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Detection of *Bartonella* in cat scratch disease using a single-step PCR assay kit

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Abstract

Purpose. *Bartonella* is an increasingly isolated emerging pathogen that can cause severe illness in humans, including cat scratch disease (CSD). The bacteria are difficult to grow and thus many detection methods have been developed, especially molecular. We previously developed a PCR method targeting *ribC* to identify *Bartonella* sp. A manufactured kit (RealCycler BART, Progenie Molecular) was commercialised shortly thereafter for the detection of *Bartonella* infection, including *Bartonella henselae*.

Methodology. We performed a comparison between this test and our in-house PCR assay on 73 lymphadenopathy samples sent to the laboratory for suspicion of CSD.

Results/Key findings. Among the 28 positive samples for *Bartonella*, 21 were identified by the two PCR assays, and seven by the commercial kit only.

Conclusion. The performance of this commercial kit suggests that it could be a suitable alternative to our in-house PCR assay, highlighting the importance of the molecular methods used to diagnose CSD.

INTRODUCTION

Debre *et al.* [1] were the first to describe the link between regional lymphadenopathy and cat scratches in 1950. In 1988, English *et al.* [2] performed the first isolation and culture of the cat-scratch disease (CSD) organism, first called *Rochalimaea henselae* [3] and renamed *Bartonella henselae* in 1993 by Brenner *et al.* [4].

Bartonella is a small, rod-shaped, fastidious, non-motile, pleomorphic, Gram-negative, aerobic, oxidase-negative bacillus, that grows slowly. It belongs to the *alphaproteobacteria*, based on its 16S rRNA sequences. This intracellular bacillus is susceptible *in vitro* to beta-lactams (except oxacillin and cephalothin), aminoglycosides, macrolides, and tetracyclines [5]. Azithromycin and, alternatively, doxycycline are the most commonly used antibiotics to treat CSD if necessary [6]. It appears to confer lifelong immunity.

The *Bartonella* genus consists of 19 currently recognised species that share 98 % homology in their 16S rRNA sequence [5, 7–10]. Eight species cause human diseases, such as verruga peruana and Carrion's disease (*B. bacilliformis*), chronic bacteremia in cats (*B. clarridgeiae, B. henselae*), bacillary angiomatosis (*B. henselae* and *B. quintana*), endocarditis (*B. henselae, B. quintana, B. elizabethae, B. vinsonii* subsp. *berkhoffi, B. vinsonii* subsp. *arupensis*), cat scratch disease (*B. henselae*), neuroretinitis *(B. grahamii),* and others, including several mostly encountered in immunocompromised patients [5, 9–14].

CSD is the most common cause of chronic lymphadenopathy in children. The diagnosis of CSD usually requires past contact with a cat and/or the presence of a cat scratch, no other cause of lymphadenopathy discovered by laboratory explorations, and characteristic histopathological findings in a lymph node biopsy [15]. However, a past injury caused by a cat is sometimes not reported by the patient, and *B. henselae* serology can lack both sensitivity and specificity [15, 16].

The initial symptoms of CSD are an erythematous papule at the site of inoculation, within seven to 12 days, and lymphadenitis within two to four weeks [3] (Fig. 1). Complications (deep lymphadenitis, osteomyelitis, arthralgia, rash, thrombocytopenic purpura, hepato-splenomegaly, neuroretinitis, and encephalopathy [17]) are rare in immunocompetent patients. CSD tends to spontaneously resolve within a few months and the prognosis for these patients is excellent.

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Keywords: *Bartonella*; cat scratch disease; real-time PCR.

Abbreviations: CRP, C reactive protein; CSD, Cat scratch disease.

