Haemolytic–uraemic syndrome caused by a non-O157:H7 *Escherichia coli* strain in experimentally inoculated dogs

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Both O157:H7 and non-O157:H7 *Escherichia coli* strains are reported to cause haemolytic–uraemic syndrome (HUS). This study was carried out to explore the pathogenicity of O157:H7 and non-O157:H7 *E. coli* strains in experimentally inoculated dogs. Twenty 40-day-old dogs were randomly divided into four groups, and the groups ($n=5$) were administered orally with *E. coli* O157:H7 strains HJ2001-1 (from a patient with serious haemorrhagic diarrhoea) and HZ2001-4 (from a domestic sheep kept in the house of a patient who died from diarrhoea and subsequent acute renal failure), HZ2001-9 (a non-O157:H7 strain, from a 6-month-old child who died from diarrhoea and subsequent acute renal failure) or a control strain, EC8099. HJ2001-1 and HZ2001-4 caused slight diarrhoea, and the dogs recovered without any complications. However, HZ2001-9 resulted in watery diarrhoea accompanied with slightly bloody stools, followed by death on the fifth or sixth day. In the fatally infected experimental animals, necrotic lesions in the liver and bacterial embolism in the kidney were observed. The primary cause of death was microvascular thrombosis caused by the bacteria, leading to renal and multiple organ failure. Therefore, the non-O157:H7 *E. coli* strain HZ2001-9 causes clinical signs and pathological lesions in dogs that are consistent with those in acute renal failure or HUS in humans.

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INTRODUCTION

Haemolytic–uraemic syndrome (HUS) is characterized by acute haemolytic anaemia, thrombocytopenia and acute renal failure. Karmali *et al.* (1983a) showed that diarrhoea-associated HUS was caused by *Escherichia coli* strains of O157:H7 serotype, which produce potent cytotoxins called Shiga-like toxins. The specific strains were described as enterohaemorrhagic *E. coli* (EHEC). Concurrently, the association between HUS and infection with verotoxin-producing *E. coli* (VTEC) was reported (Johnson *et al.*, 1983; Karmali *et al.*, 1983a; O'Brien *et al.*, 1983).

EHEC O157:H7, as a newly emerging enteric pathogen, can cause serious diseases in humans (Bell *et al.*, 1994; Boyce *et al.*, 1995; Karmali *et al.*, 1983b; Su & Brandt, 1995). Although some studies have indicated that HUS occurring in diarrhoea outbreaks is associated with *E. coli* O157:H7 infection, the pathogenesis of EHEC O157:H7 leading to acute renal failure or HUS remains unclear. Heydermen *et al.* (2001) questioned why, if the glomerular lesions were toxin-mediated, was there a delay of 5–7 days between the onset of

diarrhoea, when *E. coli* O157:H7 and its toxin were most easily detected in the stool, and the subsequent development of HUS. In addition, most HUS cases resulting in renal failure during childhood were of uncertain aetiology (Blaser, 2004).

In 2000, a few patients in Shuixian county of China died from diarrhoea and subsequent acute renal failure. Although *E. coli* O157:H7 was isolated from the stools of domestic sheep in the region, *E. coli* O157:H7 was not isolated from stools of the dead patients (Zhang *et al.*, 2002). In 2001, a 6-month-old boy died from renal failure after diarrhoea, but *E. coli* O157:H7 could not be isolated from his stools. However, a bacterium was isolated that could not be serotyped by EHEC serotyping and thus was classified as a non-O157:H7 *E. coli* strain.

According to previous studies, it is difficult to detect VTEC or other pathogens in cases with HUS (Blaser, 2004; Bonnet *et al.*, 1998; Dundas *et al.*, 2001; Willshaw *et al.*, 1994; Yamada *et al.*, 1994; Zhang *et al.*, 2002). Therefore, the present study was carried out to explore the pathogenicity of O157:H7 and non-O157:H7 *E. coli* strains in experimentally inoculated dogs using different *E. coli* strains that had been collected in our centre. We observed that the non-O157:H7 *E. coli* strain described above caused clinical signs

Abbreviations: BUN, blood urea nitrogen; EHEC, enterohaemorrhagic *E. coli*; Ehly, enterohaemolysin; HUS, haemolytic–uraemic syndrome; VTEC, verotoxin-producing *E. coli*.

and pathological lesions in dogs that are consistent with those in HUS in humans.

