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Detection of *Bartonella henselae* DNA by Two Different PCR Assays and Determination of the Genotypes of Strains Involved in Histologically Defined Cat Scratch Disease

ANNA SANDER,[1]* MIRIAM POSSELT,[1] NORBERT BÖHM,[2] MICHAEL RUESS,[1] AND MARTIN ALTWEGG[3]

Abteilung Mikrobiologie und Hygiene, Institut für Medizinische Mikrobiologie und Hygiene,[1] and Pathologisches Institut, Klinikum der Universität Freiburg,[2] Freiburg Germany, and Institut für Medizinische Mikrobiologie der Universität Zürich, Zürich, Switzerland[3]

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Cat scratch disease (CSD) is a common cause of subacute regional lymphadenopathy, not only in children but also in adults. Serological and molecular studies demonstrated that *Bartonella henselae* is the etiologic agent in most cases of CSD. Amplification of *B. henselae* DNA in affected tissue and detection of antibodies to *B. henselae* are the two mainstays in the laboratory diagnosis of CSD. We designed a retrospective study and investigated formalin-fixed, paraffin-embedded lymph nodes from 60 patients (25 female, 35 male) with histologically suspected CSD by PCR amplification. The sensitivities of two different PCR assays were compared. The first primer pair amplified a 296-bp fragment of the 16S rRNA gene in 36 of the 60 samples, corresponding to a sensitivity of 60%. The second primer pair amplified a 414-bp fragment of the *htrA* gene in 26 of the 60 lymph nodes, corresponding to a sensitivity of 43.3%. *Bartonella* DNA could be detected in a total of 39 (65%) of the 60 lymph nodes investigated. However, histopathologic findings are typical but not specific for CSD and cannot be considered as a "gold standard" for diagnosis of CSD. The sensitivity of the PCR assays increased from 65 to 87% if two criteria (histology and serology) were used in combination for diagnosis of CSD. Two genotypes (I and II) of *B. henselae* are described as being involved in CSD. Genotype I was found in 23 (59%) and genotype II was found in 9 (23%) of the 39 PCR-positive lymph nodes. Seven (18%) lymph nodes were negative in both type-specific PCR assays. Thirty (50%) of our 60 patients were younger than 20 years old (15 were younger than 10 years), 20 (33%) were between 21 and 40 years old, and 10 (17%) patients were between 41 and 84 years old. Our data suggest that detection of *Bartonella* DNA in patients' samples might confirm the histologically suspected diagnosis of CSD.

Bartonella henselae is the causative agent in most cases of cat scratch disease (CSD) a common cause of subacute regional lymphadenopathy in mostly immunocompetent children and adults. Patients are typically scratched or bitten by a cat, and after 3 to 10 days, skin lesions such as pustules or papules develop at the inoculation site. During the next 1 to 3 weeks, regional lymph nodes enlarge, remain stationary for another 2 to 3 weeks, and then resolve spontaneously over an additional period of 2 to 3 weeks (3). These typical clinical manifestations and a history of cat contact should lead to the presumptive diagnosis of CSD. The diagnosis can be confirmed by detection of antibodies to *B. henselae* in the patient's sera (13, 14, 17), by histopathological examination (10, 12, 20), and by molecular detection of *B. henselae* DNA from the patient's biopsy (1, 2, 4, 7, 10, 12, 20). Histopathological findings in the lymph nodes depend on the stage of infection. There may be lymphoid hyperplasia, arteriolar proliferation, and reticulum cell hyperplasia early in the course of infection. Granulomas with central areas of necrosis, multinucleated giant cells, and stellate multiple microabscesses may be found in later stages (3, 11). However, histopathological findings are typical but not specific for CSD. Infections caused by other agents, such as lymphogranuloma inguinale caused by *Chlamydia trachomatis*, atypical mycobacteriosis, yersiniosis, tularemia, brucellosis, certain mycoses, and chronic granulomatous disease of childhood must be considered in the differential diagnosis (11). Detection of *B. henselae* DNA in tissue samples therefore would be useful to confirm histologically suspected CSD.

