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HUMORAL AND CELLULAR IMMUNE RESPONSE INDUCED BY *E. Coli* [O157:H7 AND O157:H7:K99] VACCINES IN MICE

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Abstract-

Background- *Escherichia coli* Serogroup O157:H7 (*E. coli* O157:H7) is a zoonotic bacterial pathogen that causes symptoms ranging from self-limiting bloody diarrhea to severe hemorrhagic colitis in humans. *E. coli* O157:H7 infection can also cause extra-intestinal illness, most importantly hemolytic-uremic syndrome (HUS). The aims of this study was to evaluate the effect of two types of *E. coli* possess O157:H7 antigen and *E. coli* possess O157:H7:K99 antigen on humoral and cellular immunity in mice.

Materials and Methods- Three groups of mice (twenty in each) were used, first group was immunized twice at two weeks intervals subcutaneously (S/C)with 0.5 ml of heat killed *E. coli* O157:H7 (first vaccine) containing 1×10^8 C.F.U, second group immunized similarly with *heat killed E. coli* possess O157:H7+K99 (second vaccine), third group was injected S/C with phosphate buffer saline (PBS). Blood samples were collected at 2, 4 and 6 weeks post booster dose. Humoral immunity was detected by ELISA test, while cellular immunity detected by delayed type hypersensitivity test (DTH-skin test). Post six weeks of vaccination, the immunized and control mice groups were challenged with (1×10^{10}) of virulent *E. coli*.

Results- Antibody titers (IgG) was increased significantly ($p<0.05$) at 2,4 and 6 weeks in the immunized group and the maximum increase was determined at fourth week [O157 vaccine (751.5±21.5)] & [K99+O157:H7 vaccine (802.8±1.85)] in comparison with the control group which remained within the normal value in all times of the experiment. In DTH test, immunized groups showed a significant increase in foot-pad thickness after 24&48 hours post inoculation with soluble antigen in comparison with control group.

A significant protection was observed in immunized groups challenged with $4LD_{50}$ (4×10^{10} cells) compared with control group of mice which died within 1-3 days.

Conclusion- Immunization of mice with different *E. coli* vaccines was induced humoral and cellular immune responses against challenge with virulent *E. coli*. These vaccines of *E. coli* [O157:H7:K99] and [O157:H7] vaccine proved efficacy in inducing immunity.

Keywords- *Escherichia coli*, Immunity to *E. coli*, DTH skin test, Elisa test

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Introduction

Escherichia coli are a gram negative bacterium that is notable for the frequency and severity of infections that it causes hospitalized patients. *Escherichia coli* important zoonotic agents transmitted from horses to human especially veterinarian and persons who lived with this animals in the same place [1].

Escherichia coli O157:H7 has been responsible for multiple food- and waterborne outbreaks of diarrhea and/or hemorrhagic colitis (HC), The majority of *E. coli* O157:H7-associated fatalities results from renal failure, neurologic manifestations, or other complications of HUS. Worldwide [2], Infection with the EHEC O157:H7 serotype is highly pathogenic and fatal. Currently, there are few effective interventions to reduce the risk of this infection. Therefore, the prevalent EHEC infections in humans have become a global public health problem [3].

To facilitate development of therapeutic strategies and vaccines for humans against these agents, animal models that mimic one or more aspect of STEC infection and disease are needed. Focusing on the characteristics of various mouse models that have been developed and that can be used to monitor STEC colonization disease, pathology, or combinations of these features as well as the impact of Stx alone [3]. While antibiotics are still the most effective

treatment for O157 infection, their use promotes release of EHEC Shiga toxins (Stx), which increases the chance of complicating HUS [4,5]. Thus, finding alternative means to controlling these infections is a high priority. Vaccination is one of the most promising approaches against EHEC O157 infection.

Enterotoxigenic *E. coli* (ETEC) strains as the important cause of sever diarrhea in newborn calves carry fimbriae (such as F5 (K99) and F41) mediating binding of the bacteria to microvilli of enterocytes of the small intestine [6,7].

Different vaccines have been developed using ETEC strains producing K99 and F41. These vaccines may consist of bacterin [8,9] crude K99 and F41 extracts [10] or purified fimbriae [11].

This study was designed to evaluate the humoral and cellular immune response in mice following exposure to *E. coli* O157:H7 and E.coli O157:H7:K99 against challenge with virulent strain of *E. coli*.

Material and Methods

Bacterial Strain

Two hundred fecal samples were collected from 200 diarrheic and non-diarrheic horses at equestrian club location in Iraq, *E. coli* was isolated from these samples using different selective media, biochemical (SIM, TSI, SC, Urease, MR/VP) and KB003 Hi25^TM (Enterobacteriaceae identification Kit). Latex test was used for detection of K99 (K99 Politest) and latex test for detection of O157antigen(Mariel company) [12].

