Characterization of *Candidatus* Bartonella ancashi: A Novel Human Pathogen Associated with Carrión's Disease

By

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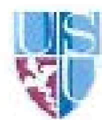

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DEDICATION

In memory of my Grandfather, Bernard Clark. I know he would be so proud.

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Kristin Mullins

May 15th, 2015

ABSTRACT

Characterization of *Candidatus* Bartonella ancashi: A Novel Human Pathogen Associated with Carrión's Disease

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Bartonella species belong to a group of emerging, yet neglected pathogens. In the past 25 years, over 20 new *Bartonella* species have been identified. *Bartonella* species are vector borne pathogens that infect a wide array of mammalian hosts, including humans. Of the *Bartonella* species, three regularly cause human disease. These species are *B. quintana, B. henselae,* and most importantly *B. bacilliformis*. *B. bacilliformis* causes a biphasic illness, called Carrión's disease, which is characterized by an acute phase, Oroya fever, and a chronic phase, verruga peruana. Infections are only seen in the Andes Mountain range of Peru, Ecuador, and Colombia. The chronic phase presents as benign red-purple skin nodules and was the subject of a 2003 clinical treatment trial in Caraz, Ancash, Peru- an area where *B. bacilliformis* is endemic. During this clinical treatment trial three isolates that were found to be disparate (based on sequencing of a 33 bp region of *gltA*) from *B. bacilliformis* were identified. To confirm these isolates were unique and to gain insight into their pathogenicity, the isolates were

further characterized using gene sequencing methods, multilocus sequence typing (MLST), multispacer sequence typing (MST), and full genome sequencing methods. Additionally, the phenotypic properties of the isolates were investigated through observations of growth characteristics, colony morphologies, biochemical utilizations, and antibiotic susceptibilities. The results indicated that these isolates were most similar to *B. bacilliformis,* yet distinct from *B. bacilliformis,* based on both MLST and full genome phylogenic analyses. More importantly, the genomes of these isolates were found to contain *Bartonella* virulence factors, such as the VirB/D4 type IV secretion system and flagellar proteins. Furthermore, the three isolates, though 99.7% identical at the sequence level, were found to contain an intriguing genomic inversion between the isolates, which is thought to play a role in flagella expression. These results confirmed that these isolates were members of a single novel Bartonella species, which we are calling *Candidatus* Bartonella ancashi, which contains virulence factors essential to the genus *Bartonella.*

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LIST OF ABBREVIATIONS

BadA	*Bartonella* adhesin protein A: Found in the *Bartonella henselae* genome and thought to be essential for adhesion, aggregation, inhibition of phagocytosis and angiogenesis
Bep	*Bartonella* effector protein: Delivered to the host cell via the VirB/D4 type IV secretion system and are found in many *Bartonella* species
BHIA	Brain heart infusion agar: Culture media used for the cultivation of microorganisms
BID	*Bartonella* intracellular delivery: Found on several Beps and are believed to play a role in translocation
BSR	BLAST score ratio: Used to estimate relatedness of proteins at the amino acid level
Ca	Candidatus: Used to denote a candidate species
EMEM	Eagle's minimum essential medium: Cell culture medium that can be used to support growth of *Bartonella* species
FBS	Fetal bovine serum: Used to supplement media for cell culture and the growth of *Bartonella* species
FIC	Filamentation induced by cAMP: Domain found on Beps that have an unknown function
ftsZ	GTP-binding tubulin-like cell division protein: Is a housekeeping gene, often used for MLST analyses, that is found in all prokaryotic cells and is homologous to the eukaryotic protein tubulin
gltA	Citrate synthase: A housekeeping gene often used for genetic analyses and is found in most prokaryotes
groEL	Molecular chaperone GroEL: A housekeeping gene, often used for MLST analyses and found many bacterial species and required for the proper folding of a variety of proteins
HIF	Hypoxia- inducible factor: Respond to changes in environmental oxygen concentrations and upregulated during Bartonella infections, promoting angiogenesis
HUVECs	Human umbilical vein endothelial cells: Used to study the functions and pathology of human endothelial cells when exposed to a variety of conditions, including microorganisms, like *Bartonella*
IL	Interleukin: Cytokines that regulate cell functions
ITS	Intergenic transcribed spacer: Often used for MST analyses, as the nucleotide sequences of ITS regions are often able to distinguish between strains of a single species
MLST	Multilocus sequence typing: A method used create more accurate phylogenies for differentiation of bacterial species and strains
MST	Multispacer sequence typing: A method used to differentiate between different strains of the same species using ITS regions
RAST	Rapid annotation using subsystem technology: A fully automated service used for genome annotation

ribC	Riboflavin synthase: A housekeeping gene often used for MLST analyses and found in a variety of bacterial species
rpoB	RNA polymerase β subunit: A housekeeping gene often used for genetic analyses and found in most bacterial species
rrs	16s ribosomal RNA: A housekeeping gene used in genetic analyses and found in all bacterial species
ssrA	SsrA transfer-mRNA: Found in bacterial species and recently used as a target for *Bartonella* species differentiation
STM	Signature-tagged mutagenesis: Used for the study of gene function and often used to uncover potential bacterial virulence factors
T4SS	Type IV secretion system: Found in Gram-negative bacteria and are involved in the translocation of effector molecules and the transfer of genetic material
TAAs	Trimeric autotransporter adhesin molecules: Found in Gram-negative bacteria and are involved in host cell invasion and adhesion
THP-1	Human monocytic cell line: This cell line was human with acute monocytic leukemia and is used to culture a variety of microorganisms
trwJ	Pilus associated components: Part of the Trw T4SS found in *Bartonella* species that lack flagella and thought to be essential to erythrocyte invasion
trwL	Pilin associated components: Part of the Trw T4SS found in *Bartonella* species that lack flagella and thought to be essential for erythrocyte invasion
TSA	Trypticase soy agar: Growth media used for bacterial culture
VEGF	Vascular endothelial growth factor: Upregulated in *Bartonella* infections and contributes to a proangiogenic environment
VEGF-R	Vascular endothelial growth factor receptor: Upregulated in *Bartonella* infections and contribute to a proangiogenic environment
VM	Virulence modulating proteins: Found in *B. australis* and *B. bacilliformis* and thought to play a role in *Leptospira* pathogenesis as they are found in greater abundance in pathogenic *Leptospira* species
Vomp	Variably expressed outer membrane protein: Adhesin proteins found in *B. quintana* believed to be important for autoaggregation and angiogenesis
WGM/ WGRM	Whole genome mapping/ whole genome restriction mapping: Used to determine the arrangement of genes within a full genome based on the order of restriction contigs

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CHAPTER 1:

Introduction

HISTORICAL BACKGROUND

The genus *Bartonella* currently consists of 31, officially recognized, species and three subspecies of fastidious, aerobic, Gram- negative cocobacilli, belonging to the α_2 subgroup of the proteobacteria class (http://www.bacterio.net/bartonella.html) (Table 1). *Bartonella* are known for their ability to invade erythrocytes. The genus *Bartonella* is truly a group of emerging pathogens. Prior to the early 1990s the genus contained only one single member, *Bartonella bacilliformis*, which was described in 1905, by the namesake of the genus, Alberto Barton and confirmed by members of the 1913 Harvard Expedition to South America, as the causative agent of the biphasic illness, Carrión's disease(183; 226; 231-233; 238). *B. bacilliformis* infection of humans date back over a thousand years. Evidence of verruga peruana skin lesions are depicted in pre-Incan ceramics and in journal entries written by Spanish conquistadors who arrived in the Andes Mountain range of what are now Peru, Ecuador, and Colombia (226). It was not until the late 1800s to early 1900s that *B. bacilliformis* gained widespread attention. This occurred following an outbreak of *B. bacilliformis* infections that killed thousands of workers involved in the construction of the Trans Andean railroad, connecting Oroya and Lima, Peru, in1870 (183). While *B. bacilliformis* was characterized over 100 years ago, it was not until the 1990s that other bacterial pathogens were added to the genus *Bartonella* (23; 34).

In 1993, members of the genus *Rochalimae* were combined with the genus *Bartonella* (34). This consolidation added four species to the *Bartonella* genus (34). Two of these species were known pathogens of human importance, *Bartonella quintana*, formerly *Rochalimaea quintana*, and the newly characterized *Bartonella henselae*,

Table 1. 31 recognized *Bartonella* species: reservoirs, human disease, and date the species was recognized.

Bartonella Species	Reservoir	Human Disease	Year Recognized	Reference
B. alsatica	Rabbits	Lymphadenitis and Endocarditis	1999	(5; 100; 198)
B. acomydis	Mice		2013	(216)
B. bacilliformis	Humans	Carrión's Disease	1909	(233)
B. birtlesii	Mice		2000	(21)
B. bovis (weissii)	Cattle		2002 (2001)	(20; 33)
B. callosciuri	Squirrels		2013	(216)
B. capreoli	Roe Deer		2002	(20)
B. chomelii	Cattle		2004	(160)
B. clarridgeiae	Cats	Cat Scratch Disease	1996	(132)
B. coopersplainsensis	Rats		2009	(97)
B. doshiae	Voles		1995	(23)
B. elizabethae	Rats	Endocarditis	1993	(57)
B. florencae	Shrews		2013	(174)
B. grahamii	Voles, Mice	Cat scratch disease	1995	(23; 105; 186)
B. henselae	Cats	Cat Scratch Disease, Bacillary Angiomatosis, Hepatic Peliosis, and Endocarditis	1992	(26; 66; 201; 249)
B. jaculi	Jerboa		2013	(216)
B. japonica	Mice		2010	(110)
B. koehlerae	Cats	Endocarditis	1999	(11; 71)
B. pachyuromydis	Gerbils		2013	(216)
B. peromysci	Mice		1942	(23; 207; 241)
B. queenslandensis	Rats		2009	(97)
B. quintana	Humans		1916	(6)
B. rattaustraliani	Rats		2009	(97)

B. rochalimae	Dogs, Foxes	Oroya Fever- like Illness	2007	(79; 103)
B. schoenbuchensis	Roe Deer		2001	(62)
B. senegalensis	Unknown		2013	(173)
B. silvatica	Mice		2010	(110)
B. talpae	Moles		1905	(93; 240)
B. taylorii	Mice		1995	(23)
B. tribocorum	Rats		1998	(101)
B. vinsonii subsp. arupensis	Mice	Endocarditis	1999	(14; 248)
B. vinsonii subsp. berkhoffii	Dogs	Endocarditis	1995	(133; 209)
B. vinsonii subsp. vinsonii	Voles		1942	(16; 247)

formerly *Rochalimaea henselae* (34). The two other members of the genus *Rochalimaea* were *Rochalimaea elizabethae*, which was first isolated from the blood of an individual suffering from endocarditis, and *Rochalimaea vinsonii*, first identified as a rickettsial agent from voles in Canada (1946) and later combined with the genus *Rochalimaea* in 1982 (16; 57; 247). *B. henselae* was first characterized in 1992, when the bacterium was isolated from a febrile HIV positive individual (201). Later that same year serologic testing linked *B. henselae* to a disease syndrome whose causative agent had eluded researchers for over 40 years (66). This disease was cat scratch disease. Cat scratch disease first gained the interest of physicians and scientists in the 1950s, but a causative agent was never isolated from patients (58). However, with the link between *B. henselae* and cat scratch disease established through serology, it was only a matter of time before *B. henselae* would be isolated from patients with cat scratch disease. Luckily, this did not take long. *B. henselae* was finally isolated from a patient with cat scratch disease the following year (1993) (66).

Unlike *B. henselae*, *B. quintana* has been known as a pathogen of human importance since World War I, although it was initially mistakenly classified with the genus *Rickettsia* (13; 244). By 1919, several researchers described seeing rickettsia-like bodies in the excrement of body lice, at which point, *Rickettsia quintana*, now *B. quintana*, was proposed as the causative agent of Trench fever (6; 13; 40). Trench fever was an illness that plagued troops during World War I (40). Sporadic reports of this disease pre-date World War I and evidence points to manifestations of disease as far back as Antiquity (69; 170). However, it was not until 1961 that *Bartonella quintana*, then named *Rickettsia quintana*, was isolated from a patient with trench fever and cultivated

on solid media (111; 244). Today, *B. quintana* is mostly seen in individuals in developed countries who suffer from homelessness, alcoholism, and poor living conditions (68; 84; 113; 170; 230).

Two years after the consolidation of *Rochalimae* into the genus *Bartonella*, the genus *Grahamella* was also combined with the *Bartonella* genus (23; 34). This consolidation added two new members to the *Bartonella* genus (23). In this same publication, three new *Bartonella* species were characterized, bringing the total number of species in the genus *Bartonella* to ten (23). The initial member of the genus *Grahamella*, *Bartonella* (*Grahamella*) *talpae* was first identified in mole erythrocytes in 1905 (93). Since the genus, *Grahamella*, was formed in 1911 and up until its unification with *Bartonella* in 1995, upwards of 40 species of *Grahamella* had been proposed (23; 38). However, unlike the genera *Rochalimaea* and *Bartonella*, the genus *Grahamella* was poorly defined and extensive work had not been done to characterize any of these proposed organisms (23; 240). Therefore, at the time of unification only two species were officially recognized and classified under the *Grahamella* genus (23). Since the unification of the genera *Grahamella* and *Rochalimaea* with *Bartonella* 20 years ago, the *Bartonella* genus has seen the addition of 21 recognized species and 3 subspecies. Many of these species were found to cause infections in immunocompromised persons, especially HIV positive individuals, the homeless, alcoholics, indigents, and populations living in poor conditions and/or in poor health (36; 37; 68; 128; 129; 249). These manifestations include bacillary angiomatosis, hepatic peliosis, septicemia, fever, endocarditis, and chronic bacteremia (26; 170; 230; 249).

CLINICAL DISEASE

Carrión's Disease

Carrión's disease, as mentioned previously, was the first disease to be associated with a member of the genus *Bartonella*. Carrión's disease is a biphasic illness, consisting of an acute phase called Oroya fever and a chronic phase referred to as verruga peruana. Oroya fever or the acute form of *B. bacilliformis* infection was named after the Oroya line of the trans Andean railroad, due to an 1870 outbreak of *B. bacilliformis* infections, which killed hundreds of workers building the railroad (183). The chronic form of *B. bacilliformis*, verruga peruana, had been well known to those living in the Andes mountains long before the 1870 outbreak of Oroya fever (183; 226). However, the association between Oroya fever and verruga peruana was not established at that point. It was not until 1885 that Oroya fever and verruga peruana were recognized to be caused by the same biological agent (88; 226). This link was made by the namesake of the disease, Daniel Carrión. Daniel Carrión was a Peruvian medical student, who theorized that Oroya fever and the verrugas were caused by the same agent, but he had no experimental data to back up his claims (88; 226). To prove his theory that the same agent caused both verruga peruana and Oroya fever he decided to inoculate himself with material from the verrugas. Daniel Carrión subsequently presented with Oroya fever and proved both Oroya fever and verruga peruana were caused by the same agent (27; 88; 226). Unfortunately, Daniel Carrión never recovered from Oroya fever and died in 1885 (183; 226).

Oroya fever is characterized as a febrile illness with a severe hemolytic anemia caused by *B. bacilliformis* invading erythrocytes (Figure 1A) (19; 60) (109). Mortality

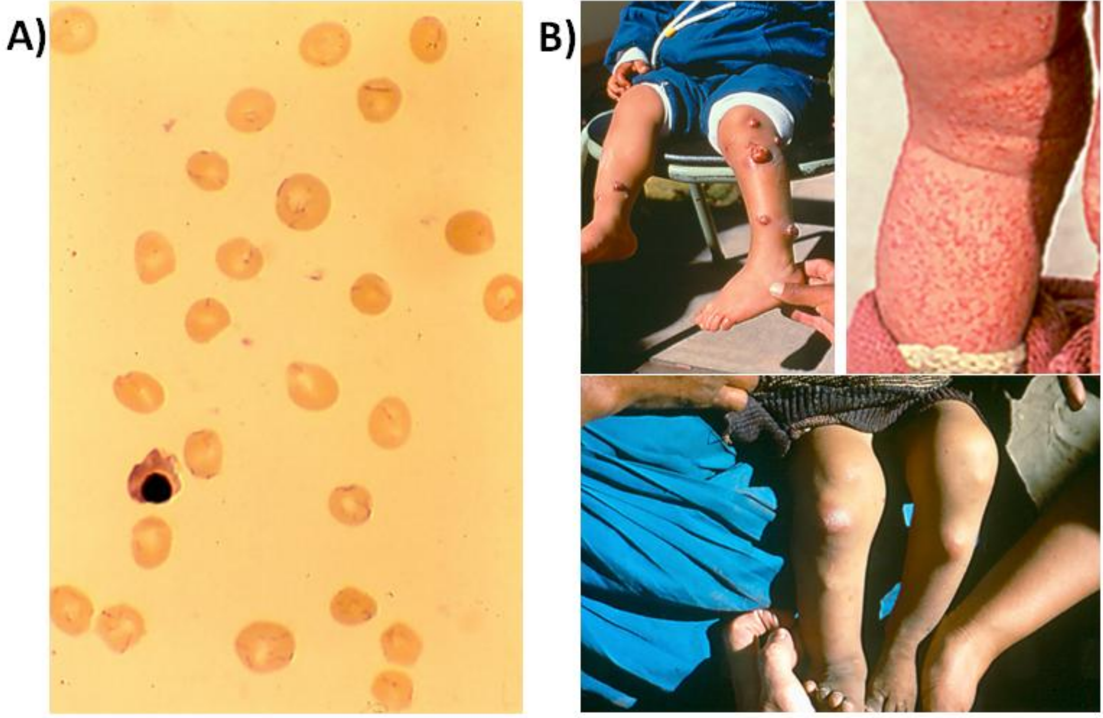

Figure 1: Pathology associated with Carrión's Disease. **A)** Blood smear from patient with *B. bacilliformis* infection. *B. bacilliformis* bacteria can be seen invading and adhered to the patient's red blood cells. **B)** Patients with varying presentations of chronic *B. bacilliformis infection* or verruga peruana.

Photos courtesy of Dr. Larry Laughlin

rates for untreated Oroya fever have been documented to reach as high as 88%, making *B. bacilliformis* one of the most deadly pathogens known to man (56; 76; 94; 157; 158; 176; 226). In contrast, the chronic form is characterized by benign, raised, red-purple skin nodules, caused by the invasion of endothelial cells by *B. bacilliformis*, also known as verruga peruana (Figure 1B) (157; 176). These skin lesions can last for as little as a few weeks up to several months (176). Unlike, the acute form, fatalities from chronic *B. bacilliformis* infections are rarely seen (176).

Today both the acute and chronic forms of *B. bacilliformis* infections are seen within the endemic region, consisting of the Andes mountain range of Peru, Ecuador, and Colombia, at 2,500- 8,000 feet above sea level (1; 76; 94; 134; 157; 158; 183; 212; 226). This corresponds to the geographical range of the suspected vector, phlebotomine sandflies, which was first suspected as the vector of *B. bacilliformis* in 1913 (236-237). The disease can manifest as a sequential progression from the acute phase to the chronic phase, or each phase can manifest individually. Oroya fever usually presents 60 days after the bite of an infected sandfly and is usually seen in children (107; 157; 176; 212). Oroya fever will last anywhere from 1-4 weeks (176). If patients recover, 4 to 8 weeks may pass before the onset of verrugas (18). However, this is not always the case. Oroya fever can manifest without latter appearance of verrugas and chronic disease can be seen in the absence of acute disease. Oroya fever affects those living in areas where *B. bacilliformis* is endemic, areas where sporadic outbreaks occur, and in individuals traveling to endemic regions from non-endemic areas of the world (48; 76; 134; 157). However, the occurrence of verruga peruana without subsequent Oroya fever is usually only seen in areas where *B. bacilliformis* is endemic (134). To complicate matters even

further, persons living in endemic regions have been found to exhibit asymptomatic bacteremia, with one study finding the incidence of asymptomatic bacteremia to be 12.7/100 person-years (48; 76; 212). This asymptomatic bacteremia supports the thought that humans are the reservoir host for *B. bacilliformis*. Interestingly, in 2007, an American traveling in Peru became ill with an Oroya fever-like illness, consisting of high fever and mild anemia (79). Blood cultures were preformed and a novel *Bartonella* species was isolated, *B. rochalimae* (79).

Trench Fever

The clinical disease, trench fever, caused by *Bartonella quintana*, was fully characterized after trench fever ravaged British troops, in France, during World War I (40; 108). During the First World War, it was estimated that 45,000 per 1 million troops exhibited symptoms of *B. quintana* infection (40; 108). At its peak during World War I, 15% of all clearing station admissions were caused by *B. quintana* infections and 90% of affected soldiers were off duty for three or more months (108). While the morbidity associated with these first cases of trench fever was high, the mortality associated with trench fever was non-existent (40; 108). Even with no mortality, the lousy conditions seen in trench warfare were perfect for spreading *B. quintana* between soldiers and the sheer number of infected troops caused trench fever to become a significant issue during the war (10).

B. quintana infection is spread when the excrement of the human body louse, *Pediculus humanus humanus*, containing *B. quintana* is inoculated into broken skin (40; 84). After an approximate 15- 25 day incubation period the symptoms of trench fever

begin (108). These symptoms begin with the acute onset of fever, severe headache, and long bone pain, with leukocytosis (40; 84; 108). The symptoms progress for approximately 5 days, at which point all the symptoms will subside (40; 108). The unique feature of this disease is that within 4-8 days symptoms will return (108). These periods of recurrent symptoms may occur for 2 – 6 weeks, with each attack less severe than the proceeding (84; 108). *B. quintana* infection usually resolves spontaneously, but in some individuals chronic relapses or asymptomatic bacteremia can occur, lending support to the conclusion that humans are the reservoir host for *B. quintana* (40; 83; 84).

More recently, trench fever has re-emerged in developed countries like the United States and France, in their homeless and indigent populations, where hygiene and health are poor. During a 6 month period in 1993, ten cases of a trench fever like illness were seen in a group of homeless, alcoholics living in Seattle (230). Upon further investigation, more evidence of *B. quintana* infections was found in those living in poverty in Seattle (114). In the follow- up study, one hundred and ninety two serum samples from patients seen at an inner city Seattle clinic were tested using serological methods looking for evidence of *B. quintana* infections (114). Of the 192 samples collected, 39 samples were seropositive for *B. quintana* (114). Additionally in 1997, 10 of 71 homeless patients who presented to an emergency room in Marseilles, France were found to be positive for *B. quintana* infection, while 20 of these 71 homeless patients were seropositive for antibodies to *B. quintana* (36). Subsequent studies done in Marseilles indicated that approximately 5.3% of the homeless people living in Marseilles are infected with *B. quintana* (37).

Symptoms that were most common for infected individuals were reminiscent of trench fever-severe headaches, fever, and leg pain (36; 230). Additional cases of what is now being referred to as 'urban' trench fever have been confirmed through serosurveys (36; 83; 185; 211; 230). Finally, studies conducted in Baltimore in 1996 found that IV drug users are also exposed to *B. quintana*, although the mode of transmission is unclear in these cases (55).

Cat Scratch Disease

A French researcher, Robert Debré, first described the condition known as cat scratch disease in 1950 (41; 58). Following Debré's initial publications on cat scratch disease in the 1950s, researchers went on to catalogue hundreds of cases of cat scratch disease (58; 112; 162; 163; 165). Clinical characteristics of cat scratch disease include lymphadenopathy and an erythematous papule at the site of inoculation (Figure 2) (4; 42; 66; 163; 166). The disease will progress to include additional lymphadenopathy and tenderness of the draining lymph nodes near the site of inoculation (4; 163). Approximately, 30% of patients will become febrile and for the large majority of patients symptoms will resolve within 2 weeks to 4 months (42; 178; 189). However, in approximately, 5 to 10% of cases additional complications can be seen (42; 178). These complications include rash, lytic bone lesions, conjunctivitis, pneumonitis, hepatic and splenic abscesses, and central nervous system involvement (42; 178).

Unlike *B. bacilliformis* and *B. quintana*, cat scratch disease caused by *B. henselae* is not associated with the presence of an arthropod vector. While *B. henselae* is

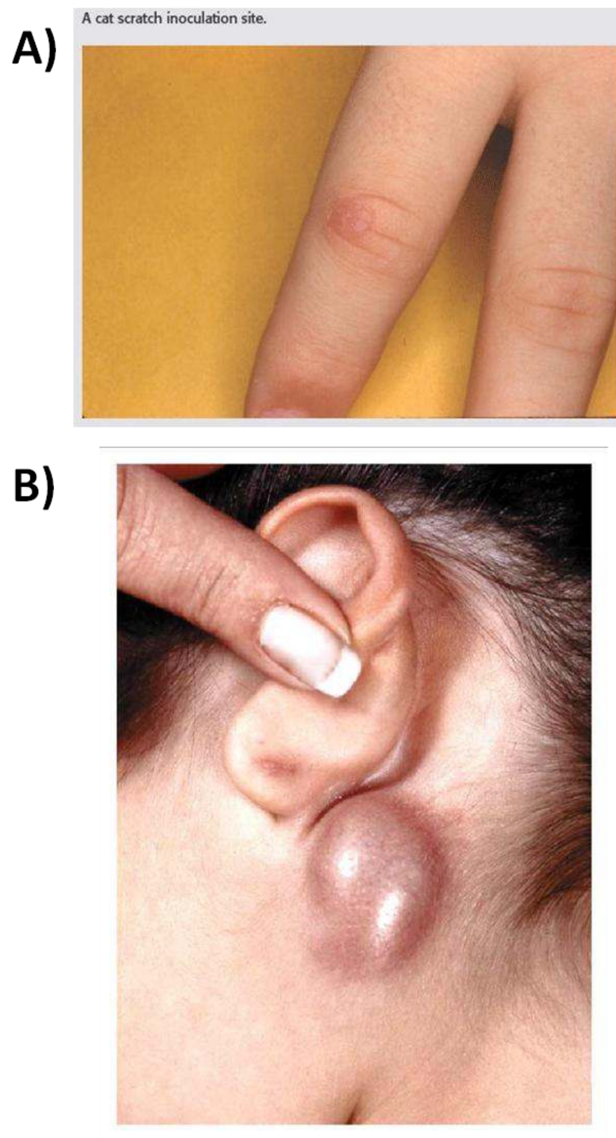

Figure 2: Clinical Characteristics associated with cat scratch disease. **A)** Papule at the site of inoculation. **B)** Lymphadenopathy seen with *B. henselae* infection.

