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## Two-Step Polymerase Chain Reaction Assay for Detection of *Yersinia* Species in General and of Pathogenic *Yersinia enterocolitica* Strains Specifically

Paul T. Odinot, MSc;\* Jacques F.G.M. Meis, PhD, MD;\* Jo H.A.J. Curfs, PhD;\* Jacomina A.A. Hoogkamp-Korstanje, MD;\* and Willem J.G. Melchers, PhD\*

### ABSTRACT

**Objectives:** Detection of *Yersinia enterocolitica* in clinical samples is still not sensitive and fast enough. Polymerase chain reaction (PCR) offers the advantages of sensitivity, specificity, and rapidity. A two-step PCR assay to detect pathogenic *Y. enterocolitica* in infected tissue has been developed.

**Method:** In the first step of the PCR assay, a general primer PCR amplification of the small subunit rDNA gene was used for the detection of a broad range of *Yersinia* species. In the second step, the virulence plasmid encoded *virF*-gene of *Y. enterocolitica* was specifically amplified in a nested PCR to identify pathogenic *Y. enterocolitica*.

**Results:** With this two-step PCR assay it was possible to detect *Yersinia* in general as well as pathogenic *Y. enterocolitica* specifically. The assay was then applied to detect *Y. enterocolitica* in spleens of experimentally infected rats.

**Discussion:** This study shows the potential use of the PCR for the detection of pathogenic *Y. enterocolitica*, and its ability to detect *Y. enterocolitica* in non-seeded samples.

**Key Words:** PCR detection, rats, 16S rRNA, *virF*, *Yersinia*, *Yersinia enterocolitica*

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Three *Yersinia* species are pathogenic for humans, namely *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*. *Yersinia pestis* is the causative agent of plague, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* are the pathogens responsible for non-

plague yersiniosis. *Yersinia pseudotuberculosis* is prominently involved in rodent infections,<sup>1</sup> and *Y. enterocolitica* may be the cause of gastroenteritis in humans.<sup>2</sup>

Pathogenic *Yersinia* harbor a plasmid (pYV) with a molecular weight of about 70 kb. Several proteins are encoded by this plasmid, including Ca<sup>2+</sup> and temperature inducible proteins, *Yersinia* outer membrane proteins (YOPs). The loss of the complete plasmid or of some YOPs always correlates with the loss of pathogenicity.<sup>3</sup> Besides plasmid-associated virulence, there are chromosomal loci involved in the pathogenicity of *Yersinia* (i.e., the *inv* and *ail* genes) which encode proteins that facilitate the invasion of epithelial cells.<sup>4</sup>

For the detection of pathogenic *Y. enterocolitica*, polymerase chain reaction (PCR) offers the advantages of maximum sensitivity, specificity, and rapidity. Recently several PCRs have been described for the detection of pathogenic *Y. enterocolitica*.<sup>5-8</sup> These methods depend on the detection of either chromosomal or plasmid sequences. With primers based only upon chromosomal sequences, all *Y. enterocolitica* strains are detected, including those that do not contain pYV. In addition, with primers based only upon pYV sequences, false-negative detection may occur. Nakajima et al overcame these problems by using a mixture of primers against the plasmid-encoded *virF* gene, the *ail* gene of *Y. enterocolitica* and the *inv* gene of *Y. pseudotuberculosis*.<sup>9</sup> However, all described PCR assays for *Yersinia* were performed on cultured bacteria and, in some cases, on *Y. enterocolitica*-seeded fecal samples.<sup>5-8</sup> To date, only Norkina et al have described a diagnostic PCR test for *Y. pestis*.<sup>10</sup> They were able to detect *Y. pestis* in blood of experimentally infected mice.

In this report, a general-primer PCR assay is described using the 16S rDNA gene as target for the detection of *Yersinia* species. To specifically detect pathogenic *Y. enterocolitica* in clinical material, a nested PCR assay was developed with primers targeted to the *virF*-gene, which is considered to be the key regulator gene for the YOPs.<sup>1</sup> First, the specificity and the sensitivity of the PCR assay was tested. Secondly, the PCR assay was further validated on experimentally infected rats.

