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IMMUNOLOGICAL STUDY ON MICE EXPERIMENTALLY INFECTED WITH SHIGA TOXIN-PRODUCING *E. COLI* O26

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ABSTRACT
STEC infection have been described in a wide range of both human and animal infections and lead serious disease such as bloody diarrhea, hemolytic uremic syndrome, hemorrhagic colitis, Thrombotic thrombocytopenic purpura, fever, vomiting, and possible death. Mice infected with STEC O26 (2.5×10^8 C.F.U/ml) to study the immunological change due to infection with STEC. The immunological study on infected mice showed both humoral and cellular immune response. The humoral immune response was detected by passive haemagglutination test and the maximum titer of antibodies reached a mean of (192.0 ±21.33) at days 21 post infection in mice, the cellular immune response was detected by using the skin test, the mean thickness of the right footpad was (3.14 ±0.13 mm) after 24 hours then declined to reach (1.80 ±0.10 mm) after 72 hours in mice infected by infectious dose. The cytokines level was measured by ELISA (Interleukin 6 and IFN- γ) which showed a significant increased ($P<0.05$) in comparison with control group at all experiment period (3, 14 and 21) days after infection. STEC O26 was important for the infection of children. The experimental infection with STEC O26 induced both cellular and humoral immune response with elevated of inflammatory cytokines (IFN- γ and IL-6).

KEYWORDS: STEC, O26, *E. coli*, cytokines, mice, Interleukin 6, IFN- γ, and immune response.

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) infection have been described in a wide range of both domestic and wild animal species, but their natural pathogenic role has been demonstrated only in young calves, pigs and dogs. The cattle recognized as the major reservoir for human infections (Caprioli *et al*., 2005, Beutin, 2006; Wang, 2015). Human infection with STEC usually causes serious diseases (Stromberg, 2015; Wang, 2015), such as bloody diarrhea, hemolytic uremic syndrome, hemorrhagic colitis, Thrombotic thrombocytopenic purpura, fever, vomiting, and possible death (Mathusa *et al.*, 2010; Etcheverria and Padola, 2013; Croxen *et al*., 2013). STEC serotypes O26 has prime importance in causing disease in humans (Tozzi *et al*., 2003; Bettelheim, 2007). Also it has been isolated from calves and lambs with diarrhea (De *et al*., 2002; Blanco *et al*., 2003; Caprioli *et al*., 2005). It has been linked to both human and animal illness for more than 25 years (Bettelheim, 2003; Valadez, 2010). It was accounted for the largest proportion of infections among STEC (Stigi *et al*., 2012; Gould *et al*., 2013), and it was ranked among the top of STEC serogroups isolated from individuals with sporadic illness reported to the CDC between 1983 and 2002 (Brooks *et al*., 2005).

Interestingly, STEC O26 possesses many of the similar virulent factors as STEC O157, but it has possess additional ability to infect animals (Campellone and Leong, 2003; Caron *et al*., 2006; Jenkins *et al*., 2008). O26 appears to survive in bovine intestinal tract, rather than other food animals (Bettelheim, 2007). Serotype O26 has been reported from healthy cattle and cattle, mainly calves, with diarrhea, also outbreak accured in Canada in cattle with HC (Rahn *et al.*, 1998; Leomil *et al*., 2005), and in Spain (Blanco *et al.*, 1997). Outbreaks associated with O26 in the U.S. have been linked to lake water (Minnesota, 2001), a daycare (Nevada, 2005 and Iowa, 2007), blueberries and strawberries (Massachusetts, 2006), raw milk (Washington, 2010), and ground beef (multistate outbreak, 2010) (USDA-FSIS, 2011). In 1997, Japan (Hiruta *et al*., 2001). In 2000, an outbreak in Germany (Weber *et al*., 2002). In 2007, in Denmark (Ethelberg *et al*., 2009). On 2016, the CDC announced two outbreak in multi-state of USA, the initial, larger outbreak was first detected in 11 state in October 2015 with the outbreak strain of STEC O26 were reported from ill people were hospitalized, and in December, 2015, a second outbreak of a different, strain of STEC O26 was identified in other three states (CDC, 2016).

