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# Detection and Identification of *Yersinia pseudotuberculosis* and Pathogenic *Yersinia enterocolitica* by an Improved Polymerase Chain Reaction Method

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**We developed a polymerase chain reaction method in order to detect and identify both *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica*. Polymerase chain reaction was performed by using a mixture of primers against the *inv* gene from *Y. pseudotuberculosis* and the *ail* gene from pathogenic *Y. enterocolitica*. Further addition of primers against the plasmid-coded *virF* gene from *Y. enterocolitica* made it possible to detect a virulence-associated gene of both species at the same time. This method was proved to be an adequate and convenient procedure for routine detection and identification of these bacilli.**

Recently, a method for the rapid detection of pathogenic *Yersinia* species, *Y. pseudotuberculosis* and *Y. enterocolitica*, by the polymerase chain reaction (PCR) technique was reported by Wren and Tabaqchali (8) and Fenwick and Murray (2). They used primers based on the *virF* gene and the *ail* gene, respectively. By these methods, it was impossible to differentiate between the two bacilli, because DNA fragments of the same size were amplified by *virF* primers and a product from the *ail* gene was detected only from pathogenic *Y. enterocolitica* and not from *Y. pseudotuberculosis*. In the case of *Yersinia* infection, however, *Y. pseudotuberculosis* and *Y. enterocolitica* cause almost the same symptoms, so it is impossible to differentiate between the bacilli on the basis of the symptoms that result from them. Therefore, we developed a rapid PCR method for the detection and differentiation of both *Yersinia* species and the detection of their virulence-associated genes directly from their cultures. The sensitivity of PCR detection was also examined.

The bacterial strains used in this study are listed in Table 1. These included 25 strains of virulence plasmid-harboring ($p^+$) *Y. pseudotuberculosis* belonging to each serotype from patients and a wild animal (raccoon dog), 6 strains of virulence plasmidless ($p^-$) *Y. pseudotuberculosis* (serotypes 2a, 2b, 4a, 5a, and 6) from river water, 12 strains of *Y. enterocolitica* ($p^+$; serotypes O:3 and O:8) from patients, 2 patient-derived strains of *Y. enterocolitica* of which the virulence plasmids were lost after long storage ($p^-$; serotypes O:3 and O:8), 13 strains of nonpathogenic *Y. enterocolitica* from river water, 11 strains of *Yersinia frederiksenii*, 17 strains of *Yersinia intermedia* from a wild animal (raccoon dog), and other isolates from patients (4 isolates of *Salmonella* spp., 1 isolate of *Campylobacter jejuni*, 1 isolate of enteroinvasive *Escherichia coli*, 2 isolates of enterotoxigenic *E. coli* producing heat-stable or heat-labile enterotoxin, and 4 isolates of enteropathogenic *E. coli* with the following serotypes: O55:H7, O111:H21, O126:H27, and O127:H21). Plasmids were isolated by the method of Kado and Liu (6) and detected on agarose gel. For PCR, we selected a 295-bp region of the *inv* gene coding for 594 to 692 amino acid residues of invasin protein on the chromosome of *Y. pseudotuberculosis* (5) as a target DNA. Since this region lacks homology with *Y. enterocolitica* (9), we could synthesize primers to amplify a 295-bp DNA fragment only from *Y. pseudotuberculosis*; the primers were 5′-TAAGGGTAC TATCGCGGCGGA-3′ and 5′-CGTGAAATTAACCGTCA CACT-3′. For the purpose of detecting pathogenic *Y. enterocolitica*, the 664- to 833-nucleotide region of the *ail* (7) gene related to its cell adhesion was selected as a target, and a 170-bp product was expected to be amplified by the following primers: 5′-ACTCGATGATAACTGGGGAG-3′ and 5′-CCCCCAGTAATCCATAAAGG-3′. The primers for the *virF* (1) gene used by Wren and Tabaqchali (8) to detect pathogenic *Yersinia* spp. harboring a 67- to 72-kb virulence plasmid (3, 4) were used for amplification of a 591-bp product as previously described (8). Each primer set for the *inv* or *ail* gene was used for the detection of each gene, and a mixture of primers against the *inv*, *ail*, and *virF* genes was used for the detection of one or more genes among the *inv*, *ail*, and *virF* genes. Template DNAs for PCR were prepared as follows. (i) From colonies, each isolate was suspended in autoclaved distilled water to achieve a concentration of $10^8$ CFU/ml and boiled for 10 min, and 5 μl of each sample ($10^5$ CFU) was applied to PCR. (ii) In order to examine the detection limit for PCR, a series of 10-fold dilutions of *Y. pseudotuberculosis* or *Y. enterocolitica* strains with river water or autoclaved water was made. Cells of each dilution were collected by centrifugation and suspended in 20 μl of proteinase K buffer (50 mM Tris-HCl [pH 7.5], 5 mM $CaCl_2$). After being freeze-thawed three times, 0.5 μl of proteinase K (5 mg/ml in 10 mM $CaCl_2$ solution) was added to the suspension, which was incubated at 45°C for 1 h, and PCR was performed with the suspension. DNA samples were amplified in a total of 50 μl of the following reaction mixture: 50 mM KCl–10 mM Tris-HCl (pH 8.3)–1.5 mM $MgCl_2$–0.001% (wt/vol) gelatin; 100 μM each dATP, dCTP, dGTP, and dTTP (Perkin-Elmer Cetus); 0.05 μM *inv* primers, 0.1 μM each *ail* and *virF* primers; 0.5 μg of RNase A (Sigma); and 0.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). The samples were overlaid with a drop of mineral oil and amplified by PCR with the Thermal Sequencer

