Rapid Identification of *Yersinia enterocolitica* in Blood by the 5′ Nuclease PCR Assay

KEYA SEN*

Division of Emerging and Transfusion Transmitted Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, Maryland 20852

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*Yersinia enterocolitica* accounts for 50% of the clinical sepsis episodes caused by the transfusion of contaminated red blood cells. A 5′ nuclease TaqMan PCR assay was developed to detect *Y. enterocolitica* in blood. Primers and a probe based on the nucleotide sequence of the 16S rRNA gene from *Y. enterocolitica* were designed. Whole-blood samples were spiked with various numbers of *Y. enterocolitica* cells, and total chromosomal DNA was extracted. When the TaqMan PCR assay was performed, as few as six bacteria spiked in 200 μl of blood could be detected. The assay was specific and did not detect other *Yersinia* species. The TaqMan assay is easy to perform, takes 2 h, and has the potential for use in the rapid detection of *Y. enterocolitica* contamination in stored blood units.

*Yersinia enterocolitica*, a gram-negative bacterium, is responsible for 50% of all the clinical sepsis episodes that occur as a result of transfusion of contaminated red blood cells (RBCs) (19). It is the major bacterial contaminant found in RBC concentrates, and the result of such contamination has proven to be fatal in 61% of all the reported cases of *Y. enterocolitica* sepsis resulting from transfusion (19). *Pseudomonas fluorescens* is responsible for a further 26% of the transfusion-associated sepsis episodes. *Serratia liquefaciens* has become another major bacterial pathogen to contaminate blood product units, and since 1992, five cases have been reported to the Centers for Disease Control and Prevention (27). Other bacteria implicated in RBC contamination are *Pseudomonas putida*, *Campylobacter jejuni*, *Enterobacter jejuni*, *Escherichia coli*, and *Flavobacterium* (32). The ability of *Y. enterocolitica* to contaminate RBCs can be attributed to its being a psychrophilic bacterium that can survive well at refrigerator temperatures, using dextrose and iron from the blood. Thus, packed red cells, which are usually stored at 4°C for up to 42 days, can allow the growth of these bacteria (32). The bacteria go through an initial lag period of 7 to 32 days during storage at 4°C and then show exponential growth, with an 18- to 20-h doubling time (3, 8, 18, 29, 32). *Y. enterocolitica* secretes an endotoxin, which is probably responsible for much of the morbidity and mortality (9). While the bacterium may lose its virulence during storage, since the plasmid that encodes virulence factors that lead to cellular invasion and resistance to the complement-mediated lysis is often lost during storage (19, 24), the endotoxin released and the bacterium itself act as sources of toxicity. Conventional methods for detection of this bacterium in blood include detection of the endotoxin it produces by the Limulus amebocyte lysate assay (30) and staining of the bacterial cells with hematologic stains such as acidine orange or Giemsa or Wright-Giemsa (10, 23). However, the identity of the bacterium cannot be established by these methods. In addition, these methods depend on the growth of the bacteria to a density of 10⁶ to 10⁹ CFU per ml, a level that may take several days to achieve. A PCR-based assay for detection of *Y. enterocolitica* in blood was described by Feng et al. (11). They could detect 500 bacteria seeded in 100 μl of blood, or 5,000 bacteria/ml. This was a significant improvement in the detection sensitivity and specificity and suggested that PCR could be useful for detecting bacterial contamination in stored blood units. This assay, however, may not be able to detect early contamination, when only a few bacteria are present in blood. Therefore, newer nucleic acid-based methodology was sought, to achieve this level of detection sensitivity.

The 5′ nuclease fluorogenic TaqMan assay has been recently described (21). The method exploits the property of *Taq* polymerase to act as a 5′-3′ exonuclease (15). Briefly, an oligonucleotide probe that has a reporter fluorescent dye attached to its 5′ end and a quencher dye attached to its 3′ end is used in the assay. Initially, the unbound probe is not able to emit a fluorescent signal because of the proximity of the reporter and quencher dyes. When the probe hybridizes to its target template, the reporter dye is cleaved by the 5′ nuclease activity of *Taq* polymerase and becomes capable of emitting a fluorescent signal without the suppression activity of the quencher dye. With increasing cycles of amplification more fluorescent signal is generated by binding of the probe to more available target, which can be detected in real time by the ABI 7700 sequence detector (PE Applied Biosystems, Foster City, Calif.). The sequence detector contains a thermocycler, laser detection system, and analysis software system. Analysis of the signal takes only about a minute after the PCR is completed. Because the generation of the fluorescent signal depends on the hybridization of the probe to a specific template, which is being amplified, there is less scope for false signals from nonspecific amplification. It is necessary to detect a very few bacteria in blood, which may have contaminated a unit, in order to avoid a sepsis reaction, and this assay seemed to have the potential for such detection. In this report, a TaqMan PCR assay is described which is rapid and shows specific and sensitive detection of *Y. enterocolitica* in blood.

