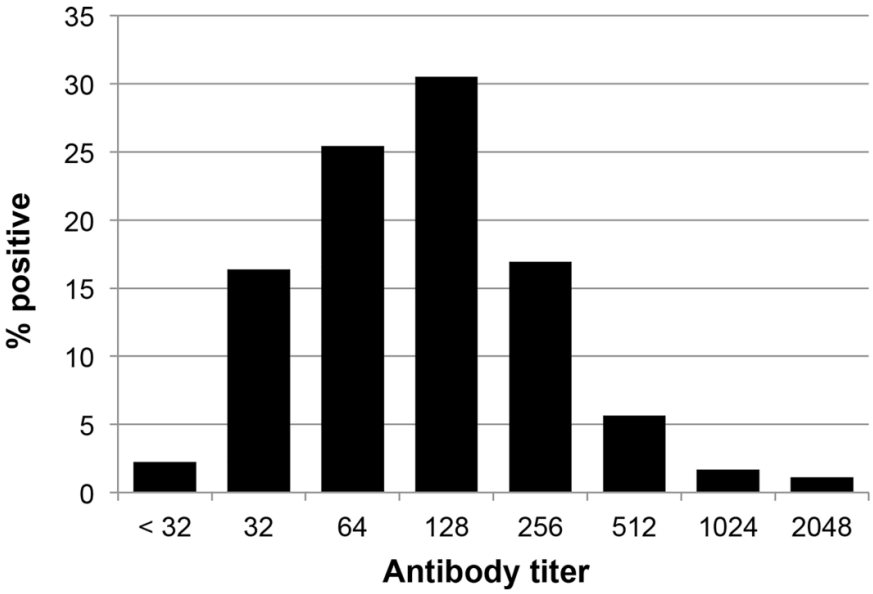

Fig 1. Distribution of *B. bacilliformis* antibodies by IFA assay among the study population (n = 177).

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for IgG anti-GroEL, but no other specific association was observed between symptoms and other IgM/IgG positive status.

Fig 5 shows the IgM and IgG levels for antigenic candidate ELISAS for all the study population (Fig 5A). Moreover, Fig 5B and 5C show the IgM results of post-outbreak localities for each antigen ELISA according to the IFA and RT-PCR results (statistically significant differences for SCS-α and SCS-β according IFA results and Pap31 according RT-PCR results). No significant differences were found for IgG when the IFA or RT-PCR results were taken into account. Fig 5D and 5E represent the results of the antigenic candidate ELISAs according to the whole cell *B. bacilliformis* ELISA assays for the post-outbreak areas (Fig 5D) and the endemic area (Huancabamba) (Fig 5E). It is of note that in the case of IgM, concordance

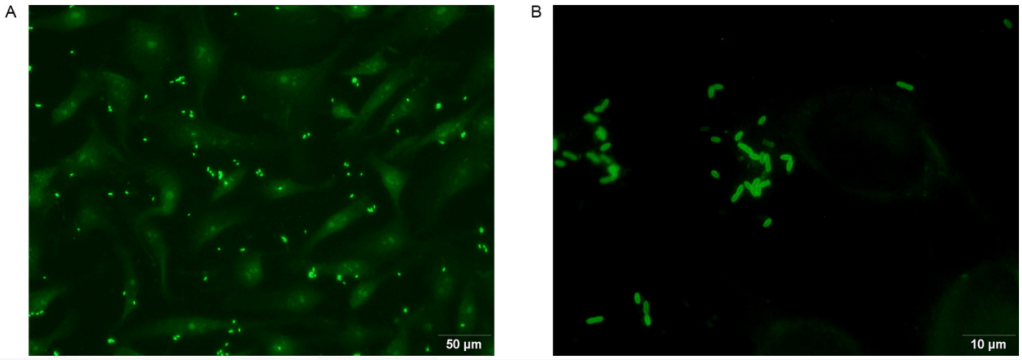

Fig 2. Example of a positive IFA test. A) 20X objective and 10X oculars. B) 100X objective and 10X oculars.

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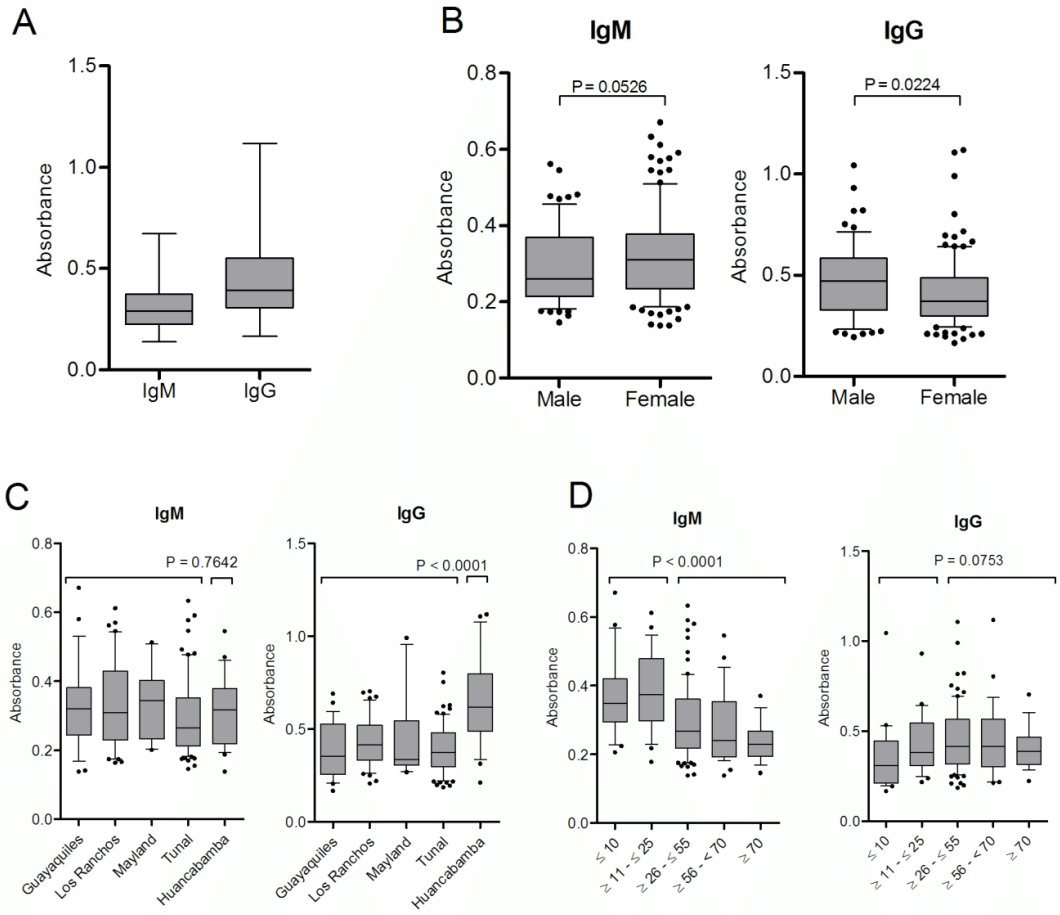

Fig 3. IgM and IgG antibodies in the study population. A) Overall data. Data are presented as boxplots that illustrate the medians and the maximum and minimum. B) IgM and IgG distribution by sex; C) IgM and IgG distribution by locality. *P*-values obtained on comparing the 4 post-outbreak areas with the endemic area. When comparing pairs of localities we obtained significance for IgG with the following analysis: Guayaquiles and Huancabamba ($P = 0.0001$); Los Ranchos and Huancabamba ($P = 0.0002$); Mayland and Huancabamba ($P = 0.0201$); Tunal and Huancabamba ($P<0.0001$). D) IgM and IgG distribution by age groups. *P*-values obtained comparing the antibody levels of subjects between $\leq$ 25 years with $\geq$ 26 years. In panels B, C and D the data are presented as boxplots that illustrate the medians and the 25th and 75th quartiles, and the whiskers represent the 10% and 90% percentiles. Outliers are marked with circles. *P*-values were calculated using the Mann-Whitney test.

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between the ELISAs for antigenic candidates and the ELISA for whole cell *B. bacilliformis* was very high and ranged from 98.3 (59/60) to 100% (60/60). By comparing the antigenic candidates ELISA results between the post-outbreak villages with the endemic area, only the IgG levels of SCS-α were significantly higher in the post-outbreak areas than in Huancabamba ($P = 0.0225$).

It seems that the positive samples for IgM ELISA of any of the antigenic candidates tested show a positive correlation with the remaining candidates (*P* varies from <0.0001 for SCS-α / GroEL to 0.0041 for SCS-β / GroEL). Regarding IgG ELISA of the antigenic candidates, only GroEL did not show a significant association with the remaining antigenic candidates. Nonetheless, a possible bias related to the antigenic-candidates selection process cannot be ruled out,

Table 5. Distribution of the positive individuals for each diagnostic technique used according to the symptoms described.

	N (%)*	RT	ELISA_IgM	ELISA_IgG	IFA
		n (%)*	n (%)*	n (%)*	n (%)*
Headache	48 (27.1)**	23 (47.9)	10 (20.8)	13 (27.1)	15 (31.3)
Joint pain	10 (5.6)	3 (30.0)	2 (20.0)	2 (20.0)	4 (40.0)
Fever	8 (4.5)	4 (50.0)	2 (25.0)	3 (37.5)	4 (50.0)
Malaise	8 (4.5)	4 (50.0)	2 (25.0)	3 (37.5)	4 (50.0)
Myalgia	8 (4.5)	4 (50.0)	2 (25.0)	4 (50.0)	3 (37.5)
Pallor	5 (2.8)	4 (80.0)	2 (40.0)	1 (20.0)	4 (80.0)
Peruvian wart	1 (0.6)	0	0	1 (100.0)	0
Any symptom	60 (33.9)	27 (45.0)	13 (21.7)	17 (28.3)	18 (30.0)

* percentage in the population (considering n = 177).

** 6 more individuals presented headache but were not considered in this table because only the blood sample were collected.

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and thus a posterior analysis using samples from different endemic areas are needed to perform an in depth analysis.

Discussion

Currently, early diagnosis of Carrion's disease remains an unsolved problem due to the socio-geographical context of the endemic areas; the illness is present in poor, rural and isolated areas with precarious access, with neither the necessary equipment nor qualified personnel to perform molecular/immunological techniques. In endemic areas, the diagnosis is usually based on clinical symptoms or thin blood smear, an expertise-dependent methodology with 96% of specificity but very low sensitivity (24–36%) [19]. This problem is further enhanced by the unspecific initial symptoms of Carrion's disease, common to a several pathologies existing in these areas, such as dengue and other arboviral diseases, malaria or tuberculosis [7,20]. All of this leads to misdiagnosis of patients [20,21] and the non treatment of asymptomatic carriers, thereby perpetuating disease transmission. The present data highlight the non-concordance between symptomatology and antibody levels. This is a relevant fact emphasizing again the challenges that exist in diagnosing the illness clinically. Bacterial culture is not clinically useful due to the culture requirements and the slow bacterial growth rate [6,20]. Molecular and serologic tools, like PCR or IFA are able to detect acute cases more efficiently but are very difficult to be implemented in routine practice in remote endemic rural areas [1,8,22]. The use of sensitive and specific rapid diagnostic tools is a way to overcome these limitations.

Table 6. Concordance among the techniques used.

		RT-PCR		IFA		ELISA IgM	
		N (%)		N (%)		N (%)	
		+	-	+	-	+	-
IFA	+	20 (11.3)	24 (13.6)				
	-	53 (29.9)	80 (45.2)				
ELISA IgM	+	24 (13.6)	36 (20.3)	14 (7.90)	46 (26.0)		
	-	49 (27.7)	68 (38.4)	30 (16.9)	87 (49.2)		
ELISA IgG	+	19 (10.7)	29 (16.4)	9 (5.1)	39 (22.0)	19 (10.7)	29 (16.4)
	-	54 (30.5)	75 (42.4)	35 (19.8)	94 (53.1)	41 (23.2)	88 (49.7)

Only individuals with both blood and serum samples were considered (n = 177).

doi:10.1371/journal.pntd.0004989.t006

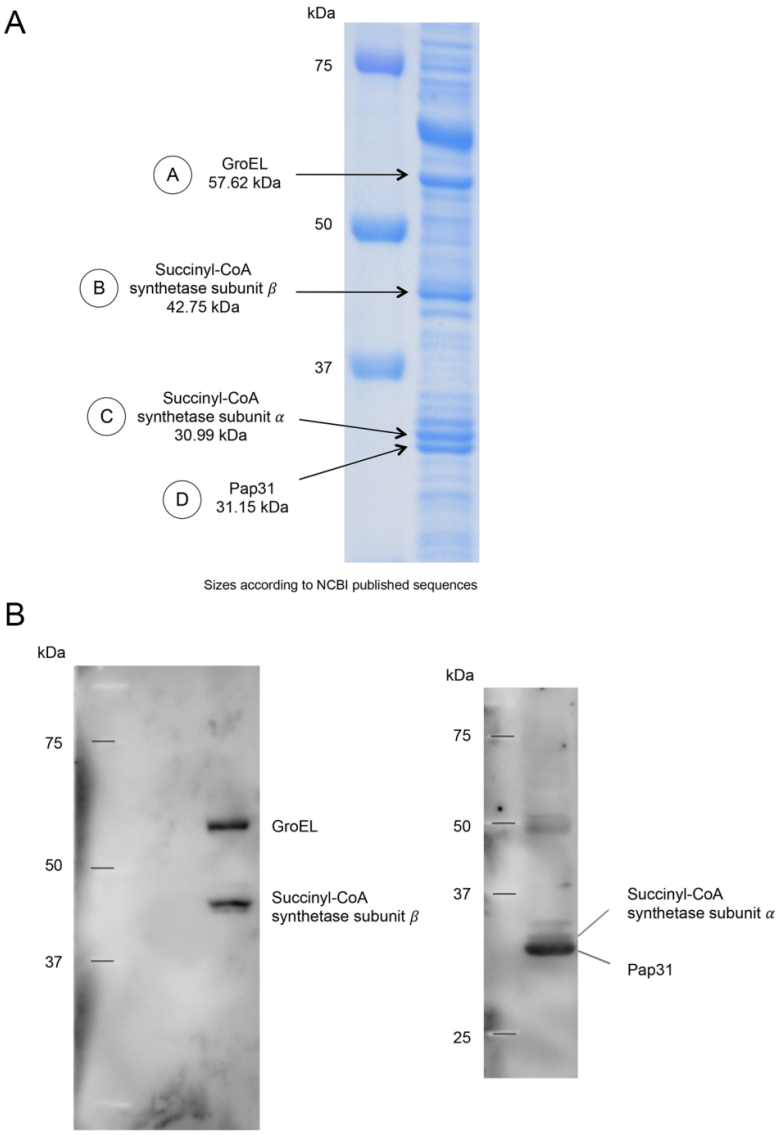

Fig 4. Antigenic candidates of *B. bacilliformis* selected. A) SDS gel with sonicated whole cell *B. bacilliformis*. B) Example of a Western blot with a positive serum by ELISA. The left panel corresponds to IgG and the right panel to IgM.

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Chamberlin *et al.* described that 82% of acute confirmed cases and 91% of convalescents showed a positive IFA test, while 74% of the volunteers with a positive IFA test had had Bartonellosis within the last year. This percentage decreased to 40% when a more distant or a non-bartonellosis episode was reported [8]. Accordingly, the IFA positivity from the samples collected in the established endemic area in our study were the lowest at 12.5%. Nonetheless, only 26.8% of the individuals from post-outbreak zones had a positive result in the IFA test. This,

Table 7. N-terminal sequencing results.