METHODS

Bacterial strains and their characteristics. The experimental strains used in this study were two O157:H7 *E. coli* strains and one non-O157:H7 *E. coli* strain, which were designated HJ2001-1, HZ2001-4 and HZ2001-9, respectively. Strain HJ2001-1 was isolated from a 17-year-old female patient who complained of severe abdominal pain, fever and serious haemorrhagic diarrhoea, but recovered after antibiotic treatment, as reported previously (Wang & Jin, 2002). HZ2001-4 was isolated from a stool of a domestic sheep in the home of a patient who died from HUS and HZ2001-9 was isolated from a stool of a 6-month-old boy who died from acute renal failure as mentioned above. He suffered acute diarrhoea at his home in the countryside and was sent to a city hospital, where he refused foods and exhibited lethargy and decreased urine volume. The patient died 5 days later of acute renal failure. A non-pathogenic *E. coli* strain, EC8099 (provided by the Chinese National Centre for Disease Control, Beijing, China), was used as a negative control.

The three strains, HJ2001-1, HZ2001-4 and HZ2001-9, were identified as *E. coli* using standard methods, based on their characteristic reactions in triple-sugar-iron agar, the ability to produce indole and to hydrolyse *o*-nitrophenyl galactoside and the inability to split urea. The serogroups of the strains were confirmed by O157-specific latex agglutination and other EHEC serum-agglutination tests (Karch *et al.*, 1996; Zhou & Zao, 2001). They were serotyped for their ‘O’ and ‘H’ antigens. The diagnostic serum was provided by the National Institute of Biological Products and Centre for Disease Control, Shanghai. Strains HJ2001-1 and HZ2001-4 were unable to ferment sorbitol on sorbitol MacConkey agar plates, while strain HZ2001-9 was able to ferment sorbitol. Strain HZ2001-9 was identified by both biochemical and serological tests. Phenylalanine decarboxylase, ornithine decarboxylase, H₂S, Voges–Proskauer and oxidase tests were all negative and lysine decarboxylase and nitrate reduction tests were positive. Washed sheep-blood agar plates were used for detection of enterohaemolysin (Ehly) (Beutin *et al.*, 1996).

Multiplex PCR assays were used to detect whether these strains possessed the *eaeA*, *rfbO157*, *stx1*, *stx2* and *hlyA* genes (Ma *et al.*, 2002; Paton & Paton, 1998). PCR primer pairs were designed with reference to published sequence data (Table 1) (Paton & Paton, 1998).

The strains were inoculated into 100 ml Luria–Bertani (LB) broth, followed by culture at 37 °C. Bacterial suspensions were obtained in the exponential growth phase and then diluted in LB broth to contain 1 × 10⁷ c.f.u. ml⁻¹.

Animal experiments and sampling procedures. All procedures were performed with the permission of the Henan Provincial Management Committee of Experimental Animals and complied with the Experimental Animal Standard of the Ministry of Health of P. R. China.

Twenty 40-day-old specific-pathogen-free domestic dogs, weighing 400–500 g, were provided by the Zhengzhou University Experimental Animal Centre, Henan Province, China. They were fed with normal cows’ milk with a supplement of essential nutrients (provided by the Experimental Animal Centre), and all animals were healthy. Before inoculation, these dogs had received a 10-day adaptation to the surrounding environment, and periodic culture of rectal swabs was performed. Dogs did not have diarrhoea, and pathogenic *E. coli* or other pathogens were absent from the gastrointestinal tract. Dogs were divided randomly into four experimental groups, five in each. Group A was inoculated with strain HJ2001-1, group B with strain HZ2001-4, group C with strain HZ2001-9 and group D with strain EC8099. Each dog was inoculated orally once only with 5 ml bacterial suspension containing 5 × 10⁷ c.f.u. through a stomach tubule (Barrow *et al.*, 1998).

