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Original Article

The effect of Verotoxin Producing *Escherichia coli* in Development of Experimental Gastroenteritis among Streptomycin Treated Murine Model: A Longitudinal Study

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***Escherichia coli* O157:H7 is a newly emerged pathogen, which has been recognized as a major cause of large scale epidemics and thousands of sporadic cases of gastrointestinal illness. This observation particularly calls for the *in vivo* examination of *E. coli* O157 candidates in an attempt to mimic various aspects of *E. coli* O157:H7 disease symptoms in humans. Healthy conventional Swiss albino mice were used for investigating the clinical manifestation exerted by *stx1A* and *stx2A* positive *E. coli* O157:H7 in various doses applied through three alternative routes (oral, intramuscular and intraperitoneal). The highest titer of orally added *E. coli* 0157:H7 (10^9 CFU ml^{-1}) among the five test doses had started symptoms at the earliest time and manifested most of the classical symptoms. The symptoms started to become visible at 14^{th} hour, increased with time and had reached moribund condition near 48^{th} hour just before death of the host. The oral way of *E. coli* O157:H7 addition at the dose of 100 µl suspension containing 1 X 10^9CFU ml^{-1} was taken as the most potent concentration in producing bacterial fatality and hence was selected as the Minimum Lethal Dose (MLD).**

Key words: *E. coli* 0157:H7 infection, streptomycin treated murine model

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is an important group of multiple food and water-borne pathogens. EHEC, including *E. coli* O157:H7 comprises a subset of Shiga toxin-producing *E. coli* (STEC). *E.coli* O157:H7 has become a worldwide threat to public health and is one of today's most troubling food-borne pathogens. It has been known to be a human pathogen for nearly 30 years[1-2].

E.coli O157:H7 is a newly emerged pathogen, which has been the focus of immense international research effort driven by its recognition as a major cause of large-scale epidemics and thousands of sporadic cases of gastrointestinal illness. At least 30 countries in six continents have reported *E. coli* O157:H7 infections in humans [3]. *E. coli* O157:H7 infection can manifest in a variety of ways. Some individuals who are infected with the microbe remain asymptomatic, others experience diarrhea, but most develop hemorrhagic colitis (HC), the hallmark of *E. coli* O157:H7 infection. Children and the elderly appear to be especially susceptible to *E. coli* O157:H7 mediated disease and for reasons that are unclear, may develop HUS (a triad of clinical manifestations including hemolytic anemia, thrombocytopenia, and renal failure[3] and other systemic problems that include central nervous system impairment[4].

The incubation period for disease caused by EHEC O157:H7 among human population ranges from 1 to 16 days. Most infections become apparent after 3-4 days; however, the median incubation period was eight days[5] in one outbreak at an institution (Figure 1).

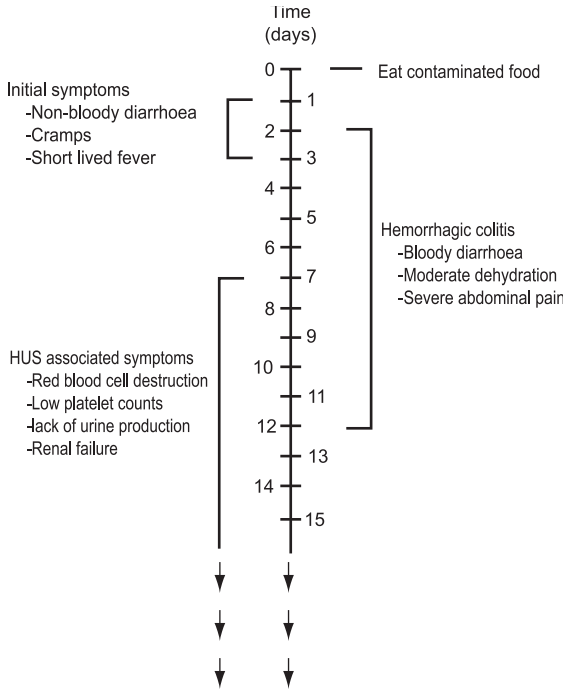

Figure 1. *Symptoms and time course of E. coli O157:H7 infection*

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The centre for disease control (CDC) has estimated that 85% of *E. coli* O157:H7 infections are food borne in origin. In fact, consumption of any food or beverage that becomes contaminated by animal (especially cattle) manure can result in contracting the disease. Foods that have been sources of contamination include ground beef, venison, sausages, dried (non-cooked) salami, unpasteurized milk and cheese, unpasteurized apple juice and cider[6-8]. The CDC has recently reported *E. coli* O157:H7 as the fourth most prevalent bacterial diarrheal pathogen after *Campylobacter* sp., *Salmonella* sp., and *Shigella* sp[9-10].