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found in cat fleas, in the case of human infections, *B. henselae* is inoculated into scratches caused by a cat, usually a kitten, rather than through the bite of a cat flea, hence the name cat scratch disease (189; 254). Therefore, a majority of patients will report recent contact with a cat, thus leading to the close contact with cats and kittens being a major risk factor *B. henselae* infection (254). Interestingly, while humans are considered an incidental host for *B. henselae*, with cats being their primary host, cat scratch disease caused by *B. henselae* is currently the most common *Bartonella* infection seen today (112). Estimates indicate that approximately 22,000 cases of cat scratch disease occur each year, with an incidence rate of 9.3 per 100,000 ambulatory patients annually (112). It is important to note that while the overwhelming majority of cat scratch disease cases can be attributed to *B. henselae*, there have been reports of another *Bartonella* species causing cat scratch disease in immunocompetent individuals, *Bartonella clarridgeiae* (131; 132; 164). Reports indicate that cat scratch disease caused by *B. clarridgeiae* is indistinguishable from *B. henselae* cat scratch disease (132; 164). Finally, *Bartonella grahamii* was recently isolated from a patient with chronic lymphocytic leukemia who was exhibiting symptoms associated with cat scratch disease (186).

Endocarditis

Besides the three classic presentations of *Bartonella* infections, Carrión's disease, trench fever, and cat scratch disease, most other infections with *Bartonella* are seen in immunocompromised individuals, with the large majority of those individuals having HIV infection or AIDS (129; 179; 191; 194; 201). However, one manifestation of *Bartonella* infection, endocarditis, has been seen in immunocompetent, HIV negative

individuals (26; 45; 68; 70; 92; 196; 197; 243). To date, six *Bartonella* species are currently associated with endocarditis in HIV negative individuals, often termed culture negative endocarditis (11; 57; 82; 196; 197; 209; 243). The overwhelmingly majority of *Bartonella* endocarditis cases are seen in males and are caused by either *B. quintana* or *B. henselae (45; 196; 197)*. Endocarditis cases, caused by *B. quintana*, are usually seen in homeless persons or alcoholics, which is consistent with data indicating that homeless persons and persons suffering from alcoholism or subject to poor living conditions are at risk for *B. quintana* infection and trench fever-like illnesses (36; 68; 113; 114; 197; 230).

The other major player in *Bartonella* endocarditis, *B. henselae*, is usually seen in individuals who have had major valve surgery (197; 243). Unlike with endocarditis due to *B. quintana*, endocarditis caused by *B. henselae* is not associated with alcoholism, homelessness, or poor living conditions (197). Additionally, *B. elizabethae, B. vinsonii subsp berkhoffii, B. vinsonii subsp arupensis, B. koehlerae*, and *B. alsatica* have all been implicated in causing culture negative endocarditis in otherwise healthy individuals (11; 57; 82; 198; 209). As with endocarditis, caused by *B. henselae*, previous major valve surgery was a risk factor associated with the development of endocarditis caused by *B. elizabethae, B. vinsonii subsp berkhoffii, B. vinsonii subsp arupensis, B. koehlerae*, and *B. alsatica* (11; 57; 82; 197; 198; 209).

Vasculoproliferative Disorders

Bartonella species can also cause two vasculoproliferative disorders, bacillary angiomatosis and peliosis hepatis, primarily in immunocompromised persons, most often those infected with HIV (128-130; 191; 249). For vasculoproliferative lesions to develop,

chronic bacterial infection needs to be established which makes HIV positive patients and other immunocompromised patients prime candidates for developing these disorders. Bacillary angiomatosis is a unique clinical disease only caused by *Bartonella* species and more specifically only seen in patients infected with *B. henselae* and *B. quintana* (128-130; 249). Bacillary angiomatosis is a unique vascular lesion that can involve multiple bodily organs (128; 129). These include the skin, gastrointestinal track, lungs, bones, and brain (Figure 3) (128; 129). Additionally, *B. quintana* bacillary angiomatosis is highly associated with lytic bone lesions (128). The second vasculoproliferative disorder, peliosis hepatis is characterized by blood filled cavities throughout the liver and sometimes spleen (128; 130; 249). Unlike bacillary angiomatosis, peliosis hepatis is caused exclusively by *B. henselae* (128).

BACTERIAL CHARACTERISTICS

As stated previously, the genus *Bartonella* consists of aerobic, pleomorphic, Gram-negative bacilli. Additionally, *Bartonella* species usually measure between 0.3 x 1.0 μm to 0.6-1.7 μm in size (65). *Bartonella* species are variable in their presence of flagella. For example *B. bacilliformis* expresses unipolar flagella, while like the majority of *Bartonella* species, *B. henselae* and *B. quintana*, do not express flagella (34; 218). Additionally, *Bartonella* are fastidious, facultative intracellular organisms. *Bartonella* species require hemin for growth and therefore, *Bartonella* species, require solid media to be supplemented with sheep or rabbit blood for growth (43; 65; 250). In addition to solid media, *Bartonella* species can grow in both liquid broth and mammalian cell culture

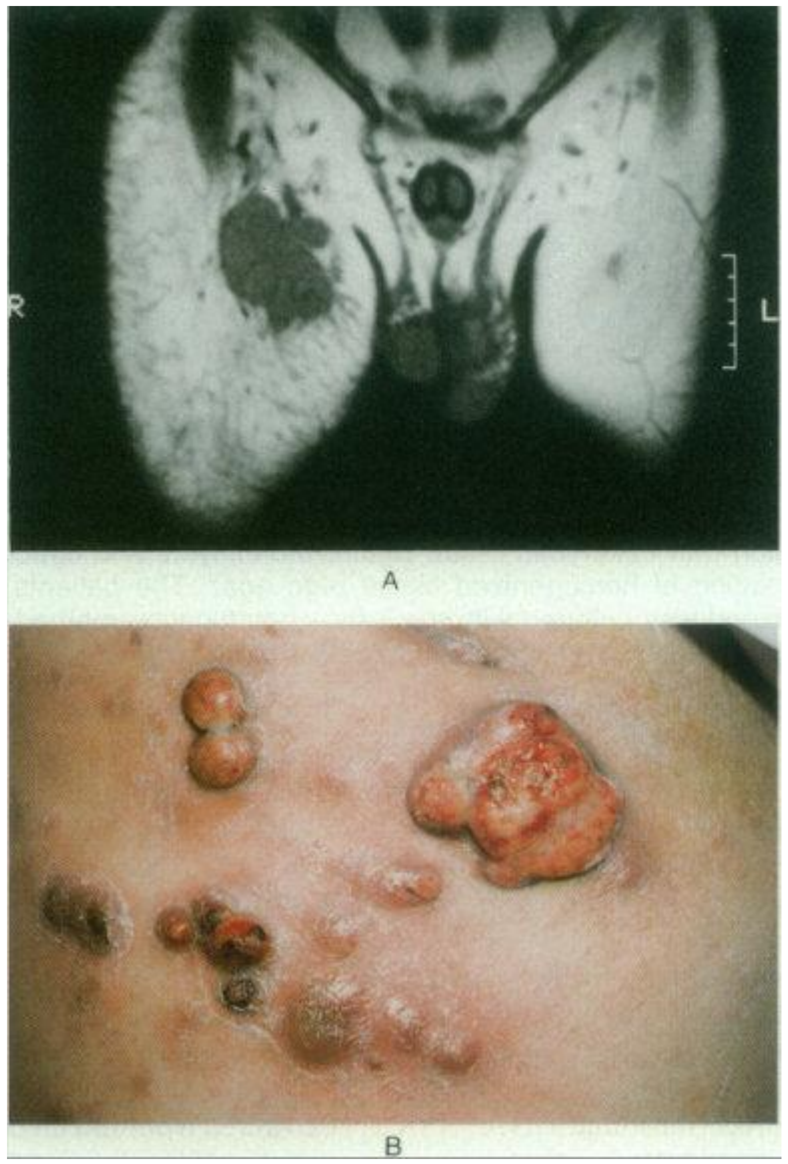

Figure 3: Cutaneous Lesions of Bacillary Angiomatosis on the Right Thigh of a Patient. **A)** Magnetic resonance imaging shows a lobulated, highly vascular soft-tissue mass measuring 7 by 4 by 5 cm anterior to the femoral vessels eight months before the diagnosis of bacillary angiomatosis. **B)** Multiple tender, fungating, vascular, oozing lesions are present at the time of the diagnosis.

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(144; 154; 250). However, broths must be supplemented with fetal bovine serum (FBS) to guarantee growth (144; 154). Most *Bartonella* species are fairly biochemically inert and are found to be urease, catalase and oxidase negative (65; 133). Although some *B. quintana* isolates have been reported to produce a weak positive oxidase test (133). *Bartonella* species, excluding *B. bacilliformis*, grow best at $35^{O}C$ with 5% CO_2, while *B. bacilliformis* grows best between 25 and $30^{O}C$ without the presence of CO_2 (65). Growth of all *Bartonella* species is slow and can take up to two weeks or possibly longer before primary colonies are visible on solid media (144; 171). The slow growing nature of *Bartonella* species can make the diagnosis of *Bartonella* infection difficult.

Additionally, *Bartonella* species have been found to be highly susceptible to most antibiotics (169; 228). All *Bartonella* species that have undergone antibiotic susceptibility testing have been found to be highly susceptible to β-lactams, macrolides, aminoglycosides, doxycycline, and rifampin (169; 228). Of the macrolides, clindamycin was least effective and of the β-lactam antibiotics, oxacillin and cephalothin were found to be least effective (169; 228). Interestingly, *Bartonella* species showed variable susceptibility to fluoroquinolone antibiotics (115; 169; 228). Unfortunately, antibiotic susceptibility testing *in vitro* does not always accurately correlate to treatment success *in vivo* (208).

DIAGNOSTICS

Diagnosis of bartonelloses is somewhat difficult. For a positive diagnosis of *Bartonella* infection clinicians will first need to suspect or consider the possibility of a *Bartonella* species as part of their differential diagnosis. This is because routine cultures

preformed in a clinical laboratory setting will not be able to identify *Bartonella* species, due to the fastidious nature of these organisms. Therefore, in addition to culture methods for diagnosis of bartonelloses, serological testing, immunohistochemical staining, and molecular methods are employed routinely.

Culture Methods

Routine culture techniques will not guarantee isolation and identification of *Bartonella* species from patient samples. While *Bartonella* species will grow on trypticase soy agar supplemented with 5% defibrinated sheep blood, which is routinely used for bacterial culture in clinical laboratories, the length of time needed to isolate *Bartonella* species from patient samples often exceeds the length of time most laboratories hold onto cultures (4). Primary isolation of *Bartonella* species can take anywhere from two to four weeks and in some cases up 45 days (144; 171). In an attempt to decrease the time it takes to culture *Bartonella* species, several types of enriched media have been created (154; 156; 250). However, these techniques require the clinician to suspect *Bartonella* infection.

Even if *Bartonella* infection is suspected and an enriched media is used, these techniques are still time consuming and positive diagnoses still cannot be made for a minimum of four days (156). Additionally, automated blood culture systems, which are now standard in most clinical laboratories, complicate matters even more. Automated blood culture systems rely on the generation of CO_2 by microorganisms growing in the blood culture bottles. Once enough CO_2 builds up in the culture bottles a sensor is activated, which will alert the clinical staff to a positive blood culture. *Bartonella* species

in blood cultures often do not generate enough CO_2 to activate the sensors and this can easily lead to a missed diagnosis (153). Luckily, in endocarditis cases, *Bartonella* is often suspected when blood cultures appear to be negative. Once *Bartonella* endocarditis is suspected, the most reliable method for positive identification of *Bartonella* in endocarditis patients is to subculture the blood cultures to a shell vial (84). However, these techniques are again time consuming and results will not be available for several weeks (84). For this reason, diagnosis is often made through alternative means.

Serology

The most widely used method for diagnosis of *Bartonella* infections is indirect immunofluorescence assays. These tests can be preformed immediately and results can be obtained much quicker than with culture methods. Serologic testing for *Bartonella* species employs assays designed to identify patients with both IgG and IgM antibodies to *Bartonella* species. These assays generally require titers of either ≥1:64 or ≥1:100 to confirm exposure to *Bartonella* (35; 36; 68; 214). Unfortunately, cross reactivity of anti-*Bartonella* IgG and IgM antibodies have been seen. Cross-reactions have occurred with *Coxeilla* and *Chlamydia* antigens and *Bartonella* infections may be missed if sera are only tested against *Coxeilla* or *Chlamydia* (143; 168).

Immunohistochemistry

Skin biopsy and silver staining can be used to identify bacilli in tissue samples and help diagnose bartonelloses that result in pathological skin lesions. Silver staining is effective for visualizing bacilli in biopsies of bacillary angiomatosis skin lesions, in skin biopsies

taken from the primary lesions of cat scratch disease, and in valve tissue biopsies (42; 89; 146; 166; 246). Bacteria will be seen within the endothelial cells located in upper reticular dermis of patients with bacillary angiomatosis (89). Additionally, bacteria can be seen within the primary lesion of cat scratch disease patients (166). In patients with suspected *Bartonella* endocarditis, bacteria are seen with in the fibrin deposits in the extracellular matrix of the valvular vegetation (146).

In addition to silver staining, hematoxylin and eosin staining can be used to identify the pathological characteristics associated with bacillary angiomatosis and cat scratch disease. Hematoxylin and eosin staining can be used to reveal the lobular proliferation of blood vessels, which is characteristic of bacillary angiomatosis (4). Lymph node biopsies from patients with cat scratch disease, stained with hematoxylin and eosin, will show follicular hyperplasia, microabscesses, and caseating granulomas, that are characteristic of cat scratch disease caused by *Bartonella* species (42). Unfortunately, either staining technique is only sufficient for presumptive diagnosis and must be used in conjunction with other information and/or diagnostic tests to confirm Bartonellosis.

Molecular Methods

Molecular techniques can be useful when trying to diagnose bartonelloses and are one of the few techniques able to identify the species of *Bartonella* responsible for the manifesting disease syndrome. DNA can be extracted from both blood samples and tissues infected by *Bartonella* species (81; 84). A variety of genus specific or species specific PCR assays have been created that are used to amplify fragments of genes such

as *rpoB* , *gltA,* ITS*,* and *ribC* (29-32; 72; 84; 117; 149). For assays that only identify *Bartonella* infections at the genus level, a subsequent sequencing analysis is usually needed to identify the species of *Bartonella* causing disease (29-32; 72; 117; 149). Another drawback to these assays is that they can produce false negative results (29; 195). This is due to the very low bacteremia associated with most *Bartonella* infections (195). To get around this issue several PCR tests performed now require that an enriched culture technique be preformed prior to DNA extraction, thereby stretching the turnaround time for molecular diagnostics to least a week (30; 195).

TREATMENT OF BARTONELLOSES

Bartonella species are highly susceptible to antibiotics *in vitro*. However, practical use of antibiotics in a clinical setting is found to be much more difficult (169; 207). Because of the ambiguity of the *in vitro* susceptibility results, antibiotic susceptibly testing does not provide a reliable basis for treatment (207). The variety of disease caused by *Bartonella* species further complicates matters. One single antibiotic is not recommended as a treatment, rather antibiotic treatment is specific to the disease state the *Bartonella* species is thought to be causing.(207).

For patients with Carrión's disease, caused by *B. bacilliformis*, the recommended antibiotic differs with the acute and chronic forms. For Oroya fever, patients should receive chloramphenicol or ciprofloxacin, while patients with verruga peruana should receive rifampin or streptomycin (157; 158). Doxycycline and gentimicin are recommended for patients suffering from trench fever (85). In cases of *Bartonella* endocarditis, gentimicin with ceftriaxone and in some cases doxycycline are

recommended for culture negative cases of endocarditis, however with culture positive *Bartonella* endocarditis, gentimicin and doxycycline are given (196). Finally, doxycycline or erythromycin is recommended for use in immunocompromised patients suffering from either bacillary angiomatosis or peliosis hepatis (130). Conversely, no antibiotic treatment is recommended for patients with cat scratch disease. Rather, due to the self-limiting nature of cat scratch disease, symptoms are monitored and allowed to run their course (126; 207). However, in extreme cases where a patient exhibits extensive lymphadenopathy or progressive syndromes such as neuroretinitis, azithromycin and doxycycline are recommended, respectively (17; 200).

ECOLOGICAL NICHES

Members of the genus *Bartonella* are vector-borne pathogens, which are transmitted to susceptible hosts by the bite of arthropod vectors, most commonly flies, fleas, ticks, and body lice (6; 40; 52; 104; 192; 236; 238; 248). Reservoir hosts consist mainly of small mammals such as rats, moles, mice, and cats, but in some cases the reservoir hosts are larger mammals like dogs, cattle, camels and in some cases humans (3; 20; 21; 52; 93; 101; 133; 248; 251) (Table 1). However, more often humans are an incidental host for *Bartonella* species.

Evidence suggests that *Bartonella* species show a degree of host specificity. This specificity is apparent in the limited ability to isolate single *Bartonella* species from multiple potential reservoir host species like cats and mice, while multiple *Bartonella* species can be isolated from a single host species (21; 50; 52; 110; 251). For example, multiple studies have shown that several species of *Bartonella* can be recovered from

wild rodents both in the United States and Asia (44; 137). This host specificity could be due to a range of factors. One obvious factor would be vector ecology. The geographic range of known *Bartonella* vectors and the subsequent specificity of these vectors for certain hosts, surely plays a role in the host specificity of *Bartonella* species. For instance, *B. henselae* and *B. quintana* have worldwide distribution, like their hosts, cats and humans, respectively, and their vectors, cat fleas and body lice, respectively . Unlike *B. quintana* and *B. henselae*, *B. bacilliformis* has a very limited geographic range, which correlates with its suspected vector the, new world sandfly, *Lutzomyia verrucarum* (158). The limited or expansive geographic range of both *Bartonella* vectors and host species is likely to have a major impact on *Bartonella* specificity. However, the selective transmission of *Bartonella* species to a single or limited number of hosts is likely not due to vector ecology alone.

Of the 31 recognized *Bartonella* species, much more is known about their reservoirs than their vectors. Studies to replicate host infection have produced persistent bacteremia in cats inoculated with *B. henselae* and *B. clarridgeiae*, in mice infected with *B. grahamii*, in dogs infected with *B. rochalimae*, and in rats infected with *B. tribocorum* (51; 95; 223; 252). A study by Kosoy et al, 2000 found that genetically distinct *Bartonella* isolated from wild rodents show host specificity when inoculated into cotton rats, Wistar rats, BALB/c mice, and white footed mice (136). Additional studies have been performed to try and establish a persistent bacterial infection of *Bartonella* species in non-reservoir hosts, yet, to date, none have been successful. These studies included attempts to infect cats, rats, and guinea pigs with *B. rochalimae*, rats with *B. henselae and*

B. quintana, and mice with *B. henselae*, as the reservoirs for *B. rochalimae, B. henselae*, and *B. quintana* are dogs, cats, and humans, respectively (51; 120).

The ecological niches of *Bartonella* have also been examined at the gene level. *Bartonella* phylogenies and genome analyses have been used to track the adaptation of *Bartonella* species to their specific ecological niche (61; 77; 78; 242). Analyses support the idea that *Bartonella* species or *Bartonella* clades have adapted to their particular hosts independently of one another, in part, due to the highly intracellular nature of these organisms (61; 77; 78). Horizontal transfer events in metabolic pathways and the evolution of virulence factors such as the type IV secretion systems have been studied to help define ecological niches and map the parallel evolution of *Bartonella* species (61; 78; 258).

VIRULENCE FACTORS

Bartonella species contain two important groups of virulence factors. These are factors that are involved in the survival of *Bartonella* species in: 1) the nucleated host cells- endothelial cells or 2) the erythrocytes of the host. Virulence factors described to be involved in nucleated host cell infection mainly consist of trimeric autotransporter adhesin molecules (TAAs), VirB/D4 type IV secretion system (VirB/D4 T4SS) proteins, outer membrane proteins, and GroEL heat shock proteins (122; 177). Described virulence factors involved in erythrocyte infection are the Trw type IV secretions system (T4SS) proteins, flagella, deformin, invasion associated loci, and hemolysins (122; 177). Of these proteins the TAAs, VirB/D4 T4SS, Trw T4SS, and the flagellar proteins are the best characterized for multiple species of *Bartonella*.

Endothelial Cell Infection

Trimeric Autotransporter Adhesins

TAAs are a class of adhesin proteins specific to Gram negative bacteria. TAAs are found in a wide assortment of Gram negative bacteria, including human pathogens like *Neisseria meningitidis* to Uropathogenic *Escherichia coli* (2; 217). TAAs play a crucial role in host cell adhesin and invasion. TAAs are extracellular filaments made up of a head domain, thought to be involved adhesin, and stalk domains, which are highly variable in length (151). All species of *Bartonella* are believed to carry genes encoding TAAs (122). However, the TAAs of *B. henselae* and *B. quintana* have received the most attention and are the most extensively studied, as compared with the TAAs found in other *Bartonella* species.

B. henselae encodes a TAA referred to as *Bartonella* adhesin protein A (BadA), which is only found in *B. henselae*. Studies have shown that BadA is responsible for autoagglutination, adhesin, inhibition of phagocytosis, and regulation of proangiogenic transcription profiles in host endothelial cells (123; 180; 204; 205). BadA is believed to be crucial for *B. henselae* endothelial cell attachment by binding to β1-intergins, which bridge BadA to extra cellular matrix proteins like collagen and fibronectin (204). Additionally, in HeLa cells, BadA has been implicated in proangiogenic transcriptional regulation based on the activation of NF-κB and hypoxia- inducible factor 1 (HIF 1), which in turn up regulate interleukin (IL)-8 via NF-κB and vascular endothelial growth factor (VEGF) via HIF-1 (125; 204). Both IL-8 and VEGF are known to stimulate angiogenesis (124; 204). Studies using HUVECs (human umbilical vein endothelial cells)

showed a similar activation profile to that seen in HeLa cells, but in addition to the activation of NF-κB and HIF-1, HIF-2 was also activated, leading to an up regulation of genes for vascular endothelial growth factor receptor 2 (VEGF-R2) in addition to IL-8 and VEGF (124; 125). Further, immunohistochemical staining for HIF-1α has successfully detected HIF-1α in lesions from patients with bacillary angiomatosis (124). While BadA is the most studied *Bartonella* TAA, the mechanisms by which BadA interacts with host cells has not be fully resolved and at the present time the mechanism by which BadA protects *B. henselae* from phagocytosis by macrophages is completely unknown (204).

The four variably expressed outer membrane proteins of *B. quintana* (VompA-D) are the next most studied group of TAAs within the *Bartonella* genus. Of the Vomps, Vomps A-C are the most highly conserved apart from their major and minor variable regions (122). In turn, Vomps A-C seem to be most similar to BadA (122). However, in terms of size the Vomps are much smaller than BadA, at around 40 nm for Vomps A-C versus 240 nm for BadA (180). Additionally, the Vomps undergo phase variation during blood stream infections and not all four Vomps are present within the different *B. quintana* strains (257). Like BadA, the Vomps are implicated in autoaggregation and angiogenesis (225; 257). Studies have shown that VompA is responsible for the autoaggregation phenotype, as *B. quintana* isolates, which lack VompA do not autoaggregate (257). Like BadA, the Vomps have been implicated in the upregulation of VEGF in HeLa cells and in a human monocytic cell line THP-1 (225). Unlike BadA, the role of Vomps in adhesin is unclear. Zhang et al, 2004 found that VompC was required for *B. quintana* to bind collagen, while Schulte et al, 2006 concluded that the Vomp

proteins were not responsible for adhesin in experiments using HeLa and THP-1 cells (225; 257). These conflicting results may be due to the specificity of the Vomps and further studies are needed to clear up these discrepancies.

VirB/D4 Type IV Secretion System

Type IV secretions systems are present in many Gram negative bacterial species. T4SS consist of a membrane spanning channel and a pilus or another filament or protein, which attaches to the host cell (119). The main functions of most T4SS are to act as delivery mechanisms for effector proteins into host cells (119). These effector proteins then subvert the host cell functions to create an environment more suitable to the bacterial pathogen (119). Some *Bartonella* species are known to lack T4SS (*B. bacilliformis*), while others possess one or more T4SS (61). Of the type IV secretion systems seen in *Bartonella* species the VirB/D4 T4SS is thought to be the T4SS mainly involved in the infection of endothelial cells and the subsequent pathology associated with these infections, such as bacillary angiomatosis (223).

Like with the TAAs of *Bartonella* species, the VirB/D4 T4SS of *B. henselae* has been the most widely studied VirB/D4 T4SS of *Bartonella*. The VirB/D4 T4SS of *B. henselae* was discovered when Padmalayam et al, 2000 were trying to characterize the 17-kDa immunodominant protein of *B. henselae* (190). Through this work and subsequent studies, the 17-kDa protein was found to be a Vir5B homolog encoded for on a virB operon, similar to the prototype VirB T4SS of *Agrobacterium tumefaciens* (190; 222). Since the discovery of the VirB/D4 T4SS in *B. henselae,* studies have shown that the VirB/D5 T4SS participates in inhibition of endocytosis and invasome formation, the

uptake of large bacterial aggregates, proinflammatory activation via NF-κB, and inhibition of apoptosis, the latter two which are associated with angiogenesis (61; 221).

The proteins translocated by the VirB/D4 T4SS of *Bartonella* are referred to as *Bartonella* effector proteins or Beps. *B. henselae* is found to have seven Beps, BepA-G (61; 224). These Beps each contain a *Bartonella* intracellular delivery (BID) domain and a positively charged tail at the C-terminus. Beps E-G contain additional BID domains, which do not function in translocation, but are thought to play a role in effector function (61). Additionally, Beps A-C have a filamentationinduced by cAMP (FIC) domain at their N-terminus and Beps D-F were found to have N-terminal tyrosine phosphorylation domains (61). While the function of the FIC domains is still unknown, phosphorylation of the tyrosine phosphorylation domain of BepD after effector translocation has occurred in human endothelial cells has been demonstrated (224).

