Molecular mechanisms of resistance to antibiotics in *Bartonella bacilliformis*

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**Objectives:** *Bartonella bacilliformis* is the aetiological agent of Carrion’s disease. Although ciprofloxacin, rifampicin and erythromycin have been successfully used in the treatment of the disease, failures and relapses have been reported. The objective of our study was to select *in vitro* mutants resistant to antibiotics in order to determine the frequency of mutations and to characterize the mechanism of resistance at the molecular level.

**Methods:** Antibiotic-resistant mutants were selected by serial passages of bacteria on blood agar plates containing antibiotics. Candidate genes involved in resistance were amplified and sequenced and compared in order to look at mutations associated with antibiotic resistance.

**Results:** Ciprofloxacin-, rifampicin- and erythromycin-resistant mutants were obtained after five, three and four passages, respectively. Conversely, no mutant was obtained with either gentamicin or doxycycline even after 16 passages. The ciprofloxacin mutant contained an amino acid change at position 87 (Asp → Asn) in its quinolone resistance-determining region of the DNA gyrase protein, whereas the rifampicin-resistant strain had an amino acid change at position 531 (Ser → Phe) in the rifampicin resistance-determining region of the *rpoB* gene. Similarly, the erythromycin-resistant mutant showed an A2058G mutation in the 23S rRNA gene.

**Conclusions:** According with the current knowledge on the treatment of human bartonellosis, we believe that doxycycline in association with gentamicin may be the preferred regimen for the treatment of the acute and eruptive stages of Carrion’s disease, but clinical trials are warranted to support our findings.

Keywords: Carrion’s disease, genomic, bioinformatics, *in vitro* selection, disc diffusion assay

**Introduction**

*Bartonella bacilliformis* is the causative agent of Carrion’s disease in humans, a biphasic disease endemic to Andean valleys. The pathogen is transmitted between humans through the bite of contaminated phlebotomine sand flies of the genus *Lutzomyia*. The acute stage of the Carrion’s disease, called Oroya fever, is characterized by a severe, life-threatening haemolytic anaemia. The majority of infected people are children or young adults. Oroya fever results from the massive invasion of human red blood cells and causes death in 40% to 85% of infected humans who do not receive treatment. The chronic stage, termed verruga peruana, results in the appearance of unique vascular proliferative lesions of the skin. The infection is characterized by benign cutaneous vascular lesions, which typically consist of round papules that are frequently pruritic and bleeding, and the infection is accompanied by osteoarticular pain. This infection occurs most commonly in the Andes of Peru, Ecuador and Colombia, especially in tourists and transient workers. In the past, the only available treatment for the acute anaemia of Oroya fever was blood transfusion, but the effectiveness of the treatment was poor and the mortality rate was high. Penicillin G, chloramphenicol, tetracycline and erythromycin have been used for the treatment of Oroya fever. Treatment with these drugs produces rapid defervescence, with disappearance of the organisms from the blood usually within 24 h, although therapeutic failures and persistent bacteraemia have been reported after withdrawal of these antibiotics.

Antimicrobial therapy varies with syndrome and includes the use of chloramphenicol for Oroya fever and the use of...
streptomycin or rifampicin for verruga peruana. Reports of successful treatment of a limited number of infected patients with fluoroquinolones (ciprofloxacin) or macrolides (erythromycin or roxithromycin) hold promise for alternative therapeutic strategies. Since 1975, rifampicin has become the drug of choice for the treatment of the eruptive phase of Carrion’s disease. However, failures of rifampicin treatment have also been reported for the treatment of verruga peruana. Fluoroquinolone compounds have been used successfully in the last 5 years in adults and children and represent an alternative to chloramphenicol for the treatment of Oroya fever.

