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AN OUTBREAK OF ACUTE BARTONELLOSIS (OROYA FEVER) IN THE URUBAMBA REGION OF PERU, 1998

BARBARA A. ELLIS, LISA D. ROTZ, JOHN A. D. LEAKE, FRINE SAMALVIDES, JOSE BERNABLE, GLADYS VENTURA, CARLOS PADILLA, PABLO VILLASECA, LORENZA BEATI, RUSSELL REGNERY, JAMES E. CHILDS, JAMES G. OLSON, AND CARLOS P. CARRILLO

Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, and Meningitis and Special Pathogens Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; Instituto Nacional de Salud, Lima, Peru; Centro de Salud de Urubamba, Ministerio de Salud, Urubamba Santa Rosa Wanchaq, Cuzco, Peru

Abstract. During May 1998, we conducted a case-control study of 357 participants from 60 households during an outbreak of acute bartonellosis in the Urubamba Valley, Peru, a region not previously considered endemic for this disease. Blood and insect specimens were collected and environmental assessments were done. Case-patients (n = 22) were defined by fever, anemia, and intra-erythrocytic coccobacilli seen in thin smears. Most case-patients were children (median age = 6.5 years). Case-patients more frequently reported sand fly bites than individuals of neighboring households (odds ratio [OR] = 5.8, 95% confidence interval [CI] = 1.2–39.2), or members from randomly selected households ≥ 5 km away (OR = 8.5, 95% CI = 1.7–57.9). *Bartonella bacilliformis* isolated from blood was confirmed by nucleotide sequencing (citrate synthase [*glt*A], 338 basepairs). Using bacterial isolation (n = 141) as the standard, sensitivity, specificity, and positive predictive value of thin smears were 36%, 96%, and 44%, respectively. Patients with clinical syndromes compatible with bartonellosis should be treated with appropriate antibiotics regardless of thin-smear results.

Bartonellosis is a diphasic illness characterized by acute bacteremia with fever and profound anemia (Oroya fever) and by a benign, chronic phase with nodular skin lesions (verruga peruana).[1] Case fatality ratios (CFRs) of untreated Oroya fever exceed 40% but may reach more than 90% when superinfection with *Salmonella* species occurs.[2] The geographic distribution of bartonellosis is traditionally restricted to remote Andean valleys in Peru, Ecuador, and Colombia situated at elevations of 500–3,000 meters,[1,3] although recent foci of bartonellosis cases reportedly occurred at lower elevations in Ecuador and Columbia.[4]

The etiologic agent of bartonellosis (Carrion's disease), *Bartonella bacilliformis,* is thought to be transmitted to humans by crepuscular and nocturnal-feeding phlebotomine sand flies of the genus *Lutzomyia.*[5] Transmission of *B. bacilliformis* has been documented after intradermal injection of infected, homogenized *L. verrucarum* into monkeys,[6] and after wild *L. verrucarum* have been allowed to feed on macaques.[5] *Lutzomyia noguchii, L. peruensis,* and other sandfly species may also be competent vectors, since there have been documented outbreaks of bartonellosis in areas devoid of *L. verrucarum.*[5] To date, a natural, non-human vertebrate reservoir has not been identified for *B. bacilliformis.*

Though the disease has apparently been recognized since the time of the Incas, described bartonellosis outbreaks have been rare since the beginning of this century. To date, no single bartonellosis outbreak has been characterized in terms of its descriptive epidemiology, risk factors, and molecular identification of the etiologic agent. An outbreak of Oroya fever in the Pomabamba Province, Peru, was reported during February–October 1987; 14 persons died and 14 others became seriously ill with fever and anemia. The CFR among untreated cases was 88%.[7] Three bacterial isolates presumed to be *B. bacilliformis* were obtained from case-patients, but nucleotide sequencing was not performed. A retrospective case-control study from 1984 to 1995 identified 17 bartonellosis case-patients from Zamora Chinchipe Province, Ecuador. An association of bartonellosis cases with the presence of sick or dead chickens was reported.[8] In both of these reports, nearly all cases were diagnosed on clinical grounds.