Animals were observed visually at 4 h intervals after inoculation (Gunzer *et al.*, 2002). Clinical symptoms were recorded in detail and diarrhoeic stools were sampled every day if present. Blood and urine samples were obtained before and 5 days after inoculation, when the animals showed severe clinical symptoms (if any). The levels of serum potassium, sodium, blood urea nitrogen (BUN) and creatinine and urine albumin, ketone bodies, red blood cells, specific gravity, sodium, creatinine and pH were also examined. Since all dogs in group C died from diseases associated with *E. coli* infection, the animals in this group were dissected immediately after death and tissue samples of different organs including kidney, liver, spleen and intestine were collected for bacterial isolation and histological examinations. Dogs in groups A, B and D were killed on the sixth day after inoculation. At necropsy, tissue samples of different organs were obtained for bacterial isolation and histological examinations.

Bacterial examinations. Any bacteria isolated from stool, blood and/or tissue samples of kidney and/or liver were inoculated into 100 ml LB broth and incubated for 6 h at 37 °C. Bacteria were then isolated by the immunomagnetic separation method and cultured

Table 1. PCR primers used in the experiments

Gene	Primer	Product (bp)
<i>stx1</i>	Forward: 5'-ATAAATCGCCATTCTGACTAC-3'	180
	Reverse: 5'-AGAACGCCCCACTGAGATCATC-3'	
<i>stx2</i>	Forward: 5'-GGCACTGTCTGAAACTGCTCC-3'	255
	Reverse: 5'-TCGCCAGTTATCTGACATTCT-3'	
<i>eaeA</i>	Forward: 5'-GACCCGGCACAAGCATAAGC-3'	384
	Reverse: 5'-CCACCTGCAGCAACAAGAGG-3'	
<i>hlyA</i>	Forward: 5'-GCATCATCAAGCGTACGTTCC-3'	534
	Reverse: 5'-AATGAGCCAAGCTGGTTAAGCT-3'	
<i>rfbO157</i>	Forward: 5'-CGGACATCCATGTGATATGG-3'	259
	Reverse: 5'-TTGCCTATGTACAGCTAATCC-3'	

Table 2. Characteristics of *Escherichia coli* strains

+, Positive serum agglutination test or presence of the gene; –, negative serum agglutination test or absence of the gene. Ehly was detected on washed sheep-blood agar plates.

Strain	Serotype		Genotype				Ehly
	O157	H7	<i>stx1</i>	<i>stx2</i>	<i>eae</i>	<i>hlyA</i>	
HJ2001-1	+	+	+	+	+	+	+
HZ2001-4	+	+	–	+	+	+	+
HZ2001-9	–	–	–	–	–	–	+
EC8099	–	–	–	–	–	–	–

on sorbitol MacConkey agar plates and CHROMagr O157 plates (Karch *et al.*, 1996). Isolated strains were identified by the bioMérieux Vitek AMS system and GNI⁺ check (Wales *et al.*, 2001; Wang & Jin, 2002; Zhang *et al.*, 2002) and confirmed by multiplex PCR assays (Ma *et al.*, 2002; Zhou & Zao, 2001). Confirmed strains were finally subject to serotyping.

Histological examinations of tissues. Tissue samples of kidney and liver were fixed in 10% neutral formalin-buffered solution, dehydrated with alcohol and embedded in paraffin. Sections (4 µm thick) were cut and stained with haematoxylin and eosin (H&E) for light microscopy (Dean-Nystrom *et al.*, 1997, 1998; Gunzer *et al.*, 2002; Wales *et al.*, 2001).

Statistical analysis. Data on serum and urine biochemical indices were expressed as means ± SD. The difference in these indices before and 5 days after inoculation was analysed by a *t* test; *P* < 0.05 was considered as statistically significant.