Protein*	N-terminal sequence	Molecular Mass (KDa)	Homologous Protein	Identity** (%)	Accession number	Organism
A	AAKEVKFGRDARERL	57.52	GroEL	100.0	CH60_BARBK	***Bartonella bacilliformis***
		57.62	GroEL	100.0	CH60_BARBA	***Bartonella bacilliformis***
		57.62	GroEL	93.3	CH60_BARHE	*Bartonella henselae*
		57.61	GroEL	93.3	CH60_BARQU	*Bartonella quintana*
		57.60	GroEL	93.3	CH60_BART1	*Bartonella tribocorum*
B	MNIHEYQAKRLLHEY	42.74	SCS-β	100.0	SUCC_BARBK	***Bartonella bacilliformis***
		42.82	SCS-β	100.0	SUCC_BARHE	*Bartonella henselae*
		43.00	SCS-β	100.0	SUCC_BARQU	*Bartonella quintana*
		42.87	SCS-β	100.0	SUCC_BART1	*Bartonella tribocorum*
		42.53	SCS-β	93.3	SUCC_BRUAB	*Brucella abortus*
		42.53	SCS-β	93.3	SUCC_BRUA1	*Brucella abortus*
		42.53	SCS-β	93.3	SUCC_BRUC2	*Brucella canis*
		42.53	SCS-β	93.3	SUCC_BRUMB	*Brucella melitensis*
		42.53	SCS-β	93.3	SUCC_BRUME	*Brucella melitensis*
		42.53	SCS-β	93.3	SUCC_BRUO2	*Brucella ovis*
		42.53	SCS-β	93.3	SUCC_BRUSU	*Brucella suis*
		42.53	SCS-β	93.3	SUCC_BRUSI	*Brucella suis*
		42.52	SCS-β	93.3	SUCC_OCHA4	*Ochrobactrum anthropi*
C	SILINKDTKVLVQGL	30.93	SCS-α	100.0	WP_024847155	*Aminobacter* spp.
		30.99	SCS-α	100.0	BG36_06620	*Aquamicrobium defluvii*
		32.92	SCS-α	100.0	B9JCF2_AGRRK	*Agrobacterium radiobacter*
		30.90	SCS-α	100.0	B9JTS6_AGRVS	*Agrobacterium vitis*
		31.07	SCS-α	100.0	E6YT34_9RHIZ	*Bartonella* spp.
		30.99	SCS-α	100.0	E6YXC2_9RHIZ	*Bartonella* spp.
		30.99	SCS-α	100.0	WP_035454724	***Bartonella bacilliformis***
		30.99	SCS-α	100.0	A1UQW0_BARBK	***Bartonella bacilliformis***
		31.01	SCS-α	100.0	E6YNR1_9RHIZ	*Bartonella rochalimae*
		30.96	SCS-α	100.0	WP_026620040	*Ensifer* spp.
		30.90	SCS-α	100.0	A9DG17_HOEPD	*Hoeflea phototrophica*
		31.08	SCS-α	100.0	WP_027835951	*Maritalea myrionectae*
		30.97	SCS-α	100.0	WP_028751285	*Rhizobium leucanae*
		30.99	SCS-α	100.0	L0LRG5_RHITR	*Rhizobium tropici*
		30.90	SCS-α	100.0	WP_026615514	*Sinorhizobium* spp.
		30.93	SCS-α	100.0	WP_028001748	*Sinorhizobium arboris*
		30.87	SCS-α	100.0	G9A287_RHIFH	*Sinorhizobium fredii*
		30.97	SCS-α	100.0	I3XDY7_RHIFR	*Sinorhizobium fredii*
		30.91	SCS-α	100.0	A6UDP1_SINMW	*Sinorhizobium medicae*
		30.93	SCS-α	100.0	F7X0D4_SINMM	*Sinorhizobium meliloti*
D	ADVMIPQEISPIISA	31.70	Pap31	100.0	Q3HM50_BARBA	***Bartonella bacilliformis***
		31.16	Pap31	100.0	WP_035454931	***Bartonella bacilliformis***
		31.61	Omp25	100.0	A1UU13_BARBK	***Bartonella bacilliformis***

* As indicated in Fig 4.

** Only those with identity levels > 90%.

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Table 8. Positive prevalence for the antigenic candidate ELISAs.

	SCS-α*	Pap31	SCS-β*	GroEL
	N (%)	N (%)	N (%)	N (%)
IgM	137 (77.4)	172 (97.2)	168 (94.9)	138 (78.0)
IgG	61 (34.5)	104 (58.8)	106 (59.9)	70 (39.5)

*Succinyl-CoA synthetase. Cut offs considered: SCS-α (IgM) = 0.076; SCS-α (IgG) = 0.130; Pap31 (IgM) = 0.088; Pap31 (IgG) = 0.212; SCS-β (IgM) = 0.056; SCS-β (IgG) = 0.107; GroEL (IgM) = 0.093; GroEL (IgG) = 0.400

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together with Chamberlin's results, suggest that IFA positivity peaks just after clinical resolution and slowly decreases thereafter. However, other factors could affect the final IFA positivity and should be taken into account, including the *B. bacilliformis* strain used for its development, and its specific levels of antigen expression as well as the subjectivity associated with the reading of the slides. In fact, in a previous study developed by Chamberlin *et al*, differences were observed in IFA positivity when two different *B. bacilliformis* strains were used [8].

Previous studies have explored the use of PCR techniques to diagnose the acute phase of Carrion's disease. Thus, del Valle *et al* [22] detected 21 acute Carrion's disease cases analyzing 113 previously diagnosed as negative patients using a *Bartonella*-specific *16S rRNA* based PCR. Nonetheless, further studies showed the potential limitation of this technique to detect asymptomatic carriers [23]. The use of RT-PCR has also been studied [16], 14 positive acute being detected cases among 63 previously negative children. It has been shown that RT-PCR has a higher sensitivity than classical PCR procedures [24], but to the best of our knowledge the use of RT-PCR to detect asymptomatic *B. bacilliformis* carriers has not been performed. Nonetheless, the positive results observed were close to the detection limit of the technique, showing that the bacterial burden present in the blood of asymptomatic carriers may be extremely low, suggesting the possible presence of bacteria in specific tissues which remain apparently undetected in blood over long periods of time. Although all volunteers received a 14-day course of ciprofloxacin during the outbreak, it is noteworthy that there was still a very high number of confirmed asymptomatic carriers by RT-PCR. This suggests that the short time elapsed since the outbreak might have favored the high number of positive samples by RT-PCR. Despite a case in which the duration of asymptomatic bacteremia was up to 3 years [25], there is a lack of in depth studies about bacteremia in asymptomatic carriers. In any case all these results highlight the enormous relevance of the need for techniques which may be implemented in endemic areas to detect asymptomatic carriers in order to adequately control and possibly eradicate this illness.

ELISA data showed high levels of antibodies against *B. bacilliformis*, which was expected because an outbreak had occurred several months earlier in 4 out of 5 of the villages analyzed. It is known that inhabitants of endemic areas develop partial immunity due to exposure to *B. bacilliformis* [5]. Accordingly, the inhabitants from the long established endemic infected area showed significantly higher IgG levels when compared with the other 4 non-endemic villages together or with each of the post-outbreak villages. Similarly, IgM and IgG levels were age-dependent suggesting the development of partial immunity throughout life. The IgM levels were significantly higher when comparing individuals $\leq$25 years-old with adults $\geq$26 years. Moreover, the IgG levels were significantly lower for the age group $\leq$10 years-old when compared with each of the age groups of adult population ($\geq$11 - $\leq$25; $\geq$26 - $\leq$55 and $\geq$56 - <70) with the exception of individuals $\geq$70 years. Regarding the differences in the antibody levels by gender, the IgG levels were higher in men probably because men often move to the neighboring

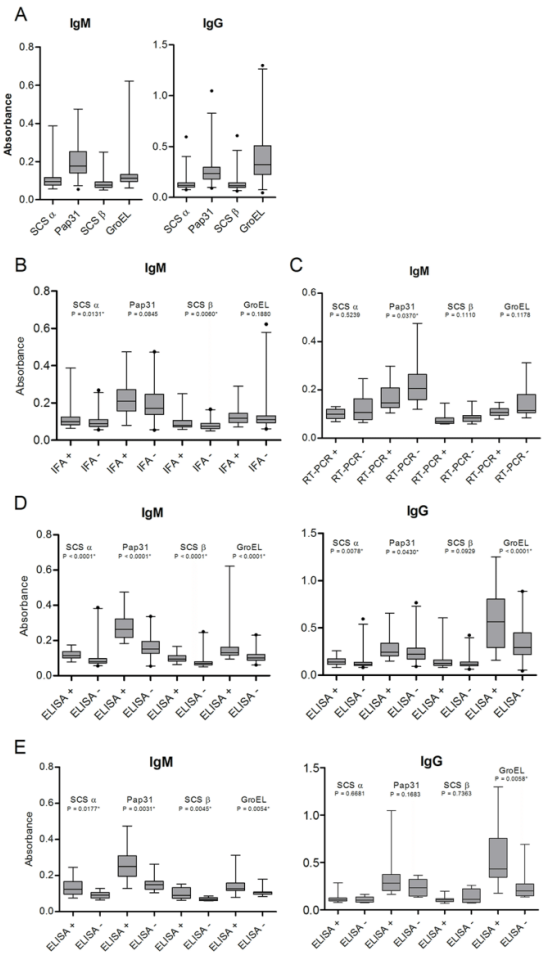

Fig 5. IgM and IgG levels for antigenic candidate ELISAs. A) Overall IgM and IgG data for the study population. Data are presented as boxplots that illustrate the medians and the 1 and 99% percentiles. Outliers are marked with circles. B) IgM ELISA results of post-outbreak localities for SCS-α, Pap31, SCS-β and GroEL according to IFA results. C) IgM ELISA results of post-outbreak localities for SCS-α, Pap31, SCS-β and GroEL according to RT-PCR results. D) IgM and IgG ELISA result of post-outbreak population for SCS-α, Pap31, SCS-β and GroEL taking into account the whole cell *B. bacilliformis* ELISA assay. E) IgM and IgG ELISA result for the endemic area (Huancabamba) for SCS-α, Pap31, SCS-β and GroEL according to the whole cell *B. bacilliformis* ELISA assay. For B, C, D and E data are presented as boxplots that illustrate the medians and the 25th and 75th quartiles, and the whiskers represent the 1% and 99% percentiles. Outliers are marked with circles. *P*-values were calculated using the Mann-Whitney test.

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endemic areas such as Cajamarca department (San Ignacio area) to work in the coffee bean harvesting. The relation between coffee plantations and the presence of *Lutzomyia* spp. has already been reported [26].

The presence of antibodies and the blood carriage of *B. bacilliformis* open the door to different possibilities such as the bacterial evasion of the host immune system by changes in the expression of bacterial epitopes which may correlate with decreased ability to invade erythrocytes and facilitate bacterial trapping in other tissues.

In the present study we have identified four potential antigenic candidates, two of which have previously been reported in the literature, GroEL and Pap31 [9,10,13], while the remaining two are described for the first time. These new antigenic candidates, SCS-α and SCS-β are involved in the tricarboxylic acid cycle, an important cytosolic metabolic pathway. Indeed, subunit α has been described as an immunogenic protein of *Brucella melitensis* [27], and the β subunit was recently reported in *Bartonella henselae* pathogenesis [28].

Pap31 or Hemin-binding protein A has shown to be a good candidate in the ELISA technique and is highly expressed in *B. bacillifomis* cultures [13]. Furthermore, Pap31 is an outer membrane protein that seems to be good candidate for the development of serodiagnostic tools for *Bartonella* infections, as proposed for *Bartonella quintana* infections [17].

Knobloch *et al.* [10] described that GroEL, a heat shock protein, never bound to *Bartonella* IgM antibodies, suggesting that GroEL may be a good antigenic candidate for the chronic phase. Similarly, in the present study GroEL was identified as reactive with IgG. However, we were also able to detect IgM anti-GroEL. Differences in the time elapsed from the infection and the sample collection may explain this finding. GroEL is present in the outer and inner membranes of *B. bacilliformis*, having also been detected in *B. bacilliformis* supernatants. Its presence is correlated with mitogenic activity against human vascular endothelial cells which leads to the development of verrucous lesions [29]. This mitogenic activity is inhibited *in vitro* by the presence of specific anti-GroEL antibodies, suggesting the protective role of specific IgG in asymptomatic carriers. Indeed, the presence of specific IgG anti-GroEL was observed in the volunteer presenting a Verruga Peruana. GroEL has also been described as a good candidate for vaccine production and the development of diagnostic kits for *Brucella melitensis* [30].

The strong correlation between the positive results for IgM ELISAs anti-*B. bacilliformis* whole cell and positive results for antigenic candidates, suggests that the antigens can be used indistinctly in a rapid diagnostic test. These are preliminary results and more studies should be done to characterize these *B. bacilliformis* antigens.

To the best of our knowledge, to date only 5 antigenic candidates of *B. bacilliformis* have been described in the literature. In this study we have identified 2 new antigens of *B. bacilliformis*. These kind of antigens are of special relevance in the development of new, easy and cheap rapid diagnostic tools, able to be implemented in remote rural areas without the need for specific expertise. The diagnosis and treatment of both patients and asymptomatic carriers, who continue to perpetuate the illness, is crucial to reduce the burden of this disease. Therefore, studies characterizing antigens expressed during *B. bacilliformis* infection are fundamental to elucidate the pathogenesis of this disease and may be useful for the development of a rapid diagnostic tool, absolutely necessary to advance towards Carrion's disease eradication.

Supporting Information

S1 Fig. The figure shows the geographical localization (district, province and department) of the study area. Three of the villages (Tunal, Guayaquiles and Mayland) are located within the Lalaquiz district, while Los Ranchos is in Canchaque district, and Huancabamba is in the homonymous district.
(TIF)

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Author Contributions

Conceived and designed the experiments: CGo JR MM.

Performed the experiments: CGo NP CTV MJP IS.

Analyzed the data: CGo AMT JR MM.

Contributed reagents/materials/analysis tools: JdVM CGu.

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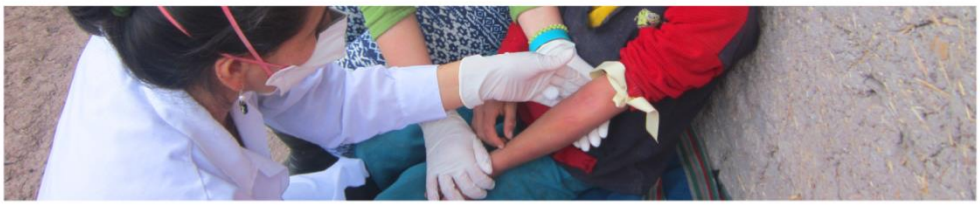
DISCUSSION



Carrión's disease is an endemic overlooked illness that affects rural areas of the Andes in Peru. This thesis was focused on two main issues: antimicrobial resistance (Chapter I) and the diagnosis of Carrión's disease (Chapter II).

CHAPTER I – ANTIMICROBIAL RESISTANCE

The current clinical guidelines on Carrión's disease include the administration of ciprofloxacin and azithromycin for the treatment of the acute and chronic phases of the disease, respectively. However, before this guideline was established, chloramphenicol and rifampicin, respectively, were the treatments of choice. Clinical failure with chloramphenicol and the presence of tuberculosis in endemic zones of Carrión's disease are the reasons for these changes [297]. Information about the mechanisms of antimicrobial resistance in *B. bacilliformis* is scarce and is mainly focused on constitutive quinolone resistance. Indeed, studies analyzing the quinolone resistance-determining region of several *B. bacilliformis* clinical isolates, including those recovered prior to the introduction of quinolones to clinical practice, showed an alanine at both position 91 of GyrA and position 85 of ParC which are responsible for the intrinsic resistance of *B. bacilliformis* to quinolones [226, 264]. This characteristic is not exclusive of *B. bacilliformis*. It has also been reported in other members of the *Bartonella* genus from which the DNA sequence encoding GyrA has been determined [261] as well as in other microorganisms having constitutive resistance to quinolones such as *Brevundimonas diminuta* [298]. This could explain the microbiological failure and the high number of asymptomatic carriers found in endemic areas. Moreover, persistent bacteremia has also been described in 50% of patients after chloramphenicol treatment [229]. This latter finding should be confirmed by additional studies. However, there is evidence that although *B. bacilliformis* resistance is rare and antibiotics continue to be effective, the guidelines for the treatment of Carrión's disease should be carefully revised and supported with further molecular studies and clinical assays.

