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# Real-Time TaqMan PCR for *Yersinia enterocolitica* Detection Based on the *ail* and *foxA* Genes

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*Yersinia enterocolitica*, a cause of emerging enteric infections, is a foodborne pathogen associated with various enteric and systemic syndromes, e.g., diarrhea, enteritis, and enterocolitis. Therefore, the detection of this pathogen has important significance. Previous real-time PCR for detection of *Y. enterocolitica* was primarily based on the *ail* gene; biotype 1A nonpathogenic strains were not included (1–3). However, recent studies (4, 5) showed that biotype 1A *ystB*-positive strains are potentially pathogenic and related outbreaks were reported. We therefore designed a TaqMan real-time PCR method for detection of both pathogenic and nonpathogenic *Y. enterocolitica* strains.

Using data from sequence analysis of the *ail* and *foxA* genes from many *Y. enterocolitica* strains (6), we designed TaqMan probes and primers (Table 1).

An experiment using the entire reaction system was performed using a 20-μl volume containing 10 μl premix (TaKaRa; China), 7.2 μl ultrapure distilled water, 0.2 μl ROXII, and 0.2 μl (100 nmol/liter) of each primer and probe. A two-step method was adopted. The cycling conditions for the use of a Rotor-Gene Q system consisted of 1 cycle of initial denaturation at 95°C for 10 s followed by 40 cycles of melting at 95°C for 5 s and elongation at 60°C for 30 s. And for the use of a ABI 7500 Fast system, cycling was performed using one initial denaturation at 95°C for 20 s followed by 40 cycles of melting at 95°C for 3 s and elongation at 60°C for 30 s.

A total of 168 pathogenic *Y. enterocolitica* strains (3 *ail* sequence patterns and 8 *foxA* sequence patterns) and a total of 41 nonpathogenic *Y. enterocolitica* strains (13 *foxA* sequence patterns) were used to assess the sensitivity and specificity of the method. Most of these strains were isolated from animals, mainly swine and mice, in China. Furthermore, 258 non-Y. enterocolitica strains were used to test the specificity of the two detection systems. Most of the strains were Gram-negative bacteria of various genera. All the strains were isolated from patients and identified by using a Vitek Compact 2 biochemical identification instrument (bioMérieux). The results showed that both the *ail* and *foxA* gene detection systems have 100% specificity.

Standard curves and sensitivity data were obtained by amplifying standard plasmid that had been serially diluted 10-fold. In parallel, we detected the sensitivity of conventional PCR. The results suggested that the slope was −3.09 and the $R^2$ was 0.99 for the *ail* system and that the slope was −3.16 and the $R^2$ was 0.99 for the *foxA* system. The detection limit was $10^2$ copies/μl for both detection systems. This represented sensitivity 10 times greater than that of the conventional PCR detection.

To exclude false-negative results caused by potential inhibitors, we used the internal amplification control (IAC) developed by Fricker et al. (7). A total of 15 pathogenic *Y. enterocolitica* strains were used to test the IAC with the *ail* and *foxA* detection systems. When the *ail* and *foxA* detection systems were mixed with the IAC, both of them amplified well with IAC and also had no nonspecific amplification, thus showing that the IAC that we used was suitable for our detection systems.

In our laboratory, combining conventional PCR and culture isolation achieves good results; currently, we first employ PCR screening to find positive samples with the *Y. enterocolitica* conserved *foxA* gene or pathogenic *ail* gene and then inoculate positive samples onto cefsulodin-irgasan-novobiocin isolation media (CIN agar; Difco).