Between November 1997 and May 1998, several cases of suspected Oroya fever occurred in the Urubamba Valley region in Peru, an area where *B. bacilliformis* was not considered endemic. We report the results of a collaborative effort by the Instituto Nacional de Salud (INS, Lima, Peru) and the Centers for Disease Control and Prevention (CDC, Atlanta, GA) that included isolation and confirmation of the etiologic agent and a case-control study to identify risk factors for acute bartonellosis.

CASES AND METHODS

Study site. The Urubamba Valley of Peru lies approximately 65 km northwest of Cuzco and 550 km southeast of Lima along the eastern slope of the Andes Mountains at an elevation of 2,825–3,250 meters (Figure 1). Individuals selected for the case-control study originated from communities as far north as Ollantaytambo (13°15′17″S, 72°15′48″W) and as far south as Calca (13°20′S, 71°57′W), approximately 35 km away. The climate is characterized by a distinct rainy season from October through May, when approximately 90% of the yearly precipitation falls, followed by a dry, cool season from June through September. Mean ± SD maximum temperatures are relatively constant throughout the year (22.4 ± 0.45°C); the lowest mean minimum temperatures are in July (1.2°C). Mean yearly precipitation totals 503 mm (Servicio Nacional de Meteorología y Geografía, Urubamba, Peru, 1988–1998, 10-year mean).

This case-control study was conducted in collaboration with the Peruvian National Institutes of Health as an emergency response outbreak investigation; therefore, the requirement for institutional review board approval was waived at CDC. The study was conducted at the request of the Peruvian National Institutes of Health and all aspects of the investigation were conducted in accordance with their

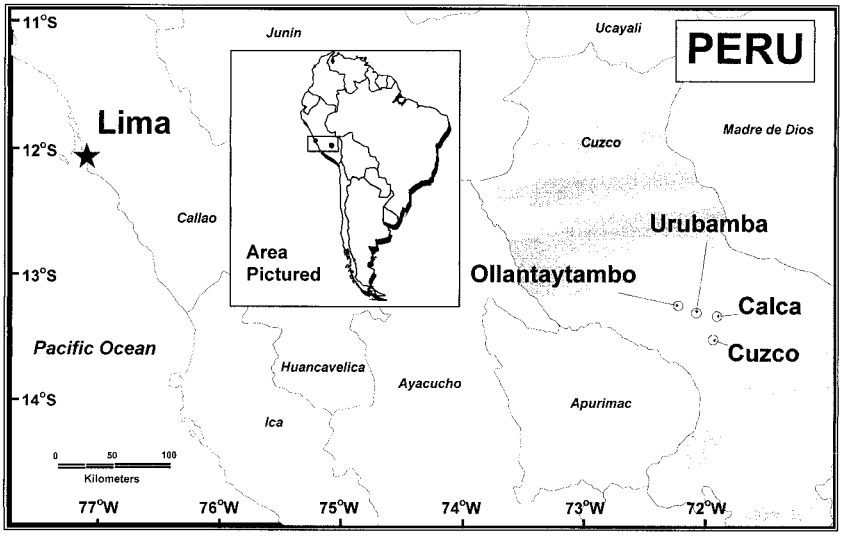

FIGURE 1. Location of the bartonellosis case-control study in the Cuzco area, Peru, May 1998.

human subject review policies. Verbal informed consent was obtained from all persons included in the study.

Study population. A confirmed case of Oroya fever was defined as fever (self-reported or documented ≥ 38°C); anemia (clinical pallor, hematocrit [Hct] ≤ 38 or hemoglobin ≤ 12.6 g/dl); and intra- or extra-erythrocytic coccobaccillary organisms seen on Giemsa-stained peripheral blood smears. Standards of anemia were adjusted for persons living at altitudes above 2,800 meters.[9] Cases were identified from clinical records of persons presenting for treatment at health centers in Urubamba, Calca, and Ollantaytambo, or at the regional hospital in Cuzco from April 3 through May 22, 1998. Though probable cases were identified from January 1 through April 3, cases could only be confirmed after April 3, since thin-smear results were only available for patients admitted after this date. Additional case-patients were prospectively enrolled as they presented to these health facilities during the case-control study period (May 12–22, 1998).

