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Culture of *Bartonella quintana* and *Bartonella henselae* from Human Samples: a 5-Year Experience (1993 to 1998)

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***Bartonella quintana* and *Bartonella henselae* are fastidious gram-negative bacteria responsible for bacillary angiomatosis, trench fever, cat scratch disease, and endocarditis. During a 5-year period, we received 2,043 samples for culture of *Bartonella* sp. We found *Bartonella* sp. to be the etiologic agent in 38 cases of endocarditis, 78 cases of cat scratch disease, 16 cases of bacteremia in homeless people, and 7 cases of bacillary angiomatosis. We correlated the results of positive cultures with the clinical form of the disease, type of sample, culture procedure, PCR-based genomic detection, and antibody determination. Seventy-two isolates of *B. quintana* and nine isolates of *B. henselae* from 43 patients were obtained. Sixty-three of the *B. quintana* isolates and two of the *B. henselae* isolates, obtained from patients with no prior antibiotic therapy, were stably subcultured. The sensitivity of culture was low when compared with that of PCR-based detection methods in valves of patients with endocarditis (44 and 81%, respectively), skin biopsy samples of patients with bacillary angiomatosis (43 and 100%, respectively), and lymph nodes of cat scratch disease (13 and 30%, respectively). Serological diagnosis was also more sensitive in cases of endocarditis (97%) and cat scratch disease (90%). Among endocarditis patients, the sensitivity of the shell vial culture assay was 28% when inoculated with blood samples and 44% when inoculated with valvular biopsy samples, and the sensitivity of both was significantly higher than that of culture on agar (5% for blood [$P = 0.045$] and 4% for valve biopsy samples [$P < 0.0005$]). The most efficient culture procedure was the subculture of blood culture broth into shell vials (sensitivity, 71%). For patients with endocarditis, previous antibiotic therapy significantly affected results of blood culture; no patient who had been administered antibiotics yielded a positive blood culture, whereas 80% of patients with no previous antibiotic therapy yielded positive blood cultures ($P = 0.0006$). Previous antibiotic therapy did not, however, prevent isolation of *Bartonella* sp. from cardiac valves but did prevent the establishment of strains, as none of the 15 isolates from treated patients could be successfully subcultured. For the diagnosis of *B. quintana* bacteremia in homeless people, the efficiency of systematic subculture of blood culture broth onto agar was higher than that of direct blood plating (respective sensitivities, 98 and 10% [$P < 10^{-7}$]). Nevertheless, both procedures are complementary, since when used together their sensitivity reached 100%. All homeless people with positive blood cultures had negative serology. The isolation rate of *B. henselae* from PCR-positive lymph nodes, in patients with cat scratch disease, was significantly lower than that from valves of endocarditis patients and skin biopsy samples from bacillary angiomatosis patients (13 and 33%, respectively [$P = 0.084$]). In cases of bacillary angiomatosis for which an agent was identified to species level, the isolation rate of *B. henselae* was lower than the isolation rate of *B. quintana* (28 and 64%, respectively [$P = 0.003$]). If culture is to be considered an efficient tool for the diagnosis of several *Bartonella*-related diseases, methodologies need to be improved, notably for the recovery of *B. henselae* from lymph nodes of patients with cat scratch disease.**

Human infections due to *Bartonella* species (formerly *Rochalimaea* species) are widely considered emerging diseases. They include long-recognized diseases such as Carrion's disease due to *Bartonella bacilliformis*, trench fever due to *Bartonella quintana*, and cat scratch disease (CSD) due to *Bartonella henselae* and *Bartonella clarridgeiae* (25, 32, 38). Newer clinical manifestations such as bacillary angiomatosis (BA) and peliosis hepatitis caused by both *B. henselae* and *B. quintana*; chronic lymphadenopathy due to *B. quintana*; and endocarditis due to *B. henselae*, *B. quintana*, and, in one case, *Bartonella elizabethae* have been recently identified (2, 38, 39). New molecular biology techniques, mainly based on 16S rRNA gene amplification and analysis, have allowed recognition of the role of *Bartonella* in an increasing number of pathological conditions. The association of *B. henselae* infection with BA is an example of such an approach for the identification of pathogens difficult to cultivate (52).

Growth of bartonellae is slow, since when blood agar is used (16, 34, 40, 60), primary isolates are typically obtained after 12 to 14 days, although prolonged incubation periods of up to 45 days are sometimes necessary (40). First subcultures of an isolate are also difficult to obtain, with colony formation again taking 10 to 15 days. Repeated subcultures, however, reduce this time to only 3 to 5 days, although colonial morphology is significantly affected. Cell coculture systems, as first described by Weiss et al. (64), have been reported to be more sensitive and allow more rapid growth of bartonellae than the blood agar-based techniques (16, 29, 43, 60).

