Description of *Bartonella ancashensis* sp. nov., isolated from the blood of two patients with verruga peruana

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Three novel isolates of the genus *Bartonella* were recovered from the blood of two patients enrolled in a clinical trial for the treatment of chronic stage *Bartonella bacilliformis* infection (verruga peruana) in Caraz, Ancash, Peru. The isolates were initially characterized by sequencing a fragment of the *gltA* gene, and found to be disparate from *B. bacilliformis*. The isolates were further characterized using phenotypic and genotypic methods, and found to be genetically identical to each other for the genes assessed, but distinct from any known species of the genus *Bartonella*, including the closest relative *B. bacilliformis*. Other characteristics of the isolates, including their morphology, microscopic and biochemical properties, and growth patterns, were consistent with members of the genus *Bartonella*. Based on these results, we conclude that these three isolates are members of a novel species of the genus *Bartonella* for which we propose the name Bartonella *ancashensis* sp. nov. (type strain 20.00^T=ATCC BAA-2694^T=DSM 29364^T).

The genus *Bartonella* contains more than 30 vector-borne, fastidious, small, Gram-negative bacilli (Birtles *et al.*, 1995; Brenner *et al.*, 1993; Kaiser *et al.*, 2011). Within this genus, three organisms are well-characterized human pathogens (*Bartonella quintana*, *Bartonella henselae* and *Bartonella bacilliformis*) and others are opportunistic pathogens that cause illness primarily in immune-compromised individuals (Harms & Dehio, 2012; Houpikian & Raoult, 2001; Kaiser *et al.*, 2011; Rolain *et al.*, 2004). The original member of the genus, *B. bacilliformis*, is known to cause Carrión's disease, which is a biphasic illness consisting of an acute phase (also known as Oroya fever) and a chronic phase (also known as verruga peruana) (Alexander, 1995; Kosek *et al.*, 2000; Sanchez Clemente *et al.*, 2012). Verruga peruana is characterized by benign yet persistent red–purple, raised skin nodules. Additionally, *B. bacilliformis* infection is seen only in the Andes mountain range of Peru, Ecuador and in Colombia (at 2500–8000 feet above sea level), where it is endemic and likely transmitted by the new world sandfly (*Lutzomyia* species) (Alexander, 1995; Kosek *et al.*, 2000; Sanchez Clemente *et al.*, 2012). Although *Bartonella rochalimae* has been reported to have caused an Oroya-fever-like illness in an American traveller returning from Peru (Eremeeva *et al.*, 2007), *B. bacilliformis* is currently the only agent identified to cause Carrión's disease.

We recently described the isolation of a novel *Bartonella* agent, designated Bartonella *ancashensis* sp. nov., during an antibiotic treatment trial, which was designed to test the efficacy of azithromycin, as compared with standard care (rifampicin), for the treatment of verruga peruana caused by *B. bacilliformis* in Caraz, Ancash, Peru (Blazes *et al.*, 2013). In total, three isolates were obtained from

Abbreviations: ITS, intergenic spacer; MLST, multi locus sequence typing; MST, multi spacer typing.

The GenBank/EMBL/DDBJ accession numbers for the *gltA*, *rpoB*, *ftsZ*, *groEL* and *ribC* housekeeping genes, the *rrs* ribosomal gene and the 16S–23S gene ITS of strain 20.00^T are KP720638–KP720644, respectively. The GenBank/EMBL/DDBJ accession numbers for the *gltA*, *rpoB*, *ftsZ*, *groEL* and *ribC* housekeeping genes, the *rrs* ribosomal gene and the 16S–23S gene ITS of strain 41.60 are KP7206345–KP720651, respectively.

