Identification of *Bartonella* Species Directly in Clinical Specimens by PCR-Restriction Fragment Length Polymorphism Analysis of a 16S rRNA Gene Fragment

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It is now established that two species of *Bartonella*, namely, *Bartonella henselae* and *B. quintana*, cause bacillary angiomatosis in human immunodeficiency virus-infected patients. In addition, *B. henselae* causes cat scratch disease and *B. quintana*, *B. henselae*, and *B. elizabethae* can cause bacteremia and endocarditis in immunocompetent persons. We have developed a PCR-restriction fragment length polymorphism-based assay for direct detection and identification to species level of *Bartonella* in clinical specimens. This is accomplished by PCR amplification of *Bartonella* DNA using primers derived from conserved regions of the gene carrying the 16S ribosomal DNA, followed by restriction analysis using *Dde*I and *Mse*I restriction endonucleases. We amplified a *Bartonella* genus-specific 296-bp fragment from 25 clinical samples obtained from 25 different individuals. Restriction analysis of amplicons showed that identical patterns were seen from digestion of *B. henselae* and *B. quintana* amplicons with *Dde*I, whereas a different unique pattern was seen by using the same enzyme with *B. vinsonii* and *B. elizabethae*. With *Mse*I digestion, *B. henselae* and *B. vinsonii* gave nearly identical patterns while *B. quintana* and *B. elizabethae* gave a different pattern. By combining the restriction analysis data generated with *Mse*I and *Dde*I, unique “signature” restriction patterns characteristic for each species were obtained. These patterns were useful in identifying the *Bartonella* species associated with each tissue specimen.

Twelve species of *Bartonella* have been identified. Four, *Bartonella henselae*, *B. quintana*, *B. elizabethae*, and *B. bacilliformis*, are recognized as human pathogens, and one, *B. clarridgeiae*, has been implicated as a human pathogen by serology (8, 13, 15). *B. bacilliformis*, the first organism in the genus to be identified, causes bartonellosis (3). Recently, *B. henselae* was detected by PCR (1) and isolated from the lymph nodes of patients with cat scratch disease (CSD) (6), as well as from the blood of pet cats (11, 12), providing strong evidence for *B. henselae* as the causative agent of CSD. Both *B. henselae* and *B. quintana* cause bacteremia and bacillary angiomatosis in both immunocompromised and immunocompetent persons (10, 11, 18). *B. quintana* is also the etiologic agent of trench fever (20, 21), and both *B. quintana* and *B. elizabethae* can cause endocarditis (4, 7, 16, 20).

The implication of these *Bartonella* species in a variety of human diseases and the difficulty in isolating them from clinical specimens underscore an urgent need for better detection and identification methods. Birtles identified *Bartonella* species by PCR-restriction fragment length polymorphism (RFLP) analysis (2). We describe the development of a PCR-RFLP assay for the identification of *Bartonella* to the species level and demonstrate the application of this method directly to clinical diagnostic specimens.

**MATERIALS AND METHODS**

*Bartonella* DNA fragments were amplified from 25 tissue specimens, including fresh or paraffin-embedded clinical specimens (lymph node or lymph node aspirates, skin, subcutaneous nodules, or other tissues), obtained from patients participating in epidemiologic studies of CSD and bacillary angiomatosis (19). Amplifications of control tissues were also run in parallel. Control isolates were *B. henselae* ATCC VR-358, *B. quintana* ATCC VR-358, and *B. elizabethae* B97-020005. The PCR amplification was performed in a PC-100 Thermal controller (MJ Research, Watertown, Mass.) for 35 cycles. Each cycle consisted of 120 s at 94°C, 60 s at 60°C, and 90 s at 72°C, and a final extension of 10 min at 72°C was done. The amplified products were detected by electrophoresis on a 1% agarose gel (14 by 14 cm) in 1× Tris-borate-EDTA buffer at 100 V for 60 min. Gels were stained with ethidium bromide and photographed. PCR controls included a known positive DNA extract and a reagent blank.

For digestion of PCR-amplified DNA from cultures and clinical specimens, 10 to 15 μl of PCR-amplified DNA was restricted with 10 U of *Dde*I and *Mse*I in a total volume of 15 to 20 μl, respectively, according to the manufacturer’s specifications (New England Biolabs, Inc., Beverly, Mass.). The restriction fragments were separated by electrophoresis on agarose gels (4% NuSieve agarose [3:1] [FMC Bioproducts, Rockland, Maine] at 60 V for 2 h. Gels were photographed, and fragment sizes were determined with interpolation by using the BioImage system whole-band software analysis (Millipore Corp., Ann Arbor, Mich.).
RESULTS