Preparation of *E. coli* Killed Vaccines for Immunization

Two isolates of *E. coli* with different antigens were selected for preparation of two vaccines, the first vaccine prepared from isolate of *E. coli* possess (O157:H7) and the second vaccine prepared from isolate *E. coli* possess (O157:H7 + K99). These vaccines prepared as follows:

The culture from each type was inoculated into brain heart infusion broth at 37°C for 14 hours and harvested with phosphate buffered saline. Cells were washed in PBS by centrifugation at 500 X g for 10 min at 4°C and then suspended to the appropriate density in PBS. Colony counts were performed to verify the number of bacteria at $1^{\times}10^{8}$ CFU/ml. Bacteria were killed by heating suspensions to 60°C for 1 hour [13]. The antigens were tested for sterility before use according to OIE [14].

Preparation of Soluble Antigen

Soluble antigen which used for DTH (skin test) prepared according to Mitov, et al [15] briefly; three to five colonies from the bacterial isolates on selective medium where inoculated into trypticase soy broth at incubated overnight. The cultures were harvested by centrifugation at 10.000Xg for 30 minutes. The sediment was sonicated for 50 minutes at intervals in a water cooled sonicator oscillator at 40 MHZ per second full power. The homogenate was centrifuged twice by using a cooling centrifuge at 8000 Xg for 30 minutes each time to remove cellular debris. The supernatants were passed through a 0.22 µm Millipore filter and stored at (-20ºC) until used. Protein content was determined by biuret protein assay.

Immunization of Mice

Sixty healthy mice aged 4 to 6 weeks were selected. All mice had negative fecal bacteriological culture for *E. coli.* They were reared in separate cages in the animal house of Veterinary College, University of Baghdad. The mice were divided equally into three groups. The first group was immunized subcutaneously with *E. coli* O157:H7 vaccine twice at two weeks intervals at adose of 0.5 ml containing $1^{\times}10^{8}$ CFU/ml. The second group was injected S/C with 0.5 ml containing $1x10^{8}$ CFU/ml of *E. coli* (O157:H7+ K99) antigen at the same time. Third group (control group) was injected S/C with 0.5 ml of PBS. Sera were collected at 2nd, 4th and 6th week post injection with booster immunization. Sera were stored at -20 ºC until use for analysis by ELISA.

Estimating the LD50

Virulent strain of *E. coli* which possess (O157:H7:K99) was used for challenge. The viable count of this bacteria was done by bacterial plate count method in eight fold dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5},10^{-6},10^{-7},10^{-8}) according to Quinn, et al [12].

The LD50 was estimated according to Reed and Muench [16]. Forty eight healthy mice of both sexes were divided into (8) groups (6 mice in each group). Seven groups of mice were injected subcutaneously with 0.5 ml calculated CFU diluents, and the eighth group was considered as a control group injected with PBS. All groups were monitored for 30 days to calculate total live and dead mice.

Enzyme-Linked Immunosorbent Assay (Elisa)

Elisa and its reagents were prepared according to manufacturer (immunological consultant's laboratory, Inc.). This test used to follow up humoral immunity by detection of IgG in the serum.

Delayed Type Hypersensitivity Test DTH (Skin Test)

This test was done according to Hundson and Hay [17]. After 21 days of immunization. Briefly, 0.1ml of soluble antigen of *E. coli* (O157:H7:K99) was injected intradermally in right footpad of the mouse while the left side was injected by 0.1 ml of sterile PBS (pH = 7.2). The thickness of skin was measured by vernier caliper before injection and at 24, 48 and 72 hours post injection.

Challenge of Immunized Mice

At 6 weeks after the first immunization, all mice were challenged intraperitonially with 5 LD50 of virulent *E. coli* (O157:H7: K99) in 0.5 ml PBS. The relative degree of protection afforded by antigens was assessed by the number of mice surviving 30 days after infection.

Ethical Approved

This study was approved by the ethical and research committee of Veterinary Medicine of College /University of Baghdad/ Minstery of High Education And scientific research.

Statistical Analysis

Statistical analysis were conducted to determine the statistical differences among different groups using ready - made statistical design statistical package for social science (SPSS).

Results

Prevalence of *E. coli* Isolation

Out of 200 samples collected from diarrheic and non-diarrheic foals and horses with different age, *E. coli* was isolated from 68 samples. 32 isolates of *E. coli* gives +ve results for K99 Latex test. 22 isolates give +ve results for latex test O157:H7. 3 isolates of *E. coli* were showed +ve results for K99 and O157 [Table-1].

