Molecular Typing of “*Candidatus Bartonella ancashi*,” a New Human Pathogen Causing Verruga Peruana

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A recently described clinical isolate, “*Candidatus Bartonella ancashi*,” was obtained from a blood sample of a patient presenting with verruga peruana in the Ancash region of Peru. This sample and a second isolate obtained 60 days later from the same patient were molecularly typed using multilocus sequence typing (MLST) and multispacer sequence typing (MST). The isolates were 100% indistinguishable from each other but phylogenetically distant from *Bartonella bacilliformis* and considerably divergent from other known *Bartonella* species, confirming their novelty.

The genus *Bartonella* consists of 29 recognized species and three subspecies of hemotropic Gram-negative bacteria that infect a wide range of mammals by transmission through various arthropod vectors, such as human body lice, fleas, and sandflies. Among them, *Bartonella henselae*, *Bartonella quintana*, and *Bartonella bacilliformis* are established as pathogens of human importance, causing cat-scratch fever, trench fever, and Carrion’s disease, respectively, while an increasing number of other *Bartonella* species have recently been found to be infectious to humans, particularly immunocompromised persons (1, 2). Additionally, *B. bacilliformis* is transmitted by *Lutzomyia* sandflies and causes a biphasic syndrome (Carrion’s disease) consisting of an acute phase, Oroya fever, and a chronic phase known as verruga peruana that is characterized by benign and persistent red-purple raised skin nodules (3). *B. bacilliformis* infections are seen only in the Andes mountain region of Peru, Ecuador, and Columbia (2,500 to 8,000 feet above sea level) (3, 4). Although *B. bacilliformis* was the first *Bartonella*

species discovered, over 100 years ago, and is a pathogen causing significant morbidity and mortality, our understanding of this pathogen and the causes of Carrion’s disease are rather limited (2, 4–6). Currently, *B. bacilliformis* is the only agent definitively identified to cause Carrion’s disease, though *Bartonella rochalimae* caused an Oroya fever-like illness in a traveler returning from Peru (2, 4, 6). Here, we further characterized a new *Bartonella* pathogen

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TABLE 1 Primers used for PCR, nested PCR, and/or sequencing^a

Gene	Primer	Sequence (5′–3′)	Use(s)	Fragment length (bp)
<i>rrs</i>	16SU17F	AGAGTTTGATCCTGGCTCAG	PCR, nPCR, sequencing	1,424
	16SU1592R	AGGAGGTRATCCAGCCGCA	PCR, nPCR, sequencing	
	16SU 833R	CTACCAGGGTATCTAATCCTGTT	nPCR, sequencing	
	16S E. coli-518F	CAGCAGCCGCGTAATAC	nPCR, sequencing	
<i>gltA</i>	BHCS 781p (F)	GGGGACCAGCTCATGGTGG	PCR, sequencing	338
	BHCS 1137n (R)	AATGCAAAAGAACAGTAAACA	PCR, sequencing	
<i>rpoB</i>	rpoB1435F	CGCATTGGTTTCTTCGTATG	PCR	589
	rpoB2327R	GTAGACTGATTAGAACGCTG	PCR, nPCR, sequencing	
	rpoB1696F	CCTACGCATTATGGTCGTATTTG	nPCR, sequencing	
<i>ftsZ</i>	Bfp1 (F)	ATTAATCTGCAYCGGCCAGA	PCR, sequencing	864
	Bfp2 (R)	ACVGADACACGAATAACACC	PCR, sequencing	
<i>groEL</i>	HSPF1d (F)	GAAGTNGAAGATAAGTTNGAA	PCR	736
	BbHS1630.n (R)	AATCCATTCCGCCCATTC	PCR	
	HSP1 (F)	GGAAAAAGTNGCAATGAAG	nPCR, sequencing	
	HSP2 (R)	GCNGCTTCTTCACCNCGATT	nPCR, sequencing	

^a *rrs* primer 16SU17F was previously described (14). The additional *rrs* primers and reaction conditions were previously described (7). *rpoB* primer rpoB2327R was previously described (13). The additional *rpoB* primers and the sequencing conditions were previously described (7). *gltA* primers were previously described (12), and reaction conditions were described by Blazes et al. (7). *ftsZ* and *groEL* primers and reaction conditions were previously described (10, 11). F, forward primer; R, reverse primer; nPCR, nested PCR.