In the first chapter of this thesis we published a study aimed to develop and analyze *B. bacilliformis* mutants highly resistant to the main antimicrobials used in the treatment of Carrión's disease. We did not limit our study to the antibiotics reported in the current guidelines because we are aware that other treatments are also used [105], instead we included the most common treatments to provide a broader view of the *in vitro* development of resistance in *B. bacilliformis*. To my knowledge only 3 previous studies have developed *B. bacilliformis* mutants, and only with the antibiotic

pressure of coumermycin, ciprofloxacin, rifampicin and erythromycin. In addition, these reports only used two well established collection strains (KC583 or KC584) alone and were limited to the search for point mutations [238, 265, 266]. In this thesis we wanted to go one step further, and in addition to developing resistant mutants to the 4 main antibiotics used in the treatment of Carrión's disease, we also evaluated the stability of antibiotic resistance, determined the possible presence of cross resistance between antibiotics and the role of efflux pumps in resistance. Moreover, we used 3 different strains (CIP 57.18; CIP 57.19; CIP 57.20) to provide new genetic variability to these studies. Thus, highly resistant mutants were obtained for azithromycin, rifampicin, ciprofloxacin and chloramphenicol highlighting the ability of *B. bacilliformis* to become resistant to the principal antibiotics used in the treatment of Carrión's disease. Our results showed that the antibiotic which most easily selected resistant mutants was ciprofloxacin while the selection of resistant mutants was the most difficult with chloramphenicol. This is in agreement with the fact that *B. bacilliformis* is intrinsically resistant to quinolones [226]. These findings also agree with a study analyzing the antibiotic susceptibility of clinical isolates of *B. bacilliformis* [233] in which it was found that among 100 strains of *B. bacilliformis* isolated from patients in endemic zones from 2005 to 2011, 26% and 1% were resistant to ciprofloxacin and chloramphenicol, respectively.

Alterations in the antibiotic-target genes were a common resistance mechanism found in 11 out of the 12 mutants obtained. The majority of amino acid substitutions, insertions or deletions observed have been previously described to confer antibiotic resistance at the same or equivalent position in other microorganisms, such as *E. coli*, *Streptococcus pneumoniae, M. tuberculosis*, or *Bartonella* spp. [238, 266, 269, 270, 273, 276, 299-304]. Nevertheless, we also found new deletions and insertions in the L4 and L22 proteins, respectively for azithromycin mutants. While the deletions located at L4 were in the same regions as those described in other microorganisms [305], to my knowledge, no insertion has previously been described at the equivalent point of L22 in other microorganisms. In the case of ciprofloxacin it is of note that with the presence of a mutation at codon 91 of the *gyrA* gene, the resulting amino acid (valine) greatly affects the hydrophobic pattern of GyrA and the interaction with ciprofloxacin. The two other mutations selected, affecting either GyrA or GyrB, are perhaps more advantageous to the bacteria because of the additive effect of the quinolone-resistance mechanisms [251]. Higher levels of quinolone resistance are probably conferred with these mutations by adding an alteration at a new position rather than changing the amino acid at position 91.

It is interesting to note that the resistance selected in the azithromycin mutants was always associated with the L4 and L22 proteins while no mutation at *23S rRNA* was observed. This may be related to a decrease in fitness associated with alterations in *23S rRNA*, as proposed for other microorganisms [301, 306]. However, this differs from findings in microorganisms with a low number of *23S rRNA* gene copies [307] and previous studies performed with *B. bacilliformis* and *B. henselae* [238, 269, 270]. Contrary to what has been described in azithromycin mutants, substitutions in *23S rRNA* were found for 2 out of 3 chloramphenicol mutants. However, for both mutants the *23S rRNA* substitution reverted, and the resistance levels substantially fell in the 3 mutants after passages without antibiotic pressure, showing a rapid reversion of this mutation. In fact, mutations at the *23s rRNA* gene or in the ribosomal proteins involved in the development of chloramphenicol resistance are rarely found [249]. It has been suggested that structural changes at the peptidyl transferase center that might prevent chloramphenicol binding are incompatible with satisfactory ribosome function [249]. This involvement at the fitness level is also coherent with the enhanced difficulty to select chloramphenicol resistance and with the high persistence of bacteremia following chloramphenicol treatment [229]. Regarding the stability of the other antibiotics, we observed that the stability of the resistance of mutants selected with rifampicin remained constant, emphasizing the serious risk of selection of stable rifampicin resistance during treatment.

As mentioned above rifampicin was largely used in the treatment of the chronic phase, but as far as I know data on therapeutic failure are scarce. Maguiña *et al.* [105] reported poor and fair response of 4% and 15% of patients during rifampicin treatment respectively. Nonetheless, it should be taken into account that the treatment of the chronic phase is relatively long, and since most patients live in remote areas of difficult access, follow up is infrequent. Moreover, the chronic phase rarely results in death, generally spontaneously reverts and subsequently is not perceived as a relevant health problem by the inhabitants of endemic areas. Notwithstanding, it is these patients together with asymptomatic carriers who make up the bacterial reservoir.

Considering the important contribution of efflux mechanisms to antibiotic resistance we evaluated 2 efflux pump inhibitors. Efflux pump inhibitors have been widely sought as adjuvants to potentiate conventional antibiotics, being PAβN one of the most studied efflux pump inhibitors [308]. PAβN competitively inhibits efflux by competing with efflux pump substrates [309], but it also permeabilizes bacterial membranes

[308]. The other efflux pump inhibitor studied was artesunate, a derivate of artemisinin. Artesunate inhibits efflux pumps by increasing antibiotic accumulation via inhibition of the multidrug efflux pump system AcrAB-TolC, which is an important multidrug efflux system for Gram-negative bacteria (belonging to the RND family) (Figure 24). The mRNA expression of AcrAB-TolC is reduced, enhancing the susceptibility of *E. coli* to β-lactams by increasing the accumulation of the antibiotic [286]. It is of note that the RND family is an important efflux pump that induces the multiple antibiotic resistance of Gram-negative bacteria [310]. Although our results showed that highly resistant mutants of *B. bacilliformis* can be obtained mainly by mutations in the target genes, resistance was also mediated by efflux pumps. Thus, in half of the mutants the susceptibility levels decreased in the presence of the PAβN and/or artesunate, demonstrating that efflux pumps play a role in the acquisition of antimicrobial resistance by *B. bacilliformis*. Moreover, the effect of efflux pumps on the acquisition of antibiotic resistance was observed in mutants selected with the different type of antibiotics under study, demonstrating that these 4 antimicrobial compounds can be extruded from the bacteria and that efflux pump inhibitors may be useful to potentiate the effect of antibiotics in Carrión's disease.

Albeit inconstant, cross effects were observed among the different mutants emphasizing the risk of co-selection of antibiotic resistance during the treatment of Carrión's disease. Taking into account that the 3 antibiotics used act at the ribosomal level, it was expected that ribosomal alterations may differentially affect other agents. For example, concomitant resistance to chloramphenicol and macrolides had already been described in *E. coli* and *S. pneumoniae* [305, 311]. The cross effect may not be directly related to the mechanisms of resistance detected but rather to the expression of different genes leading to alterations in permeability, growth rates or the overexpression of efflux pumps, among others, being reflected by slight alterations in resistance to other antimicrobials.

Overall, and taking into account the results presented in chapter I of this thesis, it seems that the most useful antibiotics to treat the acute and chronic phases of Carrión's disease should be chloramphenicol and azithromycin, respectively. However, further studies are needed to achieve definitive conclusions and to seek solutions for more effective treatment schedules.

Additionally, the present data along with information regarding antibiotic resistance in clinical isolates, antibiotic treatment failure and social attitudes, among others, may

facilitate the design of mass antibiotic treatment such as that described for trachoma or yaws [312, 313]. In the absence of a vaccine and considering the possibility of outbreaks beyond the Peruvian borders, mass treatment together with educational programs promoting self-protection such as the use of impregnated nets could be a possible approach for the eradication of Carrión's disease.

CHAPTER II – DIAGNOSIS

This chapter addresses the diagnosis of Carrión's disease. As previously mentioned throughout the thesis, the current diagnostic techniques are not sufficiently effective, and misdiagnosis is frequent. This is due to the lack of a reliable, rapid method to perform the diagnosis and also because of the unspecific symptoms of Carrión's disease. Indeed, in endemic zones the febrile syndromes may be related to a series of different bacterial, viral or parasitic pathogens, such as *Plasmodium* spp*.,* dengue or *Salmonella typhi*. Besides the clinical presentations and symptoms, the diagnosis of Oroya fever is mainly based on a blood smear stained with Giemsa and observed by microscopy. However, it is important to point out that expertise is needed to perform this technique, particularly in cases of low bacteremia. Furthermore, this situation becomes complicated when outbreaks in new areas occur because health personnel are less familiarized with the characteristics and diagnosis of Carrión's disease. In this respect it should be highlighted that the first cases of Carrión's disease in some areas of the Cusco department were diagnosed as hepatitis, leading to 12.2% of lethality due to inadequate treatment [28]. Similarly, in 2006 a case of Carrión's disease in Lima had a fatal outcome due to an incorrect diagnosis and was largely discussed in the local daily press [314-316]. The patient was presumably infected in Huarochiri, in the area of the Santa Eulalia river at approximately 40 Km from Lima [317].

All of the above has been fully covered in the first paper of this chapter in which a misdiagnosed Oroya fever outbreak is described. Our results showed the presence of *S. faeni* as the causative agent of the reported outbreak, and no evidence of *B. bacilliformis* was found. We hypothesized that the outbreak was mistakenly attributed to Carrión's disease due to the proximity with endemic areas as well as positive blood smears. To my knowledge this was the first outbreak caused by *S. faeni* and the first community-acquired outbreak related to *Sphingomonas* spp. This microorganism has an environmental nature and the results strongly suggested the association of the outbreak with the water consumed and aquatic activities. Why this *S. faeni* strain

caused an outbreak remains unclear. Nonetheless, we can speculate that some virulence factor(s) may have been acquired. On one hand, this contention is supported by the close relationship of *S. faeni* with *Sphingomonas paucimobilis* [318], which is able to infect non-compromised patients [319]. On the other hand, *Sphingomonas* spp. are considered potential virulent bacterial pathogens, possessing several virulence factors such as those related to the intracellular capacity of *Brucella* spp. [320]. The presence of these virulence factors in the *S. faeni* strain causing this outbreak could have facilitated erythrocyte adhesion or invasion leading to misdiagnosis. It has been proposed that the virulence factors in *Sphingomonas* spp. is acquired by horizontal gene transfer rather than the sharing of a common pathogenic ancestor with related microorganisms [320]. Moreover, the ability of interaction and entry into epithelial cells *in vitro* has also been described in *Sphingomonas* spp. [321]. As mentioned above, the lack of unique clinical characteristics, together with the absence of an efficient diagnostic tool and the high rotation of physicians in endemic areas can lead to a fatal outcome when a patient with Carrión's disease is misdiagnosed. The present data highlight the inverse problem that could also lead to inadequate treatment and serious problems. Fortunately, this was not the case, and the patients fully recovered. Nonetheless, these results demonstrate the imperative need for an efficient diagnostic tool allowing the identification of *B. bacilliformis* and correct diagnosis of Carrión's disease in Peru.

Molecular techniques are efficient tools for the diagnosis of Carrión's disease. For instance, a quantitative RT-PCR [203] and loop-mediated isothermal amplification have recently been described [206]. The determination of the detection limits of these techniques is extremely valuable since the bacterial load during *B. bacilliformis* varies according to the stage of the disease and also seems to vary among individuals, with infected erythrocytes ranging from 2 to 100% [105]. For instance, the loop-mediated isothermal amplification showed a detection limit of 1 and 10 copies/µL using bacterial genomic DNA alone or in the presence of human plasma, but the usefulness of this technique remains to be validated in human clinical samples. Despite the higher sensitivity of RT-PCR over the classical PCR procedures [188], conventional PCR is the most commonly used molecular technique to test clinical samples in Peru. Thus, in the second study of this chapter we evaluated the detection limit of 3 conventional PCR approaches used to detect *B. bacilliformis*. At present, the *16S rRNA* PCR approach is used in Peru for the diagnosis of Carrión's disease and in the study by del Valle *et al*. 21 acute cases of Carrión's disease were detected among 113 samples previously diagnosed as negative using blood smear [41]. With this

approach and according to an *in silico* analysis all *Bartonella* spp. could be amplified. Nonetheless, DNA sequencing is necessary to confirm the species identity. With the exception of *B. clarridgeiae*, the amplification of the *fla* gene allows the 3 *Bartonella* causing Carrión's disease to be distinguished from the remaining *Bartonella* spp. which do not possess flagella. Regarding *its,* this region has been proposed for the detection of DNA of *Bartonella* spp. in clinical samples due to the distinct sizes detected in the sequences among *Bartonella* spp. allowing the detection of different species within this genus [322]. Our results showed a detection limit of 5 CFU/µL for both the *16S rRNA* and the *fla* PCRs, increasing to 500 CFU/µL for *its* amplification. This sensitivity probably allows the diagnosis of acute cases of Carrión's disease, in which the mean percentage of erythrocytes infected is 61% [105]. In addition, a good specificity was found since none of the non-*Bartonella* microorganisms included in the study was amplified by the *fla* or *its* PCRs, and in the case of the *16S rRNA* only *B. melitensis* was amplified. Thus, the diagnosis should be performed in an adequate clinical context and the nonspecific amplification of other related species should be taken into account, especially in cases with immunosuppression and environmental samples since the majority of the related microorganisms are not human pathogens. For example, *Mesorhizobium* spp. could show a positive result in the *its* PCR [322]. A combination of the 3 PCR approaches will certainly provide more information about the infection and should be encouraged.

PCRs are usually carried out at the laboratories of the National Institute of Health or in a reference laboratory in the principal cities of Peru. One of the problems is sample transportation from the endemic areas where the samples are collected to the laboratories where the molecular techniques are performed. Moreover, to my knowledge the samples are not usually sent one by one but rather collected over time until several samples are available in order to reduce the associated shipment costs. Thus, transportation delays the diagnosis which is also associated with the time needed for the laboratory to undertake the molecular techniques and subsequently several months may be required to achieve definitive results. Thus, filter papers with DBS may be a good solution to overcome these problems in sample collection and transportation [323]. In addition, the collection volume is considerably less which should be taken into account in both the pediatric setting and anemic patients. The sensitivity and specificity of DBS in the diagnosis of tropical diseases are similar or only slightly inferior to gold standard samples [323]. Indeed, DBS have been recommended for the diagnosis of several infections (both by molecular or serological techniques) such as malaria, HIV, *Haemophilus influenzae* or rickettsial diseases

[324-328]. In light of the above we decided to test the efficacy of the same PCR approaches starting with DBS and to compare the results with those previously obtained with blood samples. The detection limits achieved with the *16S rRNA* and *its* approaches using DBS were the same as those for blood samples albeit with fainter bands being obtained in all the cases. Thus, it seems that the use of DBS may be a solution for sample transportation in acute cases of Carrión's disease in which bacteremia is usually pronounced and diagnosis is crucial. Nonetheless, DBS may not allow the identification of asymptomatic or chronic carriers. Furthermore, even in acute cases of the disease, low levels of bacteremia may compromise the results. In addition, the tropism of *B. bacilliformis* for distinct kinds of cells could also lead to a lesser amount of bacteria in blood.

Thus, paper number 2 in this chapter provides a wide view of some PCRs approaches to amplify *B. bacilliformis* from both blood samples and DBS and to identify the detection limits of these techniques which are important in order to determine the management of the samples or facilitate their use in future studies. However, the practicality of implementing these techniques in rural endemic areas, taking into account the training of health personnel and the availability of laboratory staff to process few samples (except during outbreaks) might be questioned. Moreover, other financial questions should be taken into account such as the cost of the equipment and reagents needed as well as that of each sample analyzed. On the other hand, the implementation of PCR would allow the diagnosis of acute high bacteremia cases. However, it would be necessary to also be able to detect cases with low bacteremia, including chronic and asymptomatic cases. It has been shown that the serologic techniques are closer to this objective but the same issues regarding their implementation in rural endemic zones remain [39], making the development of a rapid diagnostic tool essential.

Taking all of the above into account we performed the third study of this chapter, the main objective of which was the identification of antigenic candidates of *B. bacilliformis*. To date, several reports have described serologic techniques or immunogenic proteins of *B. bacilliformis* but more studies are needed to better characterize the antigens expressed during *B. bacilliformis* infection and to obtain good antigenic candidates for the development of a rapid diagnosis test. The development of such a test would allow the diagnosis of patients on site by non expert personnel with no need to send the sample to a reference laboratory. Moreover, a rapid test could also be of great value in blood bank sample testing. In

the blood banks of endemic zones of Carrión's disease the presence of *B. bacilliformis* is tested by blood smears. On analyzing 42 samples from blood donors in a non endemic area in the north of Peru, 2.4% of the samples were positive by PCR [329]. Indeed, a rapid technique with a higher sensitivity than PCR would likely increase the detection of infected donations. Blood donors are usually apparently healthy people, and as such those who are infected are actually asymptomatic carriers who probably have a low bacterial load in blood. Moreover, *B. bacilliformis* testing in blood banks outside endemic zones would be extremely valuable due to the natural movement of people who may provide unexpected infected donations in far away areas. There are no studies on how long a person can be an asymptomatic carrier, with 3 years having been described in a specific case of an Ecuadorian expatriate [61] .