Although the number of diagnosed cases of bartonella infections continues to increase, most diagnoses are based on either serology or PCR-based methods (1, 51), and reports of isolation of *Bartonella* from humans remain scarce. Furthermore, this finding is in contrast to the apparent ease with which *B. henselae* and *B. clarridgeiae* can be isolated from cats, their natural reservoir (8, 23, 28, 33). In this work, we summarize our 5-year experience (September 1993 to September 1998) of

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attempted isolation of bartonellae from human clinical specimens, during which time several techniques including cell culture and axenic media have been used.

MATERIALS AND METHODS

Specimen collection for *Bartonella* sp. diagnosis and case definition. As the National Reference Center for Rickettsiosis, our laboratory receives approximately 10,000 samples each year from France and abroad with presumptive diagnoses of rickettsial disease, with 40% of them from our hospital center. We have developed several protocols for collection and management, each tailored to the patient's clinical presentation. When samples were obtained from hospitals other than those directly served by our laboratory, efforts were made to contact the sender, to arrange the collection of additional or more-suitable samples if necessary, and to obtain relevant clinical and epidemiological data. At least a serum sample was obtained from all patients. Most clinical data associated with the isolates described in this work have been described elsewhere (9, 12, 16, 17, 37, 40, 43, 44).

Patients were suspected of having *Bartonella* endocarditis if standard blood culture remained sterile. Diagnosis was based on fulfillment of the Dukes endocarditis service criteria (19) in which a positive serologic result was included as a minor criterion, and detection of bartonellae by culture and/or genomic amplification from blood and/or from valvular tissue was considered as a major criterion. Complete sampling included a heparinized blood sample for shell vial and blood plating. Frozen cardiac valves were also sent when removed and inoculated onto shell vials and agar plates. When heparinized blood was not available, we asked that the inoculated blood culture bottle be submitted. Broth was further inoculated on shell vial and agar plates.

All homeless patients who presented for any reason to the emergency room of any hospital in Marseille, France, and who provided their informed consent were sampled. Samples included at least a heparinized blood sample for blood plating and a blood culture bottle for subculture on agar. *Bartonella* sp. bacteremia was defined as the isolation of a bacterium from at least one blood sample by any procedure.

Patients were defined as having CSD by fulfilling the three following criteria: (i) history of cat contact, (ii) at least one enlarged lymph node for which pathologic findings were compatible with CSD and no bacterium other than *Bartonella* sp. was isolated, and (iii) an immunofluorescence assay antibody titer to *Bartonella* sp. of ≥1:100 and/or detection by culture and/or genomic amplification of *Bartonella* sp. from blood and/or from lymph node biopsy sample. Samples used for culture attempts included lymph node biopsy samples or needle aspirates. They were inoculated onto shell vials and agar plates. In several cases, we also received a heparinized blood sample or an osteomedullar biopsy sample for culture.

Patients with suspected BA were patients with pathologic findings of BA on tissue biopsy samples (61). Cutaneous biopsy samples, frozen immediately after collection, were inoculated onto shell vials and agar plates. In several cases, we also received a heparinized blood sample or an osteomedullar biopsy sample for culture.

Isolation procedures. (i) Blood culture broth. Aerobic BACTEC PLUS blood culture bottles (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) were inoculated with samples of approximately 10 ml and then incubated in a BACTEC NR-860 machine. After 7 days, even if no bacterial growth was detected, 1 ml of inoculated blood culture broth was systematically removed from the bottle and plated onto Columbia sheep blood agar, as described below. In cases of endocarditis handled in hospitals other than those directly served by our laboratory, the same procedure was followed, but broth was also inoculated onto shell vials as described below.

(ii) Blood plating. One milliliter of blood samples collected into VACUTAIER tubes containing lithium heparin (Becton Dickinson Vacutainer Systems, Rutherford, N.J.) was plated onto Columbia sheep blood agar plates as described below under "Agar plate procedure." After publication of the study of Brenner et al. (March 1997) reporting an increase in culture sensitivity after freezing (8), whole blood was frozen at −85°C for 24 h and then thawed at room temperature before inoculation onto Columbia agar plates.

