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 food-borne pathogen 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 detection for *Y. enterocolitica* was primarily based on the *ail* gene where biotype 1A non-pathogenic strains were not included (1-3). However, recent studies (4, 5) showed that biotype 1A *ystB* positive strains are potentially pathogenic and related outbreaks are reported. We therefore designed a TaqMan real-time PCR method for detection of both pathogenic and non-pathogenic *Y. enterocolitica*.

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

The entire reaction system was performed in a 20μl volume containing 10μl premix (TaKaRa, China), 7.2μl ultrapure distilled water, 0.2μl ROX II, 0.2μl (100 nmol/L) of

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each primer and probe. Two-step method was adopted. The cycling condition for the
Rotor-Gene Q system consisted one cycle of initial denaturation at 95°C for 10 s;
followed by 40 cycles: melting at 95°C for 5 s and elongation at 60°C for 30 s. And
for the ABI 7500 Fast system cycling was one initial denaturation at 95°C for 20 s;
followed by 40 cycles: melting at 95°C for 3 s and elongation at 60°C for 30 s.

168 pathogenic *Y. enterocolitica* strains (3 *ail* sequence patterns and 8 *foxA*
sequence patterns) and 41 non-pathogenic *Y. enterocolitica* strains (13 *foxA* sequence
patterns) were used to assess sensitivity and specificity of the method. Most of these
strains were isolated from animals in China, mainly for swine and mouse.

Furthermore, 258 non-*Y. enterocolitica* strains were used to test the specificity of the
two detection systems. Most of these strains were Gram negative bacteria of various
genera. All the strains were isolated from patients and identified by using 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 were obtained by amplifying tenfold serially diluted
standard plasmid. In parallel, we detected the sensitivity of conventional PCR. The
result suggested that for the *ail* system: the slope was -3.09 and the $R^2$ was 0.99; for
the *foxA* system: the slope was -3.16 and the $R^2$ was 0.99. The detection limits of the
two detection systems were both $10^2$ copies/$\mu$l. This was 10 times more sensitive than
the conventional PCR detection.

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

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

To compare real-time PCR detection, conventional PCR detection and culture isolation methods, we tested 228 animals and patients separate specimens. DNA was extracted from 228 animals and patients specimens by using DNA nucleic acid extraction kit (Tiangen, China). They 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 primers and amplification profile for conventional PCR used the method of Huang et al (6). and culture isolation was 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: ct<31.7 was positive; ct>35 was negative; and the “gray area” was between 31.7 and 35. While, for foxA: ct<32.8 was positive; ct>36 was negative; and the “gray area” was between 32.8 and 36. If results fell in the “gray area”, the test was repeated twice. If there was one or two results still falling in the “gray area”, we defined them as positive, otherwise they were 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 *ail* gene and 86.4% (197/228) for *foxA* gene, higher than conventional PCR at 25.0% (57/228) and 60.1% (137/228), respectively. Further, all specimens positive for *ail* using conventional PCR were amplified by real-time PCR. However, 99.3% of the specimens positive for *foxA* using conventional PCR were amplified by real-time PCR detection where only one specimen (GX2013-D35) was conventional PCR positive but real-time PCR negative. We sequenced its *foxA* gene and found it 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. Additionally, it is consistent with the conventional culture method and conventional PCR method. Therefore, there are advantages to substitute the conventional PCR method with the real-time TaqMan PCR method for preliminary screening before *Y. enterocolitica* culture isolation.

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References


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Table 1. Primers and probes used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence from 5’ to 3’</th>
<th>Position</th>
<th>GenBank no.</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ail</em>-F</td>
<td>TTTGGAACCGGTTGAATTG</td>
<td>17797-17778</td>
<td>FR729477.2</td>
<td>101bp</td>
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<tr>
<td><em>ail</em>-R</td>
<td>GTCACCGGAAAGGTAAAGTCATCT</td>
<td>17697-17720</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ail</em>-probe</td>
<td>FAM-CTGCCCCGTATGCCATTGACGTCTTA-BHQ</td>
<td>17747-17772</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>foxA</em>-F</td>
<td>ACGGCCGTGATGTGAACAA</td>
<td>386606-386624</td>
<td>AM286415.1</td>
<td>85bp</td>
</tr>
<tr>
<td><em>foxA</em>-R</td>
<td>GGGTCCACTTGACGACACATT</td>
<td>386690-386671</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>foxA</em>-probe</td>
<td>FAM-ACCTTCCTTGGGCTGCGCTTACTC-BHQ</td>
<td>386626-386652</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>IAC</em>-F</td>
<td>GCAGCCACTGGTAAACAGGAT</td>
<td>1216–1235</td>
<td>L09137</td>
<td>118 bp</td>
</tr>
<tr>
<td><em>IAC</em>-R</td>
<td>GCAGAGCGACGATACCAAAT</td>
<td>1314–1333</td>
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<tr>
<td><em>IAC</em>-probe</td>
<td>HEX-AGAGCGGATGATGAGCCG-TAMRA</td>
<td>1240–1259</td>
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</table>
Table 2. Detection using real-time PCR and conventional PCR for *ail* and *foxA* genes.

<table>
<thead>
<tr>
<th>real-time PCR (ail gene)</th>
<th>conventional PCR</th>
<th>total</th>
<th>real-time PCR (foxA gene)</th>
<th>conventional PCR</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>57</td>
<td>79</td>
<td>136</td>
<td>+</td>
<td>136</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>92</td>
<td>92</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>total</td>
<td>57</td>
<td>171</td>
<td>228</td>
<td>total</td>
<td>137</td>
</tr>
</tbody>
</table>

\( \chi^2 \) (ail gene) = 51.4 > \( \chi^2 \) \_0.05,1 = 3.84; \( \chi^2 \) (foxA gene) = 48.4 > \( \chi^2 \) \_0.05,1 = 3.84