In our study several techniques including IFA, RT-PCR and ELISA were performed in order to characterize the population. The positive results obtained for IFA were lower compared to those reported in the literature [113]. These differences could be explained by the different strains used to perform the IFA slides [113], or by the subjectivity associated with slide reading. Besides, taking into account that an anti-human IgG antibody is used in this technique, it seems that the positivity peak of IFA should be achieved immediately after clinical recovery. On the other hand, our ELISA results showed similar levels of IgG antibodies against *B. bacilliformis* in the post-outbreak areas analyzed, but in the endemic area the IgG levels were significantly higher compared to those obtained by IFA. These differences can be explained by the different methodologies used; in the IFA we used intact *B. bacilliformis* cells and sonicated cells to perform the ELISA.

Our results are in accordance with the known partial immunity acquired along life, being IgM levels higher in younger subjects up to 25 years of age and IgG levels higher in individuals older than 26 years. Furthermore, IgG levels in our study were higher in men. These results can probably be explained by the usual movement of men to nearby endemic areas, increasing the risk of becoming infected. Moreover, it is frequent for men to go to work in coffee plantations in these endemic areas, which is another factor to consider since a correlation has been described between coffee plantations and the presence of *Lutzomyia* spp. [32]. Regarding the use of RT-PCR, we detected a high percentage of individuals carrying *B. bacilliformis* in blood, thereby highlighting the presence of asymptomatic carriers both in endemic areas

and in inhabitants of post-outbreak areas even after having received antibiotic treatment.

The identification of antigens inducing protective immunity would be valuable to prevent infection. It would be the initial step for future testing of antigenic candidates to develop a diagnostic tool and/or vaccines. Indeed, antigens have been described in the literature for *B. bacilliformis* as well as for other relevant *Bartonella* spp. such as *B. quintana* and *B. henselae* [215, 330-332]. In our study four potential antigenic candidates were identified, GroEL, Pap31, SCS-α and SCS-β. Pap31 and GroEL had previously been described in the literature as the main antigens of *B. bacilliformis* [208, 209, 214]. Indeed, to my knowledge, GroEL was one of the first *B. bacilliformis* antigens identified, and it was described as being a good antigenic candidate for the chronic phase of the disease [209]. In fact, we have seen that sera with the highest IgG antibody levels against *B. bacilliformis* react with GroEL in Western blot analyses, contrary to what happens with IgM. The presence of GroEL is associated with mitogenic activity against human vascular endothelial cells that lead to the development of verrucous lesions [143]. Moreover, GroEL has recently been described as being a good candidate for vaccine production and the development of diagnostic kits in other related species such as *B. melitensis* [333]. Pap31 was first identified in *B. bacilliformis* in 2005 by Taye *et al*. [214] who showed that Pap31 is a good candidate for the development of serodiagnostic tools for Carrión's disease. Nonetheless, the study only used 1 serum for the identification of Pap31 [214]. Our results corroborate that Pap31 is one of main antigens of *B. bacilliformis.* Using ELISAs with recombinant protein we found that almost all the sera studied were positive, which could be explained by the population analyzed in our study having been diagnosed with Carrión's disease several months before sample collection. Nonetheless, the possible detection of past infection and the absence of samples from healthy people never exposed to *B. bacilliformis* should also be taken into account. In other *Bartonella* spp., Pap31 is homolog of heme-binding protein A [214, 334], a family of proteins described as predominant outer membrane antigens and potent markers for serological diagnosis of infections by *B. henselae* and *B. quintana* [215, 334]. To my knowledge, our study was the first to identify SCS-α and SCS-β as antigenic candidates of *B. bacilliformis*. Nonetheless, SCS-α had previously been described as an immunogenic protein of *B. melitensis* [335], and SCS-β was recently reported to be involved in the pathogenesis of *B. henselae* [336]. Both antigens are subunits of SCS, an enzyme responsible for the reversible conversion of succinyl-CoA to succinate, an important reaction of the tricarboxylic acid cycle. We found that

all 4 antigenic candidates showed good performance in the ELISA assay, and despite the need for further studies, the use of more than one antigenic candidate may be the best option for a rapid diagnostic test. Indeed, other authors have suggested the need to evaluate other proteins of *B. baciliformis* in order to improve the sensitivity or specificity of the serological test, and in order to achieve this goal a cocktail of recombinant proteins or synthetic peptides would be necessary [213].

Carrión's disease is an illness which is restricted to small isolated rural areas far from the tourist routes of middle-income countries. It mainly affects the poorest populations and thus, may not be perceived as relevant or generate sufficient interest to receive funding or encourage scientific interest. However, this lack of visibility is probably the biggest obstacle to achieve the eradication of Carrión's disease. Notwithstanding, although the number of people affected is low compared with other infectious diseases and the clinical management of Carrión's disease is relatively easy with the administration of antibiotics, this disease is currently expanding to new areas, and albeit few for now, people are dying from this disease, especially during outbreaks. Indeed, any effort to improve the diagnosis and treatment of Carrión's disease would have a great impact on the communities living side by side with this disease and in the end, will only those who are affected benefit or will we all?

CONCLUSIONS

CHAPTER I - ANTIMICROBIAL RESISTANCE

1. *B. bacilliformis* mutants become highly antibiotic-resistant by both the development of antibiotic-target alterations as well as mediation by efflux pump overexpression, with ciprofloxacin resistance being the most easily selected and chloramphenicol resistance the most difficult to develop.

2. The instability of resistance detected in several azithromycin, chloramphenicol and ciprofloxacin mutants suggests a high biological cost, which may underlie the infrequent antibiotic-resistant *B. bacilliformis* clinical isolates. On the other hand, the selected resistance to rifampicin was stable even in the absence of antibiotic pressure.

3. Based on the study of *B. bacilliformis* mutants and in the absence of clinical assays, chloramphenicol seems to be the best treatment option *in vitro*, while the use of ciprofloxacin and rifampicin should not be encouraged.

CHAPTER II - DIAGNOSIS

4. The diagnosis of febrile syndromes by clinical criteria or microscopy may lead to misdiagnosis. An outbreak was mistakenly attributed to *B. bacilliformis* and our results strongly suggested the emergence of *S. faeni* as the causative agent of a community-acquired outbreak, probably associated with water.

5. *16S rRNA* PCR has the lowest detection limit compared to *fla* and *its* PCR approaches. It seems that the sensitivity of these techniques might allow the diagnosis of acute cases of Carrión's disease, but the applicability to detect low-bacteremia carriers' remains unclear. The use of DBS leads to a small decrease in the positivity of the technique.

6. The positive results obtained with ELISA and RT-PCR demonstrate the high levels of antibodies against *B. bacilliformis* and the relevant number of bacteria-blood carriers in the areas analyzed.

7. GroEl, Pap31, SCS-β and SCS-α are among the most dominant immunogenic proteins of *B. bacilliformis*. These proteins are potential antigenic candidates and may be useful for the development of a rapid diagnostic tool.

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ANNEX

1. ARTICLE FROM EL PAÍS NEWSPAPER

TAN LETAL COMO EL ÉBOLA Y MÁS QUE LA PESTE… PERO ERRADICABLE

Texto publicado en el *El País* a 12 Noviembre 2014
Autores: Cláudia Gomes / Pau Rubio

“Sus pueblos no figuran en nuestros mapas. Ellos no salen en la foto. Y, de entrada, el nombre de la maldición que los persigue tampoco inspira terror. Sin embargo, la enfermedad de Carrión, olvidada entre las olvidadas, ostenta el dudoso honor de figurar entre las infecciones bacterianas más agresivas: con una tasa de mortalidad en su fase aguda de entre el 44% y el 88%, resulta tan letal como el ébola y más que la peste.

La enfermedad de Carrión se hace fuerte con los más débiles: la población en mayor situación de riesgo son las personas de las zonas rurales con menos recursos, sobre todo mujeres embarazadas y niños. Originariamente endémica de los valles andinos de Perú, Ecuador y Colombia, ahora que los patógenos parecen haberle tomado el gusto a eso de viajar —y ahí están las epidemias de ébola y de chikungunya para recordárnoslo— ha iniciado su expansión hacia territorios vecinos, amenazando con llegar hasta donde el vector que la transmite, la mosca titira, consiga llevarla.

A todo ello, se añaden otras dificultades que la convierten en un reto para la ciencia:

1. La propia condición de olvidada, su circunscripción a regiones remotas, hace que no existan datos sobre el número de personas al que afecta. Los datos rigurosos son una herramienta indispensable para diseñar estrategias específicas de salud pública.

2. La existencia en las zonas endémicas de un gran número de personas que no desarrollan la enfermedad pero que son portadoras de la bacteria que la causa, *Bartonella bacilliformis*, contribuye a que siga perpetuándose en las áreas con presencia del insecto vector. Estos portadores asintomáticos, que actúan como reservorios, pasan desapercibidos para el sistema sanitario.

3. La bacteria medra en zonas remotas de países con sistemas sanitarios débiles, donde la capacidad para diagnosticarla adecuadamente es muy limitada.

Hasta ahí las malas noticias. Ahora, las buenas.

Las muertes que causa son perfectamente evitables. A diferencia de lo que ocurre con el ébola o con el chikungunya, para la enfermedad de Carrión sí que existe tratamiento. Por suerte, los antibióticos todavía son efectivos contra ella.

Por el momento y pese a que factores como el cambio climático contribuyen a su expansión, se encuentra restringida a una zona geográfica limitada. Eso, unido al hecho de que no se conoce ningún reservorio animal, permite alcanzar una conclusión esperanzadora: si se desarrolla una herramienta de diagnóstico eficaz, se mejora — y mucho — la capacidad de los sistemas de salud locales para la vigilancia epidemiológica de los portadores asintomáticos y se administra tratamiento de forma masiva a las poblaciones endémicas, la enfermedad de Carrión es — ¿lo adivinan?— potencialmente erradicable. Ahora, ¿quién debería dar el primer paso?”

2. SUMMARY OF THE THESIS IN SPANISH

RESISTENCIA ANTIMICROBIANA Y NUEVAS PERSPECTIVAS EN EL DIAGNÓSTICO DE LA ENFERMEDAD DE CARRIÓN

INTRODUCCIÓN

1. HISTORIA

Es difícil saber con exactitud el inicio de la enfermedad de Carrión en Perú. Sin embargo, existen evidencias de que la enfermedad de Carrión era conocida por las culturas precolombinas [1]. En algunos huacos antropomorfos hechos por los indígenas en la época precolombina se observan lesiones verrugosas, así como en algunas figuras de piedra de la cultura Huaylas [1]. En 1531, después de la llegada de los conquistadores españoles a Coaque, en Ecuador, Pizarro perdió un cuarto de sus hombres cuando fueron atacados por una epidemia verrugosa con dolores musculares y óseos, similar a la descripción de la enfermedad de Carrión [1, 6]. El primer informe escrito de la enfermedad de Carrión en el Perú es del siglo XVII. En 1630, Gago de Vadillo describe la primera zona verrugosa en Huaylas, departamento de Ancash, y plantea la hipótesis de que las verrugas son consecuencia del consumo de agua de la zona [10]. Sin embargo, el evento que desencadenó la primera atención de la comunidad médica para esta enfermedad fue la aparición de una enfermedad febril que mató a miles de hombres, muchos de ellos chinos y chilenos, en 1870, mientras trabajaban en el ferrocarril en Cocachacra, un pueblo ubicado entre Lima y La Oroya. La epidemia inexplicable apareció de repente, la fiebre y la anemia fueron rampantes así como el número de muertes, más de 7000. La nueva enfermedad fue conocida como fiebre de Oroya a pesar que la ciudad de La Oroya estaba bastante alejada [6].

La contribución más notable fue hecha por Daniel Alcides Carrión, un joven estudiante de medicina peruana que el 27 de agosto 1885 se inoculó a sí mismo la sangre de la verruga de un niño hospitalizado [11-13]. Veintiún días después aparecieron los primeros síntomas, con una progresión de la enfermedad rápida y completamente compatible con las características clínicas de la fiebre de Oroya [1, 6, 11]. Por desgracia, Daniel Carrión murió 35 días después de la inoculación convirtiéndose en un héroe en la medicina peruana, ya que su experimento demostró que la fiebre de Oroya y la verruga peruana son 2 fases diferentes de la misma enfermedad [1, 6, 8]. Más tarde, en 1909, Alberto Barton anuncia el descubrimiento

del agente causal de la enfermedad de Carrión [14]. En el mismo período, el entomólogo Charles Townsend estaba a cargo de encontrar el vector de la enfermedad y propuso que el vector era el *Phlebotomus verrucarum* (llamado actualmente *Lutzomyia verrucarum*) [15]. En 1927 el investigador japonés, Hideyo Noguchi, publicó el aislamiento, en sus medios para el cultivo de *Leptospira*, del agente etiológico tanto de la sangre de pacientes con fiebre de Oroya como con verruga peruana, no dejando lugar a dudas que son 2 manifestaciones clínicas de la misma enfermedad [16, 17].

2. EPIDEMIOLOGIA

2.1 Perú

Perú es el país más afectado por la enfermedad de Carrión. De hecho, esta enfermedad es considerada como una de las enfermedades de especial relevancia en el país, siendo de notificación obligatoria. De acuerdo con la clasificación del Banco Mundial, el Perú es un país de renta media-alta [21], con una población de aproximadamente 31 millones de habitantes, ocupando la 84ª de 188 posiciones del índice de desarrollo humano [22]. Se ha de reseñar el alto porcentaje de pobreza en este país, alcanzando un 22,7% de la población en el año 2014 y siendo extremadamente alto en las áreas rurales de montaña (50,4% de habitantes) [23]. El acceso a los centros de salud en las zonas rurales sigue siendo uno de los retos no resueltos de este país. La disponibilidad de hospitales y de personal técnico capacitado es baja en Perú, en especial fuera de las principales ciudades, estando concentrado de manera primordial en Lima. Otra cuestión importante es la venta incontrolada e ilegal de medicamentos [26].

2.2 Características de la población afectada

La persistencia de la enfermedad de Carrión en regiones endémicas se asocia principalmente con la pobreza, el clima cálido, las condiciones de vida, los bajos niveles de educación y las características de la región que definen la presencia del vector. Se ha demostrado que la enfermedad de Carrión afecta en general a ambos sexos de manera similar, con una prevalencia ligeramente superior entre los hombres [27-30]. Tanto los niños como las mujeres embarazadas son dos segmentos de población que pueden verse especialmente afectados. Las muertes fetales, abortos involuntarios y los partos prematuros se encuentran entre las complicaciones más graves que afectan a las mujeres embarazadas de las zonas rurales del Perú [33]. En cuanto a los niños, ellos son los más afectados por la fase aguda de la enfermedad [29, 34].

2.3 Enfermedad de Carrión en Perú

La enfermedad de Carrión es una enfermedad que afecta a los valles interandinos ubicados entre 500 y 3200 metros sobre el nivel del mar. Peru es un país endémico, siendo él más afectado por la enfermedad de Carrión. Se estima que las áreas endémicas representan alrededor de 145 000 Km^2 [38], donde viven más de 1,6 millones de habitantes [38]. En estas áreas se ha reportado una seroprevalencia de más del 60% [6]. La bartonelosis es considerada una enfermedad reemergente que se está expandiendo a nuevas áreas y está influenciada por los cambios climáticos y por fenómenos como El Niño [15, 46]. El fenómeno del Niño provoca un calentamiento de la temperatura marina cada 5-7 años, lo que indirectamente afecta favorablemente a la ecología de los vectores [39], incrementando el número de casos en las regiones endémicas, así como brotes en zonas no endémicas [39, 47].