The three Beps (Bep D-F) with tyrosine phosphorylation domains were found to interact with proteins integral in host cell signaling when phosphorylated, lending credit to the belief that these effectors are essential for subversion of the host's cellular processes (226). Additionally, BepA was found to inhibit apoptosis and induce sprout formation leading to angiogenesis in human umbilical vein endothelial cells (HUVECs) (220). BepG or BepC and BepF together, were found to elicit invasome formation by inhibiting endocytic uptake of *B. henselae* in HUVECs (203; 239). More recently, BepE was found to be essential for invasion and dissemination of *B. henselae* in HUVECs (187). Recent comparative genomic work has identified operons encoding probable VirB/D4 T4SS systems and their associated Beps in many other *Bartonella* species, but little is known about their roles (61; 77; 98). Additionally, the only other Bep studied was

a *B. quintana* ortholog of *B. henselae* BepA, which has been implicated in inhibition of HUVECs apoptosis (220). Otherwise, little research has investigated the translocation potential or *in vitro* and *in vivo* activities of the Beps in other *Bartonella* species.

Erythrocyte Infection

Trw Type IV Secretion System

Another important T4SS found in *Bartonella* species is the Trw T4SS. The Trw T4SS found to be strikingly different from any other *Bartonella* T4SS (227). Unlike the VirB/D4 T4SS, which is involved in endothelial cell adhesion, invasion, and angiogenesis, the Trw system is believe to be required for adhesion and invasion of erythrocytes in mammalian hosts (242). The Trw T4SS was discovered during a differential fluorescence screen looking for genes that were upregulated during *in vitro* endothelial cell infection by *B. henselae* (210). Initially, this data suggested a role for Trw in endothelial cell infection (210). However, latter experiments proved that Trw was not essential for endothelial cell infection, but rather for colonization of erythrocytes (227; 242). Infection studies involving *B. tribocorum* mutants lacking a functional Trw T4SS found that the Trw deficient mutants will appear in the blood stream of experimentally infected rats, but are quickly cleared and unable to establish infection within erythrocytes (63). Signature-tagged mutagenesis (STM) screens of *B. tribocorum* and *B. birtlesii*, which naturally infect rats and mice, respectively, found that the Trw system was necessary for adhesion and invasion of rat erythrocytes, in the case of *B.*

tribocorum, and of murine erythrocytes, in the case of *B. birtlesii*, in both *in vivo* and *in vitro* models (63).

Unlike the VirB/D4 T4SS of *Bartonella*, the Trw system is unable to translocate effector molecules and instead carries tandem gene duplications of *trwL* and *trwJ*, that code for the surface expressed subunits making up the pilin and pilus associated components, respectively (61; 227). TrwJ has recently been shown to play an integral role in *B. birtlesii* adhesion and invasion of mouse erythrocytes (63). *In vitro* work has shown that TrwJ binds to a major transmembrane glycoprotein found on erythrocyte membranes (63). These subunits (TrwL and TrwJ) are thought to be essential to erythrocyte colonization allowing the *Bartonella* to adhere to and invade erythrocytes (63).

The highly variable nature of these subunits is advantageous for the *Bartonella* because the surface proteins on mammalian erythrocytes are also highly variable. When *B. tribocorum* Trw was ectopically expressed in *B. henselae,* which is normally only able to invade cat erythrocytes, and in *B. quintana*, which specifically invades human erythrocytes, both, *B. henselae* and *B. quintana,* were able to invade rat erythrocytes like *B. tribocorum*, indicating the specific nature of the Trw system (242). Interestingly, not all species of *Bartonella* harbor Trw systems (99) (77) (61; 210). However, all species that do not have Trw systems are instead found to be equipped with flagellar systems, making these two systems mutually exclusive (61; 177; 210).

Flagella

Several species of *Bartonella* are found to express flagella, including *B. bacilliformis*. Early work with *B. bacilliformis* suggests that non-motile *B. bacilliformis* cannot infect erythrocytes (19; 245). Follow up work showed *B. bacilliformis* invasion of erythrocytes was resistant to trypsin treatment, which degrades most *B. bacilliformis* outer membrane proteins, excluding flagella (218). Additionally, anti-flagellin antibodies directed against *B. bacilliformis* inhibit the ability of *B. bacilliformis* to invade erythrocytes, indicating flagella must play a role in invasion of human erythrocytes, *in vitro (218)*. These results are consistent with reports that flagella from other bacterial species, such as *Escherichia coli*, *Salmonella enteric, and Burkholderia pseudomallei,* have been shown to be associated with adhesion and invasion properties (218). However, little work has been done to further characterize the adhesion and invasion properties of *Bartonella* flagella since these initial studies on *B. bacilliformis*.

GENOMICS

As the number of recognized *Bartonella* species are growing each year, gene sequencing for identification and classification of these new species has become essential. Genetic classifications can come in the form of single gene sequencing, multilocus sequence typing (MLST), multispacer sequence typing (MST), and full genome sequencing. Genes commonly used for single gene sequencing, MLST, and MST include citrate synthase (*gltA),*RNA polymerase β subunit *(rpoB),* GTP-binding tubulin-like cell division protein *(ftsZ),* riboflavin synthase *(ribC),*16s ribosomal RNA*(rrs),* molecular chaperone GroEL *(groEL),*SsrA transfer-mRNA *(ssrA)*, and the 16S-23S ribosomal RNA intergenic transcribed spacer (ITS) (64; 106; 110; 117; 142; 184; 199; 253; 255; 256).

When relying on a single gene identities for identification of *Bartonella* species, *gltA* and *rpoB* are believed to have the best discriminating power for determining new or novel species of *Bartonella* (142). A 338 bp fragment of the *gltA* is most often used to detect *Bartonella* species in specimen samples, such as blood samples from rats found in Thailand or samples from humans in areas endemic for *B. bacilliformis* (15; 48; 184). Recent work has shown that *ssrA* is useful as a target for real-time quantitative (q)PCR assays. One study used an *ssrA* qPCR assay to identify *Bartonella* in elk blood samples and a subsequent sequencing analysis, using *ssrA* as the target, was able to differentiate these *Bartonella* at the species level (64). Additionally, *ribC, ftsZ,* and *groEL* have all been shown to have the ability to resolve *Bartonella* at the species level, while *rrs* is only useful for genus level identification (117; 167; 255; 256). The 16S-23S ITS is often used for MST and can discriminate between strains of a single species (148; 253). Comparisons between 16s-23s ITS sequences is commonly used when comparing *B. bacilliformis* isolates from endemic areas (22; 148). Phylogenies created using single gene analyses are able to differentiate *Bartonella* species. However true evolutionary pictures are rarely seen with single gene analyses; instead, MLST and genome sequencing are better suited for this task (80; 159).

All eight housekeeping genes, or a combination of the eight genes mentioned previously, are often used for MLST analysis (110). The genes of choice are concatenated and then used to build a phylogenies aimed at uncovering the relationships between *Bartonella* species and between strains of *Bartonella* species, including *B. bacilliformis, B. henselae, and B. quintana* (8; 9; 46; 175). Additionally, this technique is often used for the thorough classification of novel *Bartonella* species (110; 135; 199).

Furthermore, the use of multiple genes through MLST creates a more accurate representation of the relationships between the different *Bartonella* species and has been used to create an evolutionary map for the genus *Bartonella* (77).

Bartonella species are commonly classified into four 'lineages' (Figure 4) (77). Lineage 1 contains only one species, *B. bacilliformis* and is generally referred to as the 'ancient' lineage (61; 77; 99; 177). Lineage 2 contains ruminant specific species, and lineages 3 and 4 contain *Bartonella* species that infect a diverse group of mammals, resulting from the adaptation of each species to a particular mammalian host (77; 99; 210). Lineage 1 is commonly referred to as the 'ancient' lineage based on the idea that *B. bacilliformis* is the ancestral species for all lineages, with lineages 2-4 representing more recent member of the genus *Bartonella* and commonly referred to as the 'modern' lineages (61; 78; 99; 177). Genome analyses using the core genomes of fully sequenced *Bartonella* species have confirmed these apparent lineages (Figure 4) (77). In addition, genome analyses have uncovered that lineages 3 and 4 have acquired traits through independent parallel evolution (77). The parallel evolution of the type IV secretion systems of lineages 3 and 4 have received the most attention and several studies have been published in recent years confirming initial reports (Figure 4) (77; 98; 258). As stated previously, genome analyses have been used to support the theory that *Bartonella* species have adapted to specific ecological niches (Figure 4) (77; 78; 258). These genome analyses have revealed gene losses and gains within the phospholipid pathway that suggest adaptation to the specific ecological niche of *Bartonella* lineages (258).

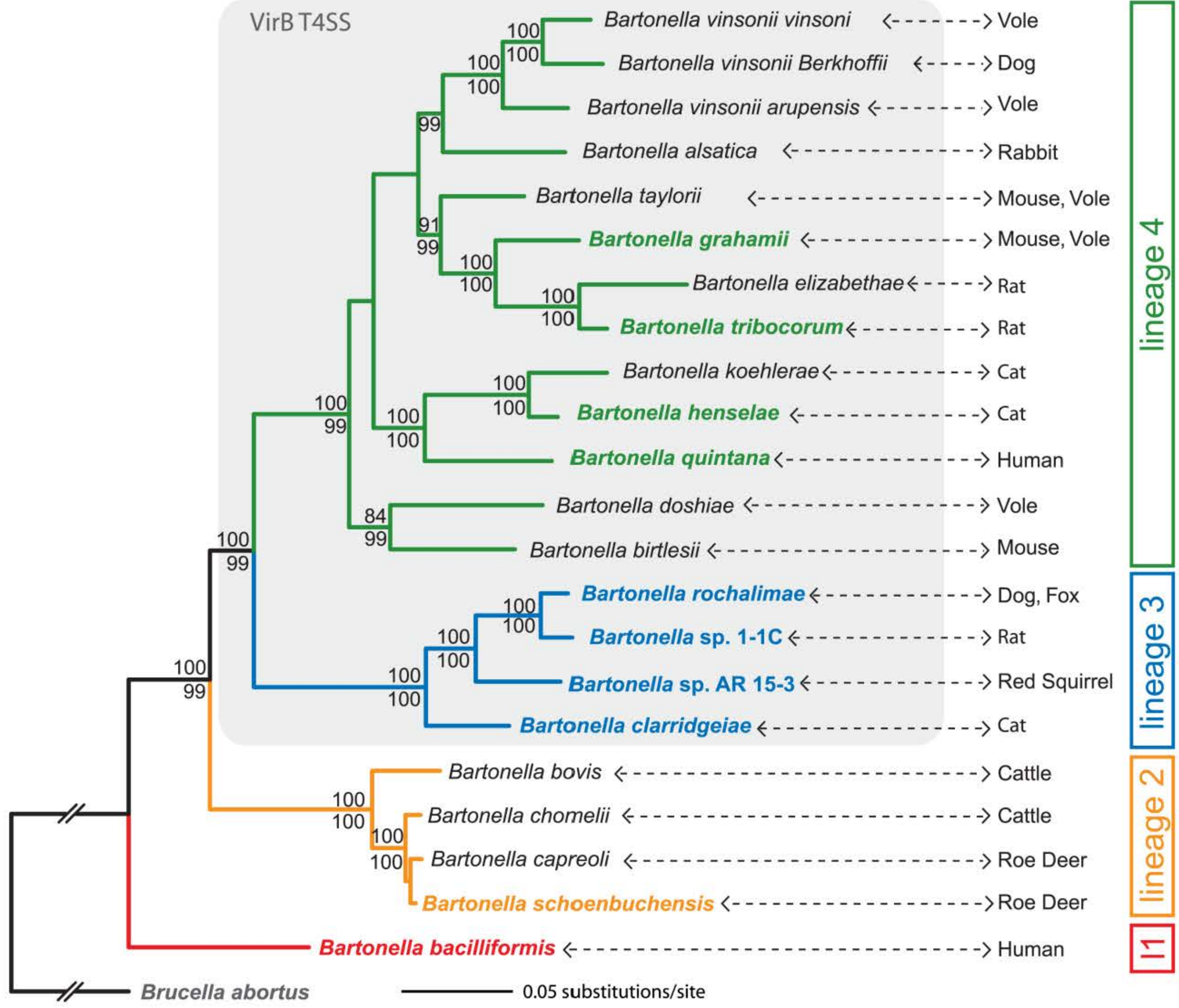
VirB T4SS
Bartonella vinsonii vinsoni
Vole
Bartonella vinsonii Berkhoffii
Dog
Bartonella vinsonii arupensis
Vole
Bartonella alsatica
Rabbit
Bartonella taylorii
Mouse, Vole
Bartonella grahamii
Mouse, Vole
Bartonella elizabethae
Rat
Bartonella tribocorum
Rat
Bartonella koehlerae
Cat
Bartonella henselae
Cat
Bartonella quintana
Human
Bartonella doshiae
Vole
Bartonella birtlesii
Mouse
Bartonella rochalimae
Dog, Fox
Bartonella sp. 1-1C
Rat
Bartonella sp. AR 15-3
Red Squirrel
Bartonella clarridgeiae
Cat
Bartonella bovis
Cattle
Bartonella chomelii
Cattle
Bartonella capreoli
Roe Deer
Bartonella schoenbuchensis
Roe Deer
Bartonella bacilliformis
Human
Brucella abortus
0.05 substitutions/site
lineage 4
lineage 3
lineage 2
l1

Figure 4: Phylogeny of *Bartonella* based on a genome-wide dataset. Maximum likelihood analysis using an alignment of 478 genes (515,751 nt) of ten sequenced *Bartonella* species (indicated by bold and color type) and *Brucella abortus*. Based on sequence data from *rpoB*, *gltA*, *ribC*, and *groEL* genes, additional *Bartonella* species were included in the analysis. Numbers above the branches represent maximum likelihood bootstraps (100 replicates); numbers below represent Bayesian posterior probabilities. Values ≥80% are shown. Lineages harboring the VirB T4SS (210) are shaded in gray; the primary mammalian hosts are indicated for each species. Phylogenetic trees inferred from either the genomic data set excluding non-sequenced bartonellae or the sequences from only *rpoB*, *gltA*, *ribC*, and *groEL* genes revealed the same four lineages for the *Bartonella* ingroup .

Figure and figure legend were reproduced from (78) with some modification to the figure legend

CLINICAL TREATMENT TRIAL (UNPUBLISHED DATA)

In 2003, a clinical trial was undertaken in Caraz, Ancash, Peru to test the efficacy of azithromycin as an alternative treatment for verruga peruana caused by *B. bacilliformis* (Figure 5). Caraz sits in the Andes Mountain range in a region where *B. bacilliformis* is known to be endemic. At the time of the study, the recommended treatment for verruga peruana was two weeks of rifampin. To test the efficacy of azithromycin as an alternative treatment, 127 patients were enrolled in the study. Those patients enrolled were divided into two groups. One group was to receive rifampin every day for two weeks and the other was to receive azithromycin once a week for two weeks. Blood samples were taken from the patients at days 0, 7, 14, 30, and 60. Of the 127 patients, 72 were positive for *Bartonella* infection by culture (Table 2). The remaining 55 patients were culture negative throughout the course of the clinical treatment trial. Of these culture positive patients, 19 were positive by culture on two or more days. The majority of patients were culture positive at day zero and then again at day 30 or 60, indicating either treatment failure or possible new infections. From these blood draws and subsequent culture, a total of 94 isolates were obtained from the 72 patients. To further confirm *B. bacilliformis* infection, the 94 isolates, from the 72 patients, were subjected to gene sequencing. A 338 bp fragment of *gltA* was sequenced and compared to *B. bacilliformis gltA* gene sequences in GenBank. The results confirmed that 91 of the 94 isolates were *B. bacilliformis* (Figure 6). Additionally, of the 19 patients who were culture positive on multiple days, six patients had a change in the strain isolated between the different blood draws. Finally, three isolates from two of the patients (at ten year old male and a three year old male)

Figure 5: Map indicating study area.

Table 2: Culture results for the 127 patients enrolled in the study over the course their 60 day follow ups.

Number of Patients	**Blood Culture Outcome** *Day of Blood Draw*				
	Day 0	*Day 7*	*Day 14*	*Day 30*	*Day 60*
55	Negative	Negative	Negative	Negative	Negative
50	**Positive**	Negative	Negative	Negative	Negative
1	Negative	**Positive**	Negative	Negative	Negative
1	Negative	Negative	**Positive**	Negative	Negative
1	Negative	Negative	Negative	**Positive**	Negative
12	**Positive**	Negative	Negative	Negative	**Positive**
3	**Positive**	Negative	Negative	**Positive**	Negative
3	**Positive**	Negative	Negative	**Positive**	**Positive**
1	**Positive**	Negative	**Positive**	**Positive**	**Positive**

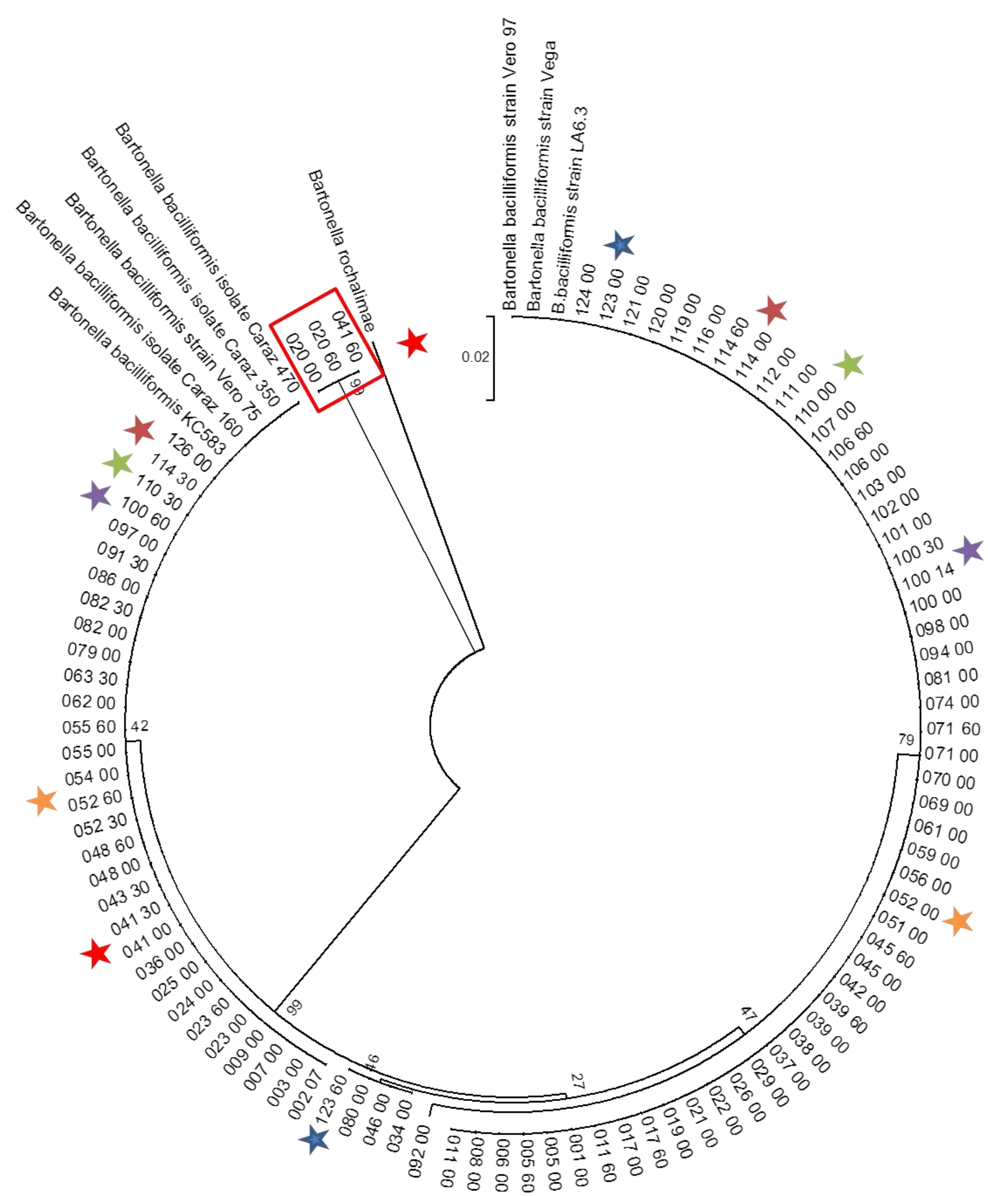

Figure 6: Phylogeny for the 94 isolated collected during the clinical treatment trial. Phylogeny was created for a 240 bp fragment of *gltA* with Mega5 software, using the neighbor joining tree method (1,000 bootstrap replicates) (231). The distances were calculated using the Jukes-Cantor method, where units are calculated as the number of base pair substitutions per site. Isolates were compared to eight *B. bacilliformis* isolates from GenBank and *B. rochalimae*. Stars denote patients with a change in the genotype of the *B. bacilliformis* cultured from their blood.

were dissimilar from *B. bacilliformis*, however, identical to one another, based on the *gltA* sequence. The results from this study indicate the complexity, diversity, and abundance of *B. bacilliformis* and possibly other *Bartonella* species in Caraz, Peru needs to be studied further. The ability to effectively treat populations is limited when there is limited data, knowledge, and understanding concerning the strains and species of *Bartonella* circulating in endemic areas like Caraz.

HYPOTHESIS

In the past 25 years over 20 new *Bartonella* species have been identified. These novel *Bartonella* species are being identified in a wide array of mammals, including small mammals like mice and rats, to larger mammals like cattle and kangaroos. Some of these new *Bartonella* species have been implicated in causing human disease, though mostly due to incidental infections. During a 2003 clinical trial aimed at investigating alternative therapeutics for chronic *B. bacilliformis* infection in humans, three isolates (identified as isolates 20.00, 20.60 and 41.60) from two patients were identified to be inconsistent with *B. bacilliformis*, but identical to one another, based on a 338 base pair sequence of *gltA*. When the sequences were compared to all known *Bartonella* species found in GenBank no matches were found.

This preliminary evidence led us to hypothesize that the three isolates (20.00, 20.60, and 41.60) derived from children suffering from apparent verruga peruana belong to a single novel species of Bartonella, *which possesses virulence factors essential to pathogens of the genus Bartonella.*

SPECIFIC AIMS

Aim 1

Investigate the genotypic and phenotypic characteristics of isolates 20.00, 20.60 and 41.60 to determine if the agents are identical and novel.

In order to determine if these isolates make up a novel species of *Bartonella* growth characteristics, colony morphology, microscopic characteristics, biochemical utilization, antibiotic susceptibilities, and genetic similarity/dissimilarity to each other, *Bartonella bacilliformis*, and other *Bartonella* spp., needed to be explored. The results from these tests will provide insight as the nature of these isolates and provide insight into how the isolates relate each other and to other species in the genus *Bartonella*.

Aim 2

Characterize the genomes of isolates 20.00, 20.60 and 41.60 using bioinformatic methodology to elucidate similarities and differences among the three isolates and between the three isolates, Bartonella bacilliformis, other Bartonella species, and other bacterial pathogens and to identify possible virulence factors.

Although isolates 20.00, 20.60 and 41.60 were cultured from the blood of two individuals presenting with verruga peruana, the pathogenicity of this new agent is unknown. Since at this time we cannot speculate as to the appropriate animal model to use to study this pathogen, full genome sequencing will be used to investigate pathogenicity using bioinformatic tools aimed at providing evidence of virulence factors similar to those of *B. bacilliformis,* other *Bartonella* species, and other bacterial

pathogens. This genomic characterization will also provide insight as to the relatedness of the three isolates to one another, to other *Bartonella,* and to other bacterial pathogens at both the sequence and protein level.

CHAPTER 2:
Novel *Bartonella* Agent as Cause of Verruga Peruana

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*Figure, table, reference numbers, and headings have been modified to follow the format of this dissertation.

ABSTRACT

While studying chronic verruga peruana infections in Peru from 2003, we isolated a novel *Bartonella* agent, which we propose be named *Candidatus* Bartonella ancashi. This case reveals the inherent weakness of relying solely on clinical syndromes for diagnosis and underscores the need for a new diagnostic paradigm in developing settings.

INTRODUCTION

Bartonellosis is a disease caused by infection with species from the *Bartonella* genus. In South America, infection with *B. bacilliformis*, an α-2 proteobacterium, may cause a life- threatening bacterial infection (157; 226). If untreated, the acute form of the illness, sometimes referred to as Oroya fever, has a high mortality rate because the bacteria invade erythrocytes, resulting in subsequent severe anemia and secondary infections. A chronic phase, termed verruga peruana, is characterized by vasculoproliferative skin lesions; some researchers have also described an asymptomatic bacteremic phase, which may contribute to the longevity of the reservoir status of infected persons (48).

In 2007, a novel species of *Bartonella* (*B. rochalimae*) was isolated from a single traveler who had an acute febrile anemia after traveling to Peru (79). We report the identification of another novel agent of *Bartonella* isolated from a patient with chronic bartonellosis (collected in 2003, fully characterized in 2011–2012). We suggest that the isolate be named *Candidatus* Bartonella ancashi in honor of the highland region of Peru.

THE STUDY

The patient was a 3-year-old boy with no known underlying medical history who was identified in 2003 as having clinical verruga peruana by the classic appearance of the eruptive nodular rash (Figure 7). He and his family lived in a rural setting near the town of Caraz, Ancash region, Peru. He lived in close proximity to numerous pets and farm animals and had experienced insect bites around the time the eruptive rash developed. His rash had been present for ≈30 days, and he had no fevers, chills, or arthralgias. Baseline laboratory studies included complete blood counts and bacterial culture for *Bartonella* species, using methods previously described (161; 245). Briefly, the media was biphasic, consisting of Bacto agar with Proteose Peptone No. 3 (Becton, Dickinson and Co., Sparks, MD, USA), dextrose, sodium chloride and 10% defibrinated sheep's blood, and RPMI supplemented with 10% inactivated fetal bovine serum.