\*Department of Medical Microbiology, University Hospital Nijmegen, Nijmegen, The Netherlands.

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Address correspondence to Mr. Paul T. Odinot, Department of Medical Microbiology, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

## MATERIAL AND METHODS

### Organisms

*Yersinia ruckeri*, *Yersinia kristensenii* (patient isolate), *Yersinia frederiksenii* (patient isolate), *Y. pseudotuberculosis* (patient isolate), and *Y. enterocolitica* 0:8 WA (ATCC 9610) were available for testing. In addition, a panel of other gram-negative microorganisms chosen as controls included *Bacteroides fragilis* (ATCC 10584), *Pseudomonas aeruginosa* (ATCC 101145), *Proteus vulgaris* (ATCC 6380), *Klebsiella pneumoniae* (ATCC 10031), *Enterobacter cloacae* (ATCC 13047), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 13311), *Shigella sonnei* (ATCC 11060), and *Shigella flexneri* (patient isolate). Clinical isolates were identified with the API 20E identification system (Bio Mérieux, Marcy l'Etoile, France). The DNA from the bacterial strains was purified as previously described.<sup>11</sup>

### Selection of 16S Recombinant RNA Gene Primers and Probe

The *Y. enterocolitica* 16S rRNA gene sequence was aligned to sequences of other microorganisms, using the sequence analysis software package (Genetics Computer Group at the University of Wisconsin, Madison, WI, USA) implemented on a Vax computer. *Yersinia* genus-specific sequences were selected. The alignment resulted in two primers and a probe: Ye16S1Q 5'-AGTCGAGCG-GCAGCGGGAAGT-3', located in variable region 1 and Ye16S3, 5'-TCTGGGCACATCCGATGGCGT-3', located in the variable region 2 of the 16S rRNA gene. Positive PCR amplification with these primers yielded a product of 179 bp. The probe: Ye16S2, 5'-TTTACTACTTCCGGGCGAGC-3', located in the variable region 1, was used for Southern blot hybridization.

### Selection of *virF* Primers and Probe

The *Y. enterocolitica virF* gene sequence,<sup>12</sup> the transcriptional activator of the *Yersinia* virulence regulon, was used to select two pair of primers and a probe. The first pair of primers: YvirF2, 5'-GCTCTGCGCCATTAG-GCAAC-3' and YvirFwt2, 5'-ACTCATCTTACCATTAA-GAAG-3', yielded a 224-bp fragment. The second pair of primers targeted to the internal portion of the first amplification product resulted in a PCR product of 94 bp: YvirF3, 5'-CCCAGAAGAACGGTTGCAGAAAT-3' and YvirF5, 5'-AGAATACGTCGCTCGCTTATCC-3'. Primer YvirFwt2 was previously described by Wren and Tabaqchali.<sup>8</sup> The probe, used for hybridization of Southern blots, was targeted to the internal portion of both amplification products: YvirF4, 5'-GTTAATCCCATGCC-GAATTCTCG-3'.

### Experimentally Infected Rats

Nine Lewis rats (Central Animal Laboratory, Nijmegen, The Netherlands) were intravenously infected with 10<sup>4</sup> colony-forming units (CFU) of *Y. enterocolitica* 0:8 WA (ATCC 9610). A non-infected Lewis rat was used as a negative control. Fourteen days after infection, all rats were killed and their spleens were removed. The tissues were cut and divided into two portions. The first was used to inoculate Cefsulon, Irgasan, novobiocin (CIN) agar, which then was incubated at 37°C for 3 days to isolate *Y. enterocolitica*; 27 mm<sup>3</sup> of the second portion was cut and homogenized with disposable pellet pestles and tubes (Kontes, Vineland, NJ, USA), in 200 µL PAS-buffer (6% *p*-aminosalicylic acid [Sigma, St. Louis, LA, USA], 1% NaCl, 10 mM EDTA, pH 8); 100 µg proteinase K (Boehringer, Mannheim, Germany) was added; and the samples were incubated for 2 hours at 37°C. DNA was purified by successive phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol extractions followed by precipitation with ethanol overnight at -20°C. The DNA was dissolved in 100 µL of distilled water.<sup>13</sup> DNA concentration was determined with a Pharmacia GeneQuant RNA/DNA calculator (Pharmacia LKB, Cambridge, UK).

