RESEARCH ARTICLE

IMMUNE RESPONSE AGAINST INFECTION BY ESCHERICHIA COLI O157:H7 IN IMMUNIZED MICE WITH TWO TYPES OF ESCHERICHIA COLI O157:H7 ANTIGENS.

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Manuscript Info

Abstract

The aim of the present study was to determine the role of whole sonicated E.coli O157:H7 antigen and culture filtrated E.coli O157:H7 antigen in protection of mice against this pathogen. To achieve this aim, sixty white mice both sexes with average age ranged 8 – 10 weeks were equally and randomly divided into four groups as following:

1st group immunized with 0.3 ml of WSECAg protein concentration (3.86 mg/ml) s/c two doses two weeks intervals.

2nd group immunized with 0.3 ml of CFECAg protein concentration (3.33 mg/ml) s/c two doses two weeks intervals.

3rd group served as control positive group.

At 27 – 30 days post immunization skin test and ELISA were done, the 1st , 2nd and 3rd groups were inoculated IP with 0.4 ml bacterial suspension containing 0.45x10^{10} cfu/ml of E.coli O157:H7 isolated from cattle fecal sample.

4th group served as control negative which inoculated with 0.3 ml of normal saline. All animals were sacrificed at ten days of age 15 – 16 weeks.

The results were showed both types of antigens which stimulated both the cellular and humoral immune response that their intensity differ according to the type of antigens, heavy to more heavy bacterial isolation with severe pathological changes were recorded in examined organs (heart, liver, spleen, lung, kidney and brain) of the control positive group while mild to moderate bacterial isolation with mild pathological changes were recorded in examined organs of mice of the immunized group with WSECAg while no growth of bacterial isolation with very mild pathological lesions were reported in examined organs of mice of immunized group with CFECAg post challenge.

The results of this study were concluded that both types of antigens produced a good protection against E. coli O157:H7 infection in mice as well as showed that CFECAg was good stimulator for cell mediated immunity (CMI) while WSECAg was a better stimulator for humoral immunity but generally CFECAg was better than WSECAg because the lesion was less in severity and showed good protection.

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Introduction:
Esherichia coli O157:H7 infection is a zoonotic disease induce a wide range of symptoms average from self-limiting watery diarrhea to severe bloody diarrhea and hemorrhagic colitis in a patient (Armstrong et al.,1996). In addition, it may cause extra-intestinal infections mostly hemolytic uremic syndrome due to kidney failure, particularly in children (Karmali et al.,1985) as well as neurological disorder and other complications of infection (Mariani-Kurkdjian and Bingen,1999).

E.coli O157:H7 is considered a main food-borne organisms in cattle and other ruminants which considered the major reservoirs for this organism (Shabana 2014). Sargeant et al., (2007) found that beef and dairy cattle have carriage rates less than 0.5 to greater than 2% of this pathogen. Therefore, beef and dairy products are the important vehicle for food-borne transmission of this pathogens for humans (Riley et al.,2005).

Manning et al., (2008) suggested that E.coli O157:H7 infections were emergency disease worldwide distribution, and E.coli O157:H7 was highly virulence pathogens due to low dose (10 - 100 organisms) can induce infection in humans (Besse et al.,2001) and the main virulence factor of E.coli O157:H7 that associated with severe infection of humans was shiga toxin (Paton and Paton, 1998 ; Nakao and Takeda, 2000).

It was suggested that treatment of E.coli O157:H7 with antibiotic was contraindicated due to this treatment may promote toxin expression from the lysogenized phage that typically carries shiga toxin (Stx) genes also using antimotility agents can lead to sustained presence, and consequent toxin expression of EHEC in the gastrointestinal tract in addition to, treatment of E.coli O157:H7 by antimicrobial agents were not efficient to kill this pathogens or diminish duration of shedding or prevent development of haemolytic uremic syndrome (HUS) (Molbak et al.,2002), therefore, vaccination strategies may provide protection against this pathogen.

In Iraq, little researches were done about the immune response against E. coli O157:H7 that isolated from cattle and humans. The aim of the study was a determination of the role of whole sonicated E. coli O157:H7 and culture filtrated E. coli O157:H7 antigens in protection of the mice from E. coli O157:H7 infection.

