Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

ELSEVIER

Veterinary Microbiology 66 (1999) 251–263

veterinary microbiology

Characterization of *eae*$^+$ *Escherichia coli* isolated from healthy and diarrheic calves

Robert E. Holland[a,*], Richard A. Wilson[d], Margo S. Holland[b], Vilma Yuzbasiyan-Gurkan[c], Thomas P. Mullaney[b], David G. White[e]

[a]*Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA*
[b]*Department of Pathology, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA*
[c]*Department of Microbiology, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA*
[d]*The Escherichia coli Reference Center, The Pennsylvania State University, University Park, PA 16802, USA*
[e]*Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND 58105, USA*

Received 9 June 1998; accepted 21 January 1999

Abstract

Strains of *Escherichia coli* from 101 healthy and 114 diarrheic calves were screened by PCR for the *eae* (intimin) gene and Shiga toxin genes (*stx*). Each *eae*$^+$ and *eae*/*stx*$^+$ strain was examined for antimicrobial susceptibility, enterohemolysin activity, and the somatic O antigen was determined. An immunoassay was used to detect Shiga toxin antigens for the *eae*/*stx*$^+$ *E. coli*. Significantly more ($p = 0.005$) of the healthy calves carried *eae*$^+$ and *eae*/*stx*$^+$ *E. coli* in their feces when compared to strains from diarrheic calves. Moreover, Shiga toxin antigens were detected significantly more ($p = 0.001$) often among the *eae*/*stx*$^+$ strains from healthy calves when compared to *eae*/*stx*$^+$ strains from diarrheic calves. However, significantly more ($p = 0.001$) of the *eae*$^+$ and *eae*/*stx*$^+$ strains from diarrheic calves were resistant to at least one of the antimicrobials tested, and the strains from diarrheic calves had a significantly ($p = 0.05$) higher rate of antimicrobial resistance to at least two different antimicrobial classes. No significant difference ($p \geq 0.05$) was detected among the *eae*$^+$ and *eae*/*stx*$^+$ strains from healthy and diarrheic calves for enterohemolysin production. Serogroups O-negative, O5, O26, and O111 were predominate among both healthy and diarrheic calves.

Keywords: Cattle-Bacteria; *Escherichia coli*; Virulence; Pathogenicity

* Corresponding author. Tel.: +1-517-432-6323; fax: +1-517-3361042; e-mail: rholland@cvm.msu.edu

0378-1135/99/$ – see front matter
PII: S0378-1135(99)00013-9

1. Introduction

Calf strains of *Escherichia coli* possessing the *eae* gene and having the capacity to induce the attaching and effacing lesion are designated as attaching and effacing *E. coli* (AEEC) (Moon et al., 1983). Intimin, a 94 kDa outer membrane protein encoded by the *eae* gene, mediates adherence of AEEC to intestinal epithelial cells; an event critical for the formation of the attaching and effacing lesion (Jerse and Kaper, 1991; Donnenberg and Kaper, 1992). Thus, the pathophysiologic mechanism by which AEEC cause the attaching and effacing lesion in the intestinal tract of calves may follow the three-stage model proposed for human strains of enteropathogenic *E. coli* (EPEC) (Donnenberg and Kaper, 1992; Donnenberg et al., 1993a, b). The three-stage model for the attaching and effacing lesion consists of localized adherence, signal transduction, and intimate adherence. There is an initial nonintimate attachment of the bacteria to intestinal epithelial cells leading to effacement of the underlying microvilli, followed by intimate attachment, and then the accumulation of cytoskeletal proteins beneath the areas of bacterial attachment.

Based on expression of intimin, Shiga toxins, and the ability to elicit the attaching and effacing lesion, some bovine AEEC strains are equivalent to human enterohemorrhagic *E. coli* (EHEC) (Levine, 1987; Knutton, 1994). Thus, two classes of bovine AEEC have been described (Mainil et al., 1993). One class, in addition to carrying the *eae* gene, carry *stx* genes; and the second class do not carry *stx* genes. In calves, the *eae* gene and intimin have a defined role in causing the attaching and effacing lesion, whereas Shiga toxins do not appear to have a role in causing the attaching and effacing lesion (Tzipori et al., 1987; Hall et al., 1990). Further, the importance of Shiga toxins in calf diarrhea and systemic disease is not well defined. In humans, Shiga toxins are an essential virulence attribute of EHEC associated with severe systemic disease such as hemolytic uremic syndrome (Karmali, 1989; Tesh and O'Brien, 1991). In pigs and rabbits, Shiga toxins have a role in inducing vascular lesions and have a direct effect on the intestinal epithelium (Gyles, 1994; Nataro and Kaper, 1998).

Numerous studies have documented the importance of AEEC in calf diarrhea (Chanter et al., 1984, 1986; Sherwood et al., 1985; Hall et al., 1985, 1990; Moxley and Francis, 1986; Mohammad et al., 1985, 1986; Pospischil et al., 1987; Blanco et al., 1988, 1993; Schoonderwoerd et al., 1988; Wray et al., 1989; Janke et al., 1990; Synge and Hopkins, 1992; Dorn et al., 1993; Mainil et al., 1993; Knutton, 1994; Fisher et al., 1994; Wieler et al., 1996; Saridakis et al., 1997). Few reports have examined the occurrence of AEEC infections concurrently in both healthy and diarrheic calves (Mohammad et al., 1985; Wieler et al., 1992; Blanco et al., 1993, 1994). The objectives in this research were to determine the presence of certain virulence properties associated with eae^{+} *E. coli* isolated from healthy and diarrheic calves and to assess the role of AEEC in calf diarrheal disease.