Personnel from the three above-mentioned health facilities, INS (Lima), and CDC visited the households of participating cases and controls. A standardized questionnaire was administered to the consenting head of household or surrogate ≥ 16 years old in his or her native language (Spanish or Quechua). Respondents provided the following information for each member of the household: name, age, sex, education, recent illnesses and hospitalizations, chronic medical conditions, routine medicines taken, alcohol and tobacco use, time spent outdoors during sand fly feeding times (crepuscular and nocturnal hours), insect bite prevention measures (clothing covering, insecticide and insect repellent use, and bed net use), history of insect bites or infestation (sand flies, fleas, and lice) and place where insect bites occurred (home or work), number and type of domestic and peridomestic animals at the home, outdoor occupational and recreational activities (fishing, swimming, animal trapping, wood gathering, and animal herding), work-related and non-work-related travel since January 1, 1998, and household hygienic facilities.

Cases were compared with three control groups: household residents, age-matched residents of houses located closest to the right of the case (near controls), and age-matched residents of houses located ≥ 5 km from the case (far controls). Near control households were selected by choosing the closest dwelling to the right of the case household with an age-matched resident in the home (0–4, 5–14, 15–30, and > 30 years old). Far control households were selected by locating the first house ≥ 5 km away in a randomly chosen direction with a similarly age-matched resident in the home.

Environmental assessment. Standardized environmental assessments were conducted to evaluate home and peridomestic conditions. Data were collected regarding the presence of running water, electricity, toilet facilities, potential sandfly breeding and harborage areas (e.g., stone walls, tree trunks), and number of open windows. Assessments of insect and small mammal infestation were made. Global positioning system units (Garmin Corp., Olathe, KS) were used to obtain latitude, longitude, and elevation of each home enrolled in the study. Arthropod vectors were collected from case and control households.

Specimen collection. A blood sample was collected by venipuncture or fingertip lancet from all consenting study participants for culture and thin smear, respectively. Additional whole blood specimens and thin smears were collected from consenting patients who presented to one of the above-mentioned health facilities with signs and symptoms compatible with bartonellosis but who were not participants in the case-control study. Thin smears were read by supervisory laboratory personnel from Cuzco Regional Hospital and by INS personnel who traveled to these health facilities during the study (CP and GV).

Blood samples were stored on dry ice in the field and transferred to -70°C when shipped to the laboratory for isolation of *Bartonella*. Peripheral smears were stained with Giemsa and examined by light microscopy for the presence of intra- or extra-erythrocytic coccobacillary bacteria.

Arthropod vectors were collected by a CDC miniature light trap fitted with a fine-mesh bag placed in the interior and exterior of consenting case and control households. Traps were set at dusk and collected the following morning.

Potential peridomestic sandfly habitats were sought and

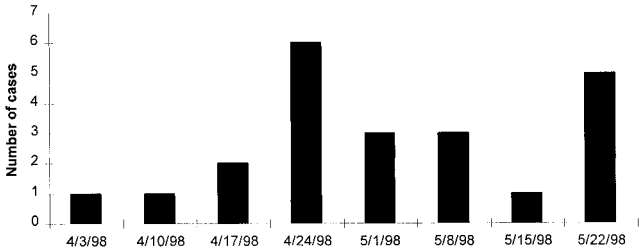

FIGURE 2. Weekly number of acute cases of bartonellosis by week, Urubamba Valley, Peru, April 3–May 22, 1998.

sand flies were collected by aspiration for approximately one hour per household. Captured arthropods were placed in vials, taken to the field laboratory where they were separated by genus and placed into 70% ethanol, then identified according to published references.[10] Samples of DNA from individual sand flies were extracted and polymerase chain reactions (PCRs) were used to detect *Bartonella.*

Bacterial isolation. Whole blood was streaked onto heart-infusion agar plates supplemented with 5% rabbit blood (BBL, Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md) for *Bartonella* isolation. Separate aliquots of 100 μl each for every specimen were incubated under two conditions: 28°C without CO_2 and at 32°C in 5% CO_2 for up to 35 days. Although *B. bacilliformis* is best isolated under the former conditions, the latter conditions were included to isolate other *Bartonella* species (e.g., *B. henselae*), if present.

