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Detection of Shiga-Like Toxin (*stx*$_1$ and *stx*$_2$), Intimin (*eaeA*), and Enterohemorrhagic *Escherichia coli* (EHEC) Hemolysin (EHEC *hlyA*) Genes in Animal Feces by Multiplex PCR

PETER K. FAGAN,[1] MICHAEL A. HORNITZKY,[1] KARL A. BETTELHEIM,[2] AND STEVEN P. DJORDJEVIC[1]*

Elizabeth Macarthur Agricultural Institute, New South Wales Agriculture, Camden, New South Wales 2570,[1] and Victorian Infectious Diseases Reference Laboratory, Fairfield Hospital, Fairfield, Victoria 3078,[2] Australia

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A multiplex PCR was developed for the rapid detection of genes encoding Shiga toxins 1 and 2 (*stx*$_1$ and *stx*$_2$), intimin (*eaeA*), and enterohemolysin A (*hlyA*) in 444 fecal samples derived from healthy and clinically affected cattle, sheep, pigs, and goats. The method involved non-solvent-based extraction of nucleic acid from an aliquot of an overnight culture of feces in EC (modified) broth. The detection limit of the assay for both fecal samples and pure cultures was between 18 and 37 genome equivalents. *stx*$_1$ and *hlyA* were the most commonly encountered virulence factors.

Enterohemorrhagic *Escherichia coli* (EHEC) is the most important recently emerged group of food-borne pathogens. It can cause severe gastrointestinal disease, including fatal infections, and is being detected more frequently worldwide. More investigations regarding the laboratory diagnosis of these organisms have been carried out in recent years than with any other group of food-borne pathogens, yet this group remains the most difficult to detect. EHEC strains not only produce potent cytotoxins (verotoxins) but have also acquired the ability to adhere to the intestinal mucosa in an intimate fashion (4, 15, 21). They are also defined by the presence of specific virulence factors; all strains produce hemolysin (most producing an EHEC-specific plasmid-encoded hemolysin, encoded by *hlyA*) (26) and at least one Shiga-like toxin (encoded by *stx*$_1$ or *stx*$_2$) (21), and many produce intimin, a 97-kDa attachment-and-effacement protein (encoded by *eaeA*) (19). Although *E. coli* O157:H− is currently the most common EHEC strain in many regions of the world (3), serotypes O5, O26, O91, O111, and O113 are also recognized as a serious threat to public health and have been recovered from infected patients (4). Strains of *E. coli* O157:H− are comparatively easy to isolate because of unique biochemical characteristics; however, the other serotypes can be differentiated from commensal *E. coli* only by specialized techniques, such as those described in this report (reference 11 and references therein).

Paton et al. (22) described a PCR for the amplification of *stx*$_1$ and *stx*$_2$ sequences in primary human fecal cultures. However, oligonucleotide hybridization probes were required to distinguish between the two toxins in a separate test. The presence of *stx*-positive fecal cultures in asymptomatic individuals (9, 22) suggested that other virulence factors besides *stx* are required to cause serious disease in humans. Fratamico et al. (12) described a multiplex PCR capable of detecting *stx*$_1$, *stx*$_2$, *eaeA*, and EHEC *hlyA* sequences. However, this PCR was not tested with fecal samples; primers for each target gene sequence showed differential sensitivities, and *stx* primers were unable to distinguish *stx*$_1$ from *stx*$_2$ by agarose gel electrophoresis. Ideally, PCR-based detection methods should be rapid and sensitive without requiring extensive sample preparation. More recently Paton and Paton (23) developed a multiplex PCR utilizing four PCR primer pairs for the detection of *stx*$_1$, *stx*$_2$, *eaeA*, and EHEC *hlyA* in human feces and foodstuffs. However, the relatively lengthy PCR template preparation protocol used was considered inappropriate for testing large numbers of samples.

Ruminants, particularly cattle (5, 7, 30) and sheep (7, 17), are natural reservoirs of EHEC, although other domestic animals, including goats, pigs, poultry, cats and dogs, can also harbor these bacteria (1, 5, 6). However, methodologies which provide comparatively rapid (24-h) and sensitive detection of *stx*$_1$, *stx*$_2$, *eaeA*, and *hlyA* gene sequences in animal feces have not been reported. The aim of this study was to develop and evaluate a multiplex PCR for this purpose.