RESULTS

Attributes of strains used

Strain HZ2001-9 was negative for *eaeA*, *rfbO157*, *stx1*, *stx2* and *hlyA* genes by the multiplex PCR and single PCR assays, but positive for Ehly as detected on washed sheep-blood agar plates (Beutin *et al.*, 1996). Strain HZ2001-9 was also negative for O157 and H7 serotyping and, thus, this strain did not belong to any identified serotype of *E. coli* (Table 2). In contrast, strain HJ2001-1 was positive for all markers tested and strain HZ2001-4 was negative only for *stx1*.

Clinical findings

All dogs in groups A and B developed acute watery or mucoid diarrhoea on the second day after inoculation of the bacteria, accompanied with slightly bloody stool. Decreased appetite, nausea and vomiting were observed. Symptoms caused by infection with *E. coli* O157:H7 strain HZ2001-1 in group A were more severe than those caused by *E. coli* O157:H7 strain HJ2001-4 in group B. All these animals recovered 1 or 2 days later without any treatment (Table 3).

Dogs in group C developed nausea and vomiting, followed by watery or mucoid diarrhoea with slightly bloody stools on the second day after inoculation with *E. coli* HZ2001-9. Diarrhoea persisted for 2 days. The symptoms then became aggravated, although diarrhoea diminished. The dogs refused any food and water, with dramatic weight loss and moderate or severe dehydration, fatigue, lethargy and a

Table 3. Clinical symptoms of dogs after oral inoculation with different *E. coli* strains

Each group contained five animals; values are number showing symptom/total number of animals. –, No clinical symptoms.

Animal group	Time after inoculation (days)	Anorexia	Nausea	Diarrhoea	Bloody stools	Coma	Spasm	Difficulty in swallowing	Urine reduction	Death
A (strain HJ2001-1)	1	–	3/5	–	–	–	–	–	–	–
	2	1/5	2/5	1/5	–	–	–	–	–	–
	3	2/5	–	5/5	4/5	–	–	–	–	–
	4	–	–	5/5	3/5	–	–	–	–	–
	5	–	–	–	–	–	–	–	–	–
B (strain HZ2001-4)	1	–	2/5	–	–	–	–	–	–	–
	2	–	1/5	3/5	–	–	–	–	–	–
	3	2/5	1/5	3/5	–	–	–	–	–	–
	4	–	–	2/5	–	–	–	–	–	–
	5	–	–	–	–	–	–	–	–	–
C (strain HZ2001-9)	1	–	–	–	–	–	–	–	–	–
	2	3/5	3/5	4/5	–	–	–	–	–	–
	3	3/5	2/5	4/5	–	–	–	–	–	–
	4	4/5	–	5/5	3/5	1/5	–	1/5	–	–
	5	4/5	–	5/5	5/5	4/5	2/5	3/5	5/5	–
	6	5/5	–	1/5	3/5	4/5	3/5	5/5	5/5	4/5
	7	–	–	–	1/5	–	1/5	1/5	1/5	1/5
D (strain EC8099)	1–6	–	–	–	–	–	–	–	–	–

noticeable decrease in urine volume. One of five animals showed a seizure and died on the fifth day. In addition, all dogs had neurological dysfunction, such as cerebral infarction, blindness and coma. All five animals in this group died within 5 days after the initial onset of diarrhoea (Table 3).

Necropsy and serum and urine biochemical changes

At necropsy, there was no visible inflammation or damage in the stomach, intestine, kidney or liver of animals in groups A and B. Some petechiae were observed on the surface of one animal's kidneys. Organs of all control animals (group D) appeared normal.

In group C, inflammation and oedema were visible in the small and large intestines of the animals. Kidneys appeared pale, with a few petechiae on the surface, and livers were also visibly inflamed and swollen. There was little urine output on the fifth day post-inoculation. The decrease in total urine output was accompanied by an increase in serum BUN concentrations and the presence of red blood cells and protein in urine, and ketone bodies were increased significantly (Table 4).

Bacteriological and pathological findings

All *E. coli* strains in groups A, B and D colonized well within 24 h after inoculation. The *E. coli* strain HZ2001-9 in group C was recovered from stools at 24 h after inoculation and during the period of diarrhoea. The bacterium was also isolated from samples of kidney, liver and colon after

dissecting the dead dogs. However, no bacteria were isolated from cystic urine.