2.4 Enfermedad de Carrión fuera de Perú

Algunos países vecinos como Ecuador y Colombia también se ven afectados por la enfermedad de Carrión [9]. La enfermedad tiene una amplia distribución en Ecuador [9] donde parece estar presente una forma menos agresiva ya que apenas hay casos en fase aguda, estando descritas formas leves de la fase verrugosa [53]. En Colombia la enfermedad era aparentemente desconocida hasta 1936, cuando se produjo un brote [9, 59]. Además, se han notificado casos esporádicos en Chile y Bolivia [27]. A pesar que la enfermedad está fuera de los destinos turísticos más relevantes, se han descrito varios casos de *Bartonella* importados en viajeros e inmigrantes procedentes de zonas endémicas [55, 61, 62].

3. *BARTONELLACEAE*

El género *Bartonella* se propuso tras la descripción de *Bartonella bacilliformis*, del cual fue la única especie descrita durante largos años. Sin embargo, estudios filogenéticos demostraron una estrecha relación filogenética y fenotípica entre *B. bacilliformis* y *Rochalimaea quintana*, que en ese momento pertenecía al género *Rochalimaea* [63, 64]. A raíz de estas observaciones, el género *Rochalimaea* se unificó con el género *Bartonella*, quedando con el nombre del último [65]. Más tarde, el género *Grahamella* se fusionó también con el género *Bartonella* [66]. Así, el género *Bartonella* pertenece a la familia *Bartonellaceae* y combina todas las especies de los 3 géneros citados: *Bartonella*, *Rochalimaea* y *Grahamella*. Son miembros del subgrupo alfa-2 de las alfa-proteobacterias [67], junto con *Rickettsia* y *Brucella* [39]. Hasta 1993, el género *Bartonella*, como se ha indicado, sólo contenía 1 especie, *B. bacilliformis* [69]. Sin embargo, actualmente, se han identificado y

reconocido internacionalmente 33 especies. Además, se han propuesto otras 13 especies y al menos 8 están clasificadas como especies *Candidatus*.

Se han descrito varios vectores artrópodos para *Bartonella* spp. tales como pulgas, garrapatas, moscas y piojos. Las coinfecciones por diferentes cepas y especies de *Bartonella* son comunes en mamíferos con una tasa de co-infección de 90% en las pulgas de ratas.

Es tal vez debido a que *Bartonella* spp. puede infectar una amplia variedad de células, incluyendo eritrocitos, células endoteliales, células dendríticas, células $CD34^+$ y varias células de tipo macrófago que las manifestaciones clínicas y patológicas de las infecciones parecen ser muy diversas tanto en animales como en seres humanos [83]. Varias especies se han asociado con diferentes enfermedades y síndromes en los seres humanos: *B. bacilliformis*, *Bartonella henselae*, *Bartonella quintana*, *Bartonella elizabethae*, *Bartonella clarridgeae*, *Bartonella rochalimae* y *Bartonella ancashensis* [54, 55, 68, 72, 84]. Por otra parte, se han asociado casos clínicos de endocarditis, con varias especies de *Bartonella*, apoyando el importante papel patológico de este género en seres humanos inmunocompetentes [83].

3.1 *Bartonella bacilliformis*

B. bacilliformis es el microorganismo responsable de la enfermedad de Carrión. Es un cocobacilo pleomórfico, Gram-negativo, no fermentador, aeróbico, con 0,2-0,5 µm por 1-2 µm, con 2-16 flagelos que le confieren una alta movilidad. La temperatura óptima de cultivo es de 28°C, precisa de sangre en los medios, y presenta crecimiento lento [15, 16]. *B. bacilliformis* puede permanecer viable a 4ºC durante largos periodos de tiempo [86, 87], tal como se describe para otras especies de *Bartonella* [88]. El genoma de *B. baciliformis* consiste en una única molécula de ADN circular de aproximadamente 1600 kpb [89] y 39-40% de contenido de GC [63].

3.2 Diversidad genética

Las relaciones filogenéticas y la diversidad genética entre los aislados de *B. bacilliformis* están escasamente estudiadas. Sin embargo, *Infrequent restriction site PCR* y comparación de las secuencias de nucleótidos de los genes *gltA* y *ialB* han demostrado variación genotípica dentro de las poblaciones de *B. bacilliformis* [97]. Más recientemente, se ha desarrollado un MLST que consiste en la amplificación y secuenciación de 7 loci genéticos diferentes [98]. Actualmente, los análisis filogenéticos han identificado hasta 12 *Sequence types* distintos entre aislados

clínicos de *B. bacilliformis* [98, 99]. Un enfoque genómico comparativo revela que la evolución de *B. bacilliformis* está conformada predominantemente por mutaciones [100].

4. Presentaciones clínica de la enfermedad de Carrión

Una vez que una persona es picada por un flebótomo infectado, se puede desencadenar una infección asintomática o una enfermedad leve a severa. La gravedad de la enfermedad de Carrión se determina probablemente por la predisposición individual y por la virulencia inherente de la cepa que causa la infección. El tiempo medio de incubación es de 61 días (entre 10 y 210 días) [68]. En la enfermedad de Carrión se han descrito dos síndromes, que se producen de forma independiente o de forma secuencial [38], una fase aguda llamada fiebre de Oroya y una fase crónica designada verruga peruana.

4.1 Fiebre de Oroya

En los seres humanos las principales consecuencias clínicas de la infección por *B. bacilliformis* son resultado de la invasión de los eritrocitos por la bacteria en la fase aguda de la enfermedad. *B. bacilliformis* infecta a los eritrocitos que posteriormente se destruyen en el bazo e hígado conduciendo a una anemia hemolítica severa e inmunosupresión transitoria [102] que predispone los pacientes a infecciones oportunistas [103, 104]. Un estudio realizado por Maguiña *et al.* mostró que la media del porcentaje de glóbulos rojos infectados en esta fase de la enfermedad fue del 61% (rango 2-100%), así como que en el 25% de los pacientes >90% de los glóbulos rojos estaban infectados en el momento de la admisión [105]. Los síntomas de la fiebre Oroya son indistinguibles de los primeros síntomas de otras enfermedades infecciosas, con malestar, fiebre, dolor de cabeza y escalofríos leves, pueden incluir palidez, hepatomegalia, dolor abdominal y otros síntomas inespecíficos [30, 34, 68, 106]. Los niños y adolescentes hasta 15 años parecen ser el grupo de edad más afectado por la fase aguda [6, 30, 36, 46, 51], lo que sugiere una inmunidad adquirida después de la exposición [36]. La fase aguda puede resultar en una tasa de letalidad del 40-85% en pacientes no tratados, pero con el tratamiento adecuado y oportuno la letalidad se reduce a valores en torno al 10% en centros de referencia [6, 15, 68, 105, 107]. Las complicaciones o infecciones secundarias oportunistas son responsables de empeorar drasticamente el pronóstico clínico, siendo por desgracia bastante comunes [30, 34, 68, 108, 109]. El patógeno más común es *Salmonella* spp. estando presente en el 90% de las muertes por fiebre de Oroya [1]. También se

han reportado complicaciones no infecciosas incluyendo complicaciones hematológicas, cardiovasculares y neurológicas [30, 34, 106].

4.2 Verruga Peruana

La fase crónica, llamada verruga peruana, se caracteriza por el desarrollo de erupciones dérmicas. Esta fase ocurre típicamente semanas o meses después del síndrome febril agudo persistiendo por periodos que varían de un mes a un año [1, 68], tendiendo a curarse espontáneamente. Las lesiones varían en tamaño y número, afectando principalmente brazos y piernas [110]. Los microorganismos se observan dentro de las verrugas como inclusiones intracelulares y como organismos libres dentro de la matriz extracelular [110]. La clasificación distingue 3 tipos de lesiones: miliares, mulares y nódulos subdérmicos [105, 110]. La fase eruptiva es una manifestación más común en los habitantes de las regiones endémicas y el riesgo de desarrollar verrugas aumenta con la edad [51].

4.3 Portadores asintomáticos

Además, se sabe que estos microorganismos pueden persistir en la sangre durante muchos años después de la infección [112]. Los portadores asintomáticos se han descrito en las zonas endémicas [113, 114] y se cree que son los perpetúadores de la enfermedad y que la pueden introducir a nuevas áreas.

5. PATOGÉNESIS Y FACTORES DE VIRULENCIA

B. bacilliformis puede invadir *in vitro* diferentes tipos de células humanas [115], pero durante la infección en humanos se sabe que la bacteria infecta respectivamente eritrocitos y células endoteliales en la fiebre Oroya y verruga peruana.

5.1 Eritrocitos / Fiebre de Oroya

El primer estudio que informó de la capacidad de *B. bacilliformis* de penetrar en los eritrocitos se llevó a cabo en 1969 [122]. Aunque haya controversia y hasta ahora ninguno haya sido identificado, algunos autores describen la colonización de un nicho primario después de la inoculación y antes de la infección de los eritrocitos [38, 116, 123, 124]. La infección de los eritrocitos por *B. bacilliformis* implica al menos 2 pasos importantes: 1) la unión de las bacterias a la superficie de los eritrocitos y 2) la deformación de la membrana de los eritrocitos, incluyendo invaginaciones profundas y fusiones de membrana que conducen a la formación de vacuolas intracelulares que contienen las bacterias [127].

Los flagelos, compuestos de múltiples subunidades de flagelina de 42 kDa, proporcionan un alto grado de movilidad a la bacteria durante la búsqueda de los eritrocitos [126, 129]. Esta motilidad bacteriana parece ser esencial para la unión a los eritrocitos y para la deformación proporcionando potencialmente fuerza mecánica [124, 127]. Más allá de los flagelos, otra proteína fundamental para la invasión de los eritrocitos es la deformina. Esta proteína es capaz de deformar los eritrocitos humanos mediante la producción de invaginaciones profundas en sus membranas [120, 131]. Además, un locus asociado a la invasión que incluye los genes *ialA* y *ialB* parece facilitar la invasión de los eritrocitos, ya que las proteínas codificadas por estos genes han demostrado la capacidad de invadir los eritrocitos humanos en ausencia de otros factores de virulencia [132]. La adherencia y la invasión a los eritrocitos implican sin duda otras proteínas y factores, algunos de ellos ya identificados. Por ejemplo la actina, la espectrina α y β, la proteína banda 3 o las glicoforinas A y B son proteínas de los eritrocitos implicadas en la unión específica de *B. bacilliformis* a la membrana de eritrocitos humanos [117, 131].

5.2 Células endoteliales / Verruga peruana

En la fase crónica de la enfermedad de Carrión, aparece la verruga peruana, con la infección de las células endoteliales y su subsiguiente proliferación descontrolada que resulta en las erupciones características. La existencia de numerosos capilares dentro de las verrugas sugiere que la infección de las células endoteliales induce una respuesta angiogénica local [137], esto es, la formación de nuevos vasos sanguíneos. La infección *in vitro* de células endoteliales humanas con *B. bacilliformis* resultó en la activación de GTPasas de la familia Rho (Rho, Rac y Cdc42 - proteínas de señalización clave en las vías de organización de la actina) y la remodelación del citoesqueleto de las células endoteliales estimuladas para formar filopodios y lamelipodios que sirven para introducir las bacterias en la célula, lo que demuestra que *B. bacilliformis* estimula su propia entrada en las células endoteliales [140-142]. Una proteína de choque termico altamente inmunogénica, GroEL, también parece jugar un papel clave en la inducción de la proliferación celular vascular. GroEL se secreta activamente en los sobrenadantes de cultivos de *B. bacilliformis* y la proliferación celular se inhibe en presencia de anticuerpos anti-GroEL [143].

6. INMUNOLOGIA

El conocimiento actual sugiere que la secreción de IL-10 es importante para la modulación inmune por los miembros del género *Bartonella* [124]. Esta citocina es un regulador clave de la inmunidad y podría estar implicada en el control de la

respuesta inflamatoria inducida por productos bacterianos [124, 146]. En consecuencia, los pocos estudios realizados sobre la enfermedad de Carrión han mostrado una elevación significativa de IL-10 e INF-γ en pacientes en la fase aguda de la enfermedad y explicarían el curso grave en algunos pacientes [148]. Los efectos de la elevada secreción de IL-10 favorecen en gran medida la infección asintomática y el estado de tolerancia inmune periférica necesaria para establecer una infección persistente [124, 150, 151].

En las primeras cuatro semanas de la fase aguda de bartonelosis los niveles de anticuerpos IgM están aumentados, mientras que los niveles de IgG e IgA permanecen normales. Los niveles de IgG se elevan después de aproximadamente la cuarta semana. En la fase eruptiva hay un aumento significativo pero no muy marcado de los niveles de IgA, IgG e IgM [46, 157]. Es de reseñar la depresión transitoria de la inmunidad celular que se produce durante la fase hemática aguda [158], con los pacientes presentando linfopenia con una disminución en los linfocitos-T $CD4^+$ y un ligero aumento de los linfocitos T $CD8^+$. La semejanza entre esta inmunosupresión y el SIDA fue establecido recientemente; en ambas enfermedades se produce inmunosupresión provocando un deterioro de la inmunidad celular [104], que de este modo podría explicar la predisposición para infecciones oportunistas [6, 104, 106]. Los datos de la respuesta inmune en la fase eruptiva son muy escasos, pero se ha descrito que los niveles de leucocitos permanecen normales con una ligera tendencia a presentar linfocitosis [6, 106].

7. TRANSMISIÓN

Hasta la fecha se han descrito o propuesto diferentes formas de transmisión de *Bartonella*. Sin embargo, la enfermedad de Carrión es una enfermedad transmitida por vectores, siendo esta la forma más común y relevante de la transmisión. Townsend identificó *P. verrucarum* como vector de la enfermedad de Carrión [15, 161], pero el papel de *L. verrucarum* como vector no fue firme hasta los estudios de Noguchi a finales de 1920s [162], y definitivamente corroborado por Hertig en 1942 [163]. El género *Lutzomyia* está compuesto por al menos 26 subgéneros y aproximadamente 400 especies [165].

La distribución de la enfermedad de Carrión y la presencia de *L. verrucarum* no siempre parecen coincidir, habiéndose propuesto la posibilidad de la participación de otras especies del género *Lutzomyia* en la transmisión de esta enfermedad [169, 171]. Así, en zonas endémicas de los departamentos de Cajamarca y Amazonas,

Lutzomyia maranonensis y *Lutzomyia robusta* se consideraron los vectores probables [169, 172]. Por otro lado, en la selva del departamento de Huánuco los autores encontraron que *Lutzomyia serrana* era el vector más probable [173]. En un brillante estudio de Noguchi, se recogieron varias especies de insectos, incluyendo 3 especies de Lutzomyia (*L. verrucarum* y dos especies que han sido nombrados *Lutzomyia noguchii* y *Lutzomyia peruensis*), de los distritos de Perú en la que prevalece la enfermedad. Los resultados mostraron que *L. noguchii* y *L. verrucarum* eran, muy probablemente, vectores de *B. bacilliformis*, pero siguieron existiendo reservas con respecto a *L. peruensis* [162]. En un estudio posterior hecho en Cusco usando PCR se ha demostrado que el 1% de las *L. peruensis* recogidas estaban infectadas con *B. bacilliformis*, siendo así este vector definitivamente implicado en la transmisión de bartonelosis a los seres humanos [37].

A pesar que la transmisión vectorial es la ruta de transmisión de la enfermedad más relevante, hay otras rutas posibles. Debido a la naturaleza de la enfermedad, todas las inoculaciones directas o el contacto con sangre humana infectada puede resultar en su adquisición y por lo tanto, las transfusiones de sangre, así como el contacto accidental con sangre infectada en laboratorios o durante las prácticas médicas deben ser consideradas como potenciales vías de transmisión. Además, la transmisión vertical y el contacto con otros fluidos humanos también deben ser tenidos en consideración.

7.1 Control y prevención

El vector es el objetivo principal para el control y prevención de la enfermedad de Carrión. El uso de pulverizaciones con DDT fue utilizado de manera eficiente para el control del vector después de la Segunda Guerra Mundial y desde entonces se ha aplicado en las zonas endémicas [1].

8. RESERVORIOS DE *B. BACILLIFORMIS*

Se han sugerido varios candidatos a ser el reservorio de la enfermedad de Carrión. Las plantas euforbiáceas fueron propuestas inicialmente como reservorios por la correlación geográfica entre este tipo de vegetación y las zonas de verrugas, así como la incidencia estacional de la enfermedad y el período de mayor crecimiento de las plantas [181]. Se han realizado varios estudios con animales para la búsqueda de otros posibles reservorios. Noguchi demostró con éxito que *Macacus rhesus* es susceptible a la infección por *B. bacilliformis* [18]. Del mismo modo, Hertig, en 1942, fue capaz de aislar *B. bacilliformis* a partir de sangre de *M. rhesus*

experimentalmente expuestos a la picadura de *L. verrucarum* salvajes [163]. Actualmente el ser humano es el único reservorio establecido para la enfermedad de Carrión pero la no existencia de otros reservorios animales o vegetales para *B. bacilliformis* nunca se ha demostrado de forma convincente.

