ABSTRACT

Escherichia coli O157:H7 is a major cause of zoonotic food-borne infections transmissible from asymptomatic animals to humans following consumption of contaminated foods. Pathogenicity of E. coli O157:H7 is attributed to possession of virulence genes such as eaeA and stx responsible for intimate adhesion to enterocytes and production of cytolethal shiga toxins. The pathogenic potentials of five E. coli O157:H7 isolates of different virulence gene profiles recovered from the faeces of slaughter cattle was compared in rabbit model. Five groups (A-E) of five rabbits were each inoculated orally with $5 \times 10^9$ colony forming units of an E. coli O157:H7 isolate possessing one of the virulence gene profiles: stx1/stx2/eaeA/hlyA (group A), stx1/stx2 (B), stx2 (C), stx1 (D), and eaeA/hlyA (E). Group F (control) received sterile broth. The mean onset and duration of clinical manifestations varied significantly among the experimental groups being earliest and shortest in group infected with E. coli O157:H7 possessing stx1/stx2/eaeA/hlyA. Infected rabbits showed clinical signs including dullness, profuse non-bloody diarrhoea, weakness, anorexia and epistaxis starting from two days post infection (p.i.). Epistaxis was observed only in rabbits inoculated with isolates that possessed stx2 either alone or in combination with other virulence genes. Mortality of 100% was recorded in isolates with stx1/stx2/eaeA/hlyA, stx1/stx2 and stx2 and 60% with stx1 and eaeA/hlyA. Test organisms were detected in the faeces of inoculated animals as from two days p.i. and persisted in survivors for 19 to 30 days p.i. This study showed that E. coli O157:H7 isolates from cattle produced fatal illness in experimental rabbits and that virulence gene profile significantly influenced the onset, duration and severity of clinical manifestation of infection in the experimental animals.

Keyword: Clinical manifestation, E. coli O157:H7, experimental infection, rabbits, virulence, gene profile.
INTRODUCTION

Escherichia coli O157:H7 is a leading cause of acute gastroenteritis leading to fatal haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) in humans (Riley et al., 1983; Rangel et al., 2005). Although food animals especially ruminants are natural reservoirs of E. coli O157:H7 (Zschöck et al., 2000; Ojo et al., 2010), the pathogenicity of the organism in food animals is low as clinical infection appears to occur only occasionally in young animals while no serious disease condition is recorded in adults (Stoffregens et al., 2004; Twardon et al., 2005; Dean-Nystrom et al., 2008). In contrast, humans are very susceptible to E. coli O157:H7 infection. Fewer than 10 bacterial cells of E. coli O157:H7 are able to establish clinical infection in humans (Twardon et al., 2005). Most cases of E. coli O157:H7 infections in humans are due to the consumption of foods and water contaminated with faeces of asymptomatic carrier animals (Tauxe, 1997; Mead et al., 1999).

The preponderance of E. coli O157:H7 in the faeces of ruminants and non-adherence to principles of hygiene during processing and marketing of meat facilitate contamination (Ojo et al., 2010). Consequently, consumers are at risk of possible exposure to this dangerous pathogen. However, infections with E. coli O157:H7 strains do not always lead to clinical disease and in cases of clinical infections, presenting signs may differ according to the strains involved. The severity of E. coli O157:H7 infections in humans is influenced by the possession of virulence genes including shiga toxin 1 and 2 (stx1 and stx2), intimin (eaeA) and enterohemolysin (hlyA) genes (Paton and Paton, 1998). The presence of these virulence genes either alone or in combination in E. coli O157:H7 strains determines the clinical outcome of infection (Dean-Nystrom et al., 2003). Pathogenic STEC colonizes the large intestine, adheres to the enterocytes and produces shiga toxins. Escherichia coli O157:H7 adherence to intestinal epithelia is enhanced by the possession of intimin (Paton and Paton, 1998). Shiga toxins are transported across the epithelial cells and into the blood circulation from where they are disseminated to other organs (Paton and Paton, 1998). The toxins have profound effect on the endothelial cells of blood vessels and cause endothelial damage. Toxins are responsible for non-bloody to bloody diarrhoea, abdominal cramp and in some cases renal damage leading to HUS (Paton and Paton, 1998).