Stained tissue sections of kidney and liver were examined by light microscopy. No pathological changes were seen in sections of kidneys or livers of animals in group A, B and D (Fig. 1a, b). In group C, the bacteria colonized and accumulated in the blood vessels of the renal medulla and cortex, and many bacterial embolisms were observed in the renal medulla (Fig. 1c, d). Significant pathological changes such as hyperaemia, oedema and inflammation were also observed in the livers. Moreover, hyperaemia of the central vein and local necroses were observed in the hepatic lobules (Fig. 1e, f).

DISCUSSION

Great efforts have been made in the investigation of the role of Shiga-toxin-producing *E. coli* (STEC), especially O157:H7 *E. coli* strains associated with HUS (Johnson *et al.*, 1983; Karmali *et al.*, 1983b; O'Brien *et al.*, 1983; Perna *et al.*, 2001; Thorpe, 2004). However, little is known about the role of Shiga-negative (verocytotoxin was not identified) non-O157:H7 *E. coli*. In recent years, sporadic outbreaks of HUS cases have been reported, but O157:H7 *E. coli* or other Shiga-toxin-positive pathogens were not isolated from some of these HUS patients (Blaser, 2004; Chandler *et al.*, 2002; Dundas *et al.*, 2001; Kaplan & Drummond, 1978; Karmali *et al.*, 1985; Thorpe, 2004). Karmali *et al.* (1985) carried out a study to determine the relationship between VTEC strains and HUS. In total, 40 patients with idiopathic HUS were examined, along with an equal number of age- and sex-matched control subjects. Thirty of the 40 patients and none of the control subjects had evidence of VTEC infection (Karmali *et al.*, 1985). In 1996, there was an outbreak of *E. coli* O157:H7 infection in central Scotland (Dundas *et al.*, 2001). Dundas *et al.* (2001) retrospectively investigated the risk factors for HUS among all hospitalized patients during this outbreak, but no *E. coli* O157:H7 or other pathogenic organisms were isolated from stool cultures of patients with HUS, which was similar to our previous study in 2000 (Zhang *et al.*, 2002).

Our study demonstrated that dogs infected with the non-O157:H7 *E. coli* strain HZ2001-9 presented with clinical symptoms that were identical to HUS in human patients, confirming that this strain is able to cause HUS and subsequent death in experimental dogs. We further postulate that this strain was the pathogen that caused HUS and subsequent death of the 6-month-old boy. In fact, we have frequently isolated Shiga-toxin-negative *E. coli* from stool samples of patients with acute renal failure or HUS. According to the literature, HUS patients who die from diarrhoea generally have several days of acute diarrhoea, and the disease then deteriorates rapidly. Acute renal failure follows when diarrhoea ceases, and the volume of urine declines. These patients commonly die within 5–14 days after the onset of diarrhoea (Goldfarb & Adler, 2001; Gunzer *et al.*,

Table 4. Serum and urine diagnostic indices in experimental dogs in group C ($n=5$)

Data are expressed as means $\pm$ SD. –, Negative; w, weakly positive; +, positive; ++, strongly positive.

Biochemical variable	Baseline	Value on day 5 post-inoculation
Serum		
Potassium (mmol l ⁻¹)	6.55 $\pm$ 1.2	5.34 $\pm$ 1.1
Sodium (mmol l ⁻¹)	150.7 $\pm$ 8.6	123.0 $\pm$ 6.3*
BUN (mmol l ⁻¹)	27.9 $\pm$ 5.7	71.0 $\pm$ 11.2†
Creatinine (μ mol l ⁻¹)	137.0 $\pm$ 9.9	158.0 $\pm$ 7.5†
Urine		
Albumin	–	w
Acetone body	–/w	++
Red blood cells	–	+
Specific gravity	1.020 $\pm$ 0.04	1.015 $\pm$ 0.03
Sodium (mmol l ⁻¹)	113.10 $\pm$ 8.5	38.40 $\pm$ 13†
Creatinine (mmol l ⁻¹)	4.2 $\pm$ 0.8	1.3 $\pm$ 0.7*
pH	5.0 $\pm$ 0.5	5.5 $\pm$ 0.6

* $P<0.05$, compared with the baseline level.