TABLE 2 Identity of “*Candidatus Bartonella ancashi*” genes to *gltA*, *rpoB*, *ftsZ*, *groEL*, *ribC*, and *rrs* of known *Bartonella* species

Species and strain	% Identity ^a					
	<i>gltA</i>	<i>rpoB</i>	<i>ftsZ</i>	<i>groEL</i>	<i>ribC</i>	<i>rrs</i>
<i>Bartonella alsatica</i> IBS 382	86.5	84.8	88.7	81.8	76.9	97.7
<i>Bartonella bacilliformis</i> KC583	84.9	86.4	86.8	84.9	79.4	99.0
<i>Bartonella birtlesii</i> IBS 325	88.1	84.0	88.3	83.0	75.7	98.2
<i>Bartonella bovis</i> 91-4	89.4	85.9	86.5	84.4	75.6	98.2
<i>Bartonella capreoli</i> IBS 193	85.5	85.6	88.3	83.0	75.7	98.2
<i>Bartonella chomelii</i> A828	87.1	85.5	87.9	83.2	75.4	97.8
<i>Bartonella clarridgeiae</i> Houston-2 cat	86.8	85.0	86.6	82.6	75.9	97.6
<i>Bartonella doshaiae</i> R18	84.6	84.9	87.3	77.9	75.2	98.2
<i>Bartonella elizabethae</i> F9251	84.9	83.2	86.2	81.7	74.4	97.9
<i>Bartonella grahamii</i> V2	83.9	83.7	85.7	81.2	75.6	97.9
<i>Bartonella henselae</i> Houston-1	87.1	84.7	87.1	81.2	75.4	98.0
<i>Bartonella japonica</i> Fuji 18-1	81.7	84.4	87.3	81.7	75.1	97.7
<i>Bartonella koehlerae</i> C-29	85.8	84.2	86.6	81.0	74.6	98.2
<i>Bartonella quintana</i> Fuller	84.9	84.8	87.3	81.5	75.4	98.0
<i>Bartonella schoenbuchensis</i> R1	86.5	85.6	87.6	83.3	75.9	98.2
<i>Bartonella silvatica</i> Fuji 23-1	81.0	84.0	85.9	81.2	75.9	98.2
<i>Bartonella taylorii</i> M6	84.9	84.2	86.0	81.6	75.7	97.9
<i>Bartonella tribocorum</i> IBS 506	85.5	83.7	86.4	80.7	76.6	97.7
<i>Bartonella vinsonii</i> subsp. <i>arupensis</i> OK 94-513	85.5	84.6	87.3	78.0	75.2	97.9
<i>Bartonella vinsonii</i> subsp. <i>berkhoffii</i> 93-CO1	85.2	84.1	87.6	82.2	74.4	98.1
<i>Bartonella vinsonii</i> subsp. <i>vinsonii</i> Baker	85.8	84.7	87.8	82.6	73.3	98.2

^a Percentages were calculated based on the full gene sequences compiled using 454 sequencing. Comparisons were then made to known *Bartonella* species in GenBank and fragments of *gltA* (312 bp), *rpoB* (825 bp), *ftsZ* (788 bp), *groEL* (1,192 bp), *ribC* (607 bp), and *rrs* (1,351 bp). Boldface type indicates the *Bartonella* species with the highest similarity to “*Candidatus Bartonella ancashi*” for each respective gene fragment.

causing verruga peruana (7) by using multilocus sequence typing (MLST) and multispacer sequence typing (MST).

The agent was initially isolated from a 3-year-old boy with verruga peruana living in Caraz, Peru (located in the Andes mountain range of Peru), who had been enrolled in a clinical trial to compare the efficacies of azithromycin and rifampin for the treatment of verruga peruana caused by *B. bacilliformis*. The child had 56 lesions, distributed mainly on the extremities, but did not have fevers, arthralgias, or malaise. He had no evidence of anemia at presentation, and his Giemsa blood smear was negative for intracellular organisms. Blood samples taken from the patient (clinical trial subject 20) were culture positive for *Bartonella* at the time of enrollment (20.00) and again at day 60 (20.60). Peripheral blood thin smears were prepared at the Ministry of Health (MOH) Hospital and later confirmed at the reference laboratory in Lima. Blood samples for culture were collected in sodium citrate tubes and transported to Lima, where they were cultured in sealed flasks, using a modified F-1 medium with liquid overlay of RPMI 1640 medium with 10% fetal bovine serum, and then were observed for 8 weeks at 28°C without additional CO₂. Confirmation of bacterial isolates as *B. bacilliformis* was done by PCR using primers for the citrate synthase gene (*gltA*).

