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Typing of Bovine Attaching and Effacing *Escherichia coli* by Multiplex In Vitro Amplification of Virulence-Associated Genes

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Attaching and effacing *Escherichia coli* is a new causal agent of diarrhea in calves. Its major virulence factors are the intimin protein, encoded by the *eaeA* gene, and the Shiga-like toxins, encoded by *slt* genes. Because the sequences of these genes are available, we selected specific primers to amplify each virulence gene so as to develop a new identification test based on multiplex amplification of virulence-associated genes. Of 30 tested strains, 14 were *eaeA*$^+$, 15 were *eaeA*$^+$ *slt-I*$^+$, 1 was *eaeA*$^+$ *slt-I*$^+$ *slt-II*$^+$, and 1 was *eaeA*$^+$ *slt-II*$^+$. The method proved in our hands to be fast and specific and in perfect correlation with the hybridization method.

In the past, *Escherichia coli* strains which were implicated in neonatal-calf diarrhea were mostly enterotoxigenic. More recently, attaching and effacing *E. coli* (AEEC) (18) has been implicated in diarrhea and dysentery, mostly in 2- to 8-week-old calves (5, 17). Attaching and effacing was the term first used by Moon et al. (18) to describe an intestinal lesion produced by *E. coli*: "attaching" indicates the intimate attachment of bacteria to the enterocyte; "effacing" indicates the localized effacement of brush border microvilli. Such a lesion appears when enteropathogenic *E. coli* (EPEC) or enterohemorrhagic *E. coli* (EHEC) infects its host. Intimate attachment of AEEC involves a 94-kDa outer membrane protein called intimin and encoded in human EPEC by the *eaeA* gene (10). An *eaeA*-related gene is also present in human EHEC (2, 24). The sequence is 83% identical to the EPEC locus, with identities of 97% for the first 2,200 bp and 59% over the last 800 bp. Therefore, the *eaeA* gene is made up of two parts: a constant 5′ region and a variable 3′ region.

The name EHEC has been applied to verotoxigenic *E. coli* (VTEC), which has been implicated in hemorrhagic colitis in humans (13). Verotoxins are *E. coli* cytotoxins which are lethal for cultured Vero cells (12). These toxins share a number of properties with Shiga toxin, produced by *Shigella dysenteriae*, and are therefore also called Shiga-like toxins (21). Verotoxins consist of two groups, with VT1 (Shiga-like toxin I [SLT-I]) constituting one group and VT2 (SLT-II) and antigenically related toxins constituting a second group (19). The SLT-I- and SLT-II-encoding operons were sequenced (8, 9). EPEC and EHEC strains from several animal species, including cattle, have been described (5). A previous study indicated that most bovine AEEC strains were actually EHEC-like strains (17).

The phenotypic diagnosis of EHEC and VTEC is based on the cytotoxicity of verotoxins for Vero cell monolayers (23) and on immunological detection. Enzyme-linked immunosorbent assays for the detection of verotoxin-producing cultures or for direct detection of VTEC in feces or food have been developed by a number of workers (23). Attaching and effacing activity can be detected by the fluorescence actin staining assay for human EPEC strains (11), but the assay seems to be more difficult for animal isolates. The genetic diagnosis of AEEC can be performed either by hybridization with probes specific for the *eaeA, slt-I*, and *slt-II* virulence genes or by specific amplification of virulence genes (23).

We report here the development of a typing assay for bovine EPEC, EHEC, and VTEC strains based on multiplex PCR assay of the virulence-associated genes.

Since the major virulence-associated genes described for AEEC are *eaeA, slt-I*, and *slt-II*, we deduced from published sequences primer pairs specific for each gene (Table 1). The *eaeA* primers were chosen for the constant part of the gene to ensure amplification of the *eaeA* gene from either EPEC or EHEC strains. These primers were first tested on reference strains (Table 2). Bacteria were grown overnight at 37°C in 5 ml of Luria broth medium (1). Three hundred microliters of culture was centrifuged for 30 s in a minicentrifuge. The pellets were resuspended in 50 μl of sterile distilled water, boiled for 10 min, and centrifuged again for 30 s in a minicentrifuge. The supernatant was collected and used as a DNA template.

