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# Detection of Pathogenic *Yersinia enterocolitica* Strains by a Rapid and Specific Multiplex PCR Assay

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A multiplex PCR assay targeting the *yst* and 16S rRNA genes of *Yersinia enterocolitica* was developed to specifically identify pathogenic *Y. enterocolitica* from pure culture. Simultaneous amplification of 145 and 416 bp fragments of the *yst* and 16S rRNA genes of *Y. enterocolitica* was obtained using the primer pairs in a single reaction. Validation of the assay was performed with the reference *Yersinia* strains and other members of the family Enterobacteriaceae. The defined primer pairs amplified the targeted sequence from only pathogenic *Y. enterocolitica* strains, whereas none of the other bacterial species yielded any amplified fragments. Within an assay time of 4 h, this assay offers a very specific, reliable, and inexpensive alternative to the conventional phenotypic assays used in clinical laboratories to identify pathogenic *Y. enterocolitica*.

**Key Words:** *Yersinia enterocolitica*, Multiplex PCR, *yst*, 16S rRNA gene

## INTRODUCTION

*Yersinia enterocolitica*, a gram-negative, oxidase-negative, and facultatively anaerobic species, is an important human enteroinvasive pathogen with a global distribution (Bottone, 1977; Ostroff, 1995; Bottone, 1999). Worldwide surveillance data show an extensive increase in the number of non-outbreak-related isolates and cases of yersiniosis reported in the last two decades. This notice has led to the referral of *Y. enterocolitica* as a potential emerging enteric human pathogen worldwide (McCarthy et al., 1990; Ostroff, 1995; Tauxe, 1997).

Pigs are regarded as a major reservoir of *Y. enterocolitica*, and in particular, the consumption of porcine tongue and tonsils is considered a risk factor (Adesiyun et al., 1995; CDC, 1998; Bottone, 1999). *Y. enterocolitica* is thought to be a significant food-borne pathogen. The clinical spectrum of *Y. enterocolitica* infections varies with age and underlying conditions (Bottone, 1977). Most commonly, yersiniosis is associated with gastroenteritis, although in children more severe clinical manifestations (peritonitis, ileitis, pseudoappendicitis) are observed and several fatalities have been reported (Bottone, 1977; Martin et al., 1982; Staatz et al., 1998). Due to the tropism for lymphoid tissues and the spread of the bacterium via the bloodstream, generalized infections may occur, resulting in meningitis, endocarditis, and aneurysm (La Scola et al., 1997; Tame et al., 1998). As a result of the host's immune response, *Y. enterocolitica* may also induce secondary, postinfectious sequelae such as acute and chronic arthritis, erythema nodosum, and septicemia (Bottone, 1977; Petrus et al., 1997; van der Heyden et al., 1997). *Y. enterocolitica* is responsible for 50% of all the clinical sepsis episodes that occurs as a result of transfusion of contaminated red blood cells (Klein et al., 1997).

In contrast to most other common bacterial enteropathogens, *Y. enterocolitica* is able to proliferate at temperature of about 4℃. This psychrophilic nature is responsible for the survival of the bacterium at temperature found in refrigerators, e.g., in food (Adesiyun et al., 1995; Bien, 1998; CDC, 1998; Bottone, 1999) or packed red blood cells needed for transfusion (Wagner et al., 1994; Klein et al., 1997; CDC, 1998). Several methods that have been recommended

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for the isolation of yersinias from biological and environmental samples take advantage of their psychrophilic nature (Greenwood et al., 1989; Feng et al., 1994). The most widely used method is cold enrichment, which requires an incubation period of up to 3 weeks, followed by subculturing onto CIN agar and biochemical confirmation (Lambertz et al., 1996; Bhaduri et al., 1997; Hoorfar et al., 1999). The main disadvantage of these culture methods is the need for an extended incubation period and a relative lack of sensitivity (Swaminathan et al., 1982; Lambertz et al., 1996).