EHEC reference strains O111:H8, O157:H7, O128:H2, O91:H−, O113:H21, and O5:H− were provided by the Victorian Infectious Diseases Reference Laboratory (Fairfield, Australia). A positive EHEC control, *E. coli* O111:H−, was provided by the Victorian Institute of Animal Science (Fairfield, Australia), and the negative control strain *E. coli* JM109 was provided by Mark Walker (Wollongong University, Wollongong, Australia). Two hundred thirty-five diagnostic fecal samples from sheep, cattle, and pigs submitted to the Regional Veterinary Laboratory, Elizabeth Macarthur Agricultural Institute (Menangle, New South Wales, Australia), for microbiological analysis and a further nine bovine fecal specimens from cattle from a dairy farm with an EHEC history were used for this study. Two hundred fecal samples were also collected from apparently healthy animals from four sheep flocks and four bovine herds (25 samples from each herd or flock).

E. coli isolates were each cultured on EC (modified) agar, which was prepared by adding 1.5% agar to EC (modified) broth (CM853; Oxoid, Basingstoke, United Kingdom) and incubated at 37°C for 18 to 20 h prior to nucleic acid extraction.

* Corresponding author. Mailing address: Elizabeth Macarthur Agricultural Institute, New South Wales Agriculture, Private Mail Bag 8, Camden, New South Wales 2570, Australia. Phone: 0061-246-406426. Fax: 0061-246-406384. E-mail: steve.djordjevic@agric.nsw.gov.au.

TABLE 1. Primer sequences and predicted lengths of PCR amplification products

Primer	Direction	Primer sequence (5′-3′)	Fragment size (bases)	Reference
EHEC *hly*	Forward	ACGATGTGGTTTATTCTGGA	165	12
	Reverse	CTTCACGTGACCATACATAT		
stx_1	Forward	ACACTGGATGATCTCAGTGG	614	13
	Reverse	CTGAATCCCCCTCCATTATG		
stx_2	Forward	CCATGACAACGGACAGCAGTT	779	13
	Reverse	CCTGTCAACTGAGCAGCACTTTG		
eaeA	Forward	GTGGCGAATACTGGCGAGACT	890	14
	Reverse	CCCCATTCTTTTTCACCGTCG		

Fecal broth cultures were prepared by inoculating 50 mg of feces into 10 ml of EC (modified) broth and incubated at 37°C for 18 to 20 h. For DNA sample preparation either a 15-μl aliquot of the overnight fecal culture or a single colony off EC agar was mixed in 1 ml of sterile water in a 1.7-ml microcentrifuge tube. Bacteria were pelleted by centrifugation at 11,000 rpm for 1 min in a Biofuge *pico* (Heraeus, Hanau, Germany). The supernatant was subsequently discarded, 200 μl of InstaGene matrix (Bio-Rad) was added to the pellet, and the mixture was incubated at 56°C for 30 min. After incubation, the mixture was vortexed for 10 s and then incubated at 100°C for 8 min, followed by vortexing and centrifugation at 11,000 rpm for 1 min prior to removal of the nucleic acid template for PCR.

Multiplex PCR for detection of stx_1, stx_2, *eaeA*, and EHEC *hlyA* gene sequences was performed with a PC-960 thermal cycler (Corbett Research). Oligonucleotide primers were manufactured commercially (GIBCO-BRL). Primers and the predicted lengths of PCR amplification products are listed in Table 1. These primers were chosen because they amplify conserved regions of the target genes and allow single-step identification of amplified DNA fragments by agarose gel electrophoresis. Each primer pair had been determined to be specific for *E. coli* and had been shown not to amplify products detectable by agarose gel electrophoresis using DNA templates derived from a range of gram-positive and gram-negative bacterial species from food and animal sources (12–14).

PCR assays were carried out in a 50-μl volume containing 2 μl of nucleic acid template prepared from fecal cultures (approximately 60 ng of DNA) or 1 μl of nucleic acid template prepared by using reference EHEC isolates (approximately 30 ng of DNA), 10 mM Tris-HCl (pH 8.4), 10 mM KCl, 3 mM $MgCl_2$; 2 mM concentrations of each primer, 0.2 mM concentrations of each 2′-deoxynucleoside 5′-triphosphate, and 4 U of AmpliTaq DNA polymerase (Perkin-Elmer). Temperature conditions consisted of an initial 95°C denaturation step for 3 min followed by 35 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 90 s. The final cycle was followed by a 72°C incubation for 5 min. Amplified DNA fragments were resolved by gel electrophoresis (25) using 2% (wt/vol) agarose. Gels were stained with 0.5 μg of ethidium bromide per ml, visualized with UV illumination, and imaged with a GelDoc 1000 fluorescent imaging system (Bio-Rad).