### Polymerase Chain Reaction Amplification of 16S Recombinant DNA Gene- and *virF*-Sequences

Polymerase chain reaction assays were performed in 100 µL of reaction mixture containing: 200 ng of DNA, 75 mM Tris-HCl (pH 9.0), 2.5 mM MgCl<sub>2</sub>, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% Tween-20, 0.2 mM dNTPs, 50 pmol sense primer, 50 pmol antisense primer, and 0.2 U *Taq* DNA polymerase (Thermopertect plus DNA polymerase, Integro, Zaandam, The Netherlands). Amplification was performed in a Perkin-Elmer Cetus 9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). For 16S rDNA amplification, primers Ye16S1 and Ye16S3 were used. The reactions were subjected to 40 cycles of amplification by means of the following cycling program: 1 minute denaturation at 94°C, 1 minute annealing at 60°C, and 30 seconds extension at 72°C. For the *virF* nested PCR, the first 40 cycles were performed with primers YvirF2 and YvirFwt2 and by means of a cycling program of: 1 minute at 94°C, 1 minute at 55°C, and 30 seconds at 72°C. After the last cycle, 10 µL of the reaction mixture was added to a new PCR mixture containing primers YvirF3 and YvirF5. Again, 40 cycles of PCR amplification were performed. Each cycle for the nested PCR-amplification reaction involved 1 minute at 94°C, 1 minute at 65°C, and 30 seconds at 72°C. All cycling programs started with an initial denaturation step of 4 minutes at 94°C.

**Analysis of Amplified Samples**

Aliquots of amplified samples (10 µL) were analyzed by gel electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. For Southern blotting, the agarose gel was depurated in 0.25 M HCl, denatured in 0.5 M NaOH-1.5 M NaCl, and transferred to a nylon membrane (Hybond; Amersham International, Amersham, England) by overnight diffusion blotting in 0.5 M NaOH-1.5 M NaCl. The DNA was bound to the nylon membrane by baking at 80°C for 2 hours. The oligonucleotide probes, Ye16S2 for detection of 16S rRNA gene PCR products and YvirF4 for detection of *virF*-PCR products, were 3'-end labelled with digoxigenin-11-dUTP, according the terminal transferase method in the manufacturer's instructions (Boehringer). Hybridization was performed by standard techniques.<sup>13</sup> The membranes were prehybridized in 6× SSC (1× SSC is 15 mM sodium citrate plus 150 mM sodium chloride), 5× Denhardt solution (1× Denhardt solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 0.1% sodium dodecyl sul-

fate (SDS), 250 µg of denatured sonicated salmon sperm DNA per mL at 42°C for 2 hours. Hybridization was performed for 16 hours at 42°C in 6× SSC, 1× Denhardt solution, 0.1% SDS, 100 µg of denatured sonicated salmon sperm DNA per mL plus 5 µL of 3'-end labelled oligonucleotide probes per 10 mL of hybridization mix. The blots were washed twice for 15 minutes at 42°C in 5× SSC, 0.1% SDS, and twice for 15 minutes at 42°C in 1× SSC, 0.1% SDS. The detection method used was in accordance with the manufacturer's instructions (Boehringer) for detection of digoxigenin-11 dUTP-labelled oligonucleotides with chemiluminescence.