2. Materials and methods

2.1. Calves

Fecal specimens were obtained from 215 Holstein calves after digital stimulation of the rectal mucosa. At the time of fecal collection, 114 calves had diarrhea as determined by

visual assessment of the specimen and the calf. On each farm, the diarrheic calves were matched to an approximate number of healthy calves. Thus, fecal specimens were collected from 101 calves that did not have diarrhea and appeared healthy. Depending on the number of diarrheic calves sampled, 10 to 18 calves (both diarrheic and healthy) were sampled on six farms, and 19 to 24 calves were sampled on six farms. The 12 dairy farms were in Michigan.

Feces were collected into sterile cups, packed on ice packs in chests, and were transported to the laboratory. Upon arrival to the laboratory, feces were immediately cultured. At the time of fecal collection, historical and clinical examination data were recorded on each calf.

2.2. Cultures

For primary culture, 1.0 g of feces was homogenized in 9.0 ml of peptone-saline solution. An inoculating loop was used to transfer 10 μl of the fecal/peptone-saline suspension to MacConkey (MAC) agar plates, and the plates were streaked for isolation of lactose fermenting colonies (LFC). The MAC plates were incubated aerobically at 37°C for 12 to 16 h. Ten LFC that exhibited *E. coli* growth characteristics were randomly harvested with sterile toothpicks from the last streaking area of each MAC plate. Total bacterial DNA was extracted and then prepared for PCR analysis. Each eae^+ and eae/stx^+ colony from each calf was verified as *E. coli* by the API 20E system (bioMerieux Vitek, Hazelwood, MO).

2.3. PCR analysis

Ten individual LFC from each calf were suspended in individual 500 μl microcentrifuge tubes (Elkay Products, Shrewsbury, MA) containing 200 μl of lysis buffer (1.0% Triton X-100, 20 mM Tris–HCl, 2.0 mM EDTA, pH 8.5), mixed by vortexing for 30 s, heated at 100°C for 3 min, then centrifuged at 12,000 × *g* for 3 min. Amplification of bacterial DNA was performed with 5 μl of bacterial DNA in 45 μl of PCR reaction mixture. The PCR reaction mixture contained 3 μl of double distilled water, 5 μl of 10 × PCR buffer, 2 mM $MgCl_2$, 10 mM dNTP mix, 0.8 μM of each primer, and 1.5 U of Taq DNA polymerase (Gibco BRL, Life Technologies, Inc., Grand Island, NY).

Prior to testing each LFC for the *eae* gene, three sets of published oligonucleotide sequences (Gannon et al., 1993; Donnenberg and Kaper, 1991; Karch et al., 1993) were evaluated for detection of *eae* sequences among well characterized strains of *E. coli* that have been determined to possess various *eae* allelic sequences or lacked the *eae* gene (Table 1). These strains were of multiple serotypes and were isolated from human and animal sources. Based on this study, the AE 13–AE 14 oligonucleotide sequences {(5′GTG GCG AAT ACT GGC GAG ACT 3′) and (5′CCC CAT TCT TTT TCA CCG TCG 3′)} were chosen to amplify the *eae* gene (Gannon et al., 1993). The size of the amplified DNA product was 890 bp. The amplification method was 94°C for 1 min for denaturing, 57°C for 1 min for annealing, and 72°C for 1 min for extension for 30 cycles.

For amplification of *stx* genes, the MK1–MK2 oligonucleotide primers {(5′TTT ACG ATA GAC TTC TCG AC 3′) and (5′CAC ATA TAA ATT ATT TCG CTC 3′)} were used

Table 1
Attributes of the *E. coli* strains used to validate the PCR and Premier EHEC assays

Strain ID	Serotype	*eae*	*stx*	Source
ECRC 933[a]	0157:H7	+	+	adult/human
MIEC[b]	0157:H7	+	+	human
C1520–77[a]	0157:H7	+	+	calf
AI2198–77[a]	0111:H$^-$	+	+	infant/human
EL2966–56[a]	0111:H2	+	−	adult/human
EF1–50[a]	0111:H2	+	−	adult/human
EE3782–62[a]	055:H6	+	−	infant/human
EQ5624–50[a]	055:H7	+	−	infant/human
807–13[b]	0149:NM	−	−	foal
6131[b]	05:NM	+	+	calf

[a] Obtained from the *E. coli* Reference Center, Pennsylvania State University.
[b] Positive control strains (MIEC and 6131) and negative control (807-13) isolated from identified sources in Michigan.

(Karch and Meyer, 1989; Schmidt et al., 1993). The MK1–MK2 primer pair was demonstrated to amplify a 230 bp fragment for both *stx*1 and *stx*2 loci (Karch and Meyer, 1989). The thermocycling program was 94°C for 1 min for denaturation, 43°C for 1 min for annealing, and 72°C for 1 min for extension for 30 cycles.