Materials and Methods:-
Bacterial Strain
The bacterial strain was obtained from zoonotic unit that confirmed diagnosis by routine examination according to Jawetz, Melnick and Adelberg 2016.

Culture Filtrated E. coli O157:H7 Antigen (CFECAg) preparation
After performing of the bacterial virulence test, ten petridishes were cultured with E. coli O157:H7 onto sorbitol mac Conkey (SMAC) agar and incubated at 37°C for 24 hrs., bacterial suspension was done by adding 1ml of normal saline (NS) to each petridish and harvested, bacterial count was performed according to Miles-Misra method after that the bacterial suspension was centrifuged at 3000 g for 20 min., the supernatant was called CFECAg that contain a bacterial secretions which used as a vaccine. The sterility test was done by bacterial culturing on SMAC agar and incubated at 37°C for 24 hours which was appeared no any growth.

Whole Sonicated E. coli O157:H7 Antigen (WSECAg) and Soluble Sonicated E. coli O157:H7 Antigen (SSECAg) preparation
The pellet or precipitate was produced after centrifugation, it was sonicated by sonifier appearatus in college of medicine Alnahairn university, a long 90 minutes of duration which worked 2 min. and paused 1 min. by using the ice and addition of NS, after that the sonicated bacterial suspension was centrifuged at 5000 g for 20 minutes. A part of the supernatant after sonication was taken from its and cool centrifugation 12000 g for 20 minutes was done for its, the new supernatant produced by cool centrifugation was called SSECAg used for a measurement of immunity by skin test, while the pellet (after cool centrifugation) was mixed with the residual supernatant and the pellet produced by sonication before cool centrifugation and this was called WSECAg used for immunization. The sterility test for WSECAg and SSECAg was done by bacterial culturing on SMAC agar and incubated at 37°C for 24 hours which was appeared no any growth.
Whole sonicated \textit{E. coli} O157:H7 antigen (WSECAg), soluble sonicated \textit{E. coli} O157:H7 antigen (SSECAg) and culture filtered \textit{E. coli} O157:H7 antigen (CFECAg) were prepared according to (Kusavadee \textit{et al.}, 2012) as well as the concentration of protein was measured in CFECAg, WSECAg and SSECAg.

**Preparation of Challenge Dose**

Challenge dose was determined according to Al-waka 2014.

**Skin Test**

It was done according to Barbaud \textit{et al.}, (2013). After measurement of the skin thickness, the left leg of immunized mice and control negative group were inoculated intradermal (I/D) with with 0.02 ml of SSECAg protein concentration 0.5 mg/ml and the right leg of the same animals were inoculated I/D with 0.02 ml normal saline.

After 24 hrs, thickness of the skin at the site of injection was measured by varnia and registered also repeated after 48 hrs and registered.

**Elisa Test**

It was done according to manufacturing company (KOMA BIOTECH) by following steps:-

1. 200 μl of washing solution were added to each well. Aspirate the wells to remove liquid and wash the plate 4 times using 300 μl of washing solution per well. After the last wash, invert plate to remove residual solution and blot on paper towel.
2. 100 μl of standard or sample were added to each well in duplicate. Cover with the plate sealer provided. Incubate at room temperature for at least 1 hour.
3. The wells were aspirated to remove liquid and washed the plate 5 times like as step 1.
4. 100 μl of the diluted detection antibody (1:10000 dilute) were added per well and covered the plate by sealer provided and incubated at room temperature for 1 hour.
5. The wells were aspirated and washed plate 5 times like as step 1.
6. 100 μl of color development reagent were added to each well. Incubate at room temperature for a proper color development (1-9 minutes). To stop color reaction, add 100 μl of stop solution to each well.
7. Using a microtiter plate reader and read the plate at 450 nm wavelength.

**Histopathological Examination**

A limited specimens with dimensions 1×1×1 cm were taken from internal organs including liver, spleen, kidney, heart, lung and brain and fixed in 10% formalin immediately after removal. After 72 hrs. of the fixation the specimens were washed with tap water and then processing was routinely done with a set of high alcoholic concentration from 70% to 100% for 2 hrs. and the water was eliminated in each concentration from the tissues, then refinement was done by xylol, then filtration of the specimens by using semi-liquid paraffin wax at 58°C on two stages, then blocks of specimens were made with paraffin wax and sectioned by rotary microtome at 5μm for all tissues. All tissues were stained with Hematoxylin and Eosin stain (H&E) and the histopathological changes were observed under the light microscope (Titford 2005; Gill 2009).

