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Haemorrhagic Colitis Associated with Enterohaemorrhagic *Escherichia coli* O165:H25 Infection in a Yearling Feedlot Heifer

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Case Report

Haemorrhagic colitis associated with enterohaemorrhagic *Escherichia coli* O165 : H25 infection in a yearling feedlot heifer

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Introduction: Enterohaemorrhagic *Escherichia coli* (EHEC) cause haemorrhagic colitis and haemolytic uraemic syndrome in humans. Although EHEC infection typically results in haemorrhagic colitis in all ages of human patients, in cattle it is usually limited to 1- to 5-week-old nursing calves.

Case Presentation: A 1-year-old feedlot beef heifer was moribund with neurological signs and bloody diarrhoea. At necropsy, the colonic mucosa contained multiple grossly visible haemorrhagic erosions, each measuring <1 mm in diameter. Histologically, foci corresponding to the gross erosions had *E. coli* O165 antigen-positive bacterial rods adherent to the apical surfaces of degenerate and necrotic colonic mucosal epithelial cells in association with attaching and effacing lesions, and also within cytoplasmic vacuoles in some of these cells. An *E. coli* O165 : H25 strain was isolated from the colonic mucosal tissue, and by microarray analysis was found to contain virulence genes corresponding to type III secretion system (T3SS) structure and regulation (*cesD*, *cesT*, *escD*, *escF*, *escN*/*escV*, *escR*, *escT*, *ler*, *sepL*, *sepQ*), T3SS effectors (*espA*, *espB*, *espC*, *espD*, *espD*, *espF*, *espH*, *espJ*, *nleB*, *nleC*, *nleD*, *nleH*, *tir*), serine proteases (*eatA*, *espC*, *espP*), Shiga toxin (stx_2), EHEC-haemolysin (*ehxA*), and adhesins [intimin-ε (*eae*-ε), type 1 fimbria (*fimA*, *fimB*, *fimH*), type IV pili (*pilA*, *pilB*, *pilC*, *pilM*, *pilP*, *pilQ*) and non-fimbrial adhesin (*efa1*/*lifA*)].

Conclusion: To the best of our knowledge, this is the first report of disease in cattle associated with EHEC O165 : H25 infection, the oldest bovine EHEC disease case with isolation of the pathogen and the first bovine case to demonstrate grossly evident, haemorrhagic, colonic mucosal erosions associated with EHEC infection.

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Introduction

Although *Escherichia coli* is a commensal of the intestine, pathogenic strains have evolved that are important causes of intestinal (diarrhoeal) and extra-intestinal disease. Seven major diarrhoeagenic pathotypes of *E. coli* are now recognized (Croxen *et al.*, 2013). One pathotype, enterohaemorrhagic *E. coli* (EHEC), is generally defined as *E. coli* that contain genes that encode Shiga toxin (Stx; *stx*) and locus of enterocyte effacement (LEE) proteins [e.g. intimin (*eae*)], but may also include LEE-negative, *stx*-positive *E. coli* strains that cause haemorrhagic colitis and haemolytic uraemic syndrome (HUS) (Croxen *et al.*, 2013). Cattle are the main reservoir host of EHEC, and people most commonly become infected through contaminated food and to a lesser extent by contact with infected animals, humans or other sources (Croxen *et al.*, 2013).

Abbreviations: A/E, attaching and effacing; EC, *Escherichia coli*; EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; FDA-ECID, US Food and Drug Administration *Escherichia coli* Identification; HUS, haemolytic uraemic syndrome; LEE, locus of enterocyte effacement; Stx, Shiga toxin; T3SS, type III secretion system.

Although HUS can develop at any age as a sequela to EHEC infection (Riley *et al.*, 1983), an age of <10 years is a risk factor (Tarr *et al.*, 2005). In contrast to humans, clinical diarrhoeal disease attributable to EHEC infection in cattle is primarily limited to 1- to 5-week-old nursing calves (Moxley & Smith, 2010). Herein, we report a unique case in which a 1-year-old heifer developed haemorrhagic colitis associated with EHEC O165 : H25 infection.

