Real-Time Multiplex PCR for Detecting Shiga Toxin 2-Producing Escherichia coli O104:H4 in Human Stools

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A real-time multiplex PCR targeting stx2, wzyO104, and fliC144 of enterohemorrhagic Escherichia coli (EHEC) O104:H4 correctly determined the presence or absence of these genes in 253 EHEC isolates and enrichment cultures of stool samples from 132 patients. It is a rapid, sensitive, and specific tool for detecting EHEC O104:H4 in human stools.

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were enriched for 4 h in GN broth Hajna (Difco Laboratories, Detroit, MI) and a 100-µl volume was plated on individual plates of sorbitol MacConkey agar (Becton Dickinson, Heidelberg, Germany), enterohemolysin agar (Sifin, Berlin, Germany), and extended-spectrum β-lactamase agar (chromID ESBL; bioMérieux, Nurtingen, Germany). The overnight growth from the plates was washed into 1 ml of 0.9% NaCl solution and boiled for 10 min; 1 µl of the total extracted DNA (diluted 1:10 in sterile water) was used per 20 µl of rtMPCR volume. Eighty-three (62.9%) of the 132 stool enrichment cultures contained the EHEC O104:H4 outbreak strain, as demonstrated by conventional multiplex PCR (3) and subsequent isolation of the strain, which was used as the gold standard to which results of the rtMPCR were compared. Each of the 83 cultures produced all three amplicons (wzy O104, stx2, and fliC H4) in the rtMPCR. The remaining 49 enrichment cultures lacked the outbreak strain in both the conventional multiplex PCR and culture on ESBL agar. Thirty-eight of them yielded none of the three amplicons, whereas 11 yielded the stx2 amplicon only; various stx2-positive non-O104:H4 E. coli strains were subsequently isolated from these 11 samples. The detection limit of the rtMPCR for identification of EHEC O104:H4 in stool cultures was determined by spiking three different human O104:H4-negative stools enriched for 4 h in GN broth Hajna with 10-fold dilutions (10^1 to 10^10 CFU/ml) of EHEC O104:H4 outbreak strain LB226692 (3, 11), growing 100 µl of the mixtures on ESBL agar and Luria-Bertani agar plates at 37°C overnight, extracting total DNA from bacteria washed from the plates by boiling for 10 min, and using 1 µl of the DNA in rtMPCR. The detection limit of the rtMPCR was 7 × 10^2 (range, 1 × 10^2 to 1 × 10^3) CFU/ml of EHEC O104:H4 on the background of 4.2 × 10^7 (range, 7 × 10^6 to 6 × 10^7) CFU/ml of normal coliform intestinal flora. The detection limit of the test for EHEC O104:H4 strain LB226692 in pure culture was 1.6 × 10^2 CFU/ml.

The rtMPCR developed here has 100% specificity and 100% sensitivity for the detection of EHEC O104:H4 in human stool samples compared to culture (i.e., isolation of the strain) and for identification of EHEC O104:H4 isolates compared to serotyping. Although the EHEC O104:H4 outbreak is over, this assay can be utilized in diagnostic laboratories in Germany, in particular, in those specialized for detection of EHEC, because sporadic cases of infection with the outbreak strain still rarely occur in this country (our unpublished data). Also, the rtMPCR represents a rapid and reliable tool for epidemiological studies to determine the prevalence of EHEC O104:H4 in the human population, which is considered the major (if not the only) reservoir of this pathogen (1). Moreover, because the rtMPCR detects stx2 and its variants present in HUS-associated as well as diarrhea-associated EHEC (see Tables S1 and S2 in the supplemental material), it will also detect non-O104:H4 EHEC causing human disease. Thus, stool samples positive only for stx2 in the rtMPCR, as was the case for the sam-

### TABLE 1 Primers for real-time multiplex PCR to identify stx2-harboring E. coli O104:H4

<table>
<thead>
<tr>
<th>Primera</th>
<th>Sequence (5’→3’)</th>
<th>Concentration (nM) per reactionb</th>
<th>Target</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O104wzy-f</td>
<td>GGTATGTTCTGCTGTCTTGC</td>
<td>225</td>
<td>wzyO104</td>
<td>154</td>
</tr>
<tr>
<td>O104wzy-r</td>
<td>GTAATACTTGTGTACGATGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-stx2F</td>
<td>GTAGACTCAAGGCCCTGGATT</td>
<td>100</td>
<td>stx2</td>
<td>106</td>
</tr>
<tr>
<td>RT-stx2R</td>
<td>GGGCAACTAGGCCTCTGGCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fliCH4-a</td>
<td>GCGCAAAGTTCCACACCAGC</td>
<td>75</td>
<td>fliC H4</td>
<td>201</td>
</tr>
<tr>
<td>fliCH4-b</td>
<td>GCACCAAGTTACCCAGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Primers for wzyO104 and stx2 were designed in this study. Primers for fliC H4 were described previously (3).

*b* The primer concentrations were optimized to obtain melting peaks of similar intensities.

FIG 1 Real-time multiplex PCR for the detection and identification of EHEC O104:H4. Data represent amplification of wzyO104, stx2, and fliC H4 in prototypic EHEC O104:H4 outbreak isolate LB226692 (3, 11), HUSEC041 (O104:H4), HUSEC037 (O104:H21), and HUSEC003 (O157:H7) by the use of the real-time MPCR. Melting peaks of the wzyO104, stx2, and fliC H4 amplicons at 75.2°C, 80.2°C, and 83.6°C, respectively, are shown. C600, E. coli K-12 C600; NTC, no-template control.
ples from the 11 patients described above, need to be further inves-
tigated for EHEC of other serotypes in order to detect both
known and possibly new, emerging EHEC strains. Preliminary
information about the presence of an EHEC strain in the stool
within 24 h, as provided by the rtMPCR, is critical for epidemi-
ological purposes, in particular, for “real-time” monitoring of
spread of the infection and tracing it back to the source. From the
therapeutic standpoint, rapid detection of evidence of EHEC
O104:H4 infection may provide a basis for applying, in addition
to general therapeutic strategies used for EHEC infections (increas-
ing the volume of intravenous fluids and avoiding antibiotic ad-
ministration) (9, 17), additional, more specialized approaches
such as were successfully used during the EHEC O104:H4 out-
break (8). The method described here extends the real-time mul-
tiplex PCRs available for detecting EHEC O104:H4 in food (5, 18)
for the first time to rapid detection of the strain in human stools.
rtMPCRs for detecting other members of the HUSEC collection
are under development.

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