**Bacterial Load**

After sacrificing all animals, pieces of internal organs (liver, spleen, kidney, lung, heart and brain) were taken for determination of the bacterial load, swabs from these pieces of seven animals from each group were cultured onto SMAC agar incubated at 37°C for 24 hrs then observed and determined the growth of \textit{E. coli} O157:H7 colonies in these organs.

**Results:**

**Skin Test**

The skin test showed that the mean thickness of skin against SSECAgs (0.59±0.09) in 2nd group was higher than those values in 1st group (0.49±0.05) at 24 hrs post-examination and these values were decline (0.27±0.08) in 1st group and 2nd group (0.21±0.09) at 48 hrs post-examination as in below table the results revealed that significant variations (p<0.05) among groups.

| Group     | Mean and S. error at 24hrs | Mean and S. error at 48hrs |
|-----------|----------------------------|----------------------------|
| 1st. WSECAg | 0.49±0.05                  | 0.27±0.08                  |
| 2nd. CFECAg | 0.59±0.09                  | 0.21±0.09                  |
**Elisa Test**

The ELISA test revealed that the mean value and standard error of serum antibody titers in the WSECAG group (10.71±142) were higher than those values in the CFECAG group (10.63±1.4) at 30 days post-immunization and these values also were higher (18.50±1.7) in the WSECAG post-challenge group than those values in CFECAG post-challenge group (14.10±0.6) post challenge with E.coli O157:H7, while the mean serum Abs titers and standard error were very low (1.90±0.8) in control negative group. The results revealed that significant variations (p<0.05) among groups.

| Group         | Mean & Standard error before challenge | Mean & Standard error after challenge |
|---------------|----------------------------------------|-------------------------------------|
| WSECAG        | 10.71±1.42                             | 18.5±1.7                            |
| CFECAG        | 10.63±1.4                              | 14.1±0.6                            |
| Control -ve   |                                        | 1.9±0.8                             |

**Clinical Signs with Bacterial Load**

The results of present study showed that animals of the control positive group showed depression, collection together, off food and died during 24-72 hrs post–infection while no signs were recorded in the immunized animals for both antigens which provided 100% protection as compared with non-immunized animals post challenge with viable E.coli O157:H7 (as in below table), however, mild to no bacterial isolated from examined organs of immunized animals while very heavy to heavy bacterial isolated from examined organs of the control positive group.

| Group         | No. of Mice | Conc. Of bacteria cfu/ml | Dose ml | Number survival mice | Number of dead mice | % of protection |
|---------------|-------------|--------------------------|---------|----------------------|---------------------|-----------------|
| vaccinated with WSECAG | 7           | 0.45x10^{10}             | 0.4     | 7                    | 0                   | 100%            |
| vaccinated with CFECAG  | 7           | 0.45x10^{10}             | 0.4     | 7                    | 0                   | 100%            |
| Control +ve    | 7           | 0.45x10^{10}             | 0.4     | 0                    | 7                   | 0%              |

**Discussion:**

The result of delayed type hypersensitivity (DTH) revealed that both CFECAGs and WSECAGs elicited cell mediated immune response, since DTH was considered one arm of cell mediated immune responses and this reaction elicited by Th1 producing cytokines particularly INF-γ; this evidence was in consistent with Mosmann et al., (1986) and Dietert et al., (2010) who showed that both CD4+ and CD8+ T cells were considered a main source of INF-γ that induced DTH reaction.

The induration at the site of SSECAgs may be indicated that this Ags were directly interaction with memory immune cells producing by previous immunization with WSECAGs and CFECAGs and this interaction was associated with releasing of immune cytokines particularly INF-γ that attracted the macrophages and other immune cells to the site of Ags inoculation result in induration of the skin in this region, this investigation was agreement with observation of Mosmann et al., (1996) who showed that DTH was dependent on the memory cells of both CD4+ and CD8+ T cells that produced IFN-γ, a main macrophage chemotaxis and activator factor.