Table 1- Prevalence of E. coli isolation from horses and foals

No. of Isolates	No of *E. coli* +ve for *K99 antigen*	No of *E. coli* +ve for *O157 antigen*	No of *E. coli* +ve for *K99 +O157 antigen*	No of *E. coli* -ve for *K99 +O157 antigen*
68	32	22	3	11

Elisa Test

All mice before immunization (at zero time) showed the same means of IgG (191±11.3). After two weeks of immunization, the serum IgG titers of immunized group of *E. coli* O157and *E. coli* +O157:H7+K99vaccines was (406.4±15.9, 580±10) respectively. The results showed significant increase of antibody titers at ($p< 0.05$) after (4 and 6) weeks post immunization with booster dose, as compared with the control group [Table-2], [Fig-1].

Table 2- Antibody titers in the immunized and control groups in Elisa test.

Time (weeks)	Immunized group with *E. coli O157 vaccine* (*first vaccine*) Mean ± SE	Immunized group with *E. coli O157+K99 vaccine* (*second vaccine*) Mean ± SE	Control group Mean ± SE
0 time	191±11.3A	191±11.3A	271.6±7.13A
2nd	406.4±15.9B	580±10A	203±11.1C
4th	751.5±21.5B	802.8±1.85A	189.1±12.1C
6th	661±21B	758.8±38.8A	191±11.3C

Means with different capital letters in the same row differ significantley ($P < 0.05$)

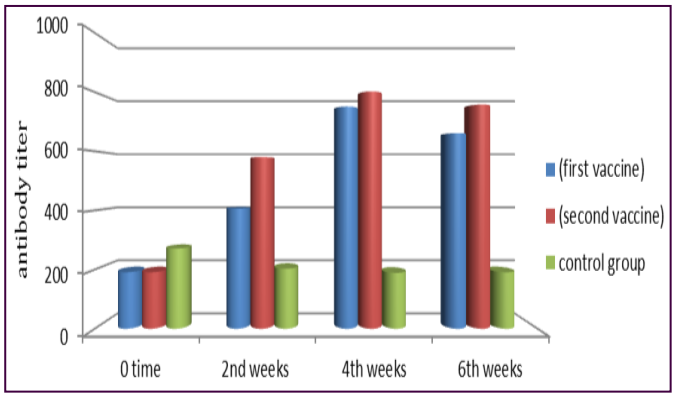

Fig. 1- Antibody titers in the immunized and control groups in Elisa test

Delayed Type Hypersensitivity Test

The results of delayed type hypersensitivity have showed increases in the thickness of the foot pad skin of the immunized mice, and the highest means of the thickness appeared after 24 hours post injection with soluble antigen.

DTH tests indicated that the values were significantly high at ($p< 0.05$) in the immunized groups compared to the control group [Table-3] [Fig-2].

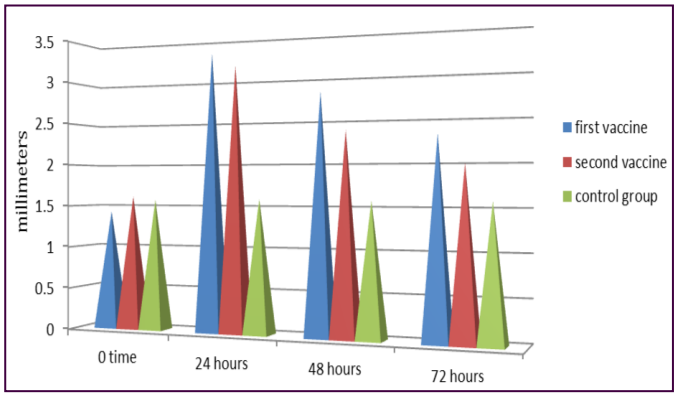

Fig. 2- Skin thickness (millimeters) of immunized and control groups in DTH test

Table 3- Skin thickness (millimeters) of immunized and control groups in DTH test

Times	Diameter of skin thickness (mm)		
	First group *E. coli* (O157) Ag	Second group *E. coli* (O157+K99) Ag	Third group (Control)
0 hours	1.428±0.067A	1.616±0.029A	1.57±0.059A
24 hours	3.321±0.207A	3.167±0.155A	1.59±0.064B
48 hours	2.829±0.136A	2.383±0.152A	1.58±0.061B
72 hours	2.3286±0.0928A	2.000±0.0522A	1.58±0.061B

Means with different capital letters in the same row differ significantley ($P < 0.05$)