*Y. enterocolitica* may be separated by serotyping into approximately 60 serogroups, of which only 11 serogroups are most frequently associated with human infections (with serogroups O:3, O:8, O:9 and O:5,27 predominating) (Wauter et al., 1991; Bottone, 1999). One group of pathogenic strains, comprising serotypes O:4, O:8, O:18, O:20 and O:21 (known as American strains), was initially mainly isolated in the United States. On the other hand, strains that were the most common causes of yersiniosis in Europe and Japan, i.e., serotypes O:3, O:5,27 and O:9 (known as European strains), were virtually unknown in the United States. Only one pathogenic serotype, i.e., O:5,27, seemed to have a global spread from the very beginning (Wauter et al., 1991; Bottone, 1999).

Of the six biotypes of *Y. enterocolitica*, five (biotypes 1B, 2, 3, 4, and 5) are considered pathogenic in humans (Miller et al., 1988; Bottone, 1997). Strains of these pathogenic biotypes contain markers associated with virulence, and these are located on the chromosome and on the pYV virulence plasmid (Miller et al., 1988; Grant et al., 1998). Expression of both plasmid and chromosomal genes is required for *Y. enterocolitica* virulence (Schiemann et al., 1982; Miller et al., 1988). However, the plasmid has been shown to be difficult to maintain during laboratory culture, which would increase the chances of obtaining a false-negative result (Fenwick et al., 1991; Kapperund, 1991). Consequently, plasmid pYV is not an ideal DNA target for the detection of pathogenic *Y. enterocolitica* strains. The chromosomal *yst* gene, however, has been shown to be a stable virulence marker limited to only pathogenic strains of *Y. enterocolitica* (Grant et al., 1998). Accordingly, amplification of *yst*-specific sequences by diagnostic PCR can be used for the unambiguous identification of invasive *Y. enterocolitica* strains. Besides the *yst* primers, we included in the PCR mixture a second primer sets, based on the *Y. enterocolitica* 16S rRNA gene, for species identification.

**Table 1.** Bacterial species included in this study

| Species | Serotype or strains | Results of PCR that targets: | |
|---|---|---|---|
| | | 16S rRNA | *yst* |
| *Yersinia enterocolitica* | O:2 | + | + |
| *Yersinia enterocolitica* | O:3 | + | + |
| *Yersinia enterocolitica* | O:5,6 | + | − |
| *Yersinia enterocolitica* | O:8 | + | + |
| *Yersinia enterocolitica* | O:9 | + | + |
| *Yersinia enterocolitica* | O:13 | + | − |
| *Yersinia enterocolitica* | O:16 | + | − |
| *Yersinia enterocolitica* | ATCC 9610 | + | + |
| *Yersinia pseudotuberculosis* | Diverse | − | − |
| *Escherichia coli* | O:157 | − | − |
| *Campylobacter jejuni* | O:1,44 | − | − |
| *Salmonell enterica Subsp. Enterica* | *typhimurium* | − | − |
| *Klebsiella pneumoniae* | | − | − |
| *Shigella dysenteriae* | O:1 | − | − |

The time- and cost-effective diagnostic tests are crucial for validation and diagnostic assay in clinical laboratories. In this report, we describe the development of a rapid and specific multiplex PCR assay that is exclusive for pathogenic strains of *Y. enterocolitica* and its applicability to pure culture and clinical specimens.

## MATERIALS AND METHODS

### 1. Bacterial species

Seven strains of *Y. enterocolitica* representing O:2, O:3, O:5,6, O:8, O:9, O:13 and O:16 serotypes obtained from the Joongang University culture collection and *Y. enterocolitica* (ATCC 9610) reference strain were studied along with 5 non-*Yersinia* strains. Details of these strains were mentioned in a previous report (Ibrahim et al., 1992). The bacterial strains tested in this study are listed in Table 1.