The pathogenicity of *E. coli* 0157:H7 is associated with several virulence factors including shiga toxins or verotoxins 1 and 2 encoded by *stx1A* and *stx2A* genes respectively. The ability to adhere to the intestinal mucosa by intimin (encoded by the *eaeA* gene)[11-12] and the production of haemolysin (encoded by the *hlyA* gene) which lyses erythrocytes release heme and globin that enhance the growth of *E. coli* 0157:H7 and serve as a source of iron[13]. This potent cytotoxin is the major factor that might lead to many symptoms or even to death in patients infected with EHEC[14].

A stool or blood culture can detect *E. coli* O157:H7 when cultured on Sorbitol-MacConkey (SMAC) agar, or the variant Sorbitol-MacConkey agar containing cefixime and potassium tellurite (CT-SMAC)[15]. Non-sorbitol fermenting colonies are tested for the somatic O157 antigen before being confirmed as *E. coli* O157. *E. coli* O157:H7 lacks the ability to hydrolyze 4-methylumbelliferyl-β-D-glucuronide (MUG) and does not grow at 45°C in the presence of 0.15% bile salts[16].

There are no currently available vaccines to prevent diseases due to EHEC, but a number of experimental approaches are being investigated in animals[17-18]. Numerous *in vitro* assays and animal models have been developed in an effort to imitate various aspects of *E. coli* O157:H7 mediated disease production in humans. These models exist in two varieties: those solely focused on the effects of Stx (in the absence of bacteria) and those that explore *E. coli* O157:H7 infection[17]. Small animals that have served as models for EHEC infection and disease include mice, rats, and rabbits. Larger animals that have been so used, even though less frequently, include chickens, pigs, cows, dogs, baboons, and macaques[18].

This work examined the clinical manifestation exerted by *stx1A* and *stx2A* positive *E. coli* O157:H7 in various doses applied through different routes into healthy conventional rodents over a period of time. Furthermore, we tried to find the most potent way of 0157 inoculation in disease production among the three test routes and to determine O157 Minimum Lethal Dose (MLD) in murine population.

Materials and Methods

E. coli O157:H7 NCTC12079 (clinical strain) was kindly provided by obtained from ICDDR,B (International Center for Diarrheal Disease Research).

Molecular characterization of Escherichia coli O157:H7 DNA by polymerase chain reaction

Bacterial chomosomal DNA was extracted and purified according to GuSCN DNA extraction method[19].

Polymerase chain reaction based detection of *stx*1A and *stx*2A gene from extracted DNA sample was performed by following the method of Brown[20].

PCR was performed in a thermal cycler (Bio-Rad, Model:C1000^{TM})

Extracted DNA from *Escherichia coli* O157:H7 NCTC 12079 was used as positive control to detect the presence of *stx*1 and *stx*2. One hundred base pair DNA (Invitrogen, Cat No: 15628-050) was used as marker to measure the molecular size of the amplified products. DNA bands were detected by staining the gel with aqueous ethidium bromide (0.5µg/ml) for 20 minutes and de-stained with ethnol for 30 minutes at room temperature and photographs were taken using gel documentation system (Bio-Rad, USA).

Mice infection protocol with increasing dose of E.coli O157:H7

One hundred and eight Swiss albino mice (*Mus musculus*) of 6-8 weeks age was purchased from Animal Division of ICDDR,B. Mice were weighed individually on day 0 and a common body weight was recorded as 18 g. Heart beat was noted as 110– 120/ minute. Each mouse was examined initially for their state of health including body temperature, appetite condition, and tidiness of fur, eye health and activity level. The mice were randomly selected on the basis of experimental scheme and to verify the reproducibility of the outcome each set of investigation was performed in duplication.