The objective of the present study is to verify the pathogenic potentials of five E. coli O157:H7 field isolates of different virulence gene profiles and compare clinical outcome of their infection in a rabbit experimental model. These isolates were originally recovered from non-diarrhoeic faeces of slaughter cattle at an abattoir in Ibadan, Nigeria.

MATERIAL AND METHODS

This experiment conformed to study protocols specified by the European Union Directive 86/609 and Council for International Organizations of Medical Sciences (CIOMS). It was approved, monitored and ethically certified by the Experimental Animal Unit of the Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria.

Bacterial isolates:
The five E. coli O157:H7 isolates possessing stx1/ stx2/ eaeA/ hlyA, stx1/ stx2, stx1, stx2, and eaeA/ hlyA were selected based on their virulence genes profiles and were all originally isolated from non-diarrhoeic faeces of slaughter cattle in an abattoir in Ibadan, Nigeria (Ojo et al., 2010). The methods of detec-
tion of virulence genes and confirmation of E. coli O157:H7 were previously described (Ojo et al., 2010).

**Preparation of inocula:**
Escherichia coli O157:H7 isolates previously preserved on nutrient agar slopes were inoculated onto sorbitol MacConkey agar (SMAC). Two to three pure colonies on SMAC were transferred into 10ml TSB and incubated for 18-24 hours at 37 °C. The TSB culture was centrifuged and the packed bacterial cells in form of sediment at the bottom of the test tube washed twice with phosphate-buffered saline (PBS). The washed bacterial cells were re-suspended in sterile TSB to a final concentration of 5 x 10^9 colony forming units per millilitre (cfu/ml) determined by pour plate count method on SMAC after a tenfold serial dilution of the bacterial suspension in TSB.

**Infection of Animals:**
Thirty weaned New Zealand rabbits of four weeks of age weighing 450 to 500g were divided into six groups (A-F) of five rabbits per group. The rabbits were apparently healthy, E. coli O157:H7-culture negative and had well-formed faeces. Each rabbit was sedated as previously described (Sherman et al., 1988) and orally infected with 5 x 10^9 (cfu/ml) determined by pour plate count method on SMAC after a tenfold serial dilution of the bacterial suspension in TSB.

Inoculated rabbits were observed 12-hourly for signs of illness. An animal was considered to be diarrhoeic if the perineum or/and the hind limbs were soiled with faeces. Rectal swabs were collected from inoculated rabbits daily (throughout the 40 days of the experiment) for the isolation and identification of E. coli O157:H7 using SMAC, E. coli O157 latex agglutination kit (DR0620M, Oxoid, Basingstoke, UK) and H7 antiserum (Difco®, USA).

**Statistical analysis:**
Mean values (± standard deviation) were calculated for animals that showed clinical signs in each group. The differences in mean values across groups were compared by Dun-can’s one way ANOVA at p<0.05 probability level using the Statistical Package for Social Sciences (SPSS) version 16 software (2007).

**RESULTS**

**Clinical findings:**
In this study, all experimental rabbits inoculated with E. coli O157:H7 showed signs of illness. Observed clinical signs were diarrhoea, dullness, anorexia, weakness, epistaxis and death (Table 1). The mean onset of clinical signs varied significantly among the experimental groups (Table 2). Diarrhoea started significantly earliest (3.4±1.1 dpi) (p<0.05) in group inoculated with stx1/stx2/eaeA/hlyA and latest (7.2±2.4 dpi) in group infected with stx1. The diarrhoea started as passage of loose faeces but progressed to profuse watery non-bloody diarrhoea in all the groups. The mean onset of dullness also differed significantly (p>0.05) among some of the experimental groups, being earliest among rabbits with stx1/stx2/eaeA/hlyA (3.2±0.5 dpi) and stx2 (3.6±0.5 dpi) but latest in groups with stx1 (5.2±0.8 dpi) and eaeA/hlyA (5.3±1.0 dpi). Similarly, anorexia started significantly earliest (p<0.05) in rabbits with stx1/stx2/eaeA/hlyA (3.4±0.5 dpi) than in all the other groups. There was no significant difference (p>0.05) in the mean onset of anorexia among groups with stx1 (9.0±
1.4 dpi), \(\text{stx}_2\) (8.8 ± 1.3 dpi) and \(\text{eaeA}/\text{hlyA}\) (8.3 ± 1.2 dpi). Three rabbits from group with \(\text{stx}_1\) and two from group with \(\text{eaeA}/\text{hlyA}\) did not develop anorexia. Weakness occurred significantly earliest (p<0.05) among rabbits with \(\text{stx}_1/\text{stx}_2/\text{eaeA}/\text{hlyA}\) (5.6 ± 0.5 dpi) than in groups with \(\text{stx}_1/\text{stx}_2\) (7.6 ± 1.7 dpi), \(\text{eaeA}/\text{hlyA}\) (9.5 ± 0.7 dpi), \(\text{stx}_2\) (10.6 ± 1.3 dpi). Only one rabbit from the group with \(\text{stx}_1\) showed signs of weakness. Epistaxis occurred in two rabbits from group with \(\text{stx}_1/\text{stx}_2/\text{eaeA}/\text{hlyA}\) at 6.5 ± 0.7 dpi and in one rabbit each from groups with \(\text{stx}_1/\text{stx}_2\) and \(\text{stx}_2\) at 7 dpi and 9 dpi respectively. All rabbits with epistaxis died within 24 hours.

