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Improved Culture from Lymph Nodes of Patients with Cat Scratch Disease and Genotypic Characterization of *Bartonella henselae* Isolates in Australia

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Over a 4-year period we detected *Bartonella henselae* isolates in 104 of 297 specimens (35.1%) from Australian patients clinically suspected of having cat scratch disease by amplification of a fragment of the *htrA* gene. We isolated 17 *B. henselae* strains (20.5%) from the 83 PCR-positive human specimens available for culture. Our culture method was based on prolonged incubation in a moist atmosphere of blood agar to which hemin was added. We obtained more *B. henselae* isolates than the number of all other isolates from lymph nodes reported in the literature. In order to identify and study the genetic variation of Australian *B. henselae* isolates, we determined the sequence of a fragment of the *pap31* gene from our 17 human isolates and also from 8 Australian cat isolates. Thirteen of the human *B. henselae* isolates belonged to the Houston genotype, variant Houston-1 (76.5%), and four belonged to the Marseille genotype, variant CAL-1 (23.5%). In contrast, seven cat isolates were classified as *B. henselae* Marseille, variant CAL-1 (87.5%), and one was classified as *B. henselae* Houston, variant Houston-1 (12.5%). Our study describes an efficient culture method for the diagnosis of cat scratch disease and contributes to the description of the genotypic distribution of *B. henselae* in Australia.

Cat scratch disease (CSD) is a frequent and mostly self-limiting zoonosis. It is mainly reported in children and young adults, in whom it usually presents as a benign enlargement of lymph nodes draining the site of a cat scratch or bite (13). In immunocompromised patients, however, it may present as bacillary angiomatosis or peliosis hepatis. Visceral involvement has also been described in immunocompetent patients, especially children (24). *Afipia felis* was the first presumed agent of CSD (11), but Dolan et al. (17) isolated *B. henselae* from patients in 1993. Since then, epidemiological, serological, and molecular studies (1, 6, 16) have implicated *Bartonella henselae* rather than *A. felis* as the main causative agent of CSD. Most often, *B. henselae* causes asymptomatic chronic bacteremia in cats. Recently, we were able to classify CSD-causing *B. henselae* isolates into two genotypes: Houston (genotype I) and Marseille (genotype II). In addition, we subclassified the strains in genotype I into variants Houston-1 and ZF-1 and the strains in genotype II into variants Marseille and CAL-1 (44). The laboratory diagnosis of CSD is hindered by various problems. Although serology, especially by immunofluorescence or enzyme-linked immunosorbent assay, is one of the most useful diagnostic tools (4, 15, 35), it lacks sensitivity and its specificity has been questioned due to cross-reactivity between *B. henselae* and other species including *Bartonella quintana*, *Coxiella burnetii* (22, 31, 40), and *Chlamydia* species (33). The fastidious nature of *Bartonella* species, especially *B. henselae*, has prevented culture from being very useful as a diagnostic tool, especially for CSD, with only 14 human isolates being reported in the literature, to the best of our knowledge (3, 16, 17, 32, 43). In order to overcome the difficulties with culture, several PCR-based detection assays have been developed for human specimens, most of which have been coupled with nucleotide sequencing or probe confirmation (29, 36, 44). Unfortunately, the sensitivities of these tests may be limited by the inactivation of many DNA polymerases by immunoglobulins in patients' lymph nodes (44).

Few data are available on CSD and *B. henselae* in Australia. The first proven case of *B. henselae*-related CSD was reported in 1995 (23). However, despite a bacteremia rate in Australian cats similar to that observed in cats in other countries, this disease appears to be infrequently diagnosed in the Australian population, possibly due to underdiagnosis (10) or underreporting. Moreover, no studies on the genotyping of CSD-causing *B. henselae* isolates in Australia have been reported in the literature.

The purpose of our work was to describe our experience with the culture and genotypic classification of *B. henselae* isolates from lymph node tissue and superficial skin specimens taken from patients with clinically suspected CSD from August 1994 to August 1998 and, when specimens were available, of *B. henselae* isolates from the blood of the patients' cats or isolates from cats whose blood had been cultured for the presence of bacteremia.