Recently, several PCR-based assays have been developed for detection of *Bartonella* DNA in clinical samples. Large differences were found concerning the sensitivities of these assays, depending on whether fresh or formalin-fixed, paraffin-embedded tissue was investigated.

In a retrospective study, we compared the sensitivities of two PCR assays: one assay was based on the amplification of a 296-bp fragment of the 16S rRNA gene as described by Relman et al. (15), and the second assay amplified parts of the *Bartonella htrA* gene encoding a 60-kDa heat shock-like protein as described by Anderson et al. (1). Additionally, a genotype-specific PCR for *B. henselae* (5) was performed with all lymph nodes to differentiate between the two different genotypes of *B. henselae* involved in CSD.

The study examined lymph nodes from 60 patients with histologically suspected CSD. From 24 of these 60 patients, serum samples taken at the time of surgery were available for serological testing.

MATERIALS AND METHODS

Lymph node samples. Paraffin-embedded lymph node biopsies from 60 patients with histopathologically suspected CSD were included in this study. The samples were obtained retrospectively for a period of 7 years, from January 1989 to December 1996, by the Institute of Pathology.

Histopathological investigation. The lymph node specimens were fixed in 10% buffered formalin, embedded in paraffin, cut at 2 to 3 μm, and routinely stained

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie und Hygiene, Hermann-Herder-Str. 11, D-79104 Freiburg, Germany. Phone: (49) 761-203-6529. Fax: (49) 761-203-6562. E-mail: sander@ukl.uni-freiburg.de.

with hematoxylin and eosin. Twelve paraffin-embedded lymph nodes without any histologic evidence of CSD were used as negative controls. Warthin-Starry staining was not performed in our study.

DNA extraction. DNA was extracted from the formalin-fixed, paraffin-embedded lymph node biopsies by using a commercially available kit (Qiagen GmbH, Hilden, Germany) as proposed by the manufacturer. The extracted DNA was used as a template in the PCR assays. Purified DNA from cultured bacterial strains of *B. henselae* (Houston-1; ATCC 49882) was used as a positive control.

Amplification of *Bartonella* DNA. The primers p24E (5′CCTCCTTCAGTTA GGCTGG3′) and p12B (5′ GAGATGGCTTTTGGAGATTA3′), previously described by Relman et al. (15), were used to amplify a 298-bp fragment of the *Bartonella* 16S rRNA gene by PCR as described elsewhere (16). The reaction mixture consisted of bovine serum albumin (8 ng/μl), deoxynucleoside triphosphates (200 μM), primers (117 nM each), *Taq* polymerase (Pharmacia Biotech [2 U]), and 2.5 μl of extracted DNA in 50.0 μl of TBE (Tris-borate-EDTA) buffer. The mixture was overlaid with two drops of light mineral oil. PCR amplifications were performed in an automated thermal cycler (Robocycler 40; Stratagene) with initial denaturation (95°C, 5 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (57°C, 1 min), and extension (72°C, 90 s), with a single final extension at 72°C for 3 min.

After the reaction, 20 μl of the product was separated on a 1.5% agarose gel, stained with ethidium bromide, visualized on a UV transilluminator, and photographed with Polaroid 667 film. The DNA molecular weight marker was (ΦX-174-RF DNA *Hin*cII DIGEST (Pharmacia Biotech).

