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Evelyn A. Dean-Nystrom  
Pre-Harvest Food Safety and Enteric Diseases Research Unit, evelyn.nystrom@ars.usda.gov

Brad T. Bosworth  
U.S. Department of Agriculture

Harley W. Moon  
Iowa State University

Alison D. O'brien  
F. Edward Hebert School of Medicine

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**Escherichia coli** O157:H7 Requires Intimin for Enteropathogenicity in Calves

EVELYN A. DEAN-NYSTROM, BRAD T. BOSWORTH, HARLEY W. MOON, AND ALISON D. O’BRIEN

Enteric Diseases and Food Safety Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010; Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011; and Department of Microbiology and Immunology, F. Edward Hebert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

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Enterohemorrhagic *Escherichia coli* (EHEC) strains require intimin to induce attaching and effacing (A/E) lesions in newborn piglets. Infection of newborn calves with intimin-positive or intimin-negative EHEC O157:H7 demonstrated that intimin is needed for colonization, A/E lesions, and disease in cattle. These results suggest that experiments to determine if intimin-based vaccines reduce O157:H7 levels in cattle are warranted.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are major causes of bloody diarrhea in humans in the United States (20). Hemolytic uremic syndrome, a life-threatening complication of EHEC O157:H7 infection, is the primary cause of acute kidney failure in children in the United States and Canada (1). Other serotypes of EHEC have also been associated with outbreaks of bloody diarrhea and hemolytic uremic syndrome (2, 3, 19).

Cattle are important reservoirs of EHEC O157:H7 strains (20, 22). The majority of cases of EHEC disease recognized in the United States are associated with ingestion of undercooked, contaminated hamburger or raw milk. Outbreaks have also been associated with produce contaminated with bovine manure. Therefore, one strategy for reducing the risk of EHEC infections in humans is to reduce the prevalence of EHEC infections in cattle.

All EHEC strains are Shiga toxin-producing *E. coli* (STEC) strains. They produce cytoxins, called Shiga toxins (Stx1 and Stx2) or verotoxins, that are considered essential for EHEC virulence in humans. EHEC strains are also characterized by the presence of a ~90-kb plasmid (12, 14, 21). Many EHEC strains have the capacity to attach intimately to host cell membranes and efface microvilli and cytoplasm in a characteristic pattern referred to as an attaching and effacing (A/E) lesion. EHEC strains cause A/E lesions in selected cell lines in vitro and in the intestines of experimental animals (13, 22).

EHEC-mediated A/E lesions are similar to those produced by enteropathogenic *E. coli* (EPEC) in humans and animals (13). In EPEC, the *eae* (for *E. coli* attaching and effacing; formerly called eaeA) chromosomal locus encodes an outer membrane adhesion protein called intimin (11). The *eae* gene is necessary, but not sufficient, for EPEC bacteria to cause A/E lesions (6, 7, 10). Some EHEC strains also carry an *eae* homolog that plays a critical role in the attachment of EHEC O157:H7 to human epithelial cells and the formation of A/E lesions in gnotobiotic pigs (8, 15, 16).

The objectives of the present study were to determine if intimin is required for EHEC-mediated enterocolitis and diarrheal disease in calves and to extend earlier studies showing its role in A/E lesion formation in neonatal piglets. We compared the pathogenicity of an intimin-negative *eae* mutant of EHEC O157:H7 (strain 86-24*eaeΔ10* [15]) with that of two isogenic intimin-positive (*eae*<sup>+</sup>) partners, one a wild-type EHEC isolate (strain 86-24) and the other the *eae* mutant complemented with the *eae* gene [strain 86-24*eaeΔ10*(pEB310) (15)], in neonatal calves and cesarean-derived, colostrum-deprived (CDCD) piglets. We also tested the pathogenicity of an intimin-negative non-O157:H7 wild-type EHEC strain (B2F1) in neonatal calves.

The bacterial strains used in this study are described in Table 1. Each of 23 colostrum-deprived calves (18 Holstein, 2 Jersey, and 3 mixed breeds; 22 male and 1 female) was inoculated at 12 h of age with 10<sup>10</sup> CFU of an EHEC strain that produces intimin (12 calves), an EHEC strain that does not produce intimin (8 calves), or the nonpathogenic control *E. coli* 123 (3 calves) as previously described (5). Calves were observed every 8 h for signs of disease and euthanized with sodium pentobarbital at 18 or 42 h postinoculation.