Case report

Clinical findings

A pen of cattle at a commercial beef feedlot in central Nebraska containing 170 animals had nine cases of frank, bloody diarrhoea (5.3 % cumulative incidence) over a 2 week period, beginning 16 February 2014. The cattle had been fed a high concentrate ration for 40 days, and the diarrhoeic cattle were separated into a hospital pen and treated with amprolium as coccidiosis was the suspected cause. Six of the nine cattle with bloody diarrhoea recovered by 1 March; two cattle died and one that was moribund was euthanatized by humane methods for diagnostic purposes, yielding a mortality rate of 1.8 % (3/170) and a case fatality rate of 33 % (3/9). The moribund animal, a 1-year-old heifer, was laterally recumbent and exhibiting neurological signs and bloody diarrhoea. This animal was clinically diagnosed with nervous coccidiosis and necropsied by the referring veterinarian.

At necropsy, a 0.6 m segment of fresh intact descending colon was ligated and placed into a sterile plastic bag on ice packs. An adjacent portion of the descending colon was opened, rinsed and placed into 10 % neutral buffered formalin. Both specimens were shipped on ice packs to the University of Nebraska-Lincoln, Veterinary Diagnostic Center, arriving within 18 h of necropsy.

Pathology

Upon arrival at the laboratory, a portion of the specimen of fresh descending colon was opened and examined grossly, and the formalin-fixed tissue was processed by routine methods for histopathology. Grossly, the mucosal surface of the descending colon contained multiple erosions, each measuring <1 mm in diameter. Some of the erosions had adherent blood clots and pale, healed lesions of the same size (Fig. 1, top). Microscopically, in haematoxylin & eosin- and Brown-and-Brenn-stained, paraffin-embedded sections of formalin-fixed colon, Gram-negative bacterial rods were found on the apical surfaces of mucosal epithelial cells in association with attaching and effacing (A/E) lesions, and within vacuoles in the cytoplasm of these cells. The colonic mucosa contained multiple foci of confluent epithelial necrosis and sloughing in association with A/E-adherent bacteria that corresponded to the gross lesions (Fig. 1, middle). Erosions (Fig. 1, bottom) and sites of re-epithelialization also corresponding to the