Additionally, a second primer pair, CAT1 [5′GATTCAATTGGTTTGAA (G/A)GAGGCT3′] and CAT2 [5′ TCACATCACCAGG(A/G)CGTATTC3′], as described by Anderson et al. (1), was used to compare the results of these two primer sets. The PCR product of CAT1-CAT2 is 414 bp in length. Internal oligonucleotides (RH1 and RQ1) were used as hybridization probes for differentiating between *B. henselae* and *Bartonella quintana* as described previously (10). For the type-specific amplification of *B. henselae*, the primers BH1 (5′-A ATCCCTCTTTCTAAATAGCC-3′) and BH2 (5′-TAAACCTCTTTCTAAAT AGCC-3′), respectively, in combination with the broad-host-range primer 16SF [5′-AGAGTTTGATCCTGG(CT)TCAG-3′] described by Bergmans et al. (5), were used. The partial 16S rRNA gene sequence differs from type I to type II in 3 bp located at positions 172 to 175 of the 16S rRNA gene. DNA amplification was carried out in 50-μl reaction volumes containing 5 μl of 10× reaction buffer (Pharmacia Biotech, Freiburg, Germany), 8 ng of bovine serum albumin per μl (Sigma, Deisenhofen, Germany), 200 μM (each) dNTPs, 20 pmol of each primer, 100 ng of genomic DNA, and 2 U of *Taq* polymerase (Pharmacia Biotech). PCR cycling consisted of 30 cycles of 20 s at 95°C, 30 s at 56°C, and 1 min at 72°C, preceded by an initial denaturation of 3 min at 95°C, and followed by a final extension of 5 min at 72°C. PCR products were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Serological testing. Serum samples taken from 24 of the 60 patients with suspected CSD at the time of lymph node biopsy were available. All sera were stored frozen at −70°C. Serological testing for immunoglobulin G (IgG) and IgM antibodies to *B. henselae* was performed with a commercially available indirect immunofluorescence antibody test (Bios, München, Germany) as described previously (17). Titers of <1:64 were regarded as negative.

RESULTS

The patients' characteristics (age, gender, and site and diameter of the infected lymph nodes) are shown in Table 1. Nineteen of the extirpated lymph nodes had been localized as cervical, 16 as axillar, and 13 as inguinal, respectively.

Histopathology. Histopathological examination of the 60 extirpated lymph nodes showed epithelioid cells next to necrotic tissue particles, necrotizing granulomatous inflammation, multiple stellate microabscesses with mixed hyperplasia, and perilymphadenitis, compatible with the diagnosis of CSD.

Amplification of *Bartonella* DNA. In 39 of the 60 lymph nodes (65%), *B. henselae* DNA could be detected by PCR. With the primer pair p12B-p24E, a positive PCR result was obtained from 36 lymph nodes (60%), whereas the second primer pair, CAT1-CAT2, amplified *B. henselae* DNA only in 26 (43.3%) of the 60 samples. Concordant positive results were obtained from 23 of the 39 lymph nodes, 13 samples were positive only with primer pair p12B-p24E, and 3 samples were positive only with primer pair CAT1-CAT2. By type-specific PCR, 23 of the 39 PCR-positive lymph nodes (59%) were found to belong to genotype I, and 9 (23%) belonged to genotype II, whereas 7 (18%) lymph nodes were negative in both type-specific PCR assays (Table 1). No specimen negative in the *B. henselae* PCR reacted with the genotype-specific primers. Only the 26 specimens positive in the PCR with primers CAT1 and CAT2 reacted with the *B. henselae*-specific oligonucleotide RH1. None of the 39 PCR-positive lymph nodes reacted with probe RQ1 (*B. quintana*).

All 12 lymph nodes used as negative controls were negative in all three PCR assays.

Serological testing. All but 1 of the 24 serum samples available showed elevated IgG antibodies to *B. henselae*. In addition, most of them contained elevated IgM antibodies. *Bartonella* DNA could not be detected by the three PCR assays of the lymph nodes in three of the patients with high antibody titers (Table 1, samples 19, 22, and 24 from the serology group). In only 1 serum sample (no. 23) were both IgG and IgM titers negative, but in this case, all PCR assays remained negative as well (Table 1). It remains to be clarified in this case if the lymphadenopathy was caused by *B. henselae* or by another agent. Of the 23 patients with CSD confirmed by both histology and serology, 20 had PCR-positive lymph nodes with a primer reaction pattern comparable to that of the unselected population.

