PCR versus Hybridization for Detecting Virulence Genes of Enterohemorrhagic Escherichia coli

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We compared PCR amplification of 9 enterohemorrhagic Escherichia coli virulence factors among 40 isolates (21 O/H antigenicity classes) with DNA hybridization. Both methods showed 100% of the chromosomal and plasmid genes: eae, stx, and stx2. PCR did not detect 4%–20% of hybridizable plasmid genes: hlyA, katP, espP, toxB, open reading frame (ORF) 1, and ORF2.

Enterohemorrhagic Escherichia coli (EHEC) pathogenicity is usually linked to a Shiga toxin (1,2) and virulence factors, including adhesins, toxins, invasins, protein secretion systems, iron uptake systems, and several unidentified functions (3,4), which are unrelated to strain phylogeny. In many laboratories, sorbitol-MacConkey medium is commonly used to screen for the slow sorbitol fermentation phenotype of the most common Shiga toxin–containing strain: O157:H7 (5), but this process does not address the pathogenic potential of the remaining sorbitol-positive E. coli. These organisms can be detected by immunologic methods or PCR evaluation of virulence factors. PCR is the most useful method for virulence factor detection, and others have made convincing arguments for its use in characterizing the virulence factor patterns of potential pathogens (6,7).

Variation in virulence factor targets and use of different PCR primers contribute to variable results in detecting the most common virulence factors: stx1, stx2, eae, and hlyA (or ehxA). Variation in amplification success is likely to increase because more virulence factor variants are certain to emerge as more EHEC and Shiga toxin–producing E. coli (STEC) strains are identified. This study addresses the potential for a broad and well-characterized set of control strains relative to virulence factor amplification and confirmed by Southern hybridization.

The Study

We used PCR amplification and Southern blot hybridization to detect 9 virulence factors among 40 EHEC type-strains from the STEC Center, National Food Safety and Toxicology Center, Michigan State University (East Lansing, MI, USA). The virulence factor targets were the following: 1 chromosomal (eae [8]), 2 phage (stx1 and 2), and 6 plasmid (open reading frame [ORF] 1, ORF2 of pO-SAK1 [1,2]; espP [9], hlyA [4,10], katP [11], and tox B [12] of pO157) (Table). DNA-DNA hybridization probes were made from virulence factors amplified from O157:H7 EDL933 genomic DNA.

PCR amplification was carried out with PCR primers (20 pmol/L each per 50 μL reaction) (Integrated DNA Technologies, Coralville, IA, USA) (Table) and 1 μL genomic DNA (extracted from overnight Luria-Bertani broth cultures according to PureGene DNA isolation kit instructions [Gentra Systems, Minneapolis, MN, USA] and dissolved in 50 μL 10 mmol/L Tris, pH 8.3) in a PCR cocktail containing 1× PCR buffer, 1.5 mmol/L MgCl2, 1 U Vent exo(–) polymerase from New England BioLabs (Beverly, MA, USA), and 200 μmol/L each dATP, dGTP, dTTP, and dCTP. The mix was incubated for 30 cycles of 94°C, 40 s; annealing (for temperatures, see Table), 45 s; 72°C, 60 s, and a final 10-min extension at 72°C. Amplification products were confirmed by DNA sequencing.

32P-labeled DNA probes were made from 2 μg PCR amplicons (purified by Montage PCR Cleanup Spin Column (Millipore Corp., Burlington, MA, USA). The DNA was denatured at 94°C, 40 sec; annealed (temperatures in Table) with 50 pmol/L of the appropriate PCR primers, 45 s extended for 2 h at 72°C. The 1× buffer contained the following: 1.5 mmol/L MgCl2, 0.4 mmol/L each dATP, dGTP, dTTP; 2.0 μL 3,000 Ci/mmol α-32P-dCTP (MP Bioscience, Buxton, UK); and 1.25 U Taq polymerase in a 50 μL final volume. Unincorporated 32P-nucleotide was removed by Sephadex G-50 in Tris-EDTA, 1% sodium dodecyl sulfate (SDS).

Bacteria (800 μL overnight cultures) were transferred to Hybond-N+ nitrocellulose membrane (Amersham Biosciences UK Ltd, Buckinghamshire, UK) by dot-blot vacuum filtration apparatus (Schleicher and Schuell, Keene, NH, USA). Lysis and binding of genomic DNA fixation were carried out by exposure to lysis solution (1.5 mol/L NaCl, 0.5 mol/L NaOH) twice for 5 min each, and twice with neutralization solution (1 mol/L Tris-Cl, pH 7.4; 1.5 mol/L NaCl) for 5 min each. The filter was then submerged in 2× SSC with gentle agitation, air dried, and the DNA UV (254 nm) cross-linked at 120,000J/cm2 (CL-1000 cross-linker, Fisher Biotech, Pittsburgh, PA, USA).

Probe hybridization was carried out in rotating hybridization bottles (Fisher Scientific Isotemp hybridization oven, Fisher Biotech) in 20 mL 6× SSC, 1% SDS at
68°C. Membranes were washed twice, for 1 min, in room temperature 2× SSC, 0.1% SDS, and twice at 45°C for 1 h in 1× SSC, 1% SDS. Hybridized membranes were exposed overnight with a phospho-imaging screen (Bio-Rad, Hercules, CA, USA) and visualized with a Personal Mo-
(228/240) of the plasmid hybridizable targets were amplified, compared to 100% (120/120) of the hybridizable chromosomal targets.

Although we detected the variable presence of genes ostensibly associated with 2 plasmids (pO157 and pO-SAK1) and the bacterial chromosome, we did not attempt to verify either plasmid or chromosomal locations for any of the amplicons or DNA:DNA hybrids. While all virulence factor targets summarized in this study are subject to change there have been reports of any of the putative chromosomal or plasmid virulence factor targets in this study being found elsewhere.

Prager et al. (7) recently reported, using PCR alone, a wide variety of 25 virulence factor combinations among 266 pathogenic *E. coli* isolates representing 81 serotypes. Such diversity speaks directly to the need to accurately assess virulence factor presence to evaluate epidemiologic and clinical correlations. A similar 5% failure of the plasmid-associated virulence factor amplifications could have implications in such virulence factor correlations. Overall, however, these results are very similar to those of this study of prospective control strains. The use of a single control, such as EDL 933, will inherently bias PCR detection schemes since a failure of amplification in a test will be read as the absence of virulence factor element because it was amplifiable in the control.

If amplification failure is a measure of template variation, we find a much greater variability among plasmid-associated virulence factors. Although pO157 has been reported in most O157 H7 strains (13), our study demonstrates a high variability in the putative virulence factor content of pO157 as well as a highly variable content of pO157-associated virulence factors among the O157 isolates screened. Finally, pO157-associated virulence factors were detected among all but 4 of the 20 *E. coli* serotypes examined.

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**References**


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