Pure cultures were collected in 5 ml of brain heart infusion broth, aliquoted into 0.5-ml quantities in cryovials, and frozen at −70°C. If several colony types were observed, or bacterial or fungal contamination was present, bacteria were serially passaged until a pure culture was obtained.

Identification of *Bartonella* from humans and sand flies. The DNA of putative *Bartonella* cultures and individual sand flies was extracted using the QIAamp Blood Kit (Qiagen, Inc., Chatsworth, CA). Cultures were centrifuged at 10,000 × *g* for 5 min and the supernatant was removed prior to addition of protease and buffers. The Boehringer Mannheim Biochemicals (Indianapolis, IN) PCR Master kit was used with primers (*Bh*CS781.p and *Bh*CS1137.n) producing a 379-basepair amplicon of the citrate synthase (*glt*A) gene in the PCR.[11] Double-distilled water and *B. henselae* [Houston-1] DNA were used in each PCR assay as negative and positive controls, respectively. Products from the PCR were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized with ultraviolet light. The PCR products of the expected size were sequenced.

Nucleotide base sequence data were obtained on purified PCR products (Wizard PCR Prep, Promega, Madison, WI) sequenced in both directions with the PRISM dye-terminator sequencing kit on an autosequencer (ABI Prism 310; Applied Biosystems Inc., Foster City, CA).

Consensus sequences were obtained with the GAP4 program of the Staden software package[12] and compared with GenBank sequences using the BLAST program of GCG software.[13] Sequences were aligned using PILEUP;[13] similarity values of sequences were calculated using length of the shorter sequences without gaps in the program OLDDISTANCES.[13]

TABLE 1
Self-reported symptoms of confirmed bartonellosis cases included in the case-control study

Symptom	# Case-patients (%)	Median duration, days (range)
Fever	20/20 (100)	7.5 (1–30)
Pallor	17/20 (85)	4 (1–30)
Headache	16/20 (80)	7 (1–21)
Cough	14/20 (70)	11 (1–30)
Emesis	13/20 (65)	2 (1–30)
Icterus	11/20 (55)	6 (3–30)
Myalgia	10/20 (50)	7 (1–20)
Dyspnea	9/20 (45)	2 (1–14)
Arthralgia	7/20 (35)	7 (1–20)
Diarrhea	6/20 (30)	2 (1–13)
Edema	4/20 (20)	4 (2–30)
Rash	1/20 (5)	2
Lymphadenopathy	1/20 (5)	NR*
Verruga	0/20	NA†

* NR = not reported.
† NA = not applicable.

Statistical analysis. Cases were compared to household, near, and far controls with respect to the above-mentioned potential risk factors using Wilcoxon's signed-rank test[14] and the Mantel-Haenszel chi-square test[15] (SPSS version 8.5 software[16] and Epi-Info version 6.0[15]).

RESULTS

Study participation. A total of 357 persons living in 60 households were enrolled in the case-control study (20 cases, 114 household controls, 113 near controls, and 110 far controls). The number of male and female participants was nearly equal for cases and all control groups. In total, 22 confirmed bartonellosis cases were identified, 16 retrospectively through clinic records, and 6 prospectively; of these, 20 case-patients (90%) agreed to participate in the case-control study. From 1–6 cases of bartonellosis occurred each week during the study period (Figure 2).

Descriptive epidemiology. Ten case-patients (50%) were male. The median age of cases was 6.5 years (range = 1.3–36 years), which was significantly lower than that of other case household members (median = 17 years; $P < 0.005$) or that of the household members in near (13 years; $P < 0.05$) or far controls (13.5 years; $P < 0.01$). Sixteen (80%) cases were ≤ 12 years old. Reported symptoms are shown in Table 1. Fifteen cases (75%) were hospitalized, and 7 (35%) required blood transfusions; one case-patient (5%) died. Of the 16 cases for which Hct values were available, the median Hct was 24.5 (range = 10–33). All of the case-patients received chloramphenicol therapy (data regarding duration of therapy and timing of onset were not available). Three case households were clustered within 200 yards, and two cases had additional family members diagnosed with acute bartonellosis within 7 days.