the blood of two patients with verruga peruana who were enrolled in the trial. Two of the isolates came from a three-year-old male: the first (20.00^T) was collected at the time of enrollment, and the second (20.60), 60 days post enrollment. The third isolate (41.60) was collected from a ten-year-old male at 60 days post enrollment. We also reported gene sequencing results, multi locus sequence typing (MLST) results, and intergenic spacer (ITS) typing results for *B. ancashensis* sp. nov. strains 20.00^T and 20.60. Five housekeeping genes, including *gltA* (citrate synthase;1341 bp), *rpoB* (RNA polymerase-beta subunit; 4149 bp), *ftsZ* (cell division protein; 1776 bp), *groEL* (60 kDa heat shock-protein; 1644 bp) and *ribC* (riboflavin synthase; 642 bp), and one ribosomal gene, *rrs* (16S rRNA gene; 1474 bp), were used for MLST, while the 16S–23S rRNA gene ITS (940 bp) was used for ITS typing (Mullins *et al.*, 2013). Sequencing analysis showed strains 20.00^T and 20.60 to be identical to one another genetically at these loci, yet distinct from all other isolates listed in GenBank. Additionally, the sequencing, MLST and ITS sequencing results showed that the isolates fall within the sequence similarity range for known species of the genus *Bartonella*. They were most closely related to, yet distinct from, even the most diverse strains of the species *B. bacilliformis*. The multi-gene sequence divergence and phylogenetic distance analysis clearly indicated that they represent a novel species of the genus *Bartonella* (Mullins *et al.*, 2013).

Here, we report on the further characterization of *B. ancashensis* sp. nov. by describing additional genotypic, phenotypic, morphological, microscopic, biochemical and growth characteristics of the three strains: 20.00^T, 20.60 and 41.60.

To assess the culture characteristics of all three strains, they were cultivated on different solid media at different temperatures and in the presence or absence of increased CO_2. The strains were grown in plates containing either trypticase soy agar (TSA) containing 5 % (v/v) defibrinated sheep blood, chocolate agar (GC agar with haemoglobin and IsoVitaleX), Columbia agar containing 5 % (v/v) defibrinated sheep blood, or deep-fill (extra volume of media to combat drying) heart-brain infusion agar (BHIA) containing 10 % (v/v) defibrinated sheep blood (BD). The cultures were incubated at 26, 30 and 35 °C with or without 5 % CO_2 in a moist atmosphere for at least 20 days. Additionally, strains were tested for their ability to grow in liquid culture (MS10 media) and in Vero and L929 cells cultured in Eagle's minimal essential medium (E-MEM) containing 10 % (v/v) fetal bovine serum at 30 °C with 5 % CO_2 (Lynch *et al.*, 2011).

The strains were subjected to Gram staining (BD) and visualized by light microscopy ($\times$1000) model B$\times$40 (Olympus). Cell morphology was visualized by transmission electron microscopy (TEM; 100 CXII; JEOL) with negative staining. Gram staining and TEM were performed on 10 and 28 day cultures passaged one to two times from frozen stocks onto BHIA with 10 % (v/v) defibrinated sheep blood at 30 °C in a moist atmosphere with 5 % (v/v) CO_2.

Biochemical analysis was carried out using the RapID ANA II system (Remel), according to the manufacturer's instructions (Clarridge *et al.*, 1995; Dehio *et al.*, 2001; Sander *et al.*, 1997). Additionally, Oxidase Reagent Droppers (BD) were used to evaluate oxidase activity, while 3 % (v/v) hydrogen peroxide (Acros) was used to evaluate the catalase activity of the three isolates. The biochemical analyses were performed on 10-day-old colonies, which were grown on BHI containing 10 % (v/v) defibrinated sheep blood.

Antibiotic susceptibility was assessed using the Equine species MIC plate (EQUINF1; Trek Diagnostic Systems). Susceptibility testing was carried out according to the manufacturer's instructions for fastidious organisms. In brief, 3–5 colonies were emulsified in 4 ml of MS10 medium and 50 µl of the suspension was transferred to 11 ml MS10 medium and vortexed. A 100 µl sample of the solution was added to each well and incubated at 30 °C with 5 % (v/v) CO_2 for up to 18 days.

For additional genotypic characterization, all three strains were subjected to full genome sequencing using a Roche GS FLX Titanium sequencing system and assembly software GSAssembler v2.5.3 (Roche 454 Life Sciences). DNA G+C content of the isolates was determined. Complete sequences of the five housekeeping genes, *gltA*, *rpoB*, *ftsZ*, *groEL*, *ribC* and *rrs* and the 16S–23S ITS, were extracted from the genome sequence of 41.60 for comparison with the respective genes of 20.00^T and 20.60. MLST analysis was employed using concatenated fragments of *rrs*, *rpoB*, *gltA* and *ftsZ* of strain 20.00^T and 30 type strains of species of the genus *Bartonella*.