### 2. DNA isolation

Genomic DNA was isolated with the QiaAmp Tissue Kit 250 (Qiagen Inc., Valencia, CA, U.S.A.), according to the manufacturer's instructions. Purified genomic DNA was diluted to a concentration of 10 ng/μl in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) and stored at -20℃.

**Table 2.** Sequence of the oligonucleotide primer sets targeting the *yst* or 16S rRNA gene

| Primer | Sequence | *Tm* (℃) |
|---|---|---|
| *yst*-pr2a | 5'-AAT GCT GTC TTC ATT TGG AGC-3' | 53.2 |
| *yst*-pr2b | 5'-ATC CCA ATC ACT ACT GAC TTC-3' | 46.9 |
| 16S rRNA-F | 5'-GCG GCA GCG GGA AGT AGT TTA-3' | 60.1 |
| 16S rRNA-EurR | 5'-CAA TCA CAA AGG TTA TTA ACC TTT ATG-3' | 53.8 |
| 6S rRNA-AmeR | 5'-CAA TCC AAC AAC GTA TTA AGT TAT TGG-3' | 56.2 |

### 3. Oligonucleotide primers

Two different primer sets for amplification of the *Y. enterocolitica* 16S rRNA gene sequences were used in the duplex PCR assay. Primer pairs, published by Trebesius et al. (1998), were (5'-GCG GCA GCG GGA AGT AGT TTA-3' and 5'-CAA TCA CAA AGG TTA TTA ACC TTT ATG-3') for amplification of the *Y. enterocolitica* European strains, (5'-GCG GCA GCG GGA AGT AGT TTA-3' and 5'-CAA TCC AAC AAC GTA TTA AGT TAT TGG-3') for amplification of the *Y. enterocolitica* American strains. On the basis of this sequence, a PCR product of 416 bp was expected. To specifically amplify the *Y. enterocolitica yst* gene, a third primer set, published by Ibrahim et al. (1997), (5'-AAT GCT GTC TTC ATT TGG AGC-3') and (5'-ATC CCA ATC ACT ACT GAC TTC-3'), was used, resulting in a PCR product of 145 bp. A mixture of primers for both the *yst* and 16S rRNA genes was used for detection of both genes in a multiplex PCR assay. The sequences of all primers used in our study are given in Table 2.

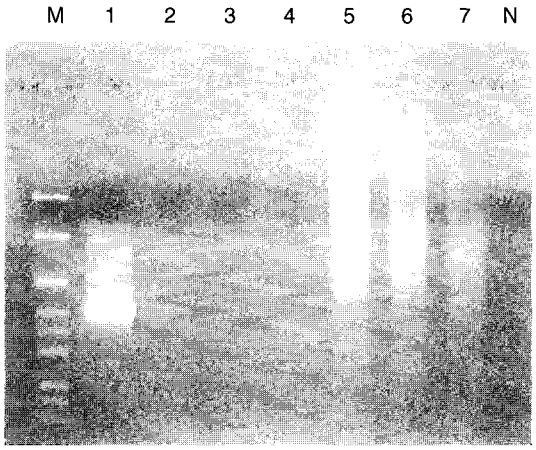


**Fig. 1.** PCR amplification of *Yersinia enterocolitica* American strain and other microorganisms of Enterobacteriaceae with American strain specific primer set. Lane M, DNA size marker (*Hinf*I digested pBH20); Lane 1, *Yersinia enterocolitica* O:8; Lane 2, *Yersinia enterocolitica* O:9; Lane 3, *Yersinia pseudotuberculosis*; Lane 4, *Escherichia coli*; Lane 5, *Campylobacter jejuni*; Lane 6, *Salmonella typhimurium*; Lane 7, *Klebsiella pneumoniae*; Lane N, Negative control.