Risk factors. Cases were more likely to report sandfly bites than near or far controls (odds ratio [OR] = 5.8, 95% confidence interval [CI] = 1.2–39.2 and OR = 8.5, 95% CI = 1.7–57.9, respectively), but not household controls (OR = 1.3, 95% CI = 0.2–9.1). Other behavioral or environmen-

tal risk factors were not identified by the case-control study. Both case and control households indicated that sand flies were more common during the period of study than during the same period in the previous year.

Environmental assessment. Case and control homes did not differ with respect to the frequency of running water, electricity, toilet facilities, potential sandfly breeding and harborage areas (e.g., stone walls, tree trunks), or number of open windows. No statistical difference in altitude between case and control households was found ($P > 0.05$, by Wilcoxon's signed-rank test).

Specimens collected. Of the 357 persons participating in the study, 250 peripheral smears (70%) and 135 whole blood specimens (38%) were obtained. An additional 11 whole blood specimens and 12 thin smears were collected from patients who presented to one of the health facilities, but who were not participants in the case-control study. Overall, 6 of 20 case-patients were acutely ill during the time the study was done; whole blood for bacterial isolation attempts was available from 5 of these 6 individuals.

All sand flies captured were identified as *Lutzomyia peruensis.* A total of 148 sand flies was collected during 107 trap nights within households (n = 52), in peridomestic settings (n = 45), and on farms (n = 10). Trapping was conducted in 95% (19 of 20) of the case households (43 trap nights); 85% of the near control households (17 of 20 households; 38 trap nights), and; 65% of the far control households (26 trap nights).

An additional 164 sand flies were collected by aspiration, 154 of which were from one case household. Mean numbers of sand flies captured within households in light traps were similar for cases and controls (case = 2.5, near = 0.3, far = 3.1; $P > 0.1$, by Wilcoxon's signed-rank test), as were those captured in peridomestic settings (case = 1.1, near control = 0.2, far control = 0.8; $P > 0.1$, by Wilcoxon's signed-rank test). The frequencies of case and control households having sand flies present either in the home or in adjacent peridomestic environments were also similar. For case households, 47% of the light traps placed within homes captured sand flies, compared with 54% of far control households and 18% of near control households. The presence of sandfly breeding, harborage, and resting areas (e.g., adobe walls, tree trunks, niches in stone or mud walls) was nearly ubiquitous: 59 (98%) of 60 study households had areas of potential sandfly harborage.

Bacterial isolation. Isolation and characterization of isolates (by *glt*A sequence) confirmed the presence of *B. bacilliformis* from 11 (8%) of 146 whole blood specimens (Table 2). Ten isolates were obtained from participants in the case-control study: 5 case-patients that had a current or past history of symptoms and disease; household controls that had no illness (n = 2), had only mild fever (n = 1), or had acute illness (n = 1); and one near control that had no symptoms. One isolate was from a patient who presented to the Urubamba Health Clinic with symptoms compatible with bartonellosis and was not enrolled in the case-control study. Overall, 6 of the 11 isolates were obtained from asymptomatic individuals with either no history of acute disease or mild disease, including 3 bartonellosis patients who had received chloramphenicol and who had terminated their treatment at least 2 weeks prior to blood sample collection. All *B. bacilliformis* isolates were obtained from cultures grown at 28°C without CO_2. Minimal to scant growth was obtained from the same samples incubated at 32°C in 5% CO_2.