The results from the genome sequencing of strain 41.60 revealed that *gltA*, *rpoB*, *ftsZ*, *groEL*, *ribC*, *rrs* and the 16S–23S ITS were 100 % identical to their respective counterparts derived from 20.00^T and 20.60. Additionally, whole genome sequencing showed the DNA G+C content of these three strains to be 38.4 mol%. This is similar to the DNA G+C content of other known species of the genus *Bartonella* (Bermond *et al.*, 2000,2002). Finally, MLST analysis confirmed *B. ancashensis* sp. nov. (represented by strain 20.00T) to be a novel species of the genus *Bartonella*, most closely related to *B. bacilliformis* (Fig. 1).

All three strains grew on TSA containing 5 % (v/v) defibrinated sheep blood, Columbia agar containing 5 % (v/v) defibrinated sheep blood and on deep-filled BHIA containing 10 % (v/v) defibrinated sheep blood, while none of the strains grew on chocolate agar. All three strains grew optimally at 30 °C with 5 % (v/v) CO_2, with visible colonies observed (for all three isolates) after 10 days. Growth was stunted at 30 °C without CO_2 and at 26 °C, with or without CO_2 (i.e. no individual colonies were visible at 10 days). Finally, no growth was observed at 35 °C with or without 5 % (v/v) CO_2. Cultures remained viable at 30 °C with 5 % (v/v) CO_2 for at least 30 days post-inoculation on BHIA with 10 % (v/v) defibrinated sheep blood. Additionally, all three strains grew in MS10 broth and in Vero and L929 cell culture.