9. DIAGNÓSTICO

9.1 Técnicas de rutina

En las zonas rurales endémicas el diagnóstico de la fase aguda de la enfermedad de Carrión se basa principalmente en los síntomas clínicos y en el frotis de la sangre periférica con tinción de Giemsa debido al bajo coste y facilidad de uso de esta técnica. Sin embargo, esta técnica requiere experiencia y a pesar de la alta especificidad (96%) muestra una sensibilidad muy baja (24-36%), especialmente en los casos leves de la enfermedad y en las infecciones subclínicas y crónicas [37, 39, 186]. Por otra parte, se debe tener en cuenta que los síntomas iníciales no son específicos de la enfermedad de Carrión ya que son comunes a varias otras patologías presentes en estas áreas, tales como dengue y otras enfermedades por arbovirus, malaria o tuberculosis, haciendo que el diagnóstico basado sólo en los síntomas clínicos sea extremamente difícil [15]. En la fase crónica de la enfermedad el diagnóstico clínico se realiza por la presencia de lesiones angiomatosas cutáneas en la piel. La sensibilidad de las técnicas microscópicas disminuye desde el 36% que se describe en la fase aguda a menos del 10% en la verruga peruana [106]. Un método más fiable es el cultivo en agar sangre pero la sensibilidad de este método también es extremadamente baja.

9.2 Técnicas moleculares

Se ha demostrado que los métodos de PCR son más eficaces que las técnicas de microscopia óptica y cultivo [41], siendo capaces de diagnosticar pacientes en fase aguda de la enfermedad de Carrión que habían sido previamente clasificados como negativos por frotis de sangre. No obstante, un problema crítico es el límite de detección de estas técnicas, generando dudas sobre su utilidad en la detección de portadores asintomáticos o portadores con baja bacteriemia. Por otra parte, hay que tener en cuenta que las técnicas moleculares son muy difíciles de aplicar en la práctica habitual en las zonas rurales endémicas [39]. Se han propuesto varios esquemas de PCR para la detección e identificación de especies de *Bartonella*, como por ejemplo la amplificación de los genes *ftsZ*, *rpoB*, *gltA*, *ialB*, *its*, *16S ARNr*, y algunos de los genes implicados en la síntesis de riboflavina [41, 190-197, 200, 202]. Por último, estudios recientes han demostrado la utilidad de técnicas moleculares

más sensibles, como la PCR cuantitativa [203], una PCR en tiempo real seguida de pirosecuenciación [204] o una amplificación isotérmica para detectar la presencia de ADN de *B. bacilliformis* [206].

9.3 Técnicas serológicas

El primer estudio de identificación de antígenos de *B. bacilliformis* fue publicado por Knobloch en 1988 [208], describiendo 24 antígenos proteicos incluyendo dos antígenos principales con 65 (BB65) y 75 kDa. BB65 es una proteína de choque térmico posteriormente identificada como GroEL, mientras que el antígeno de 75 kDa corresponde a la proteína de división celular FtsZ [208-211]. De manera posterior se han identificado 14 proteínas de la membrana externa de *B. bacilliformis* que van desde 11,2 hasta 75,3 kDa [125]. Padmalayam *et al*. describe una lipoproteína inmunogénica de 43 kDa como un antígeno [207]. Posteriormente, se desarrolló un ensayo de ELISA teniendo como antígeno la citada lipoproteína en su forma recombinante, los resultados mostraron una sensibilidad de 70,4 y 85,2% para IgG e IgM respectivamente y 90% de especificidad [212]. Se han evaluado otros ensayos de ELISA con diferentes antígenos, como por ejemplo con la proteina flagelina recombinante (*flaA*) de *B. bacilliformis* [213] y Pap31, una proteína inmunológicamente dominante expresada en los cultivos de *B. bacilliformis*, considerada una buena candidata para la técnica de ELISA [214]. El *immunoblot* usando células de *B. bacilliformis* sonicadas, es notablemente sensible a anticuerpos contra *B. bacilliformis* presentes en el suero de pacientes con enfermedad crónica (94%) [186]. Chamberlin *et al*. describe un ensayo de inmunofluorescencia indirecta. Este ensayo mostró que el 81% de los casos agudos confirmados presentaba una prueba positiva mientras que, por otro lado, el 74% de los voluntarios procedentes de zonas endémicas con una prueba positiva había tenido bartonelosis en el último año [113].

10. Tratamiento de le enfermedad de Carrión

El tratamiento varía en función de la fase de la enfermedad. Las transfusiones de sangre son quizás el tratamiento más clásico y se recomiendan para el tratamiento de la anemia severa típica en la fase aguda de la enfermedad [34, 68, 110, 223].

A partir de 2003 y de acuerdo con las recomendaciones y directrices del Ministerio de Salud del Perú, el fármaco de elección para los adultos que presentan la fase aguda de la enfermedad de Carrión es el ciprofloxacino (400 mg por vía intravenosa dos veces al día durante 3 días y luego 200 mg dos veces al día por vía oral hasta

14 días de tratamiento). En casos graves, ceftriaxona (2 g cada 24 horas durante 7 a 10 días) debe ser añadida al tratamiento [225]. Antes de la inclusión de ciprofloxacino en las directrices de tratamiento, algunos estudios reportaron resultados satisfactorios con el uso de cloranfenicol [6, 105, 230]. De hecho, después de un consenso médico en 1998 el cloranfenicol se consideraba el tratamiento de elección para los casos agudos, pero en 2003 ha sido sustituido debido a la falta de respuesta clínica en algunos pacientes [231-233].

A partir de 1969 y hasta 1975 la estreptomicina fue el fármaco de elección para el tratamiento de la fase crónica de la enfermedad de Carrión. A mediados de la década de 1970 se introdujo la rifampicina, mostrando mejores resultados en comparación con estreptomicina y convirtiéndose en el fármaco de primera línea [105, 237]. Más recientemente, el Ministerio de Salud de Perú propuso el uso de azitromicina (500 mg por vía oral por día durante 7 días) durante la fase eruptiva de la enfermedad [225].

10.1 Antimicrobianos utilizados en el tratamiento de la enfermedad de Carrión

En esta tesis se estudiaron 4 agentes antimicrobianos, azitromicina, cloranfenicol, ciprofloxacino y rifampicina. La azitromicina fue sintetizado por primera vez en 1980 [241] y pertenece a la familia de los macrólidos, compuestos que inhiben la síntesis de proteínas mediante la unión a los dominios II y IV del *23S ARNr* [243]. El cloranfenicol es un agente antimicrobiano aislado en 1947 a partir de un cultivo de *Streptomyces venezuelae* [248]. Este fármaco actúa como inhibidor de la síntesis de proteínas previniendo la elongación de la cadena peptídica mediante la unión a la subunidad ribosomal 50S [249]. El ciprofloxacino es una quinolona, una familia de antibióticos que se introdujo en la práctica clínica en 1967 [250]. Su mecanismo de acción consiste en la inhibición de las proteínas implicadas en la replicación y transcripción del ADN, en particular ADN Girasa y Topoisomerasa IV, mediante la formación de un complejo enzima-ADN-antibiotico que bloquea la enzima. Esto causa la inhibición de la síntesis del ADN bacteriano, y por lo tanto con el tiempo provoca la muerte celular [252, 253]. Por último, las rifamicinas fueron descubiertas en 1957 y se usa para el tratamiento de varias infecciones bacterianas como la tuberculosis. El mecanismo de acción consiste en la inhibición de la replicación del material genético mediante la inhibición de la ARN polimerasa bacteriana dependiente de ADN (codificada por el gen *rpoB*) [254].

11. MECANISMOS DE RESISTENCIA ANTIMICROBIANA

Para las bacterias intracelulares como *B. bacilliformis* la resistencia a los antibióticos se debe principalmente a mutaciones intrínsecas en los genes diana. Afortunadamente, las especies de *Bartonella* continúan siendo altamente susceptibles a los antibióticos [260]. Aunque escasos, los estudios *in vitro* han demostrado en general elevados niveles de sensibilidad a los antimicrobianos. No obstante, se ha reportado resistencia constitutiva al ácido nalidíxico y concentraciones minimas inhibitórias (CMIs) relativamente elevadas a clindamicina y colistina. Además, se han descrito aislamientos esporádicos que presentan resistencia al cloranfenicol o ciprofloxacino y una tendencia a la disminución de la susceptibilidad a los aminoglucósidos [226, 233, 262, 263].

Los mecanismos cromosomales de resistencia a azitromicina alteran la conformación del sitio de unión del antibiótico y se deben principalmente a alteraciones en las proteínas ribosomales L4 y L22, codificadas respectivamente por los genes *rplD* y *rplV*, así como mutaciones en el dominio V del gen *23S ARNr* [267, 268]. Por otro lado, los principales mecanismos de resistencia al cloranfenicol en microorganismos Gram-negativos son la modificación del antibiótico mediada por acetilo-transferasas y la expulsión del antibiótico mediada por bombas de expulsión transferibles como CmlA o FloR [249]. Además, otro mecanismo que confiere alto nivel de resistencia al cloranfenicol es la metilación del *23S ARNr*, aunque no es muy frecuente en microorganismos Gram-negativos. Para las quinolonas, el mecanismo de adquisición de resistencia más frecuente es la presencia de cambios aminoácidicos en las proteínas diana (GyrA, GyrB, ParC y ParE) [271, 272]. La resistencia a rifampicina se ha relacionado con diferentes mutaciones en el gene *rpoB* [275].

11.1 Bombas de expulsión

Las bombas de expulsión son proteínas implicadas en la expulsión de sustancias tóxicas de la bacteria, incluyendo, por ejemplo, antimicrobianos. Pueden ser específicas para un único sustrato o pueden afectar una variedad de compuestos, incluyendo antibióticos de diferentes familias. Esto es importante para el desarrollo de multirresistencia, ya que un solo tipo de bomba puede conferir resistencia simultánea a una amplia gama de agentes antimicrobianos [278].

Aunque ningún inhibidor de bomba de flujo se haya introducido en la práctica clínica con este fin, se han estudiado diferentes inhibidores de bombas de flujo para el futuro diseño de tratamientos en combinación con antibióticos [281]. Se cree que el

uso de tales estrategias podría facilitar la reintroducción de los antimicrobianos que no son eficaces hoy, evitando el continuo crecimiento de las cepas resistentes [282]. Entre los diferentes inhibidores de bombas de expulsión estudiados, destacan el Phenylalanine-arginine-β-naphthylamide (PAβN) que es uno de los inhibidores más frecuentemente estudiados *in vitro*, afectando especialmente a las bombas de tipo RND [283-285] y el artesunato que también inhibe bombas de la familia RND, aunque por un mecanismo de acción diferente [286].

12. ERRADICACIÓN

En 1997 fueron descritos los criterios de Dahlem sobre la viabilidad de erradicar una enfermedad [291]. En ellos 3 puntos fueron considerados como los más relevantes: 1) la eficacia de una intervención; 2) la sensibilidad de las herramientas de diagnóstico; 3) que el único reservorio vertebrado sean los seres humanos. La enfermedad de Carrión cumple con todos estos puntos y, por lo tanto, los esfuerzos de investigación deben reforzarse para diseñar y validar candidatos vacunales. Además, la alta susceptibilidad antibiótica de *B. bacilliformis* sugiere que el tratamiento antibiótico masivo podría conducir a la eliminación de la enfermedad en algunas zonas y en una disminución significativa de los portadores en otras. En ambos casos, la afectación de una zona geográficamente bien delimitada es favorable, facilitando las acciones de erradicación. Obviamente, una enfermedad restringida a zonas rurales aisladas, lejos de las rutas turísticas, en un país de ingresos medios y que afecta principalmente a la población más pobre no es percibida como relevante y como tal no genera suficiente interés para alcanzar los consensos y complicidades precisas para su erradicación. Esta falta de visibilidad es probablemente el mayor obstáculo para la erradicación de la enfermedad de Carrión.

HIPÓTESIS

1. Hasta la fecha no se han reportado aislados clínicos de *B. bacilliformis* resistentes a los antibióticos. Sin embargo, los clones resistentes a los antibióticos pueden surgir en el futuro. Se hipotetiza que es posible desarrollar *in vitro* mutantes de *B. bacilliformis* altamente resistentes a los antibióticos comúnmente utilizados para el tratamiento. Por otra parte, se seleccionarán diferentes mecanismos de resistencia. Estos mecanismos afectan la fitness bacteriana, lo que explica la aparente falta de resistencia a los antibióticos entre los aislados clínicos.

2. La forma de diagnosticar la enfermedad de Carrión está lejos del ideal, lo que resulta en un diagnóstico erróneo. Se hipotetiza que las metodologías actuales no

son suficientemente sensibles y específicas para diagnosticar correctamente la enfermedad de Carrión, y especialmente para detectar portadores asintomáticos. La identificación de candidatos antigénicos podría ser el primer paso hacia enfoques más sensibles, capaces de diagnosticar eficazmente esta enfermedad.

OBJETIVOS

1. Desarrollar y caracterizar mutantes de *B. bacilliformis*, obtenidos *in vitro*, resistentes a los principales antibióticos utilizados para tratar la enfermedad de Carrión.

1.1. Determinar la capacidad de azitromicina, cloranfenicol, ciprofloxacino y rifampicina para seleccionar mutantes de *B. bacilliformis* altamente resistentes.

1.2. Establecer los mecanismos de resistencia seleccionados, así como el papel de las bombas de expulsión en la adquisición de resistencia.

1.3 Evaluar la estabilidad de la resistencia adquirida.

2. Proporcionar nuevos conocimientos sobre el diagnóstico de la enfermedad de Carrión. Comparar varias técnicas, así como explorar nuevos candidatos antigénicos que podrían ser útiles en una futura prueba de diagnóstico para las zonas rurales endémicas.

2.1. Caracterizar un posible brote de *B. bacilliformis* en una zona no endémica.

2.2. Establecer el límite de detección y comparar diferentes esquemas de PCRs para detectar *B. bacilliformis* en muestras de sangre.

2.3. Caracterizar poblaciones que viven en zonas post-brote y endémica con varias técnicas de diagnóstico.

2.4. Identificar candidatos antigénicos de *B. bacilliformis*.

JUSTIFICACIÓN DE LA TESIS

La enfermedad de Carrión es una enfermedad desatendida restringida a las zonas más pobres de las regiones de los Andes, con sólo unos pocos grupos de investigación trabajando en este campo en todo el mundo. Hasta hace poco, se

pensaba que la enfermedad de Carrión cumplía los criterios más relevantes para poder ser erradicada, pero antes que tal suceda deben abordarse una serie de cuestiones. Uno de los problemas es la falta de programas de tratamiento eficaces bien definidos. Se necesita tanto de estudios *in vitro* de resistencia a antimicrobianos como de ensayos clínicos para determinar los mejores enfoques de tratamiento. Por otro lado, y quizás sea la necesidad más imperiosa, es preciso tener una manera fácil de realizar el diagnóstico correcto. Nuevos estudios inmunológicos y el desarrollo de pruebas diagnósticas ayudarán a identificar nuevas moléculas con potencial diagnóstico. Así, el diseño de esquemas de tratamiento que minimicen la selección de resistencia junto con el desarrollo de técnicas de diagnóstico que puedan ser implementadas en zonas rurales y aisladas, es esencial para el control, eliminación y erradicación de la enfermedad de Carrión.

Los resultados de esta tesis han sido separados en 2 diferentes aspectos de la enfermedad de Carrión; por un lado, la resistencia a los antimicrobianos (Capítulo I) y, por otra parte, el diagnóstico y caracterización de muestras clínicas (Capítulo II). El capítulo I describe un estudio sobre los mecanismos de resistencia desarrollados en presencia de los 4 antibióticos más comunes que se utilizan en el tratamiento de la enfermedad de Carrión. El capítulo 2 analiza varios aspectos de la enfermedad de Carrión que se abordan en 3 estudios. Estos estudios se realizaron en estrecha colaboración con la Dr. Juana del Valle de la Universidad Peruana de Ciencias Aplicadas y el Instituto de Investigación Nutricional en Lima, Perú y también la Dr. Mayumi Matsuoka, del Instituto Nacional de Enfermedades Infecciosas de Tokio, Japón.