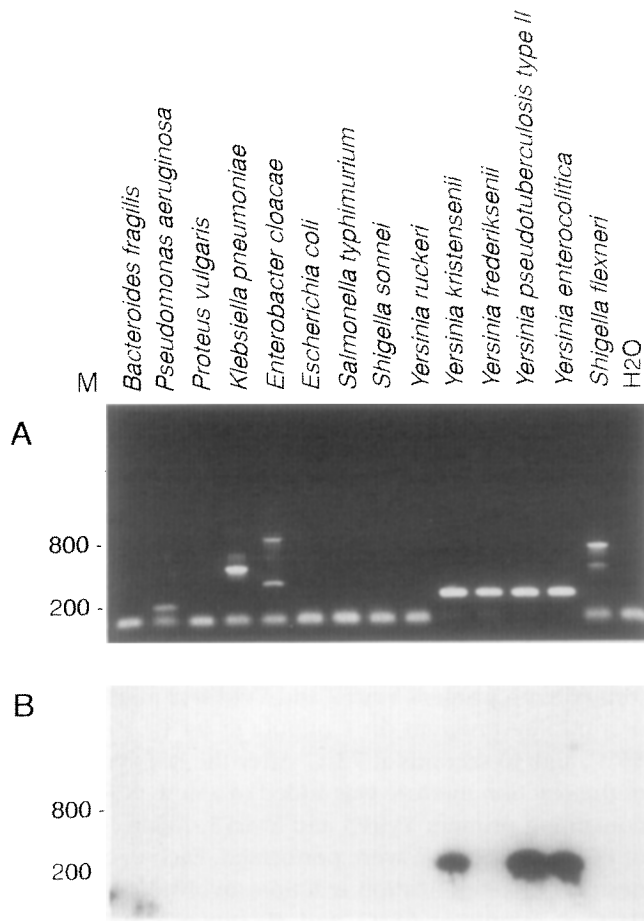
**RESULTS**

**Specificity of Yersinia 16S Recombinant DNA General Polymerase Chain Reaction Assay**

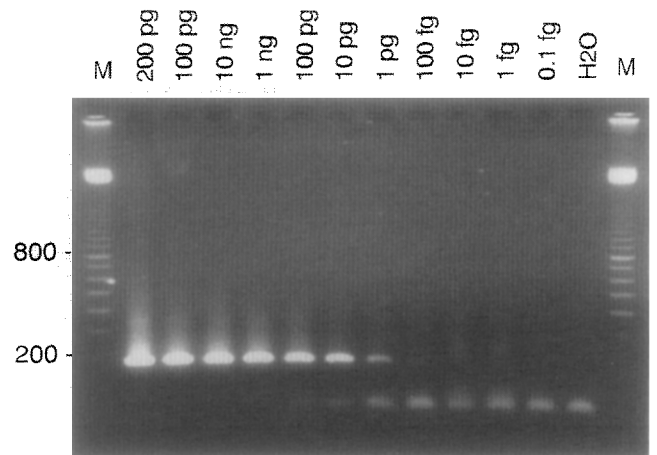
Polymerase chain reaction amplification was performed on a panel of gram-negative bacterial strains to exclude nonspecific primer annealing. Specific amplification was observed with four *Yersinia* species (*Y. kristensenii*, *Y. frederiksenii*, *Y. pseudotuberculosis*, and *Y. enterocolitica*), but not with *Y. ruckeri*. None of the other microorganisms showed a positive amplification, with the exception of *S. flexneri*, *E. cloacea*, and *K. pneumoniae* (Figure 1). Nevertheless Southern blot analysis with the oligonucleotide probe, Ye16S2, showed only clear hybridization with *Y. kristensenii*, *Y. pseudotuberculosis*, and *Y. enterocolitica* (see Figure 1). *Y. frederiksenii* did not hybridize with the oligonucleotide probe.

