Carrion's Disease: diagnostic and antibody levels in a northern endemic area of Peru

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Introduction

- **Carrion disease**, a neglected poorest-linked illness (ORPHANET 64692), affects Andean valleys of Ecuador, Colombia and Peru[1-3], and also sporadically Bolivia and Chile[4]. This illness, **endemic and widespread in Peru**, is caused **by *Bartonella baciliformis***, a Gram-negative coccobacilli which infects red blood (RBC) and endothelial cells.
- **Two well-established phases** have been described. The acute phase, so-called **Oroya's Fever,** in which *B. bacillliformis* infect the RBC, results in **severe anemia** and transient immunosuppression[1,2,5]. In absence or delay of treatment, **high levels of mortality** are achieved (44% to 88%). The chronic phase, named '**verruga peruana'**, is characterized by the development of **nodular dermal eruptions**[2]. This phase typically occurs weeks or months after the acute febrile syndrome.
- Despite clinical cure, treatment does not often result in bacteria clearance, being about **40% of people from endemic areas asymptomatic carriers,** who perpetuate this illness[4] . No other reservoir has been described out of Human.
- Current **climate changes** have a direct role in the distribution of this illness once geographical **expansion of the vector**, belonging to the genus *Lutzomyia,* occurs[4,6].
- **In rural endemic areas the diagnosis** of the acute phase is mainly based on clinical symptoms and by **peripheral blood smears**. Nonetheless, this method is expertise-dependent, showing very **low sensitivity** (24-36%)[4]. **Bacterial culture has also low sensitivity** due to slow growth of bacteria[3]. Molecular tools are able to detect acute cases more efficiently than aforementioned methods[4,7], but **no effective diagnostic tool exists nowadays**.
- Last year an outbreak was reported for first time in Tunal, Guayaquiles, Los Ranchos and Mayland. Within a project addressed to study antigenic candidates in *B. bacilliforms* we have collected samples in these areas as well as in Huancabamba, an endemic area.

The objective of this study was to compare 2 different techniques used in Peru for diagnostic and evaluate the antibody titters for *B. bacilliformis* in inhabitants of both post-outbreak and one established endemic area.

How many people continue to have symptoms? Are them infected? **?** Are the PCR for diagnostic employed in Lima enough to detect both infected people and asymptomatic carriers? **?** How many people with evidence of infection but without symptoms are living in endemic areas?

Methods and Results

- Blood and serum samples from **177** people were collected in March 2014 in 5 different localities of **Piura**, in the north of Peru. After the study explanation, a informed consent was signed and clinical data were recorded.

Figure 1: Localization of sample collection area. In the map of Peru the endemic areas of Bartonellosis are highlighted in grey.

Figure 2: Several photos of sample collection in the different endemic areas of north of Peru.

Inclusion criteria: People post-outbreak areas that were diagnosed during the outbreak were recruited. Additionally, a small number of people never diagnosed with Carrion`s disease were also included. In Huancabamba (endemic area) the volunteers were recruited at random.

The samples were collected both in sodium citrate and gel SST II advance vacuteiners (BD). This last tubes permit serum separation.

Shipment of samples to Lima, where laboratory facilities exist.

From blood, PCR for *16s rRNA Bartonella* spp. and culture were done.

Shipment of sera to Tokyo, where antibody levels were measured by ELISA.

Table 1: Demographic and clinical information.

	Tunal	Guayaquiles	Los Ranchos	Mayland	Huancabamba	Total
n	75	25	43	10	24	177
Age (mean ; SD)	46.8 ; 22.5	34.2 ; 20.3	38.4 ; 19.5	30.2 ; 25.3	31 ; 18	XX
Male (%)	29.3	32	39.5	40	62.5	37.3
Female (%)	70.7	68	60.5	60	37.5	62.7
Carrion`s Disease symptoms (%)	68	26.7	40	37.5	25.6	34.5

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

About 1/3 of population continue presenting disease symptoms!

Antibody levels

- ELISA for both IgM and IgG with whole cell antigen was also done:

How to do it?

Briefly, the antigen preparation was done by sonication of *B. baciiliformis* agar grown. The total protein concentration was done by Pierce assay. We used 1 ug/well of antigen in each well. Each sample was in triplicate and the negative control used was from DAKO (X0939). Incubations were done at room temperature for 1 hour. The secondary antibodies used were DAKO P0215 for IgM conjugated with Peroxidase and DAKO 0336 for IgG conjugated with alcaline phospatase. The result quantification was measured with o-Phenylenediamine for IgM and Sigma P104 phosphatase substrate for IgG, respectively.

In absence of an established breakpoint for ELISA we have considered as evidence of infection the samples with at least 2 times the level of a commercial negative control.

Diagnostic techniques

- PCR for amplification of specific *16SrRNA* of *Bartonella* spp. amplification were performed using the following primers:

  Forward: 5`- CCT TCA GTT MGG CTG GAT C - 3`
  Reverse: 5`- GCC YCC TTG CGG TTA GCA CA – 3`

- The presence of *B. bacilliformis* was confirmed by sequencing positive PCR amplifications.
- For PCR positive samples 100 uL of blood was cultured at 28 C in agar supplemented with 5% sheep blood.

50.3% of volunteers' present levels of IgM equivalent with evidence of infection. For IgG this only happens for 2.83%!!

Table 2: Percentage of samples with at least two times the level of negative control by ELISA. PCR amplification and culture results.

	Tunal	Guayaquiles	Los Ranchos	Mayland	Huancabamba	Total
IgM (%)	45.3	60	46.5	60	58.3	50.3
IgG (%)	-	-	-	10	16.7	2.83
PCR + (%)	4	-	-	-	-	1.7
Culture	-	-	-	-	-	0

Evidence of a lot of people with high levels of IgM against *B. bacillliformis*.

Incidence of chronic people both in Mayland and Huancabamba!

Only 3 of samples had a positive result by PCR. No growth was observed in cultures.

Table 3: Relation between the presence of symptoms and evidence of infection in each sample collection area.

		Tunal	Guayaquilles	Los Ranchos	Mayland	Huancabamba	Total
Evidence of infection (> 2 times the level of the negative control)	No symptoms	36	16	37.2	50	50	36.2
	Symptomatic	9.3	44	9.3	10	16.7	15.3
	Total	45.3	60	46.5	60	66.7	51.4
No evidence of infection	No symptoms	37.3	16	37.2	10	12.5	29.4
	Symptomatic	17.3	24	16.3	30	20.8	19.2
	Total	54.7	40	53.5	40	33.3	48.6

From people with high antibody titters (51.4% for both IgM and IgG), only 15.2% reported symptoms, suggesting that 36.2% are asymptomatic.

Conclusions

- Both PCR and culture seems not to be enough to do the diagnostic of Carrion`s disease. Meanwhile, ELISA showed the presence of a high percentage of apparently healthy population with higher levels of antibodies against *B. bacilliformis*. Present data highlight the need to develop a diagnostic tool able to be implemented in rural endemic areas.
- In Huancabamba, an endemic area for *B. bacilliformis*, the IgG levels are significantly higher than post-outbreak areas where the disease was described for the first time.
- The high levels of IgM suggests the XXX
- There are no correlation between the presence of symptoms and the high antibody levels in ELISAs showing once more the difficult of diagnose correctly this disease based on clinical data.

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