To determine the sensitivity of the multiplex PCR assay, the number of bacterial cells per milliliter in a stock suspension of *E. coli* O111:H− solution was determined with a hemocytometer. A 10-fold dilution series of the stock suspension was prepared, and from this the number of CFU was determined. Nucleic acid was extracted from a representative volume of each dilution of the titration by the InstaGene methodology. Preparation of bacterial template for PCR was carried out as described for fecal culture PCR; however, an initial 10-μl aliquot of each dilution series was washed in 1 ml of sterile water. Assay sensitivity was also determined by using the dilution series to inoculate a freshly cultured PCR-negative overnight fecal broth. Aliquots of the seeded enrichment broth were immediately prepared for multiplex PCR to determine what impact a complex coliform mixture might have on assay sensitivity. No further enrichment took place.

DNA probes for colony hybridizations (*eaeA*, stx_1, stx_2, and EHEC *hlyA*) were directly labelled with digoxigenin-11-dUTP (DIG) using the PCR as described by the manufacturer (Boehringer GmbH, Mannheim, Germany). PCR amplification was carried out as described in the previous section by using *E. coli* O111:H− as the template with the deoxynucleoside triphosphate mixture containing 10% DIG.

Overnight fecal cultures (determined to contain any combination of the four virulence factors by multiplex PCR) were diluted (to ensure single colonies), plated onto EC (modified) agar, and grown at 37°C for 18 to 20 h. Colonies (96) were picked and patched onto fresh EC (modified) agar plates prior to incubation at 37°C for 18 to 20 h. Colony lifting was subsequently used to transfer the bacteria onto nylon Hybond H+ membrane (Amersham, Little Chalfont, United Kingdom). Colony hybridization was carried out with a DIG chemiluminescence detection kit (Hyb; Boehringer Mannheim) per the manufacturer's instructions. Hybridization was carried out in a minihybridization oven (Hybaid) at 58°C by using DIG Easy Hyb (Boehringer GmbH, Mannheim, Germany) with the denatured DIG-labelled DNA probe. After incubation, membranes were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature followed by two washes in 0.5× SSC–0.1% SDS at 68°C. All washes were performed in a minihybridization oven. Bound DIG-labelled probes were detected with CSPD (Boehringer GmbH, Mannheim, Germany) by following the manufacturer's instructions. Membranes were subsequently exposed to X-ray film (Kodak) at room temperature for 10 to 60 min prior to development.

PCR products representing each of the four target EHEC virulence factors were amplified with *E. coli* O111:H− DNA template as a positive control (Fig. 1, lane 1). No amplification products were present in either the negative control (a fecal culture previously determined to lack sequences for any of the four virulence determinants by multiplex PCR) or a water control (no nucleic acid) after PCR (results not shown).

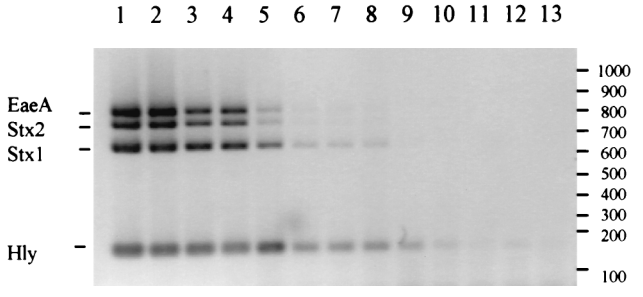

FIG. 1. Sensitivity of multiplex PCR in detecting EHEC virulence factors using serial dilutions of *E. coli* O111:H−. DNA markers are indicated on the right (numbers are molecular weights, in base pairs). Lanes 1 through 13 contain 6,250, 2,500, 630, 372, 186, 93, 46, 37, 18, 9, 3, 1.5, and <1 genome equivalents, respectively.