STEC infection can activate innate immunity by pattern recognition receptors depending on pathogen-associated molecular patterns (Jones and Neish, 2011). Activation of leukocytes to combat invading enteric pathogens mainly by Phagocytosis which involves the uptake of large foreign material by neutrophils, macrophages and dendritic cells, also mast cells are implicated in the host inflammatory response by secreting IFN-α and by recruiting neutrophils, which are involved in bacterial clearance (Wei *et al*., 2005). Antibodies to STEC virulent factors can also inhibit bacterial attachment to the epithelial cells and reduce shedding (Potter *et al*., 2004; La Ragione *et al*., 2006). Therefore, an antibody response could be an alternate to phagocytosis, as STEC could inhibit this process during infection (Marches *et al*., 2008).

This study aimed to estimate the immune response to STEC O26 infection experimentally in mice.

MATERIALS & METHODS

Bacteria Preparation and Harvest

E. coli Strain O26 which taken from child suffering from diarrhea, showed positive result in PCR analyses to the fallowing genes (*wzx*$_{O26}$, *stx1*, *stx2*, *eaeA* and *EHEC hlyA)* which has previously isolated in Ph.D. dissertation program. With a sterile wire-loop, some bacteria were streaked into a petri-dish containing MacConkey agar. A colony from harvested and cultured on an EMB agar to confirm that the colony has *Escherichia coli*. Tryptic soy broth (100 ml) to calculate bacteria according to (Ogiehor and Ikenebomeh, 2006).

Mice

One hundred seventy mice ranged from 8 to 12 weeks old which obtained from the (Iraqi Center for Cancer and Medical Genetics Research), fed clean boiled water and laboratory chow ad libitum were used in this study. After 1 day of streptomycin treatment mice (6mg/ml) according to Krystle and Alison, (2011), mice was starved from 18 to 24 h from food then divided randomly into two groups: "infected group" (85 mice) which were inoculated orally with infectious dose of STEC O26 (2.5×10^8 C.F.U/ml) and "control group" (85 mice) which were inoculated orally with phosphate buffer saline. Blood samples were collected and sera were separated from the infected mice (25 mice from each group) at 3, 14 and 21 days after induced infection and kept at (-20 ˚C) for immunological tests. Skin test were done on (10) mice from each groups at 21 days post infection.

Detection of cellular immunity (Delayed type hypersensitivity test DTH (Skin test))

This test was done according to (Hudson and Hay, 1980) after 21 days of infection. Briefly, 0.1 ml of soluble antigen of *coli* O26 was injected intradermally in the right footpad of the mouse while the left side was injected by 0.1 ml of sterile PBS (pH=7.2). The thickness of the footpad was measured by vernier caliper before and 24, 48, and 72 h after Injection.

Detection of humoral immunity (Passive haemagglutination test (PHA)

Passive haemagglutination test Preparation of coated erythrocytes Preparation of erythrocytes and test procedure was done as described by Ishag *et al*. (2014).

Estimation of cytokine (Mouse IFN-γ, and Interleukin 6) level by Eliza test

It was done according to manufacturing company (Elabscience, China) by this test uses Sandwich-ELISA method. The ELISA plate pre-coated with an antibody that were specific to (Mouse IFN-γ or Mouse IL-6). The standards or samples serum added to ELISA plate wells and react with the target specific antibody. Reading by Biochrom Asys Expert Plus ELISA Multi-Well Plate Reader (England).

Statistical Analysis: T test and One-Way ANOVA by using Statistical Package for Social Science (SPSS) system to analyze the data of our study.

RESULTS

Humoral immunity (Passive heamagglutination test) (PHA)

The anti-body titer showed a significant increase ($P<0.05$) in infected group compared with control group at all experiment time, the infected group showed significant increase ($P<0.05$) at day 21 compared with days 3 and 14 Table (1).

TABLE 1: Antibody titer against STEC O26

Groups / Time	Infected group		Control group	
Day 3	12.0 ± 2.98		0 ± 0	
	A	c	B	a
Day 14	59.2 ± 12.63		0 ± 0	
	A	b	B	a
Day 21	192.0 ± 21.33		0 ± 0	
	A	a	B	a

Values are expressed as mean ± SE

n= 25/group,

Capital letters denote significant difference ($P<0.05$) within a row, Small letters denote significant differences ($P<0.05$) within a column.

TABLE 2: The thickness (mm) of the right footpads of mice infected with STEC O26.