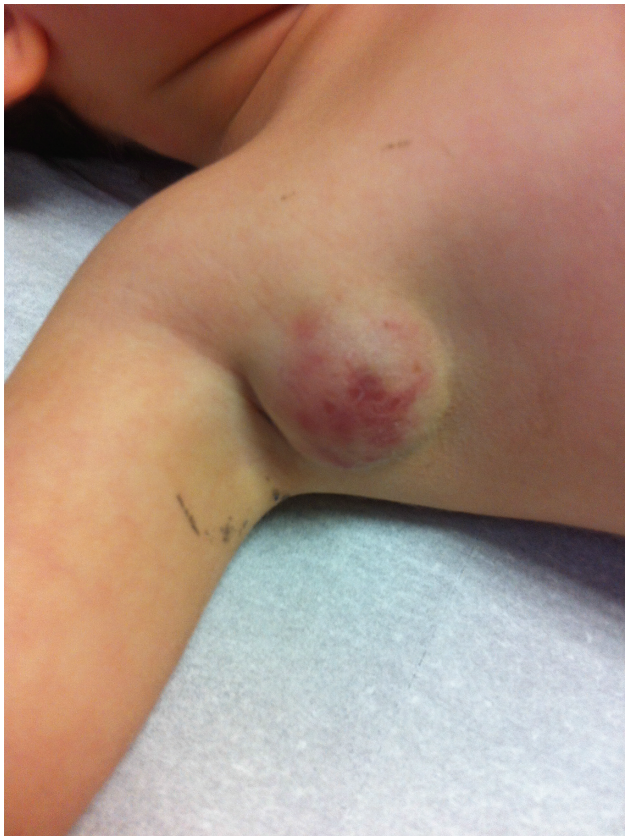

Fig. 1. Left axillary adenopathy of a 5-year-old boy.

The incidence of infestation among cats is seasonal (August-October in northern areas) and the prevalence of the disease is higher in humid and warm climates. It occurs most frequently in young patients (under 18 years old). It has been suggested that bartonellosis should be the first etiology to consider in cases of prolonged fever of unknown origin when specific recent exposure (to cats or kittens) has been reported [18].

Bartonella henselae is one of the infectious agents most often encountered in adenitis [19, 20], although other causes must be ruled out, including tuberculosis adenitis, malignant blood disease, lymphogranuloma venereum, lymphatic gland tumors, and toxoplasmosis, as well as mononucleosis, brucellosis, and sarcoidosis in cases of poly-adenitis [1]. Thus, it is critical to identify the etiological agent if present.

The culture of *Bartonella* species is difficult [10, 12, 14, 21–23], owing to their fastidious and slow growth. This bacillus was identified in 1992 by Regenery *et al.* [24] by polymerase chain reaction (PCR) amplification of the gene for bacterial 16S rRNA and PCR-restriction endonuclease length polymorphism. Since then, other groups have developed rapid detection assays [14, 25, 26], and more recently, a single-step PCR assay for the detection and differentiation of medically relevant *Bartonella* species [27], using species-specific PCR-mediated amplification of the 16S–23S rRNA intergenic region [28]. In the search for new primers, a PCR assay was developed using a single primer pair targeting the riboflavin synthase gene (*ribC*) and detected six *Bartonella* species involved in human disease [9]. Based on this work, our laboratory developed an in-house real time PCR-based test targeting *ribC* for the detection of *Bartonella* infections [9]. The *Bartonella* species is then identified by sequencing.

We report the comparison of a commercial, ready-to-use, single-step PCR kit with our in-house PCR assay for the detection of *Bartonella* in the context of high clinical suspicion of CSD. We based our interpretation of the results on clinical data, serology, and histopathology when possible.

METHODS

Study design

Between 2013 and 2015 our laboratory received 73 adenopathies from 73 patients to be tested for *Bartonella* infection using our in-house PCR assay that targets *rib*C. We analysed and retrospectively tested these samples using the ready-to-use kit developed by Progenie. We excluded all samples other than lymph node biopsies and abscess aspirates to focus only on CSD.

Clinical and biological data

Data were collected from the information file of each patient. The data analysed were the age and sex of the patient, the presence of an adenopathy, their number (single or multiple) and location, the type (abscess or lymphadenopathy), and fever. Contact with cats or scratches and the duration of the symptoms were reported when mentioned in the patient's history. When available, data concerning C-Reactive Protein (CRP) levels and the histopathology of the samples were analysed.