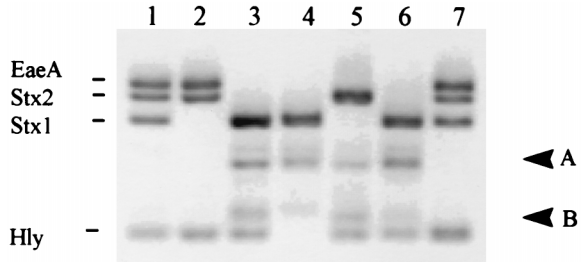

FIG. 2. Multiplex PCR analysis of EHEC reference strains. Arrows A and B refer to nonspecific PCR bands (see the text). Lanes: 1, *E. coli* O111:H8; 2, *E. coli* O157:H7; 3, *E. coli* O128:H2; 4, *E. coli* O91:H−; 5, *E. coli* O113:H21; 6, *E. coli* O5:H−; 7, *E. coli* O111:H− positive control.

DNA extracted from strains representative of each of six EHEC serotypes was the template for a range of PCR amplification products (Fig. 2). *stx* gene sequences were detected in all six reference EHEC serotypes, and one strain contained both stx_1 and stx_2. Three strains contained only stx_1, and the remaining two strains contained stx_2. All reference strains except O91:H− contained the EHEC *hlyA* gene. The genotypic results obtained by the multiplex EHEC PCR assay were in agreement with phenotypic data provided by the Victorian Infectious Diseases Reference Laboratory for 17 of the known 18 factors. stx_2, which had previously been detected in *E. coli* O91:H− by the Victorian Infectious Diseases Reference Laboratory, was not detected by multiplex PCR.

A stock suspension containing 6.3×10^7 *E. coli* O111:H− organisms per ml was determined. The sensitivity of the EHEC multiplex PCR assay was estimated to be between 37 and 18 genome equivalents. Amplified DNA bands were progressively lost in descending order from the largest (890-bp) *eaeA* product, which was lost between 37 and 18 genome equivalents, to the 165-bp EHEC *hlyA* product, which is detectable at less than 3 genome equivalents (Fig. 1). A similar level of sensitivity was achieved in fecal cultures which had previously been determined to be negative for the four virulence factors by multiplex PCR but which were seeded with titrations of stock suspension and tested without enrichment. All EHEC virulence factors were detectable between 37 and 18 genome equivalents (results not shown).

To further validate the utility of the multiplex PCR assay, 235 fecal samples were tested. Initial studies with 180 of these fecal samples used a multiplex PCR where primers for only three virulence factors, stx_1, stx_2, and *eaeA*, were tested (Table 2). stx_1 was the most commonly encountered factor, being detected in 19.4% (35 of 180) of fecal samples, while stx_2 and *eaeA* were each detected in 6.7% (12 of 180) of samples. Multiple EHEC factors were amplified in individual samples: stx_1 plus stx_2 5% (9 of 180), stx_2 plus *eaeA*, 1.7% (3 of 180); and stx_1 plus stx_2 plus *eaeA*, 2.2% (4 of 180). Of particular interest is the prevalence of stx_1 in the ovine samples (56.5%) compared to the almost equal distribution of stx_1 (17.3%) and stx_2 (21.8%) observed for cattle samples. Table 3 outlines the results for a further 64 fecal samples tested with the multiplex PCR, which included primers for the EHEC *hlyA* sequence. The EHEC *hlyA* factor was detected in 35.9% of all samples tested. The distribution of the stx_1, stx_2, and *eaeA* factors within these 64 samples was essentially the same (results not shown) as that within the 180 samples described in Table 2. Consequently, Table 3 displays EHEC factor combinations which include *hlyA*. A further 200 fecal samples from healthy cattle (four herds) and sheep (four flocks) were tested with the four-factor multiplex PCR assay (Table 4). Ovine samples from the four flocks also displayed a high prevalence of stx_1 (54%), whereas bovine samples displayed a comparatively low prevalence of stx_1 (17%) and stx_2 (7%) (Table 4). Figure 3 shows amplification products of the multiplex PCR using DNA templates recovered from nine bovine fecal samples from a dairy farm with a history of bovine EHEC excretion. In summary, *hlyA* was detected in four samples, *stx* genes were detected in four samples, and *eaeA* was detected in two samples.