The physical examination revealed that the child had 56 lesions, distributed mainly on the extremities. Laboratory values were the following: hemoglobin level 12.8 mg/dL (reference range 11–13 g/dL), hematocrit 39% (reference range 31%–43%), and platelet count of 300,000/µL (reference range 15,000–400,000); his leukocyte count was elevated at 28,000/µL (reference range 4,100–10,900) with 51% eosinophils, for which he was referred for further evaluation.

The peripheral blood smear was negative for intracellular organisms, but blood culture was positive for a *Bartonella* species. This species was further studied and found to be novel on the basis of genetic sequencing of the isolate obtained from the standard blood culture (155). His condition was treated with azithromycin, and the rash fully resolved (7; 207). To confirm the identity of the isolate from this patient, in 2011–2012 we conducted molecular analyses (including PCR, nested PCR [nPCR], and the rash fully

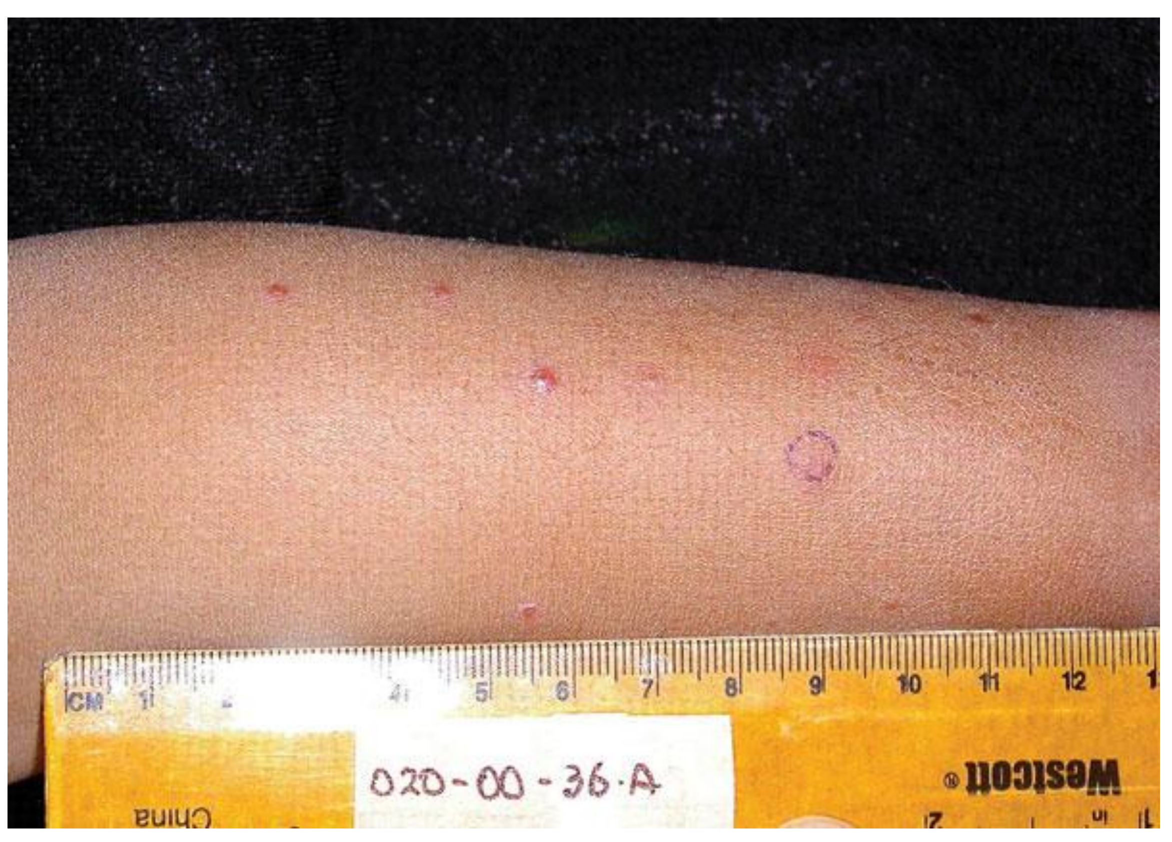

Figure 7. Clinical presentation of verruga peruana in 3-year-old boy, Peru, 2003.

resolved (7; 207). To confirm the identity of the isolate from this patient, in 2011–2012 we conducted molecular analyses (including PCR, nested PCR [nPCR], and sequencing) on the whole blood culture isolate, *Bartonella* species no. 20.00 (155).The isolate was characterized by sequencing 3 gene fragments.

The following conditions were used for PCR amplification. For *rrs*, initial denaturation was at 95°C for 1 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and elongation at 68°C for 90 s for PCR and 70 s for nPCR, and then by a final extension step at 72°C for 7 min. For *gltA*—95°C, denaturation was for 1 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and elongation at 68°C for 60 s, and then by the final extension step at 72°C for 7 min. For *rpoB*, primers were selected from the conserved regions of RNA polymerase β-subunit encoding gene (*rpoB*) after alignment of the *rpoB* from *B. quintana* and *B. vinsonni* for PCR and nPCR. PCR and nPCR were carried out by using conditions identical to those described for *gltA* (Table 3).

PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) before sequencing. PCR products were sequenced in both directions by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and run on an automated 3130*xl* Gene Analyzer (Life Technologies). Sequences were characterized by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence analysis was performed by using BioEdit version 7.1.3 (Ibis Biosciences, Carlsbad, CA, USA). The multiple sequence alignments were performed with the ClustalW multiple alignment application also in

Table 3. Primers used for PCR, nested PCR, and sequencing of novel *Bartonella* isolate from Peru, 2011–2012.

Gene	Primer name	Primer sequence, 5’ → 3’	Use	Fragment length
rrs	16SU17F	AGAGTTTGATCCTGGCTCAG	PCR, nPCR, sequencing	1,424 bp
	16SU1592R	AGGAGGTRATCCAGCCGCA	PCR, nPCR, sequencing	
	16SU 833R	CTACCAGGGTATCTAATCCTGTT	nPCR, sequencing	
	16S E. coli-518F	CAGCAGCCGCGGTAATAC	nPCR, sequencing	
gltA†	BHCS 781p (F)	GGGACCAGCTCATGGTGG	PCR, sequencing	338 bp
	BHCS 1137n (R)	AATGCAAAAAGAACAGTAAACA	PCR, sequencing	
rpoB	BrpoB1435F	CGCATTGGTTTRCTTCGTATG	PCR	589 bp
	Brpo2327R	GTAGACTGATTAGAACGCTG	PCR, nPCR, sequencing	
	Brpo1696F	CCTACGCATTATGGTCGTATTTG	nPCR, sequencing	

*nPCR, nested PCR.
†*gltA* primers were previously described by Eremeeva et al. (79).

BioEdit version 7.1.3. Phylogenetic trees were created with MEGA5 software using the neighbor-joining tree method with 1,000 bootstrap replicates (234).

Subsequent comparison with known *Bartonella* species in GenBank found no identical sequences. The 1,351-bp sequence of the *rrs* fragment was found to be 99.0% similar to the *rrs* fragment of *B. bacilliformis.* The 312- and 589-bp fragments of *gltA* and *rpoB*, respectively, were found to be most similar to their counterparts of *B. bovis* at 89.4% (*gltA*) and 85.9% (*rpoB*), respectively (Table 4). The sequence similarity ranges for the *rrs*, *gltA*, and *rpoB* for recognized *Bartonella* species are 97.7%–99.8%, 83.4%–96.1%, and 85.9%– 96%, respectively (256). In addition, *rpoB* and *gltA* are believed to have the best discriminating power for *Bartonella* species (142). La Scola et al. proposed that a new species be designated if the sequence similarities are <96% and <95.4% for a 327-bp fragment of *gltA* and a 825-bp fragment of *rpoB*, respectively (142). The sequence similarities for *Candidatus* Bartonella ancashi 20.00 to other known *Bartonella* species fall well below these suggested values, providing more evidence that this agent is unique. Phylogenetic analysis of the *rrs*, *gltA,* and *rpoB*, gene fragments provide additional evidence for identification of a unique *Bartonella* agent. The concatenated sequence of *gltA* and *rpoB* gene fragments placed the new *Bartonella* isolate in an exclusive clade that is most closely aligned with *B. bacilliformis* (Figure 8). The *rrs* fragment also placed the isolate in a clade with *B. bacilliformis*. The results from the phylogenetic analysis combined with the sequence similarity data provide evidence that this isolate, *Candidatus* Bartonella ancashi 20.00, is unique (142; 256).

Table 4. *rpoB* (top) and *gltA* (bottom) sequence similarities for *Bartonella* species.

	Percentage similarity with taxon																							
Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	**23**	24
1) *B. alsatica*		88.2	90.5	87	86.8	87	88.7	87.9	88.6	88.7	91	91.7	90.3	91.5	87.3	87	90.1	91.9	90	92.8	92.8	92.6	**84**	73.2
2) *B. bacilliformis*	84.9		86.8	88.2	88.4	87.7	88	86.8	84.2	84.5	88.2	86.8	87.2	87.7	87	88.2	85.8	87.3	85.8	87.2	85.9	86.5	**84.9**	71.9
3) *B. birtlesii*	92.9	85.8		85.9	86.5	85.9	86.5	87.3	86.1	86.6	88.4	89.4	87.7	88.6	85.1	86.6	87.5	89.4	88.9	91	90.3	91.4	**83.3**	72.5
4) *B. bovis*	87.8	87.1	90		94.7	94.3	87.3	89.3	84.9	85.6	88.2	86.3	89.3	87.5	87.2	94.9	86.6	86.5	86.1	86.5	86.1	87.2	**85.9**	73.3
5) *B. capreoli*	88.1	87.8	88.4	94.8		97.8	87.3	89.3	84.5	85.2	88.6	87	88.4	88	87.3	99.8	86.8	87.2	86.3	86.8	86.5	87.5	**85.2**	73.2
6) *B. chromelii*	87.1	85.5	88.1	95.1	97.1		87.5	88.7	84.7	85.1	87.9	87.2	88	87.7	87.2	98	86.6	86.3	86.1	86.8	87	87.3	**84.7**	73.5
7) *B. clarridgeiae*	86.2	85.2	87.8	90.7	90.3	89.4		87.2	85.1	85.6	87.5	87.2	87.9	88.4	91.7	87.5	85.1	87.9	86.5	88	87.3	87.5	**84.5**	71.9
8) *B. doshiae*	87.1	84.6	89.4	87.8	86.8	87.1	87.8		85.1	87.2	90.3	87	90.7	89.3	86.8	89.1	85.9	88.7	87.5	88	88	88.4	**85.2**	73
9) *B. elizabethae*	87.8	83.9	89.1	88.4	88.7	89.1	87.8	86.8		93.1	87	86.1	87.9	87	84.4	84.7	88.2	87.3	93.6	87	87.5	86.8	**82.4**	73.2
10) *B. grahamii*	89.4	85.8	91	90.7	90.7	91	88.1	88.1	94.8		87.9	86.8	88.2	87.9	84.4	85.4	87.5	87.3	93.6	87.9	87.7	87.7	**82.4**	74.6
11) *B. henselae*	89.4	84.2	91	90.7	87.5	88.1	87.1	87.1	86.5	89.1		88.6	95.6	93.3	86.6	88.7	87.2	89.4	88.9	89.3	89.3	90	**84**	73.7
12) *B. japonica*	85.2	83	87.1	85.5	85.8	84.9	85.5	83.9	84.6	87.1	87.1		88	88.9	85.4	87.2	89.3	88.9	89.1	91.4	91.2	91.2	**84.4**	72.3
13) *B. koehlerae*	89.7	84.9	91	89.4	87.8	88.1	87.8	85.5	87.8	89.1	94.5	86.8		91.5	86.1	88.6	86.8	89.4	88.7	88.4	88.7	89.1	**83.8**	72.8
14) *B. quintana*	88.7	83.9	88.7	89.4	88.7	87.5	88.7	85.5	85.8	87.8	92.3	87.5	91.3		87.3	88.2	87	90.8	88.6	91.2	91	91.4	**84.5**	74.7
15) *B. rochalimae*	86.2	86.2	88.7	91.9	91.6	90.7	96.1	86.5	88.4	89.4	89.1	85.5	87.8	89.1		87.2	84	85.8	85.8	85.9	85.2	85.6	**84.7**	72.5
16) *B. schoenbuchensis*	86.8	85.5	87.8	94.5	97.1	99.3	89.4	86.8	89.1	91	87.5	84.6	87.5	87.5	90.7		87	87.3	86.5	87	86.6	87.7	**85.1**	73.3
17) *B. silvatica*	85.8	82	86.5	86.5	87.1	86.8	88.7	85.5	86.2	89.1	85.8	84.2	85.2	87.5	88.4	87.1		88.6	87.7	89.3	88.9	88.9	**83.7**	72.8
18) *B. taylorii*	90.7	85.5	91.3	88.7	89.1	88.1	87.1	86.8	87.8	89.7	89.4	87.8	89.4	89.7	88.4	87.8	90.3		88	91.4	90.7	91.7	**82.8**	73.7
19) *B. tribocorum*	90.7	86.8	91.9	90	89.1	88.4	88.1	87.1	94.5	95.8	89.4	85.5	89.7	88.1	90	88.4	87.1	90		88.2	88.7	89.3	**83.3**	72.6
20) *B. vinsonii* subsp. *arupensis*	91.9	84.6	91.6	90	89.1	90	88.4	89.1	90.3	91.6	90.3	89.1	90	90.7	89.1	89.7	88.7	92.9	91		94.7	95.2	**83.5**	73.7
21) *B. vinsonii* subsp. *berkhoffii*	92.6	85.2	91.6	89.7	87.8	88.4	88.1	87.5	89.4	90.7	90.7	86.8	89.4	89.4	88.7	88.1	88.7	92.3	91.3	94.8		96.1	**82.4**	74.4
22) *B. vinsonii* subsp. *vinsonii*	92.9	86.5	91.6	89.1	89.1	89.7	88.1	88.4	90.3	91.3	90	87.8	90	89.4	89.4	89.4	89.1	94.5	91.3	95.8	96.1		**84**	74
23) Candidatus B. ancashi	**86.5**	**84.9**	**88.1**	**89.4**	**87.1**	**86.8**	**84.6**	**85.5**	**84.9**	**83.9**	**87.1**	**81.7**	**85.8**	**84.9**	**85.5**	**86.5**	**81**	**84.9**	**85.5**	**85.5**	**85.2**	**85.8**		73.3
20.00																								
24) *Brucella melitensis*	69.8	70.1	67.9	69.8	71.7	70.5	69.2	67.3	70.8	69.8	69.2	69.8	68.2	69.5	69.8	70.5	69.5	67.9	69.5	70.5	71.4	68.5	**68.2**	

*Similarities are based on a 312-bp fragment of gltA and a 589- bp fragment of rpoB. **Boldface** indicates similarities of the novel isolate.

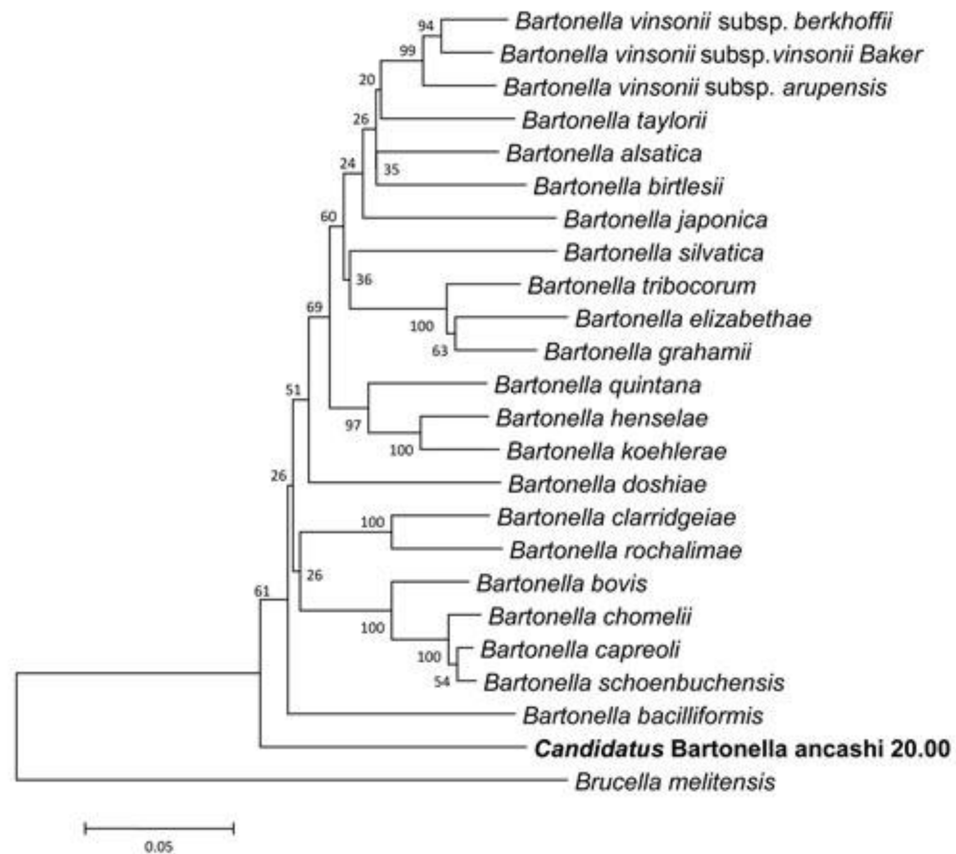

Figure 8. Phylogeny for concatenated sequences of novel *Bartonella* isolate (**boldface**), including a 312-character fragment of *gltA* and a 589-character fragment of *rpoB*. The neighbor-joining tree method (1,000 bootstrap replicates) was employed using MEGA5 software (234), and the distances were calculated by using the Jukes-Cantor method, in which units are calculated as the number of base pair substitutions per site (155). *Brucella melitensis* was used as the outgroup.

CONCLUSIONS

This case underscores the inherent weakness of relying solely on clinical syndromes for diagnosis. The variety of bacteria that have been implicated in the clinical spectrum of bartonellosis is increasing as molecular methods are applied to isolates that previously were identified by using clinical criteria or biochemical testing. The novel bacterium may have similar epidemiologic, clinical, and microbiologic properties to *B. bacilliformis*, but without relating these data to a full molecular characterization, that assumption is precarious.

To address this public health deficiency, a new diagnostic paradigm should be deployed to developing settings such as Peru. This is particularly true for areas with high biodiversity, a point identified by other investigators who have termed these regions "hot zones" for emerging infectious diseases. (118). Unfortunately, tools such as high-throughput sequencing are rare in developing settings where risk for novel pathogen emergence is highest. Investment in advanced molecular diagnostic platforms in the developing setting will be an essential tool for expanding pathogen discovery; of course, this should be accompanied by parallel investments in training in molecular laboratory techniques and analysis for resident scientists. Opportunities for grants and stable faculty positions must also be supported to encourage qualified scientists to remain in the developing setting.

Finally, evidence indicates that humans contract bartonellosis only once and that lifelong immunity results from that primary infection (54). Because of this circumstance, and the inability to identify an animal reservoir of *B. bacilliformis*, Peruvian scientists and others have identified bartonellosis as a disease that may be eradicated in the Andean region through development of a vaccine against *B. bacilliformis*, targeted treatment of

patients, and vector control programs (54). This possibility may be less feasible if multiple species of *Bartonella* cause bartonellosis. Further molecular and immunologic studies should be undertaken if this disease is to be targeted for eradication.

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We thank Jesus Gonzalez for his efforts in conducting the clinical trial in Peru.

The 3 genes have been submitted to GenBank, with the following accession numbers: *rrs*, KC178617, *gltA*, KC178618, *rpoB*. KC178619.

The original clinical trial was approved by the Institutional Review Boards of the Uniformed Services University of the Health Sciences, the Naval Medical Research Center, and the Universidad Peruana Cayetano Heredia in 2002, and compared the standard of care medication, rifampin, with azithromycin. The clinical trial was originally funded by Pfizer and the subsequent pathogen characterization was funded by the Department of Defense Global Emerging Infection System work unit no. 0000188M.0931.001.A0074. The funding agencies had no role in writing, editing, or approval of this manuscript.

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Chapter 3:

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

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*Figure, table, and reference numbers and headings have been modified to follow the format of this dissertation.

ABSTRACT

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.

INTRODUCTION

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 (79; 122). 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 (1). *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) (1; 226). 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 (46; 99; 122; 226). 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 (46; 99; 122; 226). Here, we further characterized a new *Bartonella* pathogen causing verruga peruana (25) by using multilocus sequence typing (MLST) and multispacer sequence typing (MST).

METHODS AND RESULTS

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 CO_2. 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 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" (25).

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 (46; 110; 253). MLST is useful for the detection of novel *Bartonella* species, while MST is useful for uncovering genetic differences between strains (46; 110; 253).

DNA extracted from both clinical isolates was subjected to PCR amplification and Sanger sequencing as previously described (Table 5) (25; 79; 91; 184; 202; 255; 256). 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

Table 5. Primers used for PCR, nested PCR, and/or sequencing[a]

Gene	Primer	Sequence (5′–3′)	Use(s)	Fragment length (bp)
Rrs	16SU17F	AGAGTTTGATCCTGGCTCAG	PCR, nPCR, sequencing	1,424
	16SU1592R	AGGAGGTRATCCAGCCGCA	PCR, nPCR, sequencing	
	16SU 833R	CTACCAGGGTATCTAATCCTGTT	nPCR, sequencing	
	16S E. coli-518F	CAGCAGCCGCGGTAATAC	nPCR, sequencing	
gltA	BHCS 781p (F)	GGGGACCAGCTCATGGTGG	PCR, sequencing	338
	BHCS 1137n (R)	AATGCAAAAAGAACAGTAAACA	PCR, sequencing	
rpoB	rpoB1435F	CGCATTGGTTTRCTTCGTATG	PCR	589
	rpoB2327R	GTAGACTGATTAGAACGCTG	PCR, nPCR, sequencing	
	rpoB1696F	CCTACGCATTATGGTCGTATTTG	nPCR, sequencing	
ftsZ	Bfp1 (F)	ATTAATCTGCAyCGGCCAGA	PCR, sequencing	864
	Bfp2 (R)	ACVGADACACGAATAACACC	PCR, sequencing	
groEL	HSPF1d (F)	GAACTNGAAGATAAGTTNGAA	PCR	736
	BbHS1630.n (R)	AATCCATTCCGCCCATTC	PCR	
	HSP1 (F)	GGAAAAAGTNGGCAATGAAG	nPCR, sequencing	
	HSP2 (R)	GCNGCTTCTTCACCNGCATT	nPCR, sequencing	

[a]*rrs* primer 16SU17F was previously described (90). The additional *rrs* primers and reaction conditions were previously described (25). rpoB primer rpoB2327R was previously described (202). The additional *rpoB* primers and the sequencing conditions were previously described (25). *gltA* primers were previously described (184), and reaction conditions were described by Blazes et al. (25). *ftsZ* and *groEL* primers and reaction conditions were previously described (255; 256). F, forward primer; R, reverse primer; nPCR, nested PCR.

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 6. 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" (Table 6). The analysis indicates that "*Candidatus* Bartonella ancashi" is a member of the Bartonella genus and most closely related to *B. bacilliformis* (Figure 9). 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 (Figure 10) (79). 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 (79). *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 (46). "*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 ancashi" is unique.

Table 6. Identity of "*Candidatus* Bartonella ancashi" genes to *gltA*, *rpoB*, *ftsZ*, *groEL*, *ribC*, and *rrs* of known *Bartonella* species.

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

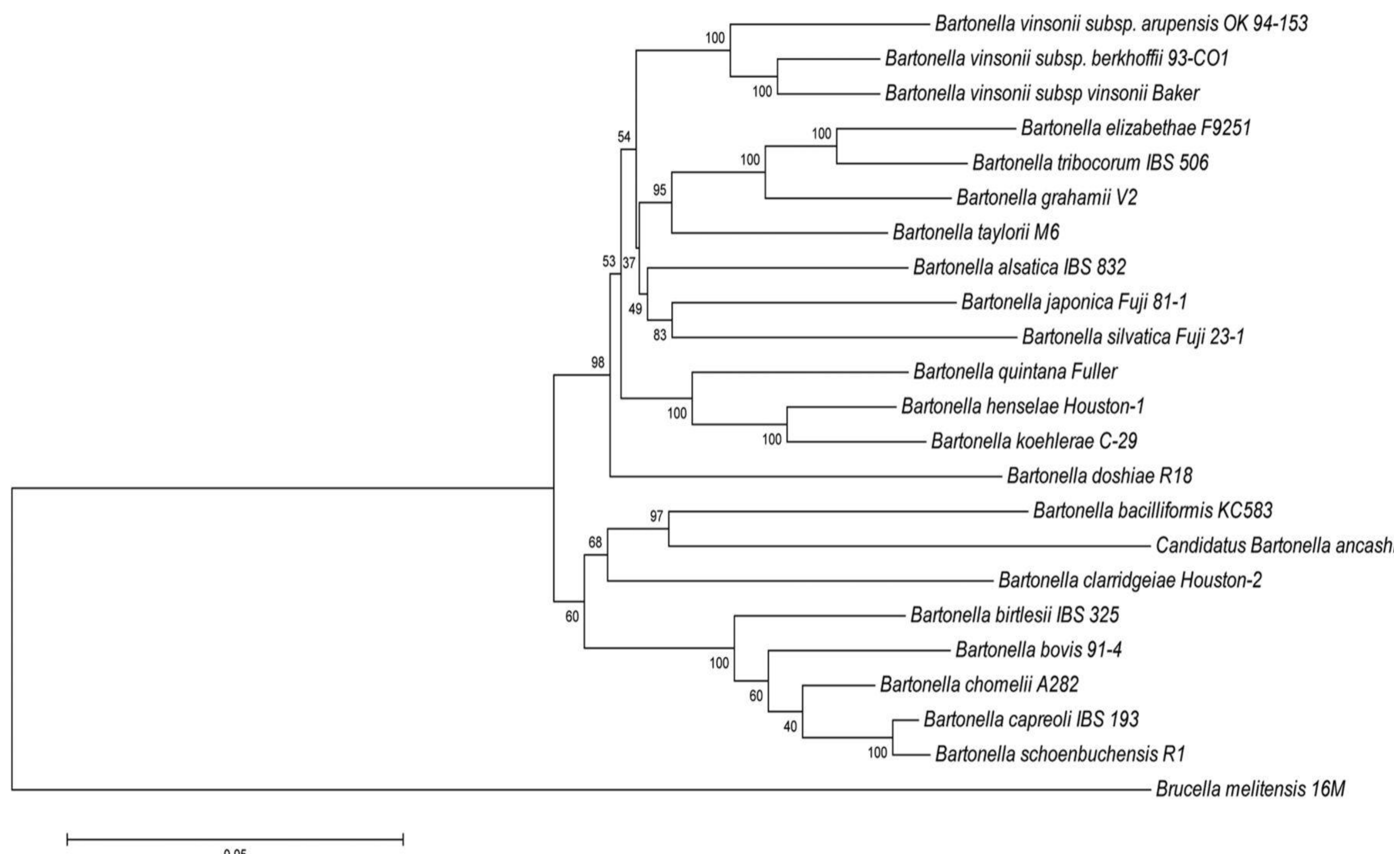

Figure 9. MLST phylogeny for a 5,108-character fragment of the concatenated gene sequences *rrs* (1,351 bp), *rpoB* (825 bp), *gltA* (312 bp), *ftsZ* (788 bp), *ribC* (607 bp), and *groEL* (1,192 bp) for the 21 *Bartonella* type strains and "*Candidatus* Bartonella ancashi" 20.60. The neighbor-joining-tree 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 (234). *Brucella melitensis* was used as the out group.

