

# *Bartonella* spp. as Emerging Human Pathogens

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## INTRODUCTION AND OVERVIEW

### *Bartonella* spp. and Disease

Microbiology or infectious disease textbooks have traditionally relegated the discussion of trench fever, caused by *Bartonella* (*Rochalimaea*) *quintana*, to a paragraph or two in a general chapter on rickettsial diseases. Until recently, trench fever was the only disease known to be caused by a member of the genus *Rochalimaea*. Some texts may have also included a brief description of the clinical manifestations of infection by another rickettsia-like organism, *Bartonella bacilliformis*. Carrion's disease, caused by *B. bacilliformis*, is a biphasic disease limited to certain regions of the Andes mountains and viewed as a medical curiosity. The acute stage of the disease, Oroya fever, is characterized by a severe, life-threatening hemolytic anemia. The chronic stage, termed verruga peruana, results in the appearance of unique vascular proliferative lesions of the skin. We now know that these two groups of organisms are closely related; consequently they have been merged into a single genus, *Bartonella* (30). In addition to causing the diseases with which they have been historically connected, members of the genus *Bartonella* have recently been associated with an increasing spectrum of clinical syndromes including bacil-

lary angiomatosis (BA) and cat scratch disease (CSD). Two additional species of *Bartonella*, *B. henselae* and *B. elizabethae*, known to be pathogenic for humans have been recently described and characterized.

The role of *Bartonella* species as modern-day pathogens was first recognized for patients with BA. BA was initially described by Stoler et al.; it is typically seen in AIDS patients and is characterized by unusual neoplasia of the microvascular tissue of the skin (182). BA lesions have a gross appearance similar to that of Kaposi's sarcoma. Relman et al. detected 16S rRNA gene sequences with a high degree of homology to those of *B. quintana* in DNA extracted from skin lesions of patients with BA (160). Simultaneously, Slater et al. described *B. quintana*-like organisms that had previously been isolated from both immunocompetent and immunocompromised patients with fever and bacteremia (174). In each case, two different organisms were subsequently identified. Both *B. quintana* and another organism later identified as a new species (*B. henselae*) were determined to be responsible. Since the initial association of *Bartonella* species with BA and fever with bacteremia, this genus has been implicated in human infections with diverse clinical presentations. Among these are serious complications resulting from bacteremia, including endocarditis and lesions of almost every organ system including the heart, liver, spleen, bone and bone marrow, lymphatics, muscle and soft tissue, and central nervous system (see references 1 and 168 for reviews). Although systemic disease is more frequent in immunocompromised patients, involvement of most of the above-mentioned systems in immunocompetent patients has been re-

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ported as well. More recently, *B. henselae* has been firmly established as the primary etiologic agent of CSD.

### *Bartonella* spp. as Bacteria

The current genus *Bartonella* was created by merging the genus *Rochalimaea* with the one existing species in the genus *Bartonella*, *B. bacilliformis*. The proposal to merge the two genera was based on DNA-DNA hybridization data and comparison of existing 16S rRNA gene sequences (30). The proposal has gained widespread acceptance in the scientific literature, and the genus designation *Rochalimaea* has been replaced by the emended and combined genus *Bartonella*. The phylogenetic relationships of the emended genus *Bartonella* to other bacteria and the rickettsiae are addressed in the section on molecular biology below. Recently, but prior to combining the two genera, two new species were identified, *B. elizabethae* and *B. henselae*. *B. elizabethae* was isolated from a single patient with endocarditis and thus far has not been shown to be a common human pathogen. *B. elizabethae* was established as a new species based on DNA-DNA hybridization data and 16S rRNA gene sequencing and was named after Saint Elizabeth's Hospital, Brighton, Mass., where the organism was isolated (49). Prior to the description of *B. elizabethae*, *Rochalimaea* (*Bartonella*)-like organisms isolated primarily from AIDS patients were described (174). Independent reports indicated that these organisms represented a new species based on 16S rRNA gene sequence (154) and DNA-DNA hybridization data (202). In addition, DNA-DNA hybridization (202) and restriction endonuclease digestions of PCR products amplified from the citrate synthase gene (154) showed that all the isolates represented a single species. The organism was named *B. henselae* in honor of Diane Hensel, a microbiologist who contributed greatly to the initial isolation of the species (154, 202).

In addition to the human pathogens, *B. henselae*, *B. bacilliformis*, *B. quintana*, and *B. elizabethae*, the genus contains several members that have not been associated with human disease. The Canadian vole agent, *B. vinsonii*, and three newly proposed species, *B. grahamii*, *B. taylorii*, and *B. doshiae*, are not known to be human pathogens (24). In addition, a proposal to combine the genus *Grahamella* with *Bartonella* resulted in the description of two additional species, *B. talpae* and *B. peromysci*, that are also apparently nonpathogenic to humans (24). An additional organism that has been shown to cause endocarditis in dogs (27) has been named a subspecies of *B. vinsonii* (subsp. *berkhoffi*) (103). Likewise, a new species of *Bartonella* that was a cause of bacteremia in cats has been isolated (42) and proposed as a new taxon (107). The members of the emended genus *Bartonella* can be described as gram-negative, oxidase-negative, fastidious, aerobic rods (24, 30). Despite the acceptance of the unified genus *Bartonella* based on phylogenetic data, there are differences in a number of the phenotypic characteristics in *B. bacilliformis* and members of the former genus *Rochalimaea* (Table 1). *B. bacilliformis* is motile by means of polar flagella. Growth is optimal at 37°C, except for *B. bacilliformis*, which has an optimal growth temperature of 25 to 28°C (29), on media containing 5 to 10% rabbit, sheep, or horse blood. Incubation in the presence of 5% CO<sub>2</sub> is preferred, except for *B. bacilliformis*, which grows best without supplemental CO<sub>2</sub>. Carbohydrates are not utilized (200, 202). The isolation of *Bartonella* species generally requires extended incubation of primary culture plates or the use of cell culture systems, as described below. Based on phylogenetic relationships and the fact that the emended genus *Bartonella* does not contain any obligate intracellular pathogens, it has been removed from the order *Rickettsiales* (30).

TABLE 1. Differential properties of *B. bacilliformis* and the pathogenic members of the recently emended genus *Bartonella* (formerly *Rochalimaea*)

Property	Occurrence in:			
	<i>B. bacilliformis</i>	<i>B. elizabethae</i>	<i>B. henselae</i>	<i>B. quintana</i>
Flagella	+	-	-	-
Optimal growth				
5% CO <sub>2</sub>	-	+	+	+
25-28°C	+	-	-	-
34-37°C	-	+	+	+
Smooth colony morphology	+	±	±	±
Colony size (>1.0 mm)	-	+	+	+
Epicellular	-	?	±	±
Infection of erythrocytes	+	-	- <sup>a</sup>	-
Vector borne	+	?	+ <sup>b</sup>	+
Fatty acids				
C <sub>16:1</sub> >15%	+	-	-	-
C <sub>18:1</sub> >40%	-	+	+	+

<sup>a</sup> Intracellular bacteria have been found in cat erythrocytes (102).

<sup>b</sup> Experimental transmission between cats by the cat flea has been demonstrated (41).

### PATHOGENESIS

Intracellular growth of *B. bacilliformis* in erythrocytes and the resulting cell lysis has been well documented. *B. bacilliformis* is also known to adhere to and invade cultured human endothelial cells (66, 83). A two-gene locus that is involved in erythrocyte invasion has been identified (133). An extracellular 67-kDa protein of *B. bacilliformis* is known to cause deformation of erythrocytic membranes (127). Although *B. quintana* has traditionally been described as epicellular (200), growth in a human endothelial cell line has been described (56). Intracellular growth of *B. henselae* in endothelial cells was not demonstrated. However, a recent publication indicated that *B. henselae* localizes inside Vero cells (205). Additionally, both *B. henselae* and *B. quintana* are capable of intracellular growth in human epithelial cells (18), and intraerythrocytic growth of *B. henselae* in cats has been reported (102). The presence of bundle-forming pili in both *B. henselae* and *B. quintana* has been demonstrated by electron microscopy. It is thought that pili are key factors in host cell attachment and are important virulence factors in these organisms. The attachment of *B. henselae* and *B. quintana* to human epithelial cells is more efficient in piliated than nonpiliated organisms (Fig. 1). It has been suggested that these two organisms possess type IV pili due to the presence of a number of properties typical of this type of pili (18). *B. henselae* and *B. quintana* may exhibit twitching motility and can pit and tenaciously adhere to agar plates (154, 174, 200, 202). This adherent (or rough) colony phenotype is common in freshly isolated organisms cultivated on blood agar plates, but the adherence property may be lost upon repeated subculture in the laboratory. Differential expression of pili appears to be, at least in part, responsible for the phase variation from the adherent rough phenotype to the mucoid smooth phenotype (18). The protein subunits of the pili found in *B. henselae* and *B. quintana* have not yet been described. A 42-kDa protein component of the flagella of *B. bacilliformis* has been identified, and antibodies raised to this protein reduce the association with and invasion of human erythrocytes by *B. bacilliformis* (166).