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TABLE 1. Results of PCR of various isolates with a mixture of primers against *inv*, *ail*, and *virF* genes

| Species and serotype | Source | No. of isolates tested | No. positive for | | |
|---|---|---|---|---|---|
| | | | *inv* | *ail* | *virF* |
| *Y. pseudotuberculosis* ($p^+$)[a] 1b, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, and 8 | Patient, raccoon dog | 25 | 25 | 0 | 25 |
| *Y. pseudotuberculosis* ($p^-$)[b] 2a, 2b, 4a, 5a, and 6 | Water sample | 6 | 6 | 0 | 0 |
| *Y. enterocolitica* ($p^+$) O:3 and O:8 | Patient | 12 | 0 | 12 | 12 |
| *Y. enterocolitica* ($p^-$) O:3 and O:8 | Patient[c] | 2 | 0 | 2 | 0 |
| Nonpathogenic *Y. enterocolitica* | Water sample | 13 | 0 | 0 | 0 |
| *Y. frederiksenii* | Raccoon dog | 11 | 0 | 0 | 0 |
| *Y. intermedia* | Raccoon dog | 17 | 0 | 0 | 0 |
| *Salmonella* spp. | Patient | 4 | 0 | 0 | 0 |
| *C. jejuni* | Patient | 1 | 0 | 0 | 0 |
| Enteroinvasive *E. coli* | Patient | 1 | 0 | 0 | 0 |
| Enterotoxigenic *E. coli* ($ST^+$)[d] | Patient | 1 | 0 | 0 | 0 |
| Enterotoxigenic *E. coli* ($LT^+$)[e] | Patient | 1 | 0 | 0 | 0 |
| Enteropathogenic *E. coli* | Patient | 4 | 0 | 0 | 0 |

[a] $p^+$, virulence plasmid harboring.
[b] $p^-$, virulence plasmidless.
[c] $p^-$ strains on account of long storage.
[d] ST, heat-stable enterotoxin.
[e] LT, heat-labile enterotoxin.

(IWAKI), which consisted of the following: predenaturation at 94°C for 1 min, 25 cycles of denaturation at 94°C for 0.5 min, primer annealing at 55°C for 1 min, extension at 70°C for 2 min, and further extension at 70°C for 5 min. In the case of the examination of the detection limit, 30 PCR cycles were repeated after the addition of AmpliTaq DNA polymerase. After PCR amplification, 10 μl of each PCR product was run on a 1.5% agarose gel at a constant voltage of 100 V in TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]). The gel was stained with ethidium bromide and photographed under UV light.

Each primer against the *inv* or *ail* gene was confirmed to amplify a 295-bp product from a *Y. pseudotuberculosis* ($p^+$) strain or a 170-bp product from a *Y. enterocolitica* ($p^+$) strain (Fig. 1, lanes 1 and 2, respectively). With a mixture of *inv*, *ail*, and *virF* primers, PCR could differentiate *Y. pseudotuberculosis* ($p^+$) from *Y. enterocolitica* ($p^+$) in suspensions containing only one or both species; 591- and 295-bp products were amplified from a *Y. pseudotuberculosis* ($p^+$) strain, while 591- and 170-bp products from *Y. enterocolitica* ($p^+$) were amplified (Fig. 2 [lanes 1 and 3] and 1 [lane 3]). The results of PCR for *Yersinia* species and other isolates are shown in Fig. 2 and Table 1. All of the strains of a variety of serotypes of *Y. pseudotuberculosis* ($p^+$) from patients and raccoon dogs gave a positive reaction for the *inv* gene and also for the *virF* gene, while *Y. pseudotuberculosis* strains ($p^-$; serotypes 2a, 2b, 4a, 5a, and 6) from water samples were positive only for the *inv* gene. *Y. enterocolitica* strains ($p^+$; serotypes O:3 and O:8) from patients gave positive reactions for both the *ail* and the *virF* genes but a negative reaction for the *inv* gene, and virulence plasmidless *Y. enterocolitica* strains ($p^-$; serotypes O:3 and O:8) from patients were positive only for the *ail* gene. Avirulent *Yersinia* strains from wild animals and water samples (*Y. enterocolitica*, *Y. frederiksenii*, and *Y. intermedia*) and other