Diarrhoea continued till death in rabbits infected with \(\text{stx}_1/\text{stx}_2/\text{eaeA}/\text{hlyA}\) and \(\text{stx}_1/\text{stx}_2\) as well as in one rabbit each from the other three infected groups. In rabbits infected with \(\text{stx}_2\)-\text{O157:H7}, diarrhoea stopped 2 to 5 days prior to death. Two rabbits each from groups with \(\text{stx}_1\) and \(\text{eaeA}/\text{hlyA}\) survived the diarrhoea episode. The mean durations of diarrhoea and weakness were not significantly (p>0.05) different among the rabbit groups (Table 3). However, the duration of dullness was significantly shorter (p<0.05) in rabbits with \(\text{stx}_1/\text{stx}_2/\text{eaeA}/\text{hlyA}\) (3.8±0.4 days) than in groups with \(\text{stx}_1\) (6.6±3.2 days) and \(\text{stx}_2\) (8.6±1.3 days) but not significantly different from rabbits with \(\text{stx}_1/\text{stx}_2\) (4.6±1.1 days) and \(\text{eaeA}/\text{hlyA}\) (5.7±1.5 days). The duration of anorexia was significantly shorter in group with \(\text{eaeA}/\text{hlyA}\) (2.7±0.6 days) than in the group with \(\text{stx}_1\) (4.5±0.7 days). There was no significant difference (p>0.05) in the duration of anorexia among groups infected with \(\text{stx}_1/\text{stx}_2/\text{eaeA}/\text{hlyA}\) (3.6±0.5 days), \(\text{stx}_1/\text{stx}_2\) (3.4±0.9 days) and \(\text{stx}_2\) (3.4±0.9 days).

Mortality was 100% in groups with \(\text{stx}_1/\text{stx}_2/\text{eaeA}/\text{hlyA}\), \(\text{stx}_1/\text{stx}_2\) and \(\text{stx}_2\) but 60% in groups with \(\text{stx}_1\) and \(\text{stx}_1\). Death occurred significantly earliest (p<0.05) in rabbits from group with \(\text{stx}_1/\text{stx}_2/\text{eaeA}/\text{hlyA}\) (7.0 ± 0.7 dpi) than in groups with \(\text{stx}_1/\text{stx}_2\) (9.2 ± 1.6 dpi), \(\text{eaeA}/\text{hlyA}\) (11.0 ± 1.0 dpi), \(\text{stx}_2\) (12.2±1.6 dpi) and \(\text{stx}_1\) (13.6 ± 1.5 dpi).

Neither signs of illness nor mortality were observed among the rabbits in the control group throughout the period of the experiment.

