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Day-old infant rabbit model for enterohaemorrhagic *Escherichia coli* induced diarrhoea

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ABSTRACT

Day-old infant rabbits inoculated with enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 strain developed severe diarrhoea and ruffled coats, usually culminating in death. All rabbits (n=8) inoculated intragastrically with either UMDL 29 or MC 110 developed severe diarrhoea two days post inoculation. None of the control rabbits inoculated with PBS developed diarrhoea. The UMDL 29 and MC 110 infected rabbits also developed ruffled coats by day two post inoculation. Mortality started to occur on day four post infection. The diarrhoea which started 24 hr post inoculation was associated with loss of mass and inflammation of the intestines. At necropsy, the ceca and colons of rabbits inoculated with either UMDL 29 or MC 110 were distended or filled with loose stool and fluids. In contrast, the ceca and colons of rabbits inoculated with PBS were not distended and contained hard, formed pellets. The intestinal contents of the infected rabbits that died and of those that were euthanatized were filled with watery content. A microscopic examination of colons showed mild inflammatory cell infiltration, thinning of the intestinal wall, or necrotic foci. The lungs of infected rabbits were congested. The limitations of current animal models led us to reexamine the day-old infant rabbit model for the study of EHEC pathogenicity. This study indicates that infant rabbits are a useful model for investigation of the intestinal stage of EHEC pathogenesis and suggest that shiga toxin and other virulence factors of *E. coli* O157 may play a critical role in causing diarrhea and inflammation in patients infected with EHEC.

Key words: pathogenicity, day-old rabbits, *Escherichia coli* O157, humans, animals

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) strains are important causes of human hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and encephalopathy (GRIFFIN and TAUXE, 1991; KARMALI et al., 1985). A common histopathological finding in patients is the destruction of endothelial cells lining the small blood vessels in the colon,

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kidneys, and central nervous system (RICHARDSON et al., 1988). The virulence of EHEC has been linked to the production of shiga-like toxins (*Stxs*) (KARMALI, 1989; KARPMAN et al., 1997; LOUISE and OBRIG, 1991). The *Stxs* act as an inhibitor of protein synthesis, enzymatically modifying the translational machinery of the host cell (DONOHUE-ROLFE et al., 1986). FUJII et al. (1994) suggested that SLT-II is toxic to both endothelial cells and neurons in the central nervous system (FUJII et al., 1994; FUJII et al., 1996).

KARPMAN et al. (1997) observed that mice inoculated with SLT-II-positive strains developed severe neurotoxic symptoms and a higher frequency of systemic symptoms, glomerular mesangial hypertrophy, and mesangial deposition than mice inoculated with SLT-II-negative strains (KARPMAN et al., 1997). However, they did not observe the glomerular vascular lesions characteristic of HUS in humans. It has been suggested that cooperation between SLT and the tumor necrosis factor (TNF) may be important in producing the pathologic changes observed in human HUS (BARRETT et al., 1989). TNF-α and SLT exhibited synergistic cytotoxic activity toward human endothelial cells (BARRETT et al., 1989). HAREL et al. (1993) suggested that local synthesis of TNF within the kidney may contribute to renal injury induced by SLT (HAREL et al., 1993).

EHEC colonization of the human colon is thought to be another key determinant of virulence. In tissue culture, EHEC, like enteropathogenic *E. coli* and *Citrobacter rodentium* (a murine pathogen), forms attaching and effacing (A/E) lesions and such lesions are believed to mediate colonization (FRANKEL et al., 1994). A/E lesions are characterized by intimate bacterial adherence to intestinal epithelial cells, localized loss (effacement) of microvilli from epithelial cells, and the accumulation of a pedestal of polymerized actin and other cytoskeletal elements beneath and around the adherent bacterium (FRANKEL et al., 1998). Intimin has also been shown to play a role in EHEC pathogenesis with animal models (DEAN-NYSTROM, 2003; MCKEE et al., 1995; TZIPORI et al., 1995). Besides *stx* and the LEE-encoded factors, several other genes are thought to contribute to EHEC pathogenicity. Of these other factors, most is known about enterohemolysin (encoded by *ehxA*), which has been shown by epidemiologic studies to be frequently associated with severe disease (BARRET et al., 1992; BOERLIN et al., 1999; SCHMIDT and KARCH, 1996).