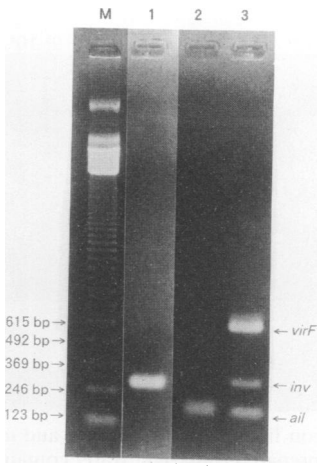


FIG. 1. Specific amplification of 591-, 295-, and 170-bp products by PCR. Lanes: 1, 295-bp product from a *Y. pseudotuberculosis* strain ($p^+$) with *inv* primers; 2, 170-bp product from a pathogenic *Y. enterocolitica* ($p^+$) strain by with *ail* primers; 3, 591-, 295-, and 170-bp products from a suspension carrying both strains used above with a mixture of primers; M, 123-bp DNA ladder marker.

isolates from patients (*Salmonella* spp., *C. jejuni*, enteroinvasive *E. coli*, enterotoxigenic *E. coli*, and enteropathogenic *E. coli*) gave negative reactions for the *inv*, *ail*, and *virF* genes. These results showed that *Y. pseudotuberculosis*, pathogenic *Y. enterocolitica*, and other isolates could be differentiated by this PCR method. Therefore, we highly recommend this assay as a method of rapid identification and differentiation of *Y. pseudotuberculosis* and pathogenic *Y. enterocolitica* strains. Furthermore, the detection limit of PCR for *Y. pseudotuberculosis* and pathogenic *Y. enterocolitica* was examined by using the *inv* and *ail* genes, respectively. The result showed that more than $10^3$ to $10^4$ CFU of bacteria in a suspension of river water was necessary for the detection of a product amplified after 30 PCR cycles (Fig. 3). However, fewer than 10 bacteria were detectable by the

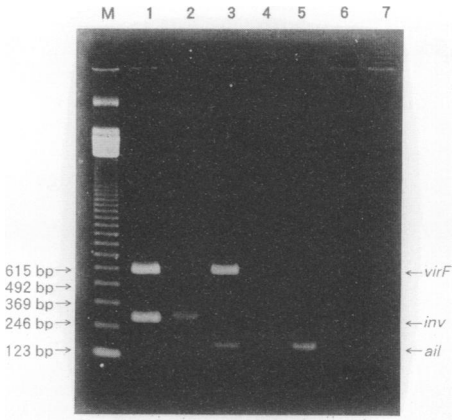


FIG. 2. Differentiation among *Yersinia* bacilli by amplified PCR products with a mixture of *inv*, *ail*, and *virF* primers. Lanes: 1, *Y. pseudotuberculosis* ($p^+$); 2, *Y. pseudotuberculosis* ($p^-$); 3, *Y. enterocolitica* ($p^+$); 4, nonpathogenic *Y. enterocolitica*; 5, *Y. enterocolitica* ($p^-$); 6, *Y. frederiksenii*; 7, *Y. intermedia*; M, 123-bp DNA ladder marker.

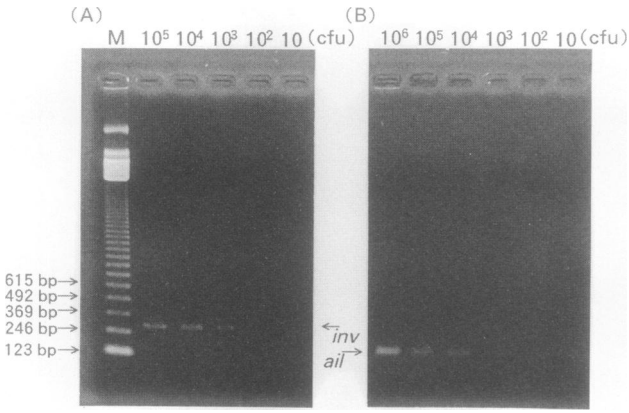


FIG. 3. Detection limit of PCR with *inv* and *ail* primers. Template DNAs were prepared from river water containing $10^5$, $10^4$, $10^3$, $10^2$, and $10^1$ CFU of *Y. pseudotuberculosis* (A) and $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, and $10^1$ CFU of pathogenic *Y. enterocolitica* (B) and used for PCR.

repetition of 30 PCR cycles after the addition of AmpliTaq DNA polymerase (data not shown). We got a similar result by using autoclaved water as a suspension medium (data not shown). Therefore, it is also suggested that this method is applicable for the detection of pathogenic *Yersinia* spp. from water samples for the purpose of epidemiological research.

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