TABLE 2

Individuals positive of those tested for *Bartonella bacilliformis* by thin smear and bacterial isolation, in the Urubamba Valley, Peru*

Case status	Thin smear	Bacterial isolation
Case	6/15 (40)	5/13 (38)
Case · household	2/80 (2.5)	4/57 (7.0)
Near control	1/83 (1.2)	1/37 (2.7)
Far control	2/72 (2.8)	0/28 (0.0)
Ill patients	3/12 (25)	1/11 (9.1)
Total	14/262 (5.3)	11/146 (7.5)

* Isolates were confirmed to be *B. bacilliformis* by the polymerase chain reaction and sequence of the 338-basepair citrate synthase (*glt*A) gene. Ill patients include those individuals presenting at local clinics with signs and symptoms compatible with bartonellosis during the investigation; these individuals were not part of the case-control study (numbers positive/total number tested; percentage given in parentheses).

Bartonella bacilliformis was isolated and confirmed by molecular characterization for 5 of the 13 cases (38%) available for testing. Two of the 5 case-patients that were bacteremic were acutely ill at the time blood was drawn.

Based on the testing of 141 individuals that had both bacterial isolation and Giemsa-stained peripheral thin-smears analyzed, the peripheral thin smear procedure had a sensitivity of 36% (95% CI = 0.09–0.63) when compared with bacterial isolation as the gold standard (4 individuals were thin-smear positive of a total of 11 isolation-positive individuals). The specificity of the thin-smear procedure when compared with bacterial isolation as the gold standard was 96% (95% CI = 0.93–0.99; 125 individuals were thin-smear negative of 130 isolation-negative individuals). The positive predictive value of the thin smear was 44% (95% CI = 0.13–0.75; 4 isolation and thin-smear positives of a total of 9 positive thin smears).

Of 312 sand flies collected during the investigation, 104 (33%) were analyzed for the presence of *Bartonella* DNA. Two PCR-positive sand flies (2%) were confirmed by nucleotide sequence analysis, including one that was identical to *B. bacilliformis* and one novel *Bartonella* that was 96% similar to *B. grahamii* (Beati L, unpublished data). Both sand flies were trapped from case households. Data regarding gender and blood meal status were not collected.

DISCUSSION

This bartonellosis outbreak is distinct from those described previously. First, it occurred in a region of Peru previously not known to be endemic. Second, most of the cases identified were children. The median age of patients in the current outbreak (6.5 years) was considerably less than the 17 years reported by other investigators in Peru.[7] These results may indicate that Cuzco is an endemic area of transmission that was previously unrecognized, and that adults in this area are at least partially immune to *B. bacilliformis* infection. Alternatively, this could be a newly susceptible population (including adults), but the findings may be due in part to selection bias, with children more likely than adults to be medically evaluated for their acute illness. Third, in this study, there was an equal gender ratio, as opposed to previous studies, which found a higher proportion (71%) of

infected males.[7] Fourth, the clinical manifestations of disease may be distinct in the confirmed bartonellosis patients from the Urubamba area, compared with elsewhere in Peru. Though it has been noted that the cutaneous phase of the disease does not always follow the anemic phase,[1] it is unusual that verruga has not been observed in any case patients to date (up to 6 months postinfection). In addition, the cutaneous phase of the disease has been documented to occur in the absence of the anemic phase, and evidence for verruga was not found while interviewing households consisting of $>$ 350 individuals. Though the isolates obtained were not genetically distinct from *B. bacilliformis* using the *glt*A gene, further characterization of the *B. bacilliformis* isolates obtained in this study may show molecular differences from isolates obtained from traditionally recognized endemic areas in Peru.

While participants in the study reported increased vector numbers over the last year, trapping of sand flies did not confirm high densities of sand flies (e.g., 20–50 sand flies[5]) in either domestic or peridomestic environments. Although this study could not define the location of exposures, the close proximity of several cases and the concurrent diagnosis of bartonellosis in more than one family member suggests that transmission may occur in or near the home. The lack of significant differences seen with other potential risk factors may be due to low case numbers and the relative homogeneity of environmental factors and behaviors in the region. In addition, the isolation of *B. bacilliformis* from asymptomatic, control participants demonstrates a problem with misclassification that may have made it difficult to detect significant risk factors for case group individuals.