Fourteen proteins ranging in size from 11.2 to 75.3 kDa have been localized to the outer membrane of *B. bacilliformis* by fractionation of the organism. On the basis of similar molec-

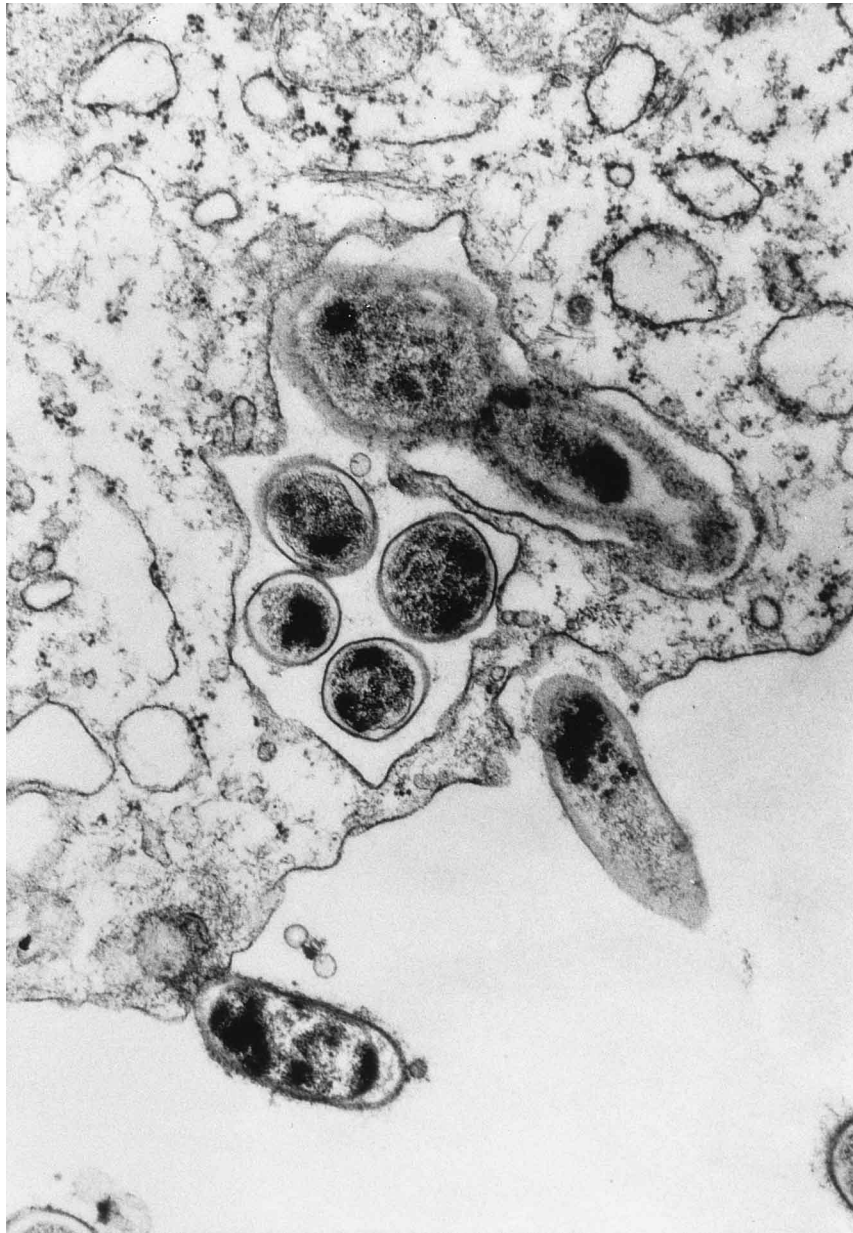


FIG. 1. Electron micrograph of low-passage-number (piliated) *B. henselae* associated with HEp-2 cells. Several intracellular bacteria can be seen inside a single vacuole. Membrane-associated bacteria can also be seen outside the cell. Reproduced from reference 18 with permission of the publisher.

ular size, 11 of those proteins appear to be labeled by using surface radioiodination (130). Knobloch found that 12 antigenic proteins of *B. bacilliformis*, ranging in size from 16 to 160 kDa, were reactive with human serum from patients with Carrion's disease (94). One particular protein, termed Bb65, has been shown to be a major antigen. Sequencing of the amino terminus of this protein suggests that it is a homolog of the GroEL class of stress response proteins (96). The identification of antigenic proteins and subunits of specific virulence factors of *B. henselae* and *B. quintana* has not progressed as far as with *B. bacilliformis*. A 17-kDa protein from *B. henselae* that is highly reactive with human sera from CSD patients has been cloned and sequenced (11). The overall predicted structure and highly antigenic nature suggest a surface location for this protein. Although this protein elicits a strong humoral immune

response in humans, a role of this protein in pathogenesis or immunity to infection with *B. henselae* has not been established. An immunogenic homolog of the HtrA stress response proteins has been cloned and sequenced from *B. henselae* (9). In other bacteria, the HtrA protein protects intracellular organisms from oxidative destruction.

Several recent studies have described selected aspects of the immune response to *B. henselae* in humans. Expression of unique surface markers on tissue macrophages in patients with CSD has been reported (143), raising the possibility of diagnostic applications. In another study, binding of *B. henselae* to peripheral blood lymphocytes from patients with CSD was demonstrated for four of five patients (147). Fumarola et al. have reported that exposure of polymorphonuclear leukocytes to *B. henselae* results in impairment of the oxidative function



TABLE 2. 16S rRNA gene sequence homology among pathogenic *Bartonella* spp. and other bacteria

Species <sup>a</sup>	% Relatedness with <sup>b</sup> :						
	<i>B. bacilliformis</i>	<i>B. elizabethae</i>	<i>B. henselae</i>	<i>B. quintana</i>	<i>Brucella abortus</i>	<i>Afipia felis</i>	<i>Rickettsia rickettsii</i>
<i>B. bacilliformis</i> (Z70003)	100						
<i>B. elizabethae</i> (L01260)	97.7	100					
<i>B. henselae</i> (M73229)	98.1	98.4	100				
<i>B. quintana</i> (M73228)	97.9	98.3	98.7	100			
<i>Brucella abortus</i> (X13695)	94.5	94.9	94.4	94.2	100		
<i>Afipia felis</i> (M65248)	86.1	88.4	87.9	87.3	89.0	100	
<i>Rickettsia rickettsii</i> (U11021)	83.4	85.1	84.9	85.0	84.3	82.9	100

<sup>a</sup> Accession numbers of the sequences used for alignment are indicated in parentheses.

<sup>b</sup> Sequences were aligned in a pairwise fashion by using the Gap program of the Genetics Computer Group package (68).

(63). However, more recently it was shown that phagocytosis and the production of oxygen radicals by polymorphonuclear leukocytes were enhanced in the presence of bacteria previously opsonized with immune sera (162). *B. henselae* was also shown to activate complement via the alternative pathway. Complement activation did not increase in the presence of specific antibodies (162).

Perhaps the most interesting observation of the interaction of *Bartonella* species with its host is the proliferation of vascular endothelial cells. This neovascularization occurs during infection with *B. henselae*, *B. bacilliformis*, or *B. quintana* and begins with the proliferation of the endothelial cells lining small blood vessels. The consequent *Bartonella*-induced angiogenesis results in the lesions observed in patients with BA and the verruga peruana of Carrión's disease. This effect can be observed in the laboratory as the proliferation and migration of cultured primary human endothelial cells, a key step in the process of angiogenesis (61, 72). Proliferation and migration of human primary endothelial cells in vitro has been demonstrated for both *B. bacilliformis* (65) and *B. henselae* (47). In addition, an extract of *B. bacilliformis* has been shown to cause angiogenesis in a rabbit model (65). The factor(s) that mediates this effect in both organisms is protease sensitive. It is not yet known whether the angiogenic factor acts directly on endothelial cells or binds receptors on endothelial cells and triggers proliferation and migration indirectly by signal transduction. Regardless, the ability of a factor from bacteria to cause proliferation of nonlymphoid cells resulting in the formation of new blood vessels is unique to the genus *Bartonella*.

### MOLECULAR BIOLOGY

The genomes of various *Bartonella* species have been estimated to be approximately  $1.6 \times 10^6$  to  $2 \times 10^6$  bp long (105, 200), and plasmids have not been described to date. The guanine-plus-cytosine content of the genomes ranges from 39 to 41% (29, 49, 188, 198, 202). A bacteriophage particle has been identified in both *B. bacilliformis* (190) and *B. henselae* (8). The particle consists of three major proteins and 14-kb fragments of double-stranded DNA that is packaged in a near-random manner reminiscent of a generalized transducing phage (8). The particle is approximately 40 nm in diameter, and tail-like structures have been visualized by electron microscopy in *B. bacilliformis* (190). The packaging of chromosomal DNA from bacteria and export of the resulting particles into the culture medium raises the possibility that this bacteriophage-like particle is a vehicle for genetic exchange among members of the genus *Bartonella*. However, the infectious nature of this particle or transduction has not yet been demonstrated, suggesting that the phage may be defective.

Initially, *B. quintana* was thought to be closely related to the

rickettsiae and was named *Rickettsia quintana*. The organism was renamed *Rochalimaea quintana* (200) and was subsequently cultivated on solid medium supplemented with horse or human blood (191). Despite the cultivation of *B. quintana* on solid medium in the absence of host cells, evidence existed that *B. quintana* was related genetically to members of the genus *Rickettsia*. DNA-DNA hybridization data indicated that both *B. quintana* and *B. vinsonii* have approximately 30% homology with *Rickettsia prowazekii* (138, 139). In addition, Regnery et al. estimated that the sequence divergence inferred from restriction fragment length polymorphisms of digested PCR products amplified from the citrate synthase gene was as similar in magnitude within the genus *Bartonella* (6.0 to 11.0%) as it was between *Bartonella* and *R. prowazekii* (10.3 to 13.6%) (154). More recent data suggest that genetic divergence between *Bartonella* species and *R. prowazekii* is greater than originally reported. Brenner et al. report that DNA-DNA hybridization levels for *Bartonella* species and *R. prowazekii* are between 6 and 14% (30). Likewise, the entire PCR product amplified from the citrate synthase gene of *B. henselae* and used previously to estimate sequence divergence has been sequenced (141). Alignment of this region of the citrate synthase gene of *B. henselae* and the corresponding region of *R. prowazekii* (141) reveals approximately 30% sequence divergence. Thus, recent data support a more distant relationship between members of the genus *Bartonella* and the true rickettsiae.