MATERIALS AND METHODS

Study design. Collection of specimens, PCR detection, and culture of clinical samples were performed at the Sullivan Nicolaides Pathology Laboratory from August 1994 to August 1998, and confirmation of the identification and genotyping of *B. henselae* isolates were done at the Unité des Rickettsies.

Patients were clinically suspected of having CSD on the basis of findings such as the presence of chronic lymphadenopathy without any other specific diagnosis

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TABLE 1. Primers used for PCR and sequencing and species-specific probes used for dot blot hybridization

Primer (reference)	Primer sequence	Position[a] (direction)	Target gene[b]
CAT1 (2)	5′-GATTCAATTGGTTTGAA(G/A)GAGGCT-3′	1181–1204 (→)	*htrA*
CAT2 (2)	5′-TCACATCACCAGG(A/G)CGTATTC-3′	1578–1598 (←)	*htrA*
PAPn1 (44)	5′-TTCTAGGAGTTGAAACCGAT-3′	437–456 (→)	*pap31*
PAPn2 (44)	5′-GAAACACCACCAGCAACATA-3′	694–713 (←)	*pap31*
536F (20)	5′-CAGCAGCCGCGGTAATAC-3′	536–553 (→)	16S rDNA
1050R (20)	5′-CACGAGCTGACGACA-3′	1050–1035 (←)	16S rDNA
RH1 (1)	5′-GGTGCGTTAATTACCGATCC-3′	1208–1227 (→)	*htrA*
RQ1 (1)	5′-GGCGCTTTGATTACTGATCC-3′	1208–1227 (→)	*htrA*

[a] Arrows indicate direction of primers (→, forward; ←, reverse).
[b] Primer positions are numbered relative to the numbering for the *htrA* gene of *B. henselae* (accession number L20127), to the *pap31* gene of *B. henselae* strain Houston-1 (accession number AF001274), and to the 16S rDNA of *Escherichia coli* (accession number J01859).

and contacts with cats (24). *Bartonella* infection was diagnosed on the basis of the results of detection of *B. henselae* from lymph node tissue by PCR with *B. henselae*-specific primers. Two hundred ninety-six specimens were obtained from 278 patients. These included 205 lymph node biopsy specimens, 47 lymph node aspirates, 21 tissue biopsy specimens including 16 skin biopsy specimens, and 23 swabs from conjunctiva or wounds inflicted by cat scratches or bites, all of which were evaluated by PCR. Of these, 83 fresh specimens (49 node biopsy specimens, 27 node aspirates, and 7 skin swabs or skin biopsy specimens) were also cultivated for the presence of *Bartonella* species as described below. The geographic origin of all patients studied was Queensland, Australia. None of them was immunocompromised. In addition, blood was collected from a patient's cat and from seven feral cats from metropolitan Sydney during the period of our study and cultivated.

Molecular detection. (i) DNA extraction. Total genomic DNA was extracted from lymph node biopsy specimens and from *B. henselae* isolates with the QIAamp Tissue kit (QIAGEN Bresatec Ltd., Adelaide, South Australia, Australia) as described by the manufacturer. Twenty-five milligrams of fresh tissue was used. The samples were handled under sterile conditions to avoid any risk of cross-contamination. Each biopsy specimen was sliced with a disposable sterile razor blade. A total of 100 μl of elution buffer was used to resuspend the DNA. Lymph node aspirates and swabs from primary lesions were extracted by a modified phenol-chloroform-isoamyl alcohol method. Briefly, 100 μl of each of the lymph node aspirates and swabs was resuspended in sterile water, mixed thoroughly, and centrifuged at 8,000 × *g* for 5 min. The pellets were resuspended in 300 μl of extraction buffer (0.075 M NaCl, 0.05 M EDTA [pH 8])–10 μl of 20 mg of proteinase K per ml–50 μl of 10% sodium dodecyl sulfate and incubated at 56°C for 1 h with regular mixing. Extraction was performed three times, with centrifugation at 8,000 × *g* for 15 min between each successive extraction with 300 μl of phenol, phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol. The DNA was precipitated with 750 μl of cold pure ethanol and then washed with 75% ethanol. The pellets were dried and resuspended in 100 μl of Tris-EDTA at 40°C for 2 h. Genomic DNAs were stored at 4°C until their use as templates in PCR assays. As a negative control of DNA extraction, we used 200-μl samples of sterile water processed as described above in a proportion of one negative control to 10 lymph node specimens.

