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Adjuvant Activity of Vero Cell on Cellular and Humoral Immunity Responses against *E. coli O157:H7* Infections in Balb/c Mice

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Abstract

Enterohemorrhagic *Escherichia coli* constitute an important current problem of public health and animal production. Extensive efforts have been made to fight the infections caused by the path types which responsible for diseases such as hemorrhagic colitis and hemolytic uremic syndrome. Different vaccines have been tested against these bacteria. Here, the current study was undertaken to evaluate the Humoral and cellular immune responses and protective immunity conferred by an *E. coli O157:H7* vaccine with vero cell as adjuvant in mice.

E. coli O157:H7 was grown in the Tryptic Soy Broth (TSB), inactivated with formalin and adjuvant with vero cell and alum. Mice were immunized with inactivated *E. coli O157:H7* vaccine to study the splenocytes suspension and serum cytokines proliferation. The serum antibody titers and cytokines Interleukin-1a (IL-1a) and TNF-y were identified by ELISA. At 28 days post immunization, the studied groups were challenged with the LD50 dose of the live bacteria.

Mice immunized with vero cell adjuvant *E. coli O157:H7* (VO group) were observed to produce high levels of serum IgG antibodies, also inducing increases in the production of lymphocyte proliferation and secretion of cytokines. Besides, this immunized group produced complete protection after challenge. Our findings introduce that VO immunized mice is capable of releasing an immunological response with several cytokines and produce complete protection after challenge. These obtained results suggested that VO group has the potential as a candidate for vaccine against *E. coli O157:H7*.

Keywords: *E. coli O157:H7*; Vero cell; Adjuvant activity; Vaccine; Humoral and cellular immunity

Introduction

Eenterovirulent *Escherichia coli* rank among the most common relevant agents of bacterial diarrhea in humans and several animal species. Five different pathotypes or groups of the *E. coli* strain were recognized which include Enterohaemorrhagic *E. coli* (EHEC), Enteropathogenic (EPEC), Enterotoxigenic (ETEC), Enteroaggregative (EAEC) and Enteroinvasive (EIEC) [1]. Enterohemorrhagic *Escherichia coli* (EHEC) is an important zoonotic pathogen in humans which causing severe diarrhea and Haemolytic-Uremic Syndrome (HUS) [2].

Konowalchuk et al. [3] found that one group of *Escherichia coli* strains produced a toxin that is very similar to the one produced by Shigelladysenteriae type 1, this group was named Shiga Toxin-Producing *E. coli* (STEC). Also, these toxins may be identified by Vero Cell Toxicity Test [4] so these bacteria are called Verocytotoxin or Verotoxin-Producing *E. coli* (VTEC). Most of the verotoxigenic strains were made anything from uncomplicated diarrhea to hemorrhagic colitis, which can led to the progress into Hemolytic Uremic Syndrome (HUS) [1]. The firmly attached bacterial colonies to the epithelial cells cause the damage to microvilli and rearrangement of host cytoskeleton proteins [5]. The basic virulence factors in EHEC are encrypted at the Locus of Enterocyte Effacement (LEE) [6]. LEE is responsible for the attaching and effacing intestinal histopathology exhibited by Enterohemorrhagic (EHEC) E. coli and related pathogens [7].

Among the STEC serotypes, *O157:H7* serotype is by far the most frequently involved in human illness [1,8]. The Centers for Disease Control and Prevention (CDC) has predicted 75,000 illnesses and 61 deaths annually in the United States which caused by *E. coli O157:H7* infections [9,10].

The Shigatoxigenic (STEC or VTEC) strains produce Shiga toxins 1 and/or 2 (stx1, stx2) while EHEC produce stx1 and/or stx2 and intimin (eaeA) [11].The Shiga toxin (Stx) is the main virulence factor of STEC/EHEC which is an optimal target to elicit neutralizing antibodies [12].

Since the cattle are the widespread reservoirs of EHEC *O157:H7* strains, reduction of the prevalence of contamination in cattle are the best method for reducing the risk of EHEC infections in the man [13].