To compare real-time PCR detection, conventional PCR detection, and culture isolation methods, we tested 228 separate an-

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**TABLE 1** Primers and probes used in this study

| Primer or probe | Sequence from 5′ to 3′[a] | Position | GenBank no. | Amplicon length (bp) |
|---|---|---|---|---|
| *ail*-F | TTTGGAAGCGGGTTGAATTG | 17797–17778 | FR729477.2 | 101 |
| *ail*-R | GCTCACGGAAAGGTTAAGTCATCT | 17697–17720 | | |
| *ail* probe | FAM-CTGCCCCGTATGCCATTGACGTCTTA-BHQ | 17747–17772 | | |
| *foxA*-F | ACGGCGGTGATGTGAACAA | 386606–386624 | AM286415.1 | 85 |
| *foxA*-R | GGGTCCACTTGCAGCACATT | 386690–386671 | | |
| *foxA* probe | FAM-ACCTTCCTTGATGGGCTGCGCTTACTC-BHQ | 386626–386652 | | |
| IAC-F | GCAGCCACTGGTAACAGGAT | 1216–1235 | L09137 | 118 |
| IAC-R | GCAGAGCGCAGATACCAAAT | 1314–1333 | | |
| IAC probe | HEX-AGAGCGAGGTATGTAGGCGG-TAMRA | 1240–1259 | | |

[a] BHQ, black hole quencher; FAM, 6-carboxyfluorescein; HEX, 5-hexachloro-fluorescein; TAMRA, 6-carboxytetramethylrhodamine.

**TABLE 2** Detection of *ail* and *foxA* genes by the use of real-time PCR and conventional PCR[a]

| Real-time PCR result | No. of samples: With indicated conventional PCR result for *ail* gene | | | No. of samples: With indicated conventional PCR result for *foxA* gene | | |
|---|---|---|---|---|---|---|
| | + | − | Total | + | − | Total |
| + | 57 | 79 | 136 | 136 | 61 | 197 |
| − | 0 | 92 | 92 | 1 | 30 | 31 |
| Total | 57 | 171 | 228 | 137 | 91 | 228 |

[a] $\chi^2_{(ail\ gene)} = 51.4 > \chi^2_{0.05,\ 1} = 3.84$; $\chi^2_{(foxA\ gene)} = 48.4 > \chi^2_{0.05,\ 1} = 3.84$.

imal and patient specimens. DNA was extracted from 228 animal and patient specimens by the use of a DNA nucleic acid extraction kit (Tiangen; China). The specimens were tested by using the *ail* and *foxA* real-time PCR detection system and conventional PCR. Then, all the specimens were inoculated onto the CIN media for identification. The determinations of the primers and amplification profile for conventional PCR used the method of Huang et al. (6), and culture isolation was performed using the method of Duan et al. (8). Positive or negative results for real-time PCR were defined as follows. For the *ail* real-time PCR detection system, a threshold cycle ($C_T$) value of <31.7 represented a positive result; a $C_T$ value of >35 represented a negative result; and the "gray area" was between 31.7 and 35. For *foxA*, a $C_T$ value of <32.8 represented a positive result; a $C_T$ value of >36 represented a negative result; and the gray area was between 32.8 and 36. If results fell in the gray area, the test was repeated twice. If one or two results still fell in the gray area, we defined them as positive; otherwise, we defined them as negative.

The detection rates for real-time PCR and conventional PCR were different (Table 2). Real-time PCR detections were 59.6% (136/228) for the *ail* gene and 86.4% (197/228) for the *foxA* gene; those values are higher than those measured for conventional PCR at 25.0% (57/228) and 60.1% (137/228), respectively. Further, all specimens positive for the *ail* gene by the use of conventional PCR were amplified by real-time PCR. However, 99.3% of the specimens positive for the *foxA* gene by the use of conventional PCR were amplified by real-time PCR detection whereas only one specimen (GX2013-D35) was positive by conventional PCR but negative by real-time PCR. We sequenced the *foxA* gene of that specimen and found that its pattern was different from other *foxA* patterns we have found. When aligned to primers and probes, it had multiple mismatches.

In conclusion, these results indicate that the real-time PCR method has 100% specificity and is more sensitive than the conventional method. Additionally, its results are consistent with those of the conventional culture method and conventional PCR method. Therefore, there are advantages to replacing the conventional PCR method with the real-time TaqMan PCR method for preliminary screening before *Y. enterocolitica* culture isolation.

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