Molecular methods

DNA was extracted from each sample using blood and tissue QIAamp kits (Qiagen EZ1), following the instructions of the manufacturer. Samples were frozen and stored at −20 °C until analysis with the commercial kit.

In-house PCR

The primers, 'BAR1' and 'BAR2', specifically target the *ribC* gene, as described by Johnson *et al.* [9]. The amplified sequence is 588 bp in length. The PCR mix contains 12.5 µl Premix Ex Taq (Takara), 4.5 µl water, 0.20 µl each of sense and anti-sense primers (25 µmol l^{-1} each), and 2.5 µl Sybr-Green for a total volume of 20 µl. DNA (5 µl) is added to the mix to obtain a total volume of 25 µl. Real-time PCR is performed with a Smart Cycler (Cepheid) using a program consisting of 600 s at 95 °C, 37 cycles of 60 s at 95 °C, 60 s at 60 °C, and 60 s at 72 °C, and a ramp up from 60 to 95 °C by 0.2 °C s^{-1} to obtain a melting peak curve. Beta-globulin detection was performed on all samples to test for the presence of inhibitors.

Sequencing of *ribC*

DNA from the patients who tested positive was sequenced and aligned using Sequencing Analysis and BioEdit software. The sequences were analyzed in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Commercial kit

The CE marked, *RealCycler* BART kit of Progenie Molecular was used. The kit uses primers targeting the *groEL* gene, specific to *Bartonella* spp., an *alr-gcvP* intergenic spacer, specific to *B. henselae*, and an internal control. Eight microliter DNA was added to the ready-to-use mix before starting the real-time PCR. We performed the PCR protocol following the manufacturer's instructions. The analytical sensitivity of the kit was 10 copies μl^{-1} for both *Bartonella* spp. and *B. henselae*, according to the data of the manufacturer. We considered the diagnosis to be positive for *Bartonella* for all patients with a positive PCR (in-house or kit).

Antibody detection

Serology was performed for 36 patients (49.3 %). The detection of antibodies against *Bartonella* was either performed using an indirect immunofluorescent assay (Bartonella IFA, Vircell), or by the National Reference Center in Marseille, France (Professor Didier Raoult).

RESULTS

We performed PCR with the commercial kit on all 73 samples which were originally tested with the in-house PCR. Of these, 28 were positive for *Bartonella* and 45 were negative. We did not detect any amplification inhibitors. Twenty-one samples were positive with both the in-house PCR and the commercial kit. Seven were positive with the commercial kit only. We sequenced the positive samples from the in-house PCR. Sequence alignment confirmed the results and allowed identification of *B. henselae* in all cases. The PCR results obtained with the commercial kit also confirmed the presence of DNA from *B. henselae* in all the samples.

The average age of the positive and negative groups for *Bartonella* was 19.4 years and was not significantly different between the two groups (Table 1). There were more male patients in the positive than negative group, but the difference was not significant (P=0.055).

Only four patients presented fever (two in the *Bartonella* positive group and two in the *Bartonella* negative group).

Sample analysed were lymph nodes and abscess puncture. Twenty-one patients (28.7 %) had poly-adenopathy (21.4 % in the positive group versus 33.3 % in the negative group) and 52 patients (71.2 %) presented a single adenopathy (78.5 % in the positive group versus 66.6 % in the negative group). Concerning the location of the adenopathy, 57.1 % (16/28) and 66.6 % (30/45) were axillary or cervical, and 17.8 % (5/28) and 20 % (9/45) inguinal for the positive and negative groups, respectively.

Table 1. Main clinical and biological characteristics of the study population (n=73)

		Positive for *Bartonella* (%) n=28	Negative for *Bartonella* (%) n=45
Age	Mean±SD	19.4±20.6	19.4±18.4
	Minimum	1	1
	Maximum	81	73
Sex	Male	18 (64.3)	18 (40)
CRP (mg l^{-1})	Mean±SD	36.3±69.4	12.5±21.7
	Minimum	1	1
	Maximum	315.8	93.9
	Not performed	7	23
Fever (°C)	Maximum	38.7	39.3
	Number of cases with fever	2 (8.3)	2 (5.4)
	No fever	22 (91.6)	35 (94.5)
	No information	4 (16)	8 (17.7)
Adenopathy	Single	22 (78.5)	30 (66.6)
	Multiple	6 (21.4)	15 (33.3)
Sample	Adenopathy biopsy	22 (78.5)	39 (86.6)
	Abscess puncture	6 (21.4)	6 (13.3)
Histopathology	No histopathology analysis	11/28 (39.2)	9/45 (20)
	Inflammation	13/17 (76.4)	25/36 (69.4)

