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 Carrión’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 (Carrión’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 Colombia (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 Carrión’s disease are rather limited (2, 4–6). Currently, B. bacilliformis is the only agent definitively identified to cause Carrión’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.

TABLE 1 Primers used for PCR, nested PCR, and/or sequencinga

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'–3')</th>
<th>Use(s)</th>
<th>Fragment length (bp)</th>
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<tbody>
<tr>
<td>rrs</td>
<td>16SU17F</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>PCR, nPCR, sequencing</td>
<td>1,424</td>
</tr>
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<td></td>
<td>16SU1592R</td>
<td>AGGGAGGTATTCCAGCCGCA</td>
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<td></td>
<td>16SU 833R</td>
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<tr>
<td></td>
<td>16S E. coli-518F</td>
<td>CAGCAGCGCCCGGTAAATC</td>
<td>nPCR, sequencing</td>
<td></td>
</tr>
<tr>
<td>gltA</td>
<td>BHCS 781p (F)</td>
<td>GGGACACAGCTATGTTGG</td>
<td>PCR, sequencing</td>
<td>338</td>
</tr>
<tr>
<td></td>
<td>BHCS 1137n (R)</td>
<td>AATGCAAAAAGAACAGTAAACA</td>
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<tr>
<td>rpoB</td>
<td>rpoB1435F</td>
<td>CCACATTGTTTTRCTTTGTATG</td>
<td>PCR, nPCR, sequencing</td>
<td>589</td>
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<tr>
<td></td>
<td>rpoB2327R</td>
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<td></td>
<td>rpoB1696F</td>
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<td></td>
</tr>
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<td>ftsZ</td>
<td>Bfp1 (F)</td>
<td>ATTAATCTGCAyCGGCCAAG</td>
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<td>864</td>
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<td></td>
<td>Bfp2 (R)</td>
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<td></td>
</tr>
<tr>
<td>groEL</td>
<td>HSP1F1d (F)</td>
<td>GAACTNGAAGATAAGTTNGAA</td>
<td>PCR</td>
<td>736</td>
</tr>
<tr>
<td></td>
<td>BbHS1630.n (R)</td>
<td>AATCCTATTCTCGCATTTT</td>
<td>PCR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HSP1 (F)</td>
<td>GGGAATGNGGCAATGGA</td>
<td>nPCR, sequencing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HSP2 (R)</td>
<td>GCNGGTTCCTCACCNGCATT</td>
<td>nPCR, sequencing</td>
<td></td>
</tr>
</tbody>
</table>

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.
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 species with the highest similarity to "Candidatus Bartonella ancashi" genes to gltA, rpoB, ftsZ, groEL, ribC, and rrs of known Bartonella species.

### TABLE 2

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>gltA %</th>
<th>rpoB %</th>
<th>ftsZ %</th>
<th>groEL %</th>
<th>ribC %</th>
<th>rrs %</th>
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<tbody>
<tr>
<td>Bartonella alastica IBS 382</td>
<td>86.5</td>
<td>84.8</td>
<td>88.7</td>
<td>81.8</td>
<td>76.9</td>
<td>97.7</td>
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<tr>
<td>Bartonella bacilliformis KC583</td>
<td>84.9</td>
<td>86.4</td>
<td>86.8</td>
<td>84.9</td>
<td>79.4</td>
<td>99.0</td>
</tr>
<tr>
<td>Bartonella birtlesii IBS 325</td>
<td>88.1</td>
<td>84.0</td>
<td>88.3</td>
<td>83.0</td>
<td>75.7</td>
<td>98.2</td>
</tr>
<tr>
<td>Bartonella bovis 91-4</td>
<td>89.4</td>
<td>85.9</td>
<td>86.5</td>
<td>84.4</td>
<td>75.6</td>
<td>98.2</td>
</tr>
<tr>
<td>Bartonella capreoli IBS 193</td>
<td>85.5</td>
<td>85.6</td>
<td>88.3</td>
<td>83.0</td>
<td>75.7</td>
<td>98.2</td>
</tr>
<tr>
<td>Bartonella chomelii A828</td>
<td>87.1</td>
<td>85.5</td>
<td>87.9</td>
<td>83.2</td>
<td>75.4</td>
<td>97.8</td>
</tr>
<tr>
<td>Bartonella claridgeiae</td>
<td>86.8</td>
<td>85.0</td>
<td>86.6</td>
<td>82.6</td>
<td>75.9</td>
<td>97.6</td>
</tr>
<tr>
<td>Bartonella doshiae R18</td>
<td>84.6</td>
<td>84.9</td>
<td>87.3</td>
<td>77.9</td>
<td>75.2</td>
<td>98.2</td>
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<td>Bartonella elizabethae F9251</td>
<td>84.9</td>
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<td>86.2</td>
<td>81.7</td>
<td>74.4</td>
<td>97.9</td>
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<td>Bartonella grahamii V2</td>
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<td>83.7</td>
<td>85.7</td>
<td>81.2</td>
<td>75.6</td>
<td>97.9</td>
</tr>
<tr>
<td>Bartonella henselae Houston-1</td>
<td>87.1</td>
<td>84.7</td>
<td>87.1</td>
<td>81.2</td>
<td>75.4</td>
<td>98.0</td>
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<tr>
<td>Bartonella japonica Fuji 18-1</td>
<td>81.7</td>
<td>84.4</td>
<td>87.3</td>
<td>81.7</td>
<td>75.1</td>
<td>97.7</td>
</tr>
<tr>
<td>Bartonella koehlerae C-29</td>
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<td>84.2</td>
<td>86.6</td>
<td>81.0</td>
<td>74.6</td>
<td>98.2</td>
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<tr>
<td>Bartonella quintana Fuller</td>
<td>84.9</td>
<td>84.8</td>
<td>87.3</td>
<td>81.5</td>
<td>75.4</td>
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<td>85.6</td>
<td>87.6</td>
<td>83.3</td>
<td>75.8</td>
<td>98.2</td>
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<tr>
<td>Bartonella silvatica Fuji 23-1</td>
<td>81.0</td>
<td>84.0</td>
<td>85.9</td>
<td>81.2</td>
<td>75.4</td>
<td>98.2</td>
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<td>Bartonella taylorii M6</td>
<td>84.9</td>
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<td>86.0</td>
<td>81.6</td>
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<tr>
<td>Bartonella tribocorum IBS 506</td>
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<td>83.7</td>
<td>86.4</td>
<td>80.7</td>
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<td>97.7</td>
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<tr>
<td>Bartonella vinsonii subsp. arupensis OK 94-513</td>
<td>85.2</td>
<td>84.1</td>
<td>87.6</td>
<td>82.2</td>
<td>74.4</td>
<td>98.1</td>
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<tr>
<td>Bartonella vinsonii subsp. berkhoffii 93-C01</td>
<td>85.2</td>
<td>84.1</td>
<td>87.6</td>
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<td>98.1</td>
</tr>
<tr>
<td>Bartonella vinsonii subsp. vinsonii Baker</td>
<td>85.8</td>
<td>84.1</td>
<td>87.6</td>
<td>82.2</td>
<td>73.3</td>
<td>98.2</td>
</tr>
</tbody>
</table>