DISCUSSION

The clinical features of CSD were described nearly 50 years ago (8), but *B. henselae* as the etiological agent of this disease was recognized only a few years ago and confirmed by serological and molecular studies (1, 2, 4, 5, 10, 12, 13, 14, 17, 20). Even today, the symptoms of CSD remain often unrecognized, and the diagnosis is based on the histological examination of a surgically removed lymph node or a biopsy. With the Warthin-Starry silver stain, the bacilli can be detected in tissue specimens, but the technique is difficult and the result is not specific for *Bartonella*. By this method, Scott et al. (20) demonstrated a few pleomorphic bacilli compatible with the CSD agent in only 14% of 42 formalin-fixed lymph node biopsies.

A better approach to a specific diagnosis is provided by DNA amplification methods. In the same study by Scott et al. (20), *B. henselae* DNA was found in 27 of 42 (64%) histologically defined lymph node biopsies and in 23 of 34 (68%) specimens from patients with CSD diagnosed both clinically and by histology (Table 2). The first primers for molecular identification of the agent of bacillary angiomatosis were described by Relman et al. (15). Using these primers and Southern blotting of the PCR products, Bergmans et al. (4) found *B. henselae* DNA in a high percentage of their CSD patients. These results were confirmed in our study. In 1994, Anderson et al. (1) described a new primer set for PCR detection of *Bartonella* DNA in specimens from CSD patients. This primer pair has been used in many studies (2, 10, 12) with good results (Table 2). However, the problem with all evaluations so far has been the lack of a defined "gold standard" for CSD or for the presence of *B. henselae*. Neither histology nor clinical symptoms or serology alone is satisfactory. Reliability, however, increased in most studies if two criteria were used in combination. Thus, in our study, the sensitivity of PCR (with both PCR assays) increased from 65 to 87% if histology was confirmed by the results of serology. A similar increase was seen by Bergmans et al. (4) (Table 2). Although not specific, the histological diagnosis of CSD appears to be quite accurate in cases confirmed by serology. Only 1 of our 24 patients for whom serum was available had no serological evidence for a *B. henselae* infection. This suggests a correct histopathological result in 96% of the cases studied.

In the absence of a gold standard for diagnosis of CSD, we compared our PCR results with the histopathological interpretation of the investigated lymph nodes. However, histopatho-

TABLE 1. PCR results for all patients with histologically CSD-positive lymph nodes in this study