Mice were housed under standard day length, temperature, and humidity conditions. Each cage contained a control mouse devoid of any treatment, maintained with normal mice feed and drinking water. The treated group after specific treatment was given normal mice feed and drinking water.

To demonstrate the infective capacity of *E. coli* O157:H7 in murine model, three sets of mice (set-1, set-2, and set-3) each containing six animals were administrated with specific dose of *E. coli* O157:H7 culture through three different routes of entry: oral, intramuscular (i.m.) and intraperitoneal (i.p.). Over the course of infection the animals were observed daily for their activity level, water intake and amount of food consumption.

Pellet to be inoculated orally was re-suspended in 1 ml of 1% sucrose solution. Pellet/bacterial seed to be administered via i.m. and i.p. route was re-suspended in 1 ml PBS solution.

Different sets of mice were inoculated intramuscularly (set 2) intraperitoneally (set 3) following an identical (Table 1) bacterial titer plan. Set 1 mice were given phage free drinking water with streptomycin (5 g/L) for one day to reduce the count of normal facultative resident flora of the intestine. All of the three groups of mice (set-1, set-2 and set-3) were starved for 24 hrs.

Table 1. *Various titers of orally administered E. coli O157:H7*

Animal model	Mouse identification number (ID No) in each set	Dose (CFU ml^{-1}) scheme	
	A1/B1	Dose 1	Negative control (1% aqueous sucrose solution)
Mice set-1	A2/B2	Dose 2	$1X10^5$
	A3/B3	Dose 3	$1X10^6$
	A4/B4	Dose 4	$1X10^7$
	A5/B5	Dose 5	$1X10^8$
	A6/B6	Dose 6	$1X10^9$

Mice of the first set were fed with 100µl of 3% sodium bicarbonate by calibrated dropper to neutralize the stomach acidic condition. All the oral doses were administrated while the mice were held in a supine position with the head up, allowing the animals to suck the fluid from the tip of micropipette as shown in Figure 2.

Figure 2. *Mouse oral administration pattern*

Mice of set-2 and 3 aimed for i.m. and i.p. treatment following the same dose plan of Table 1 were injected once with 100 µl of PBS suspended *E. coli* 0157:H7 (Figure 3 and 4).

Figure 3. *Procedure of mouse intramuscular administration*

Figure 4. *Way of mouse intraperitoneal administration*

Determination of disease severity pattern with the increasing dose of E.coli O157:H7

Treated mice of all the three sets (set 1, 2, 3) challenged with different doses of *E. coli* 0157:H7 (Table 1) along with the untreated (negative control) ones were kept under careful observation for a further 144 hrs to detect any visible change.

Health status of mice was recorded according to the grade chart[21] labeled as 0-5 which is summarized in Table 2.

Table 2. *Increasing dose of E. coli O157:H7 enhances disease severity*

Disease symptoms	Level of disease severity
A normal and unremarkable condition	level 0
Slight illness, defined as lethargy and ruffled fur	Level 1
Moderate illness, defined as severe lethargy, ruffled fur, and hunched back	Level 2
Severe illness, with all the signs plus exudative accumulation around partially closed eyes increase body temperature loss of appetite	Level 3
A moribund state	Level 4
Death	Level 5

Establishment of minimum lethal dose (MLD) of E.coli O157:H7 for murine model

E. coli 0157 suspensions in PBS were prepared for the calculation of its MLD. The health status of the mice along with the increasing dose of *E.coli* O157:H7 were recorded. The dose responsible for the death or worst situation (moribund or terminally ill) of murine model was determined to establish the MLD.

To verify if the cause of death was truly due to *E. coli* O157:H7 bacteremia, blood samples were collected from dead mice by cardiac puncture and then directly spread on CT-SMAC plate.

Results

A clinical strain of *Escherichia coli* O157:H7 obtained from ICDDR,B was confirmed by direct culture onto Sorbitol MacConkey Agar plate (SMAC) containing cefixime and potassium tellurite (CT-Supplement) where *E. coli* 0157:H7 produced colorless colonies (Fig. 6) and other *E. coli* K12 gave distinguishable pink colonies (Figure 5).

