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 20.00T = ATCC BAA-2694T = DSM 29364T).

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; Houpiikian & Raoult, 2001; Kaiser et al., 2011; Rolain et al., 2004). The original member of the genus, *B. bacilliformis*, is known to cause Carrion’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 Carrion’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...
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 (20.00T) 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 20.00T 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 20.00T
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: 20.00T, 20.60
and 41.60.

To assess the culture characteristics of all three strains, they
were cultivated on different solid media at different tem-
peratures and in the presence or absence of increased
CO2. 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) defi-
brinated sheep blood, or deep-fill (extra volume of media
to combat drying) heart-brain infusion agar (BHA) con-
taining 10 % (v/v) defibrinated sheep blood (BD). The cul-
tures were incubated at 26, 30 and 35 °C with or without
5 % CO2 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 % CO2 (Lynch et al., 2011).

The strains were subjected to Gram staining (BD) and
visualized by light microscopy (×1000) model B × 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 BHI with 10 % (v/v) defibrinated sheep
blood at 30 °C in a moist atmosphere with 5 % (v/v) CO2.

Biochemical analysis was carried out using the RapID ANA II
system (Remel), according to the manufacturer’s instruc-
tions (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 manu-
facturer’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 solu-
tion was added to each well and incubated at 30 °C
with 5 % (v/v) CO2 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 soft-
ware 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 20.00T and 20.60. MLST analysis
was employed using concatenated fragments of rrs, rpoB,
gltA and ftsZ of strain 20.00T 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 20.00T 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 (Bermont 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) defi-
brinated sheep blood, Columbia agar containing 5 % (v/v)
defibrinated sheep blood and on deep-filled BHA contain-
ing 10 % (v/v) defibrinated sheep blood, while none of
the strains grew on chocolate agar. All three strains grew opti-
mally at 30 °C with 5 % (v/v) CO2, with visible colonies
observed (for all three isolates) after 10 days. Growth was
stunted at 30 °C without CO2 and at 26 °C, with or with-
out CO2 (i.e. no individual colonies were visible at
10 days). Finally, no growth was observed at 35 °C with
or without 5 % (v/v) CO2. Cultures remained viable at
30 °C with 5 % (v/v) CO2 for at least 30 days post-inocu-
lation on BHA 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) CO₂. At 10 days, colonies of 20.00ᵀ 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, colonies of 20.00ᵀ 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 (20.00ᵀ), 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-glucosamidase 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).

Antibiotic susceptibility testing revealed sensitivity to amikacin (≤2 μg ml⁻¹), azithromycin (≤0.125 μg ml⁻¹), ceftiofur (≤0.125 μg ml⁻¹), chloramphenicol (≤2 μg ml⁻¹), enrofloxacin (≤0.125 μg ml⁻¹), ticarcillin (≤4 μg ml⁻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 20.00ᵀ 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 20.00ᵀ, 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).
After a 10 day incubation on BHIA containing 10 % (v/v) defibrinated sheep blood at 30 °C with 5 % (v/v) CO₂, 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-glucosamine 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 pyrrolidinyl. 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), 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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References