All *eae* and *stx* amplifications were performed in a programmable thermal controller (PTC-100, MJ Research, Inc., Watertown, MA). 16 μl of amplified products were analyzed by gel electrophoresis in 1.5% agarose. Products were visualized by ethidium bromide staining followed by UV transillumination. A 100 bp DNA ladder (GibcoBRL, Grand Island, NY) was used as the molecular weight marker. For each PCR run, DNA from EHEC O157:H7 and a calf strain of AEEC (serotype 05:NM) were used as positive controls, and DNA from a strain of *E. coli* and *Salmonella dublin* that lacked *eae* genes were negative controls. The control strains had been previously evaluated for *eae* and *stx* genes by colony blot hybridization and PCR.

2.4. Enterohemolysin activity

Each *eae*$^+$ and *eae*/*stx*$^+$ *E. coli* was assessed for enterohemolysin activity on 5% defibrinated washed sheep blood agar plates as described (Beutin et al., 1988). Colonies surrounded by clear zones of hemolysis were defined as exhibiting enterohemolysin activity.

2.5. Serotyping

Serotyping was performed by standard methods at The *E. coli* Reference Center, The Pennsylvania State University (Wilson and Francis, 1986). *E. coli* strains were grown overnight in tryptic soy broth, then heated to 100°C for 2 h. Agglutinations were performed by using preselected dilutions of each of 183 O group antisera in microtitre plates and the positive reactions were confirmed by microtitre titrations against monovalent antisera (Glantz, 1971). If there was no agglutination in the preliminary

screening assay, the bacterial suspension was reheated at 121°C for an additional 1 h and the microtitre agglutination assays were repeated.

2.6. Antimicrobial susceptibility testing

Susceptibility to different classes of antimicrobial agents was performed using standard disk diffusion methods in Mueller-Hinton agar (Bauer et al., 1966). Results were interpreted according to (National Committee for Clinical Laboratory Standards, 1997) guidelines. The disks containing the following amounts of antibiotics were used: amikacin 30 μg, ampicillin 10 μg, ceftiofur 30 μg, cephalothin 30 μg, chloramphenicol 30 μg, enrofloxacin 5 μg, gentamicin 10 μg, tetracycline 30 μg, trimethoprim/sulfamethoxazole 1.25/23.75 μg, and nalidixic acid 30 μg.

2.7. Premier EHEC assay

To demonstrate Shiga toxin antigens, the Premier EHEC assay (Meridian Diagnostics Inc., Cincinnati, OH) was performed on *eae*/*stx*$^+$ *E. coli* strains known to produce Shiga toxins (Table 1). As additional controls, 10 *eae*$^+$ only and 10 *stx*$^+$ only strains were examined by the enzyme immunoassay. After 12 h growth at 37°C on MAC plates, a single colony of each strain was suspended in 200 μl of sample diluent for testing. The immunoassay was performed according to the manufacturer's recommendation. The reaction mixture was read spectrophotometrically at 450 nm.

2.8. Postmortem and microbiologic examinations

Postmortem and microbiologic examinations were performed on 45 of the 114 diarrheic calves. Postmortem examinations were performed by attending veterinary pathologists. Tissue segments from the duodenum, jejunum, ileum, cecum, spiral colon, transverse colon, descending colon, and rectum were immediately fixed in neutral buffered 10% formalin. After 24 h in the fixative, tissues were trimmed, routinely processed, and embedded in paraffin. Thin sections were cut, mounted on slides, and stained with H & E. Slides were examined by light microscopy.

Intestinal contents or feces from each necropsied calf were examined for *Cryptosporidium parvum*, enterotoxigenic *E. coli*, *Salmonella* sp., enteric viruses, and BVDV by routine procedures. Briefly, *C. parvum* oocysts were detected by light microscopic examination of concentrated oocysts (Garcia et al., 1983). 1 g of intestinal contents was dispensed in 9.0 ml of peptone-saline and 10 μl was plated onto MacConkey and brilliant green agar plates. Selenite enrichment broth was inoculated with 1.0 ml of the fecal/peptone-saline suspension and was subcultured onto brilliant green agar for detection of *Salmonella* sp. Suspect colonies were confirmed as *Salmonella* after differential biochemical and serological testing. Strains of *E. coli* were screened for heat-stable enterotoxin type A by PCR using published oligonucleotide sequences (Woodward et al., 1992). The K99 fimbrial antigen was detected by agglutination in K99 monospecific antibody after enhancement on Minca Isovitalex agar. The presence of enteric viruses was detected by electron microscopy and group A rotavirus antigen was

detected by ELISA (Pathfinder Rotavirus, Kallestad, Chaska, MN). Bovine viral diarrhea virus was identified in lysed mononuclear cells or sera by the immunoperoxidase monolayer assay (Houe et al., 1995).

2.9. Statistical analysis

To determine significance of the *E. coli* properties between healthy and diarrheic calves, the chi-square test of independence was used to evaluate the association between illness and the characteristics of the *E. coli* strains (Snedecor and Cochran, 1989).