The duration of the symptoms before bacterial analysis for the 28 positive patients was under seven days (inclusive) for two (7.1 %), between 7 and 15 days (inclusive) for seven (25 %), between 15 and 30 days (inclusive) for 11 (39.2 %), and over 30 days for eight (28.5 %). The declared symptoms were sweating (two, 7.1 %), deterioration of the general status (asthenia, anorexia, and weight loss) (five, 17.2 %), abdominal pain (one, 3.5 %), purpura (one, 3.5 %), and fever (three, 10.7 %). Sixteen (57.1 %) patients had been regularly exposed to cats.

The level of CRP was higher than 20 mg l^{-1} for seven of 21 samples in the positive group, and for five of 22 samples in the negative group (P=0.5).

Histopathology and/or cytology was performed for 17/28 samples in the positive group and 36/45 in the negative group. Inflammation was described for 13 samples (76.4 %) of the positive *Bartonella* group versus 25 samples (67.5 %) of the negative *Bartonella* group, (P=0.7). Among the 16 positive samples with histology and/or cytology, epithelioid cells and/or granuloma were found in 4 samples (25 %). Among the 36 negative samples with histology and/or cytology, epithelioid cells and/or granuloma were found in 14 samples (38.9 %). There was no significant difference between these two groups (P=0.33). Amongst the positive samples, epithelioid cells were found in three (17.6 %), and granuloma was described in two (11.7 %). There were no significant differences between the positive and negative groups.

The serology results were not contributive. Only two of the 36 samples tested (5.5 %) were found to be positive, one in the positive group and one in the negative group. These results were not sufficient to be included in this study as they were associated with one positive and one negative PCR result.

Etiological diagnosis of samples negative for *Bartonella*

Twenty-four patients were diagnosed for another disease to explain their adenopathy. The other diagnoses included tuberculosis (three), lymphogranuloma venereum (two), *Francisella tularensis* infection (one), non-tuberculosis *Mycobacterium* infection (four), *Staphylococcus* infection (two), lymphadenitis abscess (two), and an adverse effect of BCG vaccine (one). We also observed malignant blood diseases [Hodgkin disease (one), and acute lymphoid leukemia (one)], and other pathologies, such as myositis ossificans (one), and sarcoidosis (two). Twenty-one patients (28.7 %) remained without a specific diagnosis up to the time of writing this paper. A secondary *Bartonella* sero-conversion was diagnosed retrospectively for one patient, considered to be negative in our study, based on two negative PCRs, and negative initial serology. Hence this patient could be a possible false negative.

DISCUSSION

Bartonellosis is an emerging pathogen and CSD is highly predominant among the various clinical presentations.

Many methods for the diagnosis and detection of *Bartonella* infections have been described. Culture is difficult due to the fastidious and slow growth of *Bartonella* species [21, 29]. Thus, the differentiation, identification, and diagnosis of an infection by a *Bartonella* species requires molecular methods [23, 28, 30]. Given their advantages, we compared a commercial ready-to-use single-step PCR kit to the in-house PCR assay used in our laboratory for the diagnosis and detection of *Bartonella.*

Some of the genes among those used for the identification and/or detection of *Bartonella* species appear to be gold standards, including the 16S–23S rRNA intergenic spacer region (ITS) for the characterisation of *B. henselae* and *B. quintana* [25–28, 31]. However, this region exhibits limited variation between members of the *Bartonella* genus [32]. Thus, some authors have described alternative methods using new primers that target the gene which encodes the RNA polymerase beta-subunit (*rpoB*) [33] or the riboflavin synthase chain (*ribC)* [9, 10, 30]. The sequence of the *ribC* gene is conserved between *B. henselae*, *B. clarridgeiae*, *B. quintana*, *B. bacilliformis* [30]. Avidor *et al.* proposed a first round of PCR targeting the citrate synthase gene, followed by rDNA16S PCR, to increase the performance of the strategy in case the first step is negative, but this approach is not currently practiced in medical diagnosis [34].