The patient was treated with azithromycin on day 0 and day 7, which typically results in effective tissue levels of antibiotic for 2 weeks. His rash resolved fully, but he had another positive blood culture at day 60.

Pure bacterial isolates (from culture) were initially subjected to microbiology testing and PCR and Sanger sequencing of a 338-bp *gltA* fragment for identification of *B. bacilliformis*. The *gltA* sequences from both isolates were identical to each other but were found to be significantly different from those of *B. bacilliformis* and other known *Bartonella* species. Fragments of *rrs* (1,424 bp) and *rpoB* (RNA polymerase beta-subunit) (589 bp) were sequenced, and a BLAST query

FIG 1 MLST phylogeny for a 5,108-character fragment of the concatenated gene sequences *rrs* (1,351 bp), *rpoB* (825 bp), *gltA* (312 bp), *ftsZ* (788 bp), *ribC* (607 bp), and *groEL* (1,192 bp) for the 21 *Bartonella* type strains and “*Candidatus Bartonella ancashi*” 20.60. The neighbor-joining-tree method (1,000 bootstrap replicates) was employed, using Mega5 software, and the distances were calculated using the Jukes-Cantor method, in which units are calculated as the number of base pair substitutions per site (19). *Brucella melitensis* was used as the out group.

FIG 2 ITS phylogeny for a 1,029-character fragment of the 16S-23S intergenic linker region of “*Candidatus Bartonella ancashi*” 20.60, *Bartonella bacilliformis* isolates, *Bartonella rochalimae*, and the next-most-closely related *Bartonella* species, *Bartonella clarridgeiae*. The neighbor-joining-tree method (1,000 bootstrap replicates) was employed, using Mega5 software, and the distances were calculated using the Jukes-Cantor method, in which units are calculated as the number of base pair substitutions per site (19).

against the NCBI nonredundant DNA database (nr/nt) confirmed the sequence dissimilarity, indicating a probable emerging *Bartonella* human pathogen that was subsequently provisionally named “*Candidatus Bartonella ancashi*” (7).

To more fully characterize “*Candidatus Bartonella ancashi*,” the initial isolate (20.00) and the second isolate (20.60) were subjected to gene sequencing, MLST, and MST. MLST and MST techniques are used in the differentiation of *Bartonella* species and strains (5, 8, 9). MLST is useful for the detection of novel *Bartonella* species, while MST is useful for uncovering genetic differences between strains (5, 8, 9).

DNA extracted from both clinical isolates was subjected to PCR amplification and Sanger sequencing as previously described (Table 1) (1, 7, 10–14). Furthermore, the genomes of both isolates were fully sequenced and assembled using a Roche GS FLX Titanium sequencing system and assembly software GSAssembler, version 2.5.3 (Roche 454 Life Sciences, Branford, CT). Complete sequences for five housekeeping genes, *gltA* (1,341 bp), *rpoB* (4,149 bp), *ftsZ* (1,776 bp), *groEL* (1,644 bp), and *ribC* (riboflavin synthase, 642 bp), and for *rrs* (1,474 bp) and the 16S-23S intergenic spacer (ITS) (940 bp) were extracted and submitted to GenBank (*ribC* [KC886734], *ftsZ* [KC886735], *gltA* [KC886736], *groEL* [KC886737], *rpoB* [KC886738], *rrs* [KC886739], and ITS [KC886740]). The sequences from both Sanger and 454 pyrosequencing are 100% identical to each other. Additionally, the gene sequences of “*Candidatus Bartonella ancashi*” isolates 20.00 and 20.60 were found to be 100% identical to one another at these loci. The full gene sequences of isolate 20.60 were then used for molecular typing, including MLST and MST.

For these isolates, the MLST analysis included the concatenation of *rrs* (16S rRNA genes), *gltA*, *rpoB*, *ftsZ* (cell division protein), *groEL* (60-kDa heat shock protein), and *ribC* (riboflavin synthase) for phylogenetic assessment, while the MST analysis was conducted using the ITS region.