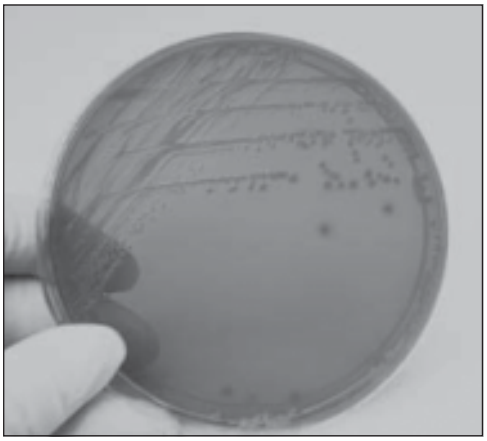

Figure 5. *Pink colonies of E. coli O157:H7 along with other E. coli colonies on MacConkey agar*

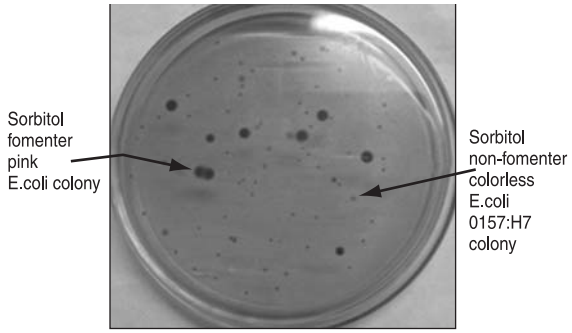

Figure 6. *Significant colorless sorbitol non-fermenting E. coli O157:H7 colonies*

Colorless colonies from CT-SMAC plate were inoculated in MUG broth where *E. coli* 0157:H7 showed MUG (4-methylunbelliferyl-β-D-glucuronide) negative (non-fluorescent) growth (E.C. Broth with MUG; Oxoid; England) after exposure to 360 nm UV light as shown in Figure 7.

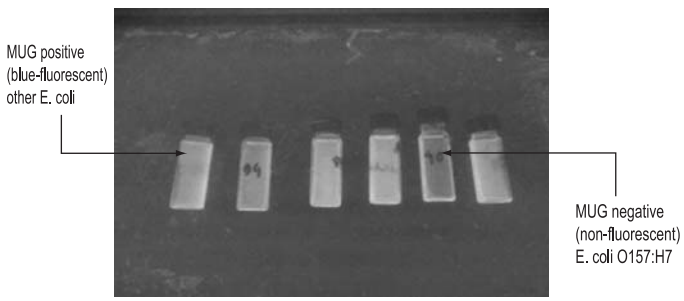

Figure 7. *MUG broth showing growth of MUG negative (Non-fluorescent) E. coli O157:H7 isolates and MUG positive (blue-fluorescent) other E. coli*

Detection of E. coli O157:H7 specific virulence genes by PCR

The result revealed that the clinical strain of *E. coli* O157:H7 used in the research was positive for the presence of *stx1A* and *stx2A* identical to the reference (Figures 8 and 9).

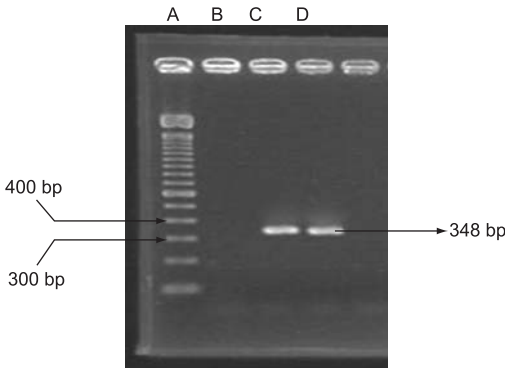

Figure 8. *Agarose gel (1%) electrophoresis pattern of PCR amplicons of E. coli O157:H7 obtained with primers specific for stx1A*

Key: Lane A- 100bp molecular weight marker; Lane B- Negative control E. coli K-12; Lane C- Positive Control E. coli O157:H7 NCTC 12079;Lane D - Test clinical isolates of E.coli O157:H7