Figure 10. 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 (234).

DISCUSSION

In the majority of cases, a patient is given a diagnosis of Carrión'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 (110; 121; 142). 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 (110; 121), while MST is based on the variable 16S/23S intergenic linker region and is valuable for genotyping strains of *Bartonella* species (253). Additionally, fragments of the *gltA* and *rpoB* genes are found to have the highest discriminating power for *Bartonella* species identification and classification (142). 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 (184). 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 *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* (8; 9). 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 6). 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 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 Carrión's disease is endemic (25; 46). 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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There are no conflicts of interest to declare.

Chapter 4:

Isolation and Analysis of Human Pathogens Causing Verruga Peruana in Rural Ancash Region of Peru Revealed a Novel *Bartonella* Phylogenetic Lineage

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ABSTRACT

Background: The genus *Bartonella* contains over 30 species, a majority of those discovered in the past 25 years. Three *Bartonella* species are recognized to cause the majority of human disease associated with the genus *Bartonella*. Those are *B. bacilliformis*, *B. quintana*, and *B. henselae*, however in recent years several other *Bartonella* species have been implicated in human disease. One such pathogen is the novel species *Candidatus* (*Ca*). Bartonella ancashi, which was isolated from the blood of two patients living in the Ancash region of Peru.

Methodology/Principle Findings: In this study, we aimed to fully sequence and characterize the genome of the novel pathogen, *Ca.* B. ancashi. Through full genome sequencing and whole genome mapping of three isolates of *Ca.* B. ancashi, obtained from two children with disease indistinguishable from verruga peruana caused by *B. bacilliformis*, we were able to assemble and annotate the *Ca.* B. ancashi genome. Comparisons were made between all *Bartonella* species with full genome sequences and *Ca.* B. ancashi. The genome of *Ca.* B. ancashi was found to be most similar to the genome of *B. bacilliformis*. The *Ca.* B. ancashi genome contains flagellar and virulence modulating protein genes like *B. bacilliformis*. However, unlike *B. bacilliformis, Ca*. B. ancashi contains type IV secretion system proteins. Additionally, whole genome mapping uncovered an intriguing genomic inversion not present in all the *Ca.* B. ancashi isolates.

Conclusion/Significance: This genomic analysis indicates *Ca.* B. ancashi shares features seen in both modern and ancient lineages of *Bartonella*. This study provides additional evidence that *B. bacilliformis* diverged from other *Bartonella* species in the distant past. While *Ca.* B. ancashi is the closest relative of *B. bacilliformis*, the divergence between *Ca.* B. ancashi and *B. bacilliformis* is much greater than what is seen

between species other *Bartonella* lineages, indicating *B. bacilliformis* may be uniquely adapted to the human host and *Ca.* B. ancashi may represent an additional *Bartonella* lineage.

AUTHOR SUMMARY

The genus *Bartonella* contains over 30 species with an ever-increasing number of those causing human disease. *Bartonella* are often overlooked by the scientific community and because of this, there is a severe deficiency in the understanding of these species at all levels, including the genomic level. In this study, we undertook a genomic characterization of a novel *Bartonella* species, *Ca.* B. ancashi, which was uncovered during a clinical treatment trial for another *Bartonella* pathogen, *B. bacilliformis*. Interestingly, *Ca*. B. ancashi was found to cause disease indistinguishable from chronic *B. bacilliformis* infection. Our work aimed to uncover both differences and similarities between *Ca.* B. ancashi and *B. bacilliformis* and other known *Bartonella* species at the genomic level. Through this work, we hope to have developed a greater understanding of the genus *Bartonella* and with the discovery of a new human pathogen; we hope to see renewed interest in the genus as a whole.

INTRODUCTION

The bacterial pathogen *Bartonella* is an important emerging infectious disease because of the high prevalence of chronic *Bartonella* infections in mammals and humans and their rising risk to immunocompromised populations (27; 28; 99; 116; 179). Bartonellosis occurs worldwide and is associated with a number of well-known diseases, such as the fatal Oroya fever and the chronic verruga peruana lesions caused by *B. bacilliformis*, cat scratch disease caused by *B. henselae*, and trench fever caused by *B. quintana* (1; 99; 122; 134; 207). More recently, *B. henselae* and *B. quintana* have been associated with chronic infections such as bacillary angiomatosis and endocarditis, while *B. quintana* chronic bacteremia occurs in homeless populations around the world (45; 67; 70; 83; 129; 179; 191; 197). In addition to these three major infectious pathogens, a growing number of new *Bartonella* spp. have been identified in recent years as zoonotic bacteria transmitted by diverse arthropod vectors (28; 99; 122). Currently there are more than 40 official and candidate *Bartonella* species listed in the Taxonomy Database of NCBI; 31 of these are recognized species (http://www.bacterio.net/*Bartonella*.html). Historical accounts suggest *Bartonella* ssp. have infected humans for hundreds to thousands years (1; 212). Over the course of evolution, *Bartonella* lineages have adapted to a variety of hosts and have developed virulence factors associated with a diverse set of disease symptoms (22; 39; 78; 96; 116; 150).

B. bacilliformis was the only *Bartonella* species found in Peru until the identification of *B. rochalimae* in 2007 (79). Since its initial discovery, *B. rochalimae* has been shown to exist in fleas and several domestic and wild animals in many countries including Peru and the U.S. (102; 193). Phylogenetically, *B. rochalimae* is closely related to *B. clarridgeiae*, which has a worldwide distribution (79). Despite the high

morbidity of Bartonellosis in Peru, systematic studies on *Bartonella* pathogens are still lacking, so the genetic diversity and the existence of non-*bacilliformis Bartonella* in this country might be underestimated. In our study, we performed genomic analysis of three non-*bacilliformis Bartonella* isolates obtained from pre- and post- therapy patient blood samples collected during a clinical trial investigating antibiotic treatment options for Bartonellosis in the Ancash mountain region of Peru. Our previous work identified the isolate as a new species - *Candidatus* Bartonella ancashi (*Ca.* B. ancashi hereafter) (25; 181). The whole genome analyses undertaken in this study revealed the unique characteristics of *Ca.* B. ancashi. The study highlights similarities and differences between *Ca.* B. ancashi and its closest relative, *B. bacilliformis*. Our study also describes an intriguing genome inversion among the three *Ca*. B. ancashi isolates and possible horizontal gene acquisition.

METHODS

Bartonellosis patients, drug trial, clinical specimens

A clinical trial to compare rifampin, the standard drug for Bartonellosis caused by chronic *B. bacilliformis* infection, with azithromycin, a possible alternative medication for the treatment of chronic *B. bacilliformis* infections, was conducted in 2003 in the Caraz District of the Ancash region of Peru (Blazes DL, clinical trial manuscript in preparation). The human subject use protocol, including the clinical specimen collection, and the consent procedure were approved in 2002 by the Institutional Review Boards of the Uniformed Services University of the Health Sciences (Bethesda, MD, USA), the Naval Medical Research Center Institutional Review Board (Bethesda, MD, USA) and the Universidad Peruana Cayetano Heredia (Lima, Peru). Patients with suspected chronic *B. bacilliformis* infection (verruga peruana) presented either to the local hospital in Caraz or were identified by home visits and referred to the local hospital in Caraz. Then, patients, greater than one year, with the characteristic verruga rash were randomly chosen to receive 14 days of antibiotic treatment. Patients either received a daily dose of rifampin (Pfizer) or two weekly doses of azithromycin (Pfizer). For the patients who participated in the trial, survey data and medical records were collected on day 0 (baseline), i.e. time of presentation to the local hospital, and patients were started on the two week antibiotic treatment, consisting of either azithromycin or rifampin. Clinical data was also collected on days 7, 14, 30 and 60. Additionally, peripheral blood specimens were collected for each patient at days 00, 07, 14 and 60 in sodium citrate tubes at the local hospital in Caraz and transported on ice to the clinical laboratory at the U.S. Naval

Medical Research Unit No. 6 (Lima, Peru) for blood cultures and PCR assays. Selected specimens, *Bartonella* isolates, and genomic DNA extracts were sent to the Naval Medical Research Center (NMRC) and the Walter Reed Army Institute of Research (WRAIR) for additional investigations.

Blood culture, microbiology and biochemistry examinations, PCR assay

Blood specimens were cultured for up to 8 weeks using the procedure previously described. Bartonella culture positive specimens were confirmed to be *Bartonella* species by microbiological observations and PCR sequencing. Nucleic acids were isolated from the blood culture positive samples and subjected to PCR amplification of the 338-bp fragment of citrate synthase gene (*gltA*) and DNA sequencing was performed using the Sanger method.

Single-locus sequence typing, *Bartonella* strain isolation and identification

Sequences generated from the PCR amplicons of the partial *gltA* gene (homologous to nucleotides 781 through 1137 of the *B. bacilliformis* KC583 *gltA* sequence) were aligned and then used for phylogenetic analyses. Samples with *gltA* sequences significantly different from *B. bacilliformis* were selected for further strain isolation and identification. In brief, the isolates were cultured on brain heart infusion agar supplemented with 10% defibrinated sheep blood (BHIA with 10% sheep blood) or trypticase soy agar with 5% sheep blood (TSA with 5% sheep blood) for 10 to 28 days (BD Diagnostics, Sparks, MD). Passages 1 through 3 from the initial stocks received

from Peru were subjected to electron microscopy using a JEOL 100 CX II transmission electron microscopy (TEM) (JEOL, Peabody, MA).

Roche 454 pyrosequencing, whole genome restriction mapping, genome sequence assembly and finishing

Genomic DNA extracts for pure *Bartonella* isolates were randomly fragmented by focused ultrasonication using Covaris S2 System (Covaris, Inc., Woburn, MA), followed by rapid shotgun genomic DNA library preparation and pyrosequencing by using Roche 454 GS FLX Titanium System (Roche 454 Life Sciences, Branford, CT).

Bartonella isolates from frozen stocks were passaged 2-3 times on BHIA with 10% sheep blood and/or TSA with 5% sheep blood at 30°C with 5% CO_2 for 10-14 days. DNA was then isolated using the Argus HMW Sample Preparation Kit as described by the manufacturer (OpGen Inc., Gaithersburg, MD). DNA quality and quantity was assessed using Argus® QCard Kit and mapped using the Argus® MapCard Kit and Argus® Enzyme Kit-AflII on the Argus system. Downstream analysis, clustering and genome alignment was carried out by MapSolver version 3.2.4. (OpGen Inc).

The Roche 454 pyrosequencing reads were de novo assembled into sequence contigs by using GS Assembler software (Newbler) version 2.5.3 followed by assembling the contigs to scaffolds with WGRM as the physical reference. PCR amplification and open reading frame annotation were used to finish genome assembling and address pyrosequencing errors in nucleotide homopolymer regions. After virtual digestion of complete genome sequence using AflII, the in silico whole genome restriction map was aligned to WGRM to ensure correct order and orientation of the final assemblies. The genome sequencing method with integrated Roche 454 pyrosequencing and WGM was

published in detail by Onmus-Leone, et al (188). The finished genome sequence of *Ca.* B. ancashi strain 20.00 has been submitted to NCBI and was given GenBank accession number CP010401.

Genome-wide phylogeny

For genome-wide phylogenetic analysis of *Bartonella* species, complete genome sequences or assembly contigs for whole genome sequences were aligned using Mauve version 2.3.1 (59) to identify single nucleotide changes in conserved genomic regions. The phylogenetic tree was constructed using the R *phangorn* package (219). The initial tree was constructed using the neighbor joining algorithm and was optimized using the parsimony maximum likelihood method. Tree stability was evaluated using 100 bootstrap replications. An in-house developed pipeline in Perl was used to run the process.

Genome annotation and comparative genome analysis

Complete genome sequences were annotated for GenBank submission and comparative analysis. The RAST (Rapid Annotation using Subsystem Technology) (http://www.nmpdr.org/FIG/wiki/view.cgi/FIG/RapidAnnotationServer) and the IGS Annotation Engine was used for structural and functional annotation of the sequences (http://ae.igs.umaryland.edu/cgi/index.cgi) (12; 87). The Genome Viewer (http://www.nmpdr.org/FIG/wiki/view.cgi/FIG/GenomeViewer) and Manatee (http://manatee.sourceforge.net/) were used to view annotations. Annotated genes were loaded on Virulence Factors Database (VFDB, www.mgc.ac.cn/VFs/) to identify genes

responsible for pathogenicity. A pairwise genome-wide comparative analysis was performed by comparing homologous proteins from pairs of *Bartonella* species. The results were presented as density distribution curves for amino acid identity as well as dot plots for pairwise amino acid identity for each homologous protein. Genes of interest were subjected to additional analysis including gene cluster comparisons.

RESULTS

Novel Bartonella isolates from two Peruvian patients

B. bacilliformis is endemic in the Ancash Region of Peru (134; 212). In a clinical trial testing the efficacy of rifampin and azithromycin for treatment of chronic *B. bacilliformis* infection (verruga peruana), *Bartonella* spp. were cultured from the blood specimens of 72 out of 127 patients. Using single-locus sequence typing, we found three isolates with a *gltA* gene sequence that diverged from that of *B. bacilliformis* reference strain KC583 (25; 134).

Sequencing the *gltA* PCR product yielded unexpected results. The novel species *Ca.* B. ancashi was cultured from patient #20 at days 0 and 60 (isolates 20.00 and 20.60). However, *B. bacilliformis* was isolated from patient #41 on days 0 and 30 (41.00 and 41.30), whereas *Ca.* B. ancashi was cultured from the patient on day 60 (41.60) (Table 6).

The *Bartonella* bacteremia levels in two patients were drastically different, although the clinical symptoms for both patients were indistinguishable from one another and from other confirmed cases of chronic *B. bacilliformis* infection (Table 6).

DNA extracts from the four original whole blood specimens, 20.00, 20.60, 41.00 and 41.60, were tested using quantitative bacterial 16S rDNA PCR, standard bacterial 16S PCR, and *Ca.* B. ancashi specific PCR assays (Table 6). Whole blood from patient #20 was PCR-negative for bacteria on both days 0 and 60, whereas very high levels of bacteremia were seen for whole blood specimens from both days 0 and 60 for patient #41. The results confirmed that *Ca.* B. ancashi was correctly isolated from whole blood

Table 6. Non-*bacilliformis Bartonella* Isolates in the study.

Isolate number	**20.00**	**20.60**	**41.00**	**41.60**
Patient	Patient 20, 3 year old male		Patient 41, 10 year old male	
Symptom	Lesions found on hands and feet, disappeared after antibiotics treatment			
Antibiotics	Azithromycin at day 0 and day 7		Rifampin daily of day 0 – 14	
Whole blood collection time	Day 0	Day 60	Day 0	Day 60
Peripheral blood smear	-	-	-	-
Blood culture for *Bartonella*	+	+	+	+
16S 321/533 TaqMan qPCR [a]	-	-	3.93×10^5 (19.24)	6.36×10^4 (22.15)
16S 27F2/533R PCR	-	-	+	+
Ca. B. ancashi specific PCR [b]	-	-	-	+
Blood culture gltA PCR / sequencing	*Ca.* B. ancashi	*Ca.* B. ancashi	B. *bacilliformis*	*Ca.* B. ancashi
Isolate pure culture sequencing	*rrs, gltA, rpoB*; whole genome	*rrs, gltA, rpoB*; whole genome	ND	*rrs, gltA, rpoB*; whole genome

[a] The data are 16S rRNA gene copy number per µl (qPCR Ct number).

[b] Primers 22RC-3F (TTCGGCTTAGCTTATCCGTTTCACAA) and 32RC-5R (CGTAAGAGCTTTGTGGCAAAATAGCAA) were used; the expected PCR amplicons size is 0.8 kb, corresponding to nucleotide 673839 to 674636 of *Ca.* B. ancashi (GenBank CP010401).

specimens belonging to patient #41 on day 60 but not on day 0. The evidence suggests the emergence of a new *Bartonella* species in Peru, which can cause its own verruga peruana-like infection in humans or possibly co-infect humans in conjunction with *B. bacilliformis*. It is particularly intriguing that the bacteremia profile, based on blood cultures, for patient #41 switched from exclusively *B. bacilliformis* at days 0 and 30 to exclusively *Ca.* B. ancashi at day 60 (Table 6, Figure 11).

Whole genome sequence analysis

The three *Ca.* B. ancashi isolates underwent genome-wide analyses with whole genome mapping (WGM) and Roche 454 next-generation sequencing (188; 206). Whole genome restriction maps (WGRMs) for isolates 20.00, 20.60, and 41.60 were compared to each other and the *in silico* map available for the *B. bacilliformis* reference genome KC583 to assess genome structure and similarity (Figure 12). The optical maps of the three strains showed greater than 99.7% similarity to each other and less than 10% similarity to the WGRM of *B. bacilliformis* KC583. WGM showed the genome of *Ca.* B. ancashi is a circular molecule of approximately 1.46 Mb. Interestingly, a region of about 0.64 Mb in the 20.00 genome was inverted as compared with the maps of 20.60 and 41.60 (Figure 12).

Analysis of draft genome sequences of the three isolates showed that they are cultures of same *Ca.* B. ancashi strain, with their only difference being the genome inversion in 20.00. The complete genome sequence for isolate 20.00 was finished using its WGRM as a scaffold (Figure 12). *Ca.* B. ancashi strain 20.00 has a circular genome

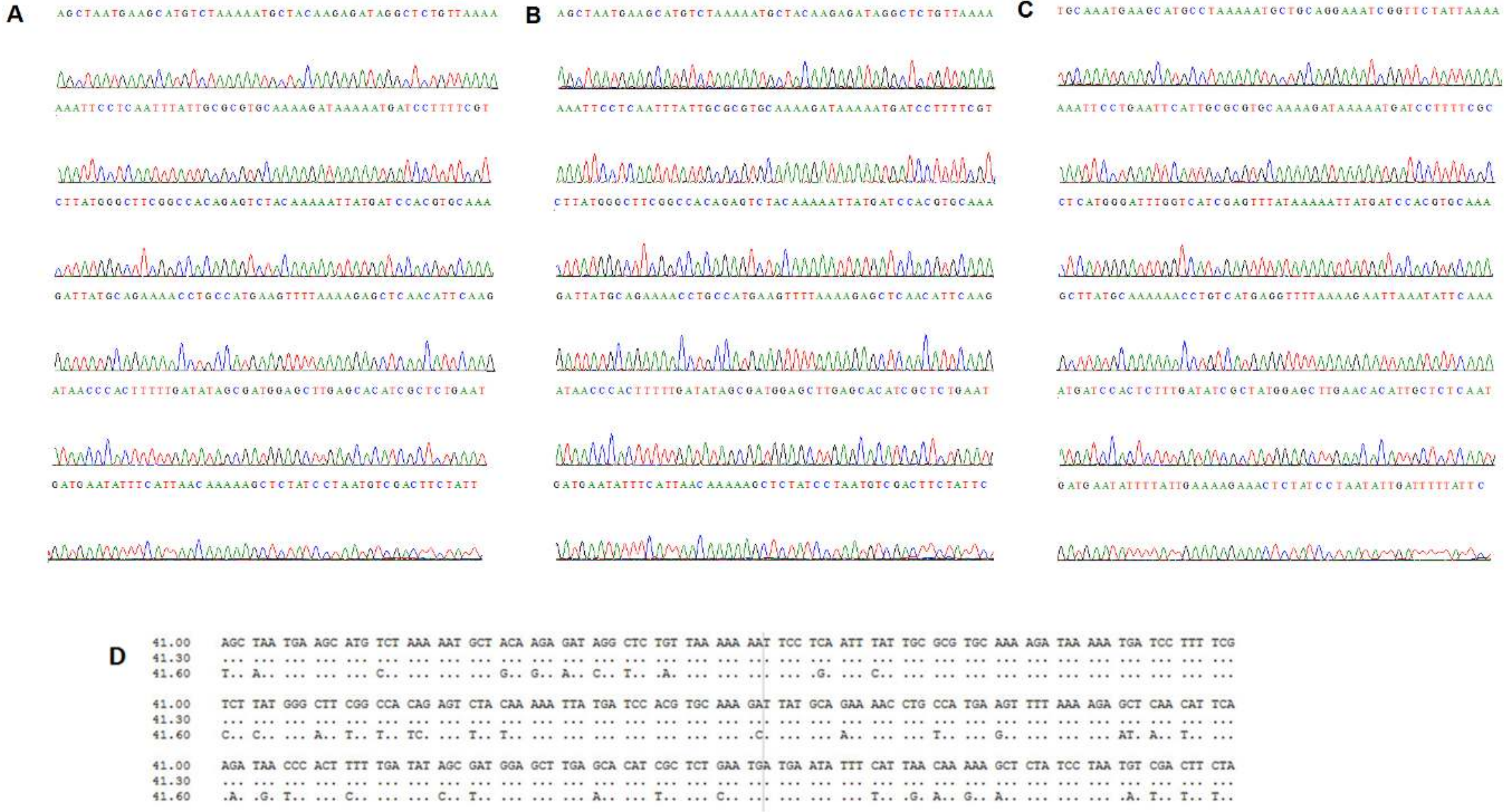
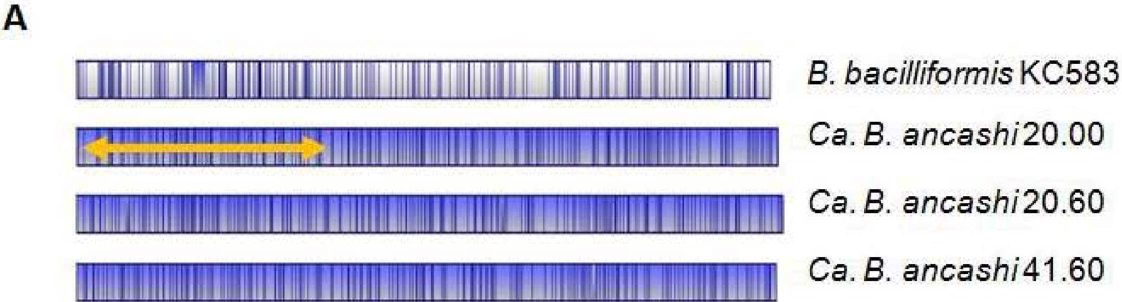


Figure 11. Sanger sequencing results for isolates 41.00, 41.30 and 41.60. Chromatographs for a 300 base pair region of the *gltA* gene for **A)** 41.00, **B)** 41.30 and **C)** 41.60. **D)** Alignment of 300 base pair region of the *gltA* gene for *Ca.* B. ancashi isolates 41.00, 41.30 and 41.60.

A



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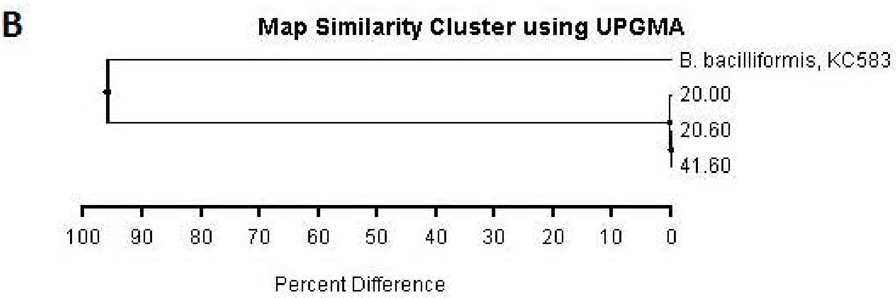

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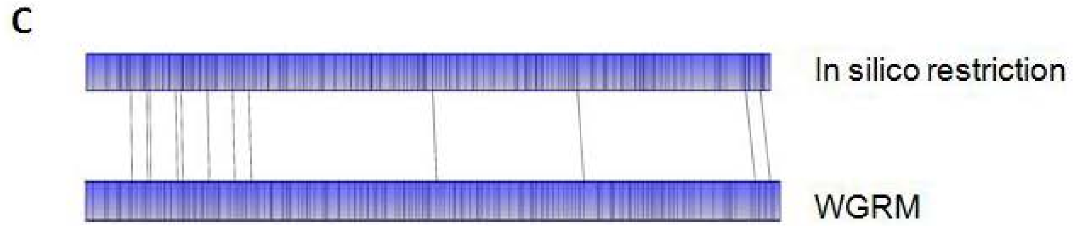


Figure 12. Genomic comparisons of *Ca.* B. ancashi isolates to each other and to *B. bacilliformis*. **(A)** Alignment of whole genome restriction maps (WGRM) and *in silico* WGRM for B. *bacilliformis*. WGRMs for *Ca.* B. ancashi isolates 20.00, 20.60 and 41.60 were generated using WGM. Blue shaded areas indicate regions of alignment, unshaded areas indicate regions where restriction maps do not align, and black horizontal lines mark restriction sites. **(B)** Phylogeny based on WGRM similarity by using UPGMA. **(C)** Alignment of the whole genome restriction map of *Ca.* B. ancashi predicted *in silico* from the complete genome sequence to the experimentally observed *Ca*. B. ancashi WGRM.