**MATERIALS AND METHODS**

**Bacterial species and culture conditions.** *Y. enterocolitica* isolates of serotypes Y:288, O:3, O:1,2,3, O:5,27, and O:20 were obtained from P. Feng (Food and Drug Administration, Washington, D.C.), Serotypes O:3, O:1,2,3, O:5,27, and O:20 were previously implicated in blood endotoxemia resulting from transfusion (30). The bacteria were grown in brain heart infusion (BHI) broth (Sigma, St.

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* Mailing address: Division of Emerging and Transfusion Transmitted Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, HFM-320, 1401 Rockville Pike, Rockville, MD 20852. Phone: (301) 594-6752. Fax: (301) 594-6989. E-mail: Senk @cber.fda.gov.
TABLE 1. Specificity of the Y. enterocolitica TaqMan assay

<table>
<thead>
<tr>
<th>Species</th>
<th>Serotype or strain</th>
<th>% Homology with primers and/or probe</th>
<th>C&lt;sub&gt;T&lt;/sub&gt; value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yersinia enterocolitica</td>
<td>O:3</td>
<td>100 with primers and probe</td>
<td>18.94</td>
</tr>
<tr>
<td></td>
<td>O:1,2,3</td>
<td>100 with primers and probe</td>
<td>19.2</td>
</tr>
<tr>
<td></td>
<td>O:5,27</td>
<td>100 with primers and probe</td>
<td>18.18</td>
</tr>
<tr>
<td></td>
<td>O:20</td>
<td>100 with primers and probe</td>
<td>20.9</td>
</tr>
<tr>
<td></td>
<td>Y. pseudotuberculosis</td>
<td>ATCC 29833</td>
<td>18.69</td>
</tr>
<tr>
<td></td>
<td>Y. frederiksenii</td>
<td>ATCC 33641</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hafnia alvei</td>
<td>ATCC 13337</td>
<td></td>
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<tr>
<td></td>
<td>Serratia entomophila</td>
<td>ATCC 43705</td>
<td></td>
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<tr>
<td></td>
<td>Serratia ficaria</td>
<td>ATCC 33105</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serratia grimesii</td>
<td>ATCC 14460</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pseudomonas fluorescens</td>
<td>ATCC 13525</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serratia liquefaciens</td>
<td>ATCC 35551</td>
<td></td>
</tr>
</tbody>
</table>

* Total DNA was isolated from the bacteria by the Puregene kit. For the Y. enterocolitica strains, 20 ng of template DNA was used per 50 μl of PCR mixture. For the other bacterial strains, 200 ng of template DNA was used per 50 μl of PCR mixture.

RESULTS

Theoretical detection limit by TaqMan PCR. In order to determine the sensitivity of the assay, pure chromosomal DNA from serotype O:3 was isolated by the Puregene kit from overnight cultures of bacteria grown in BHI medium at 30°C. TaqMan PCR was performed by using 100 ng to 1 fg of chromosomal DNA as a template and the primers and probes described above. A DNA concentration of ≥5 fg could be detected (Fig. 1). Since the size of the Y. enterocolitica genome is not known, this number could not be used to calculate the exact number of bacteria that could be detected. However, based on the size of the Yersinia pestis genome, which is 4,398 kb (22), and that of the Yersinia ruckeri genome, which is 4,460 to 4,770 kb (26), if we assume the size of the Y. enterocolitica genome to be 4,400 kb, then 5 fg of DNA would amount to approximately one genome. If there were 1 copy of the 16S rRNA gene per cell, this would translate to 1 template, or 10 templates if there were 10 copies of the gene per cell, and so on. The 201-bp PCR fragment was also cloned into E. coli using the TopoTA cloning vector pCR 2.1-Topo, of a length of 3.9 kb, from the Topo TA cloning kit (Invitrogen Corp., Carlsbad, Calif.). When the plasmid pKSY, having a length of 4,101 kb (3.9 + 0.201 kb) and containing the cloned insert, was used as a template, the detection limit was 0.0061 fg. This number amounted to a copy number of 1.3. This was calculated as follows: 4.101 kb is equal to 2.7 × 10<sup>3</sup> g/mol, and 0.006 fg of a 4,101-kb plasmid would contain 2.2 × 10<sup>−23</sup> mol. Multiplying this number by Avogadro’s number, 6 × 10<sup>23</sup>, gives the number...
of molecules or the copy number value of 1.3. Thus, the primers and the probe combination as well as the length of the amplicon which was being amplified were optimal, since the greatest TaqMan PCR sensitivity could be achieved, which was 1 CFU.

