Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/ 9365
DOI URL: http://dx.doi.org/10.21474/IJAR01/9365

RESEARCH ARTICLE

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

Khanjar A.F. and Alwan M.J.
Zoonotic diseases unit, College of Veterinary Medicine, University of Baghdad and Missan Directorate of Health, Iraq.

Manuscript Info

Manuscript History
Received: 10 May 2019
Final Accepted: 12 June 2019
Published: July 2019

Key words:-
Khanjar A.F., immune response, *E. coli* O157:H7.

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.

Corresponding Author:-Khanjar A.F..
Address:-Public Health Department, Missan Directorate of Health, Missan, Iraq.

……..

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 filterated 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 peteridish 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 Alnahrain 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 *E. coli* O157:H7 antigen (WSECAg) , soluble sonicated *E. coli* O157:H7 antigen (SSECAg) and culture filterated *E. coli* O157:H7 antigen (CFECAg) were prepared according to (**Kusavadee *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 *et al*., (2013)**.After mesurment 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 readed 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 histopathlogical 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 *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 (as in below table) the results revealed that significant variations ($p<0.05$) among groups.

Group	Mean & Standard error	
	before challenge	**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- 72hrs 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-y ,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-y 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-y 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-y, 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, 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 .IL-12 stimulated natural killer cells to secreted INF-y 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 posses 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 associted 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 reached 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 lipopolysacchride O-antigens O157 lead to reduce bacterial shedding by this animal.

References:-

1. Abdulzahra, H.N. (2010). Cryptococcosis: Immunopathological study in mice immunized with Cryptococcus neoformans antigens and diagnostic study in human. Msc. Thesis. College of Veterinary Medicine at University of Baghdad.
2. Alfredo, G.T.; Yongguo, L.I.; Christopher, B.T.; Lijun, X.; Tonyia, E.P.and Lynn, S. (2006). Outer Membrane Protein A of *Escherichia coli* O157:H7 Stimulates Dendritic Cell Activation, Infect Immun., 74(5): 2676–2685.