TABLE 2. Multiplex PCR data derived from 180 livestock fecal samples

Sample type	No. of samples positive for gene(s)								
	None	stx_1	stx_2	*eaeA*	stx_1 + stx_2	stx_1 + *eaeA*	stx_2 + *eaeA*	stx_1 + stx_2 + *eaeA*	Total
Ovine	14	25	1	4			1	1	46
Bovine	75	7	11	4	9		1	3	110
Caprine	6	3					1		10
Porcine	10			4					14
Total									180

TABLE 3. Multiplex PCR data derived from 64 livestock fecal samples

Sample type	No. of samples positive for[a]:								Total
	No *hly*	*hly*	*hly* + stx_1	*hly* + stx_2	*hly* + stx_1 + stx_2	*hly* + *eaeA*	*hly* + stx_2 + *eaeA*	*hly* + stx_1 + stx_2 + *eaeA*	
Bovine	36	8	2	1	2	1		1	51
Other[b]	5	3	2			1	1	1	13
									64

[a] No samples were positive for *hly* plus stx_1 plus *eaeA*.
[b] Includes porcine, ovine, and caprine samples.

DNA hybridization was carried out on the overnight fecal culture which possessed stx_1 and EHEC *hlyA* (Fig. 3, lane 3). Four colonies from a total of 90 were identified as positive for both stx_1 and EHEC *hlyA* (data not shown); none of the four virulence factors were observed among the other 86 colonies from this sample.

The multiplex PCR described in this study is an effective means of detecting EHEC virulence factors in bovine, ovine, porcine, and caprine feces. Although a number of different primer sequences which amplify EHEC virulence factors have been described, including those used in this study (12–14), the combination of primers reported here was chosen to generate a high level of sensitivity (37 to 18 genome equivalents) and to facilitate clear resolution of each of the four amplification products by size on a single 2% agarose gel. A multiplex PCR which amplified the four known EHEC virulence factors in human feces was recently described; however, the sensitivity levels for each primer pair were not reported, and preparation of PCR template required the use of lysozyme, proteinase K, boiling, and ethanol precipitation, a procedure requiring at least 3 to 4 h and significant cost (23). Sensitivity in an EHEC assay is essential, as the infectious dose for humans may be as low as 1 to 10 CFU. A combination of overnight culture in EC (modified) broth (which dilutes PCR inhibitors and increases the number of target bacteria) and the use of a comparatively rapid and inexpensive template preparation methodology not reliant on PCR-inhibiting solvent methodologies (8) facilitated the development of a multiplex PCR that is sensitive (between 37 and 18 genome equivalents), rapid, and amenable to high

TABLE 4. Multiplex PCR data derived from 200 fecal samples from four sheep flocks and four cattle herds

Flock or herd	No. of samples positive for gene(s)[a]												
	None	*stx*$_1$	*eaeA*	*stx*$_1$ + *stx*$_2$	*stx*$_1$ + *eaeA*	*hly*	*hly* + *stx*$_1$	*hly* + *stx*$_2$	*hly* + *stx*$_1$ + *stx*$_2$	*hly* + *eaeA*	*hly* + *stx*$_1$ + *eaeA*	*hly* + *stx*$_1$ + *stx*$_2$ + *eaeA*	Total
Flock 1	9	8	1		4		1			1	1		25
Flock 2	9	5	1		1		7				2		25
Flock 3	6	5	5			3	2		1	1		2	25
Flock 4	10	13							2				25
Herd 1	12	1				9	1	2					25
Herd 2	19	1				5							25
Herd 3	14	1		3		3	2		2				25
Herd 4	2	1				14	5						25
													200

[a] No samples were positive for *stx*$_1$ plus *stx*$_2$ plus *eaeA*.

throughput sampling compared with similar PCR-based detection assays.

Multiplex PCR *stx*$_1$ and *eaeA* profiles for the six reference *E. coli* strains were in agreement for the *stx*$_1$ and *eaeA* marker results provided by the Victorian Infectious Diseases Reference Laboratory. The *stx*$_2$ gene from *E. coli* O91:H− was the only factor not detected. This is not surprising, as Shiga-like toxins are readily lost during subculture (16); however, it should be noted that *stx*$_1$ was detected in this particular strain. EHEC *hlyA* was detected in all strains except *E. coli* O91:H−; again, this result is not surprising, as the EHEC plasmid which carries the *hlyA* gene was not previously detected in this serotype (27). The gene encoding the attachment and effacement factor, *eaeA*, was detected only in *E. coli* O157 and O111; these strains are the most common EHEC strains cultured from patients with food-borne illness in Australia (11). With single-primer PCR, nonspecific amplification (labelled A and B in Fig. 2) appeared to be associated with amplification with *eaeA* and *stx*$_2$ primers sets respectively. By altering annealing conditions in the multiplex PCR, these amplification products could be avoided; however, the sensitivity of detection of EHEC virulence factors was reduced (results not shown).