1,466,048 bp in length, with a G + C content of 38.4%, which is similar to the *B. bacilliformis* KC583 genome (NC_008783.1) – 1,445,021 bpin size with a G + C content of 38.2%. The inverted region of the 20.00 genome, 0.64 Mb in length, is flanked by two 507-bp repetitive sequences, one at each end in a reversed position. The repetitive sequence has 97% sequence identity to the gene for *B. bacilliformis* integrase (protein family HMM PF00589), a DNA breaking-rejoining enzyme that catalyzes DNA recombination.

Whole-genome phylogeny and proteomic comparisons

We used whole-genome phylogenetic analyses to examine the evolutionary relatedness between *Ca.* B. ancashi and 38 *Bartonella* strains with publicly available complete or draft genome sequences (Table 7) (Figure 13). This analysis, based on 12,740 SNPs in DNA sequences conserved across all species, showed that *Ca.* B. ancashi is most closely related to *B. bacilliformis*, *B. bovis* and *B. melophagi*.

The genome of *Ca.* B. ancashi strain 20.00 has 1346 putative protein encoding genes. The degree of similarity between *Ca.* B. ancashi and 15 other *Bartonella* species was further examined by assessing the pairwise similarity of homologous proteins using the BLAST score ratio method (147) (Figure 14). This analysis indicated that *Ca.* B. ancashi predicted proteins are most similar to *B. bacilliformis* proteins. Furthermore 1) 63.7% of *Ca.* B. ancashi genes are part of a core genome common to all 15 comparator species; 2) 10.7% of *Ca*. B. ancashi proteins did not have a homolog in any of the

Table 7. Whole genome sequences of *Bartonella* spp used in the analysis.

Species	Strain	Accession
Bartonella alsatica	IBS 382	AIME01000000
***Ca.* Bartonella ancashi**	20.00	n/a
Bartonella australis	Aust/NH1	NC_020300.1
Bartonella bacilliformis	INS	AMQK01000000
Bartonella bacilliformis	KC583	NC_008783.1
Bartonella birtlesii	IBS 325	AKIP01000000
Bartonella birtlesii	LL-WM9	AIMC01000000
Bartonella bovis	91-4	AGWA01000000
Bartonella bovis	m02	AGWB01000000
Bartonella clarridgeiae	73	NC_014932.1
Bartonella doshiae	NCTC 12862	AILV01000000
Bartonella doshiae	ATCC 700133	JAGY01000000
Bartonella elizabethae	F9251	AIMF01000000
Bartonella elizabethae	Re6043vi	AILW01000000
Bartonella grahamii	as4aup	CP001562.1
Bartonella henselae	Houston-1	BX897699.1
Bartonella henselae	JK 53	AHPI01000000
Bartonella henselae	Zeus	AHPJ01000000
Bartonella koehlerae	C29	AHPL01000000
Bartonella melophagi	K-2C	AIMA01000000
Bartonella queenslandensis	AUST/NH15	CALX01000000
Bartonella quintana	JK 31	AHPG01000000
Bartonella quintana	JK 63	AHPF01000000
Bartonella quintana	JK 67	AHPC01000000
Bartonella quintana	JK 68	AHPD01000000
Bartonella quintana	RM-11	CP003784.1
Bartonella rattaustraliani	AUST/NH4	CALW02000000
Bartonella rattimassiliensis	15908	AILY01000000
Bartonella rochalimae	ATCC BAA-1498	FN645455.1 - FN645467.1
Bartonella rochalimae	BMGH	AHPK01000000
Bartonella sp. DB5-6	DB5-6	AILT01000000
Bartonella sp. OS02	OS02	CALV01000000
Bartonella tamiae	Th239	AIMB01000000
Bartonella tamiae	Th307	AIMG01000000
Bartonella taylorii	8TBB	AIMD01000000
Bartonella tribocorum	CIP 105476	AM260525.1
Bartonella vinsonii	OK-94-513	AILZ01000000
Bartonella vinsonii	Pm136co	AIMH01000000

Bartonella vinsonii	Winnie	NC_020301.1
Bartonella washoensis	085-0475	AILX01000000
Bartonella washoensis	Sb944nv	AILU01000000

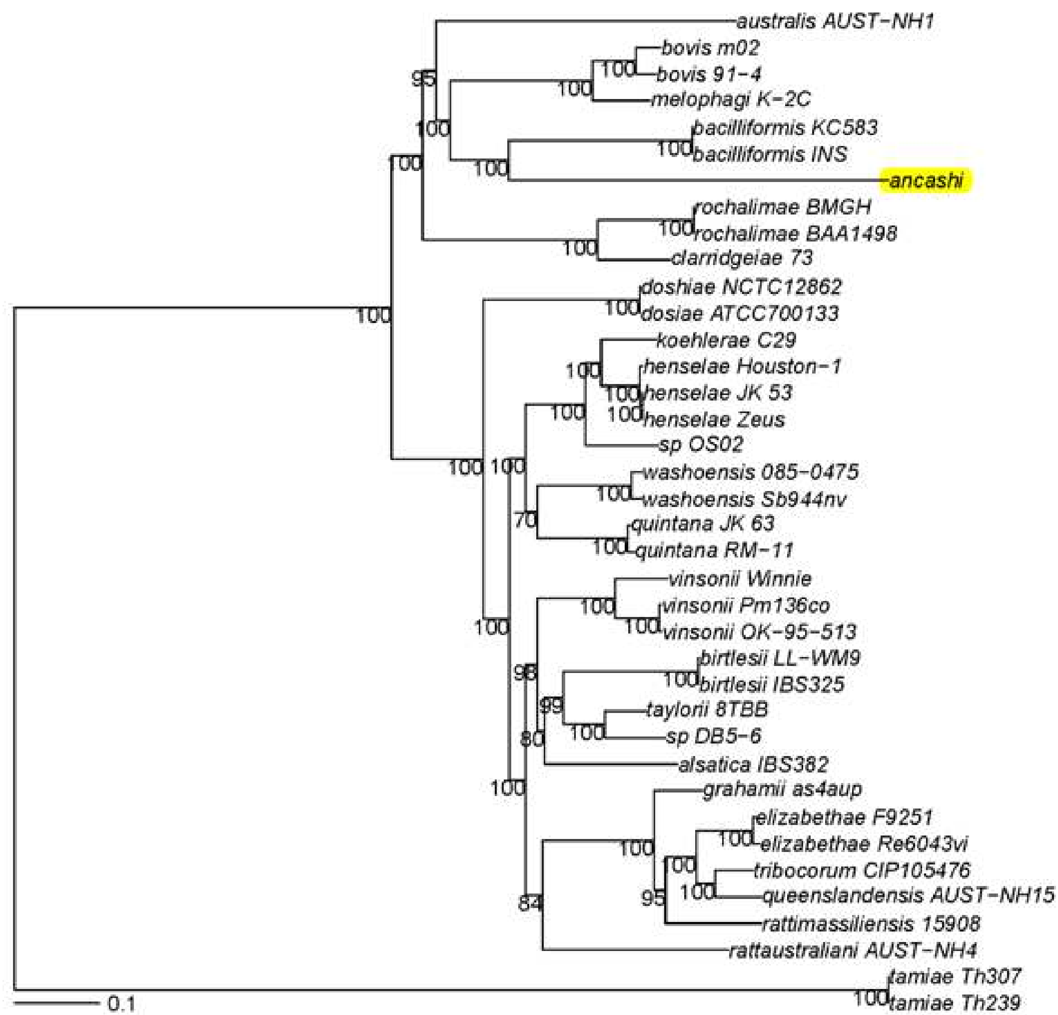


Figure 13. Phylogenetic relationship of *Ca.* B. ancashi with other *Bartonella* species based on whole genome phylogeny. The phylogenetic tree is based on SNPs identified in genomic regions common to all of the *Bartonella* strains examined.

reference *Bartonella* spp.; 3) 89.3% of *Ca.* B. ancashi proteins are present in at least one other *Bartonella* spp., with 79.1% of *Ca.* B. ancashi proteins being found in *B. bacilliformis* (Figure 14).

All the data taken together indicates *Ca.* B. ancashi is a unique *Bartonella* species that co-circulates with *B. bacilliformis* in an area of endemic Bartonellosis. While *Ca.* B. ancashi and *B. bacilliformis* are phylogenetically related and share genomic similarities, they show intriguing genomic variations.

Conserved and variable genes of *Ca.* B. ancashi

As mentioned above, the majority of the *Ca.* B. ancashi genes identified in the pan-genome analyses had homologs in one or more *Bartonella* species (Figure 14). It is notable that there were 8 proteins shared by *Ca.* B. ancashi and *B. bacilliformis*, and not present in any other *Bartonella* species, while an additional 5 proteins were shared between *Ca.* B. ancashi, *B. bacilliformis*, and *B. australis* and no other known Bartonella species. In contrast, there were also 8 proteins shared by *Ca.* B. ancashi and all 14 other *Bartonella* species, excluding B. *bacilliformis*, but including *B. australis,* which is considered to be the most divergent *Bartonella* species currently recognized, while another 129 *Ca.* B. ancashi proteins have homologs in only a subset of the non-*bacilliformis Bartonella* (86; 98).

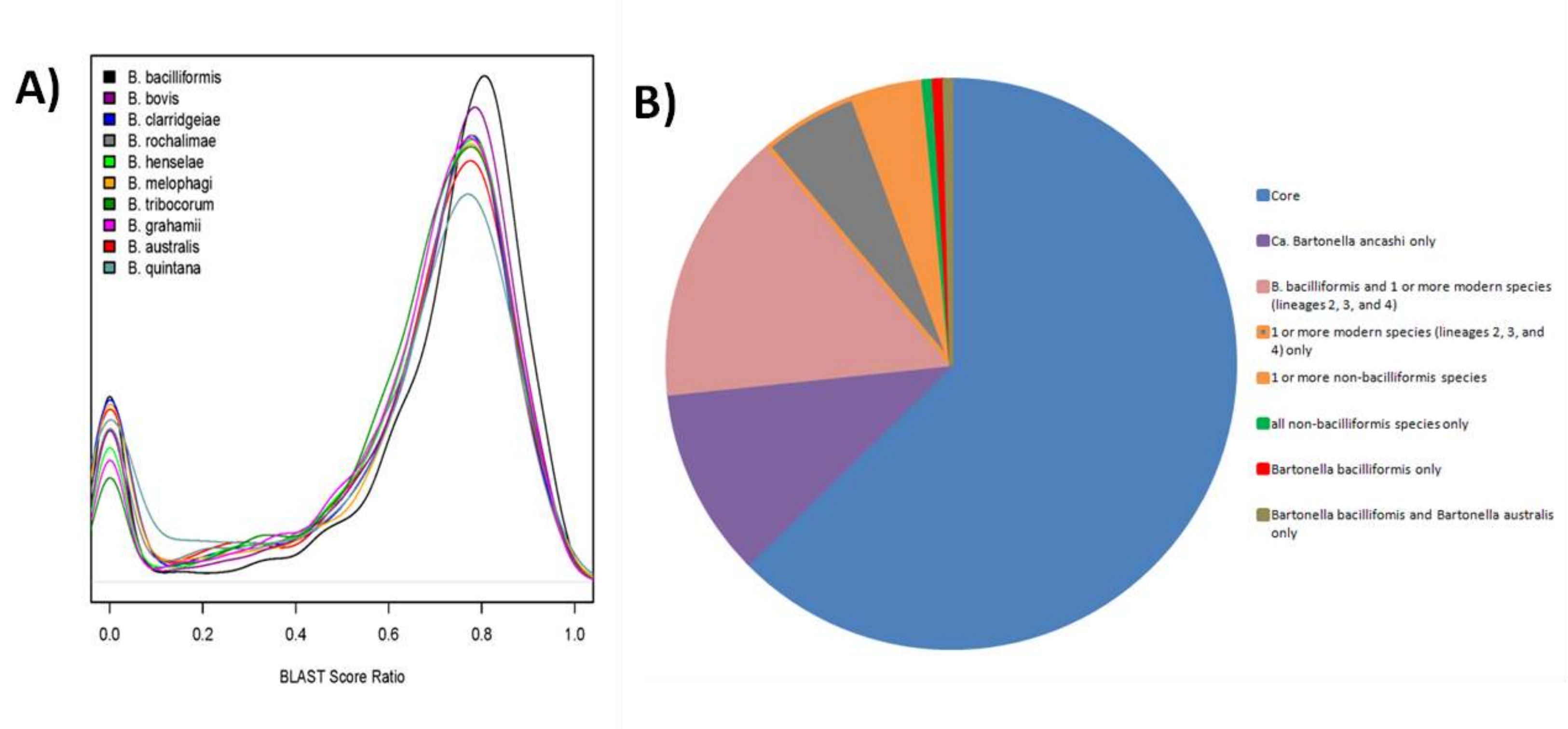


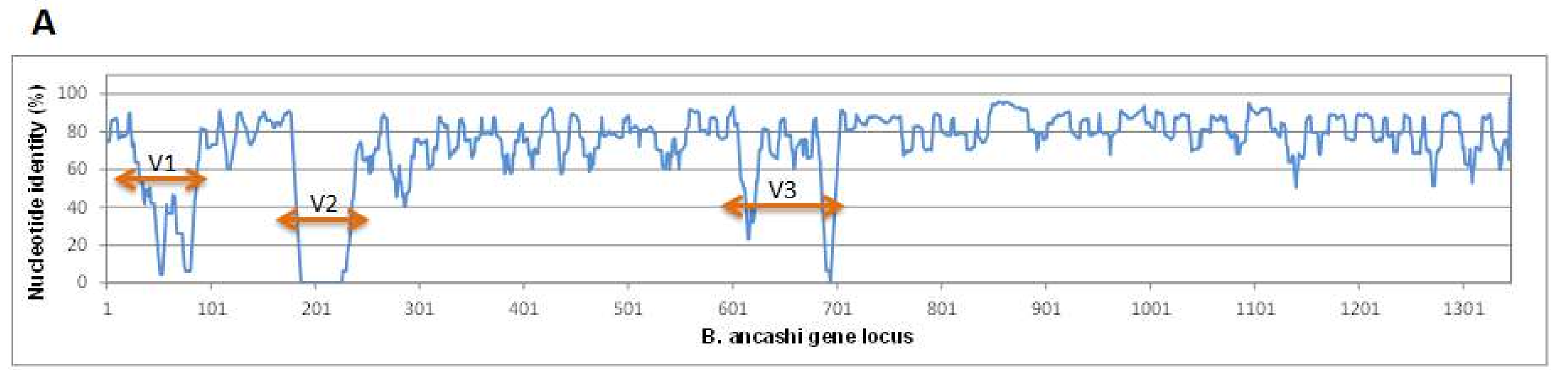
Figure 14. Proteomic analysis among *Ca.* B. ancashi and related species. **A)** Density plot representing the similarity between *Ca.* B. ancashi protein coding genes and genes from 10 of the more closely related Bartonella species. Similarity scores are based on the BLAST Score Ratio (BSR) method (147). A score of 1.0 indicates identity between two proteins, while a score below 0.3 indicates that the two proteins do not show meaningful similarity. The *Bartonella* species whose protein similarity score distribution has a peak closest to 1 (*B. bacilliformis*) has the highest overall protein similarity to *Ca.* B. ancashi. **B)** Presence of homologous protein coding genes in *Ca.* B. ancashi and 15 *Bartonella* species. Proteins from two species with a BSR score ≥ 0.3 were considered to be homologs and considered present in *Ca.* B. ancashi and 1 or more *Bartonella* species.

In three regions of the *Ca.* B. ancashi genome the similarity between *B. bacilliformis* and *Ca.* B. ancashi proteins were lower than average (Figure 15). Proteins in variable region 1 (genes 30 - 90) are homologous to non-*bacilliformis* species proteins, including phage proteins such as Hig A, and Hig B. Proteins encoded in variable region 2 (loci 180 - 240) are entirely absent from *B. bacilliformis.* Among these are proteins with high similarity to *Bartonella* Type IV secretion system proteins, hypothetical gene products with moderate identity to proteins from other non-*bacilliformis* species, and novel hypothetical proteins. Variable region 3 (genes 620-704) contains loci encoding hypothetical proteins not seen in other *Bartonella* species and several toxin proteins that are not found in *B. bacilliformis*, including the RelE/StbE replicon stabilization toxin, the RelB/StbD replicon stabilization protein and the HigB toxin protein.

From this analysis, two characteristic features of *Ca.* B. ancashi were identified - possession of type IV secretion complex (VirB2) proteins, which are not in *B. bacilliformis,* and the presence of flagella proteins, which are not seen in *Bartonella* species belonging to lineage 4, which includes the human pathogens *B. quintana* and *B. henselae* (61; 74; 78)

Genomic inversion interrupted flagella gene cluster

In isolates 20.60 and 41.60, the 31 flagellar genes encoded by *Ca.* B. ancashi are located in the identical order and distances as their homologs in the *B. bacilliformis* genome. Relative to 20.60 and 41.60, isolate 20.00 has a large genomic inversion. As a result of this rearrangement, one gene required for production of flagella, *FliJ*, lies in a



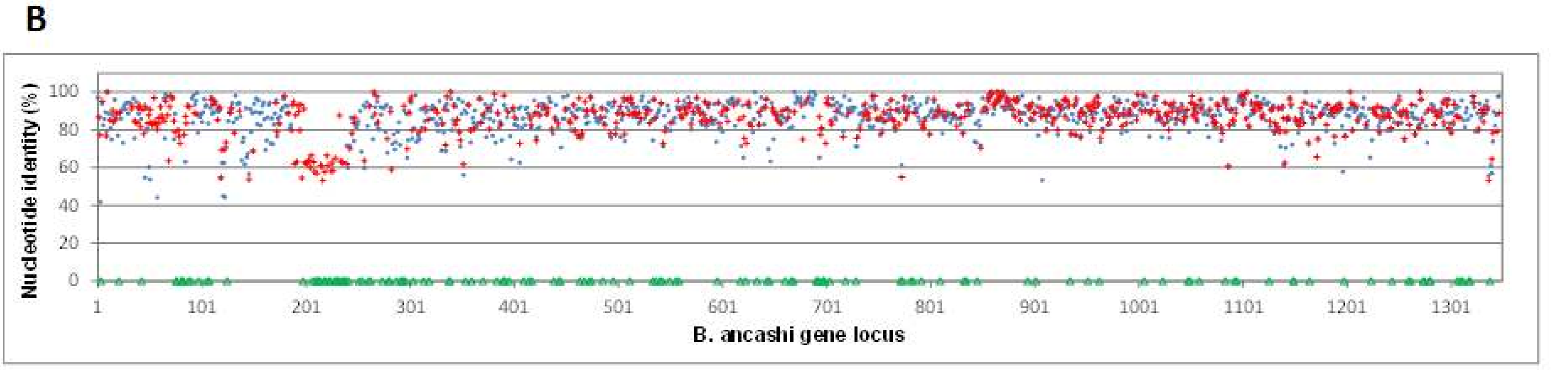


Figure 15. Pairwise comparisons of protein-coding genes. **A)** Nucleotide similarity plot. Average nucleotide identity within a window of 10 genes was plotted against gene locus number. Region I, II and III are 3 variable regions that contain genes with lower nucleotide identities or absent in the *B. bacilliformis* genome.**B))** Nucleotide similarity of *Ca.* B. ancashi protein coding sequences in comparison with *B. bacilliformis* (shown as *blue dot* ●), top hit non-*bacilliformis Bartonella* (shown as *red cross* +) and not-determined hypothetic proteins (shown as *green empty triangle* Δ).

new position relative to the remaining 30 flagellar genes. In isolate 20.00, *FliJ* is ~600 kb away from the main flagellar gene cluster, as opposed to being ~100 kb distant in the other two *Ca.* B. ancashi strains; the orientation of *FliJ* relative to the main flagellar gene cluster is also reversed in isolate 20.00 (Figure 16).

Electron microscope examination showed that isolate 20.00 cells lacked flagella (Figure 17) whereas 20.60 and 41.60 cells were variable in their expression of flagella. This variable expression of flagella seems to correlate with the genomic inversion. Both 20.60 and 41.60 lack the inversion and produced flagella, while 20.00 has an inversion and did not appear to be flagellated when viewed using electron microscopy.

Putative virulence modulating proteins

A *Leptospira* virulence attenuation study identified a group of paralogous virulence modulated (VM) genes (145). Subsequent comparative genomic analysis showed that VM proteins are present in other bacterial pathogens, including *B. bacilliformis* and *B. australis*. Interestingly, *Ca.* B. ancashi encodes five VM proteins (Figure 18). By contrast, no homologs of VM proteins were found by BLAST search in any other recognized *Bartonella* species. As was seen for the VM proteins of *Liptospira* species, the VM protein genes in these three *Bartonella* species were scattered throughout these genomes and the number of VM proteins was different for each species.

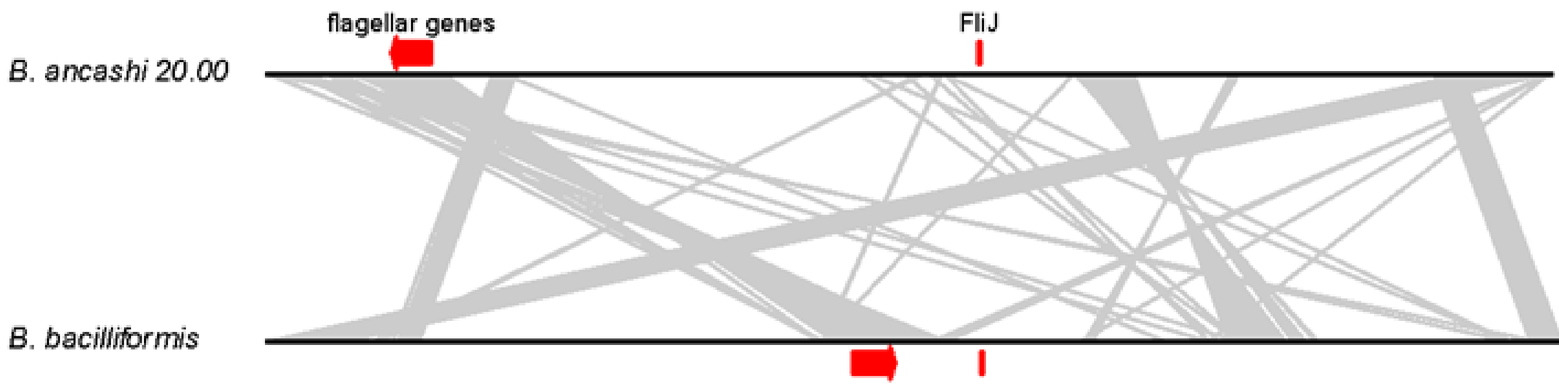


Figure 16. Genetic arrangement of the *Ca.* B. ancashi 20.00 genome compared to that of *B. bacilliformis* KC583. *Black lines* indicate the chromosomes. *Gray lines* link syntenic genomic regions that are rearranged between the two genomes. Locations of the FliJ gene and the flagellar gene cluster are indicated in *red*.

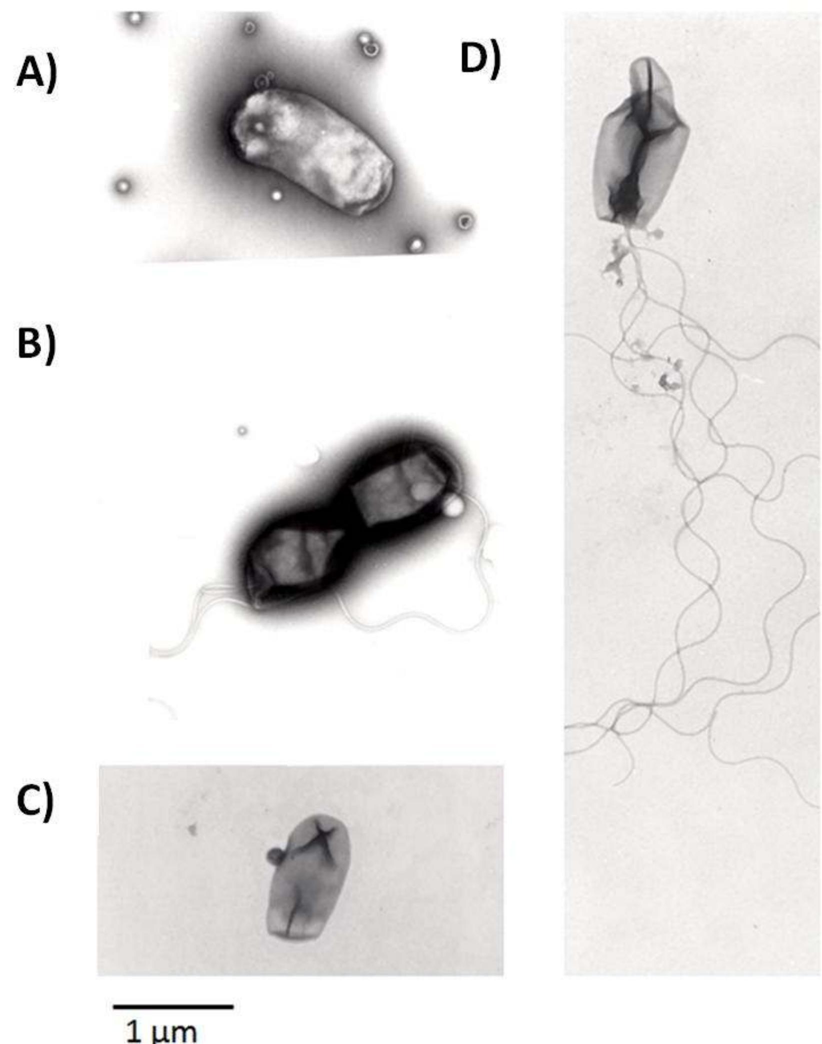


Figure 17. Electron microscope images for cells of *Ca.* B. ancashi isolates. **A)** Isolate 20.00. **B-C)** Isolate 20.60. **D)** Isolate 41.60. These images indicate variable expression of flagella between isolates of *Ca.* B. ancashi obtained from this study. Bar indicates 1 μm.