(ii) PCR detection and dot blot hybridization. The degenerate primers used to amplify a 414-bp fragment from *Bartonella* species from clinical samples were designed on the basis of the sequence of the *htrA* gene of *B. henselae* (GenBank accession number L20127) (2) and are presented in Table 1. In each case the PCR was carried out in a Rapidcycler thermocycler (Idaho Technology Inc., Salt Lake City, Utah) with a *Taq* DNA polymerase kit (Boehringer Mannheim, Mannheim, Germany). The 25-μl reaction mixture consisted of primers (12.5 pmol each), $MgCl_2$ (final concentration, 1.6 mM), each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP) at a concentration of 2 mM, 10× buffer, *Taq* DNA polymerase enzyme (0.5 U), sterile water, and 10 μl of DNA sample. PCR amplification was performed under the following conditions: an initial 3 min of denaturation at 94°C was followed by 44 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 50°C, and extension for 45 s at 72°C. The amplification was completed by holding the reaction mixture for 7 min at 72°C to allow complete extension of the PCR products. PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide. Measures were taken to prevent PCR carryover contamination. Each PCR step was performed in a different room. All amplification reactions included a negative control (sterile water) processed exactly as described above to ensure that none of the extraction buffers and reagents was contaminated with *Bartonella* DNA.

A dot blot hybridization assay with digoxigenin-labeled species-specific probes (Table 1) and a digoxigenin DNA detection kit (Boehringer Mannheim) was applied to amplified products to differentiate *B. henselae* and *B. quintana*, as described previously (1). *B. henselae* type strain ATCC 49882 and *B. quintana* strain Fuller were used as positive controls for both PCR and dot blotting.

Histological examination. Routine histological examination of lymph nodes and other tissues with hematoxylin-eosin and a Warthin-Starry stain to detect the presence of silver-staining organisms was performed if the histology was suggestive of CSD. Staining with Gram stain and acid-fast stains was also performed when indicated.

Culture procedures. When available, homogenized fresh lymph node tissue, aspirates, and swabs were cultured on laked horse blood agar slopes supplemented with 100 mg of hemin per liter. Tissue was swabbed over the slope surface and then partly embedded in the agar. The plates were sealed and incubated in a 5% CO_2 atmosphere for up to 6 weeks at 37°C. Humidity of 85% was obtained with sterile filter paper moistened with sterile saline. Cultures were examined for growth after 2 weeks and then weekly for 4 weeks or until growth became evident. Blood from cats was collected in Isolator tubes (Wampole Laboratories, Cranbury, N.J.); the lysed cells were spun down and then cultured as described above. Putative identification of clinical isolates was based on colonial appearance and the presence of nonmotile small curved gram-variable to weakly gram-negative rods which were oxidase negative, catalase negative, urease negative, indole negative, and glucose nonfermenting and which failed to grow on MacConkey agar. The RapID ANA II system (Innovative Diagnostics Systems, Norcross, Ga.) was used to confirm biochemical reactions, and all isolate identifications were confirmed by *pap31* PCR amplification and sequencing as described below.

16S rDNA detection. We used broad-range, 16S rRNA gene (rDNA)-based primers 877F and 1258R (20) (Table 1) to detect any bacterium in 10 suppurative granulomatous lymph nodes that were negative by PCR with *htrA*-specific primers.

Identification and genotyping of isolates. DNA was extracted and amplified as described above by using primers whose sequences were derived from the sequence of the *pap31* gene (Table 1) of *B. henselae* strain Houston-1 (GenBank accession number AF001275) (44). These primers were used to amplify a 238-bp fragment that allows the subtyping of *B. henselae* isolates. Amplification was carried out in a PTC-200 automated thermocycler (MJ Research, Waltham, Mass.) under the conditions described above, except that a 58°C temperature was used for the annealing steps, and the PCR products were purified with the QIAquick PCR purification kit (QIAGEN), as proposed by the manufacturer. PCR products were sequenced in both directions with PCR primers and the d-Rhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Coignieres, France) according to the instructions of the manufacturer. The sequences of the products were resolved with an ABI 310 automated sequencer (Perkin-Elmer). Sequence analysis was performed with the ABI Prism DNA sequencing analysis software package (version 3.0; Perkin-Elmer) and with CLUSTAL W software (version 1.8) (42). The sequences were aligned with the *pap31* sequences available in GenBank.