A variety of animal models have been used to study the symptoms and histopathologic changes associated with human EHEC infection. EHEC strains caused gastrointestinal, neurological, or systemic symptoms and death in gnotobiotic piglets (FRANCIS et al., 1986), rabbits (PAI et al., 1986), and mice (LINDGREN et al., 1993; WADOLKOWSKI et al., 1990a; WADOLKOWSKI et al., 1990b). Acute tubular necrosis of the kidneys was found in inoculated animals, but glomerular pathology was not observed (WADOLKOWSKI et al., 1990a; WADOLKOWSKI et al., 1990b). A variety of animal species, including mice, pigs, baboons, macaques, rabbits, ferrets, and cows, have been used as models to study the pathogenicity of *E. coli* O157:H7 isolates (DEAN-NYSTROM, 2003; MELTON-CELSA and

O'BRIEN, 2003; MOXLEY and FRANCIS, 1998), but no model reproduces all aspects of EHEC-related disease. Several of these models are limited by high cost (e.g., nonhuman primates) and/or by the requirement for complex animal facilities (e.g., gnotobiotic piglets). Day-old infant rabbits may provide a less expensive, more readily available animal model in which to examine the pathogenicity of EHEC isolates from humans and animals and their contribution of virulence factors.

Materials and methods

Preparation of bacterial suspension. The Inoculation was done according to the method previously described by POTTER et al. (1985). Two VTEC O157:H7 isolates (UMDL 29 and MC 110) were tested for pathogenicity in the infant rabbit model. The choice of the isolates was based on the results from PCR, which showed that the strain UMDL 29, was positive for *Stx1, eae A, EhlyA* toxins. UMDL 29 was isolated from a patient with diarrhoea. The MC 110 strain used was *Stx1, Stx2, eae A* and *EhlyA* positive by PCR and was isolated from carcasses of cattle from the Morogoro abattoir.

Inoculation of infant rabbits. Using a Pasteur wire pin, a colony was picked and inoculated into mTSB then incubated at 37 °C for 24 hrs. This bacterial suspension was diluted 10-fold with mTSB to make a suspension of $5x10^8$ and $4x10^8$ and CFU/mL for the human and cattle isolates respectively (RICHARDSON et al., 1992). An aliquot of 1mL of the suspension was given orally to three-day-old infant rabbits using a pipette. For the bacteria count of the inoculums, the bacterial suspension was serially diluted, 10 fold, and the pour plate method was used (PAI et al., 1986).

Animal protocols. Litters of 1-day-old New Zealand-White rabbits were obtained from the Small Animal Laboratory Unit of Sokoine University of Agriculture. The litters were divided into three groups, (A, B and C) consisting of 8 rabbits each and housed in a clean plastic cage. The rabbits were fed with Lactogen^R commercial infant milk formula. Sterile, commercially produced and bottled spring water (Maji Africa^R) was used for reconstitution of the milk powder.

Experimental design. Feeding of the infant rabbits was carried out twice a day at 10:00 am and 6:00 pm, from day 1 after birth until the morning of day 7. Each infant rabbit in groups A, B and C was fed with 3.0 mL per fed on days 1, and 2; 5 mL on day 3 and 4; and 7.0 mL on day 5 and 6; and once in the morning on day 7. At the age of 3 days the animals in groups B and C were also individually inoculated intragastrically with 1 mL of the VTEC 5×10^8 and 4×10^8 CFU respectively using a sterile flexible pipette; Group A animals were not inoculated with VTEC isolate but were fed with PBS. The animals were then weighed daily and examined for clinical signs of diarrhoea until day 7 after infection. Diarrhoea was categorized into the following levels of severity: no diarrhoea, (normal pellets which are dark green, hard and formed); mild, when diarrhoea consisted

of a mixture of soft yellow-green unformed and formed pellets, resulting in light staining of the hind legs; and severe, when diarrhoea consisted of unformed or liquid stools, resulting in significant staining of the perineum and hind legs, wet tail and prolapsed rectum. Animals that died after 1, 4, and 7 days of VTEC inoculation were examined for histopathologic lesions. The gastrointestinal tract was dissected asceptically for isolation of VTEC. All experimental procedures were carried out in accordance with the standards set forth in the Guide for the care and use of laboratory animals (ANONYMOUS, 1985).

Histopathological examination. Segments of ileum, caecum and distal colon were surgically removed, washed and then fixed in 4% buffered formalin, blocked in paraffin, and sectioned. Sections were stained with hematoxylin and eosin, and examined under a microscope for edema, VTEC cell attachment to the epithelial cells and for pseudoeosinophil infiltration**.**

Bacteriological examination. Intestinal faecal material was spread onto SMAC agar plates and incubated at 37 ^{O}C for enumeration of non-sorbitol fermenting VTEC and EMB agar for confirmation that non-sorbitol fermenting organisms identified on SMAC agar were *E. coli* O157.