(iii) Shell vial assay. Samples of heparinized blood were sedimented ($1 \times g$) for 1 h, and 1 ml of the supernatant was collected for inoculation into shell vials. One milliliter of 7-day-old inoculated blood culture broth was used in cases of blood culture bottles. Tissue samples, including cardiac valves, skin biopsy samples, lymph nodes, and osteomedullar biopsy samples, were triturated in cell culture medium prior to inoculation into shell vials. Culturing was performed by the centrifugation-shell vial technique with ECV 304 human endothelial cell monolayers (29). ECV 304 cells were grown in RPMI medium with 10% fetal calf serum and 1 mM L-glutamine. Shell vials (Sterilin, Feltham, England; 3.7 ml) with 12-mm-diameter round coverslips were seeded with 1 ml of medium containing 50,000 cells and incubated in a 5% CO_2 incubator at 37°C for 3 days to obtain a confluent monolayer. One milliliter of supernatant of each sample was mixed with 1 ml of RPMI medium. Six confluent shell vials were inoculated for each blood or tissue sample with this mixture and centrifuged at $700 \times g$ for 1 h at 22°C. The inoculum was removed, and the shell vials were washed twice with sterile phosphate-buffered saline (PBS) and incubated in 1 ml of medium at 37°C under a 5% CO_2 atmosphere. Medium was changed at 15 and 30 days. Detection of *Bartonella* sp. on coverslips was carried out directly inside the shell vial by immunofluorescence. After fixation with cold acetone, vials were washed twice with PBS. One hundred microliters of a locally produced anti-*Bartonella* sp. rabbit antibody previously demonstrated to react with *B. henselae* serotypes Houston and Marseille and *B. quintana* (titer of 1/3,200), diluted 1:200 in PBS with 3% nonfat dry milk, was added, and vials were incubated in a moist chamber at 37°C for 30 min. After three washes in PBS, vials were incubated for 30 min at 37°C with 100 μl of a goat anti-rabbit immunoglobulin fluorescein-conjugated serum (Jackson Immunoresearch Laboratories, West Grove, Pa.) diluted 1:200 in PBS containing 0.2% Evans blue. After three washes with PBS, the coverslips were mounted (cells facedown) in phosphate-buffered glycerol medium (pH 8) and examined at 400× with a Zeiss epifluorescence microscope. Growth was assessed after 15, 30, and 45 days of incubation. If immunofluorescence was positive, identification to species level was performed by using the PCR-based methods outlined below.

(iv) Agar plate procedure. Tissue samples, including cardiac valves, skin biopsy samples, lymph nodes, and osteomedullar biopsy samples were triturated in brain heart medium prior to inoculation onto Columbia 5% sheep blood agar plates (BioMerieux, Marcy l'Etoile, France). Plates were placed in polyethylene bags and were incubated at 37°C in 5% CO_2 (Genbag CO_2 system; BioMerieux). Plates were examined weekly for evidence of growth for 3 months. In cases of whole blood and inoculated blood culture broth, the plates were briefly held at a 45° angle to allow the blood to flow across the agar. Plates were set agar side down initially and were then inverted after 24 h. When *Bartonella*-like colonies were observed, confirmation was achieved by the PCR-based methods outlined below.

Strain establishment. The supernatants of positive shell vials and the colonies obtained on agar plates were inoculated on confluent layers of ECV 304 cells in 150-cm^2 culture flasks and onto fresh Columbia sheep blood agar plates in order to establish isolates of *Bartonella* sp. A strain was considered established after two subcultures were obtained.

Identification of isolates. When bacteria were obtained on Columbia sheep blood agar plates, presumptive identification of isolates was made by determination of oxidase and catalase reactions and by microscopic examination after Gram and Gimenez staining. When bacteria were obtained on shell vials, presumptive identification was made by immunofluorescence. DNA extracts were prepared from suspect colonies or from immunofluorescence-positive shell vials for use as templates in PCR amplification with the QIAmp blood kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. During the first 3 years of this study, molecular detection and identification were based on 16S rRNA gene and citrate synthase gene amplification and sequencing as previously described (26, 53). Subsequently, a new procedure for citrate synthase gene amplification was designed. This PCR incorporated the primers CS 443 (6) and CS 979 (TGC ATG ATT TTT GCA CGT GG), designed by R. J. Birtles in our laboratory, allowing amplification of a citrate synthase (*gltA*) fragment. The identity of *gltA* gene products derived from test samples was determined by sequence determination and comparison as previously described (26, 53). Further confirmation of identity was achieved by intergenic spacer region amplification and sequence comparison. This PCR incorporated the primers QHVE1 (TTC AGA TGA TGA TCC CAA GC) and QHVE3 (AAC ATG TCT GAA TAT ATC TTC), designed by V. Roux in our laboratory.

Molecular detection of *Bartonella* sp. from clinical samples. DNA extracts suitable for use as templates in PCRs were prepared from crushed tissues and from EDTA blood samples by using the reagents of the QIAmp tissue kit (Qiagen) according to the manufacturer's instructions. The effectiveness of the extraction and the absence of PCR inhibitors were assessed by PCRs incorporating human β-globin gene primers, as previously described (22). Positive control reaction mixtures for each assay incorporated DNA extracted from *B. elizabethae*. Negative control reaction mixtures were sterile distilled water. The procedures for DNA amplification and sequence determinations were the same as those used for the identification of isolates described above.