Unfortunately, the risk of HUS has been increased by antibiotics [14] because of the induction of the bacterial SOS response (an abundant response to DNA damage) which induce the transcription of type 2 Shiga toxin genes [15,16] and increase the extracellular concentrations of Shiga toxin [17]. There are no definitive treatments for EHEC human infections, and vaccines to prevent the disease are not yet accessible other than common supportive procedures [9].

Global efforts are underway to develop vaccines for a potential EHEC infection and to reduce the contamination of cattle and its environmental dissemination of EHEC *O157:H7* [18]. Different factors make complicate the development of a vaccine to prevent EHEC/STEC human infections include; the type of immune response may confer protection, multiple pathways of infection include cattle-containing food products, green vegetables, drinking water [19].

Several vaccines with different immunogenic, adjuvants, inoculation pathways, number of doses have been synthesized [18]. Besides, other efforts continue to develop new adjuvants to improve the immunogenicity and efficacy of mucosal delivered vaccines. So far different adjuvants were used for this purpose, such as Freund's incomplete adjuvant [20], aluminum hydroxide, oil-based adjuvant, lipids, peptidoglycans, different toxins [21] and protein based adjuvant [9] which lacks the capability to produce both Humoral and cellular immunity.

For example, as mentioned before, one of the most important proteins for the vaccination of ruminant targets is the LEE-encoded type III secreted proteins. Several researchers investigated the vaccine based on this protein. Immunization of the cattle with *E. coli O157:H7* type III secreted proteins motivate the inhibition of attachment of *E. coli O157:H7* to HEp-2 cells by the immunized cattle sera [22,23].

Until now, current vaccination strategies only partially successful in reducing *E. coli O157:H7* excretion. Awareness of the host cell interactions of *E. coli O157:H7* and differences between the pathogenesis in the ruminant reservoir and in humans, can progress current control strategies [5].

We have introduced a vaccine against Enterohemorrhagic *Escherichia coli* (EHEC) with new adjuvant. For the first time we used the vero cell as adjuvant for *E. coli O157:H7* which increase the Humoral and cellular immunity other than pervious adjuvants. Vero cells are a tissue culture cell line which obtained from monkey kidney epithelial cells. Vero cells have the potency for the adherence and contamination with other organisms [24,25] and extensively studied for the propagation and intracellular bacteria [26]. So, bacteria can attach to the vero cells and may be inter the cell. The vaccines were administered at 7 weeks and serum antibodies and Humoral and cellular immunity were assessed.

Materials and Methods

Animals

In this experiment, 40 female Balb/c mice (weight 18-22 g) were randomly chosen from the Laboratory Animal Center of Razi Vaccine and Serum Research Institute (RVSRI), Shiraz, Iran. The mice were randomly divided into 4 groups each containing of 10 mice. All experiments have been performed in accordance with the guideline for care and use of experimental animal of the National Counsel of Animal Experimentation Control.

Cultivation and purification of *E coli O157:H7*

Frozen *E coli O157:H7* were cultured in defibrinated sheep blood agar 6% (Merck). The purity of the isolates were confirmed by morphological characteristics on smears stained with Gram's staining and by growth on the Eosin Methylene Blue Agar (EMB) (Merck) and MacConkey agar (MA) (Merck). Afterward, this strain were inoculated in the Tryptic Soy Broth (TSB) (Merck) and incubated at 37°C for 24 hr. According to other our studies, multiplex variation polymerase chain reaction (MV-PCR) was applied for molecular analysis of *E. coli* isolates [27,28].

DNA extraction and PCR procedure

DNA extraction for molecular techniques has been done by boiling method [29]. 25 µl reaction mixtures included 0.25 µl TaqDNA polymerase, 1.25 µl MgCl2, 0.5 µl deoxynucleotide triphosphates 10 mM, 2.5 µl PCR buffer (10x), 1 µl primer 20 and 2 µl of the DNA template. Thermocycler (Master cycler Eppendorf, Germany) parameters included 94°C (5 min) one cycle and 94°C (55 s), 55°C (30 s), and 72°C (60 s) for 35 cycles, followed by a final extension time for 72°C (4 min). The oligonucleotides used as primers in PCR to detect *E. coli O157* and Stx1, Stx2 with variants are shown in **(Table 1)**.