Fluoroquinolone antibiotics exert their antibacterial effects by inhibition of certain bacterial topoisomerase enzymes, namely, DNA gyrase (bacterial topoisomerase II) and topoisomerase IV. These essential bacterial enzymes alter the topology of double-stranded DNA within the cell. Resistance to fluoroquinolone antibiotics is typically conferred by point mutations in the quinolone resistance-determining region (QRDR) located near the N terminus of the A subunits of both gyrase (GyrA) and topoisomerase IV (ParC). Macrolide compounds such as erythromycin inhibit protein synthesis by binding to domains II and V of 23S rRNA. The inhibitory action of erythromycin, and probably that of the other 14-member-ring macrolides, is affected at the early stages of protein synthesis. In certain bacterial species, macrolide resistance can be due to mutations in highly conserved regions of ribosomal proteins L4 and L22. Resistance to rifampicin is almost exclusively associated with mutations in the rpoB gene encoding the RNA polymerase β-subunit. In the great majority of the rifampicin-resistant isolates, mutations occurred within an 81 bp hotspot region [the rifampicin resistance-determining region (RRDR), encoding 27 amino acids and corresponding to codons 507–533 or cluster I according to Escherichia coli numbering] in the centre of the 3516 bp rpoB gene. Tetracycline compounds such as doxycycline act by binding to the 30S ribosomal subunit, resulting in the inhibition of protein synthesis. Tetracycline resistance is often due to the acquisition of new genes, which code for energy-dependent efflux of tetracyclines or for a protein that protects bacterial ribosomes from the action of tetracyclines (tet and oxy genes). Aminoglycosides such as gentamicin and streptomycin bind to the ribosomes and thus interfere with protein synthesis. Resistance to these antimicrobials is widespread with various aminoglycoside-modifying enzymes and mutations in the 16S rRNA gene or the rpsL gene encoding ribosomal protein S12.

Given the growing potential of ciprofloxacin, rifampicin and erythromycin for the treatment of Carrion’s disease, this study was undertaken to select in vitro mutants resistant to antibiotics in B. bacilliformis in order to determine the specific target and frequency of mutations that confer resistance to these drugs in vitro and to propose new antibiotic treatment regimens for Carrion’s disease.

Materials and methods

The B. bacilliformis strain used (ATCC KC384) in this study was grown on Columbia 5% sheep blood agar plates (BioMerieux, Marcy L’Étoile, France). The plates were placed in polyethylene bags and incubated at 28°C for 2 weeks. Selection of antibiotic-resistant mutants was performed by serial passages of B. bacilliformis on a blood agar plate containing an antibiotic disc [ciprofloxacin (5 μg), rifampicin (30 μg), erythromycin (15 μg), doxycycline (30 μg) and gentamicin (15 μg)] (Mast Diagnostics, Amiens, France) initially placed in the corner or in the centre of the plate according to the measurement of diameter of growth inhibition. The plates were incubated at 28°C and diameters of growth inhibition (in millimetres) were measured every 2 weeks. The confluent growth outside the zone of inhibition was harvested with an inoculation loop and subcultured for the disc diffusion assay every 2 weeks until the strain became completely resistant.

The antibiotic-resistant strains of B. bacilliformis obtained in vitro were screened by PCR amplification and DNA sequencing using specific oligonucleotidic primers described in Table 1. These primers were designed according to the available complete genome sequence of other Bartonella species (http://www.genome.jp/kegg/). Methods for PCR amplification and DNA sequencing were similar to those previously reported for Ehrlichia spp. and Anaplasma spp. and for Bartonella quintana, except that annealing temperatures were different according to the nature of the primers.

The nucleotide sequences of candidate genes were compared using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/) to look at possible mutations known to be associated with antibiotic resistance. The known original sequences of E. coli (K-12 MG1655; for gyrA gene, rpoB gene and 23S rRNA gene comparison) and Streptococcus pneumoniae (TIGR4; for L4 gene and L22 gene comparison) were retrieved from the KEGG web site (www.genome.jp) and used for sequence alignments. The three-dimensional (3D) structure of the QRDR region of DNA gyrase A protein of E. coli (GenBank accession number 1AB4) was retrieved at the NCBI Structure web site and used for amino acid numbering and 3D construction using the Cn3d version 4.1 software downloadable at http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml to show the critical positions 81, 83 and 87 known to confer resistance to fluoroquinolone compounds.

Results

The ciprofloxacin-resistant strain of B. bacilliformis was obtained in vitro after five passages (diameter of inhibition fell

