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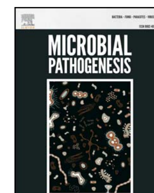

Prevalence, quantification and isolation of pathogenic shiga toxin *Escherichia coli* O157:H7 along the production and supply chain of pork around Hubei Province of China

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ABSTRACT

Shiga toxin *Escherichia coli* (STEC) O157:H7 is an important zoonotic food borne pathogen causing gastroenteritis that may lead to life threatening hemorragic colitis (HC) and hemorrhagic uremic syndrome (HUS). 325 meat and tissue samples were tested for enumeration of O157:H7 strains using most probable number (MPN)-PCR targeting their specific genes *flicH7* and *rfbO157* followed by isolation, sereotyping and pathogenicity testing. The overall prevalence of O157:H7 was 41.3% (134/325) along the production and supply chain of pork (PSCP), being higher in supply chain (59%, 118/200) as compared to pig farms (12.8%, 16/125). Along the PSCP, the highest prevalence was found in slaughter houses (86.25%, 69/80) followed by wet- (53.3%, 32/60) and super-markets (28.3%, 17/60). The MPN values ranged from 3 to 1100 MPN/g in overall positive samples, being higher in slaughter houses followed by wet and super markets. Except from intestine and meat samples of slaughter house, the MPN was found higher in summer as compared to winter samples. Eight STEC O157:H7 isolated from meat and liver samples were tested in Balb/C mice for pathogenicity. After development of clinical signs and symptoms, 50–83.3% mortality was produced in the infected mice. Histopathological investigations revealed visible necrosis of intestinal epithelial cells, shedding of cellular debris in the intestine, while in the kidney, necrosis of renal cortical portion of tubular epithelial cells was observed. STEC O157:H7 is prevalent along PSCP around Hubei of China in different proportions being alarmingly higher in supply chain and markets which is a matter of concern for public health.

1. Introduction

Shiga toxin *Escherichia coli* (STEC) are food-borne pathogens implicated in human infections. There are more than 400 serotypes of STEC, and among them O157:H7 is the principal serotype [3] and [4]. In 1999, the first ever severe outbreak of *E. coli* O157:H7 occurred in Xuzhou of China which caused the death of 177 people [36]. In countries like Germany, Australia and the UK, non-O157 STEC infections prevail [2] and [34]. STEC strains are characterized by their ability to cause severe life threatening risks in humans, such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura(TTP). Bloody diarrhea and hemorrhagic colitis (HC) are other symptoms of STEC. Young children, elderly people and immunocompromised individuals are more prone to STEC infections than healthy adults [11]; [25]. Globally, it is one of the principal causes of acute renal failure in young children [28,35] and [14]. Globally, outbreaks and sporadic cases of STEC infections have been reported that pose a great threat for human health [8] [29]; and [33]. Different prevalence of STEC in pigs production system is reported previously in different countries, such as 7.5% in Switzerland [15], 78% in China [24], 0.2–2% in Hong Kong [20], 15% in France [6], 4.8% in Canada [5] and 4.2% in Czech republic [17]. Similarly different prevalence of STEC in pork sale markets

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is reported by different countries including 50% in USA [22], 14% in Germany [23] and 15% in Korea [19].

Keeping in view the importance of O157:H7, we carried out this study to probe prevalence, quantification of O157:H7 using MPN PCR followed by isolation along the PSCP. For pathogenicity, O157:H7 strains were tested in Balb/C mice.

2. Materials and methods

2.1. Sample collection and processing

A total of 325 samples were used in the study. From the pig production system we collected 125 tonsil swabs from 4 to 6 weeks old clinically healthy pigs from different herds of five intensive pig farms (25 sample/farm) located in the vicinity of Hubei province. Tonsil swab was taken by scraping the tonsil surface thrice with a sterile bamboo tongue-spatula after gently opening the pig mouth with a mouth gag, and immediately put into a sterile plastic tube and transported to laboratory. From the pork supply chain, samples were purchased from different markets in Hubei province, including 80 samples from two different slaughter houses (20 each meat, liver, intestine and kidney), 60 each from six different wet and super markets (30 each meat and liver). Meat samples in wet and super markets were from the same slaughter houses. Samples were collected in sterile plastic bags and subjected for further process immediately upon arrival to the laboratory. Upon arrival to the laboratory, tonsil swabs were immediately washed with 0.5 ml PBS, and 0.1 ml of them was inoculated into 9 ml Brain heart infusion broth (BHIB; BD Difco, USA) at 37 °C for 24 h. Out of 200 g meat and tissue samples purchased from the different slaughter houses and markets, 50 g was cut into small pieces and mixed with 450 ml BHIB (BD Difco, USA) in homogenizer (250 Watt, Type MJ-25BM05A, Guangdong Midea Electrical Appliance Co., Ltd., Foshan, China) for 2 min. One ml of homogenized sample was mixed with 9 ml of BHIB and incubate at 37 °C for 24 h for further bacterial isolation. Sample homogenate was further used for detection, MPN-PCR and isolation of *E. coli* O157:H7 as described below.