**Recovery of E. coli O157:H7 from experimentally infected rabbits:**

E. coli O157:H7 was detected in faeces of all the inoculated animals as from 48 hours p.i. The shedding continued till the rabbits died. The two surviving animals from the group infected with \(\text{stx}_2\)-\text{O157:H7} shed the organism for 16 and 19 days post-diarrhoea while surviving animals from group infected with \(\text{eaeA}/\text{hlyA}\)-\text{O157:H7} continued to shed the organisms until the experiment was terminated (30 days post-diarrhoea). None of the faecal samples collected from the control group yielded E. coli O157:H7.
Table 1: Clinical signs observed in rabbits experimentally inoculated with *E. coli* O157:H7 of different virulence gene profiles

<table>
<thead>
<tr>
<th>Observed clinical signs</th>
<th>Frequency of occurrence per group</th>
<th>Group B (stx1/stx2)</th>
<th>Group C (stx1)</th>
<th>Group D (stx2)</th>
<th>Group E (eaeA/hlyA)</th>
<th>Group F (Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dullness</td>
<td>3/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Anorexia</td>
<td>5/5</td>
<td>5/5</td>
<td>2/5</td>
<td>5/5</td>
<td>3/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Weakness</td>
<td>5/5</td>
<td>4/5</td>
<td>1/5</td>
<td>5/5</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>2/5</td>
<td>1/5</td>
<td>0/5</td>
<td>1/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Death</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
<td>5/5</td>
<td>3/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

Table 2: Onset of clinical signs in rabbits experimentally infected with *E. coli* O157:H7 isolates of different virulence gene profiles

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>Mean ± standard deviation (range) days post infection in rabbits showing Signs within groups*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A (stx1/stx2/eaeA/hlyA)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>3.4 ± 1.1a (2.0 – 5.0)</td>
</tr>
<tr>
<td>Dullness</td>
<td>3.2 ± 0.5a (3.0 – 4.0)</td>
</tr>
<tr>
<td>Anorexia</td>
<td>3.4 ± 0.5a (3.0 – 4.0)</td>
</tr>
<tr>
<td>Weakness</td>
<td>5.6 ± 0.5a (5.0 – 9.0)</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>6.3 ± 0.6 (6.0 – 7.0)</td>
</tr>
<tr>
<td>Death</td>
<td>7.0 ± 0.7a (6.0 – 8.0)</td>
</tr>
</tbody>
</table>

* The mean values were determined only for the rabbits that manifested clinical signs out of the five rabbits inoculated in each group. Values with the same superscripts are similar while those with different superscripts differ significantly at p<0.05.
<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>Mean ± standard deviation (range) days post infection in rabbits showing Signs within groups*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Group A (stx1/stx2/ eaeA/ hlyA)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>3.6±0.9a (3.0-5.0)</td>
</tr>
<tr>
<td>Dullness</td>
<td>3.8±0.4a (3.0-4.0)</td>
</tr>
<tr>
<td>Anorexia</td>
<td>3.6±0.5a, b (3.0-4.0)</td>
</tr>
<tr>
<td>Weakness</td>
<td>1.6±0.5a (1.0-2.0)</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>1.0±0.0 (1.0)</td>
</tr>
<tr>
<td>Survival time</td>
<td>7.0±0.7a (6.0-8.0)</td>
</tr>
</tbody>
</table>

*The mean values were determined only for the rabbits that manifested clinical signs out of the five rabbits inoculated in each group. Values with the same superscripts are similar while those with different superscripts differ significantly at p<0.05.
DISCUSSION

Escherichia coli O157:H7 is an important zoonotic pathogen associated with foodborne gastroenteritis in humans. Natural infections often manifest as non-bloody to bloody diarrhoea.

In this study, all the E. coli O157:H7 isolates induced severe non-bloody diarrhoea in weaned rabbits regardless of the virulence genes profile. The mean onset of diarrhoea was shortest in rabbits inoculated with \( \text{stx}1/\text{stx}2/\text{eaeA}/\text{hlyA} \)-O157:H7 (2.6±0.6 dpi) and longest in those inoculated with \( \text{stx}1 \)-O157:H7 (6.5±2.5 dpi). Earlier studies have shown that E. coli O157:H7 induced non-bloody diarrhoea in rabbits (Pai et al., 1986; Sherman et al., 1988; Raji et al., 2009). Raji et al. (2009) reported that day-old infant rabbits inoculated with E. coli O157:H7 developed severe diarrhoea 2 days post infection. In another study, E. coli O157:H7 induced diarrhoea in experimentally infected post-weaning rabbits as from 5 days p.i. (Sherman et al., 1988). In humans, E. coli O157:H7 infection typically begins with an onset of non-bloody watery diarrhoea between 2 and 12 days p.i. which may last 1 to 3 days (Tarr et al., 2005). In most cases, this progresses to bloody diarrhoea which is the hallmark of E. coli O157:H7 infection in humans. However, it appears oral inoculation of E. coli O157:H7 does not induce bloody diarrhoea in rabbits as observed in this study and those by other workers (Pai et al., 1986; Sherman et al., 1988; Raji et al., 2009).