Serologic procedure. *B. quintana* Oklahoma, provided by D. F. Welch (University of Oklahoma, Norman), and *B. henselae* Houston (ATCC 49882) were propagated in 150-cm^2 culture flasks containing ECV 304 human endothelial cell monolayers to be used for antigens for an immunofluorescence assay. All procedures for the *B. henselae* and *B. quintana* immunofluorescence assays have been described in detail elsewhere (16, 40).

Statistical tests. Pearson's chi-square test was used to compare data. A difference was considered significant when P was <0.05. When arms were lower than 30, a difference was considered significant when P was <0.1.

RESULTS

Results are summarized in Tables 1 and 2. For each procedure studied, the sensitivity of the procedure was calculated by dividing the number of positives obtained by this procedure by the overall number of positives (number of positive detections by genomic amplification or any culture procedure in any sample).

TABLE 1. Sensitivities of culture techniques used for the diagnosis of *Bartonella* infections

Disease (*n*)	Result by:			No. of patients	No. with:	
	Culture	PCR	Serology		*B. henselae*	*B. quintana*
BA (7)	+	NT[a]	−	2		2
	+	NT	+	1		1
	−	+	−	4	4	
Endocarditis (38)	+	+	+	18	4	14
	+	NT	+	1		1
	−	NT	+	5		
	−	−	+	6		
	−	+	+	7	2	5
	−	+	−	1	1	
CSD (78)	+	+	+	1	1	
	+	+	−	3	2	1
	+	−	−	1		1
	−	−	+	53		
	−	+	+	15	15	
	−	+	−	5	5	

[a] NT, not tested.

Patients with *Bartonella* sp. endocarditis. We received samples from 369 patients with endocarditis, of which 65 had an antibody titer to *Bartonella* sp. of ≥1:100 and 35 had an antibody titer to *Bartonella* sp. of ≥1:1,600. We received mostly heparinized blood samples (350 samples) but also 19 blood culture bottles from 19 patients and 52 cardiac valves from 52 patients. Among these 369 patients, 38 were diagnosed as having definite *Bartonella* sp. endocarditis, and 19 had at least one positive culture. Of these 19 culture-positive patients, 16 also had an antibody titer to *Bartonella* sp. of ≥1:1,600 and tested positive by PCR-based methods. Only two had a low antibody titer to *Bartonella* sp. of 1:400. Among the 19 culture-negative patients, 18 had an antibody titer to *Bartonella* sp. of ≥1:1,600, of which 7 also tested positive by *Bartonella*-specific PCR methods. A single patient was diagnosed solely on the basis of PCR testing. Among the 38 endocarditis cases, 7 were caused by *B. henselae* and 20 were due to *B. quintana* (Table 1).

In total, seven patients with endocarditis had a positive blood culture, obtained by inoculation of shell vials. Two isolates from two patients were obtained from heparinized blood inoculated onto a shell vial; however, only one strain was established. Six isolates from five patients were obtained from blood culture broth, five after subculture on shell vials and one after subculture on agar. Four of these strains were established. Comparison of isolation procedures was possible for samples from two patients on which all isolation procedures were used. An isolate was obtained on shell vial for both, by subculture of broth in one, and by inoculation of heparinized blood in the second, demonstrating the complementarity of these procedures. No isolate was obtained by direct plating of blood onto agar. The sensitivities of each isolation protocol are presented in Table 2. The overall sensitivity of cell coculture procedures (28%) was significantly higher than that of agar procedures (5%) ($P = 0.045$). With the exception of one patient who was treated for less than 24 h, none of the patients who had begun an antibiotic regimen, including rifampicin, fluoroquinolones, or aminoglycosides, had a positive blood culture. In the group of patients with PCR-positive detection on valvular tissue who received an antibiotic for more than 24 h prior to blood sampling, seven of seven had negative blood culture. Previous antibiotic therapy significantly affected results of blood culture, since none of the 32 patients with previous antibiotic therapy had a *Bartonella* strain established (one strain was isolated) from blood cultures, whereas four of five patients with no previous antibiotic therapy had their strain isolated and established from blood culture ($P = 0.00006$).

TABLE 2. Sensitivity of procedures used to isolate *Bartonella* sp. from blood of patients with *Bartonella* endocarditis

Procedure (*n*)[a]	No. positive	% Sensitivity
Heparinized blood-shell vial (20)	2	10
Blood culture broth-shell vial (7)	5	71
Total shell vial (25)	7	28
Whole blood-agar plate (20)	0	0
Blood culture bottle-agar plate (6)	1	17
Total agar (20)	1	5

[a] For each procedure used, *n* is the number of positive detections by genomic amplification or any culture procedure in any sample (overall positive). The significance (*P*) of total shell vial versus total agar is 0.045.