Specificity of TaqMan PCR. The specificity of the TaqMan PCR assay for *Y. enterocolitica* strains was examined by isolating genomic DNA from five different *Y. enterocolitica* strains. In PCR assays, 20 ng of this pure DNA was used. A C<sub>T</sub> value in the range from 18.2 to 20.9 was obtained with each of the *Y. enterocolitica* strains (Fig. 2; Table 1). When 200-ng samples of chromosomal DNA from the related species *Yersinia frederiksenii* and *Yersinia pseudotuberculosis* were used, the C<sub>T</sub> values obtained were 45 (Fig. 2; Table 1). The C<sub>T</sub> values were also 45 when two other major bacteria that contaminate RBC units, *P. fluorescens* and *S. liquefaciens*, were examined by this set of primers and probe (Fig. 2; Table 1). The TaqMan probe YE1, in addition to recognizing all *Y. enterocolitica* strains, also showed 100% homology with 16S rDNA of *Hafnia alvei*, some *Serratia* spp., and *Erwinia* spp. TaqMan PCR was therefore done with chromosomal DNA isolated from *H. alvei*, *Serratia ficaria*, and *Serratia grimesii*. The C<sub>T</sub> values obtained were 45.

The primers and probe could not be tested with *Erwinia* species because a sample chromosomal DNA could not be obtained. But it is unlikely that the assay would recognize *Erwinia* species because a set of three oligonucleotides is used in the TaqMan assay, and for a positive reaction, all three would have to show substantial homology. Thus, even though *Y. frederiksenii* showed 100% homology with the forward primer and 84% homology with the reverse primer, since the probe did not show any major sequence homology, the C<sub>T</sub> value was 45 (Fig. 2; Table 1).

Blood samples. Logarithmic-phase *Y. enterocolitica* O:3 cells were spiked into blood at different concentrations. Six different kits were tried initially for extraction of the total DNA, containing DNA from blood and bacteria. The DNA extracted by the QiAamp kit and the Dynal kit proved to be equivalent for the TaqMan assay (Fig. 3). In addition, both methods were fast; they took only 20 min for extraction of the DNA. In four different spiking experiments, the minimum threshold of detection was six bacteria spiked into 200 µl of blood. Represenative data, of a dilution series with 6 to 6,000 bacteria spiked into 200 µl of blood, are shown in Fig. 3. The C<sub>T</sub> values obtained typically were between 36 and 37. Optimization experiments were performed initially, to choose the right probe concentration and magnesium concentration, so that the highest ΔR<sub>n</sub> values were obtained without compromising the specificity of the signal. The cutoff C<sub>T</sub> value was taken to be 39, and any signal higher than this was not considered a positive signal because the unspiked blood sometimes gave a signal at a C<sub>T</sub> value of 40. However, in these samples the ΔR<sub>n</sub> value was less than 0.05.

DISCUSSION

The need to identify a very small number of bacteria in blood that is to be transfused is critical. In this report the development of a PCR-based 5′ nuclease assay to detect a small number of *Y. enterocolitica* organisms in blood is described. The use of PCR to identify the presence of microbial DNA in a variety of clinical specimens has been reported by several laboratories (4, 17, 25). A factor that has limited the use of PCR-based diagnostic methods to detect microbial contamination in blood directly is the inhibitory effects of blood on Taq polymerase. This includes the hemoglobin in blood itself and the preservatives used to store blood (1, 33). In this study several DNA extraction methods have been evaluated. Furthermore, since the DNA extracted was to be used in an assay...
measuring fluorescence, some of the extraction methods needing the use of reagents that quenched fluorescence had to be eliminated. Both the QIAamp blood kit and the Dynal DNA kit proved to be superior in providing DNA suitable for the assay. The results with the QIAamp blood kit appeared to be more reproducible from day to day.