At necropsy, rectal contents and sections from the rectum, colon, cecum, and ileum were collected and frozen at −80°C for bacteriological examination (5). Tissues from the rectum, colon, cecum, and ileum were fixed in formaldehyde, sectioned, and stained with hematoxylin and eosin or immunohistochemically with goat anti-O157:H7 as the primary antibody, biotinylated anti-goat immunoglobulin G (heavy and light chains) as the secondary antibody, and an avidin-biotin conjugate (5). Some sections were removed from formaldehyde and postfixed in glutaraldehyde for transmission electron microscopy (5).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th><em>eae</em> gene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Stx type</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>86-24</td>
<td>O157:H7</td>
<td>+</td>
<td>Stx2</td>
<td>Phil Tarr</td>
<td>15</td>
</tr>
<tr>
<td>86-24*eaeΔ10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>O157:H7</td>
<td>−</td>
<td>Stx2</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>86-24*eaeΔ10&lt;sup&gt;c&lt;/sup&gt;(pEB310)</td>
<td>O157:H7</td>
<td>+</td>
<td>Stx2</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>B2F1</td>
<td>O91:H21</td>
<td>−</td>
<td>Stx2d</td>
<td>Mohamed Karmali</td>
<td>9, 17</td>
</tr>
<tr>
<td></td>
<td>O43:H28</td>
<td>−</td>
<td></td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>

* Corresponding author. Mailing address: USDA, ARS, National Animal Disease Center, P.O. Box 70, Ames, IA 50010-0070. Phone: (515) 239-8376. Fax: (515) 239-8458. E-mail: enystrom@nadc.ars.usda.gov.

<sup>a</sup> Presence (+) or absence (−) of *eae* gene.

<sup>b</sup> In-frame deletion in *eae* gene in strain 86-24.

<sup>c</sup> *eae* mutant complemented with the *eae* gene.

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Three of five calves inoculated with wild-type EHEC strain 86-24 developed watery diarrhea by 18 h postinoculation (Table 2). Two of these calves had blood-tinged diarrhea at 42 h postinoculation, and one of these calves died on day 2, about 2 h prior to the scheduled necropsy. Postmortem observations were compatible with enteritis as the cause of death. Similarly, two of seven calves inoculated with the complemented mutant strain 86-24eaeΔ10(pEB310) developed diarrhea by 18 h, and three of four had diarrhea (blood tinged in two calves) by 42 h postinoculation. One such infected calf died on day 2, about 2 h prior to the scheduled necropsy. Again, postmortem observations were compatible with enteritis as the cause of death. In contrast, all calves inoculated with mutant strain 86-24eaeΔ10, with B2F1, or with nonpathogenic control strain 123 remained healthy throughout the experiment.

Hyperemia, focal petechiae, and fibrinous exudates in the intestines were common postmortem observations in calves inoculated with strain 86-24 or 86-24eaeΔ10(pEB310) but were not noted in any of the calves inoculated with mutant strain 86-24eaeΔ10, strain B2F1, or control strain 123. A/E lesions containing O157:H7+ bacteria were identified by immunostaining in the ileum and large intestines of five of five calves inoculated with strain 86-24 and six of seven calves inoculated with strain 86-24eaeΔ10(pEB310). In addition to A/E lesions, a diffuse mucosal neutrophil infiltration with accompanying hemorrhage, edema, atrophy of ileal villi, and fibrinous to fibrinohemorrhagic exudates in the intestinal lumen was noted in histologic sections from some of these calves. Neutrophil infiltrates also occurred in the one calf that had no A/E lesions. Examination of sections of ileum from two calves [18 and 42 h postinoculation with strain 86-24eaeΔ10(pEB310)] by electron microscopy confirmed the in vivo A/E activity of the complemented mutant (Fig. 1). No A/E lesions or histopathological abnormalities were detected in any calf inoculated with mutant strain 86-24eaeΔ10, strain B2F1, or control strain 123. However, there were patchy layers of O157:H7+ bacteria on the epithelium in the cecum, colon, and ileum of one calf necropsied 18 h after inoculation with mutant strain 86-24eaeΔ10, but these bacteria were not associated with A/E lesions.