The multiplex PCR identified 26 of 46 (56.5%) ovine fecal samples containing *stx*$_1$ gene sequences, compared to a comparatively equal distribution of *stx*$_1$ (7 of 110 [6.4%]), *stx*$_2$ (11 of 110 [10%]) and *stx*$_1$ plus *stx*$_2$ (9 of 110 [8.2%]) sequences in cattle feces. Similar finding were demonstrated in the samples collected from healthy sheep and cattle. A recent study (28) described the presence of *stx* sequences in feces of cattle, sheep and pigs in Queensland, Australia. This study identified 19 of 105 (18%), 70 of 101 (69%), and 27 of 129 (21%) bovine, ovine, and porcine fecal samples, respectively, to be positive for *stx* sequences, similar to the results presented in this study. Kudva et al. (18) reported that approximately 75% of isolates from a single sheep flock were *stx*$_1$, *stx*$_2$, and *eaeA* positive by colony hybridization while a further 22.9% of isolates were positive for only *stx*$_1$ and *eaeA*. This high prevalence of the *eaeA* and *stx*$_2$ was not observed among fecal samples collected from 46 sheep in our study, with only 2.2 and 8.7% of fecal samples being positive for *stx*$_2$ and *eaeA* sequences, respectively. Beutin et al. 1997 (7) reported that the vast majority of bovine isolates were positive for *stx*$_2$, all but one isolate were negative for *eaeA*, and only one isolate was positive for *stx*$_1$. While these results vary considerably from our data, it has been shown that patterns of shedding of Shiga-toxin-producing *E. coli* are affected by diet, age, stress, and seasonal variation (17, 18).

Although PCR can detect EHEC virulence factors with a relatively high degree of sensitivity amongst fecal *E. coli* strains, the detection of a gene does not indicate whether that factor is being expressed. Kudva et al. (18) showed that 9 of 11 *stx*-positive isolates were capable of expressing Shiga-like toxin(s) in Vero cultures, indicating the presence of the appropriate gene regulatory sequences. While combinations of the four EHEC virulence factors typically are present in most EHEC strains which have been recovered from symptomatic patients, a small proportion of *stx*-positive *E. coli* isolates do not possess *eaeA* or EHEC *hlyA* and are still able to cause hemolytic-uremic syndrome (20). Furthermore, it has been suggested that *stx*-positive *E. coli* strains which lack *eaeA* sequences may be less virulent for humans than *eaeA*-positive EHEC isolates, although it is not known if all Shiga toxin-producing strains are equally pathogenic in this regard (6). These observations suggest that other factors may enable a small proportion of *stx*-positive *E. coli* isolates to induce symptoms associated with typical EHEC isolates. Several studies have demonstrated that EHEC virulence factors are mobile within bacterial populations (2, 24, 31), and the assortment of genes between *E. coli* organisms may lead to pathogenic strains. A multiplex PCR approach is advantageous in rapidly detecting EHEC pathogenicity factors in the natural environment while the concomitant use of colony hybridization enables the identification of specific EHEC isolates. Future studies will focus on applying the multiplex PCR in conjunction with colony hybridization to livestock fecal samples collected from different geographic lo-

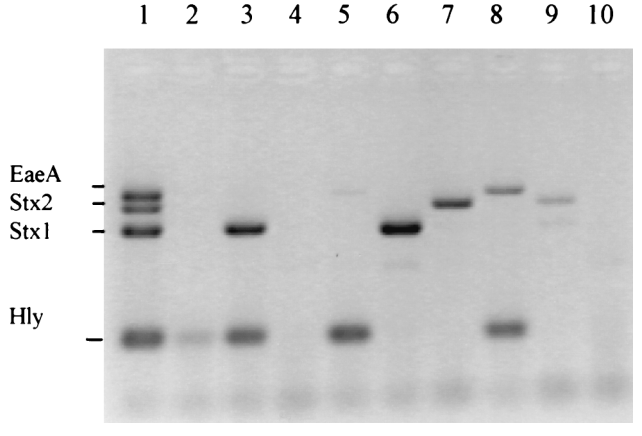

FIG. 3. Multiplex PCR analysis of bovine fecal samples. Lanes: 1, O111:H− positive control; 2 through 10, fecal samples collected on a farm previously identified as containing EHEC-positive animals.

cations within Australia and among herds and flocks utilizing different farm management practices.

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