As with many rickettsiae and fastidious bacteria, determination of the 16S rRNA gene sequences has proven useful in defining new species and determining phylogenetic and taxonomic relationships among members of the genus *Bartonella*. Weisburg et al. first reported that *B. quintana* exhibited significant levels of homology of the 16S rRNA gene to *Brucella abortus* and *Agrobacterium tumefaciens* yet had no significant homology to *Rickettsia* spp. (197). Sequencing of the 16S rRNA gene of the type strain of *B. henselae* (154) indicated that this organism was the same as the agent previously associated with BA (160). Phylogenetic relationships based on 16S rRNA gene sequence analysis indicated that *B. bacilliformis*, *B. quintana*, and *Brucella abortus* were the closest relatives to *B. henselae* and that a new species designation was warranted (154). Similar studies have supported the phylogenetic relationship of *Bartonella* spp. to other bacteria (23, 24, 27, 29, 30, 48, 142, 159), as originally proposed by Weisburg et al. (197). The sequence homology between members of the emended genus *Bartonella* known to be pathogenic for humans is depicted in Table 2. In addition to the 16S rRNA, 5S and 23S rRNA genes and intergenic regions between rRNA genes have been sequenced (131, 132) and are being exploited for strain typing and phylogenetic comparisons of the genus *Bartonella* to other bacteria (123, 126, 164).

## SEARCH FOR THE ETIOLOGIC AGENT OF CAT SCRATCH DISEASE

### Background and *Afipia felis*

Since the first description of CSD by Debré et al. in 1950 (51), the search for the etiologic agent of CSD has been the source of much controversy and confusion. Efforts to identify the etiologic agent of CSD have yielded numerous publications, often adding to the confusion (26, 58, 59, 69, 91, 167, 187, 196). Our current knowledge regarding the etiology of CSD has been recounted in recent reviews (1, 7, 153). The most recent developments are worth mentioning here. Although CSD had been previously attributed to viruses and chlamydiae, bacterial agents became the central focus in 1983, when researchers using the Warthin-Starry stain, a specialized silver stain normally used for spirochetes, detected bacilli in lymph nodes from patients with CSD (196). The bacteria were described as delicate, pleomorphic, gram-negative bacilli that were shown to be gram negative by using a Gram stain modified for tissue (196). In 1988, the presumed agent was isolated and cultured from the lymph nodes of 10 patients with CSD (59). This bacterium was termed the cat scratch disease bacillus and named *Afipia felis* (28). However, subsequent research has failed to provide a strong link between *A. felis* and the vast majority of CSD cases. In fact, recent reports indicate no serologic response to *A. felis* in patients with CSD (5, 144, 184). In a separate study with PCR primers specific for either *Bartonella* spp. or *A. felis*, Bergmans et al. found that 96% of lymph node specimens from patients with CSD who were skin test positive contained *Bartonella* DNA (19). They also found that specimens from 60% of patients with suspected CSD cases (not skin tested) contained *Bartonella* DNA. None of the samples tested contained detectable *A. felis* DNA (19). Although it is unlikely that *A. felis* plays a significant role in causing CSD, occasional reports persist describing the detection of this organism (or antibodies to it) in CSD patients (4, 55, 64, 147). Recently, both *Bartonella* and *A. felis* DNA were found in the lymph nodes of two patients with CSD by using PCR, and a dual role for these agents in the etiology of CSD was suggested (4). In that report, a thorough evaluation of primer specificity was not described and it was not clear if the primers used would amplify DNA from bacteria other than *A. felis* or *B. henselae*.

### *Bartonella henselae* Causes Cat Scratch Disease

Recently, a new bacterial pathogen, *B. henselae*, was isolated and identified from immunocompromised patients with BA. Similar properties between the agents causing CSD and BA, primarily Warthin-Starry staining patterns, had prompted speculation that the agents of these two diseases might be the same (13, 79, 99, 110, 167). Other authors noted similarities between the agent causing BA and *B. bacilliformis* (44). Upon Warthin-Starry staining, *B. henselae* had a morphology similar to that of the bacilli observed in the lymph nodes of patients with CSD, prompting studies aimed at assessing the role of this agent in CSD. Subsequently, Dolan et al. isolated *B. henselae* from the lymph nodes of two patients with CSD (54). Two serologic studies reported that between 84 and 88% of patients with clinically diagnosed CSD had antibodies to *B. henselae* in the indirect fluorescent-antibody (IFA) assay (156, 204). Molecular analysis of CSD skin test antigens that have been used for CSD diagnosis for many years was used to determine the bacteria present. In one such study, *Bartonella* sequences were found in skin test antigen preparations (148), and in another, *B. henselae* was detected, suggesting that an antigen(s) from

this organism is likely to be responsible for eliciting the delayed-type hypersensitivity reaction that is diagnostic for CSD (10). More recent studies involving PCR have reported the specific detection of *B. henselae* in 21 (84%) of 25 lymph nodes of patients with CSD (12). The results, taken together, provide strong evidence supporting the central role of *B. henselae* in causing CSD. Despite the association of *B. quintana* with recently described disease syndromes primarily affecting immunocompromised patients, no evidence has been found linking this organism with CSD.

It is now widely accepted that *B. henselae* is the primary etiologic agent of CSD; however, at least three questions remain unanswered. First, despite a recent barrage of new diagnostic tests to detect evidence of *B. henselae* or other *Bartonella* spp., a small but significant percentage of CSD patients show no evidence of *Bartonella* infection. When the IFA assay is applied to serum samples from patients with the most strictly defined cases of CSD, 5 to 15% of the patients are negative (49, 156, 204). Percentages of CSD patients without evidence of *Bartonella* infection may be higher when other tests are used or when patient populations are not rigidly restricted. This may be due to the somewhat nonspecific criteria used for the case definition of CSD, which may not exclude other causes of lymphadenopathy. Second is the fulfillment of Koch's postulates for *B. henselae* and CSD or BA. While infectivity of *B. henselae* isolates can be demonstrated with cats, reproduction of either CSD or BA disease states in a suitable host has not been demonstrated. However, it should be emphasized that the tools available to the modern microbiologists are far more powerful than those available in Koch's time. Accordingly, a reconsideration of Koch's postulates in the modern era of molecular biology has been proposed (62). By using these revisions of Koch's postulates, "evidence of causation" can be documented for BA (62), as well as CSD. Finally, the role of *A. felis* in CSD should be addressed. Despite the isolation of *A. felis* from the lymph nodes of patients diagnosed with CSD (59), few data have supported a role for this organism in causing CSD. Serologic evidence is lacking, and neither the organism nor antibodies to it have been found in cats. Hence, the association of *A. felis* with the reservoir of CSD has not been made. It is likely that a number of studies attempting to associate *A. felis* with CSD have not been published in the scientific literature because of negative results. The association of *A. felis* with CSD must be questioned in light of extensive laboratory and clinical data implicating *B. henselae* in CSD. It is possible that *A. felis* is capable of causing lymphadenopathy and clinical symptoms that are similar to CSD. Although more studies are needed to completely rule out any role for *A. felis* (or perhaps another as yet unidentified organism), in at least a small percentage of CSD cases, it is clear from recent data that *B. henselae* is the primary agent causing CSD.

### EPIDEMIOLOGY

Among the disease syndromes attributed to *Bartonella* spp., trench fever and bartonellosis (or Carrión's disease), caused by *B. bacilliformis*, were the first to be described in the literature. Carrión's disease is a biphasic disease consisting of an acute hemolytic anemia (Oroya fever) and a chronic form (verruca peruana) that presents with vascular proliferative skin lesions similar to those seen with BA. Carrión's disease is thought to be confined to limited areas of South America (Peru, Ecuador, and Colombia) within the Andes mountains. The presumed vector, the sand fly (*Lutzomyia verrucarum*), has been found only in Peru, suggesting that an additional vector may be involved in transmission within Ecuador and Colombia (31).

Although the disease is confined to the regions of endemicity, occasional cases among travelers to South America have been reported in other countries (124). Evidence from the artifacts of pre-Columbian cultures suggests that the verruga peruana form of Carrión's disease was present in Ecuador at least 1,000 years prior to arrival of Europeans (3).

The causative agent of trench fever, *B. quintana*, is transmitted by the human body louse (*Pediculus humanus*). It was estimated that over 1 million troops were affected by louse-borne trench fever during World War I (192). Trench fever is characterized by fever, rash, bone pain, and splenomegaly and may occur as a single episode lasting 4 to 5 days (hence the name 5-day fever) as well as a single longer episode or multiple paroxysms (192). Reports of *B. quintana* infections became rare after World War II until the description of infections in human immunodeficiency virus (HIV)-infected individuals. Patients with fever and bacteremia and/or endocarditis have been reported in the United States and Europe during the 1990s (56, 176, 177, 178). In addition, *B. quintana* (as well as *B. henselae*) has been isolated from patients with BA (100). Chronic alcoholics and homeless individuals, as well as HIV-infected patients, seem to be at greatest risk of infection with *B. quintana* (56, 100, 176, 178). It is not clear if transmission of modern-day urban trench fever involves the human body louse or another as yet unidentified vector. The reason for the re-emergence of *B. quintana* infections after many years of apparent absence remains unclear (158).