Figure 1 shows the 296-bp fragment as well as the various RFLP patterns derived from digestion of amplicons of the four species with \textit{DdeI} and \textit{MseI}, and Table 1 lists the observed and predicted sizes of the fragments from the digested amplicons. \textit{B. henselae} and \textit{B. quintana} gave nearly identical patterns with \textit{DdeI}, whereas \textit{B. vinsonii} and \textit{B. elizabethae} gave a different and unique pattern. With \textit{MseI}, \textit{B. henselae} and \textit{B. vinsonii} had nearly identical patterns, and \textit{B. quintana} and \textit{B. elizabethae} had patterns different from those of \textit{B. henselae} and \textit{B. vinsonii} but nearly identical to each other. Composite types generated from restriction analysis using \textit{DdeI} and \textit{MseI} were unique for each species (Table 1).

Table 2 shows the distribution of \textit{Bartonella} species among 25 clinical specimens. Twenty-one of 26 (81\%) of the isolates gave the restriction pattern for \textit{B. henselae}, 4 (15.38\%) gave that for \textit{B. quintana}, and 1 (4\%) gave that for \textit{B. elizabethae}.

The RFLP patterns of \textit{B. henselae} and \textit{B. quintana} obtained from clinical specimens are shown in Fig. 2. All 21 \textit{B. henselae} amplified PCR products have the same composite restriction pattern with \textit{DdeI} and \textit{MseI}. This pattern is identical to that found for the \textit{B. henselae} type strain (B91-002000). All four \textit{B. quintana} amplified PCR products have the same composite restriction pattern, which is identical to that of the \textit{B. quintana} type strain (ATCC VR-358). It should be noted that minor differences in fragment lengths among isolates within the same species may be encountered (5). The sizes of digested DNA fragments are shown in Table 1.

DISCUSSION

We were able to differentiate among the different \textit{Bartonella} species implicated in human disease by extending the 16S rDNA-based detection method for \textit{Bartonella} species by using restriction analysis of the 296-bp amplicons. This was achieved by generating species-specific composite patterns based on digestion of 16S rDNA amplicons with \textit{DdeI} and \textit{MseI} restriction enzymes. The RFLP method was applied to amplicons from clinical specimens, allowing direct species identification for these specimens. The predicted and observed fragment lengths for all four species matched each other. Predicted fragments 28 for \textit{B. vinsonii} and \textit{B. elizabethae} cut with \textit{DdeI} were not observed because they were small and were not visualized on gels.

Our method was shown to be more advantageous than a previously described method (1) because only one set of primers was used in this study, followed by restriction analysis, allowing for direct identification in a variety of clinical specimens of the \textit{Bartonella} species implicated in human disease (Table 2). Although the method described by Anderson et al. (1) allows for the detection of \textit{B. quintana} and \textit{B. henselae} in lymph nodes and lymph node aspirates, it requires multiple steps and the use of radioactive probes.

Our PCR-RFLP-based assay may offer the advantage of early diagnosis of suspected \textit{Bartonella} species infections and can be used to differentiate among three species causing human disease in North America. This rapid and specific test is

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**TABLE 1. Identification of \textit{Bartonella} species by PCR-RFLP analysis of a 16S rDNA fragment**

<table>
<thead>
<tr>
<th>\textit{Bartonella} sp.</th>
<th>\textit{DdeI} Lengths of fragments* (bp)</th>
<th>\textit{MseI} Lengths of fragments (bp)</th>
<th>PCR-RFLP composite pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{O} \hspace{1cm} \textit{P}</td>
<td>\textit{O} \hspace{1cm} \textit{P}</td>
<td></td>
</tr>
<tr>
<td>\textit{B. henselae}</td>
<td>190, 115 \hspace{1cm} 187, 109</td>
<td>214, 81 \hspace{1cm} 217, 79</td>
<td>I</td>
</tr>
<tr>
<td>\textit{B. vinsonii}</td>
<td>158, 114 \hspace{1cm} 159, 109, 28</td>
<td>218, 82 \hspace{1cm} 217, 79</td>
<td>II</td>
</tr>
<tr>
<td>\textit{B. elizabethae}</td>
<td>154, 110 \hspace{1cm} 159, 109, 28</td>
<td>166, 79, 52 \hspace{1cm} 173, 79, 44</td>
<td>III</td>
</tr>
<tr>
<td>\textit{B. quintana}</td>
<td>180, 106 \hspace{1cm} 187, 109</td>
<td>168, 75, 47 \hspace{1cm} 173, 79, 44</td>
<td>IV</td>
</tr>
</tbody>
</table>

* \textit{O}, observed; \textit{P}, predicted (GenBank sequences).
an alternative to culture that provides more-timely information to clinicians, who can then direct antibiotic therapy and suggest prevention strategies to their patients based on species-specific test results (11).

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REFERENCES