**Sensitivity of Yersinia 16S Recombinant DNA General Polymerase Chain Reaction Assay**

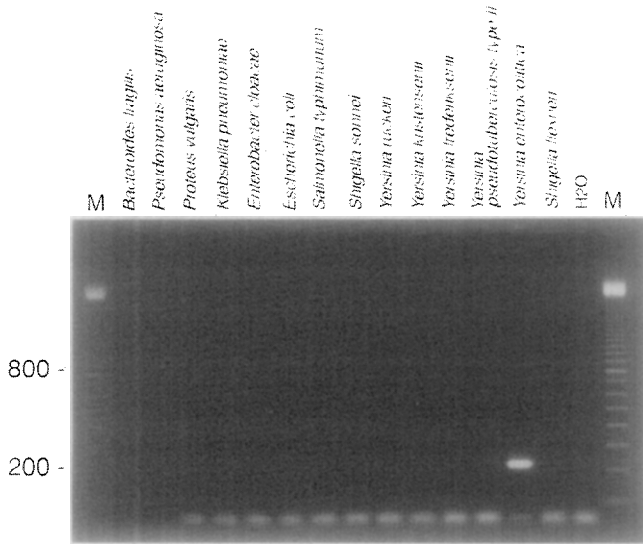
Serial tenfold dilutions of purified *Y. enterocolitica* DNA were tested by PCR. A sensitivity of 100 fg of DNA was obtained, as detected by gel electrophoresis (Figure 2).



**Figure 1.** Specificity of the 16S rRNA-gene PCR for Yersinia. Polymerase chain reaction was performed on a number of bacterial species as indicated in the figure. The reactions were analyzed by gel electrophoresis (A) and Southern blot hybridization (B). M indicates the molecular weight marker (100 bp ladder).



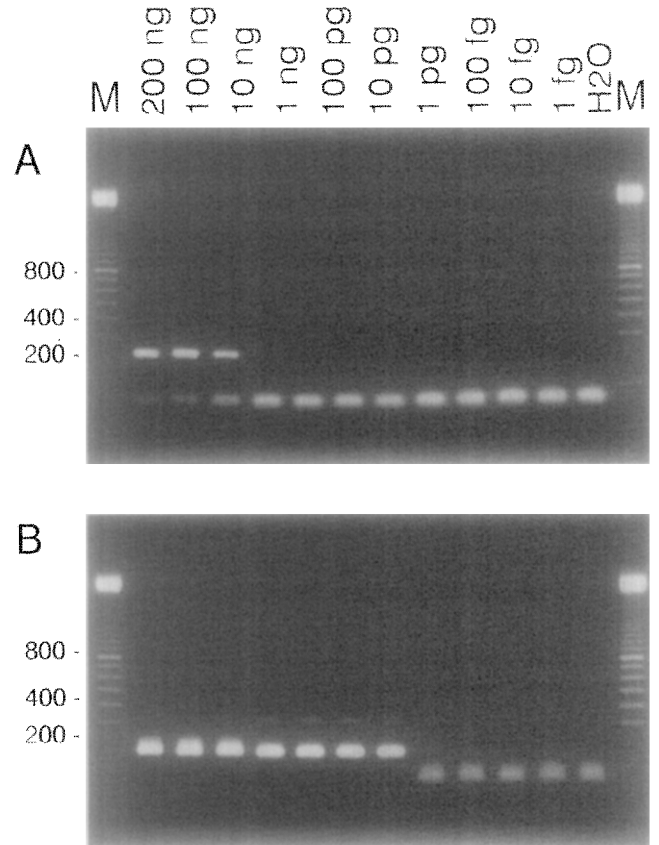
**Figure 2.** Sensitivity of the 16S rRNA-gene PCR for Yersinia. Sensitivity of the detection of tenfold diluted purified *Y. enterocolitica* DNA. The DNA concentrations are indicated above the lanes. M indicates the size marker (100 bp ladder).