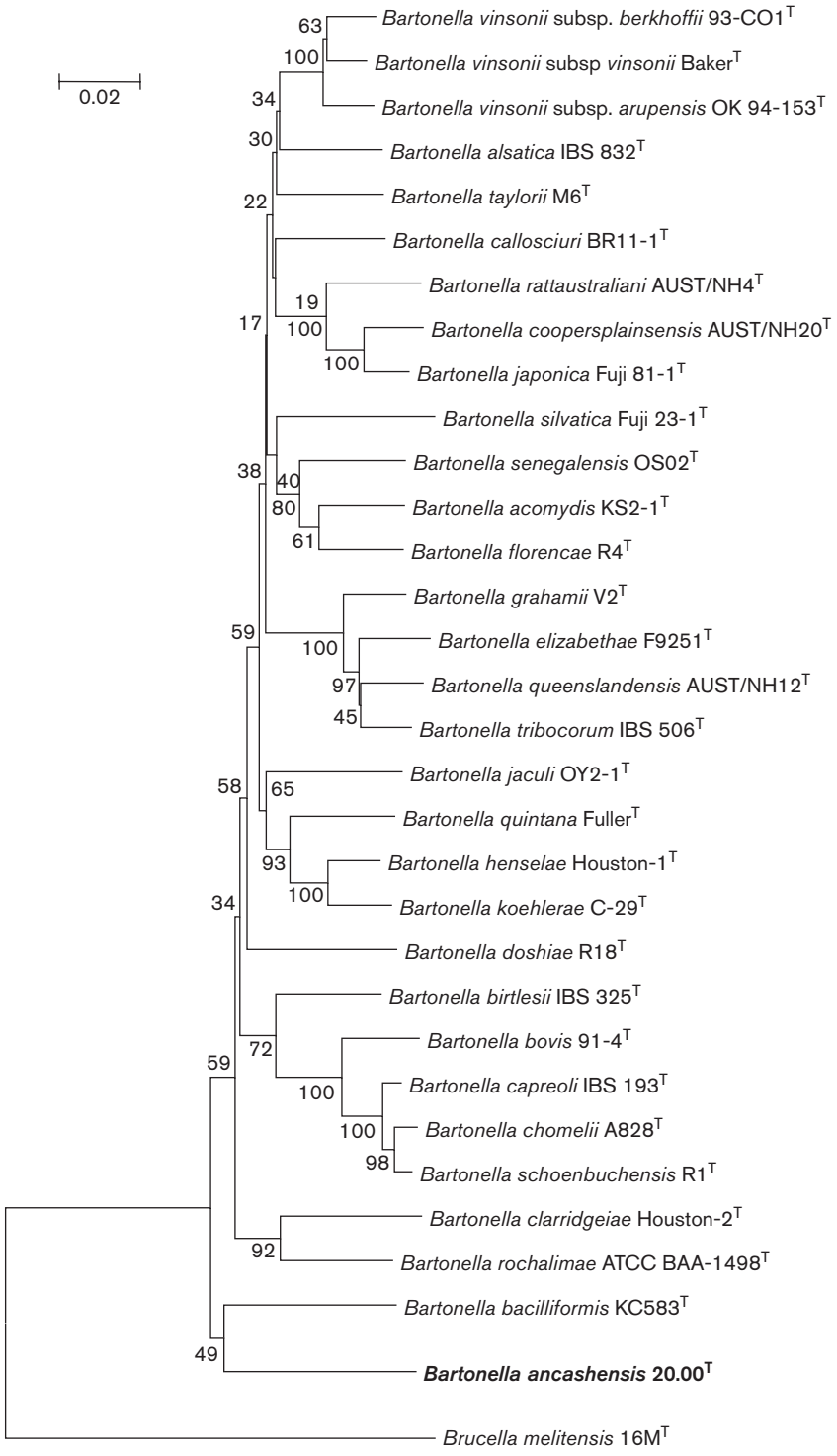

Fig. 1. MLST phylogeny for a 3277 character fragment consisting of concatenated gene fragments *rrs* (1352 characters), *rpoB* (825 characters), *gltA* (312 characters) and *ftsZ* (788 characters) of 30 type strains of species of the genus *Bartonella* with *Brucella melitensis* as the out group. The phylogeny was created with (Tamura *et al*., 2013) using the neighbour-joining tree method and distances were calculated using the Jukes–Cantor method. Bar, number of base pair substitutions per site.

Colonies passaged four times from frozen isolates were visualized at 10 and 14 days on deep-fill BHIA containing 10 % (v/v) defibrinated sheep blood at 30 °C in a moist atmosphere with 5 % (v/v) CO_2. At 10 days, colonies of 20.00^T were iridescent-grey, opaque, smooth and 1–2 mm in diameter size, while 20.60 appeared as pinpoint colonies. Additionally, the appearance of 41.60 was of a heterogeneous population of iridescent-grey, opaque and smooth colonies, which were 0.8–1 mm in size, or less than 0.5 mm in size at 10 days. At 14 days, 20.00^T colonies were iridescent-grey and opaque, with a depressed centre and 2–3 mm in size. At day 14, colonies of 20.60 were iridescent-grey, translucent and ≤0.5 mm in size, while 41.60 now exhibited a more homogeneous population of iridescent-grey, opaque and smooth colonies, largely 0.8–1 mm in size.

Gram staining and electron microscopy revealed small Gram-stain-negative coccobacilli/bacilli, while TEM revealed 1.27 μm × 0.54 μm (20.00^T), 0.99 μm × 0.64 μm (20.60) and 1.51 μm × 0.62 μm (41.60) bacilli with variable expression of polar flagella (Fig. 2).

All three strains were catalase-, oxidase-, urease- and indole-negative. Additionally, all three strains were negative for the hydrolysis of *p*-nitrophenyl-β-D-disacharide (BLTS), α-L-arabinoside, ONPG, β-D-glucoside, α-D-glucoside, L-fucoside, D-glucosamide and *p*-nitrophenylphosphate. Arylamidase activity was weakly to moderately positive for proline and phenylalanine and positive for leucine, glycine, arginine and serine, but negative for pyrrolidonyl. These results are consistent with those for other members of the genus *Bartonella* (Bermond *et al.*, 2000). In addition, the results gave a 000 671 score on the RapID ANA II system, which is identical to the results obtained for many species of the genus *Bartonella*, yet differs from the score obtained for *B. bacilliformis* (000 641) (Clarridge *et al.*, 1995).

Antibiotic susceptibility testing revealed sensitivity to amikacin (≤2 μg ml^{-1}), azythromycin (≤0.125 μg ml^{-1}), ceftiofur (≤0.125 μg ml^{-1}), chloramphenicol (≤2 μg ml^{-1}), enofloxacin (≤0.125 μg ml^{-1}), ticarcillin (≤4 μgml^{-1}), ampicillin (≤0.125 μg ml^{-1}), clarithromycin (≤0.5 μg ml^{-1}), gentamicin (≤0.5 μg ml^{-1}), erythromycin (≤0.125 μg ml^{-1}), doxycycline (≤1 μg ml^{-1}), rifampicin (≤1 μg ml^{-1}) and tetracycline (≤1 μg ml^{-1}). In addition, strains 20.00^T and 20.60 had an MIC of >2 μg ml^{-1} for oxacillin +2 % (w/v) NaCl, while 41.60 was sensitive to ≤0.125 μg ml^{-1} oxacillin +2 % (w/v) NaCl. All three strains produced MICs of >8 μg ml^{-1} for cefazolin, 1 μg ml^{-1} for ceftazidime, 0.5 μg ml^{-1} for penicillin and up to 1 μg ml^{-1} for imipenem.