ÁREA DE ESTUDIO

Los estudios realizados en esta tesis se realizaron tanto con cepas de colección adquiridas al Instituto Pasteur (París, Francia) (artículos 1, 3 y 4), como con muestras de sangre y suero recogidas en diferentes zonas del norte de Perú (artículos 2 y 4). A continuación una breve descripción de las zonas donde se recolectaron las muestras:

Departamento de Amazonas, provincia Rodríguez de Mendoza: área en la que nunca se ha descrito la enfermedad de Carrión, pero en la que se informó de un brote de esta misma enfermedad en marzo de 2013. Las muestras de sangre fueron recogidas por nuestros colaboradores en Perú, y los ADN fueron enviados a Barcelona para realizar estudios moleculares (artículo 2).

Departamento de Piura, provincia de Huancabamba: se recogieron de forma activa muestras de sangre y suero en esta área para llevar a cabo los estudios mencionados en el artículo 4 de esta tesis. Piura se divide en 8 provincias, entre ellas la de Huancabamba, que a su vez se subdivide en 8 distritos: Canchaque, El Carmen de la Frontera, Huancabamba, Huarmaca, Lalaquiz, San Miguel de El Faique, Sondor y Sondorillo. Varios estudios han descrito la presencia de la enfermedad de Carrión en la provincia de Huancabamba, pero sólo en los distritos de Sondor, Sondorillo, Carmen de la Frontera, Huarmaca y Huancabamba [30, 264, 294]. En esta tesis fueron incluidos 5 pueblos en el muestreo; cuatro de ellos (Tunal, Guayaquiles, Los Ranchos y Mayland) ubicados en el distrito de Lalaquiz, seleccionados debido a que unos meses antes hubo un brote de *B. bacilliformis* [43], mientras que el pueblo de Huancabamba, sito en el departamento homónimo, fue seleccionado porque es una zona endémica de *B. bacilliformis*. Las muestras de sangre se procesaron en el campo y posteriormente se enviaron a Lima y Tokio.

RESULTADOS

Capítulo I – Resistencia a los antimicrobianos

Artículo 1: Desarrollo y caracterización de mutantes de *Bartonella bacilliformis* altamente resistentes a antibióticos

Los datos sobre la resistencia a los antimicrobianos en aislamientos clínicos de *B. bacilliformis* es actualmente escaso y se centra principalmente en la resistencia constitutiva a quinolonas. Además, hasta la fecha, sólo hay 3 estudios en la literatura que caractericen mutantes de *B. bacilliformis*. Más aún, ningún estudio ha seleccionado mutantes resistentes a cloranfenicol, ha determinado la estabilidad de la resistencia adquirida o ha establecido el papel de las bombas de expulsión en la selección de resistencia a antimicrobianos.

El objetivo de este trabajo fue obtener información sobre el desarrollo *in vitro* de mutantes de *B. bacilliformis* resistentes a antibióticos con el fin de responder a las siguientes preguntas: ¿Cuando aparece la resistencia a antibióticos? ¿Cuál es el mejor enfoque para tratar la enfermedad de Carrión? ¿Qué mecanismos de resistencia son seleccionados con mayor facilidad? ¿Es la resistencia estable en el tiempo o desaparece cuando se elimina la presión antibiótica? ¿Tienen las bombas de expulsión un papel en la adquisición de resistencia en *B. bacilliformis*?

Para responder a nuestro objetivo hemos desarrollado 12 (3 para cada antibiótico) mutantes de *B. bacilliformis* altamente resistentes a los antibióticos más comunes para tratar la enfermedad de Carrión en la práctica clínica (azitromicina, cloranfenicol, ciprofloxacino y rifampicina). Las cepas se cultivaron durante 35 ó 40 pases consecutivos en presencia de presión antibiótica. La estabilidad de la resistencia adquirida se evaluó mediante el cultivo durante 5 pases consecutivos, en ausencia de presión antibiótica, de los mutantes obtenidos. La posible presencia de mutaciones en los genes diana se determinó por PCR y secuenciación. Además, se utilizaron dos inhibidores de bombas de expulsión, PAβN y artesunato, para evaluar el papel de las bombas de expulsión en el desarrollo de resistencia.

En todos los casos excepto en él de un mutante resistente a cloranfenicol, las CIM finales fueron de >256 mg/L para azitromicina, cloranfenicol y rifampicina y >32 mg/L para ciprofloxacino. En general, los mutantes fueron seleccionados con mayor facilidad por ciprofloxacino. Por otro lado, en nuestro estudio la resistencia a cloranfenicol fue la más difícil de obtener y los mecanismos seleccionados revertieron, probablemente debido al alto coste biológico del desarrollo de la resistencia a cloranfenicol. Solamente los mutantes seleccionados con rifampicina mostraron, consistentemente, estabilidad de la resistencia tras la retirada de la presión antibiótica.

La resistencia a los antibióticos se relacionó en 11 de los 12 mutantes obtenidos con mutaciones encontradas en los genes diana de cada antibiótico (tabla 1), que se encuentra.

Tabla 1: Mecanismos de resistencia obtenidos para los mutantes en el estudio.

Ciprofloxacino		Azitromicina		Cloranfenicol	Rifampicina
gyrA	*gyrB*	*rplD*	*rplV*	*23S ARNr*	*rpoB*
Val91	Lys475	Δ62-65	83::VSEAHVGKS	A2372	Arg527
Gly95		Arg70			Tyr540
		Lys66			Phe545
		Tyr74			Tyr588

Además, se comprobó que las bombas de expulsión tienen un rol relevante en el desarrollo de la resistencia en *B. bacilliformis*. Así se observó el efecto del PAβN y/o artesunato en la CMI de 1 mutante resistente a azitromicina, 1 a cloranfenicol y 1 a rifampicina.

El presente estudio evidencia la capacidad de *B. bacilliformis* de volverse resistente a los principales antibióticos utilizados para tratar la enfermedad de Carrión, tanto por el desarrollo de alteraciones en la diana de los antibióticos, como por la sobreexpresión de bombas de expulsión. Sin embargo, la reversión total o parcial de los niveles de resistencia adquiridos sugiere la existencia de un elevado coste biológico derivado de la selección de resistencia a antimicrobianos. Este hallazgo fue especialmente notorio en el caso del cloranfenicol en él que la selección de resistencia fue más difícil y siempre regresó a los niveles basales en ausencia de presión antibiótica. Aunque los estudios de mutantes obtenidos *in vitro* no puedan ser trasladados directamente a la comunidad, el cloranfenicol parece ser la mejor opción de tratamiento *in vitro*.

Capítulo II - Diagnóstico

Artículo 2: Un brote infeccioso no identificado en la región de la Amazonia Peruana

En marzo de 2013 se reportó un brote de fiebre de Oroya en una zona rural no endémica del noreste de Perú. El diagnóstico se hizo basado en la sospecha clínica, así como en técnicas de microscopía. En este artículo el objetivo fue caracterizar el brote mediante técnicas moleculares.

En el estudio se incluyeron 53 muestras de sangre de personas diagnosticadas con fiebre de Oroya. La revisión de los datos clínicos mostró que los síntomas más comunes fueron dolor de cabeza y malestar general (50,9%), seguido de escalofríos (32,1%) y fiebre (24,5%). Se realizó PCR con cebadores universales para el gen*16S ARNr* como específicos para el gen*16S ARNr* de *Bartonella* spp. Además, todas las muestras de sangre se cultivaron de acuerdo con las condiciones de *B. bacilliformis*, pero no se observó crecimiento de este microorganismo. En la PCR específica para el *16S ARNr* de *Bartonella* spp. no se obtuvo ninguna amplificación. Sin embargo, con los cebadores del*16S ARNr* universales todas las muestras amplificaron y se identificó la presencia de *Sphingomonas faeni* mediante secuenciación.

Sin embargo, la cuestión en cuanto al origen de la infección se mantuvo. Sabiendo que *S. faeni* es un microorganismo ambiental y que el 17% de los pacientes reportaron actividades acuáticas, se analizaron tanto muestras de agua de la red de suministro, como de pozos de agua. Los resultados mostraron la presencia de microorganismos acuáticos pertenecientes al género *Sphingomonas*.

Así, proponemos que este brote se atribuyó erróneamente a *B. bacilliformis* y nuestros resultados sugieren que el agente causante fue *S. faeni*. Hasta donde se, este es el primer reporte de un brote causado por *Sphingomonas* spp. adquirido en la comunidad.

Artículo 3: Evaluación del límite de detección de esquemas de PCRs para la detección de *Bartonella bacilliformis* en muestras de sangre

A pesar de no ser aplicable en la mayoría de las zonas rurales endémicas, la técnica de PCR es un método rápido para diagnosticar *B. bacilliformis* en los centros de referencia. Sin embargo, el límite de detección de esta técnica y su utilidad para el diagnóstico y detección de personas asintomáticas o con baja bacteriemia nunca se ha tenido en cuenta.

En este artículo se presenta un estudio comparativo de 3 métodos de PCR diseñados para detectar *B. bacilliformis*. Se determinó el límite de detección de las PCRs tanto de sangre infectada artificialmente como de papel de filtro (DBS). Se verificó la especificidad y se discutió las diferentes posibilidades del uso de las PCRs en el ámbito clínico. Las PCRs elegidas fueran fragmentos del gen *16S ARNr* específico de *Bartonella*, flagelina (*fla*) y la región variable-intergénica (*its*).

El esquema de PCR *16S ARNr* presentó el límite de detección más bajo, 5 unidades formadoras de colonias (UFC)/µL, con estos cebadores todas las especies de *Bartonella* pueden ser amplificadas. Sin embargo, este esquema de PCR también mostró un resultado positivo para *Brucella melitensis*. La amplificación del gen *its* aumentó el límite de detección (500 UFC/µL), pero es específico para *Bartonella* spp. y los productos amplificados tienen diferentes tamaños, permitiendo así la diferenciación de las 3 especies que causan síndromes similares a la enfermedad de Carrión (*B. bacilliformis*, *B. rochalimae* y *B. ancashensis*). En cuanto al gen *fla*, también es específico para *Bartonella* spp., permitiendo distinguir las diferentes especies de *Bartonella* que causan enfermedad de Carrión de las restantes que causan infección en humanos, con la excepción de *B. clarridgeiae*. En todos los casos se observó que la sensibilidad de las PCRs disminuye cuando se hicieron a partir de DBS.

Parece que la sensibilidad de estas técnicas podría permitir el diagnóstico de casos agudos de la enfermedad de Carrión, pero su aplicabilidad para detectar a los

portadores sanos o con una baja bacteriemia sigue sin estar clara. En este artículo proponemos el uso concomitante de estos esquemas de PCR en combinación con la información clínica.

Artículo 4: Succinil-CoA sintetasa: nuevo candidato antigénico de *Bartonella bacilliformis*

El correcto diagnóstico, tanto de los pacientes como de los portadores asintomáticos de la enfermedad de Carrión en zonas endémicas, sigue siendo una de las cuestiones sin resolver. En la actualidad no se ha desarrollado una herramienta diagnóstica suficientemente sensible y rápida. El uso de antígenos para el desarrollo de tiras u otras pruebas diagnósticas podría ser una alternativa, pero hay pocos informes en la literatura sobre los antígenos de *B. bacilliformis*. El objetivo de este estudio fue caracterizar una colección de muestras biológicas de personas que viven en áreas expuestas e identificar y caracterizar candidatos antigénicos de *B. bacilliformis*.

Así, se recogieron 198 muestras de sangre y 177 muestras de suero de voluntarios diagnosticados con la enfermedad de Carrión durante un brote, que había tenido lugar unos meses antes en 4 localidades no endémicas de Perú. Un área endémica se incluyó también en el estudio. Las muestras se caracterizaron por PCR en tiempo real, IFA y ELISA IgM / IgG donde el antígeno fueron células enteras de *B. bacilliformis* sonicadas.

Para identificar las proteínas inmunogénicas, se separaron por SDS-PAGE las proteínas totales de *B. bacilliformis* sonicando el crecimiento de cultivos en agar sangre, realizándose la técnica de Western blot para cada suero tanto para IgG como IgM. Teniendo en cuenta los niveles de anticuerpos obtenidos para cada suero en el ELISA desarrollado con células enteras de *B. bacilliformis*, se eligieron las proteínas inmunogénicas candidatas secuenciándose la región N-terminal de las mismas para proceder a su identificación.

Las proteínas inmunogénicas candidatas identificadas se clonaron, expresaron y se purificaron. Para determinar y caracterizar la respuesta de los candidatos inmunogénicos a los sueros recogidos se realizó ELISA tanto para IgM como para IgG usando las proteínas inmunogénicas como antígenos.

Como se muestra en la tabla 2, aproximadamente la mitad de los voluntarios del área endémica y alrededor del 40% de los individuos en las áreas de post-brote tuvo un resultado positivo por PCR en tiempo real, lo que demuestra el alto número de portadores asintomáticos en estas zonas. Aunque no se puedan descartar posibles re-infecciones, los fallos microbiológicos parecen evidentes ya que todos los voluntarios de las áreas de post-brote fueron tratados con ciprofloxacino durante el brote. En todos los casos la positividad observada en la PCR en tiempo real estaba cerca del límite de detección de la técnica, lo que demuestra que la presencia de bacterias en la sangre puede ser muy baja. En cuanto a la técnica del IFA, nuestra tasa de positividad fue menor de lo esperado; siendo del 26,8% en las áreas post-brote en comparación con el 74% que se ha descrito previamente en la literatura. Además, los niveles de anticuerpos IgG anti-*B. bacilliformis* obtenidos por ELISA para los individuos de la zona endémica fueron significativamente más elevados que los encontrados en los voluntarios de las áreas post-brote donde la enfermedad no había sido descrita con anterioridad. Esto evidencia la presencia de un alto porcentaje de personas con elevados niveles de anticuerpos contra *B. bacilliformis* viviendo en las zonas endémicas. Por otra parte, se encontró que los niveles de IgM / IgG fueron dependientes de la edad, lo que sugiere el desarrollo de la inmunidad parcial a lo largo de la vida. La presencia de anticuerpos y de *B. bacilliformis* en sangre puede apoyar la evasión bacteriana del sistema inmune del huésped, por la expresión diferencial de epitopos bacterianos y la facilidad para invadir diferentes tejidos.

Tabla 2: Resultados positivos para cada una de las técnicas en las áreas post-brote y endémica.

		Áreas post-brote n (%)	**Área endémica** n (%)	***P***	**Total n/N (%)**
PCR tiempo real		64/173 (37)	13/25 (52)	0.1883	77/198 (38.9)
IFA		41/153 (26.8)	3/24 (12.5)	0.0575	45/177 (25.4)
ELISA	**IgM**	50/153 (32.7)	10/24 (41.7)	0.4869	60/177 (33.9)
	IgG	32/153 (20.9)	16/24 (66.7)	0.0001	48/177 (27.1)
Positivo al menos por una técnica*		119/153 (77.8)	22/24 (91.7)	0.1715	141/177 (79.7)

* Sólo se consideraron individuos con muestras de sangre y suero (n = 177).
Se utilizó el test de Fisher en todos los casos.

Cuatro proteínas inmunogénicas de *B. bacilliformis* se identificaron mediante Western blot: 2 con un anticuerpo secundario anti-IgM humana, Pap31 y Succinil-CoA sintetasa subunidad α (SCS-α) y otras 2 con anticuerpo secundario anti-IgG humano, GroEL y Succinil-CoA sintetasa subunidad β (SCS-β).

Hasta donde se, esta es la primera descripción del SCS-α y SCS-β como proteínas inmunogénicas de *B. bacilliformis*. Estos nuevos candidatos antigénicos están implicados en el ciclo del ácido tricarboxílico y se han descrito también como proteínas inmunogénicas de *B. melitensis* (SCS-α), estando asimismo involucradas en la patogénesis de la infección por *B. henselae* (SCS-β).

La positividad para las ELISAs IgM de las proteínas inmunogénicas fue en todos los casos mayor del 77% mientras que para IgG los valores variaron desde 35% para SCS-α a 60% para SCS-β. Es resaltable que en el caso de IgM se observó una casi total concordancia entre las ELISAs para los candidatos antigénicos y las ELISA para las células totales sonicadas de *B. bacilliformis*, lo que parece sugerir que los antígenos identificados se pueden utilizar indistintamente en un futuro test de diagnóstico rápido.