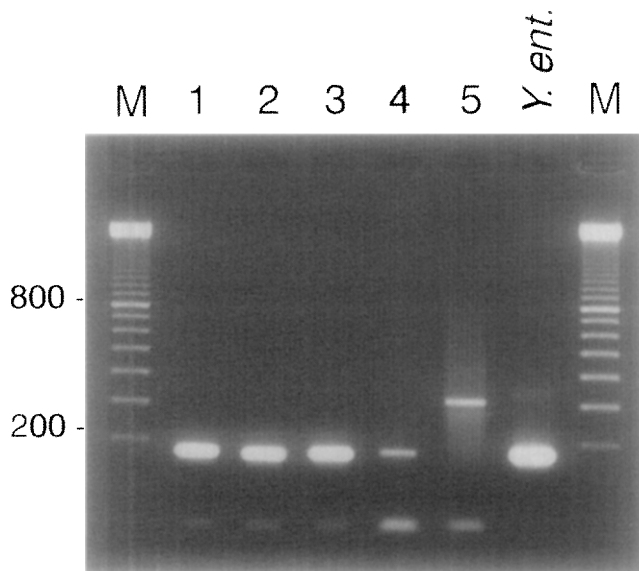
**Figure 3.** Specificity of the *virF*-gene PCR of *Y. enterocolitica*. Polymerase chain reaction was performed on a number of bacterial species as indicated in the figure. The reactions were analyzed by gel electrophoresis. M indicates the molecular weight marker (100 bp ladder).

**Specificity of *Y. enterocolitica*-Specific Polymerase Chain Reaction Assay**

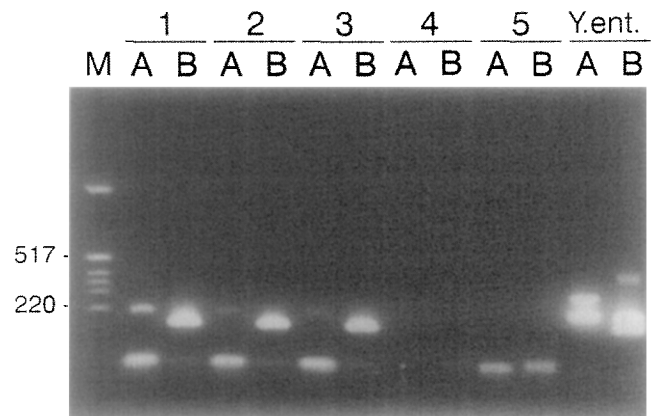
Specificity testing for PCR amplification was performed on all previously mentioned gram-negative bacterial strains. Only *Y. enterocolitica* showed positive PCR amplification (Figure 3).



**Figure 4.** Sensitivity of the *virF*-gene PCR of *Y. enterocolitica*. Sensitivity of the detection of tenfold diluted purified *Y. enterocolitica* DNA. The DNA concentrations are indicated above the lanes. A, Results of PCR amplification of the "single" PCR; B, results of PCR amplification of the "nested" PCR.



**Figure 5.** 16S rDNA PCR on infected rat spleens. Polymerase chain reaction analysis of experimentally infected rats. DNA was extracted from spleens and subjected to a general primer PCR assay for *Yersinia*. 1, Spleen of rat number 1; 2, spleen of rat number 2; 3, spleen of rat number 3; 4, spleen of rat number 4; 5, spleen of rat number 5 (uninfected control rat); *Y. ent.*, *Y. enterocolitica* DNA; M, marker lanes (100 bp ladder).



**Figure 6.** *virF* PCR on infected rat spleens. PCR analysis of experimentally infected rats. DNA was extracted from spleens and subjected to *Y. enterocolitica*-specific PCR amplification. 1, spleen of rat number 1; 2, spleen of rat number 2; 3, spleen of rat number 3; 4, spleen of rat number 4; 5, spleen of rat number 5 (uninfected control rat); *Y. ent.*, *Y. enterocolitica* DNA. The A and the B above the lanes indicate a single PCR and a nested PCR, respectively. M indicates the molecular weight marker (pBR322 *Hinf*).

### Sensitivity of *Y. enterocolitica*-Specific Polymerase Chain Reaction Assay

To determine the sensitivity of the *Y. enterocolitica*-specific nested PCR, serial dilutions of purified *Y. enterocolitica* DNA were tested by PCR amplification. With the "single" PCR, a sensitivity on the gel of 100 pg of *Y. enterocolitica* DNA was obtained. With the "nested" PCR assay a sensitivity on the gel of 1 pg of target DNA was obtained (Figure 4).