The numbers of the inoculated organisms (expressed as CFU per gram) recovered from tissues and feces of calves at 18 or 42 h postinoculation with eae+ or eae strains of E. coli are shown in Fig. 2. Sorbitol-negative EHEC O157:H7 bacteria were quantitated on sorbitol MacConkey agar containing 100 μg of streptomycin per ml (strain 86-24), 100 μg of streptomycin and 20 μg of nalidixic acid per ml (strain 86-24eaeΔ10), or 100 μg of ampicillin and 34 μg of chloramphenicol per ml [strain 86-24eaeΔ10(pEB310)]. Samples from which the inoculated strain were not recovered were recorded as having <10⁵ CFU/g. Selected sorbitol-negative isolates were tested for O157:H7 antigen by a latex agglutination assay (5). Strain B2F1 (O91:H21) and strain 123 (O43:H28) bacteria were quantitated on MacConkey agar containing 100 μg of streptomycin or 20 μg of nalidixic acid per ml, respectively. Colonies were tested for O91 and O43 antigens to identify strains B2F1 and 123, respectively, by filter blot immunoperoxidase assay (4), using anti-O91 and anti-O43 sera (E. coli Reference Center, Pennsylvania State University, University Park) and peroxidase-conjugated anti-rabbit immunoglobulin G (heavy and light

### TABLE 2. Clinical and histological findings in calves 18 and 42 h after inoculation with an eae+ EHEC strain [86-24 or 86-24eaeΔ10(pEB310)], an eae mutant EHEC strain (86-24eaeΔ10), or a strain lacking the eae gene (B2F1 or nonpathogenic E. coli 123)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time of determination (h postinoculation)</th>
<th>n</th>
<th>No. positive for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diarrhea</td>
</tr>
<tr>
<td>86-24</td>
<td>18</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>86-24eaeΔ10</td>
<td>18</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>86-24eaeΔ10(pEB310)</td>
<td>18</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>B2F1</td>
<td>42</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>123</td>
<td>42</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

* A/E bacteria stained with E. coli O157:H7 antibody by immunoperoxidase technique.

* Calf found dead at ca. 40 h postinoculation; samples were collected for histopathological studies.

![FIG. 1. Electron micrograph of absorptive cells from the ileum of a calf 18 h after inoculation with EHEC O157:H7 strain 86-24eaeΔ10(pEB310). This strain is an eae mutant which has been complemented with the eae gene. The intestinal lumen is to the left. Bacteria are intimately attached to absorptive-cell membranes with subjacent electron-dense filaments in absorptive-cell cytoplasm. Most of the absorptive-cell microvilli have been effaced. There are pedestals beneath bacteria to the upper left.](image-url)
Greater numbers of the inoculated organisms were recovered from the intestines of calves inoculated with strain 86-24 or strain 86-24\textit{eae}\textit{D10}(pEB310) than from those of calves inoculated with strain 86-24\textit{eaeD10}, strain B2F1, or control strain 123 at 42 h postinoculation (Fig. 2). Because of the large degree of variation among animals and the small number of animals, there was no significant difference among the numbers of bacteria at the individual tissue level. However, when we took the group average for each tissue and treated the tissues as a block, the mean CFU per gram for all samples obtained at 42 h postinoculation from the group of calves inoculated with \textit{eae} mutant strain 86-24\textit{eaeD10} was lower ($P < 0.05$; analysis of variance and least significant difference test) than the means for calves inoculated with \textit{eae} strain 86-24 or 86-24\textit{eaeD10}(pEB310). The only exception was that the one calf that did not develop clinical signs or have A/E lesions after inoculation with strain 86-24\textit{eaeD10}(pEB310) had bacterial levels comparable to those in calves inoculated with strains that lacked the \textit{eae} gene. The numbers for strains 86-24\textit{eaeD10} and B2F1 were similar to those for control strain 123. The number of strain 86-24\textit{eaeD10} organisms recovered from feces was similar to that of the \textit{eae} strains. The inoculum strain accounted for a larger percentage of the total number of coliforms isolated from calves inoculated with \textit{eae} EHEC than from calves inoculated with \textit{eae} mutant EHEC, strain B2F1, or the control \textit{E. coli} strain (Fig. 3). The presence and severity of A/E lesions in tissues from calves inoculated with \textit{eae} strains correlated with the number of inoculated bacteria recovered.