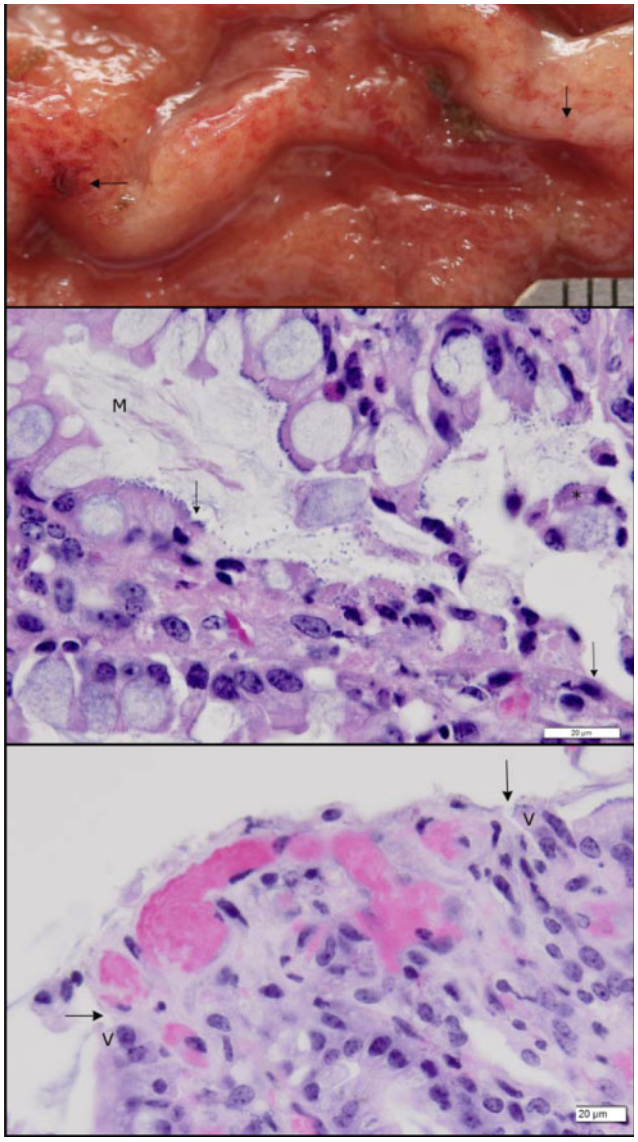

Fig. 1. Gross photograph and photomicrographs of colonic erosions. (Top) Grossly, acute erosions are hyperaemic, some with adherent blood clots (horizontal arrow), whereas re-epithelialized erosions are paler and nearly the same colour as that of surrounding mucosal epithelium (vertical arrow). Distance between each ruler mark is 1 mm. (Middle) Photomicrograph of colonic mucosa showing necrosis of bacteria-colonized enterocytes, representing the stage immediately prior to erosion. The lower half of the crypt in the photo includes a contiguous segment of necrotic epithelial cells (area bounded by arrows), some of which have sloughed into the lumen (asterisk). Individual necrotic enterocytes have pyknotic nuclei and eosinophilic cytoplasm. The area bounded by arrows measures ~130 µm in the plane of section. The crypt lumen contains increased mucus extrusion (M). Haematoxylin and eosin stain. Bar, 20 µm. (Bottom) Photomicrograph of colonic mucosa showing acute erosion. Acute punctate erosion (area bound by arrows, measuring ~100 µm in diameter in this plane of section), characterized by exposure of the basal lamina and hyperaemia of the underlying microvasculature. Viable hyperplastic enterocytes (V), some of which are colonized by bacteria, are seen at the edge of the erosion. Haematoxylin and eosin stain. Bar, 20 µm.

gross lesions were detected histologically. Occasional re-epithelialized crypts with rare intraepithelial coccidial oocysts were seen. Based on the bacteriology and serotyping results (see below), additional tissue sections from the paraffin blocks were stained with five different serotype-specific rabbit polyclonal anti-*E. coli* sera (Staten Serum Institute) using an immunohistochemical test procedure. Microscopically, the bacteria associated with A/E lesions on the apical surfaces and those in vacuoles (Fig. 2A) stained positive for *E. coli* O165 antigen (Fig. 2B, C), whereas they did not stain with anti-*E. coli* O26, O103, O145 or O157 sera.

Bacteriology

Upon arrival at the laboratory, the remaining unopened descending colon specimen was processed for bacterial culture targeting *Salmonella* and other *Enterobacteriaceae* (Table 1). An aseptic scraping of mucosa was obtained and subjected to direct culture on MacConkey agar and tryptic soy agar with 5 % sheep blood (Remel) and Salmonella Chromogenic Agar (Oxoid) at 37 °C in an aerobic environment supplemented with 5 % CO_2. In addition, the mucosal scraping was subjected to stationary enrichment culture in *E. coli* (EC) broth (Oxoid) for 6 h at 40 °C in atmospheric oxygen. Additional aseptic mucosal scraping samples from the same colonic specimen were subjected to stationary enrichment culture in tetrathionate broth (Remel) in atmospheric oxygen for 18 h at 41 °C. The tetrathionate broth enrichment culture was subcultured onto XLT4 and brilliant green agars (Remel), and incubated at 37 °C in an aerobic environment supplemented with 5 % CO_2. Enrichment and selective cultures for *Salmonella* did not yield evidence of this organism.