Several studies and publications have suggested that cats, and in particular kittens, are the reservoir for *B. henselae* (39, 40, 53, 76, 89, 98, 104, 186, 189, 204). The role of cats in the transmission of the agent causing CSD is well established. Zangwill et al. found that patients with CSD were more likely to own a kitten 12 months or younger, to have been scratched or bitten by a kitten, and to have at least one kitten that was infested with fleas (204). Contact with kittens and cats can be documented in the vast majority of CSD cases. However, in a small percentage of CSD patients, no history of animal contact can be elicited (36, 50, 179). *B. henselae* was cultured from 41% of pet and impounded cats in San Francisco and was recovered from the blood of all seven pets belonging to four patients with BA (98). The study by Demers et al. in Hawaii emphasized the primary role of kittens in the transmission of CSD (53). These investigators found that older cats seldom have detectable levels of bacteremia with *B. henselae* as determined by isolation but that they often have serologic evidence of past infection. Serologic evidence also links cats and kittens with CSD; 81% of serum samples obtained from cats living in households of patients with CSD, as compared to 38% of control cats, have elevated antibody titers to *Bartonella* species (204). The seroprevalence of antibodies to *Bartonella* species in randomly tested cats is estimated to be 15 to 44% (39, 40, 204). Cats that transmit the infection are asymptomatic and do not show evidence of illness. In fact, Koehler et al. found that all pets of the four patients diagnosed with BA were bacteremic but asymptomatic (98). Long-term bacteremia of cats with *B. henselae* has been demonstrated (98, 104, 155). Chomel et al. found that in a convenience sample of 205 cats, 81 (39.5%) were bacteremic for *B. henselae* (40). They also found that bacteremic cats were more likely to be infected with fleas than were nonbacteremic cats. The role of fleas as a possible vector for the transmission has been speculated, and experimental infection of the cat flea (*Ctenocephalides felis*) with *B. henselae* has recently been demonstrated by using an artificial feeding device (82). In that study, *B. henselae* was detected in the flea gut and feces 9 days after infection, indicating that the organism was replicating and persisting in the flea host (82). It is likely that

flea feces or infected blood from flea feeding provides the inoculum by which cats spread *B. henselae* to humans. Experimental transmission of *B. henselae* between cats by way of a cat flea vector has recently been demonstrated (41). Direct transmission of *B. henselae* from cat fleas to humans, while theoretically possible, has not been demonstrated. Patients have reported tick bites prior to infection with *B. henselae* (113). However, in that report, no ticks were found and identified when the patients sought medical care related to the *B. henselae* infection. There have been no reports of the isolation or detection of *B. quintana* in cats. It has been suggested that cats without evidence of *B. henselae* infection may be more appropriate pets for immunocompromised individuals, who are at greater risk for developing severe *B. henselae* disease (14, 40, 98).

There are an estimated 24,000 cases of CSD yearly in the United States, resulting in 2,000 hospital admissions (87). Eighty percent of cases occur in children, with a peak in the incidence of cases between ages 2 and 14 years (35, 204). The incidence of the disease is higher in males (60%) and in whites (93). CSD is seasonal, with most cases recognized in the second half of the year (35). In temperate zones, the disease occurs predominantly in the fall and winter. Seasonal variation in the incidence of the disease may be accounted for by temporal patterns of breeding of house cats and the acquisition of kittens as family pets. Based on analysis of three national databases, the incidence of patients discharged from hospitals with a diagnosis of CSD is between 0.77 and 0.86 per 10,000 population per year, while the incidence of the disease in ambulatory patients is 9.3 per 10,000 population per year. The incidence is lower in the west and higher in the south than in the nation as a whole (87). Individual reports from numerous countries suggest a worldwide distribution (60, 189, 193). The estimated health-care cost of CSD in the U.S. is \$12 million per year (87). Clustering of cases within families has been noted in association with the acquisition of new pets.

#### CLINICAL PRESENTATIONS OF *BARTONELLA* INFECTION

Infection with *Bartonella* spp. results in disease syndromes with varied severity ranging from lymphadenopathy only (CSD) to systemic disease. The severity and presentation of disease is related to immune status. In general (excluding *B. bacilliformis* infections), immunocompetent patients who are otherwise healthy tend to present with classic CSD when infected with *B. henselae*. Patients who are immunocompromised by having AIDS, chronic alcoholism, immunosuppression, or other compromising health problems tend to have systemic disease. However, there have been reports of systemic disease such as persistent bacteremia (113), endocarditis (49, 77, 85, 88, 176, 177), and bacillary angiomatosis (43, 185) in immunocompetent patients. Conversely, CSD-like symptoms have been found in patients with AIDS (150). While the immune status clearly affects clinical presentation, differences in virulence among various strains of *Bartonella* may also be responsible for the varied disease presentation.

#### Infections in the Immunocompetent Patient: Cat Scratch Disease

CSD occurs primarily in children and young adults, with 80% being younger than 21 years of age (87, 117). In a patient with a history of cat contact or scratch, CSD typically presents as lymphadenopathy usually preceded by an erythematous papule at the inoculation site. The reported percentage of CSD



TABLE 3. Signs and symptoms associated with CSD<sup>a</sup>

Sign or symptom	% of patients affected (n = 268)
Adenopathy plus other(s).....	100
Adenopathy only .....	45
Fever (38.3 to 41.2°C).....	31
Malaise, fatigue.....	30
Anorexia, emesis.....	15
Headache.....	14
Splenomegaly .....	11

<sup>a</sup> Adapted from reference 117 with permission of the publisher.

patients with papules varies from 25% (204) to 94% (36). The higher percentage was reported by Carithers et al., who noted that small papules are often found on the scalp and that a search of 5 to 10 minutes may be necessary to find the primary lesion (36). It is also possible that in some patients the primary lesion resolved before the patient sought medical care. Lymph nodes draining the site of inoculation become enlarged and tender. Involved lymph nodes undergo sequential changes characterized by lymphoid hyperplasia, granuloma formation, microabscess development, and in some cases suppuration. Low-grade fever and malaise are seen in approximately 30% of patients (Table 3). Uncomplicated CSD-mediated lymphadenopathy usually resolves spontaneously in 2 to 6 months (117). Laboratory studies have shown that *B. henselae* isolates are susceptible to a number of different antibiotics in vitro (54, 125). However, antibiotic treatment is of questionable value for CSD and is not generally recommended in the absence of systemic complications (7, 25, 117, 120). Less often, patients experience rash, hepatosplenomegaly, lytic bone lesions, granulomatous conjunctivitis, pneumonitis, and central nervous system involvement (119, 121). Of patients with CSD, 5 to 20% present with manifestations other than regional lymphadenopathy. Complications resulting from CSD involve almost every organ system.

#### Complications of Cat Scratch Disease

Parinaud's oculoglandular syndrome (POS) is the most common unusual presentation of CSD (119). POS is manifested either as conjunctivitis with parotid area swelling caused by lymphadenitis or as an ocular granuloma (119). This unusual manifestation of CSD occurs in up to 2 to 17% of patients (33, 116). Although direct inoculation of the organism is possible, autoinoculation of the eye by rubbing it with the hands after cat contact is known to occur. Findings include conjunctival granuloma and preauricular adenopathy. The involved eye may show impressive swelling and discoloration, but pain and conjunctival discharge are usually lacking. POS usually resolves in 2 to 4 months without residual sequelae, although in rare cases it can result in temporary blindness (117).

Hepatic and splenic abscesses can occur in CSD patients, who usually present with fever of unknown origin (25, 52, 73, 111, 115, 161). Most of these patients complain of abdominal pain. Hepatosplenomegaly and lymphadenopathy may be absent. Almost all patients have normal liver function tests, normal to slightly elevated leukocyte counts, and elevated erythrocyte sedimentation rates. Ultrasound and computed tomography may be used to reveal lesions representing microabscesses (161). Microscopic examination of biopsied lesions usually reveals necrotizing granuloma, and the organisms are occasionally visualized by Warthin-Starry silver staining. Treatment with gentamicin results in prompt resolution of fever; however,

the abscesses resolve slowly (2 to 3 months) and may also resolve spontaneously without antimicrobial therapy.

Encephalopathy is the most frequent neurological manifestation of CSD, followed by cranial or peripheral nerve involvement (37, 38, 78, 112, 146, 171, 181). Other forms of central nervous system involvement with CSD may include encephalitis, meningitis, and myelitis (35). These neurological manifestations occur in 1 to 7% of patients with CSD. Recently, a cluster of cases of encephalitis associated with CSD among family members was described in South Florida (38, 140). The occurrence of multiple cases of encephalitis as a complication of CSD raises the question whether differences in virulence exist among strains of *B. henselae*. Alternatively, host factors may predispose some individuals to neurological involvement during infection with *B. henselae*. Children between the ages of 7 and 12 years appear to be more prone to encephalopathy (37, 112). The onset of neurological symptoms in CSD encephalopathy is sudden and is accompanied by fever in 50% of patients (35). Convulsion is often the first presenting sign. It occurs in 40 to 50% of patients and varies from mild twitching to status epilepticus (78). Detection of *B. henselae* antibodies in the spinal fluid suggests the possibility of direct invasion of the central nervous system tissue rather than a vasculitis resulting in encephalopathy (145). Recovery is usually rapid and occurs within 2 to 14 days of the illness; 78% of patients recover within 1 to 12 weeks, and in general all patients recover completely within 1 year.

A number of other complications can occur in association with CSD, including ophthalmic manifestations (74); pneumonia and pleural effusion, which occur in 0.2% of patients (101, 118); and musculoskeletal manifestations. Osteomyelitis is a rare manifestation, occurring in 0.3% of patients; it may affect any bone (2, 34, 36, 46, 172). Occasionally there is a direct extension from an adjacent involved lymph node. Patients are usually symptomatic, presenting with fever, malaise, and pain at the site of bone involvement. Bone biopsy may reveal granulomatous inflammation and central necrosis (136). CSD osteomyelitis usually resolves spontaneously in 4 to 20 months. Paravertebral abscess (21) has been reported and can present with back pain and few constitutional symptoms.