Statistical analysis. Weight gain was expressed as a percentage of rabbit weight at the start of the experiment. Histological scores were ordinal non-parametric data (RITCHIE et al., 2003).

Results

The results of rabbits inoculated with VTEC isolates SHO 29 and MC 110. All rabbits (n = 8) inoculated intragastrically with either UMDL 29 or MC 110 developed severe diarrhoea 2 days post inoculation. None of the control rabbits inoculated with PBS developed diarrhoea. The UMDL 29 and MC 110 infected rabbits also developed ruffled coats by day 2 post inoculation. The mortality and mass loss in rabbits are presented in Appendix 2. Mortality started to occur on day 4 post infection. The diarrhoea was associated with loss of weight and inflammation of the intestines. At necropsy, the ceca and colons of rabbits inoculated with either UMDL 29 or MC 110 were distended or filled with loose stool and fluids. In contrast, the ceca and colons of rabbits inoculated with PBS were not distended and contained hard, formed pellets. The intestinal contents of the infected rabbits that died and of those that were euthanatized were oedematous and infiltrated with blood. The lungs of rabbits inoculated with both human and animal isolates were congested. The kidneys of inoculated and uninoculated rabbits were normal.

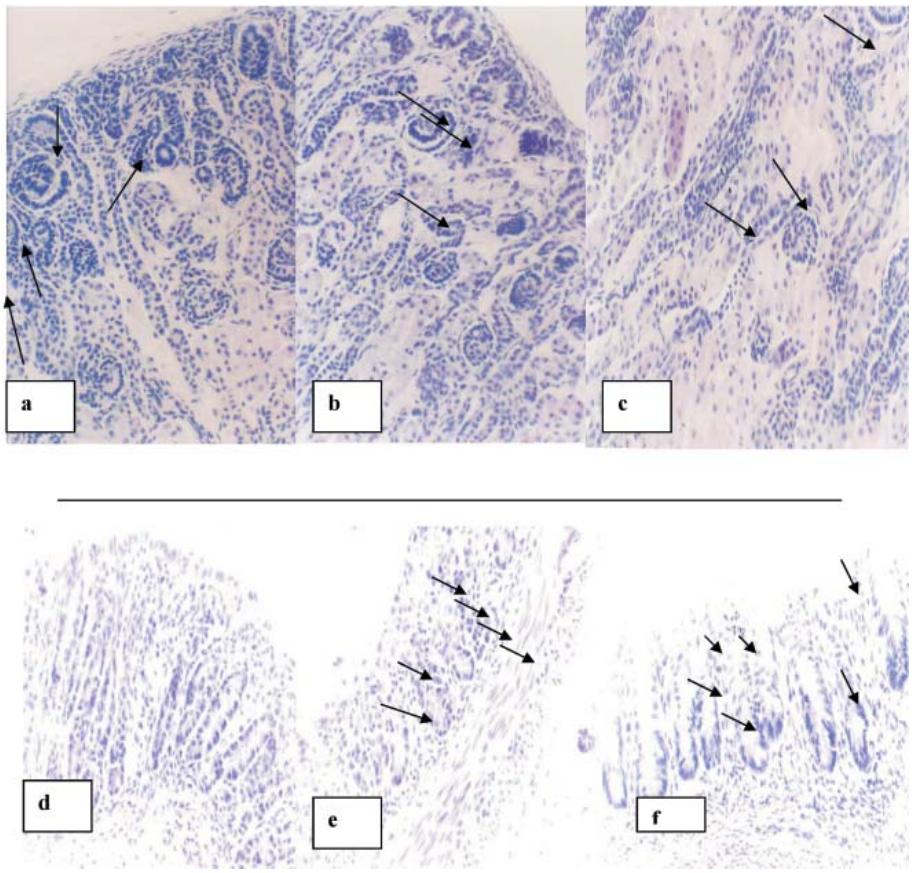

Fig. 1. Histopathology of the kidney and colon of infected infant rabbits. (H&E; ×20).

a, b and c - photomicrography of renal corpuscle surrounded by proximal convoluted tubes; a - control group A; b - group C infected with SHO 29; c - group B infected rabbit with MC 110;

d, e and f - photomicrography of colon. d - control group A; e - group B infected rabbit with MC 110, f - group C infected with SHO 29. Both e and f were characterized by irregular mucosa surface (arrow heads) and inflammatory cell infiltrate within the lamina propria.