Table 1. Sequences of primers for Bartonella bacilliformis used in this study

<table>
<thead>
<tr>
<th>Primera</th>
<th>Sequence (5’→3’)</th>
</tr>
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<tbody>
<tr>
<td>rpoB gene</td>
<td>GATGATATTGATAATCTTGCA</td>
</tr>
<tr>
<td>BarpoBF</td>
<td>AACCATTATTAAACACGCGC</td>
</tr>
<tr>
<td>BarpoBR</td>
<td>AGTATTGTAGAATTGACCAAC</td>
</tr>
<tr>
<td>gyrA gene</td>
<td>AAAATCGAGCAGGAAATGATT</td>
</tr>
<tr>
<td>BagyrAF</td>
<td>TTCAATTATGAGATTGAG</td>
</tr>
<tr>
<td>BagyrAR</td>
<td>ACCGTAACATCTCATATG</td>
</tr>
<tr>
<td>16S rRNA gene</td>
<td>GATTCTTCGAGTGTAGAG</td>
</tr>
<tr>
<td>Ba16SF1</td>
<td>GAATTCGGATGGATCAG</td>
</tr>
<tr>
<td>Ba16SR1</td>
<td>AGAAAGGAGGTGATCCAG</td>
</tr>
<tr>
<td>Ba16SF2</td>
<td>ATAAACAATACGCTCAGC</td>
</tr>
<tr>
<td>Ba16SR2</td>
<td>BAIRACAGCAGCGCTACG</td>
</tr>
<tr>
<td>S12 gene</td>
<td>TTGGTATGGATGAGATTG</td>
</tr>
<tr>
<td>BaS12F</td>
<td>TTTTAGCGGGTAGACTG</td>
</tr>
<tr>
<td>BaS12R</td>
<td>TTTTAGCGGGTAGACTG</td>
</tr>
</tbody>
</table>

|a Primers for the 23S rRNA gene, and genes encoding the L4 and L22 ribosomal proteins were described in a previous study of B. quintana. |
Bartonella bacilliformis and antibiotic resistance

from 70 to <6 mm, MIC of >32 mg/L), and the QRDR region was characterized by the sequence analysis. When compared with the parental strain, the ciprofloxacin-resistant strain contained a transition from C to T at position 549 (E. coli numbering) of the gyrA gene (Figure 1a), encoding predicted amino acid change Asp-87→Asn (Table 2) in the gyrase A protein (Figure 2). We did not find any change in nucleotide sequence in the gyrB gene or parC gene for the ciprofloxacin-resistant strain of B. bacilliformis.

The rifampicin-resistant strain of B. bacilliformis was obtained after three passages (diameter of inhibition fell from 74 to <6 mm, MIC of >32 mg/L). DNA sequencing analysis of the rifampicin-resistant strain showed one nucleotide change from G to A at position 2868 (E. coli numbering), which leads to the mutation at the serine 531 codon (Ser→Phe) in the RRDR region of the rpoB gene (Figure 1b).

A mutant resistant to erythromycin was obtained after four subcultures (2 months) of the parental strain of B. bacilliformis. The diameter of inhibition slowly decreased after each passage and after four passages, the strain became completely resistant to erythromycin (diameter of inhibition fell from 82 to <6 mm, MIC of >256 mg/L) with growth directly in contact with the disc (Figure 3). The erythromycin-resistant mutant had a homogenous single nucleotide substitution at position 549 (A2058G, E. coli numbering) in the 23S rRNA gene (Figure 1c). We did not find any nucleotide substitution, deletion or insertion in the genes encoding the L4 and L22 ribosomal proteins for the erythromycin-resistant mutant of B. bacilliformis.

With doxycycline and gentamicin, we did not obtain resistant mutants of B. bacilliformis even after 16 passages (8 months). For gentamicin, from passages 8 to 16, the bacterial growth was very close to the antibiotic disc, but not exactly in contact with the disc (diameter of growth inhibition ~10–12 mm). The sequence of 16S and ribosomal protein S12 encoding genes did not show any changes when compared with the original strain of B. bacilliformis (Table 2).

Discussion

In the present study, we have described the molecular characterization of mutant strains obtained in vitro, conferring resistance to ciprofloxacin, rifampicin and erythromycin for B. bacilliformis. The method used in our study for the selection of mutants has been previously used for selection and molecular identification of macrolide-resistant mutants of B. quintana and Bartonella henselae. Although this in vitro selection assay was somewhat long in order to obtain mutants, it is a very simple method to perform that could be applied to other facultative intracellular bacteria such as Brucella spp. or Francisella tularensis.