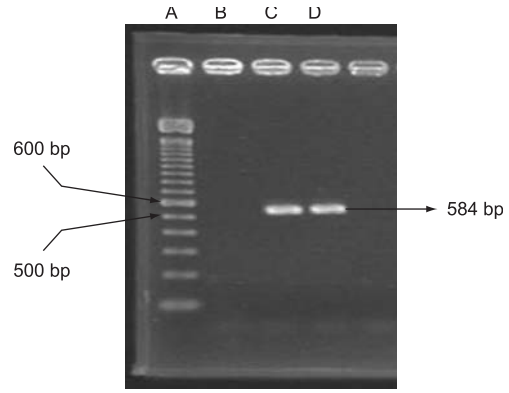

Figure 9. *Agarose gel (1%) electrophoresis pattern of PCR amplicons of E. coli O157:H7 obtained with primers specific for stx2A*

Key: Lane A- 100bp molecular weight marker; Lane B- Negative control E. coli K-12;

Lane C- Positive Control E. coli O157:H7 NCTC 12079; Lane D - Test clinical isolates of E. coli O157:H7

Effect of Escherichia coli O157:H7 on the outcome of disease severity in murine model

Mice challenged with 10^5 CFU ml^{-1} and 10^6 CFU ml^{-1} of *E. coli* 0157:H7 developed no visible symptoms while examined for 144 hrs since infection, whereas doses 3 (10^7 CFU ml^{-1}) and dose 4 (10^8 CFU ml^{-1}) produced specific symptoms at specific times but eventually the experimental animals recovered from illness. The highest titer of orally added *E. coli* O157:H7 (10^9 CFU ml^{-1}) caused symptoms at the earliest (at 14^{th} hr) time among the five test doses and manifested all the symptoms described in Table 2.

Moreover, at this particular dose, the inoculated rodent was severely ill at 36th hr and was dead at 48th hr. The mice fed with 1% sucrose solution developed none of the above mentioned symptoms and acquired adequate body weight (31.65 g) within this time frame. It could be worthwhile to mention that, all the infected mice (group A2/B2 to A5/B5) did not gain body weight as they should have gained with time and their average weight was measured as 20.15 g when followed for six days.

Consequence of intramuscularly and intraperitoneally inoculated Escherichia coli O157:H7 on the outcome of disease severity in experimental animal model

To determine the survival pattern of Swiss albino mice of set-2 and 3 (group An/Bn) the mice were injected with increasing dose of *E. coli* O157:H7 according to the list (Table 1) . For both routes 10^5 CFU ml^{-1} and 10^6 CFU ml^{-1}of *E. coli* O157:H7 developed no remarkable symptoms when examined for 144 hrs since infection, but in case of dose 4 (10^7 CFU ml^{-1}) intramuscularly infected mice gave no visible symptoms whereas intraperitoneally infected mice produced minor symptoms. For dose 5 (10^8 CFU ml^{-1}) and dose 6 (10^9 CFU ml^{-1}) mice produced minor symptoms graded as 1 and 2 (Table 2) at different point of time which was similar for both routes. Afterwards these infected animals gradually recovered from illness and regained normal health. The mice injected with PBS solution developed none of the above mentioned symptoms and acquired adequate body weight (31.15 g) within this particular period of time.

It could be mentioned that none of the diseased mice (group A2/B2 to A5/B5) gained body weight as they should have gained with time and their average weight was measured as 22.65 g at 144th hr.

Determination of minimum lethal dose (MLD) of E.coli O157:H7

To determine the minimum lethal dose (MLD) of *E.coli* O157:H7 infection in conventional murine model the diseased, moribund and deceased condition of mice were recorded at various time points (Table 3,4 and 5) after bacterial inoculation (in increasing dose) in a six day long experimentation. The disease severity of mice was found to be correlated with the augmented dose of *E.coli* O157:H7. All the three routes of bacterial inoculation shared this observation. However, the oral route of *E.coli* O157:H7 addition at a dose of 1X10^{9}CFU ml^{-1} proved to be the most potent in developing bacterial pathogenesis and hence was selected as the MLD.