The nucleotide identities between the five housekeeping genes and *rrs* of “*Candidatus Bartonella ancashi*” and known *Bartonella* species are summarized in Table 2. The results show 90% or less sequence similarity between all the housekeeping genes from “*Candidatus Bartonella ancashi*” and those of known *Bartonella* species, while the *rrs* identity was 99% or less between the isolate and known *Bartonella* species. MLST phylogeny was assessed using concatenated sequences for these 6 loci of 21 *Bartonella* type strains and “*Candidatus Bartonella ancashi*” (Fig. 1). The analysis indicates that “*Candidatus Bartonella ancashi*” is a member of the *Bartonella* genus and most closely related to *B. bacilliformis* (Fig. 1). To further explore the extent of their relatedness, complete ITS sequences for “*Candidatus Bartonella ancashi*,” *B. bacilliformis* isolates, *B. rochalimae*, and *Bartonella clarridgeiae* were aligned for the MST analysis (Fig. 2) (1). ITS sequences are highly variable between strains of the same species and are able to provide more insight into the genetic diversity and relatedness of genotypes within a species (1). *B. bacilliformis* isolates cluster in close proximity to each other, with the exception of *B. bacilliformis* isolate LA6.3, which belongs to a highly divergent group of *B. bacilliformis* isolates, strain type 8 (5). “*Candidatus Bartonella ancashi*” is located on an isolated branch away from the *B. bacilliformis* isolates, including *B. bacilliformis* isolate LA6.3, and away from *B.*

rochalimae, providing more evidence that “*Candidatus Bartonella ancashii*” is unique.

In the majority of cases, a patient is given a diagnosis of Carrion’s disease, caused by *B. bacilliformis*, based on clinical characteristics, blood smears, and microbiological culture, which are usually insufficient to distinguish novel *Bartonella* species causing either verruga peruana or Oroya fever from *B. bacilliformis*. Additionally, *Bartonella* has proved to be a difficult organism to culture from clinical samples. For these reasons, PCR and sequencing-based genotyping methods, such as single-gene sequencing and MLST/MST, need to be used for identifying new species of *Bartonella* from rodents, arthropods, and clinical specimens (9, 15, 16). MLST methods, which involve concatenating a predetermined number of gene sequences, generally include the housekeeping genes (*ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*) and *rrs* for phylogenetic analysis (9, 15), while MST is based on the variable 16S/23S intergenic linker region and is valuable for genotyping strains of *Bartonella* species (8). Additionally, fragments of the *gltA* and *rpoB* genes are found to have the highest discriminating power for *Bartonella* species identification and classification (16). La Scola et al. proposed that a *Bartonella* agent be considered a new species if a 327-bp *gltA* gene fragment and an 825-bp *rpoB* gene fragment were <96% and <95.4% similar, respectively, to a known *Bartonella* species (12). The similarity ranges for the *rpoB* and *gltA* gene fragments for “*Candidatus Bartonella ancashii*” fall well below these values, thereby providing evidence that the isolates represent a novel *Bartonella* species.

Furthermore, very low sequence divergence of genetic loci for sequence types (ST) within a species was demonstrated in MLST studies, for instance, 0 to 0.4% for *B. quintana* and 0.3 to 1% for *B. henselae* (17, 18). The sequence data obtained from the *gltA* and the *rpoB* gene fragments for the isolates (20.00 and 20.60) show notably low identity and high divergence from *B. bacilliformis* and other known *Bartonella* species (Table 2). Phylogenetic analyses using MLST and the more strain-specific ITS sequences indicate that “*Candidatus Bartonella ancashii*,” while most closely related to *B. bacilliformis* by MLST analysis, is in fact highly dissimilar from *B. bacilliformis* by MST analysis, thereby providing clear insight into the unique evolution of this new *Bartonella* agent.

The results from this study, along with the recent discovery of *B. rochalimae* and the genetic diversity of *B. bacilliformis* (up to 5.3% divergence), indicate the possibility of a diverse group of *Bartonella* species or novel *B. bacilliformis*-like agents able to cause human disease circulating in areas of Peru where Carrion’s disease is endemic (5, 7). Further microbiological, microscopic, and functional characterization of the isolates and whole-genome comparative analyses with *B. bacilliformis* and related species will further our understanding of the taxonomy of the *Bartonella* genus and, more importantly, provide insights into the genomic structure variations and dynamics, mechanisms for the high host adaptability, and pathogenesis of these endemic and opportunistic pathogens.

Nucleotide sequence accession numbers. The full gene sequences for the 6 housekeeping genes and the ITS were submitted to GenBank under the following accession numbers: *ribC*, KC886734; *ftsZ*, KC886735; *gltA*, KC886736; *groEL*, KC886737; *rpoB*, KC886738; *rrs*, KC886739; ITS, KC886740.

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