Rapid Identification and Differentiation of Clinical Isolates of Enteropathogenic Escherichia coli (EPEC), Atypical EPEC, and Shiga Toxin-Producing Escherichia coli by a One-Step Multiplex PCR Method

Daniel Müller,1 Peter Hagedorn,1 Sabine Brast,1 Gerhard Heusipp,1 Martina Bielaszewska,2 Alexander W. Friedrich,2 Helge Karch,2 and M. Alexander Schmidt1*

Institut für Infektiologie, Zentrum für Molekularbiologie der Entzündung, Westfälische Wilhelms-Universität/Universitätsklinikum Münster,1 and Institut für Hygiene und Nationales Konsiliarlaboratorium für Hämolysitisch-Uramisches Syndrom, Universitätsklinikum Münster,2 D-48149 Münster, Germany

Received 28 April 2006/Accepted 3 May 2006

Enteropathogenic Escherichia coli (EPEC), atypical enteropathogenic E. coli, and Shiga toxin-producing E. coli differ in their virulence factor profiles, clinical manifestations, and prognosis, and they require different therapeutic measures. We developed and evaluated a robust multiplex PCR to identify these pathogroups based on sequences complementary to escV, bfpB, stx1, and stx2.

Pathogenic Escherichia coli strains are responsible for a broad spectrum of intestinal and extraintestinal diseases, including diarrhea, urinary tract infections, septicemia, and neonatal meningitis (11). Enteropathogenic E. coli (EPEC) and the majority of clinical isolates of Shiga toxin (Stx)-producing E. coli (STEC) harbor the “locus of enterocecy effacement” (LEE), a pathogenicity island that is responsible for the phenotype of attaching-and-effacing (A/E) lesions (6, 11).

EPEC are a major cause of human infantile diarrhea predominantly in less-developed countries but are also identified with increasing frequency in industrialized areas (1, 11, 17). These pathogens colonize the small intestine, induce the degeneration of epithelial microvilli, and intimately adhere to the host cell. Comparable to a “molecular syringe,” the chromosomally encoded type III secretion system injects “effector” proteins into the host cell, inducing a characteristic rearrangement of the actin cytoskeleton resulting in the formation of “pedestals.” These characteristic histopathological alterations are summarized as “A/E lesions.”

The genes responsible for the A/E lesions are located on an ∼35-kb pathogenicity island, known as the locus of enterocecy effacement (LEE). Typical EPEC harbor an additional 60-MDa plasmid, the EPEC adherence factor (EAF) plasmid (16), that is not present in atypical EPEC (here abbreviated as ATEC) strains (3, 28). The EAF plasmid harbors the bundle-forming pilus (bfp) operon, encoding the type IV pili responsible for localized adherence and the formation of microcolonies on host cells. ATEC strains harbor homologues of the LEE pathogenicity island but, due to the lack of the EAF plasmid (3, 8), they mostly adhere in a diffuse pattern to epithelial cells. Recent epidemiological evidence indicates an increasing prevalence of ATEC particularly in developed countries (see, for example, references 1, 17, 21, and 28) but also in developing countries (see, for example, references 9 and 27). This also indicates that in the field the EAF plasmid is not essential to cause disease.

Like ATEC strains, the closely related STEC responsible for sporadic infections as well as serious outbreaks worldwide, mostly harbor the LEE pathogenicity island and lack the BFP-encoding EAF plasmid. STEC strains differ geno- and phenotypically from ATEC by their production of Stx. These pathogens cause an acute inflammation of the colon, resulting in hemorrhagic colitis with rare but serious sequelae including neurological disorders and the hemolytic-uremic syndrome (HUS), the leading cause of acute renal failure in children (11, 12).

Identification of EPEC, ATEC, and STEC strains is currently usually based on serotyping with specific antisera in a time-consuming process demanding some technical expertise. To facilitate diagnostic and therapeutic measures, we developed a single multiplex PCR (MPCR) for the simultaneous and rapid identification and differentiation of diarrheagenic E. coli belonging to EPEC, ATEC, or STEC pathotypes.

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Target gene</th>
<th>Primer sequence 5′ to 3′</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-escV-F</td>
<td>escV</td>
<td>GGCTCCTCTTCTTTATAGGCTT</td>
<td>534</td>
</tr>
<tr>
<td>MP-escV-R</td>
<td>escV</td>
<td>CTTTTTAAACTCACTCAGCC</td>
<td></td>
</tr>
<tr>
<td>MP-bfpB-F</td>
<td>bfpB</td>
<td>GATAAAAAGATGATGCCAGAGC</td>
<td>826</td>
</tr>
<tr>
<td>MP-bfpB-R</td>
<td>bfpB</td>
<td>AGTGACTGGTCCGGAAGACAG</td>
<td></td>
</tr>
<tr>
<td>MP-stx1A-F</td>
<td>stx1</td>
<td>GCGGTCTCTATATGAATCCTGTC</td>
<td>250</td>
</tr>
<tr>
<td>MP-stx1A-R</td>
<td>stx1</td>
<td>ATCCCAAGGACTTTCCTCAG</td>
<td></td>
</tr>
<tr>
<td>MP-stx2A-F</td>
<td>stx2</td>
<td>CGTTTTGACCATCTTCGTCT</td>
<td>325</td>
</tr>
<tr>
<td>MP-stx2A-R</td>
<td>stx2</td>
<td>AGCGTAAGGCTTCTGTCTG</td>
<td></td>
</tr>
</tbody>
</table>

* Corresponding author. Mailing address: Institut für Infektiologie, Zentrum für Molekularbiologie der Entzündung (ZMBE), Von-Esmarch-Str. 56, D-48149 Münster, Germany. Phone: 49 251 835 6466. Fax: 49 251 835 6467. E-mail: infekt@uni-muenster.de.