FIG. 2. CFU of \textit{E. coli} per gram of tissue or feces in necropsy samples from neonatal calves 18 h (■) or 42 h (□) after inoculation with \textit{eae} EHEC strain 86-24 or 86-24\textit{eaeD10}(pEB310), \textit{eae} mutant EHEC strain 86-24\textit{eaeD10}, strain B2F1, or nonpathogenic \textit{E. coli} 123. Data are means ± standard deviations. See Table 2 for the number of calves in each group. Sp., spiral.

FIG. 3. Numbers of CFU of inoculated strain (■) and total coliforms (□) per gram of tissue or feces recovered at 42 h postinoculation from calves inoculated with \textit{eae} EHEC strain 86-24 or 86-24\textit{eaeD10}(pEB310), \textit{eae} mutant EHEC strain 86-24\textit{eaeD10}, strain B2F1, or \textit{E. coli} control strain 123. Sp., spiral.
test the efficacy of anti-intimin vaccines. Such vaccines could help re-

onization is necessary for intestinal lesion formation in CDCD piglets. The

eae gene of the eae mutant. In contrast to calves, the A/E lesions occurred

mainly in the ceca of the piglets. The numbers of inoculated bacteria recovered from the cecum or ileum at 18 h postinoculation (5). In this study, we

inoculated E. coli O157:H7 infection in neonatal calves is similar to that

of A/E lesions. These results indicate that intimin plays a critical role in EHEC O157:H7 pathogenesis in CDCD piglets and extend the findings of earlier studies with these strains in gnotobiotic piglets (15).

In this study we have clearly demonstrated that the eae gene locus is required for E. coli O157:H7 strain 86-24 to intensively colonize the intestines and cause diarrhea and A/E lesions in neonatal calves and to cause colonic edema and A/E lesions in CDCD piglets. The eae mutant and B2F1 data indicate that eae-mediated adherence to the intestinal mucus is critical for EHEC to cause fibrinohemorrhagic enterocolitis and diarrhea in calves. Similarly, the results confirm that eae-mediated colo-

nization is necessary for intestinal lesion formation in CDCD piglets. These results suggest that anti-intimin vaccines might interfere with EHEC infections. Such vaccines could help re-

duce the levels of EHEC in cattle and thus reduce the number of EHEC infections in humans. The CDCD piglet EHEC infection model will be useful for preliminary experiments to test the efficacy of anti-intimin vaccines.

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Table 3. Findings in CDCD piglets at 18 h after inoculation with an eae + strain (86-24 or 86-24eaeΔ10[pEB310]) or an eae mutant strain (86-24eaeΔ10) EHEC strain or nonpathogenic E. coli 123

<table>
<thead>
<tr>
<th>Inoculated strain</th>
<th>No. of piglets tested</th>
<th>No. with colonic edema</th>
<th>No. with A/E bacteria</th>
<th>Mean log10 CFU ± SD of E. coli O157:H7/g of tissue in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>86-24</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>86-24eaeΔ10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7.1 ± 0.8</td>
</tr>
<tr>
<td>86-24eaeΔ10[pEB310]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5.6 ± 1.4</td>
</tr>
<tr>
<td>123</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>ND†</td>
</tr>
</tbody>
</table>

† A/E bacteria found only in the cecum.

‡ A/E bacteria found in the cecum (three of three) and the ileum (one of three).

ND, not determined.

(data not shown). A/E lesions were only seen in tissues contain-

ning ≥10^6 CFU of eae + HEC/g of tissue.

In earlier studies we showed that the histopathology of EHEC O157:H7 infection in neonatal calves is similar to that in CDCD piglets, but O157:H7 bacteria do not cause diarrhea in CDCD piglets by 18 h postinoculation (5). In this study, we compared the pathogenicity of isogenic eae + and eae mutant derivatives of EHEC O157:H7 strain 86-24 in <8-h-old CDCD piglets (8). As shown in Table 3, CDCD piglets developed colonic edema and A/E lesions by 18 h after inoculation with the eae + strain 86-24 or 86-24eaeΔ10(pEB310) but not with the eae mutant. In contrast to calves, the A/E lesions occurred mainly in the ceca of the piglets. The numbers of inoculated bacteria recovered from the cecum or ileum at 18 h postinoculation were similar in all experimental groups (Table 3), and bacterial counts did not correlate with the presence or absence of A/E lesions. These results indicate that intimin plays a critical role in EHEC O157:H7 pathogenesis in CDCD piglets and extend the findings of earlier studies with these strains in gnotobiotic piglets (15).

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