Base pair	Primer sequence	Primer	Generation
420	CGT GAT GAT GTT GAG TTG	F	O157
	AGA TTG GTT GGC ATT ACT G	R	
555	TTC GCT CTG CAA TAG GTA	F	Stx1
	TTC CCC AGT TCA ATG TAA GAT	R	
118	GTC CCT GTT ACT GGG TTT TTC TTC	F	Stx2
	AGG GGT CGA TAT CTC TGT CC	R	

Table 1: Characteristic of the oligonucleotides used as primers in PCR.

Preparation of formalin-inactivated antigen

Formalin inactivated *E. coli O157:H7* was achieved by the method of Sunwoo [30]. A typically isolated colony on blood agar plate was chosen and cultured in TSB at 37°C for 24 hr with shaking. The growth of the bacteria was extended by inoculating of 1 ml into one liter of TSB broth at 37°C for an overnight. Cultivated bacteria were treated with 0.3% formalin at 72 hr. Then, the inactivated bacteria were centrifuged, washed four times with PBS, suspended in the PBS and stored at 4°C until use.

Vero cell culture

Vero cell was achieved from the cell bank of the Razi Vaccine and Serum Research Institute of Shiraz. Vero cells were propagated in the DMEM medium (Dulbecco's modified Eagle's Medium) which supplemented with 10% FBS. Cell culture flask incubated in 5% CO^2 incubator for 48 hr at 37°C to make cells monolayer. After the quality of the monolayer was checked by inverted microscopy, the cells were collected for the preparation of Vero Cell Extracted Medium (VCEM).

Inoculation of *E coli O157:H7* on the vero cell line

For each vero cell, 5 × 105 CFU/ml purified bacteria were placed into a 25 cm^3 cell culture flask (Corning) containing 10 ml of DMEM medium and were incubated at 37°C in the presence of 5% CO^2 for 48 hr. After that, the changes in the number of bacteria and cytopathic effect in the cells were

Collection of treated cell with bacteria

After 48 hr incubation, vero cells were washed twice with PBS to remove nonattached bacteria, and treated with 500 µl trypsin 0.25% for 10 min at 37°C. After separation of treated cell from the culture flask, these treated cells were killed with 0.3% formalin at 37°C for 7 days.

Safety test

The safety of the vaccines was evaluated according to the general behavior of the mice after vaccination. The mice were observed for any symptoms of mental disorders, fatigue, depression, anorexia, the effect on skin conditions, and body temperature during the experiments.

Immunization of mice

For the investigation of the vaccine, different four groups of mice were inoculated twice by subcutaneously at days 0 and 14 at the dose of 500 µl.

Also, the hyper immune serum appropriate for this infection was used to immunize animals with Freund's adjuvant. The characteristics of the groups are given in **Table 2**.

Number	Symbol	Type of experiment
Group 1	Negative control group (TSB)	This group was injected with TSB medium alone.
Group 2	(V)	Mice in this group received only formalin-inactivated Vero cells.
Group 3	(VO)	This group received formalin inactivated vero cells inoculated with bacteria.
Group 4	(AO)	Mice in this group were injected with formalin-inactivated bacterium along with alum gel.

Table 2: The characteristics of the studied groups.

Collection of serum

From two weeks after the first immunization, blood was collected from the facial vein of mice (sub mandibular) [31]. Blood was kept in a sterile micro centrifuge tube without anticoagulant for serum separation. After centrifugation at 3000 rpm for 10 minutes, the serum supernatant was collected carefully. Serum samples were stored frozen at -20°C until used.

Spleen lymphocyte transformation test of mice

28 days after the first immunization, splenocyte suspensions were obtained from the spleens of immunized mice. Mice were euthanized and their spleens were aseptically separated and placed in a sterile disposable Petri dish containing 10 ml of incomplete Dulbecco's Modified Eagle Medium (DMEM) medium. Then, erythrocyte lysis by hypo-osmotic shock and the remaining cells were washed three times with DMEM medium, after than the supernatant fluid was discarded by centrifuge. The obtained pellet suspended in 5-10 ml of completed medium (DMEM medium supplemented with 2% heat-inactivated fetal calf serum) per spleen.