Results of LD50 Estimation

The result of LD50 estimation for *E. coli* (O157:H7:K99) in mice injected subcutaneously with bacteria have revealed that the LD50 is (1×10^{10} cells) [Table-4]. The estimation was done by calculating the dead and alive mice in each group during (30) days, using the following equation.

$$\text{Percent of Mortality} = \frac{\text{Total Dead}}{\text{Sum of (Total a Live} + \text{Total Dead)}}$$

Table 4- LD50 estimation for E. coli (K99,O157) in mice

*Groups	Dose	Alive	Dead	Total alive	Total dead	Percent
1	1×10^{13}	0	6	0	21	100%
2	1×10^{12}	0	6	0	15	100%
3	1×10^{11}	2	4	2	9	81%
4	**1×10^{10}**	**3**	**3**	**5**	**5**	**50%**
5	1×10^{9}	4	2	9	2	18%
6	1×10^{8}	6	0	15	0	0%
7	1×10^{7}	6	0	21	0	0%
8	BPS	6	-	-	-	0%

**6 mice in each group*

Results of Experimental Challenge

According to the dose resulted in experiment of Estimating the LD_{50} (1×10^{10}), Experimental challenge was done by administration all groups intraperitonially with 4 LD_{50} (4×10^{10}) of virulent *E. coli* (O157:H7:K99).

Post challenge, the immunized mice showed mild signs of illness and depression for 2-3 days without signs of diarrhea and returned normal within 7 days without mortality.

The non-immunized mice (control group) exhibited the clinical sings post challenge as followed: anorexia, diarrhea, the respiration rate increased, sever dehydrated and recumbent till death in 1-3 days after challenge.

Discussion

In the current study, immunization of mice with two doses of *E. coli* vaccines containing O157 or O157:H7: K99 resulted in stimulation of immunity in the immunized group compared with control group. This is in agreement with study of Figueiredo, et al [18] that used two doses of bacterin from Enterotoxigenic *Escherichia coli* (ETEC), containing fimbriae K99 and F41 to induce anti-K99 and anti-F41 antibodies in colostrum of vaccinated cows and in calf serum, the animals which received two vaccine doses induce efficient immune response than do one dose. Previous studies have shown that anti-CFA/I fimbriae Abs are protective against ETEC infection [19-21].

Our results showed that the first and second vaccines induce detectable humoral immune response detected by ELISA with signifi-

cance difference (P<0.05) post two weeks to six weeks, 4 weeks and continued to six weeks. A similar results suggested by Xinghong, et al [22] that parenteral immunization with a live attenuated *Salmonella* vaccine vector expressing CFA/I fimbriae can induce protective systemic IgG and mucosal IgA Abs and may represent an alternative method to elicit protective Abs for passive immunity to ETEC. Also agreement with study of Hong-Ying, et al [23] who found that mice immunized intranasally with purified Tir proteins produced higher IgG and IgA titers in serum and feces, resulting in significant reductions in fecal shedding of EHEC O157 and higher a survival rate (92.9%), compared with subcutaneous or control immunizations. These results demonstrate the potential for the use of Tir proteins in mucosal vaccine formulations to prevent colonization and shedding of *E. coli* O157:H7. Therefore, purified Tir protects mice against EHEC challenge after intranasal immunization.

It is obvious that *E. coli* vaccines containing (O157, O157+K99) antigens are able to induce cellular immune response as detected by DTH-skin test in this study is in agreement with Kshash, et al [24] who used purified LPS of *E. coli* 0111:B4 in immunization of mice and record a highly significant increase of skin thickness of right footpad after 24 hrs and became at peak after 48 hrs when compared with control group.

DTH 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 chemoattractive factor to attract macrophage around the area of activated T-cell which also secrete INF- that enhancing the cytolytic activity of accumulated macrophages leading into skin thickness [25].

Our study revealed that the vaccination with *E. coli* vaccines protected mice against challenge with intraperitonially rout, this incompatible with study of Xinghong, et al [22] who give a challenged mice with wild-type ETEC by the oral, intranasal (i.n.), and intraperitoneally (i.p.) routes. Naive mice did not succumb to oral challenge, but did to i.n. challenge, as did immunized mice; however, vaccinated mice were protected against i.p. ETEC challenge. Two intramuscular (i.m.) immunizations with CFA/I fimbriae without adjuvant conferred 100% protection against i.p. ETEC challenge, while a single 30 μg dose conferred 88% protection.

Conclusion

These results demonstrate the potential for the use of *E. coli* possess O157:H7 plus K99 antigens or O157antigen alone in subcutaneous vaccine formulations to induce cellular and humoral immune response against challenge. Therefore, these antigens protect mice against challenge with virulent strain of *E. coli* O157:H7:K99 and is worth further clinical development as a vaccine candidate.

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