2.2. MPN series for enumeration of E. coli O157:H7

E. coli O157:H7 in sample homogenate was enumerated using three-tube MPN method as follows. For each sample homogenate, a tenfold serial dilution series was prepared using Butterfield's buffered phophate diluent (BBPD). Then 1 ml of each original homogenate was transferred to 9 ml BHIB in each of the three MPN tubes. Inoculated tubes and uninoculated control tube were incubated at 37 °C for 24 h. After incubation, 1 ml of each MPN tube was processed for total DNA extraction using E.Z.Nce.A bacterial DNA kit (Omega Bio-Tek, USA). Using multiplex PCR, *rfbO*157 and *flicH7* genes were detected. The primers and other PCR conditions used for *rfbO*157 and *flicH7* genes are given in Table 1.

2.3. Bacterial isolation and DNA extraction

The positive samples were processed for bacterial isolation. From the original homogenate stored at −20 °C, a loop full was inoculated onto MacConkey sorbitol agar (Difco, USA) followed by incubation at 37 °C for 24 h. Typical white color colonies (three colony/sample) were selected for further analysis. *E. coli* O157:H7 was confirmed using standard bacteriological biochemical tests using an API 20E system (bioMerieux, France). Genomic DNA was extracted from the *E. coli* O157:H7 isolates using E.Z.Nce.A bacterial DNA kit (Omega Bio-Tek, USA). The genomic DNA was tested for *rfbO*157 and *flicH7* and shiga toxins genes (*Stx1* and *Stx2*) using the specific primers as described. The details of genes and primers are given in Table 1. PCR amplifications were performed using 2 × Es Taq PCR master mix providing a concentration of 3 mM of $MgCl_2$, Taq DNA polymerase, 2 × Es Taq PCR buffer and 400 μM dNTP mix (CWBIO, China), 200 ng template DNA and 10 μM of each primer in a total reaction volume of 25 μl. The thermal cycling condition include an initial incubation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min and final extension at 72 °C for 10 min. PCR products were run on 2% agarose E-gels pre-stained with Gel red (Invitrogen A/S, Taastrup, Denmark) and visualized by UV-lighting.

2.4. Pathogenicity testing of E. coli O157:H7 using Balb/C mice

For pathogenicity testing of STEC O157:H7 strains isolated along PSCP, 5–6 weeks old Balb/c female mice were used. Animals were housed in filter top cages in air condition facility of animal house centre of Huazhong Agriculture University, Wuhan, China according to institutional guidelines for the use of experimental animals. Streptomycin treated mouse model was used in this study as previously described [31]. In this model, mice were provided with streptomycin sulphate in their drinking water as a means to reduce their normal facultative flora so as to decrease bacterial competition for the infecting O157:H7 strain. A total of 8 O157:H7 strain were tested. Because only 8 strain were isolated positive for *Stx1* and *Stx2* genes. That's why only 8 strains were tested for pathogenecity in balb/C mice. A group of 6 mice were used for each strain. The mice were provided with streptomycin sulphate 5 g/L in drinking water and were kept off fed for 12 h. After 12 h, mice were infected with 10^9 CFU of O157:H7 in 20% sucrose solution intragastrically (I/G). Proper dose of the pathogen is required to produce infection in mice which can only be achieved through intragastric route while oral route cannot guarantee proper dose administration as there is possibility that the pathogen may come outside from the oral cavity. Colonization, morbidity including typical signs and symptoms, and mortality were noted.

2.5. Histopathology

The intestine and kidney were removed surgically from the dead mice/or and immediately after the mice were killed. Both the intestine and kidney were examined for pathological changes. The tissues were fixed in 10% formalin. The perfused tissue (intestine and kidney) was

Table 1
Primers and other PCR conditions used for target genes.

Target class	Target gene	Primers(5′–3′)	Amplicon size (bp)	Annealing temp(°C)	Ref.
Flageller antigen	*flicH7-F*	GCG CTGTCGAGTTCTATCGAG	625	57	[26]
	flicH7-R	CAA CGGTGACTTTATCGCCATTCC			
Somatic antigen	*rfb O157-F*	CGG ACATCCATGTGATATGG	259	57	[13]
	rfb O157-R	TTGCCTATGTACAGCTAATCC			
Shiga toxin 1	*stx1-F*	AGTTAATGTGGTGGCGAA	817	55	[16]
	stx1-R	GACTCTTCCATCTGCCG			
Shiga toxin 2	*stx2-F*	TTCGGTATCCTATTCCCG	474	55	[16]
	stx2-R	TCTCTGGTCATTGTATTA			

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