#### Clinical Diagnosis of Cat Scratch Disease

Diagnosis of CSD has traditionally required the presence of three of four criteria: contact with a cat resulting in a primary lesion, a positive skin test, regional lymphadenopathy in the absence of other causes of lymphadenopathy, and the presence of characteristic histopathologic features (35, 36, 195). Margileth recently published a modification of the second criterion indicating that serologic testing for antibodies to *B. henselae* is a suitable alternative to skin testing (119). The CSD skin test is prepared from heat-inactivated material obtained from a node of a patient fulfilling the diagnostic criteria of the disease. The skin test antigen is injected intradermally. The appearance of a delayed-type hypersensitivity response is evaluated at 48 to 96 h and is positive in 95 to 98% (134) of patients with a clinical diagnosis of CSD. Safety concerns about the use of these human-derived reagents and the lack of widespread availability has resulted in limited use of skin testing for CSD diagnosis. However, skin test antigens of laboratory-grown *B. henselae* have not yet been described. The identification and characterization of such antigens would be of great value in simplifying the diagnosis in the clinical setting and would eliminate the theoretical possibility of transmission of infectious agents in skin test antigen preparations of human origin. A history of animal contact, especially cat scratch, is the key to diagnosis.

The presence of a primary inoculation papule or pustule at the scratch site strengthens the diagnosis. Establishing a diagnosis of CSD can be challenging if the primary inoculation site has healed or is inapparent and no history of animal contact is elicited.

### Infections in the Immunocompromised Patient

The benign and generally self-limited nature of CSD may become life-threatening in the immunocompromised host. The spectrum of complications associated with *Bartonella* infection in patients who are immunocompromised by AIDS (79, 92, 99, 110, 149, 150, 167, 176), chronic alcoholism (56, 178), or immunosuppressive therapy (15, 32, 137) appears to be even greater than that observed in the immunocompetent patient. The atypical manifestations of CSD or infection with *Bartonella* species in adult patients with AIDS have been reported with increased frequency. Manifestations include cutaneous bacillary angiomatosis, extracutaneous lesions, bacillary peliosis hepatis, and fever with bacteremia (see reference 1 for a review). Many of these disease syndromes have been attributed to both *B. henselae* and *B. quintana*. Involvement of the central nervous system by infection with *B. henselae* may play a role in AIDS encephalopathy (80, 145, 170). *B. henselae* infection in some patients with AIDS-related dementia has been demonstrated based on both serologic testing and PCR (145, 170).

BA is a common clinical presentation of *Bartonella* infection in the immunocompromised individual. The characteristic lesions are reddish vascular papules or nodules (92, 109, 183). They may be solitary but are usually multiple and tender. Patients may also have other symptoms indicating systemic involvement, such as fever, chills, malaise, headache, anorexia, and weight loss. Clinically these lesions may be indistinguishable in physical appearance from those of Kaposi's sarcoma (especially atypical forms), epithelioid hemangioma, and pyogenic granuloma. Examination of skin biopsy specimens by light microscopy is the most common method for confirming the diagnosis (109). Alternatively, PCR can be used to detect *Bartonella* in DNA extracted from skin lesions. The most characteristic histologic feature is proliferation of vascular endothelial cells. Neutrophils are scattered throughout the lesion, especially around eosinophilic granular aggregates, which consist of masses of bacteria when examined by Warthin-Starry staining. Unlike CSD, which is thought to be caused by *B. henselae*, both *B. henselae* and *B. quintana* have been isolated from patients with BA. BA has been found in immunocompetent and immunodeficient individuals. The length of treatment of this form of the disease varies according to the immune status of the patient. In immunocompetent patients, treatment for 2 to 4 weeks may be sufficient, while in HIV-positive patients, treatment should be extended to months or longer if a relapse occurs. Erythromycin appears to be the drug of choice based on the excellent response reported (165). Doxycycline is also effective in some cases (135). Occasionally, patients with BA undergo spontaneous regression (45).

Cutaneous BA may also present with extracutaneous manifestations varying from subclinical to life-threatening infection. *Bartonella* infection may be associated with local or generalized complications (45) or overwhelming disseminated infection (1, 45). In addition to extracutaneous lesions, multiple cutaneous lesions may develop (128). BA may involve the respiratory and gastrointestinal mucosa (45, 183) or the heart, liver, spleen, bone marrow, muscles, or lymph nodes (67, 92, 128, 150, 160, 167, 174, 180, 185). Several reports have described bone infection related to BA (17, 100, 110). Bacillary peliosis hepatis is a common extracutaneous presentation of BA that may occur as

an isolated condition or in the course of disseminated disease (67, 175, 202). Symptoms include fever, nausea, vomiting, diarrhea, and abdominal distension. Hepatosplenomegaly is usually present on physical examination. Diagnosis is aided by histopathologic examination including Warthin-Starry staining or PCR. Successful treatment with erythromycin has been reported (149).

Fever with bacteremia is another common clinical presentation of *Bartonella* infection and has been found in both immunocompetent and immunodeficient individuals (113, 174, 202). Patients usually present with fatigue, anorexia, and fever without an obvious focus of infection. Blood cultures can be positive for *B. henselae*. Relapsing fever with bacteremia can occur in both immunocompromised and immunocompetent patients (113). Endocarditis caused by infection with *B. henselae* (77, 85), *B. quintana* (176, 177), or *B. elizabethae* (49) may result from bacteremia. The infecting species of *Bartonella* can usually be isolated by using direct plating of blood, blood culture broth, or lysis-centrifugation techniques. Extended courses of antibiotic therapy are usually required to prevent relapses.

### LABORATORY DIAGNOSIS

Until recently, little attention has been paid to existing species of *Bartonella* (i.e., *B. quintana* and *B. bacilliformis*), and some species have only recently been described (i.e., *B. henselae* and *B. elizabethae*). As a result, laboratory diagnostic procedures are only now being optimized and standardized. Among these, isolation, PCR, and serologic testing have all been used successfully by clinical and research laboratories. The type of specimen collected and the facilities and equipment available may influence the choice of laboratory procedures used to confirm *Bartonella* infections. Direct detection of the organisms or their nucleic acids is a viable option if fresh or frozen tissue or blood is available, whereas serologic testing or isolation is probably the most practical means of diagnosis.

Isolation of *Bartonella* is typically time-consuming, often requiring a 2- to 6-week or longer incubation for primary isolation. The resulting isolate must then be identified by biochemical or genetic methods. However, the time required is offset by the fact that isolation does not require special media or equipment not commonly found in the clinical microbiology laboratory and is not technically difficult. In general, isolation or detection of *B. henselae* from blood is not successful for CSD patients who have no evidence of systemic disease. Conversely, isolation of *B. henselae* or *B. quintana* from the blood of immunocompromised patients, chronic alcoholics, or patients with evidence of systemic disease is usually possible.

PCR offers a rapid and specific means to detect the organism directly from clinical samples. PCR is more sensitive than isolation when performed on suitable clinical samples such as fresh or frozen lymph node tissue or blood samples collected in EDTA from patients with systemic disease. Obviously, PCR is an alternative only for large clinical or research laboratories that have the necessary equipment (thermal cycler, electrophoresis apparatus, etc.) and expertise. Direct detection of *Bartonella* spp. by immunochemical staining has also been described. Although this technique may also be limited to use by larger laboratories, it has been applied successfully directly to tissue samples (129, 152).

Perhaps the most practical means of confirming current or prior infection with *Bartonella* species is serologic testing. Apparently, patients with uncomplicated CSD and patients with systemic disease mount both immunoglobulin G (IgG) and IgM antibody responses by the time they present to the physician with clinical symptoms. Serologic assays to detect IgG



and/or IgM antibodies that are genus specific have been described in the literature (16, 81, 84, 97, 145, 151, 170, 184). Commercially available serologic tests for anti-*Bartonella* antibodies in human serum have recently become available. Serologic methods provided the first laboratory data supporting a *Bartonella* species as an etiologic agent of CSD (156). In addition to the three common methods of serologic testing, PCR, and isolation, other methods for laboratory-based diagnosis or presumptive diagnosis, such as immunostaining and specialized silver staining, are described in the following sections.

#### Histopathologic Examination

*B. henselae* or *B. quintana* infection resulting in BA usually produces cutaneous or subcutaneous lesions. Histopathologic examination of skin biopsy specimens by light microscopy may be used to confirm the diagnosis. Characteristic lobular proliferation of blood vessels can be seen in the lesions upon staining with hematoxylin and eosin. Granuloma formation is not usually observed in specimens from patients with BA. Neutrophils are observed and may appear to contain granular aggregates consisting of clumps of bacteria upon Warthin-Starry staining. Both *B. henselae* and *B. quintana* have been cultured from the lesions of patients with BA (100). The histopathologic findings of affected lymph nodes from patients with CSD typically include stellate caseating granulomas, microabscesses, and follicular hyperplasia (35). The primary inoculation lesion in patients with CSD usually exhibits necrosis and, like affected lymph nodes, may reveal bacilli upon Warthin-Starry staining (122, 196).

Warthin-Starry silver staining has proven valuable in the detection of the etiologic agents of both BA and CSD. The stain was originally used to visualize spirochetes and reveals brown to black bacilli against a background of pale yellow to amber. Warthin-Starry staining of lymph node material provided the first concrete evidence that the etiologic agent of CSD was in fact a bacterium (196). The finding of similar bacillary forms in the skin lesions of patients with BA to those previously observed in CSD patients provided the first hint that the etiologic agents of these diseases might be the same or similar bacteria (99, 110). We now know that both *A. felis* and *B. henselae* stain well by the Warthin-Starry method.