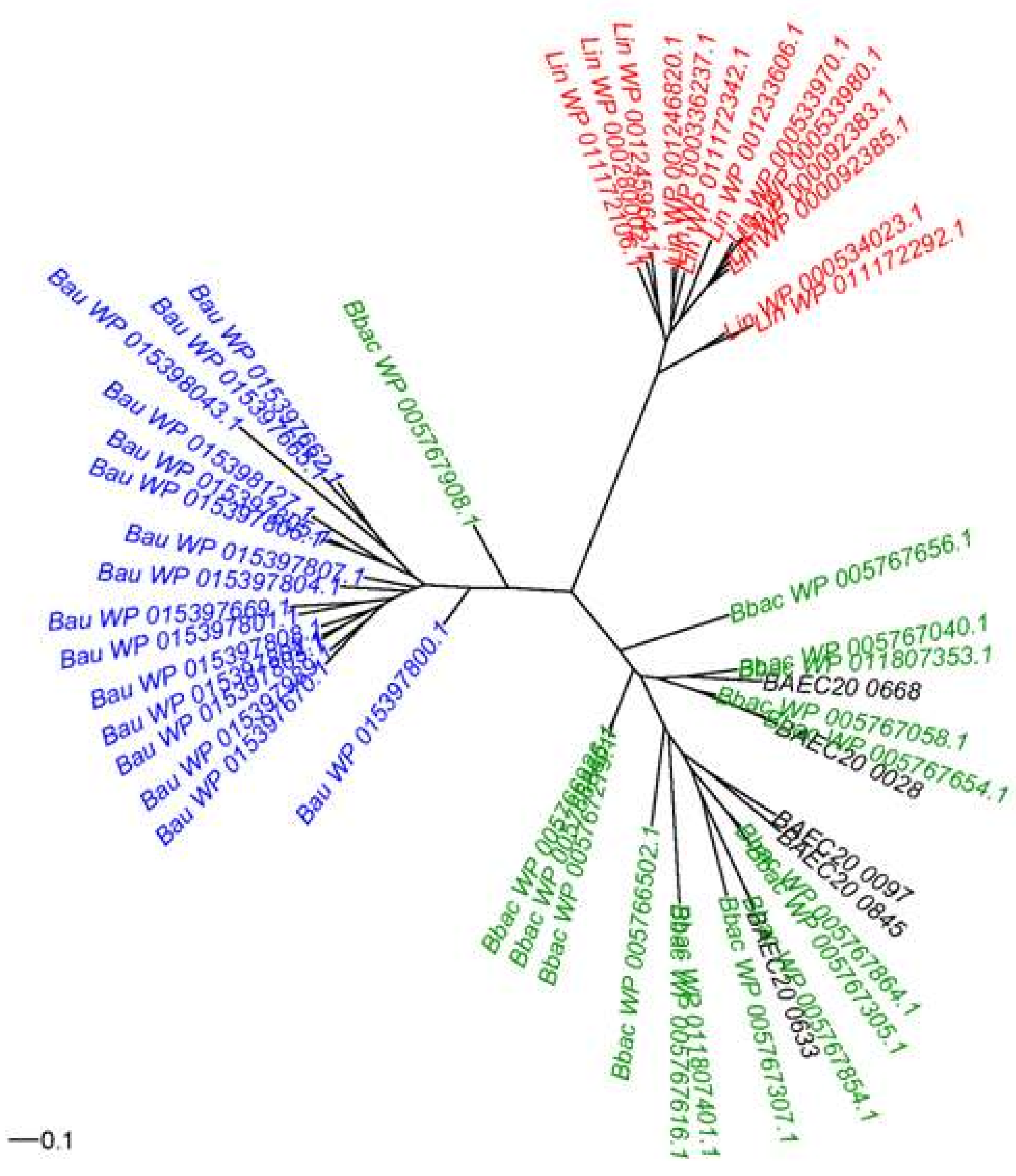


Figure 18. Virulence modulating (VM) proteins in *Leptospira* and *Bartonella*. Unrooted phylogenetic tree of VM proteins from *Leptospira interrogans* (*red*), *B. australis* (*blue*), *B. bacilliformis* (*green*), and *Ca.* B. ancashi (*black*). VM proteins from *L. interrogans*, *B. bacilliformis*, and *B. australis*, cluster by species; the five VM proteins encoded by *Ca.* B. ancashi group with their *B. bacilliformis* homologs.

DISCUSSION

B. bacilliformis was discovered in Peru in 1909 as the causative agent for Oroya fever and verruga peruana. Since then *B. bacilliformis* has been the primary subject in the Bartonelloses studies in South America. However, it has long been speculated that there might be multiple *Bartonella* spp. circulating in the region. Our previous work identified a novel *Bartonella* species, *Candidatus* (*Ca.)* B. ancashi in three blood samples from two unrelated child patients in the Ancash region of Peru, which has a high prevalence of *B. bacilliformis* (47; 48; 212).

While *Ca.* B. ancashi is most closely related to *B. bacilliformis*, it has several novel features at the genome level. Like *B. bacilliformis*, this species encodes flagellar genes. The flagellum is believed to be essential for erythrocyte invasion by *B. bacilliformis*, its main route of pathogenesis; human pathogens such as *B. quintana* and *B. henselae*, in contrast, do not express flagella and pathogenicity is thought to be linked to the type IV secretion system (T4SS) gene cluster (19; 61; 63; 74; 218). "Modern" (lineage 4) *Bartonella* species lack flagellar genes and possess either the VirB or Trw T4SS. *Ca.* B. ancashi, like *B. clarridgeiae* and *B. rochalimae*, possesses both flagellar and T4SS gene clusters (61; 63; 78; 98). *Ca.* B. ancashi encodes the VirB type IV secretion system.

Genomic analysis and microscopic examinations of the three *Ca.* B. ancashi isolates revealed that 20.00 differs from the other two isolates by a large genomic region inversion and the absence of flagella. Future analysis of *Ca.* B. ancashi gene expression profiles will allow us to test the hypothesis that the genomic inversion among the *Ca.* B. ancashi isolates alters the regulation of genes required for the production of flagella and whether the genome rearrangement serves as a switch to modulate flagellar production.

Moreover, it is also intriguing to explore whether the genomic inversion (20.00 versus 20.60) is related to the administration of antibiotics, as both isolates 20.60 and 41.60 were recovered from patients after antibiotic treatment.

A striking feature of *Ca.* B. ancashi is that it encodes VM genes – a family of homologous virulence related genes originally identified in *Leptospira interrogans*. This gene family is present in *B. bacilliformis* and *B. australis* (145). VM proteins in *Ca*. B. ancashi are most similar to homologous genes in *B. bacilliformis*. Further studies will be needed to elucidate the precise role of these genes in *Bartonella* spp. Of the bacterial species that encode VM proteins, *Ca.* B. ancashi has the fewest (five). The in-depth comparative analysis and functional studies on the large number of hypothetical proteins in *Bartonella* spp. will shed light on pathogenesis of Bartonellosis, which are sophisticated and largely unknown.

Intensified tropical disease surveillance and advances in scientific methodology led to an increasing number of new *Bartonella* species to be identified in recent years (46; 79; 96; 116). This work has revealed the extensive phylogenetic diversity of the genus *Bartonella* and suggests the necessity of systematic and more accurate taxonomic classification and species nomenclature rules (142). Microbial genome sequences obtained through next-generation sequencing technologies will greatly facilitate these efforts. The whole genome phylogenetic analysis described in this study included all *Bartonella* spp. with available complete or draft genome sequences. This work and other genomic studies demonstrate, *B. bacilliformis* - which was historically regarded as the ancestral *Bartonella* spp. - probably diverged from other species in the distant past and evolved as a species uniquely adapted to the human host (61; 78; 98; 177). Additionally,

Ca. B. ancashi represents a novel species most closely related to *B. bacilliformis*. Despite the apparent phylogenetic proximity, *Ca.* B. ancashi has a nucleotide divergence of ~20% with *B. bacilliformis* within conserved genomic regions, which is exceedingly high and comparable to the distances among the nominated *Bartonella* lineages (78; 98; 142). Therefore it is rational to designate *Ca.* B. ancashi as an independent lineage in parallel to the *B. bacilliformis* lineage. Our study provided strong evidence that there may be many more *Bartonella* species and subspecies in regions of South America that have yet to be discovered.

Bartonellosis has affected human health for hundreds to thousands years and remains persistent in several areas and continues to cause outbreaks in other regions. A systematic review indicated the severe deficiency on a wide range of aspects for Bartonellosis research and countermeasures. The discovery of a novel *Bartonella* sp. in this study not only provided long-awaited evidence of species diversity in *B. bacilliformis* endemic areas, but also indicates the importance of the acquisition of sufficient genomic data, which will allow for pathogenomics studies. Studies like these will allow for a comprehensive understanding and effective control of the diseases like Bartonellosis.

Chapter 5:

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

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ABSTRACT

Three *Bartonella* isolates 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 *Bartonella* species, including its closest relative *B. bacilliformis*. Other characteristics of the isolates, including morphology, microscopic, biochemical, and growth patterns were consistent with those of members of the *Bartonella* genus. Based on these results, we conclude that these 3 isolates are members of a novel species of *Bartonella* for which we propose the name *Bartonella ancashi* sp. nov. (type strain 20.00^{T} =ATCC BAA-2694 =DSM 29364)

INTRODUCTION

The genus *Bartonella* contains more than 30 vector-borne, fastidious, small, Gram-negative bacilli (23; 34; 122). In 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 (99; 106; 122; 207). 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 (1; 54; 134). 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 Colombia (2,500-8,000 feet above sea level), where it is endemic and likely transmitted by the new world sandfly (*Lutzomyia* species) (1; 54; 134). Although *Bartonella rochalimae* has been reported to have caused an Oroya fever-like illness in an American traveler returning from Peru (79), *B. bacilliformis* is currently the only agent definitively identified as causing Carrión's disease.

We recently described the isolation of a novel *Bartonella* agent, designated as *Bartonella ancashi*, during an antibiotic treatment trial testing the efficacy of azithromycin as compared to standard care (rifampin) for the treatment of verruga peruana caused by *B. bacilliformis* in Caraz, Ancash, Peru (25). In total, three isolates were obtained from the blood of two patients with verruga peruana enrolled in the trial. Two of the isolates came from a three-year-old male: the first was collected at the time of enrollment (20.00^{T}), and the second at 60 days post enrollment (20.60). The third isolate was collected from a ten-year-old male at 60 days post enrollment (41.60).

We also reported gene sequencing results, multi locus sequence typing (MLST), and multi spacer sequence typing (MST) results for *B. ancashi* samples 20.00 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 rDNA: 1474 bp) were used for MLST, while the 16S-23S intergenic spacer (ITS) (940 bp) was used for MST (Mullins *et al*., 2013). Sequencing analysis showed isolates 20.00^T and 20.60 to be identical genetically to one another at these loci, yet distinct from all other isolates listed in GenBank. Additionally, the sequencing, MLST and MST results showed that the isolates fall within the sequence similarity range for known *Bartonella* species, and most closely related to *B. bacilliformis*. Nevertheless, the multi-gene sequence divergence and phylogenetic distance analysis clearly indicated that they represent a novel *Bartonella* species (181).

In the present study, we further characterize this novel species *Ca. B. ancashi* by describing additional genotypic, phenotypic, morphological, microscopic, biochemical, and growth characteristics of the three isolates 20.00^T, 20.60, and 41.60.

METHODS

To assess culture characteristics of all three isolates they were cultivated on different solid media, at different temperatures and in the presence or absence of increased CO_2. The isolates were grown in plates containing either trypticase soy agar (TSA) containing 5% defibrinated sheep blood, chocolate agar, Columbia agar containing 5% defibrinated sheep blood, or deep-fill heart-brain infusion agar (BHIA) containing 10% defibrinated sheep blood, (BD diagnostics, Sparks, MD). 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, isolates were tested for their ability to grow in liquid culture (MS10 media) and in Vero and L929 cells cultured in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum at 30°C with 5% CO_2 (Lynch *et al*., 2011).

The agents were subjected to Gram staining (BD diagnostics, Sparks, MD) and visualized at 1000X by light microscopy (Olympus, Hicksville, NY). Cell morphology was visualized by electron microscopy using a JEOL 100 CXII electron microscope (JEOL, Peabody, MA) with negative staining. Gram staining and transmission electron microscopy, 10 and 28 day cultures passaged one to two times from frozen stocks on BHIA with10% defibrinated sheep blood at 30°C in a moist atmosphere with 5% CO_2.

Biochemical analysis was carried out using the Rapid ANA II system (Remel, Lenexa, KS), according to the manufacturer's instructions (53; 62; 213). Additionally, Oxidase Reagent Droppers (BD, Sparks, MD) were used to evaluate oxidase activity, while 3% hydrogen peroxide (Acros, Atlanta, GA) was used to evaluate the catalase activity of the three isolates. The biochemical analyses were performed on 10 day old colonies grown on BHIA containing 10% defibrinated sheep blood.

Antibiotic susceptibility was assessed using the Equine species MIC plate (EQUINF1, Trek Diagnostic Systems, Oakwood Village, OH). 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 media, 50 µl of the suspension was transferred to 11 ml of MS10 media, vortexed, and 100 µl of the solution was added to each well and incubated at 30°C with 5% CO_2 for up to 18 days.

For additional genotypic characterization, all three isolates 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, Branford, CT). G+C content for the isolates was determined. Complete sequences of five housekeeping genes, *gltA, rpoB, ftsZ, groEL,* and *ribC*, and *rrs* and the 16S-23S ITS, were extracted from the genome sequence of 41.60 for comparison to the respective genes of 20.00^{T} and 20.60 (Table 1).

Table 8. Accession numbers for *gltA, rpoB, ftsZ, groEL, ribC, rrs*, and the 16s-23s ITS of isolates 20.00 and 41.60

Isolate	Target	Accession Number
20.00	*gltA*	KP720638
	rpoB	KP720639
	ftsZ	KP720640
	groEL	KP720641
	ribC	KP720642
	rrs	KP720643
	16s-23s ITS	KP720644
41.60	*gltA*	KP720645
	rpoB	KP720646
	ftsZ	KP720647
	groEL	KP720648
	ribC	KP720649
	rrs	KP720650
	16s-23s ITS	KP720651

RESULTS

The results from the genome sequencing of 41.60 revealed *gltA, rpoB, ftsZ, groEL,* and *ribC*, *rrs,* and16S-23S ITS to be 100% identical to their respective counterparts of 20.00^T and 20.60. Additionally, whole genome sequencing showed the G+C content of these three isolates to be 38.4 mol%. This is similar to the G+C content of other known *Bartonella* species (Bermond *et al.*, 2002, Bermond *et al.,* 2000).

All three isolates grew on TSA containing 5% defibrinated sheep blood, Columbia agar containing 5% defibrinated sheep blood, and deep-fill heart BHIA containing 10% defibrinated sheep blood, while no isolates grew on chocolate agar. All three isolates grew best at 30°C with 5% CO_2, with visible colonies observed (for all three isolates) after 10 days growth was stunted for all three isolates 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% CO_2. Cultures remained viable at 30°C with 5% CO_2 for at least 30 days post inoculation on BHIA with 10% defibrinated sheep blood. Additionally, all three isolates grew in MS10 broth and in Vero and L929 cell culture.

Colonies passaged four times from frozen isolates, were visualized at 10 and 14 days on deep-fill BHIA containing 10% defibrinated sheep blood at 30°C in a moist atmosphere with 5% CO_2. At 10 days colonies of 20.00^T were iridescent-gray, opaque, smooth, 1-2 mm in size, while 20.60 appeared as pinpoint colonies. Additionally, 41.60 appeared as a heterogeneous population of iridescent-gray, 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 colonies were iridescent-gray, and opaque, with a depressed center, and 2-3 mm in size.

At day 14, 20.60 colonies were iridescent-gray, translucent, and ≤0.5 mm in size, while 41.60 now exhibited a more homogenous population of iridescent-gray, opaque, and smooth colonies which were largely 0.8-1 mm in size.

Gram staining and electron microscopy revealed small Gram-negative coccobacilli/ bacilli, while transmission electron microscopy, revealed 1.27 µm by 0.54 µm (20.00^T), 0.99 µm by 0.64 µm (20.60) and 1.51 µm by 0.62 µm (41.60) bacilli, with variable expression of unipolar flagella (Figure 19).

All three isolates were catalase, oxidase, urease and indole negative. Additionally, all three isolates were negative for the hydrolysis of D-dissacharide (BLTS), L-arabinoside (αARA), D- galactoside (ONPG), D-glucoside (β-GLU and αGLU), L-fucoside (FUC), D-glucosamide (NAG) and ρ-nitrophenylphosphate (PO4). Arylamidase activity is weakly to moderately positive for proline (PRO) and phenylalanine (PAL) and positive for leucine (LGY), glycine (GLY), arginine (ARG), and serine (SER), but negative for pyrrolidonyl (PYR). These results are consistent with other members of the genus *Bartonella* (Bermond *et al.*, 2000). In addition, the results give a 000 671 score on the RapID ANA II system, which is identical to results seen for many *Bartonella* species, yet differs from the score seen for B. *bacilliformis* (000 641) (Clarridge *et al.,* 1995).

Antibiotic susceptibility testing revealed sensitivity to Amikacin (≤2 µg/ml), Azythromycin (≤0.125 µg/ml), Ceftiofur (≤0.125 µg/ml), Chloramphenicol (≤2 µg/ml), Enofloxacin (≤0.125 µg/ml), Ticarcillin (≤4 µg/ml), Ampicillin (≤0.125 µg/ml), Clarithromycin (≤0.5 µg/ml), Gentamicin (≤0.5 µg/ml), Erythromycin (≤0.125 µg/ml), Doxycycline (≤1 µg/ml), Rifampin (≤1 µg/ml), and Tetracycline (≤1 µg/ml). In addition,

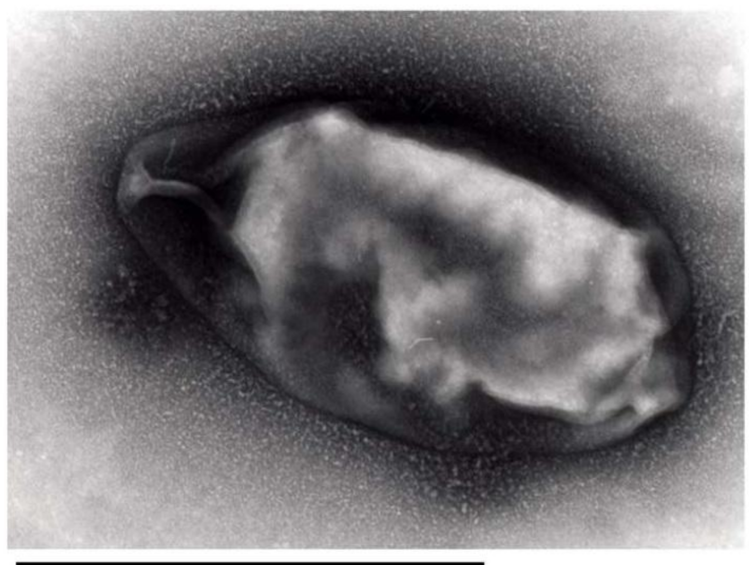

Figure 19. Electron micrograph of isolate 20.00T297 . Bar indicates 1 μm.

isolates 20.00 and 20.60 had an MIC of >2 µg/ml for Oxacillin + 2% NaCl, while 41.60 was sensitive to ≤0.125 µg/ml of Oxacillin + 2% NaCl. All three isolates produced MICs of >8 µg/ml for Cefazolin, 1 µg/ml of Ceftazidime, 0.5 µg/ml of Penicillin, and up to 1 µg/ml Imipenem.

DISCUSSION

In conclusion, the sequencing results, MLST, and MST analysis, along with the phenotypic, morphological, microscopic, biochemical, and growth characteristics of the three isolates (20.00^T, 20.60, and 41.60) indicate these three isolates belong to a single novel species that belongs to the genus *Bartonella* for which the name *Bartonella ancashi* has been proposed.

Description of *Bartonella ancashi*

Bartonella ancashi (an.cash.i) *ancashi* from Ancash, a region of Peru, bordered by La Libertad region to the north, the Lima region to the south, the Huánuco and Pasco regions to the east, and the Pacific Ocean to the west. The name of the region is derived from a Quechua word, anqash, meaning blue. The Ancash region is where the patients, from whom the first isolates were obtained, lived.

After a 10 day incubation on BHIA containing 10% defibrinated sheep blood at 30°C with 5% CO_2 colonies appear round, iridescent-gray, opaque, and smooth at 1-2mm in size. Additionally, cells are Gram negative bacilli, which lack flagella and are 1.27 µm by 0.54 µm. Oxidase, catalase, urease, and indole negative. Hydrolysis of D-dissacharide (BLTS), L-arabinoside (αARA), D- galactoside (ONPG), D-glucoside (β-GLU and αGLU), L-fucoside (FUC), D-glucosamide (NAG) and ρ-nitrophenylphosphate (PO4) is negative. Arylamidase activity is weakly positive for proline (PRO) and phenylalanine (PAL) and positive for leucine (LGY), glycine (GLY), arginine (ARG), and serine (SER), but negative for pyrrolidonyl (PYR). This gives a 000671 code using the RapID ANA II system. This species can be distinguished from other species of the genus *Bartonella* by

the *rrs, ftsZ, gltA, groEL, ribC* and *rpoB* genes and 16S-23S ITS region sequences. Additionally, this species has a G+C content of 38.4 mol%.

The type strain is 20-00^T, which was the first isolate and collected prior to antibiotic treatment. 20-00^T was isolated from a 3-year-old male living in a rural setting outside the town of Caraz, located in the highlands of the Ancash region of Peru.

Chapter 6:

Discussion

DISSERTATION SUMMARY

The main goal of this dissertation project was to characterize three bacterial isolates (20.00, 20.60, and 41.60), obtained from the blood of two children with verruga peruana. To this end, two Specific Aims were developed to characterize these isolates, using both genomic and microbiological methods and to identify probable virulence factors for these isolates using bioinformatic tools. The belief that the three isolates might be a novel species of *Bartonella* was first suggested when the isolates were obtained during the 2003 clinical treatment trial conducted in Caraz, Peru (summarized in Chapter 1). Sequencing of a 338 base pair fragment of the *gltA* gene was preformed as part of the protocol to confirm *Bartonella bacilliformis* infections, for the patients enrolled in the trial. Unexpectedly, the sequencing results for isolates 20.00, 20.60, 41.60 indicated that the organisms isolated from two patients had *gltA* gene sequences dissimilar with the known *gltA* sequences of *B. bacilliformis*. Moreover, the 338 base pair *gltA* sequence was identical for all three isolates from the two patients, thus indicating that infection with this unique agent might not be an isolated event.

Both Aims 1 and 2 were developed to prove the hypothesis that 'the three isolates (20.00, 20.60, and 41.60) derived from children suffering from verruga peruana belong to a single novel species of *Bartonella*, which possess virulence factors essential to pathogens of the genus *Bartonella*.' To prove our hypothesis, Aim 1 set out to elucidate the isolates genotypic and phenotypic characteristics using gene sequencing methods, MLST and MST, and an array of microbiologic methods including: microscopy (both light and transmission electron microscopy), growth characteristics (on solid media, in liquid media, and in cell culture), biochemical analyses, and antibiotic susceptibilities

(Chapters 2, 3, and 5). Aim 2 was developed to provide a deeper understanding as to the relationship between the three isolates at the genome level and to provide essential *in silico* derived information surrounding the virulence of these three isolates (Chapter 4).

SUMMARY OF CHAPTERS IN THE CONTEXT OF THE SPECIFIC AIMS

Synopsis of Chapter 2

The story of *Candidatus* Bartonella ancashi begins with the clinical description of a three year old boy living in Caraz, Peru, who presented with verruga peruana, thought to be caused by *B. bacilliformis*. The child was subsequently enrolled in the clinical treatment trial outlined in Chapter 1. Briefly, his clinical symptoms were hallmarked by the appearance of 56 verrugas mainly on his extremities, which appeared to be the result of *B. bacilliformis* infection. The initial work up, as part of the clinical treatment trial, included blood cultures and a subsequent sequencing analysis of a 338 base pair fragment of *gltA* from isolate 20.00, cultured from the child's blood at day zero. Results from the sequencing of *gltA* indicated the isolate might be distinct from *B. bacilliformis*.

To confirm these initial results, fragments of *rrs* and *rpoB* were PCR amplified and sequenced. The results from the sequencing of these two additional genes were consistent with what was seen after sequencing *gltA* – no matches to either gene sequence was found in GenBank. Since *rrs*, *rpoB*, *gltA* are widely used for *Bartonella* sequencing analyses; sequences of *rrs*, *rpoB* and *gltA* are available for the vast majority of recognized *Bartonella* species and for a number of candidate species (90; 142). Concatenated sequences for *gltA* and *rpoB* placed isolate 20.00 in a clade with *B.*

bacilliformis, indicating isolate 20.00 is in fact most similar to *B. bacilliformis*. Furthermore, *rpoB* and *gltA* are believed to have the best discriminating power for *Bartonella* species, allowing us to make conclusions regarding the novelty of isolate 20.00 (142). Therefore, based on these preliminary findings we were able to conclude that isolate 20.00 belonged to a novel *Bartonella* species, initially referred to as *Candidatus* Bartonella ancashi.