The current result revealed that the degree of DTH reaction induced by CFECAGs were higher than those induced by WSECAGs, in this result may be due to the protein nature of CFECAGs since they contain all secreted protein types of E.coli O157:H7, this evidence was in agreement with Belkaid and Rouse, (2005) who found that the cell mediated immune response was better stimulated by antigenic proteins also Alfredo et al., (2006) demonstrated that Outer membrane protein A (OmpA) of Escherichia coli O157:H7 stimulated dendritic cells to secreted IL-12 and IL-1 IL12 stimulated natural killer cells to secreted INF-γ that facilitated proliferation and differentiation of Th0 into Th1 that mediated CMI (Fraifeld et al., 1995).

Also the current finding was in consistence with Mohmammed, (2013) who recorded high cell mediated immunity in immunized animals with Culture filtrate Brucella melitensis antigens also the same result was observed by Abdulzahra, (2010), and Aziz, (2012) who reported high DTH reaction elicited by Candida CFAGs.
The current study demonstrated that CFECAgS and WSECAgs stimulated both cellular and humoral immune response, this result may be indicated that both antigens stimulated production cytokines of T-helper 1 (TH1) subsets which responsible for innate and cell mediated immunity through activated phagocytosis and T-helper 2 (TH2) cytokines that associated with antibodies (Abs) production, this idea was agreement with Kidd (2003) who investigated that TH1 cytokines triggered innate immune response such as phagocytosis and antimicrobial activity and TH2 cytokines such as IL-4 and IL-5 can regulate antibody production.

The present study revealed high serum antibody titers in immunized animals with WSECAgs as compared with those level in immunized animals with CFECAgS, this result may be due to the first Ags contain all structure of the pathogen such as LPS which considered a good stimulator of Abs production while the CFECAgS containing mainly protein antigens (AgS) that was considered a good stimulator for cell mediated immune response.

This evidence may be supported by result of ELISA assay which showed high level of serum Abs titers in immunized animals with WSECAgs postchallenge as compared with immunized animals with CFECAgS. However, this result may be due to the challenge dose was considered a third poster dose and the degree of its immune stimulation was dependent on memory immune cells or may be due to high immune stimulation by CFECAgS completely protective the animals against challenge dose with viable E. coli O157:H7.

The current study investigated that all non-immunized animals were died during 24-72hrs post infection by E. coli O157:H7 with heavy bacterial growth isolated from internal organs, this result may be indicated that strain was highly virulence that disseminated to all internal organs including brain, this result was in agreement with the observation of Poolman (1996) and Prasadarao (2002) who explained that E. coli O157:H7 possesses virulence factor such as translocated intimin receptor (Tir) which mediated bacterial adhesion and invasion the target cells such as brain microvascular endothelial cells, also the death of all animals of the control positive group may be due to multiorgans damage by Stx, this result was in agreement with the results of Luisa et al. (2013) who suggested that Stx2 distributed through the kidney and brain and induced damage in these organs.

The ratio of protection of immunized animals post-challenge in the present study may be indicated that both humoral and cellular immune response elicited by both immunized AgS which act against virulence factors of E. coli O157:H7, this evidence was agreed with Panton et al. (1998) who found that antibodies specific to Stx inhibited adherence of E. coli O157:H7 in vitro also Li et al. (2000) explained that successful vaccination against E. coli O157:H7 associated with induced humoral response against their virulence factors such translocated intimin receptor (Tir).

Also Luisa et al. (2013) suggested that the Stx2 specific monoclonal antibodies can neutralize Stx2 either by blocked enzymatic activity subunit of this toxin or by competing for receptor binding site on B subunit with the cell receptor and result in reduced toxin enter into host cells.

Mild to absent bacterial isolated from internal organs of immunized animals may be indicated that few bacteria reach to these organs and antibodies against attachment virulence factors and Stx prevent bacterial colonization in target organs this idea was supported by observation of Mohawk et al. (2010) who recorded that anti Stx antibodies can protect the mice against systemic E. coli O157:H7 infection as well as prevent bacterial attachment due to Stx facilitated bacterial colonization.

WSECAgs contain surface polysaccharide of E. coli O157:H7, therefore may be led to stimulate Abs against bacterial LPS which prevent bacterial colonization, this idea was in consistence with Xi et al. (2014) who found that vaccinated mice against Stx and surface polysaccharide of E. coli O157:H7 provide a protective against broad range of STEC serogroups also Gao et al. (2011) found that vaccinated cows with surface protein or lipopolysaccharide O-antigens O157 lead to reduce bacterial shedding by this animal.

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