### 4. PCR condition

The multiplex PCR was performed by the method of Eom et al. (1998). PCR reaction mixtures contained *yst*-specific primers at a concentration of 0.75 pmol/μl and the *Y. enterocolitica* 16S rRNA-specific primers at a concentration of 1.5 pmol/μl; dATP, dCTP, dGTP, and dTTP each at a concentration of 200 μM, 1.5 mM $MgCl_2$, 0.5 U of thermostable *Taq* polymerase (Takara shuzo, Kyoto, Japan), 1× PCR buffer (the buffer was supplied at 10×), and 2.5 μl (i.e., 25 ng) of template DNA. The final volume was filled to 25 μl with sterile distilled water. The amplification reaction was performed in a thermal cycler (GeneAmp® PCR System 2700, Perkin-Elmer Cetus, Boston, MA, U.S.A.). Cycling conditions started with a denaturation step at 94℃ for 2 min, which was followed by 35 subsequent cycles consisting of heat denaturation at 94℃ for 1 min, primer annealing at 53℃ for 1 min, and extension at 72℃ for 2 min. A final extension was performed at 72℃ for 15 min to complete the synthesis of all strands. The PCR products were visualized by ultraviolet illumination of ethidium bromide-stained 1.5% agarose gels (NuSieve GTG agarose; BMA, Rockland, ME, USA).

## RESULTS

### 1. Single and duplex PCR amplification

The amplified fragment of 416 bp for the *Y. enterocolitica*-specific 16S rRNA gene was obtained only the *Y. enterocolitica* American strain (serogroup O:8) and European strains (O:3, O:9) using their specific primer set in a single PCR, respectively (Fig. 1, 2). Amplification was successful with *Y. enterocolitica* American and European strains specific primer pairs in a duplex PCR (Fig. 3). Only

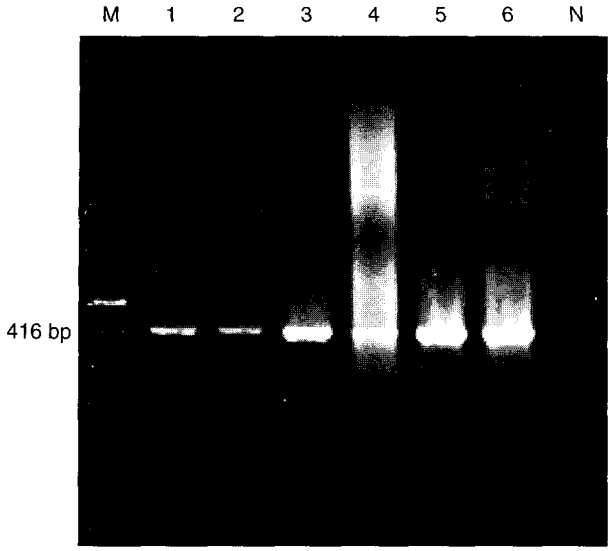


**Fig. 2.** PCR amplification of *Yersinia enterocolitica* European strains with their specific primer set. Lane M, DNA size marker (*Hinf*I digested pBH20); Lane 1, *Yersinia enterocolitica* O:2; Lane 2, *Yersinia enterocolitica* O:3; Lane 3, *Yersinia enterocolitica* O:5,6; Lane 4, *Yersinia enterocolitica* O:16; Lane 5, *Yersinia enterocolitica* O:9; Lane 6, *Yersinia enterocolitica* O:13; Lane N, Negative control.