Estudios de identificación y caracterización de antígenos de *B. bacilliformis* son esenciales para avanzar hacia el desarrollo de una herramienta de diagnóstico rápido que se pueda implementar en las zonas rurales donde esta enfermedad es endémica.

DISCUSIÓN

Capítulo I - Resistencia a los antimicrobianos

En el primer capítulo de esta tesis hemos publicado un estudio donde se obtuvieron mutantes resistentes a azitromicina, rifampicina, ciprofloxacino y cloranfenicol, destacando la capacidad de *B. bacilliformis* de volverse resistente a los principales antibióticos utilizados en el tratamiento de la enfermedad de Carrión. Los resultados mostraron que el antibiótico que selecciona más fácilmente mutantes resistentes es ciprofloxacino y que la resistencia más difícil de seleccionar es con cloranfenicol. Esto está de acuerdo con el hecho de que *B. bacilliformis* tiene resistencia intrínseca a las quinolonas [226] y con un estudio que analizó la susceptibilidad a antibióticos de varias cepas clínicas de *B. bacilliformis* [233]. Las alteraciones en los genes codificantes para las dianas de los antibióticos fueron un mecanismo común de resistencia, encontrando en 11 de los 12 mutantes obtenidos. La mayoría de las sustituciones aminoácidicas observadas se han encontrado previamente en la misma, o equivalente, posición en otros microorganismos, tales como *Escherichia coli*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis* o *Bartonella* spp. [238, 266, 269, 270, 273, 276, 299-304]. Sin embargo, para los mutantes de azitromicina hemos descrito nuevas deleciones e inserciones en las proteínas L4 y L22,

respectivamente. En el caso de ciprofloxacino se observó una mutación en el codón 91 del gen *gyrA* en la que el aminoácido resultante (valina) afecta en gran medida el patrón hidrofóbico de GyrA y la interacción con ciprofloxacino. Las otras dos mutaciones seleccionadas, que afectan a GyrA y GyrB son tal vez más ventajosas para la bacteria debido al efecto aditivo de los mecanismos de resistencia a quinolonas [251]. Es interesante observar que la resistencia seleccionada en los mutantes de azitromicina se asoció en todos los casos con las proteínas L4 y L22, mientras que no se observó ninguna mutación en el *23S ARNr*. Esto puede estar relacionado con la disminución del *fitness* asociada a alteraciones en el *23S ARNr*, como se ha propuesto para otros microorganismos [301, 306]. Sin embargo, difiere de lo descrito en los microorganismos con un bajo número de copias del gen *23S ARNr* [307] y observado en estudios previos realizados con *B. bacilliformis* y *B. henselae* [238, 269, 270]. Al contrario de lo sucedido en los mutantes de azitromicina, en 2 de los 3 mutantes seleccionados con cloranfenicol se han encontrado sustituciones en el *23S ARNr*. Sin embargo, en ambos mutantes la sustitución en el *23S ARNr* revertió después de los pases sin presión antibiótica, lo que muestra una rápida reversión de esta mutación. Se ha sugerido que los cambios estructurales en el centro peptidil transferasa que pueden impedir la unión del cloranfenicol son incompatibles con la función satisfactoria del ribosoma [249]. En cuanto a la estabilidad de los otros antibióticos, se observó que los mutantes seleccionados con rifampicina mostraron estabilidad constante de la resistencia, resaltando el grave riesgo de selección de resistencia a la rifampicina durante los tratamientos. A pesar de que nuestros resultados muestran que es posible obtener mutantes de *B. bacilliformis* altamente resistentes debido principalmente a mutaciones en los genes codificantes para las dianas de los antibióticos, la resistencia ha estado también mediada por incremento de la actividad de las bombas de expulsión. Así, en la mitad de los mutantes obtenidos los niveles de sensibilidad disminuyeron en presencia de PAβN y/o artesunato, mostrando que las bombas de expulsión tienen un papel fundamental en la adquisición de la resistencia antimicrobiana en *B. bacilliformis*. Por otra parte, el efecto de las bombas de expulsión en la adquisición de resistencia a los antibióticos se observó en mutantes seleccionados con los diferentes tipos de antibióticos en estudio, lo que demuestra que estos 4 compuestos pueden ser expulsados por la bacteria así como la posible utilidad de inhibidores para potenciar el efecto de los antibióticos en la enfermedad de Carrión.

En general, y teniendo en cuenta los resultados que se presentan en el capítulo I de esta tesis parece que cloranfenicol y azitromicina son los antibióticos más útiles para el tratamiento de las fases aguda y crónica de la enfermedad de Carrión, respectivamente. Sin embargo, se necesitan más estudios para llegar a una conclusión inequívoca y que permitan diseñar planes de tratamiento eficaces.

Capítulo II – Diagnóstico

En el primer estudio del segundo capítulo de esta tesis se describe un brote febril erróneamente diagnosticado como fiebre de Oroya. Nuestros resultados mostraron la presencia de *S. faeni* como agente causal del brote reportado. Nuestra hipótesis es que el brote se atribuyó erróneamente a la enfermedad de Carrión debido a la proximidad con zonas endémicas, así como por los frotis de sangre positivos. Hasta donde se, este fue el primer brote causado por *S. faeni* y el primer brote adquirido en la comunidad por *Sphingomonas* spp. Este microorganismo tiene carácter ambiental y los resultados sugieren una asociación del brote con el agua consumida y actividades acuáticas. Se puede especular con la adquisición de algún(os) factor(es) de virulencia. Por un lado, esta posibilidad es apoyada por la estrecha relación de *S. faeni* con *Sphingomonas paucimobilis* [318], que es capaz de infectar a pacientes inmunocompetentes [319]. Por otra parte, las especies de *Sphingomonas* se han considerado potenciales patógenos bacterianos que poseen varios factores de virulencia relacionados con la capacidad intracelular de *Brucella* spp. [320]. En el presente estudio se muestra la necesidad imperiosa de desarrollar una herramienta de diagnóstico eficaz que permita la identificación de *B. bacilliformis* y el correcto diagnostico de la enfermedad de Carrión en el Perú.

Las técnicas moleculares son herramientas más eficientes para hacer el diagnóstico de la enfermedad de Carrión. Así, la determinación del límite de detección de estas técnicas es extremadamente valiosa para definir su utilidad práctica, ya que la carga bacteriana varía según la etapa de la enfermedad y también entre individuos, yendo, en la fase aguda, de 2 a 100% de eritrocitos infectados [105]. En el segundo estudio de este capítulo decidimos evaluar el límite de detección de 3 PCRs utilizadas para detectar *B. bacilliformis*. La amplificación del *16S ARNr* se utiliza en Perú para el diagnóstico de la enfermedad de Carrión; tal como se indica por del Valle *et al*. se detectaron 21 casos de la enfermedad de Carrión entre 113 muestras de pacientes previamente diagnosticados como negativos utilizando un frotis de sangre [41]. Con la excepción de *Bartonella clarridgeiae*, la amplificación del gen *fla* permite distinguir las 3 *Bartonella* causantes de la enfermedad de Carrión de las restantes especies de

Bartonella causantes de infecciones en humanos. En cuanto a la amplificación del gen *its*, esta región se ha propuesto para la detección e identificación de especies de *Bartonella* en muestras clínicas debido a los distintos tamaños de amplificación obtenidos entre las especies de *Bartonella* [322]. Nuestros resultados mostraron un límite de detección de 5 CFU/µL, tanto para la PCR de *16S ARNr* como para la de *fla*, aumentando a 500 CFU/µL para la amplificación del gen *its*. Esta sensibilidad permite probablemente el diagnóstico de los casos agudos de la enfermedad de Carrión en el que el porcentaje medio de los eritrocitos infectados es del 61% [105]. Además, se encontró una buena especificidad ya que ninguno de los microorganismos diferentes a *Bartonella* incluidos en el estudio se amplificó por las PCRs del *fla* o *its*, y en el caso de la amplificación del *16S ARNr* sólo hubo amplificación para *B. melitensis*. Por lo tanto, el diagnóstico debe realizarse en un contexto clínico adecuado y la amplificación de otras especies relacionadas se debe tener en cuenta, especialmente en casos de depresión inmunológica y en el estudio de muestras ambientales ya que la mayoría de los microorganismos relacionados no son patógenos humanos. Por lo general, en Perú las PCRs se llevan a cabo en los laboratorios del Instituto Nacional de Salud o en algún laboratorio de referencia en las principales ciudades. Uno de los problemas es el transporte de la muestra entre las áreas endémicas donde se recogen a los laboratorios donde se realizan las técnicas moleculares. Los DBS podrían ser una buena solución para superar estos problemas [323]. Así, decidimos probar la eficacia de las mismas PCRs a partir de DBS y comparar con los resultados obtenidos anteriormente para las muestras de sangre. Los límites de detección obtenidos para *16S ARNr* e *its* fueron los mismos que para las muestras de sangre, pero en todos los casos se obtuvieron bandas más débiles. Por lo tanto, parece que los DBS podrían ser una solución para el transporte de muestras en los casos agudos de enfermedad, pero se debe prestar atención si el objetivo es identificar portadores asintomáticos o crónicos.

Sin embargo hay que tener en cuenta la dificultad de implementar técnicas moleculares o serológicas en las zonas rurales, siendo realmente esencial el desarrollo de una herramienta de diagnóstico rápido. Así, el objetivo principal del tercer estudio de este capítulo fue la identificación de candidatos antigénicos de *B. bacilliformis*. Una técnica rápida de diagnóstico con una mayor sensibilidad que la PCR podría aumentar la detección de casos y de donaciones infectadas en los bancos de sangre tanto de las zonas endémicas como fuera de estas. El análisis de 42 muestras de donantes de sangre en una zona no endémica en el norte de Perú, mostró que el 2,4% de las muestras fueron positivas por PCR [329]. Es necesario

tener en cuenta que los donantes de sangre son aparentemente personas sanas, y, si están infectados, son portadores asintomáticos con baja carga bacteriana en la sangre. En nuestro estudio se realizaron varias técnicas, tales como IFA, RT-PCR y ELISA, con el fin de caracterizar la población en estudio. Los resultados positivos obtenidos para IFA fueron bajos en comparación con los reportados en la literatura [113], pero estas diferencias podrían explicarse por las diferentes cepas utilizadas [113] o por la subjetividad asociada con la lectura del test. Nuestros resultados están de acuerdo con la inmunidad parcial adquirida a lo largo de la vida, siendo los niveles de IgM más elevados en la población más joven hasta los 25 años y los niveles de IgG más elevados en individuos mayores de 26 años. Además, los niveles de IgG fueron más altos en los hombres. Estos resultados pueden probablemente explicarse por el movimiento habitual de los hombres a zonas endémicas cercanas. Por otra parte, en estas zonas endémicas es frecuente que los hombres vayan a trabajar a las plantaciones de café, donde se ha asociado la presencia de *Lutzomyia* spp. [32]. Por la técnica de RT-PCR se detectó un elevado porcentaje de individuos portadores de *B. bacilliformis* en la sangre destacando la presencia de portadores asintomáticos, tanto en el área endémica como en los habitantes de las zonas post-brote, incluso después de haber recibido tratamiento con antibióticos.

La identificación de antígenos será el primer paso para una futura herramienta de diagnóstico y/o vacunas. En nuestro estudio se identificaron cuatro potenciales candidatos antigénicos, GroEL, Pap31, SCS-α y SCS-β. Pap31 y GroEL ya fueron descritos en la bibliografía como antígenos principales de *B. bacilliformis* [208, 209, 214]. GroEL fue uno de los primeros antígenos de *B. bacilliformis* identificado y se describe como un buen candidato antigénico para la detección de la fase crónica de la enfermedad [209]. De hecho, hemos visto que los sueros con los niveles más altos de anticuerpos IgG contra *B. bacilliformis* reaccionaban con GroEL en los análisis de *Western blot*, al contrario de lo que sucedía para IgM. La presencia del GroEL se asocia con la actividad mitogénica contra las células endoteliales vasculares humanas que conduce al desarrollo de lesiones verrugosas [143]. Pap31 se identificó por primera vez en *B. bacilliformis* en 2005 por Taye *et al*. [214]. Sus resultados mostraron que Pap31 es un buen candidato para el desarrollo de herramientas de serodiagnóstico para la enfermedad de Carrión [214]. Nuestros resultados corroboran que Pap31 es efectivamente uno de los principales antígenos de *B. bacilliformis* y por la técnica de ELISA, realizada con la proteína recombinante, vimos que casi todos los sueros en estudio tenían un resultado positivo, lo cual tiene sentido ya que la población analizada en nuestro estudio fue diagnosticada con la

enfermedad de Carrión algunos meses antes de la recogida de muestras. No obstante, es necesario mencionar tanto la posible detección de infecciones pasadas y la ausencia de muestras de personas sanas no expuestas a *B. bacilliformis*. En otras especies de *Bartonella,* Pap31 fue descrito como perteneciente a una familia de proteínas que son antígenos predominantes de la membrana externa y potentes marcadores para el diagnóstico serológico de las infecciones por *B. henselae* y *B. quintana* [215, 334]. Hasta donde se SCS-α y SCS-β fueron identificados como candidatos antigénicos de *B. bacilliformis* por primera vez en nuestro estudio. Sin embargo, SCS-α ya fue descrito como una proteína inmunogénica de *B. melitensis* [335] y se reportó recientemente que SCS-β está involucrado en la patogénesis de *B. henselae* [336]. Ambos antígenos son subunidades del SCS, un enzima responsable de la conversión reversible de succinil-CoA a succinato, una reacción importante del ciclo del ácido tricarboxílico. Nuestros resultados mostraron que los 4 candidatos antigénicos tienen un buen rendimiento en el ensayo de ELISA y, a pesar ser necesario más estudios, parece que el uso combinado de más de un candidato antigénico debe ser la mejor opción para una prueba de diagnóstico rápido.

CONCLUSIONES

Capítulo I - Resistencia a los antimicrobianos

1. Los mutantes de *B. bacilliformis* son altamente resistente a los antibióticos tanto por el desarrollo de alteraciones en sus dianas, como por la sobreexpresión de bombas de expulsión; con la resistencia a ciprofloxacino siendo la más fácil en ser seleccionada y la resistencia al cloranfenicol la más difícil de desarrollar.

2. La inestabilidad de la resistencia detectada en varios mutantes seleccionados con azitromicina, cloranfenicol y ciprofloxacino sugiere un elevado coste biológico, lo que puede ser la base para la infrecuencia de aislados clínicos de *B. bacilliformis* resistentes a los antibióticos. Por otro lado, la resistencia seleccionada para rifampicina fue estable aún en ausencia de presión antibiótica.

3. Con base en el estudio de mutantes de *B. bacilliformis* y en ausencia de ensayos clinicos, el cloranfenicol parece ser la mejor opción de tratamiento *in vitro*, mientras que el uso de ciprofloxacino y rifampicina no debería promoverse.

Capítulo II - Diagnóstico

4. El diagnóstico de síndromes febriles por criterios clínicos o microscopia puede conducir a un diagnóstico erróneo. Un brote infeccioso se atribuyó por error a *B. bacilliformis* y nuestros resultados sugieren la emergencia de *S. faeni* como el agente causante del brote adquirido en la comunidad, probablemente asociado con el agua.

5. La PCR de amplificación del gen *16S ARNr* presenta el límite de detección más bajo en comparación con las PCRs para la amplificación de los genes *fla* e *its*. Parece que la sensibilidad de estas técnicas podría permitir el diagnóstico de casos agudos de la enfermedad de Carrión, pero la aplicabilidad para detectar portadores con baja bacteriemia sigue sin estar clara. El uso de papeles de filtro conduce a una disminución de la positividad de las técnicas.

6. Los elevados resultados de positividad obtenidos con las técnicas de ELISA y PCR en tiempo real demuestran elevados niveles de anticuerpos contra *B. bacilliformis* y el alto número de portadores asintomáticos de *B. bacilliformis* en las zonas analizadas.

7. GroEL, Pap31, SCS-α y SCS-β se encuentran entre las proteínas de *B. bacilliformis* más dominantes inmunogenicamente. Estas proteínas son potenciales candidatos antigénicos y pueden ser útiles para el desarrollo de una herramienta de diagnóstico rápido.