The accidental discovery of this probable human pathogen, through the sequencing of isolates thought to be *B. bacilliformis*, hits home the major weaknesses that exist when relying on clinical symptoms and cultures for the diagnosis of bartonelloses and in particular for the diagnosis of verruga peruana, caused by *B. bacilliformis* (54). Additionally, this preliminary work suggested that *B. bacilliformis* might not be the only *Bartonella* species causing disease in Caraz or other regions in Peru. However, further characterization of the isolate, *Candidatus Bartonella* ancashi, needed to take place before any conclusions could truly be made at this stage.

Synopsis of Chapter 3

The second isolate (20.60) was cultured from the blood of the same three year old boy as isolate 20.00. In contrast, isolate 20.60 was cultured 60 days after isolate 20.00. Additionally, isolate 20.60 was cultured from the child's blood after the child had received a full course of antibiotic therapy consisting of a single dose of azithromycin on day 0 and on day 7, which should have been sufficient to clear infection (54). As with isolate 20.00, a sequencing analysis was performed on a 338 base pair *gltA* gene fragment. Like isolate 20.00, isolate 20.60 was found to be disparate from *B. bacilliformis*.

Genotype characterizations are frequently performed for *Bartonella* species using single gene sequencing methods, MLST, and MST. Single gene sequencing and MLST commonly target *rrs*, *gltA*, *rpoB*, *ftsZ*, *groEL*, and *ribC* (24; 90; 117; 142; 167; 255; 256). Therefore, to further Aim 1, housekeeping genes (*gltA*, *rpoB*, *ftsZ*, *groEL*, and *ribC*), *rrs*, and the 16s-23s ITS were sequenced using Sanger sequencing methods and the Roche GS FLX Titanium sequencing system. The sequences obtained from both isolates 20.00 and 20.60 were found to be 100% identical to one another at each locus, while disparate from any known *Bartonella* species. Additionally, all five housekeeping genes and *rrs* of isolate 20.60 were concatenated and an MLST phylogeny, which included 21 *Bartonella* species type strains and isolate 20.60, was created. MLST analyses provide valuable insight into the phylogenetic relationships and the evolutionary relatedness of *Bartonella* species and often employ concatenated sequences of a combination of housekeeping genes and *rrs* (77; 110; 159).

MLST analyses indicate that *Bartonella* species belong to four distinct lineages (77; 99). Interestingly, the MLST analysis we preformed placed isolate 20.60 in a clade with *B. bacilliformis*, though the distance between isolate 20.60 and *B. bacilliformis* is great. The distance between isolate 20.60 and *B. bacilliformis* is greater than the distance seen within other *Bartonella* clades and lineages (77; 98; 142). Finally, an MST analysis comparing the 16s-23s ITS of isolate 20.60, nine *B. bacilliformis* strains, *Bartonella rochalimae*, which was found to cause an Oroya-like fever illness in an American traveling in Peru, and *Bartonella clarridgeiae*, the next closest relative of *B. rochalimae* was preformed. MST analyses are often performed using the 16s-23s ITS and are crucial in elucidating differences between strains of a single species (22; 106; 148; 253). Data

from these analyses provide valuable information as to the genetic diversity of individual *Bartonella* species. Again, the results indicated that isolate 20.60 is related to, yet distinct from *B. bacilliformis*. Taken together the results from the sequencing analysis, MLST, and MST further support the hypothesis that isolates 20.00 and 20.60 are members of a novel species of *Bartonella* (i.e. *Candidatus* Bartonella ancashi).

The additional isolate, *Candidatus* Bartonella ancashi 20.60, isolated 60 days after the initial isolate, *Candidatus* Bartonella ancashi 20.00, gives more credibility to the thought that *Bartonella* species causing verruga peruana, in areas endemic for *B. bacilliformis*, might be more diverse that previously recognized. While, the MLST and MST results are sufficient to prove that *Candidatus* Bartonella ancashi isolates 20.00 and 20.60 are distinct from known *Bartonella* species and support our hypothesis, our studies have yet to provide any phenotypic data or genotypic information for isolate 41.60. Additionally, these studies did not provide enough data to elucidate any differences between these isolates at a genomic level or provide insight into the presence of absence of *Bartonella* virulence factors.

Synopsis of Chapter 4

The genus *Bartonella* contains a rather diverse group of pathogens. This diversity is ascribed to the adaptation of *Bartonella* species to unique ecological niches, the intracellular environment of endothelial cells and erythrocytes, of a wide array of mammalian hosts (77; 98; 258). Species within the different *Bartonella* lineages possess distinct combinations of virulence factors, like the type IV secretion systems and flagella (61; 77; 98; 177; 242). Furthermore, not only is the genetic diversity seen between

species of *Bartonella*, but profound genetic diversity among strains within a species has been observed (22; 148). This is especially apparent in the case of *B. bacilliformis* (22; 46). Studies indicate that *B. bacilliformis* isolates can be categorized into at least 8 sequence types with up to 3% dissimilarity between *gltA* gene fragments for *B. bacilliformis* genotypes (22; 46). As with other *Bartonella* species, the genome analyses, preformed on isolates 20.00, 20.60, and 41.60, showed marked differences amongst the three isolates and between the three isolates and other *Bartonella* species. At the whole genome sequence level isolates 20.00, 20.60, and 41.60 were 99.7% identical. However, when the arrangement of the sequences within the genomes of these three isolates was compared the results were unexpected. This comparison lead to the discovery of an intriguing genomic inversion found among isolates 20.00, 20.60, and 41.60, in which almost half the genome is inverted in isolates 20.60 and 41.60 as compared to isolate 20.00. Phenotypic studies showed that 20.00 did not express flagella, while flagella expression was seen with both 20.60 and 41.60, when isolates grown on solid media were observed using electron microscopy, thus indicating that this genomic inversion may have an effect on flagella expression.

Genome and proteomic comparisons between isolate 20.00 and other *Bartonella* genomes provided more evidence that these isolates were in fact novel members of the *Bartonella* genus and most closely related to *B. bacilliformis*. Just like with the MLST analysis, the whole genome phylogenic analysis, which included 38 *Bartonella* strains, placed isolate 20.00 closest to *B. bacilliformis*. Interestingly, isolate 20.00 possesses an important virulence factor not found in *Bartonella bacilliformis*, the VirB/D4 T4SS. However, like *B. bacilliformis*, isolate 20.00 does possess flagellar genes. As with other

Bartonella species that contain flagella genes, the Trw T4SS secretion system was absent from the genome of isolate 20.00, thus providing more evidence that flagellar components must play a crucial role in the erythrocyte infection (19; 215; 218). Unlike other *Bartonella* that posse flagella, it seems *Candidatus* B. ancashi may be unique in that it might have the ability to modulate its expression of flagella. This idea is based on evidence showing variable flagella expression in electron micrographs taken of 20.00, 20.60 and 41.60 even thoughthe genomes of all three isolates encode flagellar associated genes. Finally, we uncovered evidence that isolate 20.00 encodes Virulence Modulating (VM) proteins, which are not thought to be traditional *Bartonella* virulence factors, as they are only seen in *B. bacilliformis* and *B. australis*. Rather, these VM proteins are associated with *Leptospira* (145). Additionally, their functionality is unknown, however, homologs of these proteins in *Leptospira* play a critical role in determining the virulence of *Leptospira* species, thereby indicating these proteins might be more important than previously recognized (145).

The wide genetic disparity between *Candidatus* B. ancashi and *B. bacilliformis* and the presence of the VirB/D4 T4SS indicates that this novel agent belongs in a *Bartonella* taxon separate from *B. bacilliforms*. Furthermore, its genetic distance from all other *Bartonella* species indicates *Candidatus* Bartonella ancashi belongs to its own unique clade within *Bartonella* (77; 98). Additionally, the analysis of all three genomes and the 99.7% similarly found between 20.00, 20.60, and 41.60 indicate that all three isolates are members of a single species despite the large genomic inversion that was reported. While, implications of the genomic inversion cannot be fully understood at this point, the work presented in this chapter support our hypothesis that these clinical isolates

belong to a single *Bartonella* species, *Candidatus* B. ancashi and by whole genome sequencing and the identification of unique and important virulence factor genes.

Synopsis of Chapter 5

Although the data from the genotyping and genome analyses indicate that these clinical isolates are members of a unique *Bartonella* species, a phenotypic characterization was still necessary to solidify how these isolates fit into the genus *Bartonella*. *Bartonella* species are known to be fastidious, requiring hemin and several days to several weeks for culture, before growth is apparent (43; 115; 122; 144). In terms of *Bartonella* phenotypes, two groups emerge and correlate well with their lineages. *Bartonella bacilliformis*, the only currently recognized member of the ancient lineage grows best between 28 and 30°C without CO_2, and expresses flagella (77; 99; 122). Additionally, the majority of *Bartonella* species in the modern lineages grows at 35°C in the presence of CO_2 and are variable in their expression of flagella- although most do not have flagella (99; 122). Another defining feature of *Bartonella* species is there high susceptibility to antibiotics *in vivo*(207). Understanding how isolates 20.00, 20.60, and 41.60 fit into the *Bartonella* genus phenotypically is just as important as understanding their relationships at the genome level.

We found that like *B. bacilliformis*, all three *Candidatus* Bartonella ancashi isolates grow best at 30°C, yet like all other *Bartonella* species they prefer a CO_2 rich environment, which *B. bacilliformis* does not, thereby providing an interesting link between *Candidatus* B. ancashi to both the ancient lineage and to the modern lineages. Like other *Bartonella* species, *Candidatus* B. ancashi grows on solid media, in cell

culture, and in liquid broth. Interestingly, isolate 20.00 seemed to grow faster than isolates 20.60 and 41.60 on both solid media and in liquid culture. Biochemical testing indicated a utilization pattern that was consistent with other *Bartonella* species and antibiotic testing indicated sensitivity to a wide range of antibiotics, however sensitivities varied between the isolates.

The phenotypic differences seen between the isolates could not have been uncovered through genomic comparisons alone, thus highlighting the importance of providing a full phenotypic work up for novel bacterial isolates. Furthermore, these final analyses provide the last pieces of data needed to support our hypothesis and complete Aim 1. Through the work provided in this Chapter and Chapters 2-4, we have fully characterized the three isolates using genotypic and phenotypic methods and the isolates were compared at the genome level to detect any differences between the isolates and to identify punitive virulence factors, thereby completing Aims 1 and 2. The completion of Aims 1 and 2 allowed us to prove our hypothesis these three clinical *Bartonella* isolates are members of a novel *Bartonella* species, i.e. *Candidatus* Bartonella ancashi.

FUTURE DIRECTIONS

Given that the work presented characterizes a novel bacterium, which is a member of a neglected group of pathogens, the genus *Bartonella*, the future directions are almost endless. However, in the immediate future, work should progress in two directions: 1) the collection of epidemiological data associated with presence, prevalence and distribution of *Candidatus* B. ancashi within and outside of Peru: and 2) the further in

depth study of *B. ancashi* genomic characteristics (e.g. inversions) and the role of *Candidatus* B. ancashi virulence factor genes in the pathophysiology of this bartonellosis.

Epidemiological Studies

At this moment in time we only have three isolates of *Candidatus* B. ancashi, from two human patients. Additional studies need to be undertaken to identify other cases of *B. ancashi* in humans. Currently, we do not know if the infection of these two children with *B. ancashi* was an isolated event or if a small proportion of cases of verruga peruana seen in Caraz are actually caused by *B. ancashi*. Furthermore, it is possible that persons living in Caraz could have asymptomatic infections with *B. ancashi* or could have antibodies to *B. ancashi*. PCR techniques and serologic assays could be employed to look for persons with evidence of exposure to *B. ancashi*. Considering that out of 92 suspected *B. bacilliformis* isolates, three (3.3%) were actually *Candidatus* B. ancashi, the odds of finding additional *Candidatus* B. ancashi cases in Caraz seems likely.

While it might be the true that humans are the reservoir host for *Candidatus* B. ancashi, for most *Bartonella* species, humans are an incidental host. Identification of potential hosts as well as potential vectors is critical for understanding the pathology of *Candidatus* B. ancashi infections. The logical place to start would be in Caraz, Peru. Through the collection of arthropods, such as fleas, flies, ticks, and/or lice, and blood samples from small mammals in the region, possible vectors and hosts could be identified, using simple DNA extraction techniques and PCR based assays.

Investigation into sandflies as a vector for Candidatus B. ancashi

Based on the evidence that *Candidatus* B. ancashi produced a clinical syndrome identical to verruga peruana, caused by *B. bacilliformis,* and is likely circulating in areas where *B. bacilliformis* is endemic, we perused the possibility that sandflies could play a role in *Candidatus* B. ancashi transmission, as they are suspected to be the vector for *B. bacilliformis* (183; 235; 237). To this end, we extracted DNA from 99 ethanol preserved sandfly pools (pools contained between 2 and 50 sandflies; 1264 total sandflies) collected between 1998- 2000. Sandflies (*Lutzomyia* species) were collected in Caraz, Peru. DNA from the pools was analyzed using a *Candidatu*s B. ancashi, species specific, real-time quantitative (q)PCR assay. The qPCR assay we developed targets *gltA* (forward primer- 5'-GTATATCCACGTAATGACCTTG-3'; reverse primer- 5'-GATGTTGATGCATTTTGTTCATGG-3'; probe- 5'-[6-FAM]-TGTTGTAAATCCTGTTCTTTCTCAAGC-[BHQ1a-6FAM]-3')(Eurofins MWG Operon; Huntsville, AL) and is sensitive down to 1 copy/µl. When tested against *Candidatus* B. ancashi and a panel of 16 *Bartonella* species, including *B. bacilliformis*, the assay was specific for *Candidatu*s B. ancashi (unpublished).

The results from the *gltA* qPCR assay indicated *Candidatus* B. ancashi was not present in any of the sandflies tested, as no pools were positive. These results indicate sandflies might not be the vector for *Candidatus* B. ancashi and that further studies need to be carried out to attempt to identify other possible vectors. Those vectors could include ticks, fleas, keds, and/or lice as all have been implicated in transmission of *Bartonella* species (6; 37; 52; 67; 73; 104; 121; 173). Considering we now have a set of tools that can be used to screen for *Candidatus* B. ancashi, future studies are one step closer to being completed. This novel *gltA* qPCR assay can be used to screen samples from

arthropods, to look for other possible vectors, samples from rodents and small mammals, to identify possible reservoirs, and samples from humans, to identify other cases of *Candidatus* B. ancashi infection. While understanding the epidemiological dynamics of *Candidatus* B. ancashi is important, elucidating the genomic characteristics of Candidatus B. ancashi is equally important to understanding its pathogenicity and ultimately its ecological niche and epidemiological characteristics.

Genomics

In particular, we need to elucidate the importance of the genomic inversions seen between the isolates of *Candidatus* B. ancashi. This finding is unique to *Candidatus* B. ancashi and has not been seen in other *Bartonella* species, although expression of pili and VOMPs are known to be modulated by phase variation in *B. henselae* and *B. quintana*, respectively (141; 257). Furthermore, *Salmonella enterica* has been shown to modulate flagella expression through the inversion of segments of the genome (138). However, with *Candidatus* B. ancashi, it remains unclear as to the true nature of this inversion. At this point, we are unsure if these isolates are stable in their genetic arrangement or if this is a dynamic system leaving us with many questions as to the true nature of these isolates.

Questions that remain unanswered include:

Are isolates 20.00 and 20.60 the same agent recovered at different time points during infection?

<u>AND</u>

Did isolate 20.00 undergo a genomic inversion as a result of the antibiotic treatment the patient received and/or due to prolonged exposure to the human host?

Or

Are 20.00 and 20.60 completely different agents?

AND

Were isolates 20.00 and 26.00 co-circulating at day zero? Alternatively, did the patient become infected with 20.60 at some later point in time?

These questions can never be definitively answered, but if we could understand the properties of this inversion we could make more solid conclusions.

Significant genomic inversions and rearrangements have been uncovered in bacterial pathogens such as *Salmonella typhi, Bacteroides fragilis*, and Group A streptococcus (140; 152; 182). These inversions have been associated with increased virulence and/or survival in a niche environment (140; 152; 182). Given that variable expression of *B. quintana* Vomps have been observed after prolonged exposure to the human host, it is possible that the genomic inversion seen among the three isolates could be due to similar phenomena (225; 257). Prolonged exposure to the human host could have triggered an inversion event, which in turn could be linked to enhanced survival or virulence. Alternatively, this genomic inversion could have occurred as a stress response to the antibiotic therapy the patients received. Bacterial pathogens are known to regulate their flagella expression in response to environmental factors, including the desire to move towards environments that would be more favorable to their survival or away from environments detrimental to survival (49; 229). Therefore, it is also feasible to

hypothesize that *Candidatus* B. ancashi underwent a genomic inversion that allowed for the expression of flagella, in an attempt to limit antibiotic exposure.

Proof of flagellar phase variation, due to genomic inversions, in bacterial pathogens such as *Salmonella,* indicate that *Candidatus* B. ancashi could also regulate flagella expression (138; 139). However, as stated previously, this has not been seen in other *Bartonella* species, though it seems reasonable that *Candidatus* B. ancashi could regulate flagella expression, given that phase variation of pili occurs in B. *quintana* and *B. henselae* (141; 257). Additionally, the Trw gene cluster was absent from the genome of *Candidatus* B. ancashi indicating the presence of flagella are likely necessary for host erythrocyte invasion (61; 63; 177; 218; 242). Therefore, it would seem unlikely that isolate 20.00 would be able to establish infection without the ability to express flagella.

Based on our initial studies we theorized that flagella expression might be linked to the genomic inversion, which is flanked by repeat regions. Our reasoning was based on the orientation of the flagella genes within the genome of isolate 20.00, when compared to isolates 20.60 and 41.60. The genomes of all three isolates encode for flagellar components. However, the arrangement of these genes is different in 20.00, when compared to 20.60 and 41.60, which have identical arrangements of their flagellar gene cluster. Furthermore, the arrangement of the flagella genes in 20.60 and 41.60 is identical to the arrangement of the flagellar gene cluster in *B. bacilliformis*. This difference in the arrangement of the flagellar gene clusters between 20.00 and 20.60 and 41.60 is due to the position of the flagellar genes within the inverted region. Additionally, when grown on solid media, 20.00 lacks flagella expression, while isolates 20.60 and 41.60 showed expression of flagella, thereby providing evidence that flagella expression might be

linked to the genomic inversion seen between the three isolates of *Candidatus* B. ancashi. While these observations provide evidence to support the hypothesis that flagella expression is linked to the genomic inversion, further investigations are necessary to provide insight into the true properties of the genomic inversion and to provide experimental evidence linking the genomic inversion to flagella expression, in the three isolates.

To increase our knowledge as to the properties of this genomic inversion and flagella expression, we need to create assays that can be used to monitor any changes at the breakpoints for the inversion and monitor flagella expression. We would then want to systematically change culture conditions to see if a genomic inversion and/or expression of flagella can be induced. These changes could include growth in liquid and cell culture (both isolates were previously analyzed using colonies grown on solid media), presences of antibiotics, changes in growth temperature and CO_2 levels.

Preliminary investigations into flagella expression

In an attempt to gain a better understanding of flagella expression in *Candidatus* B. ancashi, we conducted an experiment to determine if isolates 20.00, 20.60 and 41.60 express flagella in cell culture, using Immunohistochemistry techniques directed at *Bartonella* flagellin (218). Isolates 20.00, 20.60 and 41.60 were inoculated at an MOI of 20 onto confluent monolayers of Vero cells grown in Eagle's minimal essential medium (EMEM) with 10% fetal bovine serum (FBS). Cultures were incubated at 30°C for 19 days at which point cells were scraped, washed, and re-suspended in phosphate buffered saline (PBS). The cells were then fixed, with methanol, onto chamber slides and stained

with rabbit anti-flagellin antibodies (218), as the primary antibody (1:100), and goat anti-rabbit- IgG conjugated to Alexa Fluor 488 (Invitrogen; Grand Island, NY)(1:50) as the secondary antibody and visualized. Interestingly, this preliminary data (unpublished) showed that all three, *Candidatus* B. ancashi, isolates expressed flagella in Vero cell culture (Figure 20). These results suggest that flagella expression can be modulated, at least in the case of isolate 20.00, which previously did not express flagella, when grown on solid media. Given that flagella are thought to be necessary for erythrocyte invasion, it is not surprising that isolate 20.00 is in fact able to express flagella, since the Trw type IV secretion system was not found to be encoded within the *Candidatus* B. ancashi genome. Unfortunately, at this point we do not know if an inversion took place within the genome of isolate 20.00 when exposed to cell culture conditions, as we currently do not have the tools to tract the genomic inversion. Therefore, the results indicate that further studies must take place to gain a true understanding of the dynamics of *Candidatus* B. ancashi flagella expression and its link to the genomic inversion seen between the isolates and it role in *Candidatus* B. ancashi virulence and pathogenicity.

OVERALL CONCLUSIONS

The genus *Bartonella* consists of a group of emerging pathogens associated with human and animal diseases. The discovery of yet another novel *Bartonella* species, especially one that is linked to human disease, underscores the importance of this genus. The lack of knowledge and understanding that surrounds *Bartonella*, is astounding given that fact that three species regularly cause human disease (one of which can be fatal) and that a growing number of species are being implicated in causing human infections (114;

121). The work within this dissertation produced an abundance of data proving *Candidatus* B. ancashi to be a novel *Bartonella* pathogen. The characterization of

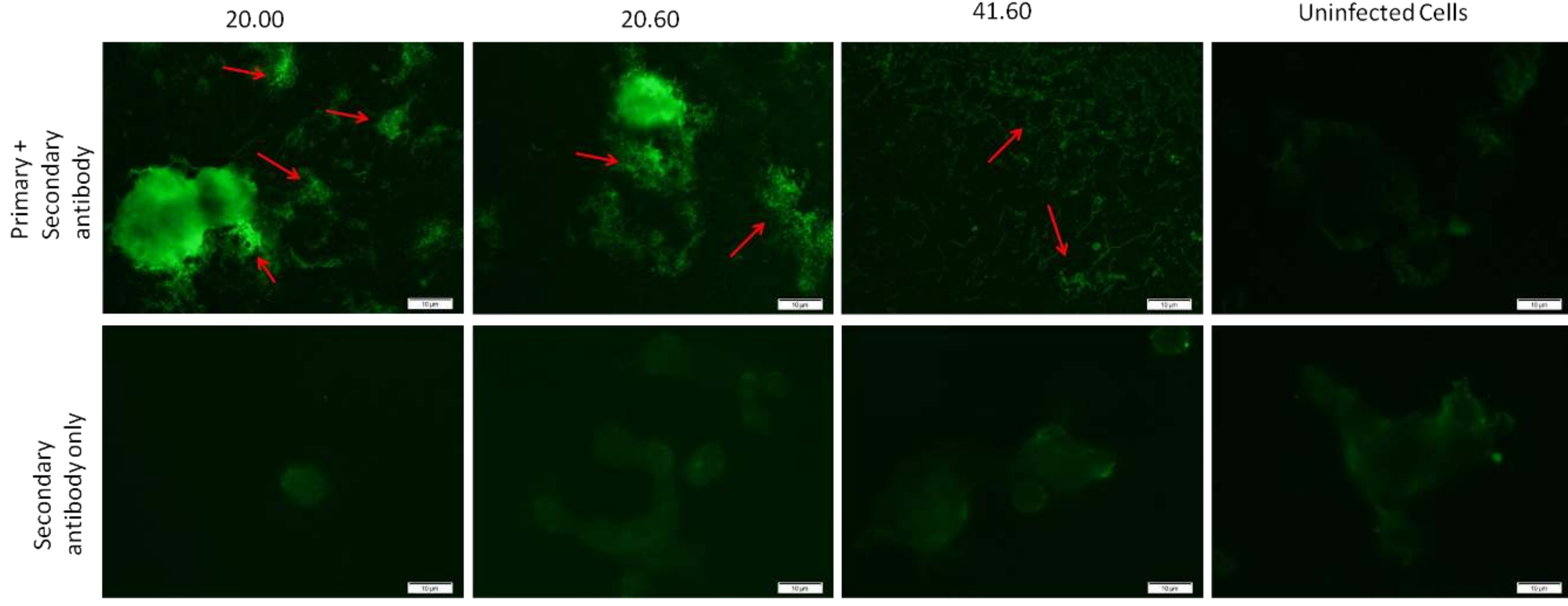

Figure 20: Flagella expression for isolate 20.00, 21.60, and 41.60 when grown in Vero cells. Isolates 20.00, 20.60, and 41.60 were labeled using rabbit anti- flagellin antibodies as the primary antibody (diluted 1:100) (19) and goat Alexa Flour 488 conjugated to anti-rabbit IgG, as the secondary antibody (diluted 1:50). Flagellar structures can be seen in the panels for isolates 20.00, 20.60, and 41.60 labeled with the primary and secondary antibodies. Flagellar structures are absent in uninfected control cells labeled with both the primary and secondary antibody and in infected controls (20.00, 20.60 and 41.60) and uninfected controls which were labeled with secondary antibody only. The gray bar is equal to 10µm

Candidatus B. ancashi not only expands our understanding of this novel species, but it also expands our knowledge base of the genus *Bartonella* as a whole. We now have a novel species that is much more closely related to the 'ancestral' *B. bacilliformis* than any other 'modern' *Bartonella* species currently recognized (76; 98). Like *B. bacilliformis*, *Candidatus* B. ancashi grows best at 30°C and possess flagella, but unlike *B. bacilliformis*, the *Candidatus* B. ancashi genome contains the VirB/D4 T4SS seen in other more recent *Bartonella* lineages (4; 77; 99; 122). Discoveries like this should expand our understanding of the genus and provide insight into the evolution of these pathogens. Furthermore, the discovery of a second *Bartonella* species in Peru, implicated in causing verruga peruana, helps to strengthen claims that the *Bartonella* species causing disease in areas endemic for *B. bacilliformis* is more diverse that currently recognized (22; 46). Hopefully, the discovery of *Candidatus* B. ancashi will help spark a renewed interest in Bartonelloses in Peru and possibly around the world.

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