† $P<0.001$, compared with the baseline level.

Fig. 1. (a, b) Light micrographs of normal tissues of kidney (a) and liver (b) from a dog inoculated with the non-pathogenic *E. coli* strain EC8099. H&E staining; original magnification $\times 200$. (c, d) H&E-stained sections of the renal medulla of a dog inoculated with non-O157:H7 *E. coli* strain HZ2001-9, showing bacterial infarctions within the tubules. Original magnification $\times 400$. (e, f) H&E-stained section of the liver of a dog inoculated with strain HZ2001-9, showing expansion and hyperaemia of the central vein in the hepatic lobule (e) and focal liver necrosis (f). Original magnification $\times 400$.

2002; Paton & Paton, 1998; Zhang *et al.*, 2002). In addition, our previous study demonstrated that the patients had severe liver, brain and neurological dysfunction in the disease process, such as increased pressure in the brain, coma and liver function damage. These patients entered into the multiple organ failure phase quickly when they had a reduced urine volume (Zhang *et al.*, 2002). In our present study, the clinical symptoms of the experimental animals resembled those of human patients.

In the present study, both bacterial culture and stained tissue sections showed that bacteria had invaded the blood of the

animals. The bacteria colonized and accumulated in the blood vessels of the renal medulla and cortex. However, no bacteria were isolated from the cystic urine in our study, which is in agreement with the previous observation by Chantrey *et al.* (2002), indicating that the bacterium did not enter into the cystic urine. In another study, by Holloway *et al.* (1993), *E. coli* was isolated in stools of only one of three dogs with HUS. However, the faecal isolate could not be serotyped, and a verocytotoxin was not detected.

Our present study showed that a Shiga-toxin-negative *E. coli* led to the death of the animals. Evolutionary studies have

revealed that the continuing emergence of EHEC O157:H7 as a pathogen accounts for its rapid genetic change (Eisen, 2001; Hacker *et al.*, 1997; Kaplan & Drummond, 1978). In the present study, although multiplex PCR assays did not detect *eae*, *hlyA* or other virulence genes in this pathogen, Ehly was positive, as detected on washed sheep-blood agar plates. We hypothesize that *E. coli* strain HZ2001-9 is a mutant strain originating from *E. coli* O157:H7 and that the pathogen still contains virulence genes, but these virulence genes cannot be detected by present PCR assays due to translocation or deletion within the genes. This needs to be further confirmed.

We also demonstrated that the two tested *E. coli* O157:H7 strains (HJ2001-1 and HZ2001-4) did not cause the death of animals in the experiment, although strain HJ2001-1 contained virulence genes *stx1*, *stx2*, *hlyA* and *eaeA*. Thus, we believe that not all *E. coli* O157:H7 strains lead to HUS and subsequent death in humans and animals. In fact, only approximately 2–15% of people with STEC infection develop HUS, of which 10% die or have permanent renal failure (Dundas *et al.*, 2001; Thorpe, 2004).

Finally, different animal models of infection with O157:H7 pathogens have been reported (Dean-Nystrom *et al.*, 1998; Gunzer *et al.*, 2002). However, the animals did not develop clinical symptoms and thus had to be killed for pathological examinations. Therefore, our studies may help in understanding the aetiological and pathological role of non-O157:H7 *E. coli* or *E. coli* O157:H7 in HUS and thus in the prevention and treatment of the syndrome.

In conclusion, the non-O157:H7 *E. coli* strain HZ2001-9 is a pathogen that can cause HUS and subsequent death of experimental dogs, and was most likely responsible for the death of the 6-month-old boy. Although it is widely believed that Stx exerts a direct toxic effect on the kidney, our result suggests that the microvascular thrombosis of bacteria is an obvious pathological lesion, which may be closely associated with acute renal failure or HUS.

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