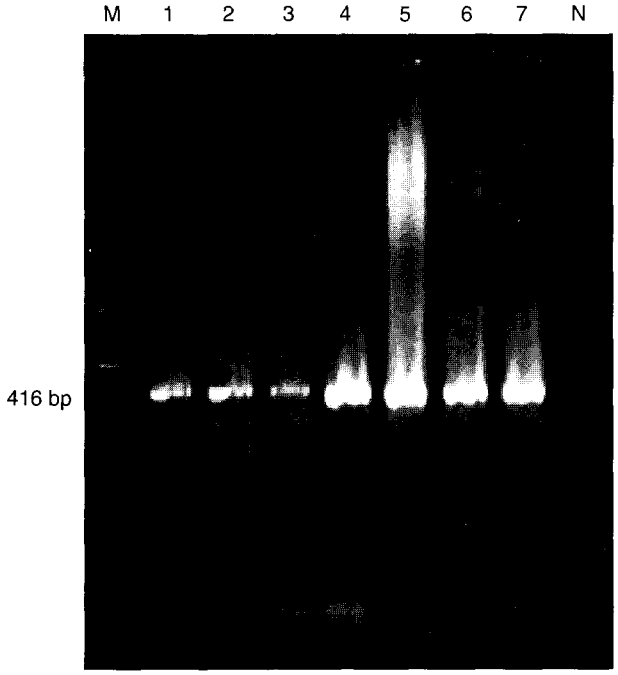


**Fig. 3.** Duplex PCR amplification of *Yersinia enterocolitica* American strain (Lane 1) and European strains (Lane 2~7). Lane M, DNA size marker (*Hinf*I digested pBH20); Lane 1, *Yersinia enterocolitica* O:8; Lane 2, *Yersinia enterocolitica* O:2; Lane 3, *Yersinia enterocolitica* O:3; Lane 4, *Yersinia enterocolitica* O:5,6; Lane 5, *Yersinia enterocolitica* O:16; Lane 6, *Yersinia enterocolitica* O:9; Lane 6, *Yersinia enterocolitica* O:13; Lane N, Negative control.

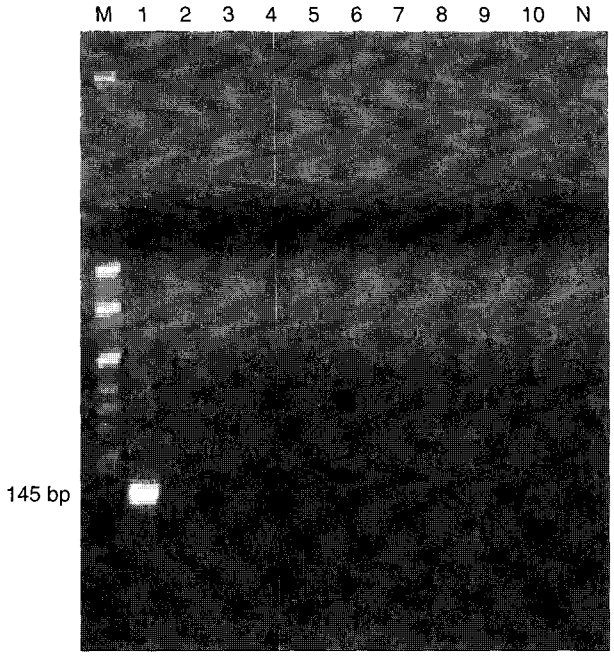


**Fig. 4.** PCR amplification of the *Yersinia* heat-stable enterotoxin (*yst*) gene. Lane M, DNA size marker (*Hinf*I digested pBH20); Lane 1, *Yersinia enterocolitica* O:8; Lane 2, *Yersinia pseudotuberculosis*; Lane 3, *Yersinia enterocolitica* O:16; Lane 4, *Yersinia enterocolitica* O:13; Lane 5, *Yersinia enterocolitica* O:5,6; Lane 6, *Escherichia coli*; Lane 7, *Campylobacter jejuni*; Lane 8, *Salmonella typhimurium*; Lane 9, *Klebsiella pneumoniae*; Lane 10, *Shigella dysenteriae*; Lane N, Negative control.

the pathogenic *Y. enterocolitica* strain (serogroup O:8) gave an PCR product of 145 bp of the *Yersinia* heat-stable enterotoxin (*yst*) gene, none of the other bacterial strains listed in Table 1 yielded any amplified products (Fig. 4).