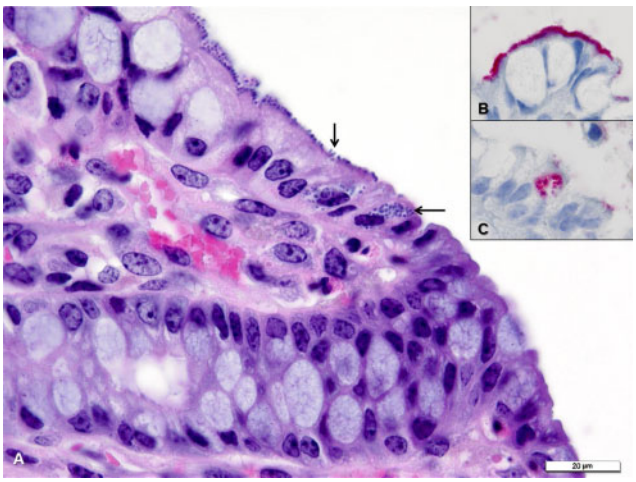

Fig. 2. Photomicrographs of colonic mucosa showing *E. coli* O165-positive bacteria adherent to apical surfaces and within cytoplasmic vacuoles of colonic mucosal epithelium. (A) Photomicrograph of colonic mucosa showing an area of colonization by bacteria intimately adherent to the apical surfaces of enterocytes (vertical arrow) and bacteria within vacuoles in the cytoplasm (horizontal arrow). Haematoxylin & eosin stain. (B, C) Subsequent tissue sections from the same block stained immunohistochemically with rabbit polyclonal antiserum against *E. coli* O165 antigen showing positive (red) staining of bacteria on apical surfaces (B) and in cytoplasmic vacuoles (C). Bar (20 µm) is representative for (A)–(C).

Table 1. Overview of culture protocol and bacterial isolates

Enrichment	Agar	Isolate
None	MacConkey agar	*E. coli* O165
None	Tryptic soy agar with 5 % sheep blood	*E. coli* O165
None	Salmonella Chromogenic Agar	Negative
Tetrathionate broth	XLT4	Negative
Tetrathionate broth	Brilliant green agar	Negative
E. coli broth	CHROMagar O157	*E. coli* O145
E. coli broth	Modified Possé agar	*E. coli* O145

The EC broth enriched culture was subcultured onto CHROMagar O157 (DRG International), Possé differential agar (Possé *et al.*, 2008), and a modified form of this agar (mPossé) containing reduced novobiocin (5.0 mg l^{-1}) and potassium tellurite (0.5 mg l^{-1}; Stromberg *et al.*, 2015), with all three media incubated in an aerobic environment for 18 h at 37 °C. Red-purple, blue-purple and green colonies from the Possé and mPossé plates, and mauve colonies from the CHROMagar O157 plate, were picked for molecular testing as described below.

From the direct culture of the colonic mucosal scraping on MacConkey and blood agars, a heavy growth of colonies consistent with *E. coli* was isolated after 24 h incubation. Eight isolated lactose-fermenting colonies of varying size and colony type on the MacConkey plate were subcultured onto blood agar to ensure purity, visualize colony phenotype and further test by molecular methods as described below. All isolates were A/A with gas and no H_2S on triple-sugar iron, indole-positive and oxidase-negative, and confirmed to be *E. coli* using a commercial identification system for Gram-negative organisms [Sensititre GNID; Thermo Scientific (Trek Diagnostics)].

Molecular typing

Colonies picked from Possé, mPossé and CHROMagar O157 plates were tested by an 11-plex PCR that detected genes specific for *E. coli* O26, O45, O103, O111, O121, O145 and O157 serogroup synthesis (*wzx*, *wbq* or *rfbE*), plus stx_1, stx_2, *eae* and EHEC-haemolysin (*ehxA*; Bai *et al.*, 2012). No *stx*-positive colonies were recovered from the Possé, mPossé or CHROMagar O157 agars; however, O145 *eae*-positive colonies were recovered from CHROMagar O157 and mPossé agars.