TABLE 3. Sensitivity of the isolation procedures used in this study to isolate *Bartonella* sp. from valvular tissues of patients with *Bartonella* endocarditis

Procedure (*n*)[a]	No. positive	% Sensitivity
Shell vial (27)	12	44
Agar (27)	1	4
Both (27)	12	44

[a] For each procedure used, *n* is the number of positive detections by genomic amplification or any culture procedure in any sample (overall positive). The significance (*P*) of shell vial versus agar is <0.0005.

Thirteen isolates from 12 patients were obtained from cardiac valves, 12 by using shell vial and 1 by using the agar procedure. All patients had positive serology and positive PCR results. All had received antibiotics for more than 2 days prior to sampling. None of these isolates could be successfully established. Among this group, the sensitivity of the agar procedure and that of the shell vial procedure were significantly different, being 4 and 44%, respectively ($P < 0.0005$) (Table 3).

Homeless patients with *B. quintana* bacteremia. From 132 homeless people, of whom 52 had an antibody titer to *B. quintana* of ≥1:100, we received 311 blood culture bottles and 382 heparinized blood samples. Forty-eight isolates of *B. quintana* from 15 patients were obtained by using a blood culture bottle, of which four were also obtained by using direct blood plating. For one patient, bacterial growth was detected by an automated system for two samples at 5 and 7 days. For another patient, isolation of *B. quintana* was achieved only by blood plating. All isolates obtained by plating samples directly onto agar were from blood samples which had been frozen. None of these patients received antibiotic therapy before sampling, and in most cases (75%), these patients had negative serology or were seronegative when bacteremic and seroconverted during the study period. For blood cultures, the sensitivities for direct plating and blood culture broth subcultured on agar plate were significantly different, being 10 and 98%, respectively ($P < 10^{-7}$). When these two techniques were both used, sensitivity reached 100% (Table 4).

Patients with suspected CSD. A total of 80 heparinized blood samples, 250 lymph node biopsy samples, and 3 osteomedullar biopsy samples were obtained from 290 patients with suspected CSD. Among these patients, 78 were diagnosed as definitely having CSD. Only 5 patients yielded isolates, whereas the 73 remaining patients were diagnosed by serology or genomic detection (53 by serologic findings, 5 by genomic

TABLE 4. Comparison of the sensitivities of the two methods used to isolate *Bartonella* sp. from the blood of bacteremic homeless patients

Procedure (*n*)[a]	No. positive	% Sensitivity
Whole blood plating (49)	5	10
Blood culture broth plating (49)	48	98
Both (49)	49	100

[a] For each procedure used, *n* is the number of positive detections by any culture procedure (overall positive). The significance (*P*) of whole blood versus blood culture broth is $<10^{-7}$.

detection of *B. henselae* from lymph nodes, and 15 by both). *B. henselae* isolates were obtained from the lymph node biopsy samples of three patients with positive genomic detection for *B. henselae*. Only one had positive serology. One isolate was obtained by using both shell vial and agar, but this strain could not be subcultured. One isolate was obtained only in shell vial but could not be established. The third isolate was obtained on agar and was successfully passaged. For the two patients with negative serology, the isolated strains were *B. henselae* serotype Marseille, and when this strain was used as antigen in immunofluorescence assay both had significant titers of antibody to this strain. Two *B. quintana* isolates were obtained from two patients with chronic lymphadenopathy and contact with cats and cat fleas. One strain was obtained from blood by using the shell vial (17). The second strain was isolated from an osteomedullar biopsy sample. PCR-based detection for *B. quintana* was positive in lymph node biopsy and blood samples (43). Both strains were successfully established. None of these patients had detectable antibodies against *B. quintana*. The sensitivities of agar plate and shell vial isolation procedures were estimated with 23 patients for whom *B. henselae* had been detected in lymph nodes by PCR-based methods. Each isolation approach had a sensitivity of 9%, and their combined sensitivity was 13%.

Patients with BA. We received samples from 52 patients with clinically suspected BA, of which one had an antibody titer to *Bartonella* sp. of ≥1:100. In total, 37 heparinized blood samples, 52 skin biopsy samples, and 3 osteomedullar biopsy samples were tested. Among these patients, seven were diagnosed as definitely having BA by pathologic findings. With the exception of a patient with acute myeloblastic leukemia, all were human immunodeficiency virus infected. Samples from four patients were found to contain *B. henselae* DNA by PCR methods, but none yielded a culture *B. quintana* was isolated from the remaining three patients. One isolate was obtained by using the whole-blood agar plate procedure, another was obtained following the inoculation of a skin biopsy sample onto agar plates, and a third was isolated from an osteomedullar biopsy sample. This last sample, obtained from another center, was inoculated onto a blood culture bottle and subcultured after 15 days onto an agar plate. The blood culture bottle (Hémoline Performance Biphasique; BioMerieux) and the agar medium (Chocolat Polyvitex) differed from those used for the other samples of this study. All three strains were established after subculture.