Patient in group	Age (yr)	Gender[a]	Site of lymph node	Lymph node diam (cm)	PCR result with primer pair:		Type-specific PCR result		Serologic result (titer)[b]	
					p12B-p24E	CAT1-CAT2	Type 1	Type 2	IgG	IgM
With serology										
1	5	F	Axillar	1.5	+	−	+	−	8,000	128
2	20	F	Submandibular	4.0	+	+	+	−	256	<64
3	29	M	Inguinal	7.5	+	+	+	−	2,048	128
4	55	M	Axillar	1.7	+	+	+	−	512	128
5	46	M	Inguinal	4.5	+	−	+	−	128	128
6	12	F	Submandibular	1.0	+	+	+	−	512	128
7	53	F	Cervical	1.0	+	−	−	+	256	<64
8	23	M	Cervical	1.2	+	+	+	−	128	128
9	19	F	Femoral	3.5	+	+	−	−	4,000	512
10	26	M	Submandibular	2.0	+	+	−	+	2,048	128
11	21	F	Cervical	NA[c]	+	−	+	−	1,024	256
12	41	M	Supraclavicular	1.8	+	−	+	−	1,024	128
13	13	F	Submandibular	5.0	+	−	−	+	1,024	128
14	14	M	Axillar	3.3	+	−	−	+	2,048	128
15	9	M	Axillar	2.5	+	−	−	+	512	<64
16	9	F	Supraclavicular	3.0	+	+	+	−	16,000	1,024
17	81	F	Submandibular	3.5	+	+	−	−	4,000	128
18	60	F	Submandibular	4.0	+	+	−	+	8,000	256
19	8	M	Inguinal	5.5	−	−	−	−	1,024	128
20	3	F	Axillar	NA	−	+	−	+	4,000	<64
21	17	F	NA	NA	−	+	−	+	4,000	512
22	44	M	Axillar	4.0	−	−	−	−	4,000	128
23	22	F	Cervical	5.0	−	−	−	−	<64	<64
24	14	F	Axillar	NA	−	−	−	−	512	<64
Without serology										
1	10	M	Retroauricular	NA	+	−	−	−	NA	NA
2	35	F	Inguinal	2.0	+	+	+	−	NA	NA
3	59	M	Cervical	1.8	+	+	+	−	NA	NA
4	4	M	Inguinal	2.8	+	+	+	−	NA	NA
5	24	M	Inguinal	2.0	+	+	+	−	NA	NA
6	18	F	Forearm	4.2	+	+	+	−	NA	NA
7	40	M	NA	NA	+	+	+	−	NA	NA
8	10	M	Submandibular	4.5	+	+	+	−	NA	NA
9	4	F	NA	2.5	+	+	−	−	NA	NA
10	26	M	Inguinal	1.3	+	+	+	−	NA	NA
11	63	F	Cervical	1.5	+	−	+	−	NA	NA
12	84	F	Axillar	3.0	+	−	+	−	NA	NA
13	26	F	Inguinal	5.0	+	−	−	−	NA	NA
14	20	M	Axillar	4.0	+	+	+	−	NA	NA
15	36	F	Axillar	3.0	+	+	+	−	NA	NA
16	32	M	Inguinal	5.0	+	+	−	−	NA	NA
17	38	M	NA	2.5	+	−	−	−	NA	NA
18	17	M	Axillar	3.0	+	+	+	−	NA	NA
19	12	M	Cervical	2.0	−	−	−	−	NA	NA
20	0.25	M	Axillar	2.0	−	−	−	−	NA	NA
21	10	F	NA	3.5	−	−	−	−	NA	NA
22	15	M	NA	3.0	−	−	−	−	NA	NA
23	26	M	Preauricular	0.7	−	−	−	−	NA	NA
24	1	F	Axillar	1.6	−	−	−	−	NA	NA
25	8	M	Axillar	2.4	−	−	−	−	NA	NA
26	3	F	NA	2.8	−	−	−	−	NA	NA
27	35	M	Cervical	4.6	−	−	−	−	NA	NA
28	16	M	Upper arm	3.0	−	−	−	−	NA	NA
29	0.7	M	Inguinal	2.8	−	−	−	−	NA	NA
30	25	M	Inguinal	2.5	−	−	−	−	NA	NA
31	30	M	Submandibular	4.0	−	−	−	−	NA	NA
32	33	M	Inguinal	3.5	−	−	−	−	NA	NA
33	34	M	Inguinal	3.0	−	−	−	−	NA	NA
34	31	M	Axillar	6.0	−	+	−	+	NA	NA
35	11	M	Cervical	2.0	−	−	−	−	NA	NA
36	8	F	Axillar	2.4	−	−	−	−	NA	NA

[a] F, female; M, male.
[b] Serologic analysis was performed with an indirect fluorescent-antibody test as described previously (17).
[c] NA, information or serum sample not available.

TABLE 2. Literature summary of the PCR results with different primers in suspected CSD