In conclusion, the sequencing results, MLST and ITS analysis, along with the phenotypic, morphological, microscopic, biochemical and growth characteristics of the three isolates (strains 20.00^T, 20.60 and 41.60), indicate that they represent a single novel species belonging to the genus *Bartonella*, for which the name Bartonella *ancashensis* sp. nov. is proposed.

Description of Bartonella *ancashensis*

Bartonella *ancashensis* (an.cash.en′sis. N.L. fem. adj. *ancashensis* pertaining to the Ancash region of Peru).

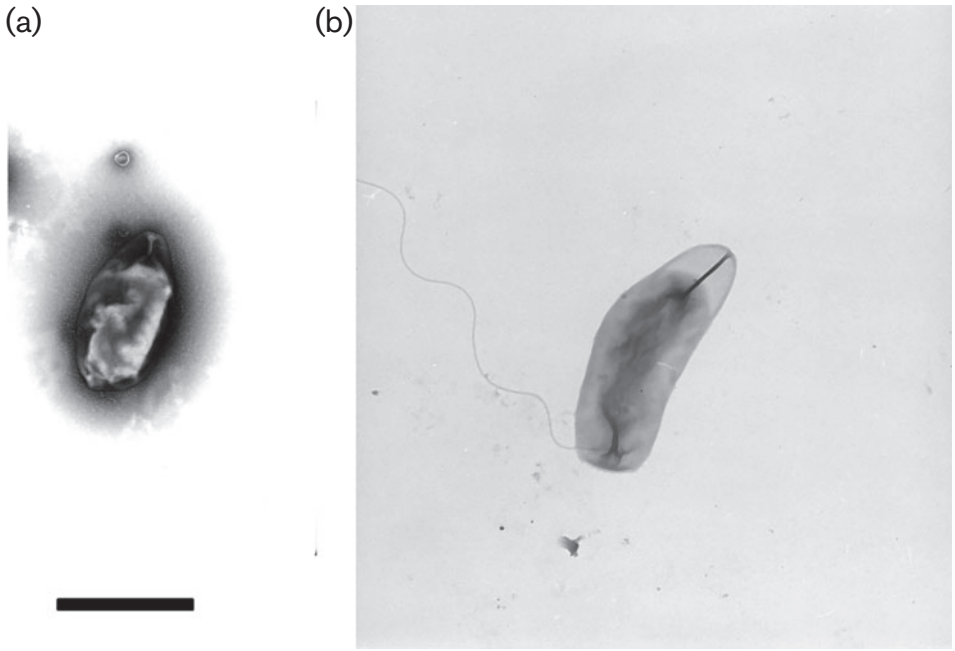

Fig. 2. Electron micrograph of isolates 20.00^T (a) and 41.60 (b) showing the variable expression of flagella. Bar, 1 μm.

After a 10 day incubation on BHIA containing 10 % (v/v) defibrinated sheep blood at 30 °C with 5 % (v/v) CO_2, colonies appear round, iridescent-grey, opaque, smooth and 1–2 mm in size. Cells are Gram-stain-negative bacilli, which lack flagella and are 1.27 μm × 0.54 μm. Oxidase, catalase, urease and indole tests are negative. Hydrolysis of *p*-nitrophenyl-β-D-disacharide (BLTS), α-L-arabinoside, ONPG, β-D-glucoside, α-D-glucoside, L-fucoside, D-glucosamide and *p*-nitrophenylphosphate is negative. Arylamidase activity is weakly positive for proline and phenylalanine and positive for leucine, glycine, arginine and serine, but negative for pyrrolidonyl. This gives a 000 671 code using the RapID ANA II system. This species can be distinguished from other species of the genus *Bartonella* by *rrs*, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* gene and 16S–23S ITS region sequences.

The type strain is 20-00^T (=ATCC BAA-2694^T=DSM 29364^T), the first isolate collected and isolated prior to antibiotic treatment from a three-year-old male living in a rural setting outside of the town of Caraz, in the highlands of the Ancash region of Peru. The type strain has a DNA G+C content of 38.4 mol%.

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