Immunohistochemical staining of *B. henselae* in lymph node biopsy specimens from patients with CSD has been described (129). Polyclonal rabbit serum raised to *B. henselae* was used to detect bacilli of two different morphologic types in the lymph node biopsy specimens. Some cross-reactivity between the anti-*B. henselae* polyclonal rabbit serum and *B. quintana* was noted. This cross-reactivity was reduced by absorbing the rabbit anti-*B. henselae* serum with *B. quintana* prior to use for immunocytochemical staining. Reed et al. had previously used similar techniques to detect *Bartonella* spp. in formalin-fixed, paraffin-embedded tissue from patients with BA and bacillary peliosis hepatis (152).

#### Isolation and Culture

Suitable samples for isolation of *Bartonella* species may include blood, lymph node tissue or aspirates, or biopsy specimens of skin or other involved organs. For patients with CSD without systemic involvement, lymph node samples are preferred over blood. Successful isolation of the organisms from lymph node tissue may depend on collecting specimens for isolation early in the course of disease. By the time the involved lymph node is suppurative, viable organisms may not be found because the lymphadenopathy seen with CSD is believed to be due primarily to an aggravated cellular immune response

to bacterial antigenic components rather than an invasive bacterial process (70). For patients with relapsing fever, endocarditis, BA, peliosis hepatis, or other presentations of systemic disease caused by *Bartonella* spp., the organisms can usually be isolated from blood samples. Several methods of isolation have been described; however, a comparison of the relative sensitivity of each method has not been published.

The first published description of the isolation of *B. henselae* was of patients having fever and bacteremia but lacking vascular proliferative lesions. In that study, organisms that were described as *B. quintana*-like were isolated from blood following lysis-centrifugation (174). These isolates were later identified as *B. henselae* (202). Material derived from centrifugation after blood cell lysis was plated on chocolate agar and sheep blood agar. The plates were incubated for a minimum of 14 days at 35°C in 5% carbon dioxide. Adherent white iridescent colonies that displayed morphological heterogeneity were noted after extended incubation. Upon subculturing on heart infusion agar supplemented with 5% rabbit blood, colonies grew more rapidly and appeared after 4 days (202). These colonies were shown to contain small, curved gram-negative rods measuring 0.6 by 1.0 µm. The organisms exhibited autoadherence and twitching motility, yet no flagella were observed.

In subsequent studies, both direct plating of blood collected in EDTA and lysis-centrifugation were used successfully to isolate both *B. henselae* and *B. quintana* (100, 113, 154, 174). Lysis-centrifugation treatment of blood (ISOSTAT system; Wampole Laboratories, Cranbury, N.J.), while requiring more extensive manipulation of specimens, has been reported to be more sensitive than direct plating for isolating *Bartonella* spp. (113). Regardless of which method is used, extended incubation of the primary isolation plates is required. A number of different plating media have been used, including Trypticase soy agar with 5% sheep blood, chocolate agar, and heart infusion agar with 5% rabbit blood. In addition, biphasic medium has been used to isolate *Bartonella* spp. (113). Isolations have also been made from the lymph nodes of patients with CSD by dispersing excised lymph nodes with a tissue grinder. The resulting homogenate was plated directly onto chocolate agar. In that study, isolates were obtained from two patients after extended incubation of the primary isolation plated for 13 and 33 days, respectively (54).

Cell culture systems have also proven valuable in isolating *Bartonella* spp. from both blood and tissue samples from infected patients. Koehler et al. described the isolation of both *B. henselae* and *B. quintana* from skin lesions of patients with BA by using a bovine endothelial cell line (CPA; ATCC cell line 207) (100). In that study, tissue biopsy specimens were minced and homogenized and inoculated onto CPA monolayers. After 9 to 36 days of incubation, the cell culture supernatant became turbid and was subcultured onto solid medium. More recently, Drancourt et al. also isolated *B. quintana* from the blood of homeless men with endocarditis. In that study, an immortalized human endothelial cell line (ECV 304) was used to isolate *B. quintana* by cocultivation (56). In that study, *B. quintana* was isolated from two patients by using the endothelial cell line and from one patient by plating of blood culture broth onto solid culture medium.

Recently, a defined RPMI 1640-based liquid medium supplemented with pyruvate, hemin, and amino acids was used successfully to isolate *B. henselae* (203). In that study, *B. henselae* was isolated from lymph node tissue and blood of patients suspected of having CSD as well as from the blood of a cat associated with one of those patients. The authors noted that the liquid medium yielded more isolates than did the

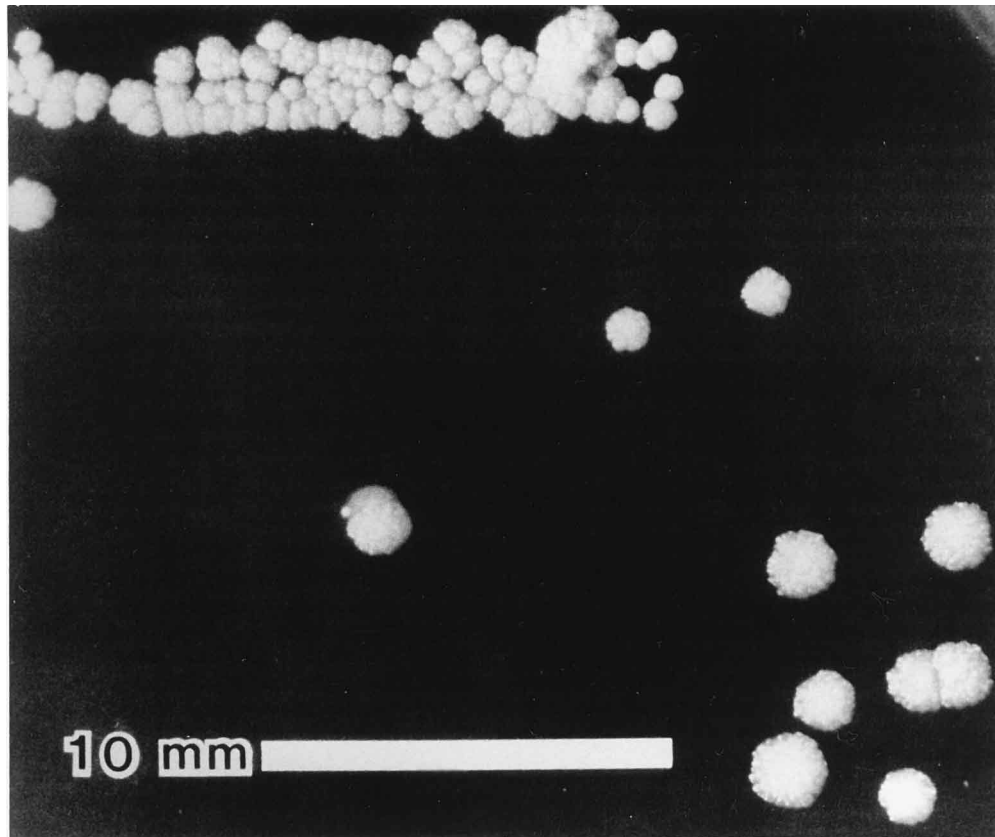


FIG. 2. Colony morphology of *B. henselae* on blood agar. Colonies depicted are low passage number (fourth) after primary isolation and were incubated for 24 days. Reproduced from reference 154 with permission of the publisher.

solid-phase media used in their study. Other liquid media for the cultivation of *Bartonella* spp. have been reported (169, 198) but have not been used for isolation purposes.

Automated blood culture systems have also been used to isolate different species of *Bartonella*. However, automated detection of CO<sub>2</sub> production does not work well with *Bartonella* spp., because these organisms do not generate sufficient CO<sub>2</sub> to give a positive signal (113). Additionally, the process of isolation and identification of *Bartonella* spp. in standard blood culture bottles is lengthened by the need to subculture them onto solid medium (106, 113). *Bartonella* spp. can be detected in blood culture broth by acridine orange staining after approximately 8 days of incubation; however, the organisms must be subcultured for identification. It must be emphasized that isolation of *Bartonella* spp. by using routine blood culture bottles, like all other methods of isolation or growth, requires extended incubation (more than 1 week) beyond what is routine for most clinical laboratories.

#### Identification of Isolates

The identification of *Bartonella* isolates to the genus level is relatively straightforward based on the properties listed below. Identification of *Bartonella* isolates to species level is somewhat more difficult. Genetic methods including DNA-DNA hybridization, PCR-based methods, reactivity with specific mouse serum, biochemical testing, and fatty acid analysis have been used for this purpose with varying results. Some of these methods are not practical for most clinical laboratories, but it is important to remember that species-level identification is not

required in all clinical situations. For instance, classic CSD has been associated only with *B. henselae*, and it is reasonable to assume that the presence of *Bartonella* spp. in patients with CSD can be interpreted as the presence of *B. henselae*.