For PCR, the following mixture was used: 1 U of Dynazyme (Finnzymes, Espoo, Finland), 5 μl of 2 mM deoxynucleoside triphosphates (Pharmacia, Uppsala, Sweden), 5 μl of 10X buffer (100 mM Tris-HCl [pH 8.8], 15 mM $MgCl_2$, 500 mM KCl, 1% Triton X-100), 0.5 μl of each primer (40 mM), and 5 μl of a DNA template in a total volume of 50 μl. Thirty microliters of mineral oil (Sigma-Aldrich, Bornem, Belgium) was added at the top of the PCR mixture. The following conditions were applied: 94°C for 5 min followed by 30 cycles at

TABLE 1. Primers pairs used in this study

Gene	Primer and sequence	Size of PCR product (bp)	Optimal annealing temp (°C)[a]	Reference(s)
eaeA	B52, AGGCTTCGTCACAGTTG B53, CCATCGTCACCAGAGGA	570	51.9	2, 24
slt-I	B54, AGAGCGATGTTACGGTTTG B55, TTGCCCCCAGAGTGGATG	388	52.9	9
slt-II	B56, TGGGTTTTTCTTCGGTATC B57, GACATTCTGGTTGACTCTCTT	807	53.4	8

[a] Calculated by using Oligo software (National Biosciences, Plymouth, Minn.).

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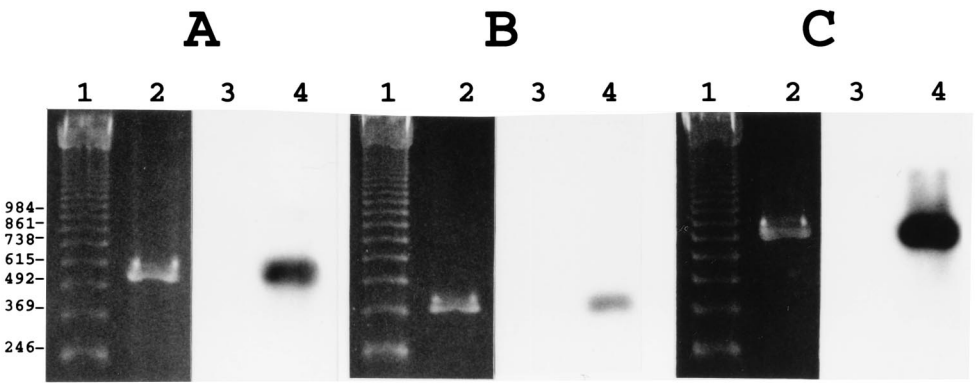


FIG. 1. Specific amplification of AEEC virulence genes. DNA from AEEC strains was used as a template in a PCR experiment with primers corresponding to virulence genes. The amplicons were analyzed by electrophoresis on a 2% agarose gel, blotted onto a nylon membrane, and hybridized with a specific probe in order to check the specificity of amplifications. (A) DNA from strain E2348/69 was used to amplify the *eaeA* gene with primers B52 and B53. Lane 1, 2 μl of a 123-bp ladder (Gibco BRL, Paisley, Scotland); lane 2, 10 μl of amplification products; lane 3, DNA from lane 1 blotted onto a nylon membrane and hybridized with the *eaeA* probe; lane 4, DNA from lane 2 blotted onto a nylon membrane and hybridized with the *eaeA* probe. (B) DNA from strain 193 was used to amplify the *slt-I* gene with primers B54 and B55. Lane 1, 2 μl of a 123-bp ladder (Gibco BRL); lane 2, 10 μl of amplification products; lane 3, DNA from lane 1 blotted onto a nylon membrane and hybridized with the *slt-I* probe; lane 4, DNA from lane 2 blotted onto a nylon membrane and hybridized with the *slt-I* probe. (C) DNA from strain 211 was used to amplify the *slt-II* gene with primers B56 and B57. Lane 1, 2 μl of a 123-bp ladder (Gibco BRL); lane 2, 10 μl of amplification products; lane 3, DNA from lane 1 blotted onto a nylon membrane and hybridized with the *slt-II* probe; lane 4, DNA from lane 2 blotted onto a nylon membrane and hybridized with the *slt-II* probe. The molecular sizes (in base pairs) are indicated at the left.