a -F, forward primer; -R, reverse primer.
MPCR development: selection of target genes and primer design. For the development of a single MPCR, we designed primer pairs (Table 1) that share similar temperature-related properties and that give rise to DNA fragments of sufficiently different sizes to be unequivocally resolved by agarose gel electrophoresis (Fig. 1). Therefore, for the specific identification of EPEC, ATEC, and STEC strains, we chose four marker genes (escV, bfpB, stx1, and stx2) exhibiting the highest degree of homology among the corresponding sequences found in the databases. The MPCR was performed in a 25-μl reaction mixture consisting of 1 U of Taq DNA polymerase with the corresponding Taq polymerase buffer (Segenetic; Borken), a 0.3 mM concentration of each deoxynucleoside triphosphate, and a 0.4 μM concentration of each PCR primer. Thermocycling conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. The identity of the amplified fragments was determined by sequencing (SEQLAB; Göttingen).

Specificity and evaluation of the MPCR. The specificity of the MPCR was tested on LE positive and LE negative reference strains (EPEC E2348/69, ATEC 3431–4/86, STEC EDL933, STEC 493/89, uropathogenic E. coli K552, and apathogenic E. coli C600). As shown in Fig. 1, the specific DNA fragments corresponding to the genes defining the appropriate LE-harboring phenotypes (EPEC carrying LEE and bfp but not stx, ATEC carrying LEE but not bfp or stx, and STEC lacking bfp and/or LEE but carrying stx) were easily detected by MPCR in a single experiment using the listed primer pairs (Table 1). The escV gene was detected in reference strains encompassing all of the intimin subtypes (α, β, γ, ε, ζ, η, θ, τ, and σ) known thus far (30) and validated by sequencing.

Moreover, the MPCR primer pairs MP2-stx1A-F/R and MP2-stx2A-F/R detected STEC belonging to each of the four different seropathotypes that have been identified among human STEC isolates by Karmali et al. (12). Most of the clinical STEC isolates possess genes encoding Stx1, Stx2, or their variants (4, 7, 14, 23, 24). These include stx1c, stx1a, stx2c, stx2a, and stx2b that were all correctly detected using the primers MP2-stx1A-F/R and MP2-stx2A-F/R included in the MPCR. As expected, stx2e, which is the prevalent stx gene in STEC strains isolated from pigeons (22, 25), could not be detected by the novel MPCR because its sequence identity to stx2e of E. coli O157:H7 strain EDL933 is only 63.4% and 57.4% for the A- and B-subunit genes, respectively (22). Although a single case of diarrhea caused by an stx2e-harboring E. coli strain has been recently reported (24), this stx allele appears to be extremely rare among human isolates (7).

Validation of MPCR with clinical isolates comprising different pathogroups. The comparison of the analysis of 184 of 281 well-defined clinical isolates comprising EPEC, ATEC, and STEC by MPCR and single PCRs targeting eae, stx1, and
TABLE 3. Comparison of marker gene detection by single PCR and MPCR in stool enrichment cultures of patients with diarrhea and HUS

<table>
<thead>
<tr>
<th>Isolated strain serotype (no.)</th>
<th>Pathogen designation</th>
<th>No. of samples</th>
<th>Single PCR</th>
<th>MPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>eae stx1 stx2 escV bfp stx1 stx2</td>
<td></td>
</tr>
<tr>
<td>ND*</td>
<td>NA*</td>
<td>35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O157:NM* (15)</td>
<td>STEC</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>O26:H11 (7)</td>
<td>STEC</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>O145:NM (6)</td>
<td>STEC</td>
<td>6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>O111:NM (4)</td>
<td>STEC</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>O26:NM (3)</td>
<td>ATEC</td>
<td>3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Other non-O157 serotypes (5)**</td>
<td>STEC</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

* ND, serotype not determined.

** NA, not applicable.

† NM, nonmotile; SF, sorbitol-fermenting.


§ ND, serotype not determined.

As with numerous well-characterized clinical isolates, the eight primers developed in the present study proved to be specific for the corresponding four genes, since we could not observe any cross-priming or the amplification of nonspecific DNA fragments. Further evaluation of the tetrameric MPCR with 281 clinical Escherichia coli isolates and 150 stool samples demonstrated that the MPCR is highly specific and reliable. We conclude that the newly designed MPCR is a specific method for the identification and differentiation of EPEC, ATEC, STEC, andLEE-negative STEC strains. As a fast, straightforward, and robust technique, it might be introduced into routine diagnostic in clinical microbiological laboratories.

We are indebted to A. Cravioto, C. Jallat, K. Jann, S. Knutton, C. Le Bouguenec, S. L. Moseley, F. and I. Ørskov, G. Peters, L. W. Riley, G. Schmidt, H.-G. Sonntag, L. R. Trabulsi, H. Tschäpe, and K. Wachsmuth for generous donations of clinical Escherichia coli isolates. We also thank the Nationale Referenzzentrum für Enteri-bacteriaceae (K. Tschäpe, RKI Wernigerode) for serotyping the clinical isolates. Furthermore, we thank Jennifer Schilling, Gunnar Sander, and Nicole Brandt for valuable technical help.

This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG SFK293 TP B5) and from the Bundes-ministerium Bildung und Forschung and Forschungs-Netwerk of Communicable Pathogenomics Alliance "Functional Genomics Research on Enterohemorrhagic, Enteropathogenic and Enterogaeggative Escherichia coli"; PG Karch/Schmidt; Universitäts-klinikum/Universität Münster [BD no. 119523/20780] PTJ-BIO/03U213B VBIIIIPG3).

REFERENCES