Fresh isolates of both *B. henselae*, *B. elizabethae*, and *B. quintana* have characteristic colony morphology and associated properties. Colonies grown on chocolate or blood agar plates appear as small white autoadherent colonies, irregular in both size and shape (Fig. 2). Optimal growth conditions appear to be 34 to 37°C with 5% supplemental CO<sub>2</sub>. Hemin is required for growth. Upon primary isolation and before extensive subculturing, isolates of both organisms adhere very strongly to both the agar surface and other cells. A corrosive pitting of and adherence to the agar surface has been reported for fresh isolates of *B. elizabethae* (49), *B. quintana* (100), and *B. henselae* (154). It is thought that this autoadherence is due to the presence of type IV pili on the surface of the organisms (18). Subculturing of the organisms appears to result in the loss of the autoadherent phenotype and in more rapid colony growth, with visible colonies appearing after 2 to 3 days of incubation. A correlation between autoadherence of *B. henselae* and increased expression of pili has been demonstrated (18). The colony morphology of *B. bacilliformis* is somewhat different, with colonies appearing small and translucent. Among the few existing isolates, autoadherence like that seen with *B. henselae* and *B. quintana* is not observed. However, two morphologically distinct phenotypes for *B. bacilliformis* have been described (194). It is not known if the autoadherent phenotype is lost upon subculturing of the *B. bacilliformis* strains. In addition, *B. bacilliformis* favors different

growth conditions including lower temperature (25 to 28°C) and no supplemental CO<sub>2</sub>. Clarridge et al. recently reported that colony morphology and growth characteristics can be used by those experienced with handling *Bartonella* spp. to differentiate all the pathogenic species (42).

Isolated colonies of *Bartonella* spp. contain cells that have been described as coccobacilli or bacilli measuring approximately 0.6 by 1.0 μm (200). These somewhat pleomorphic cells can be visualized by light microscopy with a number of different stains. Upon Gram staining, *Bartonella* spp. stain lightly to reveal curved pleomorphic rods (49, 174, 200). Acridine orange has also been used to detect *Bartonella* spp. in blood culture bottles. Larson et al. determined that acridine orange staining of BACTEC system blood cultures is a sensitive alternative to the lysis-centrifugation system for the isolation of *B. quintana* (106). In that study, it was noted that while the organisms may be detected by acridine orange staining before colonies appear on solid medium in the lysis-centrifugation technique, they must be subcultured on solid medium for definitive identification (106). Gimenez stain, a stain used primarily for rickettsiae, is also useful for visualizing *Bartonella* spp. in cell culture systems (71). In the Gimenez procedure, *Bartonella* organisms stain with carbol fuchsin and host cells and debris counterstain with malachite green (154).

In general, *Bartonella* spp. are biochemically inert (200). Carbohydrate utilization cannot be demonstrated by conventional testing or the use of nitrophenyl substrates (202). *B. quintana* and *B. vinsonii* apparently lack glycolytic enzymes but metabolize succinate, pyruvate, and glutamine (86, 199). The oxidative metabolic activity of *Bartonella* spp. is further supported by the presence of the citrate synthase gene (141). Welch et al. reported the hydrolysis of L-arginyl-L-arginine and L-lysyl-L-alanine by *B. henselae* and *B. quintana* but not *B. vinsonii* by using rapid test methods to measure preformed enzyme activity (202). *B. quintana* and some *B. henselae* isolates weakly hydrolyzed L-seryl-L-tyrosine (202). However, others have reported slightly different patterns of peptidase activity (42, 57), suggesting that strain variation or subtle differences in methods (such as the addition of hemin to the bacterial suspension) may give different results. Welch et al. have noted that the MicroScan Rapid Anaerobe Panel can be used to distinguish between *B. henselae* and *B. quintana* (201).

Fatty acid composition has been assessed for *Bartonella* spp. by gas-liquid chromatography of methyl ester derivatives (49, 106, 113, 202). Organisms were cultivated on blood agar plates and harvested after 5 to 7 days of cultivation. The fatty acid profiles of *B. henselae*, *B. quintana*, and *B. vinsonii* were similar to each other but significantly different from that of *B. bacilliformis* (202). Minor differences were observed between the C<sub>18:0</sub> composition of *B. henselae* (22 to 25%) and *B. quintana* (16 to 18%). *B. vinsonii* was also different from the other species with respect to C<sub>18:0</sub> composition (202).

A number of genetic methods to allow species-level identification of *Bartonella* isolates are available. Of these, DNA-DNA hybridization is probably the most definitive but is clearly not practical in all but the largest laboratories with access to reference strains. PCR amplification of various genes or intergenic regions and restriction endonuclease analysis of the resulting amplicons has been used successfully. Analysis of the citrate synthase gene has been used to identify isolates to the species level. In that procedure, an amplified fragment of the citrate synthase gene from a given isolate is digested with certain endonucleases and characteristic patterns are obtained for each species (154). The resulting pattern must then be compared to those of existing reference strains for identification. Joblet et al. recently sequenced the amplified citrate syn-

these PCR product to identify *Bartonella* spp. (90). Matar et al. used primers in the 16S rRNA and 23S rRNA genes to allow the amplification of a region between these two genes and a portion of the 23S rRNA gene (123). When digested with the restriction endonucleases *AluI* and *HaeIII*, characteristic patterns were obtained for *B. quintana*, *B. vinsonii*, and *B. bacilliformis*; however, two different patterns were obtained with *B. henselae* strains, suggesting that the technique may be of value for subtyping (123, 178). More recently, Birtles described a method of differentiating all species (including existing human pathogens and newly described isolates from animals) by using a segment of the 16S rRNA gene as a substrate for digestion with the restriction endonucleases *DdeI* and *MnII* (22). Recently, repetitive extragenic palindromic PCR has been shown to be a useful tool to identify isolates of *Bartonella* to the species level (42, 163). In addition, differentiation of variants within species by this technique has also been reported (163). A PCR-based method was recently used in The Netherlands to determine that two variants can be found in lymph node tissue of CSD patients (20).

### PCR

The use of nucleic acid detection techniques, specifically broad-range PCR coupled with nucleotide sequencing, has been of paramount importance for the initial characterization of *B. henselae* and for the association of both *B. henselae* and *B. quintana* with a variety of disease syndromes. In broad-range PCR, primers to highly conserved regions of the bacterial 16S rRNA gene are constructed (6, 157). Since the gene coding for the 16S rRNA gene is highly conserved, it is possible to design primers that theoretically permit PCR amplification of this gene from any eubacterium. Hence, the primers and the technique are often referred to as "universal" or eubacterium specific. Broad-range PCR coupled with sequencing has been used successfully to detect and identify bacterial pathogens that are refractory to culture (6, 157, 160).

Relman et al. first used broad-range PCR to detect bacterial 16S rRNA gene sequences in DNA extracted from formalin-fixed tissue obtained from the skin lesions of four patients with BA. The sequences that were obtained from the bacterial 16S rRNA gene fragment from three of the four patients were close to but slightly different from that of *B. quintana* (160). The fourth sequence was identical to the corresponding sequence of *B. quintana*. This description of bacterial 16S rRNA sequences in skin lesions provided, in the absence of culturing, the first evidence that an organism(s) closely related to *B. quintana* was the etiologic agent of BA. Additionally, the data of Relman et al. provided the first hint that both *B. henselae* and *B. quintana* are capable of causing BA, although the authors attributed the slight differences noted between the sequences to *Taq* polymerase incorporation errors at positions where DNA was damaged by formalin fixation (160). Both organisms have subsequently been isolated from BA lesions (100). Variations of the technique of broad-range PCR plus sequencing have been used by a number of other investigators to detect and identify *B. henselae* in skin lesions (100, 185), blood (113), CSD skin test antigen preparations (10), and cultured organisms (154). Likewise, similar methods have been used to identify *B. quintana* in blood, tissue, and cultured organisms (56, 88, 100, 126). The determination of the 16S rRNA gene sequence from the subsequently isolated type species of *B. henselae* (Houston-1) permitted comparison with the partial sequence found by Relman et al. (160) in BA skin lesions. Although speculated to be the case from partial-sequence analysis, the 16S rRNA gene sequence obtained from



the type strain of *B. henselae* was identical to the sequence obtained from the patients with BA (154).

Broad-range PCR plus sequencing remains an effective way to identify *Bartonella* spp. from both culture and clinical specimens, although this method has inherent drawbacks. Because of the lack of specificity in working with PCR primers that are reactive with virtually all bacteria, problems with contamination are magnified. Careful reagent preparation and isolation of setup and preparation areas of the laboratory are needed (6, 157). In addition, because of the high level of conservation of the 16S rRNA gene among bacteria, the need to carefully sequence each amplified product limits the use of this technique to larger clinical laboratories or research facilities. Accordingly, a number of investigators have used PCR primers that are *Bartonella* specific and allow the detection of organisms directly in clinical samples.

The first such PCR primers designed by Relman et al. allow the amplification of a 241-bp fragment from the 16S rRNA gene (160). The primers used in that study were designed before the sequence of the 16S rRNA gene was available for members of the genus other than *B. quintana*, and the specificity of the technique with known strains of *Bartonella* has not been evaluated. Subsequently, primers that were derived from the less highly conserved *htrA* gene and allowed specific amplification of a 414-bp fragment from both *B. henselae* and *B. quintana* but not *B. elizabethae*, *B. vinsonii*, or *B. bacilliformis* were described (12). The resulting PCR amplicon can then be used as a target for species-specific oligonucleotide probes to differentiate the major pathogens of the genus, *B. henselae* and *B. quintana* (12). This method was used to specifically detect *B. henselae* in 21 of 25 lymph node samples from patients with suspected CSD and was shown to correlate well with serologic testing (12) (Table 4). In addition to lymph node biopsy specimens or lymph node aspirates (12, 75), this method has been successfully applied to a conjunctival swab sample obtained from a patient suspected of having CSD (108). More recently, Bergmans et al. described primers derived from the 16S rRNA gene that allow amplification of *Bartonella* spp. They also used an oligonucleotide probe to confirm the presence of *B. henselae* or *B. quintana* in clinical samples; however, the probes they described fail to differentiate these two organisms (19). The detection of *B. bacilliformis* in blood samples and skin biopsy specimens has previously been described (114). That procedure was described before gene sequences were available for other species within the genus *Bartonella*, and accordingly, it is likely that the primers described in that report also amplify other *Bartonella* spp.