### Experimentally Infected Rats

*Yersinia enterocolitica* was cultured from the dissected spleens of all nine infected rats. With the 16S rDNA PCR all spleens were positive (Figure 5), whereas the *virF*-PCR was positive for eight animals (Figure 6). The control spleen was negative in culture and in both PCR assays.

### DISCUSSION

The detection of *Y. enterocolitica* in clinical samples is still hindered by the limitations of the available diagnostic methods. Immunofluorescence microscopy either alone or in combination with culturing is rather insensitive. Culturing the bacterium must be preceded by a cold enrichment, in case of fecal contamination, to obtain a higher sensitivity and is therefore time consuming. Since infection with *Y. enterocolitica* may lead to severe clinical sequelae and bacteria may persist in different organs of the human body, it is important to detect the bacterium in an early phase of the infection to start treatment. As with many other microorganisms, PCR has the potential to contribute to a more efficient diagnosis. Indeed, several PCR assays for the detection of *Y. enterocolitica* already have been described. However, what they all have in common is that they have only been tested on cultured bacteria or seeded clinical samples.<sup>5-8</sup> Apart from this, these assays only detect either pathogenic Yersiniaceae alone or *Yersinia* species in general. This is why the clinical value of the PCR for the detection of *Y. enterocolitica* remains to be evaluated.

By contrast, in this report, the authors described a two-step PCR assay for the detection of both Yersiniaceae in general and pathogenic *Y. enterocolitica* specifically. Using the general small subunit ribosomal RNA (ssrRNA) primer PCR assay for the detection of Yersiniaceae, the authors were able to amplify four of five *Yersinia* species tested. No amplification was observed with *Y. ruckeri*. This may be because a limited chromosomal relatedness exists between *Y. ruckeri* and other Yersiniaceae,<sup>14</sup> which suggests an apposition of the taxonomy of this *Yersinia* species. Indeed, alignment of the ssrRNA sequence of *Y.*

*ruckeri* with other sequenced Yersiniaceae revealed only a limited homology. Moreover, *Y. ruckeri* is not considered to be a human pathogen, but is found mostly in fish.<sup>15</sup> The amplification product of *Y. frederiksenii* did not hybridize with the ssrRNA oligonucleotide probe Ye16S2. Alignment of the probe sequence revealed four mismatches, excluding hybridization under stringent conditions, similarly as has been observed with *Aspergillus*.<sup>16</sup> However, *Y. frederiksenii* is more closely related to *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* in comparison to *Y. ruckeri*.<sup>17</sup> No hybridization of the *Y. frederiksenii* amplification product after general ssrRNA gene PCR with the ssrRNA probe can be accepted, since *Y. frederiksenii* is not seen as a human pathogen.<sup>1,2</sup> Although *Y. pestis* was not available for testing, alignment of the ssrRNA of this species with the other *Yersinia* species predicted that *Y. pestis* can be detected equally well by the primer set described.

The *virF* PCR, in contrast to the ssrRNA PCR, detects only pathogenic *Y. enterocolitica* isolates. No amplification with any other *Yersinia* isolates was observed. The sensitivity of the *virF*-PCR assay, however, was 1 pg, which equals approximately 500 genome equivalents, whereas the sensitivity of the 16S rDNA PCR is 100 fg, which equals approximately 50 genome equivalents. This difference in sensitivity was expressed when the two-step PCR assay was evaluated in experimentally infected rats. All nine spleens from the *Y. enterocolitica*-infected rats were found positive by the general PCR assay, but only eight were found positive when the *virF*-gene PCR assay was used. In contrast other studies showed a detection rate of 10 to 30 bacteria in seeded samples.<sup>7,9</sup> Although no information is available yet concerning the amount of bacteria present in tissue samples from patients, it cannot be excluded that the sensitivity for the detection of pathogenic *Y. enterocolitica* is not sufficient. Therefore, further evaluation and validation of this technique on human biopsy samples should be performed.

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