3. Results

3.1. Historical data

Based on interviews with calf caretakers and review of individual calf records (when available), 4 of the 101 healthy calves and 32 of the 114 diarrheic calves had received antimicrobial therapy. Twenty of these 32 calves had received antimicrobial therapy for their diarrheal disease and 12 calves had been treated for medical problems unrelated to and prior to the onset of diarrhea. Eight of the 23 diarrheic calves that carried *eae*$^+$ or *eae/stx*$^+$ *E. coli* had received antimicrobial therapy. Gentamicin sulfate, ceftiofur sodium, oxytetracycline (LA 200), and procaine penicillin were the antimicrobials most often used.

The mean age of the 101 healthy calves was 22.0 ± 15.3 days (range 1 to 90 days) and the mean age of the 40 healthy calves infected with *eae*$^+$ *E. coli* was 25.5 ± 14.6 days (range 6 to 79 days). The mean age of the 114 diarrheic calves was 14.4 ± 11.6 days (range 1 to 83 days), whereas, the mean age of the 23 diarrheic calves infected with *eae*$^+$ *E. coli* was 24.3 ± 19.0 days (range 3 to 83 days).

3.2. Preliminary PCR and Premier EHEC assays

In preliminary experiments, correlation between previously determined *eae* and *stx* profiles for select strains (Table 1) and our PCR results was 100%. Based on these results, the selected primers and the PCR assays were considered definitive for detection of *E. coli eae* and *stx* genes.

In this study, the Premier EHEC assay was used to detect Shiga toxin antigens associated with *eae/stx*$^+$ strains. The assay accurately detected Shiga toxin antigens for the *eae/stx*$^+$ strains (Table 1), no false positives of *eae*$^+$ only strains were detected, and 9 of 10 *stx*$^+$ only strains were detected. Under the conditions used in this study, the Premier EHEC assay was considered a sensitive assay to detect Shiga toxin antigens.

3.3. eae, eae/stx, and Shiga toxins

Overall, 63 of the 215 (29.3%) calves carried *eae*$^+$ *E. coli* in their feces. Forty of the 101 (40.0%) healthy calves and 23 of the 114 diarrheic calves (20.2%) carried *eae*$^+$

Table 2
Enterohemolysin, antimicrobial resistance and Shiga-toxin antigens associated with *eae*$^+$ and *eae/stx*$^+$ *E. coli* isolated from calves

	Healthy calves $N = 40$			Diarrheic calves $N = 23$		
	eae$^+$	*eae/stx*$^+$	Total (%)	*eae*$^+$	*eae/stx*$^+$	Total (%)
	20	20	40	12	11	23
Enterohemolysin	10	10	20 (50.0)	8	4	12 (52.2)
Single antimicrobial resistance	4	7	11 (27.5)	3	5	8 (34.8)
Multiple antimicrobial resistance[a]	1	8	9 (22.5)	6	6	12 (52.2)
Shiga-toxin antigens	0	20	20(100.0)	0	8	8 (73.0)

[a] Displaying resistance to at least two separate antimicrobial classes.

E. coli in their feces. Infection with *eae*$^+$ *E. coli* was significantly higher ($p = 0.001$) in the healthy calves when compared to the diarrheic calves.

The *stx* gene was identified in approximately equal percentages of *eae*$^+$ *E. coli* in healthy and diarrheic calves (Table 2). Among the 40 *eae*$^+$ strains from healthy calves, 20 (50.0%) possessed *stx* genes, whereas, among the 23 *eae*$^+$ strains from diarrheic calves, 11 (48.0%) possessed the *stx* gene. When examined in the immunoassay, Shiga toxin antigens were detected for all 20 *eae/stx*$^+$ (100%) strains from healthy calves and in 8 of 11 (73.0%) strains from diarrheic calves.

3.4. Enterohemolysin activity

The enterohemolytic phenotype was expressed by 50.0% of the *eae*$^+$ and *eae/stx*$^+$ strains from healthy calves and by 52.0% of the strains from diarrheic calves. Enterohemolysin activity was not restricted to one particular serogroup and was detected in both *eae*$^+$ and *eae/stx*$^+$ strains (Table 2).

3.5. Serogroups

Distribution of *eae*$^+$ and *eae/stx*$^+$ *E. coli* among serogroups is shown in Table 3. Forty six of the 63 (73.0%) *eae*$^+$ and *eae/stx*$^+$ *E. coli* strains belonged to 17 different typable serogroups and 17 (27.0%) *eae*$^+$ *E. coli* and *eae/stx*$^+$ strains were O-negative. Serogroups O5, O8, O26, O50, and O111 were detected in both healthy and diarrheic calves. However, strains belonging to serogroups O5, O26 and O111 accounted for 45.0% and 39.0% of the *eae*$^+$ and *eae/stx*$^+$ *E. coli* from healthy and diarrheic calves, respectively. The O-negative strains accounted for 22.5% and 35.0% of the strains from healthy and diarrheic calves, respectively.

Shiga toxin antigens were detected among *E. coli* belonging to serogroups 05, O26, O84/172, O98, O103, O111, O157 and O171 from healthy calves, and from serogroups 05, 055, 069, and 0111 from diarrheic calves.