The presence of 9–29% asymptomatic individuals infected with *B. bacilliformis,* both with and without a previous history of disease, has been previously noted in endemic areas.[17,18] Our study corroborates these findings (6 of 11 bacteremic individuals were asymptomatic at the time blood was drawn) and are in contrast to research by Gray and others,[7] who found no evidence of *Bartonella* in 97 asymptomatic, non-case individuals. At least in the Urubamba Valley area, the importance of asymptomatic individuals (including those previously treated) as potential reservoirs for *B. bacilliformis* infections should be considered further. Household contacts of bartonellosis patients should also be considered for screening and treatment.

Sandfly densities in the study area were low during our investigation, probably because of the onset of cool evening temperatures encountered toward the end of the rainy season when this investigation was undertaken. The only sandfly species encountered in the Urubamba area was *Lutzomyia peruensis,* known to feed on dogs and humans.[5] This is the first report of sequence-confirmed *B. bacilliformis* from this species of *Lutzomyia,* though a report from 1942 suggested that *L. peruensis* may be associated with transmission of bartonellosis.[5] The development of *B. bacilliformis* has not been studied in any species of *Lutzomyia.* Until careful laboratory studies can show *L. peruensis* is a competent vector, these results only tentatively incriminate this species in the transmission of bartonellosis to humans. We found approximately 1% of *L. peruensis* to be infected with *B. bacilliformis.* This is comparable to the reported frequency of infection for *L. verrucarum* (0.4–3%).[5] Favored harborage and breeding areas for *Lutzomyia* include adobe walls inside homes, and stone walls outdoors.[5] Many of the homes in the area had these constructions, and we collected *Lutzomyia* by aspiration from adobe and stone walls in case and control households.

This study is the first to examine the sensitivity and specificity of Giemsa-stained peripheral smears compared with bacterial isolation. Giemsa-stained peripheral smears were not found to be a sensitive assay for the presence of *Bartonella.* Currently, however, the thin smear is the only widely available method for confirming a diagnosis of bartonellosis. In contrast, isolation is highly sensitive (theoretically detecting the presence of one bacterium) but typically requires 2–5 weeks to culture and confirm the identity of the isolate. Given these findings and the high CFR of this illness, patients with clinical syndromes compatible with bartonellosis should be treated with appropriate antibiotics regardless of thin-smear results, especially in the setting of confirmed outbreaks. In addition, these results underscore the importance for the development of rapid diagnostic tests for *B. bacilliformis* infections. Other molecular techniques based on extraction and amplification of DNA directly from whole blood specimens, as well as serologic tests, are currently being explored as a means to provide a more timely diagnosis of bartonellosis.

Peru receives more than 700,000 tourists yearly, most of which visit the Cuzco area. Visitors going to this region should be aware of preventive measures to guard against acquiring bartonellosis, including protection against exposure to sand flies (e.g., wearing long-sleeve shirts and pants, using insect repellent, and reducing outdoor activities around dusk and dawn when sand flies are most active). Health care providers caring for travelers should include bartonellosis in the differential diagnosis for tourists returning from this area of Peru. Currently, surveillance has been intensified and public education initiated regarding bartonellosis and insect bite prevention measures in the Urubamba Valley. Additional information to define exposure areas is needed before targeted insecticide campaigns or other possible interventions can be initiated.

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Authors' addresses: Barbara A. Ellis, Lisa D. Rotz, Lorenza Beati, Russell Regnery, James E. Childs, and James G. Olson, Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Mailstop G-13, 1600 Clifton Road, Atlanta, GA 30333. John A. D. Leake, Meningitis and Special Pathogens Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Mailstop C-23, 1600 Clifton Road, Atlanta, GA 30333. Frine Samalvides, Gladys Ventura, Carlos Padilla, Pablo Villaseca, and Carlos P. Carrillo, Instituto Nacional de Salud, Capac Yupanqui 1400, Apartado 451, Jesus Maria, Lima, Peru. Jose Bernable, Centro de

Salud de Urubamba, Ministerio de Salud, Psje. Gibaja 340, Urubamba Santa Rosa Wanchaq, Cuzco, Peru.

Reprint requests: Barbara A. Ellis, Division of Viral and Rickettsial Diseases, Viral and Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, Mailstop G-13, 1600 Clifton Road, Atlanta, GA 30333.

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