3. Al-Wgaa, A. (2014).M.Sc. Thesis. Diagnosis of *E.coli* O157H7 isolated from urine of humans and sheep by PCR technique and it's pathogenesis in mice. College of Vet. Medicine, University of Baghdad, Iraq.
4. Armstrong, G.L.; Hollingsworth, J. and Jr-Morris, JG. (1996). Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. Epidemiol. Rev., 18:29-51.
5. Aziz, (2012). Influence of culture filtrate of Candida albican and *Cryptococcus neoformans* antigen on pathogenesis of candida albican isolated from mastatic milk and milkers. Thesis of Master, College of Vet. Medicine, University of Baghdad, Iraq.
6. Barbaud, A.; Collet, E.; Milpied, B.; Assier, H.; Staumont, D. andAvenel- Audran, M.(2013). A multicentre study to determine the value and safety of drug patch tests for the three main classes of severe cutaneous adverse drug reactions. Br. J. Dermatol .,168(3):555-562.
7. Belkaid, Y. and Rouse, B. T. (2005). Natural regulatory T cells in infectious disease. Nat. Immunol., 6(4): 353-360.
8. Besse, P.; Combourieu, B.; Boyse, G.; Sancelme, M.; De Wever, H. and Delort, AM. (2001). Long-range (1)H-(15)N heteronuclear shift correlation at natural abundance: a tool to study benzothiazole biodegradation by two rhodococcus strains. Appl Environ Microbiol., 67(4):1412-1417.
9. Dietert, R.R. and DeWitt, J. (2010). Developmental immunotoxicity (DIT): The why, when, and how of DIT testing. Methods Mol. Biol., 598:17–25.
10. Dietert, R.R.; DeWitt, J.C.; Germolec, D.R. and Zelikoff, J.T. (2010). Breaking patterns of environmentally influenced disease for health risk reduction: Immune perspectives. Eviron. Health Perspect 118(8): 1091– 1099.
11. Fraifeld, V.; Blaicher-Kulick, R.; Degen, A.A. and Kaplanski, J. (1995). Is hypothalamic prostaglandin E2 involved in avian fever? Life Sciences 56(16): 1343—1346.
12. Gao, X.; Cai, K.;Li, T.;Wang, Q.; Hou, X.;Tian, R.; Liu, H.; Tu, W.; Xio, L.; Fang, L.; Luo, S.; Liu, Y. and Wang, H . (2011). Novel fusion protein protects against adherence and toxicity of enterohemorrhagic *Escherichia coli* O157:H7 mice. Vaccine, 29:6656- 6663.
13. Gill, G.W. (2009). Gill hematoxylins – first person account. Biotechnic &Histochemistry. 84:1-12.Health, 54(6-7):260-277.
14. Jawetz , Melnick and Adelberg's Medical Microbiology 27th. Edition, 2016
15. Karmali, M.A.; Petric, M.; Lim, C.; Fleming, P.C.; Arbus, G.S. and Lior, H. (1985). The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing Escherichia coli. J. Infect. Dis., 151(5): 775-782.
16. Kidd, P. (2003). Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease.Review Altern. Med. Rev., 8(3): 223- 246.
17. Kusavadee, S.; Petcharat, T. and Aphidech, S. (2012), Evaluation of Antigen Preparation Methods for Polyclonal Antibody Production against Streptomyces spp., British Microbiology Research Journal, 2(3): 137-145.
18. Li, Y.E.; Frey, A.M.; Mackenzie, A.M. and Finlay, B.B. (2000). Human response to *Escherichia coli* O157:H7 infection: antibodies to secreted virulence factors. Infect. Immun., 68(9):5090-5095.
19. Luisa, W.; Cheng, ; Thomas, D.; Henderson, I.I .; Stephanie, P. ;Larry, H.; Stanker, and Xiaohua, H.(2013). Mouse in Vivo Neutralization of *Escherichia coli* Shiga Toxin 2 with Monoclonal Antibodies, Toxins(Basel) , 5(10): 1845-1858
20. Manning, S.D.; Motiwala, A.S.; Springman, A.C.; Qi, W.;Lacher, D.W.; Ouellette, L.M.; Mlaclonicky, J.M.; Somsel, P.; Rudrik, J.T. and Dietrich, S.E. (2008). Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. Proceedings of the National Academy of Sciences of the United States of America, 105(12): 4868-4873.
21. Mariani-Kurkdjian, P. and Bingen, E. (1999). *Escherichia coli* O157 : H7 an emerging pathogen. Presse Medicale, 28(37): 2067–2074.
22. Mohammed, A. M. (2013). Immunopathological study of *Brucella melitensis* isolated from aborted sheep in mice. Msc. Thesis. College of Veterinary Medicine at University of Baghdad. pp: 55-70.
23. Mohawk, K.L.; Melton-Celsa, A.R.; Robinson, C.M. and O'Brien, A.D. (2010) Neutralizing Antibodies to Shiga Toxin Type 2 (Stx2) Reduce Colonization of Mice by Stx2-Expressing *Escherichia coli* O157:H7, NIH Public Access J., 28(30): 4777–4785.
24. Molbak, K.; Smith, P.G. and Wegener, H.C. (2002).Increasing quinolone resistance in Salmonella enteric serotype enteritidis. Emerg. Infect. Dis., 8:514-551

25. Mosmann, T.R.; Cherwinski, H.; Bond, M.W.; Giedlin, MA. And Coffman, R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol., 136(7):2348-2357.
26. Nakao, H. and Takeda, T. (2000). Escherichia coli Shiga toxin. J. Nat. Toxins, 9(3):299-313.
27. Paton, A.W. and Paton, J.C. (1998). Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E. coli hlyA, rfbO111, and rfbO157. J. Clin. Microbiol., 36(2):598-602.
28. Poolman, J.T. (1996). Bacterial outer membrane protein vaccines. The meningococcal example. Adv. Exp. Med. Biol. 397:73-77.
29. Prasadarao, N.V. (2002). Identification of *Escherichia coli* outer membrane protein A receptor on human brain microvascular endothelial cells. Infect. Immun.,70(8):4556-4563.
30. Riley, R. (2005). Inferring protein domain interactions from databases of interacting proteins. Genome Biol., 6(10): 89.
31. Sargeant, J.M, Amezcua, M.R, Rajic, A, Waddell, L.(2007) Pre-harvest interventions to reduce the shedding of *E. coli* O157 in the faeces of weaned domestic ruminants: a systematic review. Zoonoses Public
32. Shabana, I.I.(2014). Escherichia coli pathotypes associated with diarrhea in human and domestic animals , American Journal of Animal and Veterinary Sciences 9(3): 155-161.
33. Titford, M. (2005). The long history of hematoxylin. Biotech Histochem., 80:73-78.
34. Xi, L.; David, S.; Clarissa, P.; Damien, R.; Colette, C. ; Jennifer, M. ; Diana, M.; Marina, L. ; Yury, E. ; Nikolay, E. ; Matthew, K. and Gerald, B. (2014). A Poly-N-Acetylglucosamine−Shiga Toxin Broad- Spectrum Conjugate Vaccine for Shiga Toxin-Producing *Escherichia coli*, mBio, 5(2): 974.