Table 2. FDA-ECID microarray results on O165 EHEC and O145 EPEC isolates from the case

Product/gene	O165 isolate	O145 isolate	Product/gene	O165 isolate	O145 isolate
T3SS			**Shiga toxin**		
Structure and regulation			*stx*$_1$	A	A
cesD	P	A	*stx*$_2$	P	A
cesT	P	P			
escD	P	P	**Haemolysin**		
escF	P	P	*ehxA*	P	P
escN/*escV*	P	P	*hlyE*	P	P
escR	P	P			
escT	P	P	**Intimin**		
ler	P	P	*eae-ε*	P	A
sepL	P	A	*eae-γ*	A	P
sepQ	P	A			
Effectors			**Fimbria**		
espA	P	A	Type I		
espB	P	A	*fimA*	P	P
espD	P	A	*fimB*	P	P
espF	P	A	*fimH*	P	P
espH	P	A	**Type IV**		
espJ	P	A	*pilA*	P	P
nleA	A	A	*pilB*	P	P
nleB	P	A	*pilC*	P	P
nleC	P	P	*pilM*	P	P
nleD	P	A	*pilP*	P	P
nleH	P	P	*pilQ*	P	P
tir	P	A	**Non-fimbrial adhesin**		
			efa1/*lifA*	P	A
Serine protease			O-antigen		
Mucinase			*wzx*$_{O145}$	A	P
eatA	P	A	*wzx*$_{O165}$	P	A
Enterotoxin					
espC	P	A	**H-antigen**		
Multifunctional enzyme			*fliC*$_{h25}$	P	A
espP	P	P	*fliC*$_{h28}$	A	P

A, Absent; P, present.

The eight *E. coli* isolates originating from the MacConkey plate were tested by a four-plex PCR method conducted as described previously (Fagan *et al.*, 1999), except that the total reaction volume was reduced to 40 µl and the mixture included 4 µl template DNA. Three of these isolates were positive for *stx*$_2$, *eae* and EHEC-haemolysin (*ehxA*).

One of the three PCR-positive isolates selected at random (designated 7050-2014) was serotyped at the *E. coli* Reference Center (Pennsylvania State University) and determined to be O165 : H25. This same isolate was tested by the US Food and Drug Administration *E. coli* Identification microarray (FDA-ECID) which incorporates genetic signatures of >250 whole-genome sequences, >40 000 *E. coli* genes and ~9800 single nucleotide polymorphisms (Jackson *et al.*, 2011; Lacher *et al.*, 2014). Via the FDA-ECID, the *E. coli* isolate in this case was confirmed to be a serotype O165 : H25 EHEC and to contain virulence genes associated with type III secretion system (T3SS) structure and regulation, T3SS effectors, Stx (*stx*$_{2a}$), EHEC-haemolysin (*ehxA*), serine proteases (*eatA*, *espC* and *espP*), and adhesins [intimin-ε (*eae-ε*), type 1 fimbria, type IV pili and non-fimbrial adhesin (*efa1*/*lifA*)] (Table 2). By FDA-ECID, the O145 isolate was confirmed to be an O145 enteropathogenic *E. coli* (EPEC) and also found to have the H28 flagellin structural gene (*fliC*$_{h28}$), but had lost many of the T3SS genes, including *espA*, *tir* and others (Table 2).

Ethical statement

The euthanasia method and study of specimens were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee (protocol 1075).

Discussion

To the best of our knowledge, this is the first report of disease in cattle associated with EHEC O165 : H25 infection, the oldest bovine EHEC case in which the bacterial pathogen was isolated, and the first bovine case to demonstrate grossly evident, haemorrhagic, colonic mucosal erosions associated with EHEC infection. Clinical illness in cattle due to EHEC infection is usually limited to young nursing calves between the ages of 1 and 5 weeks (Moxley & Smith, 2010), but this case involved a 1-year-old animal, which indicates that veterinarians should include EHEC in the differential diagnosis in cases of bloody diarrhoea in adult cattle and include O165 : H25 as another serotype capable of causing bovine disease. In addition, with this serotype being a known pathogen of humans, this case provides yet another example of cattle being a zoonotic reservoir host for EHEC. Furthermore, with the finding of EHEC bacteria in cytoplasmic vacuoles of enterocytes, it suggests that this particular serotype may have the potential for enterocyte invasion – a virulence mechanism not altogether typical of EHEC.