Statistical analysis. Fisher's exact test was used to compare the genotypic distribution in humans and cats. A P value <0.05 was considered significant.

RESULTS

Molecular detection. Of the 296 clinical specimens tested, *htrA* amplicons were obtained from 104 samples (35.1%) from 96 patients: 64 of the 205 (31.2%) lymph node biopsy specimens; 26 of the 47 (55.3%) lymph node aspirates; 10 of the 23 (43.5%) swabs, including swabs from 6 primary skin papules, 2 conjunctival swabs from a patient with Parinaud's oculoglandular syndrome, and 2 sinus swabs from a discharging suppurative lymph node; and 4 of 21 other tissue biopsy specimens (2 skin biopsy specimens of a primary papule and 2 excised granulomatous lesions from the conjunctiva). No amplicons were obtained from the negative controls. Dot blot hybridization identified all PCR amplicons as belonging to *B. henselae*.

Histological examination. Fifty-eight of the 64 *htrA* PCR-positive lymph nodes had suppurative granulomatous changes, including 2 with caseous features (25). The remaining lymph nodes were reported to have reactive lymphadenitis. Thirteen of 26 (50%) PCR-positive lymph nodes tested had argyrophilic organisms visible by Warthin-Starry staining.

Another 27 lymph nodes negative for *htrA* by PCR had similar histological characteristics. Of these, 6 were culture positive for *Mycobacterium tuberculosis*, 9 grew other atypical mycobacteria, 1 grew a *Corynebacterium pseudotuberculosis* isolate, and 1 grew a *Rhodococcus equi* isolate. The remaining 10 suppurative granulomatous lymph nodes were culture negative. Using broad-range primers, we identified *B. henselae* in 4 of these 10 specimens, *Ochobactrum anthropi* in 1 specimen, *Leptotrichia sanguinegens* in 1 specimen, *Ralstonia solanacearum* in 1 specimen, and *Methylobacterium* in 2 specimens. No amplicon was obtained for the remaining specimen.

Culture of specimens. Smooth, iridescent, umbilicated, and indole-, oxidase-, and catalase-negative colonies of pleomorphic gram-negative bacteria were grown from 13 of the 49 lymph node biopsy specimens (26.5%), from 3 of 27 lymph node aspirates (11.1%), from 1 of 7 cutaneous or superficial mucosal swabs or biopsy specimens, and from the 8 cat blood samples. The median incubation time to obtain sufficient growth was 21 days. By use of the RapID ANA II kit, all 17 isolates exhibited biochemical profile 000671, which is not provided in the database of this identification kit, and were identified as *Bartonella* spp.

Identification and genotyping of *B. henselae* isolates. *pap31* amplicons were obtained from all 17 human isolates and all 8 cat isolates. The sequences obtained from these PCR products were identical to that of *B. henselae* variant Houston-1 (genotype I) for 13 human isolates but only 1 cat isolate. In contrast, the sequence was identical to that of *B. henselae* variant CAL-1 (genotype II) for four human isolates and seven cat strains. The difference in the distribution of genotypes between humans and cats was statistically supported ($P < 10^{-2}$).