CSD diagnosis[a]	No. of specimens	No. positive	% Sensitivity	Specimen preparation[b]	Primer pair used	Target gene[c]	Product size (bp)	Reference
Histol	42	27	64	FF-PE	CAT1-CAT3	16S rRNA	153	Scott et al. (20)
Histol + Clinic	34	23	68	FF-PE	CAT1-CAT3	16S rRNA	153	Scott et al. (20)
Histol + Clinic	23	14	61	FF-PE	CAT1-CAT2	*htr*A	414	Goldenberger et al. (10)
Clinic	25	21	84	None	CAT1-CAT2	*htr*A	414	Anderson et al. (1)
Histol	13	7	54	FF-PE	CAT1-CAT2	*htr*A	414	Mouritsen et al. (12)
Histol	60	26	43	FF-PE	CAT1-CAT2	*htr*A	414	This study
Histol + Serol	23	12	52	FF-PE	CAT1-CAT2	*htr*A	414	This study
Clinic	32	22	69	None	CAT1-CAT2	*htr*A	414	Avidor et al. (2)
Clinic	32	30	94	None	BhCS.781p-BhCS.1137n	*glt*A	379	Avidor et al. (2)
Clinic	32	32	100	None	p93E-p13B	16S rRNA	480	Avidor et al. (2)
Skin test positive + Clinic	89	85	96	Frozen	p12B-p24E	16S rRNA	296	Bergmans et al. (4)
Clinic	137	82	60	None	p12B-p24E	16S rRNA	296	Bergmans et al. (4)
Histol	60	36	60	FF-PE	p12B-p24E	16S rRNA	296	This study
Histol + Serol	23	18	78	FF-PE	p12B-p24E	16S rRNA	296	This study
Clinic	68	42	62	None	Nested PCR	16S rRNA	990	Dauga et al. (7)

[a] Histol, histologically; Clinic, clinically; Serol, serologically.
[b] FF-PE, formalin-fixed, paraffin-embedded specimens.
[c] *htr*A, 60-kDa heat shock protein gene; *glt*A, citrate synthase gene.

logical findings are typical but not specific for CSD. Especially in cases with negative PCR results and lack of serological testing, we have to consider that the histopathological findings might be caused by other agents and that these patients had been suffering from a disease other than CSD. Although in our study two different primer pairs were used, only 65 and 87% of the samples without and with serology, respectively, were positive, and the results were even less satisfying if the percentages for the primers were considered separately. A small number of bacteria, below the detection limit, is a possible cause. However, we assume that the false-negative reactions are more likely due to the various steps of fixation and embedding of the tissues known to damage DNA. The variable results obtained with the three primer pairs (including genotype-specific PCR) suggest random destruction of the DNA, with the smallest target (type-specific) resulting in the highest sensitivity (82%). The assumption is supported by the fact that with untreated or frozen lymph nodes, PCR often showed a higher detection rate (1, 2, 4). However, the sensitivity of the PCR assays increased from 65 to 87% in our study when two diagnostic criteria (histopathology and serology) were combined.

The results of our study and those of others (5, 9, 19) indicate that at least two genotypes of *B. henselae* are involved in CSD. Bergmans et al. (5) demonstrated that the majority (32 of 41 samples) of the lymph nodes from patients with CSD in The Netherlands contained *B. henselae* genotype I (78%), 7 of 41 belonged to genotype II (17%), and 2 samples (5%) were found to be negative in both type-specific PCRs. Similarly, in our study, 59% (23 of 39) of the PCR-positive patients were infected with *B. henselae* genotype I, 23% (9 of 39) were infected with *B. henselae* genotype II, and 7 (18%) of the lymph nodes were negative in both type-specific PCRs. In contrast, a study in Switzerland of 34 human clinical specimens containing *B. henselae* DNA had revealed 9 infections with type I but 25 infections with type II (6).

Furthermore, 16 of 17 *B. henselae* isolates from Southern German cats belonged to genotype II, and only 1 isolate was of genotype I (18). These results suggest that different genotypes of *B. henselae* are prevalent in different geographic regions (e.g., The Netherlands, Germany, and Switzerland) or that *B. henselae* genotype I could be more pathogenic to humans than genotype II (genotype of cat isolates versus genotypes in human lymph nodes in Germany).

We conclude that the detection by PCR of *B. henselae* in tissues of patients with suspected CSD is an useful diagnostic method complementing histopathological and serological analysis. At least two different primer pairs should be used for higher sensitivity, especially in prefixed materials. The different distributions of the two genotypes in cats and humans have yet to be explained sufficiently. In addition, whether the clinical presentation is somehow dependent on the type of the infecting strains remains to be analyzed.

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