Comparison of culture with serology and genomic detection. PCR was attempted on 31 valves taken from patients with confirmed *Bartonella* endocarditis. Twenty-five (81%) yielded a positive result, including seven which failed to yield an isolate. Among BA patients, PCR was also able to detect *Bartonella* DNA in all four skin biopsy samples tested. Of lymph node biopsy samples taken from 76 patients with evidence of CSD, 23 (30%) were PCR positive. In five patients, this was the only confirmation of infection. Sera were available from 38 patients with *Bartonella* endocarditis. Thirty-seven of these had elevated antibody levels (35 had a titer of ≥1:1,600). Among CSD patients, sera were available for 76. Sixty-nine (90%) had detectable antibodies against *B. henselae*. Of the BA patients for whom sera was available, only one of seven had detectable antibodies against *Bartonella* spp. Among the 16 bacteremic homeless patients, none had detectable antibodies against *B. quintana* at the time that positive blood cultures were taken, but 10 seroconverted later in the study period.

DISCUSSION

The reliable diagnosis of *Bartonella* infections remains a difficult problem even for specialized laboratories. The isolation of infecting bacteria serves, therefore, not only as a means of diagnosis but also as a basis for the evaluation of much-needed improved diagnostic assays and as a route to enhanced understanding of the diversity and epidemiology of the bartonellae and the infections that they cause. Genotypic and phenotypic differences between *B. henselae* strains have now been widely encountered (5, 15, 28, 37, 52), and antigenic variation may explain the inconsistent results in the serological diagnosis of diseases such as CSD (15, 18). The presence of at least two different genotypes of *B. henselae* in cats in Europe, whereas only one appears to be present in North America (5), resembles the situation for *Borrelia burgdorferi* sensu lato (63). The discovery that *Borrelia garinii*, *Borrelia afzelii*, and *B. burgdorferi* sensu stricto are prevalent in Europe, whereas only the latter is prevalent in North America, allowed the understanding that each species of the *B. burgdorferi* complex was responsible for specific clinical presentations. Furthermore, *B. quintana* and *B. clarridgeiae* are responsible for some cases considered to be CSD (32, 38). The isolation of previously unrecognized pathogenic species, such as *B. clarridgeiae*, further demonstrates the potential shortfalls of specific serological or PCR-based assays. Interestingly, it is also becoming clear that for "natural" vertebrate hosts of certain *Bartonella* species, be they humans (9, 17, 43) or small mammals, an immunological response to bacteremia appears to be absent. The clinical isolation rates of *Bartonella* species and in particular of *B. henselae* appear excessively low, especially if estimates of disease prevalence are accurate. Despite there being about 22,000 clinically diagnosed cases of CSD per year in the United States alone (24), and more and more rigorous and widespread attempts to obtain isolates during the past decade, only 13 *B. henselae* strains (including those of the present study) have been reported to be isolated from CSD patients (3, 13, 68). The present study includes 72 isolates of *B. quintana* and 8 isolates of *B. henselae* from 42 patients, of which 63 *B. quintana* and 2 *B. henselae* isolates were successfully established. None of these patients was infected by both species. The fact that *B. henselae* and *B. quintana* were the sole species isolated is probably due to the specificity of the antiserum that we used for the identification of the shell vials. Previously reported isolations by other laboratories included only *B. henselae* in 39 patients (47 isolates) and *B. quintana* in 22 patients (53 isolates) (10, 11, 13, 14, 20, 27, 29, 34–36, 42, 47–50, 55–60, 62, 65–68). However, Koehler et al. reported recently, in the largest series to date, the isolation of 36 *Bartonella* strains from 25 patients with BA, of which 9 were infected by *B. henselae* and 16 were infected by *B. quintana* (30).

The media and procedures which have been employed for the clinical isolation of *Bartonella* species have been highly variable and have given discrepant results. From published data, it appears that *Bartonella* spp. are well isolated on choc-