DISCUSSION

Although serology and molecular analysis-based detection methods have proved efficient for the diagnosis of CSD, recovery of *B. henselae* from patients is crucial for the characterization of human strains by comparison with cat isolates. This may contribute to the understanding of the discrepancies of the genotypic distributions of isolates observed in cats and humans in various areas. In particular, it may allow the study of the pathogenic capacities of the respective strains. *B. henselae* is a fastidious microorganism which requires prolonged incubation. Several attempts have been made with various culture systems to cultivate this bacterium from clinical specimens. In cats, which suffer from chronic *Bartonella* bacteremia most often without obvious clinical abnormalities, blood has been demonstrated to be the best sample for analysis. Among the blood culture techniques tested, those that include erythrocyte lysis with saponin or by freezing prior to plating on blood-enriched and/or hemin-enriched agar and prolonged incubation have proved particularly efficient (12, 38). When these techniques are applied to epidemiological studies with cats, a *B. henselae* prevalence of between 10 and 40% was found in the populations in America, Asia, Australia, and Europe tested (6, 10, 14, 16, 26, 28, 30, 34, 37). In contrast, *B. henselae* is infrequently grown from the lymph nodes of humans. To the best of our knowledge, only 14 isolates from patients with CSD have been reported to date (3, 17, 32, 43). The role of immunity in limiting the disease to a localized infection or the role of antibiotics has been hypothesized to explain this difference. Another difference may be the technique used. Indeed, various methods have been described, including liquid culture (18), agar culture (16), and cell culture (32), but the sensitivity varied from 2.5 to 6.4%. In our study, we obtained 17 *B. henselae* isolates from patients' specimens, which represents the largest collection of strains from patients with CSD to date. This demonstrates the efficiency of our culture protocol, which was adapted from the blood culture protocol for cats (10). In particular, hemin was added to blood agar, and the period of incubation in a humid atmosphere was prolonged. We have previously demonstrated the importance of hemin as a growth factor for *B. henselae* (21), and Sander et al. (38) have shown that the heme compounds are essential for the growth of this bacterium because it cannot synthesize it itself and because the organism also uses it as an iron source. Another major point is the length of incubation. *B. henselae* is a slow grower when cultivated from clinical material (24), and several investigators (10, 18) have highlighted the necessity of prolonged incubation. However, prolonged incubation requires a moist atmosphere to avoid desiccation of agar plates, and the agar plates are at risk of contamination by yeasts. In our protocol, we used sterile filter paper humidified with sterile saline, and no contamination was observed. Finally, our protocol also allowed us to isolate *B. henselae* from cat blood, demonstrating its versatility.

In Australia, Flexman et al. (23) were the first to report a case of proven *B. henselae*-related CSD in 1995. However, very few cases have been reported in Australia, and Branley et al. (10) and Ng and Yates (34), who have observed a *B. henselae* bacteremia rate in Australian cats similar to that in other geographic areas, have hypothesized that CSD was underdiagnosed. In addition, no report of the isolation of *B. henselae* from patients with CSD is available from Australia. Therefore, our collection of 17 isolates is an important contribution to the knowledge of this bacterium in Australia. Using the *pap31* gene, which encodes a major protein associated with a phage that has been isolated from *B. henselae* and that is probably implicated in its pathogenicity (8), we have recently demonstrated a high degree of heterogeneity among *B. henselae* iso-

lates from humans with CSD and cats and classified isolates into four genotypic variants: variant Houston-1 and a new variant, ZF-1, in genotype I and variants Marseille and CAL-1 in genotype II (19, 44). In our study, only the variant Houston-1 variant of genotype I and the CAL-1 variant of genotype II were detected, with Houston-1 infecting 76.5% of humans and CAL-1 infecting 87.5% of cats. These results are discordant with those obtained for French patients, among whom four variants were identified, with 58.9% of French patients being infected with the CAL-1 variant, 0.9% being infected with the Marseille variant, 37.4% being infected with the ZF-1 variant, and 2.8% being infected with the Houston-1 variant (44). Such a discrepancy in the genotypes occurring between geographic areas has already been described between Germany and The Netherlands, where genotype I isolates occur more frequently than genotype II isolates (7, 39), and France (27, 28) and Switzerland (9), where genotype II isolates are the most common. Moreover, when the prevalence of *B. henselae* variants in cats and humans is compared, Australian cats were mostly infected with a genotype different from that infecting Australian patients. A similar distribution has also been described in The Netherlands and Germany, where the majority of *B. henselae* isolates from cats belong to genotype II, whereas isolates from humans are mostly identified as *B. henselae* genotype I (5, 7, 39, 41). In contrast, in Switzerland and France, both cat and human *B. henselae* isolates mainly belong to genotype II (9, 27, 28, 44). The discrepancy in genotype distribution observed in our study may be linked either to a difference in pathogenicity between genotypes in Australia or to a sampling artifact, as the number of cat isolates studied was small.

In this study we have reported on an efficient culture method which allowed us to grow the largest collection of *B. henselae* isolates ever recovered from humans with CSD. Further studies of isolates from patients with CSD and cats will reinforce the knowledge and the understanding of the genotypic distribution of *B. henselae* in Australia.

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