Table 3
Distribution of *eae*$^+$ and *eae/stx*$^+$ *E. coli* among serogroups and relationship to the attaching and effacing lesion

Serogroup	Healthy calves		Diarrheic calves		
	eae$^+$	*eae/stx*$^+$	*eae*$^+$	*eae/stx*$^+$	A/E lesion
0	9	0	7	1	5
05	2	8	0	5	2
06	0	0	1	0	0
08	1	0	0	1	0
010	2	0	0	0	0
026	2	3	2	0	0
050	1	0	1	0	0
055	0	0	0	1	1
069	0	0	0	1	1
080	0	0	1	0	1
084, 172	0	1	0	0	0
098	0	2	0	0	0
0103	1	1	0	0	0
0111	0	3	0	2	0
0118	1	0	0	0	0
0156	1	0	0	0	0
0157	0	1	0	0	0
0171	0	1	0	0	0
Total	20	20	12	11	10

3.6. Antimicrobial susceptibility

All strains were sensitive to amikacin, enrofloxacin, and nalidixic acid (Table 4). Significantly more of the *eae*$^+$ and *eae/stx*$^+$ strains from diarrheic calves, when compared to strains from healthy calves, were resistant to at least one of the antimicrobials tested. Moreover, significantly more of the strains from diarrheic calves had multiple antibiotic resistance patterns. Four *eae/stx*$^+$ strains recovered from diarrheic calves were resistant to chloramphenicol. Antimicrobial resistance was not restricted to any one serogroup, nor was resistance associated with enterohemolysin production. Most resistance patterns included resistance to tetracycline, ampicillin, and gentamicin (Table 4).

3.7. Postmortem examination data and concurrent infections

Among the 45 calves that had postmortem examinations, 15 (33.3%) had *eae*$^+$ or *eae/stx*$^+$ *E. coli* in their feces. In five calves infected with *eae*$^+$ or *eae/stx*$^+$ *E. coli*, and in the tissue sections examined did not have histopathologic evidence of the attaching and effacing lesion, *C. parvum*, coronavirus, and rotavirus were identified in one calf each. *C. parvum* and coronavirus were identified in one additional calf. Histopathologic lesions consistent with the attaching and effacing phenotype (Moon et al., 1983; Hall et al., 1985, 1990; Chanter et al., 1984, 1986; Moxley and Francis, 1986; Pospischil et al., 1987; Janke et al., 1990) were detected in 10 calves. The lesion was detected in the colon only of six calves and in the ileum and colon of four calves. Among these 10 calves, *C. parvum* was

Table 4
Antimicrobial resistance pattern of *eae*$^+$ and *eae/stx*$^+$ *E. coli* from calves

Antimicrobial	Percent resistant strains			
	Healthy ($N = 40$)		Diarrheic ($N = 23$)	
	eae$^+$	*eae/stx*$^+$	*eae*$^+$	*eae/stx*$^+$
Amikacin	S	S	S	S
Ampicillin	5.0	12.5	26.0	17.4
Ceftiofur	S	S	4.3	13.0
Cephalothin	2.5	2.5	13.0	22.0
Chloramphenicol	S	S	S	17.4
Enrofloxacin	S	S	S	S
Gentamicin	7.5	10.0	4.3	30.4
Tetracycline	17.5	20.0	26.0	35.0
Trimethoprim/sulfamethoxazole	S	S	13.0	17.4
Nalidixic acid	S	S	S	S

S = Sensitive to the antimicrobial listed.

detected in five calves, *Salmonella typhimurium* in one calf, and BVDV and *C. parvum* were detected in one calf. The pathogens screened for by our assays were not detected in feces from 3 of the 10 calves.

4. Discussion

In this study, significantly more of the healthy calves (40.0%) carried *eae*$^+$ and *eae/stx*$^+$ *E. coli* in their feces when compared to diarrheic calves (20.0%). However, significantly more *eae*$^+$ and *eae/stx*$^+$ *E. coli* that exhibited single and multiple antimicrobial resistances were isolated from diarrheic calves. Selection for resistance among the strains from diarrheic calves may have been due to a greater use of antimicrobials among the diarrheic calves. Based on limited observations made with 10 calves on one farm, a relation between antimicrobial use and selection for resistance was observed. Three of 10 calves developed diarrhea after being treated for respiratory disease. All three calves were subsequently positive for *eae*$^+$ (one calf) and *eae/stx*$^+$ *E. coli* (two calves). Two of the three strains were resistant to the antimicrobial used to treat the respiratory disease. Examination of *E. coli* from the seven healthy calves did not reveal any *eae*$^+$, *eae/stx*$^+$ *E. coli* or commensal *E. coli* strains that were resistant to that antimicrobial. In this case, exposure to the particular antimicrobial appeared to select for resistance. However, environmental sources of the resistant strains could not be excluded (Linton, 1986).

Our data show that the *stx* genes were equally distributed among *eae*$^+$ *E. coli* from both healthy (50.0%) and diarrheic calves (48.0%). Furthermore, Shiga toxin antigens were detected in more strains from healthy calves (100.0%) than in strains from diarrheic calves (73.0%). Although the immunoassay used detects Shiga toxin antigens, the data are consistent with that of others who have demonstrated the prevalence of Shiga toxin producing *E. coli* in the feces of young calves (Mainil et al., 1993; Blanco et al., 1988, 1993; Butler and Clarke, 1994; Dorn et al., 1993; and Wieler et al., 1992).