A case of mucohaemorrhagic diarrhoea in a 19-month-old Holstein cow infected with bacteria that stained positive for *E. coli* O15 antigen had been reported in Japan (Wada *et al.*, 1994). The O15-positive bacteria were associated with A/E lesions, and necrosis, sloughing and haemorrhage in the colon, but the causative organism was not detected in culture. Similarly, EHEC O26 : K60 infection associated with dysentery and A/E colonic lesions was reported in 8- to 12-month-old heifers in the UK (Pearson *et al.*, 1999). However, in that report, no EHEC organisms were isolated from the one animal (an 8-month-old heifer) in which histological lesions associated with O26 antigen-positive A/E bacteria were detected. An EHEC O26 : K60 organism was isolated from another heifer in the group with haemorrhagic diarrhoea. The present case was similar to these two reports in that a combined infection with coccidia had occurred and appeared to have contributed to the clinical demise (e.g. neurological signs and rare colonic crypt lesions). In adult cattle in the USA, coccidia (e.g. *Eimeria* spp.), other pathogens [e.g. *Salmonella*, *Clostridium perfringens*, winter dysentery (bovine coronavirus), bovine viral diarrhoea virus] and metazoan parasites (e.g. different nematodes) are clinical causes of haemorrhagic diarrhoea (Gelberg, 2012); however, the mucohaemorrhagic erosions affecting the mucosal surface were inferred to be the direct result of EHEC O165 : H25 infection based on the detection of antigen-specific bacteria attached to confluent necrotic sheets of colonic epithelial cells that matched the size of the erosions, isolation of the organism in culture, serotyping results and molecular confirmation of virulence factors.

The EHEC O165 : H25 isolate (7050-2014) in the present case was shown by multiplex PCR and more extensively by the FDA-ECID microarray to contain virulence genes known to encode products that contribute to intestinal colonization and colonic epithelial cell death by apoptosis or necrosis (Coombes *et al.*, 2008; Franzin & Sircili, 2015; McWilliams & Torres, 2014; Sánchez *et al.*, 2015; Stevens & Frankel, 2014; Stevens *et al.*, 2002; Vossenkämper *et al.*, 2011). The O145 EPEC isolate had lost many of its type III-secreted effector genes and apparently had thereby lost the potential to cause lesions. Hence, based on a lack of detection of the organism in the tissue along with microarray confirmation of loss of many effectors involved in A/E lesion development, it was considered to be inconsequential in this case.

We hypothesize that initial intestinal epithelial adherence with isolate 7050-2014 may have involved any combination of type 1 fimbria, type IV pili and the non-fimbrial adhesin Efa1. Type III secreted proteins are well substantiated to cause A/E lesion development. Non-LEE-encoded (Nle) proteins, such as NleB, NleC, NleD and NleH, are known to enhance colonization by decreasing pro-inflammatory signalling, and NleH inhibits apoptosis and thereby allows for bacterial growth on host cells (Coombes *et al.*, 2008; Franzin & Sircili, 2015; Vossenkämper *et al.*, 2011).

EHEC O165 : H25 or EHEC O165 (with H type not stated) have been isolated from the faeces (Geue *et al.*, 2006) and beef carcasses of cattle (Arthur *et al.*, 2002), but to the best of our knowledge, prior to this case, these organisms had not been associated with disease in bovines. The zoonotic implication associated with bovine EHEC O165 : H25 infection is of concern as this serotype causes sporadic cases of HUS in children and is classified as seropathotype C (Karmali *et al.*, 2003). Seropathotype C EHEC are capable of causing severe disease, but of relatively low incidence, and are rarely involved in disease outbreaks (Karmali *et al.*, 2003). EHEC O165 : H25 is an emerging foodborne EHEC pathogen of humans (Sánchez *et al.*, 2015).

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