94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. The cycles were carried out in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.).

A tenth of the PCR mixture was loaded onto a 2% agarose gel (Fig. 1). The expected sizes were 570, 388, and 807 bp for the *eaeA*, *slt-I*, and *slt-II* amplicons, respectively. The sizes deduced from the gel were in agreement with the expected sizes. In order to confirm the specificity of the amplification, the amplicons were blotted onto a nylon membrane and hybridized with probes specific for *eaeA* (10), *slt-I* (19), and *slt-II* (19), respectively. The results (Fig. 1) indicated that each amplification product was derived from the expected gene.

The pairs of primers were chosen on the basis of two criteria: first, they gave amplification products of different sizes, and second, their optimal annealing temperatures were closed. These conditions allowed us to try a multiplex PCR with the three pairs of primers in the same PCR mixture. The multiplex PCR was first used for six reference strains (Table 1) harboring different pathotypes as determined by colony hybridizations (15). Therefore, after amplification the patterns of bands with the three pairs of primers should be different for each strain. Indeed, the result of such an experiment (Fig. 2) indicated that it was possible to recognize the strain corresponding to each pathotype by looking at the band patterns.

The multiplex PCR was applied to *eaeA*-probe-positive wild-type strains isolated from calves dead of diarrhea. In parallel, we determined the pathotypes of these *E. coli* strains by colony hybridization with *slt-I*- and *slt-II*-specific probes. The hybridization and PCR patterns were in total agreement (Fig. 3). Of 30 tested strains, 14 were $eaeA^+$, 15 were $eaeA^+$ $slt\text{-}I^+$, 1 was $eaeA^+$ $slt\text{-}I^+$ $slt\text{-}II^+$, and 1 was $eaeA^+$ $slt\text{-}II^+$. As previously described (17), a majority of EHEC strains were positive for the *slt-I* gene probe only. On the other hand, only half of the $eaeA^+$ *E. coli* strains were EHEC-like.

In conclusion, we tested a multiplex PCR method for typing AEEC strains. The results obtained are in agreement with those observed by colony hybridization. However, PCR is easier and more rapid to perform. Several investigators have de-

TABLE 2. Reference strains used in this study

Strain	Description	Serotype	Pathotype	Reference
HB101	Laboratory strain	K-12	Nonpathogen	3
E2348/69	Human EPEC	O127	$eaeA^+$	10
211	Bovine VTEC	Rough H7	$slt\text{-}II^+$	16
193	Bovine EHEC	O26	$eaeA^+$ $slt\text{-}I^+$	16
309S89[a]	Bovine VTEC	O20	$slt\text{-}I^+$ $slt\text{-}II^+$	This study
374S89[a]	Bovine VTEC	O8	$slt\text{-}I^+$	This study
317S89[a]	Bovine EHEC	Rough	$eaeA^+$ $slt\text{-}I^+$ $slt\text{-}II^+$	This study

[a] Provided and serotyped by the National Institute for Veterinary Research, Brussels, Belgium.

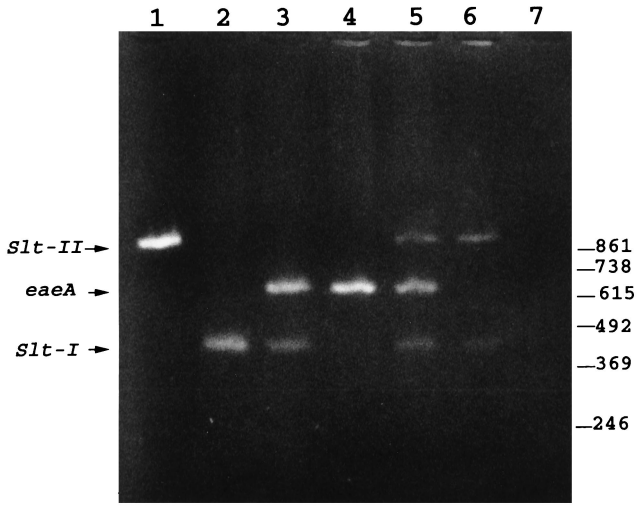
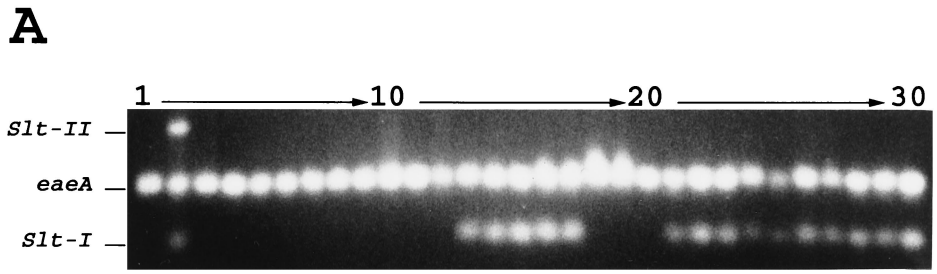