Periods after injection of soluble antigen	Infected group		Control group	
0 hours	1.55±0.05		1.54±0.04	
	A	c	A	a
24 hours	3.14±0.13		1.58±0.03	
	A	a	B	a
48 hours	2.56±0.18		1.56±0.02	
	A	b	B	a
72 hours	1.80±0.10		1.56±0.02	
	A	c	A	a

Values are expressed as mean ± SE

n= 10/group,

Capital letters denote significant difference ($P<0.05$) within a row, Small letters denote significant differences ($P<0.05$) within a column.

Cellular immunity (skin test response)

The results of delayed type hypersensitivity showed significant increases in the thickness (mm) of the right footpads of the mice of infected group (P<0.05). The highest mean of the thickness was after 24 hours post infection then declined significantly (P<0.05) after 48 hours and returned to normal thickness after 72 hours post injection of soluble antigen (Table 2).

Measurement of cytokines levels

1- Interleukin 6

The cytokines (IL-6) levels (pg/mL) showed a significant increased (P<0.05) in comparison with control group in the same period whereas the infected group revealed a significant increase (P<0.05) in IL-6 at day 24 and 21 compared to days 3, table (4-13).

TABLE 3: serum levels of IL-6 (pg/mL) in infected and control groups

Groups / Time	Infected	Control
Day 3	353.65±16.26	80.63±4.31
	A b	B a
Day 14	466.93±14.51	81.81±3.48
	A a	B a
Day 21	483.30±7.89	83.78±1.94
	A a	B a

Values are expressed as mean ± SE

n= 25/group,

Capital letters denote significant difference (P<0.05) within a row, Small letters denote significant differences (P<0.05) within a column.

2- IFN- γ

The cytokine (IFN- γ) level (pq/ml) showed a significant increase (P<0.05) in infected group in comparison with control group at same periods meanwhile the results of infected group showed a significant increase (P<0.05) in the IFN- γ level at days 14 and 21 post infection compared to result of day 3, Table (4).

TABLE 4: serum levels of IFN- γ (pg/mL) in infected and control groups.

groups / time	Infected	Control
Day 3	304.83 ± 11.03	32.63 ± 1.09
	A b	B a
Day 14	569.68 ± 21.63	33.25 ± 0. .91
	A a	B a
Day 21	593.25 ± 6.08	33.71 ± 1.03
	A a	B a

Values are expressed as mean ± SE

n= 25/group,

Capital letters denote significant difference (P<0.05) within a row, Small letters denote significant differences (P<0.05) within a column.

DISCUSSION

Humoral immunity (Passive heamagglutination test)

The mice which was infected by infectious dose, after 3 day showed antibody titers with a mean (12.0 ±2.98) but after 14 days, it showed significant increase in antibody titers (59.2 ±12.63) while in the 21 days post infection, there was reach to peak significantly increased in the antibody titers to reach a mean (192.0±21.33). The present study showed that the experimentally infected mice were able to induce humoral immune response which was represented by producing antibody against STEC O26 which was elevated after 3 days post infection, reaching the peak after 21 days post infection. The antibody response to Tir, intimin, Esp A and EspB, proteins that are secreted by the type III secretion system after STEC infections and also by lipopolysaccharide (Cornick *et al.*, 2002; Potter *et al.*, 2004; Cristancho *et al.*, 2008; Joris, 2012) and this is agreement with that mentioned by Kerstin and colleges who indicated that patients with HUS associated with infection by non-OI57 STEC (O26 lipopolysaccharide) strains could develop a robust, O group-specific immune response against lipopoly saccharide response. This study supported the evidence that mentioned by other researchers Studies have shown that infection of STEC and EPEC leads to elevation of humoral immunity (Li *et al*., 2000; Dana, 2003).

Larrie-Bagha *et al*. (2013), showed that the important role of antibody producing B cell in protection against *E.coli* O157:H7. Cataldi *et al.* (2008) showed that intimin was a target for humoral immune response in mice. Recently there has been a lot of interest in the use of EspB, EspD, NleA, EspA, intimin and stx to produce vaccine against STEC (Dziva *et al*., 2007; van Diemen *et al*., 2007; Gu *et al*., 2009). No antibody titers have detected in the mice of the control group during the same intervals.

Cellular immunity (skin test)

The result of skin test showed that STEC O26 elicited a cell mediated immune response, since delayed type hypersensitivity is the principle pattern of cellular mediated immunity (Ramzi *et al*., 1994). skin test depend on ability and activity of Tdh cells to recognize antigen and secrete IL-1 which enhanced proliferation and differentiation of other T-cell into Th-cells which secrete IL-2 as a chemoatractive factor to attract macrophage around the area of activated T-cell which also secrete INF- that enhancing the cytolytic activity of accumulated macrophages leading to skin thickness (Rosenthal and Tan, 2007). T cells activated during the sensitization phase

are CD4+ T cell primarily of the Th1 sub type but in a few cases CD8+T cells has also been showed to induce a DTH response (Mastroeni *et al*., 2001).