### 2. Multiplex PCR results

Simultaneous amplification of 145 and 416 bp fragments of the *yst* and 16S rRNA genes of *Y. enterocolitica* was obtained using the primer pairs in a multiplex PCR (Fig. 5). Amplification was successful whether the template was derived from a single colony of the pathogenic *Y. enterocolitica* strains. The amplified fragment sizes were 145 bp for the *yst* gene and 416 bp for the *Y. enterocolitica*-specific region of the 16S rRNA. Non specific products were never detected. These results indicate that the multiplex PCR was the most reliable and specific assay for detecting the pathogenic *Y. enterocolitica*.

### 3. Theoretical detection limit of PCR

In order to determine the sensitivity of the assay, chro-

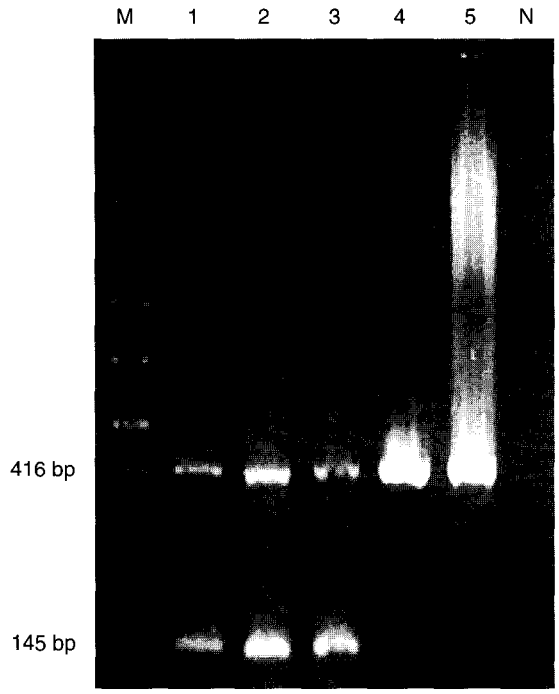


**Fig. 5.** Multiplex PCR amplification of the *yst* and 16S rRNA genes of *Yersinia enterocolitica* (Lane 1~3). Lane M, DNA size marker (*HinfI* digested pBH20); Lane 1, *Yersinia enterocolitica* O:8; Lane 2, *Yersinia enterocolitica* O:3; Lane 3, *Yersinia enterocolitica* O:9; Lane 4, *Yersinia enterocolitica* O:5,6; Lane 5, *Yersinia enterocolitica* O:16; Lane N, Negative control.

mosomal DNA from *Y. enterocolitica* serotype O:3 was isolated with the QiaAmp kit from overnight cultures of bacteria grown in CIN medium at 30℃. Multiplex PCR was performed by using 100 ng to 1 fg of chromosomal DNA as a template and the primers described above. A DNA concentration of ≥500 fg could be detected (data not shown). On the basis of an average yersinial genome size of 4,500 kb (Sen, 2000), 5 fg of DNA would amount to approximately one genome. Thus, the primers as well as the length of the amplicon which was being amplified were optimal, since the multiplex PCR sensitivity could be achieved, which was $1\times10^2$ CFU.

### 4. Specificity of PCR

The specificity of the multiplex PCR assay was examined by isolating genomic DNA from 8 different *Y. enterocolitica* serogroups, 1 other *Yersinia* spp. and 5 different non-*Yersinia* bacteria pathogenic for humans (Table 1). Among the 9 different *Yersinia* strains, only *Y. enterocolitica* strains gave a PCR product of 416 bp (Fig. 1~3). Of these, only the pathogenic strains (mainly serogroup O:8, O:3 and O:9) gave an additional PCR product of 145 bp (Fig. 5). No PCR products were observed when non-*Yersinia* strains were used. The results of the 16S rRNA gene PCR correlated well with the results obtained by Neubauer et al. (2000). The retrieval of the PCR product derived from the *yst* gene only from pathogenic *Y. enterocolitica* strains indicates the uniqueness of this virulence gene among this yersinial species.