*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.*

### 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 Mega3 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.
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), fisZ (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], fisZ [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, fisZ (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 an-
cashi" 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 insuffi-
cient 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 concat-
enateing a predetermined number of gene sequences, generally in-
clude the housekeeping genes (*ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*)
and *rrs* for phylogenetic analysis (9, 15), while MST is based on the vari-
able 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 ancashi" fall well below these values,
thereby providing evidence that the isolates represent a novel *Barto-
nella* 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
*rrs* 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 ancashi," while most closely related to
*B. bacilliformis* by MLST analysis, is in fact highly dissimilar from
*B. bacilliformis* by MST analysis, thereby providing clear in-
sight 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*
pecies or novel *B. bacilliformis*-like agents able to cause human dis-
ease circulating in areas of Peru where Carrion's disease is endemic (5,
7). Further microbiological, microscopic, and functional characteriza-
tion 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, mech-
nanisms for the high host adaptability, and pathogenesis of these en-
demic 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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necessarily reflect the official policy or position of the Department of the
Navy, Department of the Army, Department of Defense, or the U.S. Gov-
ernment. As employees of the U.S. Government, this work was prepared
as part of our official duties and therefore, under Title 17 USC paragraph
105, copyright protection is not available.

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**REFERENCES**

1. Erremeeva ME, Geras HL, Lydy SL, Goo JS, Ryan ET, Mathews SS,
Bacteremia, fever, and splenomegaly caused by a newly recognized *Barto-

bol. 301:7–15.


profound genetic diversity among isolates of the human pathogen *Bartonella ba-

6. Harms A, Debro C. 2012. Intruders below the radar: molecular patho-

7. Blazes D, Mullins K, Smook BL, Jiang J, Canal E, Solorzano N, Hall E,
peruana caused by a novel Bartonella agent: implications for pathogen
discovery and disease eradication in resource limited settings. Emerg. In-

Multispacer typing of *Bartonella henselae* isolates from humans and cats.

H-J, Maruyama S. 2010. *Bartonella japonica* sp. nov. and *Bartonella sivatua*

fication of *Bartonella* species by comparing groEL sequences. Int. J. Syst.

tiation of *Bartonella* species based on comparison of partial ftsZ gene se-

Differentiation of *Bartonella*-like isolates at the species level by PCR-
restriction fragment length polymorphism in the citrate synthase gene. J.

rpoB gene analysis for detection and identification of *Bartonella* species. J.

14. Lane DJ. 1991. 16S/23S rRNA sequencing, p 115–175. In Stackebrandt E,
Goodfellow M (ed), Nucleic acid techniques in bacteriological systems. John
Wiley and Sons, New York, NY.

species detected in fleas and ticks from the yellow-footed antechinus (*Antechinus

criteria for species definition in bacteriology: the *Bartonella* paradigm.

17. Arvand M, Raoult D, Feil EJ. 2010. Multi-locus sequence typing of a geograph-
ically and temporally diverse sample of the highly clonal human pathogen *Barto-

typing of *Bartonella henselae* isolates from three continents re-
veals hypervirulent and feline-associated clones. PLoS One 2:e1346. doi:
10.1371/journal.pone.0001346.

MEGA5: Molecular Evolutionary Genetics Analysis using maximum like-
lihood, evolutionary distance, and maximum parsimony methods. Mol.