None of the described methods [9, 15, 16, 30, 33, 34] is yet considered to be an international gold standard for the microbiological diagnosis of *B. henselae.* Based on these studies, our laboratory has developed and uses primers that target the *ribC* gene to identify *Bartonella* infections, via PCR. Sequencing of the amplification products allows identification of the *Bartonella* species. This procedure is time consuming and requires many steps. We thus tested a new commercialised, ready-to-use, kit for PCR-based detection of *Bartonella* spp., focusing on *B. henselae*. This kit targets the *alr-gcvP* intergenic spacer, considered to be a region specific to *B. henselae* [35], and also targets *groEL*, tested previously by other teams [7, 32, 36].

Here, 21 of the 73 patients tested positive with the home-made PCR assay versus 28 with the commercial kit. We consider that the in-house assay gave seven false negative results as the *RealCycler* BART kit targets two different genes: the *groEL* gene and the *alr-gcvP* intergenic spacer and both were positive with these seven samples.

The clinical presentation and patient histories were compatible with a *Bartonella* infection for most of the samples that were positive only with the manufactured kit. We focused our study on lymph node samples only and our results cannot be generalised to all *Bartonella* species, as we specifically evaluated the 'ready-to-use' kit on *B. henselae*, without testing for other *Bartonella* species.

Serological and/or PCR assays specific for *B. henselae* can be negative in definitive cases of CSD. Indeed, the timing of the biopsy may influence the PCR results, especially if they are performed after the first 6 weeks of infection [37]. Thus, many other diagnoses may also be suspected (including malignant blood diseases) and it is necessary to rely on other laboratory methods, irrespective of whether the results of our test are negative or positive. Furthermore, Bergmans *et al.* [38] reported variable sensitivity for the PCR hybridisation assay, depending on the number of fulfilled criteria for CSD.

Histological and cytological analyses are also important for differential diagnosis, but, did not appear to be specific for the CSD diagnosis in our study. Indeed, we reported epithelioid cells, granulomas, and necrosis in both groups.

Alternatively, serological methods have been used to complement PCR and culture for the diagnosis of *Bartonella* infections. This method is useful for the detection of antibodies against *Bartonella* in the diagnosis and evolution of endocarditis caused by *Bartonella* [21], but is of no interest in CSD.

This study also highlights the importance of analysing clinical and biological data as complementary sources of information for diagnosis. *Bartonella* infection in humans can be severe, and its diagnosis can be difficult. We tested a new 'ready-to-use' kit to detect *Bartonella* infection and specifically identify the *B. henselae* species, and compared it to our in-house method. In addition to offering clinical utility, the manufactured kit is easier to use, quicker, and involves less manipulation, hence less risk of contamination. It also has

the capacity to distinguish a *Bartonella* spp. infection from a *B. henselae* infection in the same step, and moreover, it appears to be more sensitive than our method. We did not test formalin-fixed paraffin-embedded tissue samples in this study and this remains to be tested. The identification of the etiological diagnosis of CSD linked to *B. henselae* is still based on the combination of clinical manifestations, history of the disease, histopathological analysis, biological results, and especially multiplex PCR kits that can identify species of *Bartonella* other than *B. henselae*, which is the main cause of CSD. We did not manage to find significant differences on clinical symptoms only or histology only. Furthermore, the serology did not seem to be contributive in our study, for CSD. Therefore, we would like to underline the importance of the PCR method, considered as major tool to confirm the diagnosis of CSD, in case of clinical or biological compatible with this diagnosis. However, complementary studies are necessary to evaluate the benefit of these commercial kits for the diagnosis of CSD or other clinical manifestations due to *Bartonella* infection.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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