Table 3: *Disease severity pattern[a] in mice with increasing dose of orally administered E. coli O157:H7*

	Various doses of *E. coli* O157:H7					
Hours (h) post infection	Dose 1 Negative Control(1% Sucrose solution) Group A1/B1	Dose 2 (10^5 CFU ml^{-1}); Group A2/B2	Dose 3 (10^6 CFU ml^{-1}) Group A3/B3	Dose 4 (10^7 CFU ml^{-1}) Group A4/B4	Dose 5 (10^8 CFU ml^{-1}) Group A5/B5	Dose 6 (10^9 CFU ml^{-1}) Group A6/B6
10	0					0
14	0					1
16	0					2
24	0	0	0	0	0	3
36	0	0	0	2	2	4
48	0	0	0	0	1	5
72	0	0	0	0	0	
96	0	0	0	0	0	
120	0	0	0	0	0	
144	0	0	0	0	0	

[a] Disease severity (0 to 5) was recorded according to the grade chart (Table 2)

Table 4: *Illness pattern*[a] *in murine model for augmented dose of intramuscularly administered E. coli O157:H7 with time* various doses of *E. coli* O157:H7

	Various doses of *E. coli* O157:H7					
Hours (h) post infection	Dose1 Negative Control (PBS) Group A1/B1	Dose 2 (10^5 CFU ml^{-1}) Group A2/B2	Dose 3 (10^6 CFU ml^{-1}) Group A3/B3	Dose 4 (10^7 CFU ml^{-1}) Group A4/B4	Dose 5 (10^8 CFU ml^{-1}) Group A5/B5	Dose 6 (10^9 CFU ml^{-1}) Group A6/B6
16	0	0	0	0	0	0
24	0	0	0	0	0	0
36	0	0	0	0	0	0
48	0	0	0	0	1	1
72	0	0	0	0	0	2
96	0	0	0	0	0	0
120	0	0	0	0	0	0
144	0	0	0	0	0	0

[a] Disease severity (0 to 5) was recorded according to the grade chart (Table 2)

Table 5. *Survival pattern* [a] *of mice with augmented dose of intraperitoneally administered E. coli O157:H7 over time*

	Various doses of *E. coli* O157:H7					
Hours (h) post infection	Dose1 Negative Control (1% sucrose solution) Group A1/B1	Dose 2 (10^5 CFU ml^{-1}) Group A2/B2	Dose 3 (10^6 CFU ml^{-1}) Group A3/B3	Dose 4 (10^7 CFU ml^{-1}) Group A4/B4	Dose 5 (10^8 CFU ml^{-1}) Group A5/B5	Dose 6 (10^9 CFU ml^{-1}) Group A6/B6
16	0	0	0	0	0	0
24	0	0	0	0	0	0
36	0	0	0	0	0	1
48	0	0	0	1	1	2
72	0	0	0	0	2	2
96	0	0	0	0	0	0
120	0	0	0	0	0	0
144	0	0	0	0	0	0

[a] Disease severity (0 to 5) was recorded according to the grade chart (Table 2)

Discussion

Although it has been nearly 30 years since the discovery of *E. coli* O157:H7 as an enteric pathogen and despite the recent rise in the rate of severe diseases associated with infection by this organism, no treatment exists[18,22]. Understanding the pathogenesis of *E. coli* O157:H7-mediated disease in humans is fairly limited. The use of human subjects to investigate the steps required for *E. coli* O157:H7 to evoke intestinal pathology is considered unprincipled because of the likelihood that a volunteer could develop HUS. A range of *in vitro* assays and animal models have been developed in an effort to mimic a range of aspects of *E. coli* O157:H7 disease production in humans and thus to improvise the potential strategies for prevention and control of *E. coli* O157:H7 mediated infections in human population.

Large animal models, such as the gnotobiotic piglet, exhibit a number of features of *E. coli* O157:H7 pathogenesis, but their breeding and maintenance require considerable veterinary skill, space, and ûnancial support[17]. Therefore small animal model systems are preferable for general use. Mouse models in particular offer a number of beneûts that include: low relative costs for

purchase and maintenance, ease of care and handling, ready availability of numerous immunological reagents, variations in genetic background among inbred mouse strains as well as access to transgenic and recombinant inbred animals, and, very importantly, the feasibility of using sufficient numbers of animals in a single study to perform meaningful statistical analyses on the resultant data. Several popular mouse models of *E. coli* O157:H7 infection have been used and suggested before[23-24]. We have chosen streptomycin-treated mice containing reduced normal ûora for our research because these animals have proven to be amenable to EHEC colonization.