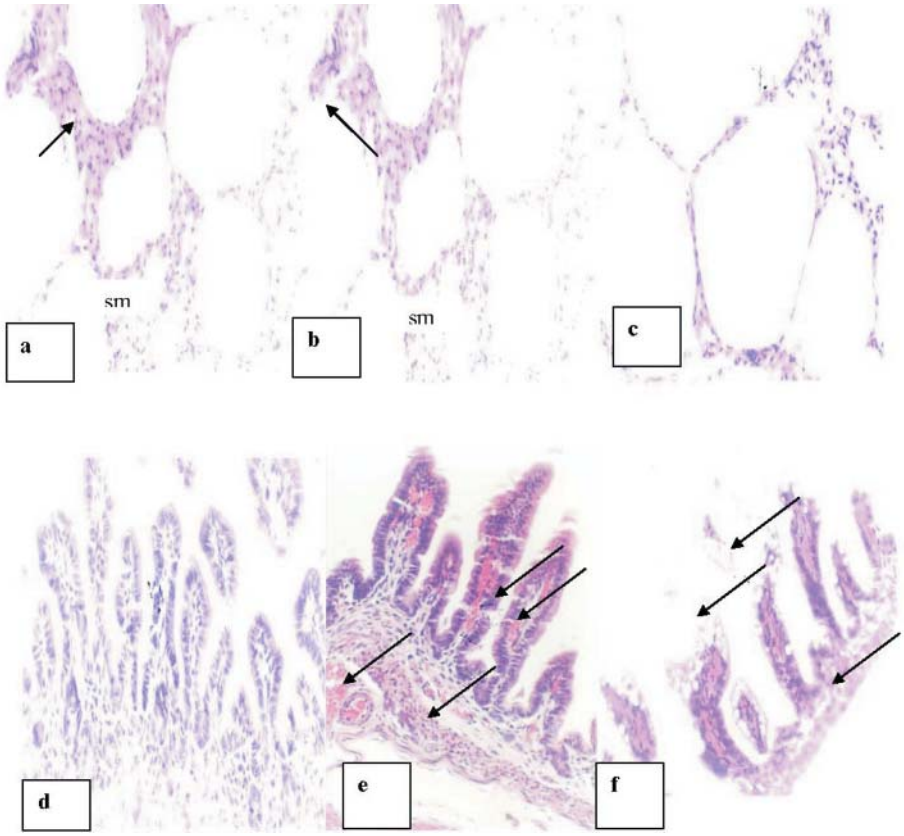

Fig. 2. Histopathology of the lungs and ileum of infected infant rabbits (H&E; ×20).

a, b and c - photomicrography of section of the lung; a and b - showing thicken wall of alveolar duck of infected rabbits with SHO 29 and MC 110 respectively; c - control group A with alveolar wall flattened.

d, e, and f - photomicrography of small intestine; d - control group A; e and f - infected rabbit with MC 110 and SHO 29 respectively.

Table 1. Diarrhoea in rabbits inoculated with VTEC strains SHO 29 and MC 110

Inoculums origin	Total Nº of rabbits	Number of infant rabbits with Diarrhea		
		Severe	Mild	None
Human (SHO 29)	8	100% (8/8)	0	0
Cattle (MC 110)	8	100% (8/8)	0	0
Control group	8	0	0	8/8 (100%)

Bacterial colonization. Strains UMDL 29 and MC 110 colonized all regions of the distal small intestines by two days post inoculation. High numbers of UMDL 29 and MC 110 bacteria were consistently found in the stool samples. PBS treated rabbits were free from *E. coli* in their small intestinal tracts.

Histopathology. Pathological lesions in UMDL 29 and MC 110 inoculated rabbits were confined in the distal colon of the large intestine of infant rabbits (Plate 2 (a, b). There were histological changes (oedematous and infiltration with hemorrhages) in the distal colon when isolates possessing both *Stx1* and *Stx 2* or *Stx 1* only were used for inoculation of infant rabbits. No histological changes were observed in the kidneys of rabbits inoculated with VTEC UMDL 29 and MC 110 (Fig. 1b and 2c) and 1a and 1b). Control rabbits exhibited no inflammatory changes.