olate agar, especially when enriched with 20% sheep blood for *B. quintana* (13, 14, 27, 29, 34, 36, 47, 55–59, 62). *B. henselae* is also better isolated on CDC blood agar (13). Nevertheless, strains have also been isolated on heart infusion blood agar (14, 29, 34, 50), Trypticase soy blood agar (29, 50, 62), brucella blood agar (14), and Columbia blood agar (56) (present study). *B. quintana* has never been recovered with buffered charcoal yeast extract or brain heart infusion blood agar (10, 14, 34), whereas *B. henselae* has been isolated on brain heart infusion blood agar (10, 14, 50). The results of culture on buffered charcoal yeast extract agar give discrepant results (11, 14, 56). Most strains have been isolated at 35 to 37°C in 3 to 9% CO_2 atmosphere. On subculture, growth may be obtained at 30°C (55). Some authors report that no isolates are obtained at 30°C or without CO_2 (56), whereas others report isolation and better growth at 30°C than at 35 to 37°C (11). Only one study has compared subculture media (10). The authors concluded that better growth was obtained on brain heart infusion blood agar and Columbia blood agar than on brucella blood agar, Shaedler's blood agar, and Trypticase soy agar (on which cultures were nevertheless obtained). Recently, Koehler et al. reported that optimal growth of *B. henselae* was obtained on heart infusion agar with 5% rabbit blood, whereas for *B. quintana* the best growth was obtained on chocolate agar (30). We tested two *B. quintana* strains, two *B. henselae* strains, the *B. elizabethae* strain, and a *Bartonella vinsoni* strain on various media including house-made Columbia agar, heart infusion agar, and brain heart infusion agar, all enriched with 5% horse blood, sheep blood, or rabbit blood, together with three commercial Columbia sheep blood agars. All plates were incubated at 35°C with 5% CO_2 or under a microaerophilic atmosphere. All these media under both atmospheres supported the growth of all the strains tested, with no evident superiority of a specific medium (unpublished data). Nevertheless, for each medium the best growth was obtained when it was freshly prepared. In fact, these works are hampered by the fact that media are compared by subculture of well-established strains for which visible colonies are obtained after 3 or 4 days of incubation, whereas primary isolation is harder and may require up to 45 days. Although cocultivation of infected material with eukaryotic cell cultures has been shown to be an alternative to, and in some instances a more sensitive isolation method than, agar procedures (29, 30, 42), including for two *B. quintana* isolates of this study, the sole use of this approach cannot yet be advised as reports of agar growth success and concurrent cell coculture failure continue, as reported in the present study for a *B. henselae* isolate.

We found that previous antibiotic therapy significantly adversely affected the outcome of blood culture attempts for patients with endocarditis, since all together, none of 32 patients with previous antibiotic therapy had a *Bartonella* strain established (one strain was isolated) from blood cultures, whereas 4 of 5 patients with no previous antibiotic therapy had their strain isolated and established from blood culture ($P = 0.00006$). Conversely, previous antibiotic therapy did not prevent isolations from being made from heart valve tissue but did prevent establishment of strains. In the 43 culture-positive patients of this study, among the 18 patients sampled with previous antibiotic therapy, only 2 had their strain established, of whom 1 was treated for less than 24 h when sampled, whereas among the 25 patients sampled with no previous antibiotic therapy, a sole of *B. henselae* strain isolated from lymph node patients with CSD was lost on subculture. Difficulties in the successful subculture of *Bartonella* isolates have also been encountered elsewhere (62). This finding may be more frequent than is currently recognized; several groups whom we contacted in order to obtain reported strains told us of losses on subculture.

For patients with endocarditis, inoculation of blood culture broth and heparinized blood onto shell vial was significantly more effective for the recovery of isolates than were the agar procedures employed. Furthermore, as inoculation of blood culture broth and inoculation of heparinized blood onto shell vial appeared complementary, they should both be attempted. Isolation of *B. quintana* from the blood of homeless patients appears to be straightforward, with up to 11 isolates for a single patient during a 1-month period. This apparent ease, compared to the situation for patients with endocarditis, may result from the lack of previous antibiotic therapy in the former group. For the diagnosis of bacteremia in homeless individuals, the use of an automated blood culture system followed by systematic blood culture broth was demonstrated to be highly effective and was significantly more sensitive than direct blood plating. Nevertheless, these procedures should be used together, since one bacteremic homeless individual yielded isolates only on direct plating. In previous studies, the lysis-centrifugation procedure and blood culture bottles with further subculture have been used for isolation of *B. quintana* and *B. henselae* from blood (10, 14, 20, 29, 34, 35, 47, 49, 50, 55, 56, 58, 59, 62, 68). Both procedures were used together in two studies but gave discrepant results. In one study, isolation was assessed by the lysis-centrifugation procedure only (55), whereas in the other, higher isolation rates were obtained by using blood culture bottles with further subculture (34). However, as detection of these slowly growing bacteria on conventional broth blood culture is difficult because they produce little or no CO_2 or visible growth, it is important to systematically subculture broth on solid medium for at least 1 month. In one study, the authors report that the optimum isolation rate was obtained by a combination of inoculation of blood culture bottle and lysis-centrifugation of blood culture broth (three isolates) rather than direct subculture of blood culture bottle (one isolate) (62). In this study, two isolates were detected in blood culture bottles but were not established. We observed the same problem for two endocarditis cases when subcultures on agar plates remained negative whereas subcultures onto ECV 304 monolayers yielded two *B. quintana* strains. The isolation rate of *B. quintana* from bacteremic homeless individuals was found to be higher when blood was frozen prior to inoculation, a methodology previously reported for blood samples from cats (8), which may be related to the intracellular location of bacteria in blood (31).

