JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

*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.

*J Infect Dev Ctries* 2015; 9(5):524-529. doi:10.3855/jidc.6235

(Received 09 November 2014 – Accepted 10 February 2015)



## 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.

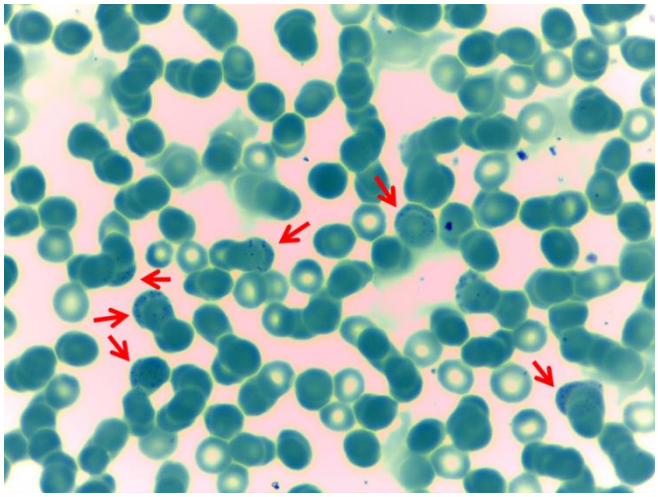
*Bacterial culture conditions*

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<br>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<br>Cough<br>Diarrhea<br>Hyporexia | 3 (5.7) |
| Low back pain<br>Epigastralgy | 2 (3.8) |
| Jaundice<br>Expectoration<br>Itching<br>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.

### Acknowledgements

This study was funded by the Instituto de Salud Carlos III (ISCIII, Spain) (grant number: FI12/00561, which included FEDER funds), by the Spanish Network for the Research in Infectious Diseases (REIPI RD12/0015) and Generalitat de Catalunya, Departament d'Universitats, Recerca i Societat de la Informació (2014 SGR 26) (JR) and by internal funds of the Universidad Peruana de Ciencias Aplicadas (UPC), Lima-Peru (JdV).

JR has a fellowship from the program I3, of the ISCIII (grant number: CES11/012). CG has a PhD fellowship of the ISCIII (FI12/00561). MJP has a postdoctoral fellowship from CONCYTEC.

We thank Donna Pringle for language and idiomatic corrections.

### 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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**Conflict of interests:** No conflict of interests is declared.