We found a ciprofloxacin-resistant strain of B. bacilliformis after five passages and sequence analysis showed an Asp-87→Asn substitution in its gyrA gene (Table 2). The association of DNA gyrA mutations with fluoroquinolone resistance has been established for both Gram-negative and Gram-positive organisms. GyrA-mediated natural resistance to fluoroquinolones has been well described in E. coli and several Mycobacterium species. High-level resistance to fluoroquinolones has been described in Mycobacterium tuberculosis because of amino acid substitution in the wild-type gyrA sequence at the critical positions 83, 84 and 87, with MICs being more than 100-fold those for wild-type strains when these mutations accumulate. In E. coli, a single point mutation in gyrA results in decreased susceptibility to fluoroquinolones, and high-level resistance is associated with double amino acid substitutions in the gyrA protein. Although additional factors, such as mutations in the ParC subunit of topoisomerase IV and decreased intracellular drug accumulation by drug efflux, have

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**Figure 1.** (a) Nucleotide sequences of ciprofloxacin-susceptible (BBSGYRA) and ciprofloxacin-resistant (BBRGYRA) strains of B. bacilliformis with transition from C to T at position 549 (E. coli numbering) encoding D87N change in the QRDR region of the gyrA gene. (b) Nucleotide sequences of rifampicin-susceptible (BBSRPOB) and rifampicin-resistant (BBRRPOB) strains of B. bacilliformis with mutation from G to A at position 2868 (E. coli numbering) in the RRDR region of the rpoB gene. (c) Nucleotide sequences of erythromycin-susceptible (BBS23S) and erythromycin-resistant (BBR23S) strains of B. bacilliformis with transition from A to G at position 2058 (E. coli numbering) in domain V of the 23S rRNA gene.
been shown to play a complementary role by increasing the level of resistance, in vitro studies with E. coli suggest that the first step in selection for decreased susceptibility to fluoroquinolones is an alteration of Ser-83. Other critical positions in the gyrA QRDR have been described as positions 84 and 87 in E. coli. The mutation Asp-87—>Asn found in the ciprofloxacin-resistant strain of B. bacilliformis is commonly observed in E. coli and the homologous Asp-94 and Asp-95 substitutions reported for several other bacteria.11 Our results are worrying because fluoroquinolone compounds are now widely used for the treatment of Oroya fever and selection of ciprofloxacin-resistant isolates may be very quick and could explain failures and relapses reported during the treatment with this drug.8

Table 2. Number of passages required to obtain resistant mutants, MICs and molecular cause of resistance to different antibiotics for Bartonella bacilliformis

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of passages for resistant strain</th>
<th>MIC (mg/L)</th>
<th>gene</th>
<th>nt mutation</th>
<th>amino acid mutationa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>3</td>
<td>32</td>
<td>rpoB</td>
<td>G2868A</td>
<td>S5231F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>23S</td>
<td>A2058G</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>5</td>
<td>32</td>
<td>gyrA</td>
<td>C549T</td>
<td>D87N</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>no resistant strain (16 passages)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>no resistant strain (24 passages)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aE. coli numbering.

In comparison with the erythromycin-susceptible strain of B. bacilliformis, the erythromycin-resistant mutant had a homogenous A2058G mutation in the 23S rRNA gene. We did not find any nucleotide substitution, deletion or insertion in the genes encoding the L4 and L22 ribosomal proteins for the B. bacilliformis erythromycin-resistant mutant. Mutation at A2058, for certain macrolides, confers the highest levels of resistance.16 The erythromycin-binding mode indicates that increased bulkiness at position 2058 should impose spatial constraints and hamper macrolide binding. The A2058—>G transition was the first rRNA mutation shown to confer erythromycin resistance and is presently the most frequent clinically isolated substitution.16 Similar mutations (A2058G) have been identified in erythromycin-resistant strains belonging to a wide variety of species including clinical isolates and in vitro mutants of S. pneumoniae, Streptococcus pyogenes, several Mycobacterium spp., Helicobacter pylori, Mycoplasma pneumoniae,16,18,31 and B. henselae.26 In our previous study, we found that resistance to erythromycin in erythromycin-resistant mutants selected in vitro was associated with transition A2058G and also with A2059C transition in domain V of the 23S rRNA gene.26 Of more interest, we were able to demonstrate that such a mutation also exists in nature with one human lymph node harbouring organisms with such a mutation. Finally, other mutations, especially in the L4 ribosomal protein, were also associated with resistance to macrolides. Similarly, we have recently reported that the B. quintana erythromycin-resistant mutant strain, obtained in vitro using the same disc diffusion method, harboured a 27 base insert in the highly conserved region of the L4 ribosomal protein resulting in a nine amino acid repeat.25 In the present study, no mutation, insertion or deletion was observed with the erythromycin-resistant B. bacilliformis, but only one strain was used for the selection of mutants.