Perhaps the simplest method of differentiating *Bartonella* spp. is with the use of specific antibodies. Slater et al. described the production in mice of antibodies specific for *B. henselae* and *B. quintana* (173). They were able to use the specific murine antibodies in an immunofluorescence assay to differentiate between *B. henselae* and *B. quintana*; cross-reactivity was noted only at the lowest dilutions of mouse serum. Application of this technique to a broad range of *B. henselae* and *B. quintana* isolates has not been described. Regardless, the use of specific polyclonal antiserum or monoclonal antibodies would be of value in simplifying species-level identification of *Bartonella*.

### Serologic Testing

Detection of antibodies to *Bartonella* spp. for diagnosis is advantageous in that it avoids many of the problems associated with other methods, such as lengthy incubation periods (for isolation), collections of samples by invasive means (lymph

TABLE 4. Comparison of PCR plus dot blot hybridization and serologic testing on samples from patients diagnosed with CSD<sup>a</sup>

Patient no.	State	Sample	Result of:	
			<i>B. henselae</i> PCR <sup>b</sup>	Serologic testing <sup>c</sup>
1	Mass.	Aspirate	+	+
2	Mass.	Biopsy	+	+
3	Mo.	Biopsy	+	+
4	Fla.	Biopsy	+	+
5	Fla.	Biopsy	+	+
6	Ohio	Biopsy	+	+
7	S.C.	Biopsy	-	+
8	N.J.	Biopsy	+	+
9	Va.	Biopsy	-	+
10	N.J.	Biopsy	+	+
11	N.J.	Biopsy	+	+
12	Pa.	Biopsy	+	+
13	Mass.	Aspirate	+	+
14	W.Va.	Biopsy	-	+
15	Maine	Biopsy	+	+
16	N.C.	Biopsy	+	+
17	Wash.	Biopsy	+	+
18	Mass.	Aspirate	+	+
19	Ga.	Biopsy	-	-
20	Tenn.	Aspirate	+	+
21	Tenn.	Aspirate	+	+
22	Tenn.	Aspirate	+	+
23	Fla.	Aspirate	+	ND <sup>d</sup>
24	Tenn.	Aspirate	+	ND
25	Va.	Aspirate	+	ND

<sup>a</sup> Adapted from reference 12 with permission of the publisher.

<sup>b</sup> PCR followed by dot blot hybridization with a *B. henselae*-specific probe (12).

<sup>c</sup> Serologic testing performed by the IFA test, with an anti-*Bartonella* titer of 64 or higher considered positive.

<sup>d</sup> ND, not done.

node excision or aspiration), or the use of specialized equipment (DNA sequencing, gas-liquid chromatography) or techniques (DNA hybridizations). Serologic methods played a pivotal role in implicating *Bartonella* spp., rather than *Afpia felis*, as the primary agent of CSD. In addition, serologic methods, specifically the IFA assay, have been the most thoroughly evaluated and applied means of laboratory diagnosis of *Bartonella* infection (48, 156, 204). Drawbacks of serologic testing for diagnosis include an apparent lack of species-specific antibody response in humans. Cross-reactivity among *Bartonella* spp. and between *Bartonella* spp. and *Chlamydia psittaci* has been described (84, 95). Another limitation inherent with serologic testing is the inability to determine if antibody levels represent active or prior infection. Both the IFA assay and immunoassays for detecting the IgG and IgM antibody response to *Bartonella* antigens in serum have been described.

The IFA assay was developed at the Centers for Disease Control and Prevention (CDC) for the serodiagnosis of BA. In the process, "control" serum samples from patients with CSD were found to be reactive with *Bartonella* antigen preparations, leading investigators to assess the role of *Bartonella* spp. in causing CSD (153). Subsequently, 88% of serum samples from patients diagnosed with CSD were shown to be reactive with *B. henselae* antigen by the IFA assay (156). None of the BA or CSD serum samples were reactive with the type strain (Fuller) of *B. quintana*, which had been repeatedly subcultured and apparently had a smooth phenotype. Thus, it was assumed that the human antibody response measured by the IFA assay was species specific. When a more recently isolated (rough) strain of *B. quintana* was used, strong cross-reactivity between serum

samples from patients infected with *B. henselae* or *B. quintana* was observed (193). These results are suggestive of a major immunodominant antigenic determinant being lost during phase variation or, alternatively, of coordinate regulation of a number of antigenic proteins that are lost in the transition to the smooth colony phenotype. Apparently, few antigenic proteins of *B. henselae* are recognized by sera from patients with CSD as determined by immunoblot analysis (11).

In addition to the phase variation problem, development of the IFA assay was hampered by the autoagglutination of *B. henselae*. Since the organisms tend to form clumps when cultivated on solid medium, the detection of specific immunofluorescent bacilli was difficult. The use of rough colony phenotypes of either *B. henselae* or *B. quintana* resulted in clusters of organisms when viewed by fluorescence microscopy and it was difficult to differentiate specific from nonspecific immunofluorescence of the *Bartonella* bacilli. To avoid this problem, Regnery et al. cocultivated *B. henselae* with Vero cells. The organisms were found to adhere to the surface of the Vero cells, providing individual bacilli to examine for specific immunofluorescence (156). The organisms apparently adhered to a greater extent to the Vero cells than to each other.

In the first report evaluating the diagnostic potential of the IFA assay, IgG titers to *B. henselae* in serum of 64 or greater were demonstrated in 38 (84%) of 45 of patients diagnosed with CSD (204). Only 4% of age-matched controls of patients who owned cats had titers of 64 or higher. Dalton et al. describe the use of the IFA assay for over 3,000 serum samples submitted to the CDC for *Bartonella* serology (48). In that study, antigen preparations of *B. henselae*, *B. quintana*, and *B. elizabethae* were used to determine IgG levels in serum. When their sample was restricted to 91 patients meeting a strict clinical definition of CSD, 86 (95%) had antibody titers to either *B. henselae* or *B. quintana* of 64 or higher. In patients for whom paired serum samples were available, 87 (66%) of 132 had a fourfold (or greater) rise or fall in titer (48). Thus, even with paired serum samples, it may be difficult to discern active from prior infection by the IFA assay, since antibody titers may be above those of controls for a year or more (48). The evaluation of the IFA assay on large numbers of patients with BA has not been reported. The CDC provides limited amounts of *B. henselae* and *B. quintana* antigens prepared in Vero cells for diagnostic serologic testing, and commercial testing is also available (Microbiology Reference Laboratories, Cypress, Calif.). Other researchers have found that IFA-based assays have marginal predictive values for the diagnosis of CSD (151). The difference in performance among different laboratories using the IFA assay may reflect different methods and techniques or a less rigid case definition of CSD resulting in the inclusion of patients with other diseases in the later study.

A number of enzyme immunoassays (EIA) to detect antibodies to various *Bartonella* spp. have been developed. The first was developed by Herrman et al. to diagnose trench fever (81) and was later modified by Hollingdale et al. (84). For that EIA procedure, a soluble antigen was prepared by sonication and centrifugation. Cross-reactivity with both the typhus group and the scrub typhus group rickettsiae was observed (84). Knobloch et al. have described the IFA assay, indirect hemagglutination, and EIA for the detection of antibodies in the serum of patients infected with *B. bacilliformis* (97). Since the identification of *B. henselae* and the recognition of *B. henselae* and *B. quintana* as human pathogens, a new wave of EIA has appeared. Patnaik et al. described an EIA that utilizes whole formalin-fixed *B. henselae* as the antigen (145). They noted no cross-reactivity with serum samples with high antibody titers to a number of other pathogens including *A. felis* and suggested

that the EIA is more sensitive than the IFA assay; however, few data are provided to support the claim. The EIA was used to demonstrate significant antibody levels in both serum and cerebrospinal fluid from HIV-infected patients with suspected central nervous system involvement (145, 170). More recently, Szalc-Kelly et al. developed an EIA for detecting both IgG and IgM with whole *B. henselae* cells as an antigen. They found that in their assay for anti-*Bartonella* IgM, the response was slightly less sensitive than that reported for the IFA assay (184). Additionally, they concluded that their IgG EIA is not sensitive enough to use for the diagnosis of CSD.

## CONCLUSIONS

The genus *Bartonella* contains two newly described human pathogens as well as a number of organisms that have been isolated strictly from animals. As these organisms become more thoroughly characterized, we may begin to understand their biology and the natural history of the diseases that they cause. Undoubtedly, questions about the role of animal reservoirs and vectors in the transmission of these agents will be answered. It is also possible and perhaps even likely that new species within the genus *Bartonella* will be described and that species currently viewed as nonpathogenic for humans will be shown to cause human disease. This is remarkable, since diseases caused by *Bartonella* spp. only 5 years ago were viewed as medical curiosities. *Bartonella* spp. (with the exception of *B. bacilliformis*) were viewed as bacteria from the past. With the recent identification of *Bartonella* spp. as emerging infectious agents, clinicians and microbiologists are becoming aware of the need for more efficient and practical means of laboratory diagnosis. Prompt laboratory confirmation of *Bartonella* infections could lead to effective antibiotic treatment in the immunocompromised host or in cases involving systemic infection. Currently, serologic methods offer the most practical and effective means of diagnosis. Isolation and identification of *Bartonella* spp. from clinical specimens is an effective alternative but requires additional time due to the lengthy incubation periods required. Diagnosis of uncomplicated cases of CSD by laboratory methods could eliminate the need for the excision or aspiration of lymph nodes (and the associated invasive procedures). Clearly, as our knowledge of the characteristics of *Bartonella* organisms increases, we will be better prepared to develop more effective means of diagnosis, treatment, and prevention of diseases caused by these unique organisms.

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