In the present study, 100% mortality was recorded in experimental groups inoculated with \( \text{stx}1/\text{stx}2/\text{eaeA}/\text{hlyA} \)-O157:H7, \( \text{stx}1/\text{stx}2 \)-O157:H7 and \( \text{sstx}2 \)-O157:H7 but 60% in groups inoculated with \( \text{stx}1 \)-O157:H7 and \( \text{eaeA}/\text{hlyA} \)-O157:H7. Survival time was longest in group inoculated with \( \text{stx}1 \)-O157:H7 (13.7±1.5 dpi) and shortest in group inoculated with \( \text{stx}1/\text{stx}2/\text{eaeA}/\text{hlyA} \)-O157:H7 (7.0±0.7). Previous studies have shown that E. coli O157:H7 caused mortality in infant rabbits 4 days p.i. (Raji et al., 2009). Pai et al. (1986) also reported mortality in infant rabbits inoculated with E. coli O157:H7. The earlier onset of mortality observed by Raji et al. (2009) than in the present study could be...
due to the age difference in the experimental rabbits used. It has been observed that the susceptibility of rabbits to *E. coli* O157:H7 is dose and age-dependent (Pai et al., 1986). The quantity of toxin produced by inoculated *E. coli* O157:H7 could also be a factor contributing to the outcome of infection (Wagner et al., 2002; Gamage et al., 2003). However, in contrast to observation in the present study, Ritchie et al. (2003) reported that *E. coli* O157:H7 did not produce mortality in experimentally inoculated infant rabbits. The present study showed that virulence gene profile of inoculated *E. coli* O157:H7 significantly influenced the survival of infected rabbits.

The present study showed that rabbits infected with stx-deficient *E. coli* O157 isolate (eaeA/hlyA-O157:H7) developed diarrhoea with 60% mortality. This *E. coli* strain, with no gene for shiga toxin production, cause significant clinical manifestation in rabbits similar to observations in humans suggesting that stx does not appear to be crucial in the manifestation of clinical diseases following *E. coli* O157:H7 infection (Schmidt et al., 1999). *Escherichia coli* O157:H7 strains possessing eaeA and hlyA genes without stx genes can be grouped into a similar pathogroup as enteropathogenic *E. coli* (EPEC) as opposed to STEC/EHEC (Schmidt et al., 1999). Non-shiga toxin producing *E. coli* strains have been associated with human outbreaks of diarrhoea and HUS (Schmidt et al., 1999; Allerberger et al., 2000).

The results of this study suggest that all the virulence genes present in the *E. coli* O157 isolates investigated are important in pathogenicity. However, the severity and duration of *E. coli* O157 infection may be influenced by differences in the combination of the virulence genes. Clinical manifestations observed in the present study were most profound with stx1/stx2/eaeA/hlyA-O157:H7 isolate followed by stx1/stx2-O157:H7, eaeA/hlyA-O157:H7, stx2-O157:H7 and least with stx1-O157:H7. Synergy among the virulence factors may contribute to make the animals succumb early to infection and influence the severity of infection. It is conceivable that the *E. coli* O157:H7 isolates used in this study may possess unidentified virulence factors that may contribute to their pathogenicity. A combination of bacterial and host factors may contribute to the spectrum and severity of lesions observed in *E. coli*-infected rabbits (Garcia et al, 2002). The *E. coli* O157:H7 isolates used in this study which were recovered from the faeces of apparently healthy cattle in abattoir, induced systemic disturbances similar to those observed in STEC infections in humans. This emphasizes the importance of strict adherence to biosecurity measures and hygienic practices during meat processing and marketing to forestall transmission of these pathogens to humans and other susceptible animals.

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(Manuscript 3rd October; 2012; accepted: 11th April, 2013).