Multiplex PCR for Enterotoxigenic, Attaching and Effacing, and Shiga Toxin-Producing *Escherichia coli* Strains from Calves

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Diarrhea in calves is commonly caused by enterotoxigenic *Escherichia coli* (ETEC). More recently, attaching and effacing *E. coli* (AEEC) and Shiga toxin-producing *E. coli* (STEC) have also been identified as causes of diarrhea and dysentery in calves (2, 15).

ETEC has two groups of virulence factors: fimbriae (pili) and enterotoxins. K99 and/or F41 fimbriae mediate adherence to the ileum and are found on most calf ETEC (1, 9, 12). Calf ETEC produces heat-stable enterotoxin a (STa), which causes hypersecretion into the gut lumen (2).

Intestinal lesions caused by AEEC are termed attaching and effacing (AE) because of their intimate attachment to the enterocyte and effacement of the microvillus border (18). A chromosomal gene, *eaeA*, encodes the protein intimin, which is involved in AE activity (10). AEEC which causes disease and does not produce enterotoxins or Shiga toxins is referred to as enteropathogenic *E. coli* (EPEC).

STEC produces two types of *E. coli* Shiga toxins, those that are immunologically similar to the Shiga toxin produced by *Shigella dysenteriae* and those that are immunologically distinct from *Shigella dysenteriae* Shiga toxin (Stx2) (11). Bovine STEC produces either Stx1, Stx2, or both. These toxins act by inhibiting protein synthesis and are lethal for in vitro cultured Vero cells (8). Some *E. coli* strains, such as the zoonotic pathogen *E. coli* O157:H7, which produces Shiga toxins and has AE activity, are often termed enterohemorrhagic *E. coli* (EHEC) because of their association with hemorrhagic colitis in humans.

Diagnosis of *E. coli* infection currently relies on the phenotypic differentiation of pathogenic strains from nonpathogenic normal flora *E. coli* via bioassays or immunoassays for toxins and fimbriae. These tests can be time-consuming and complicated and are not routinely used in many clinical laboratories.

Histological examination of the intestine may indicate involvement of ETEC or AEEC, but this is a postmortem diagnosis. Genotypic diagnosis may be accomplished by DNA colony blot hybridization to identify genes encoding virulence factors (17). However, the use of radioactive isotopes and the time required make this method unsuitable for many diagnostic laboratories.

PCR is a useful diagnostic tool because it is quick, specific, sensitive, and relatively inexpensive. A multiplex PCR which detects genes encoding intimin and Stx1 and Stx2 has been developed (4). This paper reports the identification and differentiation of ETEC, AEEC, and STEC via multiplex PCR amplification of virulence-associated genes commonly found in these *E. coli* strains. Primers specific for genes encoding the fimbrial subunits of K99 and F41, STa enterotoxin, intimin, and the A subunits of Shiga toxins Stx1 and Stx2 were incor-

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<th>Virulence factor</th>
<th>Accession number</th>
<th>Primer sequence 5'-3'</th>
<th>Position in open reading frame</th>
<th>Size of product (bp)</th>
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<td></td>
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<td>992-1013 of eaeA</td>
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<td>30-53 of A subunit</td>
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</table>

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porated into a multiplex PCR format. This multiplex PCR was then tested on 99 E. coli strains obtained from the E. coli collection at the National Animal Disease Center in Ames, Iowa, and from the Center for Vaccine Development, University of Maryland. Strains were previously probed by colony blot hybridization for genes encoding these virulence factors.

Primers were chosen from published sequences with the aid of the Primer Select software (DNASTAR Inc, Madison, Wis.). Table 1 includes the primer sequences, the position of the primer in the open reading frame of the gene, and the predicted sizes of amplified products. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa). Because the EHEC and EPEC genes which encode intimin have considerable heterogeneity on the 3' end, the eaeA primers were made to amplify a constant 5' region of the EHEC and EPEC genes (14). Other primers were chosen from the open reading frames of the published sequences. The primers were designed to have similar annealing temperatures and minimal interactions and resulted in different-sized products.

The 20-μl PCR mixture contained 0.5 μM concentrations of each primer, 0.2 mM concentrations of each dNTP (di-deoxy nucleotides), 1× AmpliTaq Gold buffer, 2.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, N.J.), and bacterial DNA. All reagents except AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, N.J.), and bacterial DNA. All reagents except AmpliTaq Gold were designed to have similar annealing temperatures and minimal interactions and resulted in different-sized products.

The 20-μl PCR mixture contained 0.5 μM concentrations of each primer, 0.2 mM concentrations of each dNTP (di-deoxy nucleotides), 1× AmpliTaq Gold buffer, 2.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, N.J.), and bacterial DNA. All reagents except AmpliTaq Gold were designed to have similar annealing temperatures and minimal interactions and resulted in different-sized products.

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with different phenotypes as illustrated in Fig. 1 and Table 2. These strains had been characterized phenotypically and by colony blot hybridization previously by others. The multiplex PCR assay correctly determined the presence or absence of the genes of interest in all of the reference strains.

Further isolates from the National Animal Disease Center and the Center for Vaccine Development were chosen to verify the multiplex PCR could detect genes with a high level of agreement with colony blot hybridization (Table 3). The isolates tested included the following groups: a, 20 ETEC isolates from calves, pigs, or humans; b, 16 EPEC isolates from humans; c, 29 STEC isolates from calves or humans; d, 14 isolates representing various combinations of the six genes of interest; and e, 11 nonpathogenic E. coli isolates containing none of the six genes of interest. The multiplex PCR correctly detected the presence or absence of all genes of interest in all 90 isolates from groups a to e.

This multiplex PCR was highly specific, because it did not detect any genes which were determined to be absent by probing. The multiplex method was also highly sensitive, as it correctly detected all genes of interest in 100% (88 of 88) of the strains and isolates containing them. This assay will be useful in diagnostic situations for identification and characterization of E. coli isolated from calves with diarrhea. This multiplex PCR may also prove useful in detecting any of the genes of interest in E. coli from other host species.

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REFERENCES