The major virulence factor and a defining characteristic of *E. coli* O157:H7 is the production of Shiga toxin. This potent cytotoxin is the factor that leads to death and many other symptoms in patients infected with EHEC[13]. Our work involves the exploitation of *stx1A* and *stx2A* positive *E. coli* O157 in healthy streptomycin-treated Swiss albino mice to investigate the clinical manifestation exerted by this clinical strain.

Various doses of *E. coli* O157 in (Table 1) were applied through three alternative routes (oral, intramuscular and intraperitoneal) into the rodents. The mice were housed and maintained under *E. coli* O157 free condition and were followed for six subsequent days after deliberate microbial infection.

The highest titer of orally added *E. coli* 0157:H7 (10^9 CFU ml^{-1}) among the five test doses started to show symptoms earlier than other doses and manifested all the classical symptoms *i.e.* decreased physical activity, ruffled fur, general lethargy, hunchback posture, exudative accumulation around partially closed eyes, bloody stool, fever, being moribund and finally death. Death was attributed to septic shock due to toxins present in the circulation of mice after challenge with the bacteria. All mouse models receiving the control treatment (PBS only) did not show any sign of bacteremia or illness. This *in vivo* assessment suggests that *E.coli* O157:H7 evoked a range of illness in rodent model, which partially resemble the symptoms exerted by O157 infection in humans. For the orally inoculated mice population with highest dose, symptoms started to manifest at 14^{th} hr, increased with time and the mice reached moribund condition near at 48^{th} hr (Table 3) just before being dead. It is worthwhile to mention that these clinical features expressed by the murine model in this study coincide with the finding by other researchers[21]. However, it is suggested that there is no animal model system that mimics the full spectrum of 0157-evoked illness in humans specially the development of HC and HUS[17].

Oral route provoked disease severity earlier and of greater degree than other routes. The orally infected mice died near 48^{th} hour whereas mice inoculated by i.m. or i.p. developed experimentally induced gastroenteritis near 48^{th} hr but recovered partially by 72 hrs and hence escaped death. Therefore, it can be speculated that for i.m. and i.p. routes, MLD in mice would be greater than 1 x 10^9CFU ml^{-1} for *E. coli* O157:H7. Considering all these results, the oral route of *E .coli* O157:H7 addition at the dose of 100 µl suspension containing 1 x 10^9CFU ml^{-1} was taken as the most potent concentration in producing O157 fatality and hence selected as the MLD. The present investigation well agrees with the previous findings where it was concluded that the test rodents inoculated by nonhaemorrhagic *E. coli* through intraperitoneal route at different concentrations (1 x 10^5CFU ml^{-1}, 1 x 10^7CFU ml^{-1}, 1 x 10^9CFU ml^{-1}) gave the MLD value of 10^7 CFU/ml as it induced fatality in all replicates within 24 hrs [21]. The experiment was not prolonged after 6 days to avoid the likelihood of contamination with other prevailing microorganisms, which would have interfered with the clinical picture. To verify if the cause of death was truly due to *E. coli* bacteremia, blood samples were collected from dead mice by cardiac puncture and were streaked on CT-SMAC agar to compare the bacteria recovered from the blood to the original inoculum, clinical isolate of *E. coli* O157:H7.

By comparing the pattern of body weight change with the degree of disease severity in this work, it could be concluded that weight decrease in infected rodents is proportional to the increase rate of symptom acquisition and severity. This observation was common for all the three routes of bacterial inoculation investigated in this research. The untreated mice group developed none of the aforementioned symptoms (Table 2) and acquired adequate body weight within the studied time frame.

As a result of the increased rate of HC and HUS over the last several years and the lack of therapies for treatment of HUS, further research is necessary to deûne mechanisms involved in the pathogenesis of *E. coli* O157:H7 and to identify potential disease prevention strategies and therapeutics. The application of animal model systems is vital to achieve these goals. Despite the fact that no one animal model recapitulates all features of *E.coli* O157:H7 infection, mouse models have been explored in this research for understanding *E. coli* O157:H7 pathogenesis better. The findings from this study can help to pinpoint the means by which *E. coli* O157:H7 infection and/or disease can be controlled or prevented.

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