## DISCUSSION

The objective of this study was to design a rapid and specific single-reaction multiplex PCR assay for the detection of pathogenic *Y. enterocolitica* strains and to use the assay as a diagnostic tool for the rapid typing of pure yersinial cultures. In this study, a multiplex PCR assay was developed to detect the presence of the *yst* and 16S rRNA genes of pathogenic *Y. enterocolitica* simultaneously, quickly and accurately.

Difficulties associated with the isolation of pathogenic *Y. enterocolitica* stem from the small number of pathogenic strains in the samples and the large number of organisms in the background flora, especially in food and environmental samples. The multiplex PCR assay provides a more rapid means of accurate identification of pathogenic *Y. enterocolitica* than present standard methods (i.e., biotyping combined with serotyping), which are time-consuming and laborious. The entire diagnostic assay, including a simplified technique for DNA extraction, the amplification process, and gel electrophoresis, could be completed within 4 h, which is better than the time required for the time-consuming traditional techniques used in clinical laboratories. Use of the multiplex PCR would significantly reduce the amount of time required to identify pathogenic *Y. enterocolitica* strains and can be used directly after primary selective culture of this pathogen, with the biotyping and serotyping steps omitted, if necessary.

Lantz et al. (1999) invented a multiplex PCR method to concurrently detect the plasmid borne *yadA* gene and a *Yersinia*-specific region of the 16S rRNA gene. The diagnostic value of the primers that target plasmid-encoded sequences, however, has been questioned on the following grounds: (i) the accidental loss of the plasmid during isolation would give false-negative results, and (ii) the target sequences of these primers are not exclusive for *Y. entero-*

*colitica* but are highly conserved in the DNAs of *Y. pseudotuberculosis* and *Y. pestis* as well (Skurnik et al., 1989). The need for a reliable and rapid diagnostic assay to detect pathogenic strains of *Y. enterocolitica* specifically is clinically and epidemiologically important. Because of possible plasmid loss on subculture and storage (Blais et al., 1995), so we use the primers that target the chromosomally encoded *yst* gene (*Y. enterocolitica* heat-stable enterotoxin) in this assay.

Some small differences in amino acid composition at the 16S rRNA gene locus between the American and non-American serotypes have been discussed (Harnett et al., 1996) and could in theory interfere with primer annealing. So two different primer sets for amplification of the *Y. enterocolitica* 16S rRNA gene sequences were used in our assay. The multiplex PCR described in this report is able to unambiguously identify both American and non-American pathogenic *Y. enterocolitica* strains.

The primer pair for amplification of the *Yersinia*-specific region of the 16S rRNA gene differentiated *Y. enterocolitica* from a broad spectrum of non-*Yersinia* bacteria. The primer pair for amplification of the *Y. enterocolitica yst* gene sequences intrinsically differentiated pathogenic from nonpathogenic *Y. enterocolitica* bacteria.

Thus far, only separate 16S rRNA gene and *yst* gene PCRs have been described for *Y. enterocolitica* (Harnett et al., 1996; Jourdan et al., 2000; Neubauer et al., 2000; Sen, 2000). Without the 16S rRNA gene PCR, species confirmation must be obtained by biotyping and serotyping; with our assay it is possible to omit these steps, and therefore, the assay can save time. To our knowledge, this is the first time that a PCR assay that makes use of combined primer sets for both *Yersinia* species identification and detection of pathogenicity has been described.

In summary, the multiplex PCR assay described in this report appears to be a convenient and user-friendly tool for the rapid, sensitive, and specific detection of pathogenic *Y. enterocolitica*. In addition, it can be applied in each laboratory with PCR facilities, even without prior biotyping and serotyping, and it is powerful tool in epidemiological studies for establishing the precise etiology of an enteric infection.

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