In PCR methods, if the template targeted is in several copies then the sensitivity of the assay should increase, which in this case would translate into detection of fewer CFU of bacteria. In bacteria there are several copies of the 16S rRNA gene (28), and therefore this gene was chosen over the invasion gene _ail_, the _virF_ gene present in the virulence plasmid pYV, or the heat-stable enterotoxin (_yst_) gene, which have been used by other groups (13, 16, 20). The detection threshold in blood using the _virF_ and _ail_ genes was 5,000 bacteria/ml (11). The pYV plasmid is often lost during storage and growth and therefore is not suitable as a target (24). Although the _ail_ and the _yst_ genes are specific for virulence and would be present only in a pathogenic strain, it was argued that the presence of any species of _Y. enterocolitica_ would be reason for removing a blood unit. The goal was to achieve the greatest sensitivity with respect to the number of bacteria detected, regardless of whether the strain was pathogenic or not. Targeting of the 16S rRNA gene by a seminested PCR approach has also been used by another group recently (31). Their method’s detection level was 100 CFU/ml. However, their samples were from pure bacterial cell suspensions. The present assay was able to detect 5 fg of _Y. enterocolitica_ bacterial DNA from pure cultures, which would be equivalent to 1 CFU and to 30 CFU of _Y. enterocolitica_ per ml of blood. The assay could be developed further by increasing the efficiency of the purification of DNA from blood or by concentrating the extracted DNA. As of now, 20 μl of DNA out of a final volume of 100 μl, extracted by the QIAamp kit, was used per PCR. This would amount to 1.2 bacteria per 50 μl of PCR mixture, if originally 6 bacteria were spiked into 200 μl of blood. The question of whether the in vitro spiking experiments represent the true viability of bacteria in blood has not been addressed in this assay. The TaqMan PCR assay, using DNA as a template, cannot distinguish between live and dead bacteria. Use of the TaqMan assay in reverse transcription-PCR, using the 16S rRNA as a template, may be able to detect live bacteria. With this reverse transcription-PCR assay, one could then hope to study the viability of bacteria in blood.

Since the 16S rRNA gene has regions of conserved sequences in all bacterial species, targeting the ubiquitous sequences on this template by PCR has some inherent problems. Contamination of samples by bacteria from the laboratory environment or by translocation of bacterial DNA from the gut to the blood could lead to false positives. Precautions were taken to use sterile reagents and conditions wherever possible. Dedicated pre- and post-PCR pipettes and rooms were used. The PCR reagents were added in a UV-irradiated hood. It is unlikely other bacterial species would be detected by this assay, since the assay uses three oligonucleotides; all of them would have to have substantial homology with the template being amplified. Thus, even though the TaqMan probe and the reverse primer show 100% homology with the corresponding region of 16S rDNA of _H. alvei_, no signal was generated when the TaqMan PCR was performed with this set of oligonucleotides. The same was true with _S. grimesii_. However, the unspiked blood sometimes showed an unspecific amplification around cycle 40. Increasing the annealing temperature to 62°C or changing the forward and reverse primer set did not solve the problem. However, the ΔRn was very small in the unspiked blood.

Although a detection sensitivity of 6 bacteria/200 μl (30 bacteria/ml) of blood is better than that for any previously published methods, this assay still perhaps cannot be used to test donors or donated blood on day 0 or 1. However, it can be used for testing of blood shortly after it is processed or during

FIG. 2. Specificity of the TaqMan assay for _Y. enterocolitica_. Chromosomal DNAs (20 ng each) from five _Y. enterocolitica_ serotypes (Y288; O:1,2,3; O:3; O:5,27; and O:20) were used as templates in TaqMan PCR, and their amplifications are represented in plots 1 to 5, respectively. The gel inset shows the 201-bp product from each PCR, and lanes 1 to 5 correspond to plots 1 to 5. The arrow indicates the position of the 201-bp product. Chromosomal DNAs (200 ng each) from seven other bacterial species listed in Table 1 were also tested with primers 16SF and 16SR and probe YE1, and their amplifications are represented in plots 6 to 12. The cycle numbers are on the x axis.
the early days of storage. The earliest time at which this detection level would be useful remains to be established.

The initial cost for equipment may pose a problem for the widespread use of this method. However, technological advances are being made, and smaller, field-oriented thermocyclers and spectrofluorometers, which use silicon chips, are being developed (6). The advanced nucleic acid analyzer recently described by Belgrader et al. (7), besides cutting the assay time to minutes and being portable, would also help decrease the cost. It is also easily adaptable to automation. Furthermore, with increased use of nucleic acid testing for detection of viral markers in blood, the technical base for such molecular testing is already being developed and will soon be in place.

In conclusion, the TaqMan assay is rapid and eliminates the use of multiplex PCR, Southern blotting, or agarose gel electrophoresis. The entire test can be completed in 3 h, which includes the DNA extraction step. In addition, only 100 to 200 μl of blood is needed for analysis. Coupled with the current development of automated systems for DNA preparation that are capable of handling DNA from 96 blood samples, such as the QIAamp BioRobot 9604 and QIAamp 9600 Biorobot kits, this assay could be considered for incorporation into high-throughput testing.

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REFERENCES