Discussion

Earlier results using infant rabbits as model hosts for the study of VTEC pathogenicity were published many years ago (FARMER et al., 1983; POTTER et al., 1985; PAI et al., 1986; ELLIOTT et al., 1994). Studies of intragastric inoculation of infant rabbits with human and animal VTEC isolates caused diarrhoea and colitis but there was no sign of HUS. *Stx 2, Stx 1* and *eaeA* and *ehly A* increased the severity and duration of diarrhoea as well as mortality and the host inflammatory response to VTEC was modulated. Colonization of the infant rabbit intestine by VTEC depended on *eae A* but was not influenced by *stx2* or *stx 1* and *ehx A*. The findings of this study indicate that infant rabbits are a useful animal model for the study of the intestinal manifestations of VTEC pathogenicity. If the results from this model apply to human VTEC infections, then our observations suggest that VTEC-induced diarrhoea is primarliy caused by *Stx1, Stx2, eaeA*, and e*hly A* in combination.

An advantage of infant rabbits over mice for the study of VTEC pathogenicity is that human VTEC isolates colonize the infant rabbit intestine without the requirement for additional treatments. Mice must be given streptomycin, presumably to eliminate the normal intestinal flora, to facilitate VTEC colonization. Furthermore, mice do not develop diarrhoea, colitis or A/E lesions following VTEC inoculation (WADOLKOWSKI et al., 1990a). A limitation of the infant rabbit model is that rabbits do not develop HUS

or other evidence of microangiopathy. The reason(s) for this are not known, although the absence of GB3, the *Stx* receptor, in rabbit kidneys may explain the absence of HUS in this model (ZOJA et al., 1992). Intravenous injecton of *Stx* in adult rabbits can cause microvascular lesions in the brain that resemble thrombotic microangiopathy in humans (ZOJA et al., 1992). The lack of this pathological finding in VTEC-infected infant rabbits may be due to insufficient absorption of *Stx* from the intestine, or alternatively, GB3 may not yet be expressed in the endothelium of the developing central nervous system in young rabbits.

The mechanism(s) by which *Stx* causes diarrhoea in infant rabbits are not known. The enterotoxigenic effect of *Stx* on the ligated ileal loop of adult rabbits is well documented (KEENAN et al., 1986) and is thought to be due to selective damage and loss of villus absorptive epithelial cells. Studies by KEUSCH et al. (1996) suggest that these effects are due to stx binding to GB3 on these cells (MOBASSALEH et al., 1994; KEUSCH et al., 1996). However, GB3 doest not appear in the rabbit small intestine until day 16 of life (MOBASSALEH et al., 1988). The pathological changes reported in this study are in agreement with those of previous studies (FARMER et al., 1983; POTTER et al., 1985; PAI et al., 1986; ELLIOTT et al., 1994). This study further confirmed the previous study that infant rabbits are a suitable animal model for demonstrating diarrhoea in VTEC O157 infections and that rabbits should not necessary need to be three days before they can be used.

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SAŽETAK

Pokusna zaraza enterohemoragičnim sojem bakterije *Escherichia coli* (EHEC) O157:H7 u jednodnevnih se kunića očitovala teškim proljevom, kovrčavom dlakom i uginućem. Teški proljev u kunića (n=8) zaraženih u želudac sojem označenim UMDL 29 ili sojem MC 110 razvio se dva dana nakon inokulacije. Proljev se nije javio u kontrolnih kunića inokuliranih puferiranom fiziološkom otopinom. Kovrčavost dlačnog pokrivača javila se također dva dana nakon infekcije. Uginuća su se počela javljati četiri dana nakon infekcije. Proljev koji je započeo 24 sata nakon inokulacije javio se kao posljedica upale crijeva, a doveo je do gubitka tjelesne mase. Razudbom je ustanovljeno da su slijepa crijeva u inokuliranih kunića bila naduta ili ispunjena rijetkim sadržajem odnosno tekućinom. Suprotno, slijepa crijeva i debelo crijevo kunića koji su dobili puferiranu fiziološku otopinu nisu bila naduta, a sadržavala su tvrdo formirani feces. Crijevni sadržaj uginulih kao i eutanaziranih kunića bio je vodenast. Mikroskopska pretraga tkiva kolona pokazala je blagu infiltraciju upalnih stanica, stanjenu stijenku crijeva i nekrotična žarišta. Ustanovljena je kongestija pluća u zaraženih kunića. Ograničene mogućnosti rabljenja dosadašnjih životinjskih modela potaknule su na ponovnu uporabu jednodnevnih kunića kao modela za istraživanje patogenosti EHEC. Može se zaključiti da se jednodnevni kunići mogu rabiti za proučavanje patogeneze crijevne infekcije uzrokovane EHEC te da šiga toksin i drugi čimbenici virulencije bakterije *E. coli* O157 imaju kritičnu ulogu u nastanku proljeva i upale u pacijenata inficiranih EHEC.

Ključne riječi: patogenost, jednodnevni kunići, *Escherichia coli* O157, čovjek, životinje