Enterohemolysin, a distinctively different hemolysin from α-hemolysin, is synthesized by calf strains of *E. coli* that produce Shiga toxins (Beutin et al., 1986, 1988, 1989). Although the influence of enterohemolysin on calf intestinal disease has not been defined, it has been suggested that enterohemolysins may compliment the effects of Shiga toxins (Nataro and Kaper, 1998). Furthermore, screening for enterohemolysin has been proposed as a diagnostic marker for Shiga toxin producing *E. coli* (Beutin et al., 1989; Wieler et al., 1995), since its presence is strongly correlated with Shiga toxins. Among bovine Shiga toxin producing *E. coli*, enterohemolysin activity has been reported to range from 57.6% to 70.8% (Beutin et al., 1989; Wieler et al., 1992). Our data show that 50.0% and 52.0% of the *eae*$^+$ and *eae/stx*$^+$ strains from healthy and diarrheic calves, respectively, had enterohemolysin activity.

Strains of AEEC belonging to the same serogroups were often isolated from healthy and diarrheic calves. Strains belonging to serogroups O5, O26, and O111 accounted for 43.0% of all the typable strains, and for 45.0% and 39.0% of the typable strains from healthy and diarrheic calves, respectively. Characterization of AEEC by O group only is less powerful than O:H typing since strains of the same O group but possessing different H antigens may have different virulence properties. Nevertheless, determination of specific virulence attributes and the association with specific O groups provide valuable epidemiologic information (Wieler et al., 1992). Attaching and effacing *E. coli* belonging to serogroups O5, O26, and O111 are widely distributed among calves and have been demonstrated in natural outbreaks and experimentally (with some strains) to cause diarrhea and dysentery (Chanter et al., 1984, 1986; Hall et al., 1985, 1990; Schoonderwoerd et al., 1988; Wray et al., 1989; Moxley and Francis, 1986; Mainil et al., 1993; Janke et al., 1990; Knutton, 1994; Butler and Clarke, 1994; Dorn et al., 1993). As calf AEEC have a similar pathophysiologic mechanism to human EPEC and EHEC, strains carrying both *eae* and *stx* genes, and belonging to serogroups O negative, O5, O8, O26, O55, O69, O84/172, O98, O103, O111, O118, O157 and 0171 would be of concern to human health.

The age at which calves are most susceptible to colonization by *eae*$^+$ *E. coli* and the age at which they are most likely to develop attaching and effacing lesions are not known. Based on the distribution of the *eae* gene among calves of various ages, it appears that an age-associated resistance does not occur during the first week of life as is recognized with ETEC. Furthermore, the mean ages of the healthy (25.5 days) and diarrheic (24.3 days) calves infected with *eae*$^+$ *E. coli* would support the notion that calves older than 21 days are more susceptible to infection with *eae*$^+$ *E. coli*. However, this does not mean that the infected calves would develop attaching and effacing lesions. The age of calves infected with *eae*$^+$ *E. coli* and those that had demonstrable attaching and effacing lesions ranged from 8 to 83 days. In natural infections, calves as young as 2 days and as old as 4 months have been described with attaching and effacing lesions (Janke et al., 1990).

Acknowledgements

Financial support from The Agricultural Experiment Station, National Food Safety and Toxicology Center, and The College of Veterinary Medicine, Michigan State University.

References

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45, 493–496.

Beutin, L., Montenegro, M.A., Ørskov, I., Ørskov, F., Prada, J., Zimmermann, S., Stephan, R., 1989. Close association of verotoxin (shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. J. Clin. Microbiol. 27, 2559–2564.

Beutin, L., Montenegro, M., Zimmermann, S., Stephan, R., 1986. Characterization of hemolytic strains of *Escherichia coli* belonging to classical enteropathogenic O-serogroups. Zbl. Bakt. Hyg. A 261, 266–279.

Beutin, L., Prada, J., Zimmermann, S., Stephan, R., Orskov, I., Orskov, F., 1988. Entero-hemolysin, a new type of hemolysin produced by some strains of enteropathogenic *E. coli* (EPEC). Zbl. Bakt. Hyg. A 267, 576–588.

Blanco, M., Blanco, J., Blanco, J.E., Gonzalez, E.A., Gomes, T.A.T., Zerbini, L.F., Yano, T., Pestana de Castro, A.F., 1994. Genes coding for Shiga-like toxins in bovine verotoxin-producing *Escherichia coli* (VTEC) strains belonging to different O : K : H serotypes. Vet. Microbiol. 42, 105–110.

Blanco, J., Gonzalez, E.A., Garcia, S., Blanco, M., Regueiro, B., Bernardez, I., 1988. Production of toxins by *Escherichia coli* strains isolated from calves with diarrhoea in Galicia (North-western Spain). Vet. Microbiol. 18, 297–311.

Blanco, M., Blanco, J., Blanco, J.E., Ramos, J., 1993. Enterotoxigenic, verotoxigenic, and necrotoxigenic *Escherichia coli* isolated from cattle in Spain. Am. J. Vet. Res. 54, 1446–1451.

