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

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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 ≥ 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 predominately among both healthy and diarrheic calves.

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

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Pii: S 0378-1135(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; Syne 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 MgCl2, 10 mM dNTP mix, 0.8 mM 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 ACA GAT GAC TTC TCG AC 3′} and {5′ CAC ATA TAA ATT ATT TCG CTC 3′} were used
The MK1–MK2 primer pair was demonstrated to amplify a 230 bp fragment for both \textit{stx} \textsubscript{1} and \textit{stx} \textsubscript{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 \textit{eae} and \textit{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 \textit{E. coli} and \textit{Salmonella dublin} that lacked \textit{eae} genes were negative controls. The control strains had been previously evaluated for \textit{eae} and \textit{stx} genes by colony blot hybridization and PCR.

2.4. Enterohemolysin activity

Each \textit{eae} \textsuperscript{+} and \textit{eae}/\textit{stx} \textsuperscript{+} \textit{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 \textit{E. coli} Reference Center, The Pennsylvania State University (Wilson and Francis, 1986). \textit{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 \textit{eae}/stx\textsuperscript{+} \textit{E. coli} strains known to produce Shiga toxins (Table 1). As additional controls, 10 \textit{eae}\textsuperscript{+} only and 10 \textit{stx}\textsuperscript{+} 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 ml 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 \textit{Cryptosporidium parvum}, enterotoxigenic \textit{E. coli}, \textit{Salmonella} sp., enteric viruses, and BVDV by routine procedures. Briefly, \textit{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 \textit{Salmonella} sp. Suspect colonies were confirmed as \textit{Salmonella} after differential biochemical and serological testing. Strains of \textit{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⁺
E. coli in their feces. Infection with eae \(^*\) E. coli was significantly higher (\(p \leq 0.001\)) 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 O5, O26, O84/172, O98, O103, O111, O157 and O171 from healthy calves, and from serogroups O5, O55, O69, and O111 from diarrheic calves.
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
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*<sup>+</sup> and *eae/stx*<sup>+</sup> *E. coli* in their feces when compared to diarrheic calves (20.0%). However, significantly more *eae*<sup>+</sup> and *eae/stx*<sup>+</sup> *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*<sup>+</sup> (one calf) and *eae/stx*<sup>+</sup> *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*<sup>+</sup>, *eae/stx*<sup>+</sup> *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*<sup>+</sup> *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).

### Table 4

| Antimicrobial                  | Percent resistant strains | Healthy (N = 40) | Diarrheic (N = 23) |
|-------------------------------|--------------------------|------------------|-------------------|
|                               | *eae*<sup>+</sup> | *eae/stx*<sup>+</sup> | *eae*<sup>+</sup> | *eae/stx*<sup>+</sup> |
| 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.*
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 complement 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 O171 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.
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