FIG. 2. Multiplex PCR of reference strains. DNA from reference strains was used as a template in a PCR experiment with primers B52, B53, B54, B55, B56, and B57. After PCR, 10 μl of the reaction mixture was analyzed by electrophoresis on a 2% agarose gel. Lane 1, strain 211; lane 2, strain 374S89; lane 3, strain 193; lane 4, strain E2348/69; lane 5, strain 317S89; lane 6, strain 309S89; lane 7, strain HB101. The molecular sizes (in base pairs) are indicated at the right. Bands corresponding to *slt-I, slt-II*, and *eaeA* are indicated.



B

STRAINS

Probes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
eaeA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
slt-I	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
slt-II	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

FIG. 3. Typing of AEEC strains isolated from calves dead of diarrhea. (A) Multiplex PCR. DNA from AEEC strains was used as a template in a PCR experiment with primers B52, B53, B54, B55, B56, and B57. After PCR, 10 μl of the PCR mixture was analyzed by electrophoresis on a 2% agarose gel. Lane 1, strain 47638MF1; lane 2, strain 47638MF2; lane 3, strain 47638MF3; lane 4, strain 48369MF1; lane 5, strain 48369MF2; lane 6, strain 48369MF3; lane 7, strain 45986MF1; lane 8, strain 45986MF3; lane 9, strain 45990MF1; lane 10, strain 45990MF2; lane 11, strain 45990MF3; lane 12, strain 46039MF2; lane 13, strain 46285MF1; lane 14, strain 46285MF2; lane 15, strain 46285MF3; lane 16, strain 46639MF1; lane 17, strain 46639MF2; lane 18, strain 46919MF2; lane 19, strain 46919MF3; lane 20, strain 47187MF3; lane 21, strain 47311MF2; lane 22, strain 45183MF1; lane 23, strain 45183MF4; lane 24, strain 45183MF6; lane 25, strain 45183MF7; lane 26, strain 45183MF8; lane 27, strain 45920MF1; lane 28, strain 46640MF1; lane 29, strain 46640MF2; lane 30, strain 46640MF3. (B) Colony hybridization. The AEEC strains listed for panel A were analyzed by colony hybridization with the *eaeA, slt-I*, and *slt-II* probes.

veloped genetic tests to identify AEEC strains isolated from biological materials. These techniques are based on colony hybridization (17, 19) or on PCR. The PCR was destined to the amplification of either *slt* genes (4, 14) or *eaeA* (7). Fratamico et al. (6) also developed a multiplex PCR for both *eaeA* and *slt* amplification. Nevertheless, *eaeA* amplification is specific to O157 serotype strains, and *slt* amplification was unable to discriminate between *slt-I* and *slt-II*. In this study, we developed a multiplex PCR for amplification of the *eaeA* and *slt* genes and for distinguishing between the *slt* genes. Because the *eaeA* primers were derived from the constant part of the gene, we could amplify *eaeA* genes from different strains. For *slt* genes, we chose the primers in variable regions, allowing discrimination between the *slt-I* and *slt-II* genes. As the reagents can be kept stable for a long time, a typing kit including the primers, polymerase, and reference DNA as a control could be useful.

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