Butler, D.G., Clarke, R.C., 1994. Diarrhoea and dysentery in calves. In: Gyles C.L. (Ed.), *Escherichia coli* in Domestic Animals and Humans. CAB International, Wallingford, UK, pp. 91–116.

Chanter, N., Morgan, J.H., Bridger, J.C., Hall, G.A., Reynolds, D.J., 1984. Dysentery in gnotobiotic calves caused by atypical *Escherichia coli*. Vet. Record 114, 71.

Chanter, N., Hall, G.A., Bland, A.P., Hayle, A.J., Parsons, K.R., 1986. Dysentery in calves caused by an atypical strain of *Escherichia coli* (S102-9). Vet. Microbiol. 12, 241–253.

Donnenberg, M.S., Kaper, J.B., 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. 59, 4310–4317.

Donnenberg, M.S., Kaper, J.B., 1992. Enteropathogenic *Escherichia coli*. Infect. Immun. 60, 3953–3961.

Donnenberg, M.S., Tacket, C.O., James, S.P., Losonsky, G., Nataro, J.P., Wasserman, S.S., Kaper, J.B., Levine, M.M., 1993a. Role of the *eaeA* gene in experimental enteropathogenic *Escherichia coli* infection. J. Clin. Invest. 92, 1412–1417.

Donnenberg, M.S., Yu, J., Kaper, J.B., 1993b. A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. J. Bacteriol. 175, 4670–4680.

Dorn, R.C., Francis, D.H., Angrick, E.J., Willgohs, J.A., Wilson, R.A., Collins, J.E., Jenke, B.H., Shawd, S.J., 1993. Characteristics of verocytoxin producing *Escherichia coli* associated with intestinal colonization and diarrhea in calves. Vet. Microbiol. 36, 149–159.

Fisher, J., Maddox, C., Moxley, R., Kinden, D., Miller, M., 1994. Pathogenicity of a bovine attaching effacing *Escherichia coli* isolate lacking shiga-like toxins. Am. J. Vet. Res. 55, 991–999.

Garcia, L.S., Bruckner, D.A., Brewer, T.C., Shimizu, R.Y., 1983. Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens. J. Clin. Microbiol. 25, 119–121.

Gannon, V.P.J., Rashed, M., King, R.K., Thomas, E.J.G., 1993. Detection and characterization of the *eae* gene of shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. J. Clin. Microbiol. 31, 1268–1274.

Glantz, P.J., 1971. Serotypes of *Escherichia coli* associated with colibacillosis in neonatal animals. Ann. N.Y. Acad. Sci. 176, 67–79.

Gyles, C.L. (Ed.), 1994. *Escherichia coli* verotoxins and other cytotoxins. *Escherichia coli* in Domestic Animals and Humans. CAB International, Wallingford, UK, pp. 365–398.

Hall, G.A., Reynolds, D.J., Chanter, N., Morgan, J.H., Parsons, K.R., Debney, T.G., Bland, A.P., Bridger, J.C., 1985. Dysentery caused by *Escherichia coli* (S102-9) in calves: natural and experimental disease. Vet. Pathol. 22, 156–163.

Hall, G.H., Dorn, C.R., Chanter, N., Scotland, S.M., Smith, H.R., Rowe, B., 1990. Attaching and effacing lesions in vivo and adhesion to tissue culture cells of vero-cytoxin-producing *Escherichia coli* belonging to serogroup O5 and O103. J. Gen. Microbiol. 136, 779–786.

Houe, H., Baker, J.C., Maes, R.K., Wuryastuti, H., Wasito, R., Ruegg, P.L., Lloyd, J.W., 1995. Prevalence of cattle persistently infected with bovine viral diarrhea virus in 20 dairy herds in two counties in central Michigan and comparison of prevalence of antibody-positive cattle among herds with different infection and vaccination status. J. Vet. Diagn. Invest. 7, 321–326.

Janke, B.H., Francis, D.H., Collins, J.E., Libal, M.C., Zeman, D.H., Johnson, D.D., Neiger, R.D., 1990. Attaching and effacing *Escherichia coli* infection as a cause of diarrhea in young calves. J. Am. Vet. Med. Assoc. 196, 897–901.

Jerse, A.E., Kaper, J.B., 1991. The *eae* gene of enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid. Infect. Immun. 59, 4302–4309.

Karch, H., Bohm, H., Schmidt, H., Gunzer, F., Aleksic, S., Heesemann, J., 1993. Clonal structure and pathogenicity of shiga-like toxin-producing, sorbitol-fermenting *Escherichia coli* O157:H. J. Clin. Microbiol. 31, 1200–1205.

Karch, H., Meyer, T., 1989. Single primer pair for amplifying segments of distinct shiga-like toxin genes by polymerase chain reaction. J. Clin. Microbiol. 27, 2751–2757.

Karmali, M.A., 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. 2, 15–38.

Knutton, S., 1994. Attaching and effacing *Escherichia coli*. In: Gyles C.L. (Ed.), *Escherichia coli* in Domestic Animals and Humans. CAB International, Wallingford, UK, pp. 567–591.