Interestingly, we were not able to select in vitro doxycycline- or gentamicin-resistant mutants of B. bacilliformis by disc diffusion assay after 16 passages (Table 2). We found similar results for other Bartonella species such as B. henselae and B. quintana with doxycycline (data not shown). For gentamicin, the B. bacilliformis strain did not become completely resistant, although the diameter of growth inhibition was very small (~10 mm), and sequence results with candidate genes for gentamicin resistance did not show any mutation when compared with the original strain.

Although Bartonella spp. were highly susceptible to most antibiotics tested previously,32–33 failures of monotherapy using a β-lactam, a macrolide, a tetracycline, rifampicin or a fluoroquinolone for Bartonella-related diseases have been reported.32–34 Relapses are also frequent following withdrawal of treatment. We have previously demonstrated in vitro that aminoglycosides alone are bactericidal against Bartonella species grown in liquid medium or in endothelial cells.35 or in erythrocytes.36 Such discrepancies between in vitro and clinical data may be explained by the lack of bactericidal activity of most antibiotics against Bartonella spp. These in vitro results have been later corroborated by in vivo results that confirm the superiority of a combination therapy with an aminoglycoside in Bartonella endocarditis37 or in bacteremic homeless people.38

Figure 2. Three-dimensional structure of QRDR domain of gyrE A protein of E. coli (GenBank accession number 1AB4) showing critical positions 81, 83 and 87; at which amino acid substitution could confer high-level quinolone resistance. The picture was obtained using the Cn3D version 4.1 software.
chloramphenicol with fluoroquinolone compounds as alternatives even if failures or relapses have been reported. In a large series of acute cases of Oroya fever reported recently, all 23 patients who received chloramphenicol with another antibiotic were cured, whereas 6 of 42 patients treated with chloramphenicol alone failed therapy and needed penicillin (3 patients) and 3 developed chronic verruga peruana lesions within the first 3 months of recovery after the acute phase. Therapeutic failures and persistent bacteraemia have been reported when chloramphenicol was used, and successful treatment with this drug does not appear to eliminate the patient’s risk for the development of the eruptive phase of B. bacilliformis infection. Because chloramphenicol is effective in most but not all patients with Oroya fever, simultaneous treatment with another antibiotic (especially a β-lactam compound) is recommended. Moreover, in verruga peruana, chloramphenicol is an ineffective treatment for this eruptive stage of infection with B. bacilliformis. Fluoroquinolone compounds have been used successfully in the first 10 years in adults and children over 6 years of age and represent an alternative to chloramphenicol for the treatment of Oroya fever. However, our results indicate that fluoroquinolones used in monotherapy may be ineffective because fluoroquinolone-resistant mutants may be selected easily.

The treatment used for verruga peruana has traditionally been streptomycin for 10 days, but the use of the intramuscular (im) route remains problematic, especially in children. Thus, rifampicin has become the first-line antibiotic for the treatment of the eruptive phase of Carrion’s disease. In a recent study, 55 of 77 patients with the eruptive phase of Carrion’s disease received antimicrobial therapy; 46 of the 55 patients received oral rifampicin (10 mg/kg/day for 10–14 days) and 9 received im streptomycin (15 mg/kg/day for 10 days). Thirty-seven (80%) of the 46 patients treated with rifampicin had a good response, and 5 (56%) of the 9 patients treated with streptomycin had a good response. The efficacy of rifampicin has been found to be comparable to that of streptomycin, with the disappearance of cutaneous lesions within a month of therapy. However, failures of rifampicin treatment have also been reported. Rapid resistance to rifampicin can develop when rifampicin is used alone, and, thus, rifampicin alone is not recommended for the treatment of any Bartonella infection except verruga peruana. We found mutation at the serine 531 codon (Ser→Phe) in the RRDR region in the case of the rifampicin-resistant B. bacilliformis strain (Table 2). As it was very easy to obtain the rifampicin-resistant strain in vitro in our study, we believe that rifampicin alone cannot be recommended for the treatment of the eruptive phase of Carrion’s disease. Failures of treatment have not been related to resistance in vitro, because in vitro susceptibility testing data on fresh isolates of B. bacilliformis are scarce in the literature. However, our study suggests that this may occur in nature and testing new strains with the Etest assay should be performed in the future.

In conclusion, according to the current in vitro and in vivo data for susceptibility and resistance to antibiotics in Bartonella spp., we believe that doxycycline in association with gentamicin may be the preferred regimen for the treatment of the acute and eruptive stages of Carrion’s disease, like in other Bartonella-related diseases. Clinical trials are warranted to support our in vitro results.

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Transparency declarations

None to declare.

References