Shell vials were significantly more effective than agar medium for the recovery of *Bartonella* sp. from cardiac valves. For BA patients, the recovery of *B. quintana* from biopsy material was efficient, whereas the recovery of *B. henselae* was not. Difficulty in isolating *B. henselae* from BA patients was also observed in the study by Koehler et al. (30). By reviewing all cases of BA in which the infecting *Bartonella* organism was identified to species level (4, 7, 20, 21, 30, 41, 52, 54, 55, 57), we found that *B. quintana* was cultured from 21 of 33 cases (sensitivity, 64%), whereas *B. henselae* was cultured from only 10 of 36 cases (sensitivity, 28%) ($P = 0.003$). The recovery of *B. henselae* isolates from the lymph nodes of patients with CSD is extremely difficult. In our study, of 78 CSD patients, 69 had elevated antibody titers against *B. henselae* and 22 were positive by PCR amplification of *B. henselae* from DNA extract prepared from lymph nodes, but *B. henselae* was isolated in only three cases, two isolates belonging to the Marseille serogroup (15, 37). Furthermore, only one of these isolates could be established. Similar results were obtained by Avidor et al., who isolated two *B. henselae* strains from 29 PCR-positive

TABLE 5. Comparison of the sensitivities of culture for the diagnosis of *B. henselae* infection in three clinical syndromes[a]

Syndrome	No. genomic detection positive and isolation positive	No. genomic detection positive and isolation negative
BA	10	26
Endocarditis	4	3
CSD	3	20

[a] Data regarding CSD and endocarditis patients were obtained during this study, whereas data regarding BA patients have been drawn from both this study and previously reported cases (4, 7, 20, 21, 30, 41, 52, 54, 55, 57). Culture sensitivity for BA and endocarditis groups combined was 33%; culture sensitivity for the CSD group was 13%. The values for the BA and endocarditis groups were significantly different from those for the CSD group ($P = 0.084$).

lymph nodes of patients with CSD (3). These observations cannot solely be due to the fastidious nature of *B. henselae*, which is harder to isolate than *B. quintana*, as *B. henselae* is significantly harder to isolate from lymph nodes of CSD patients than from the blood of patients with *B. henselae* endocarditis or from skin biopsy samples of patients with BA (Table 5). The presence of pus in affected lymph nodes could explain the lack of viability of *B. henselae*. The difficulty in isolating *B. henselae* from lymph nodes is also starkly contrasted with the apparent ease of its isolation from cat blood (8, 28, 31, 33). This finding closely resembles that observed for homeless patients with *B. quintana* in our study and another study (60), for whom isolation of *B. quintana* appeared to be simple. This paradox raises several questions including (i) the viability of the bacterium when out of circulating blood, (ii) the presence in blood of a soluble and labile factor essential for growth of some isolates, and (iii) the heterogeneity of culture requirements of *Bartonella* strains.

An alternative procedure for diagnosing *Bartonella* infections is the use of PCR-based detection methods. These had a sensitivity of 81% on cardiac valves, and diagnosis was made for seven patients for whom all cultures remained negative. However, among CSD patients, the sensitivity of PCR methods in detecting DNA of *B. henselae* was lower (30%), including five cases in which both serology and culture were negative. The sensitivity of genomic detection of *Bartonella* from tissue biopsy samples of BA patients was 100% and allowed the identification of the responsible species. facilitating epidemiological investigations (30). Genomic detection was not performed with blood of bacteremic homeless patients due to the low sensitivity of this procedure on blood in our experience, but a good procedure to detect such patients should be the genomic detection of *B. quintana* from body lice as previously demonstrated (9, 45, 46).

The most effective tool for the diagnosis of endocarditis and CSD is specific serology. For patients with endocarditis, the serology had a sensitivity of 97%. Furthermore, 95% of these patients had an antibody titer against *Bartonella* sp. of $\geq$1:1,600. The sensitivity was 90% for patients with *B. henselae* CSD. We found, however, that serology has no value in the diagnosis of BA, as only one of seven was positive. This result is probably due to the immunocompromised status of these patients (30). Furthermore, although seroconversion was observed for 10 of the homeless patients with bacteremia, all had no detectable antibodies at the time at which samples which yielded cultures were taken. Serology is therefore reaffirmed in this case as a tool for retrospective rather than for acute-phase diagnosis.

We can now conclude from all these data that there is to date no universal optimal procedure for the isolation of *B. quintana* and *B. henselae*, but rather several techniques should be combined according to the observed disease. In the future, only prospective studies using several media and procedures will allow a definition of optimal conditions for isolating *Bartonella* species, and the most pressing problem is perhaps improved methods for the recovery of isolates from CSD patients.

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