Cellular immune response induced by STEC O26 was similar to that recorded by many researchers who found the same result of cellular immune response induced by the some serovars belong to the genus STEC challenged of mice with specific antigen of O157 (Eko *et al*., 2011; Mayr *et al*., 2012). Oral inoculation of EHEC in mice also showed similar results (Cai *et al*., 2010). The present study agreed with Kshash and Habasha, (2009), who used a purified lipopolysaccharide of *E. coli* O111 in immunization of mice and recorded a high significant of right footpad skin thickness after 24 hrs with a peak level at 48 hrs as compared with control group. These result disagree with Hoffman *et al*. (2006), he recorded that STEC infections could suppress the development of an antigen-specific cellular immune response in cattle.

Measurement of cytokines levels

1- Interleukin 6 (IL-6)

The IL-6 target was chosen in response to the endotoxin (Husain *et al*., 2003). The height level and significant increase agreed with Kim *et al*. (2002) and Salazar-Mather *et al*. (2003), their studies indicated that IL-6 plays a big role in the immune response against virulent STEC. Numerous investigations described that elevated plasma concentrations of IL-6 have been observed among HUS with children suffering from gastroenteritis and in TTP patient due to *E. coli* O157:H7 infection (Inward *et al*., 1997; Murata *et al*., 1998; Van Setten *et al*., 1998; Westerholt *et al*., 2000; Proulx *et al*., 2001).

The increase synthesis and release of considerable amounts of IL-6 by may be attributed to the effect of lipopolysaccharide and Flagella (Giro´n, 2005; Oladejo and adebolu, 2013) also to stx1 that binds to monocytes via different Gb3 subtype on endothelial cells of proximal tubular epithelial and leads to the secretion of IL-6 (Hinsbergh, 1996**;** Kohan *et al*., 1997; Hughes *et al*., 2001); also by action of stx2 when it was given intravenously to mice, showing an increase in urinary IL-6 levels (Siegler *et al*., 2003). Infected IL-1R-/- mice had reduced levels of IL-6 and IFN-γ, and increased intestinal damage, including gangrenous mucosal necrosis, colonic bleeding and mortality in infected mice with STEC. The IL-1R is involved in inducing IL-6 and IFN-γ levels, which when combined are important in resolving enteric infections (Asper, 2009). These results of this study do not agree with Westerholt *et al*. (2000), who do not find any significant difference in IL-6 levels in infection with STEC.

2- Interferon gamma (IFN- γ)

The significant increase of IFN- γ were in agreement with the result found by Hayashi *et al*. (2001); Mayr *et al*. (2005) and Gobert *et al*. (2007), in mice; also, Higgins *et al*., 1999 and Vallance *et al*., 2002) demonstrated that in mouse model infection with EHEC strains, they suggested that INF- γ plays important role in defense mechanism against pathogens in vivo. Other researcher explained that the level of IFN- γ increased in humans following *E. coli* infection (Long *et al*., 2010). And in *E. coli* O157:H7 enteritis and HUS, had a significant increase of IFN- γ in infected children (Proulx *et al*., 2000). IFN-γ play essential role in both cellular and humoral immune responses against invasive pathogens, this cytokine ,found only in vertebrates (Stark and Darnell, 2012), it is an important mediator of endotoxemia and gram negative bacterial sepsis in a number of mammalian species (Parmely and Wang, 2001).

In addition to its role against viral infection, IFNγ has been shown to play an important roles in bacterial infection, antigen processing, immunoglobulin (Ig) class switching, adhesion, leukocyte homing, cell cycle regulation, apoptosis, tumor immunity, and autoimmunity (Boehm *et al*., 1997; Schroder *et al*., 2004). Many of these functions are critical for host defense, as humans lacking IFNγ, its receptors, or key signaling components, display increased susceptibility to both viral and bacterial infections (Jouanguy *et al*., 1999) Infected IL-1R-/-mice had reduced levels of IL-6 and IFN-γ, and increased intestinal damage, including gangrenous mucosal necrosis, colonic bleeding and mortality in infected mice with STEC.

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