Levine, M.M., 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent. J. Infect. Dis. 155, 377–389.

Linton, A.H., 1986. Flow of resistance genes in the environment and from animals to man. J. Antimicrob. Chem. 18, 189–197.

Mainil, J.Q., Jacquemin, E.R., Kaeckenbeeck, A.E., Pohl, P.H., 1993. Association between the effacing (*eae*) gene and the shiga-like toxin-encoding genes in *Escherichia coli* isolates from cattle. Am. J. Vet. Res. 54, 1064–1068.

Mohammad, A., Peiris, J.S.M., Wijewanta, E.A., Mahalingam, S., Gunasekara, G., 1985. Role of verocytogenic *Escherichia coli* in cattle and buffalo calf diarrhoea. FEMS Microbiol. Lett. 26, 281–283.

Mohammad, A., Peiris, J.S.M., Wijewanta, E.A., 1986. Serotypes of verocytotoxigenic *Escherichia coli* isolated from cattle and buffalo calf diarrhoea. FEMS Microbiol. Lett. 35, 261–265.

Moon, H.W., Whipp, S.C., Argenzio, R.A., Levine, M.M., Giannella, R.A., 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. 41, 1340–1351.

Moxley, R.A., Francis, D.H., 1986. Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect. Immun. 53, 339–346.

Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11, 142–201.

National Committee for Clinical Laboratory Standards, 1997. Performance standards for antimicrobial disk susceptibility tests. Approved Standard M2-A4. National Committee for Clinical Laboratory Standards, Villanova, PA.

Pospischil, A., Mainil, J.G., Baljer, G., Moon, H.W., 1987. Attaching and effacing bacteria in the intestine of calves with diarrhea. Vet. Pathol. 24, 330–334.

Saridakis, H.O., El Gared, S.A., Vidotto, M.C., Guth, B.E.C., 1997. Virulence properties of *Escherichia coli* strains belonging to enteropathogenic (EPEC) serogroups isolated from calves with diarrhea. Vet. Microbiol. 54, 145–153.

Schmidt, H., Montag, M., Bockemuhl, J., Heesemann, J., Karch, H., 1993. Shiga-like toxin II-related cytotoxins in *Citrobacter freundii* strains from humans and beef samples. Infect. Immun. 61, 534–543.

Schoonderwoerd, M., Clarke, R.C., van Dreumel, A.A., Rawluk, S.A., 1988. Colitis in calves: Natural and experimental infection with a verotoxin-producing strain of *Escherichia coli* O111:NM. Can. J. Vet. Res. 52, 484–487.

Sherwood, D., Snodgrass, D.R., O'Brien, A.D., 1985. Shiga-like toxin production from *Escherichia coli* associated with calf diarrhea. Vet. Record. 116, 217.

Snedecor, G.W., Cochran, W.G., 1989. Analysis of frequencies in one-way and two-way classifications. Statistical Methods, 8th ed. Iowa State University Press, Ames, Iowa, pp. 196–216.

Synge, B.A., Hopkins, G.F., 1992. Verotoxigenic *Escherichia coli* O.157 in Scottish calves. Vet. Record 130, 583.

Tesh, V.L., O'Brien, A.D., 1991. The pathogenic mechanisms of Shiga and Shiga-like toxins. Mol. Microbiol. 5, 1817–1822.

Tzipori, S., Karch, H., Wachsmuth, K.I., Robins-Browne, R.M., O'Brien, A.D., Lior, H., Cohen, M.L., Smithers, J., Levine, M.M., 1987. Role of a 60-megadalton plasmid and Shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* O157:H7 in gnotobiotic piglets. Infect. Immun. 55, 3117–3125.

Wieler, L.H., Bauerfeind, R., Baljer, G., 1992. Characterization of shiga-like toxin producing *Escherichia coli* (SLTEC) isolated from calves with and without diarrhea. Zbl. Bakt. 276, 243–253.

Wieler, L.H., Bauerfeind, R., Weiss, R., Pirro, F., Baljer, G., 1995. Association of enterohemolysin and non-fermentation of rhamnose and sucrose with Shiga-like toxin-genes in *Escherichia coli* from calves. Zbl. Bakt. 282, 265–274.

Wieler, L.H., Vieler, E., Erpenstein, C., Schlapp, T., Steinruck, H., Bauerfeind, R., Byomi, A., Baljer, G., 1996. Shiga toxin-producing *Escherichia coli* strains from bovines: association of adhesion with carriage of *eae* and other genes. J. Clin. Microbiol. 34, 2980–2984.

Wilson, R.A., Francis, D.H., 1986. Fimbriae and enterotoxins associated with *Escherichia coli* serogroups isolated from pigs with colibacillosis. Am. J. Vet. Res. 47, 213–217.

Woodward, M.J., Carroll, P.J., Wray, C., 1992. Detection of entero- and verocyto-toxin genes in *Escherichia coli* from diarrhoeal disease in animals using polymerase chain reaction. Vet. Microbiol. 31, 251–262.

Wray, C., McLaren, I., Pearson, G.R., 1989. Occurrence of 'attaching and effacing' lesions in the small intestine of calves experimentally infected with bovine isolates of verocytotoxic *E. coli*. Vet. Record 125, 365–368.