The number of spleen cells was adjusted to 1 × 106 cells/ml. Each splenic cell suspension was divided into two holes of the 24-well culture plate with 1 ml per hole. 100 µl *E. coli O157:H7* antigen was added in one hole and another hole was used as a control. After 48 hr incubation at 37°C in a 5% CO2 conditions, the supernatants were removed and kept at -20°C for cytokines assayed [32].

ELISA test for the antibody detection of *E. coli O157: H7*

ELISA is a quantitative test that detects and measures antibodies and proteins. In this study, two different ELISA have been done for the analysis of the results, for the evolution of antibody titer in the mice and for the detection of splenocytes culture supernatant and serum TNFy and IL-1a.

The evolution of antibody from mice was analyzed by an indirect ELISA using *E. coli O157: H7* as antigen. The polystyrene 96-well microtiter plates (Jet Corporation, Toronto, ON, Canada) were coated overnight at 4°C with the purified *E. coli O157: H7* with 105 CFU per well in coating buffer (PH=7.6). The plates were then blocked with 1% bovine serum albumin (BSA) in PBS/ Tween 20 (PBST) for 1 hr at 37°C and washed 3 times by PBST. Serum samples were diluted with PBST at a proper

concentration and were added for checkerboard titration. The plates were washed as before the conjugated antibody was incubated for 1 hr at 37°C. After the addition of conjugated antibody (Polyclonal Rabbit-Anti Mouse immunoglobulins/ HRP),plates were washed with the PBST, then the TMB substrate was added with 10 min at 37°C and stopped with 2 M H^2SO^4. The Optical Density (OD) of each well was measured at 450 nm using a universal micro plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The parameters of the indirect ELISA based on antigen was modified by the optimized experiments.

ELISA test for the detection of splenocytes culture supernatant and serum TNF-y and IL-1a

Cytokines Interleukin-1a (IL-1a) and TNF-y in the splenocytes culture supernatant and serum were identified by ELISA kits according to the manufacturer's protocol. ELISA kits for the determination of IL-1a and TNF-y were purchased from East biopharm (USA). For IL-1a detection, briefly, IL-1a was added to the wells which pre-coated with IL-1a monoclonal antibody and then incubated for 1 h at 37°C. After that, anti-IL-1a antibodies tagged with biotin was added to combine with streptavidin-HRP (Horseradish peroxidase).

After incubation, unbound enzymes removed and washed five times. Supplement chromogen solution (substrate) 3, 3', 5, 5'-Tetramethylbenzidine (TMB) was added and shakes gently to mix them up. Stop solution (H^2SO^4) was added to each well to stop the reaction (the blue color changes into yellow immediately at that moment). For evaluation, blank well-adjusted as zero, the absorbance (OD) of each well was measured one by one at 450 nm wavelengths, which should be read within the 10 minutes after adding the stop solution. To diagnose TNF-y, this instruction exactly repeats with explicit substances supplied in the Elisa TNF-y mouse kit.

Protection against lethal challenge conferred by *E. Coli O157:H7*

The mice in the VO, AO and control groups were challenged after two weeks of final immunization with LD50 dose of live *E. coli O157:H7* containing 1 × 104 CFU/ml. Survival and death of the mice were monitored daily for two weeks post challenge.

Statistical analysis

Statistical Analysis was performed using the Graph-Pad program PRISM 6.0. Interleukin titers were resembled by variance analysis (ANOVA) with repeated measurements. The significant differences were observed between the means in the similar group of the paired Student's t-test. The P value of <0.05 was taken as the level of significance.

Results

Detection of *E.coli O157: H7* with PCR

Multiplex PCR was led to detect the presence of the shiga-toxin genes (stx1 and stx2) in the *E.coli O157:H7* isolates. The results of multiplex PCR for the detection of stx1 and stx2 genes are shown in **Figure 1** these genes were appeared at 555 and 118 bp, respectively.

Result of vero cell culture

As shown in **Figure 1**, after 48 hr of incubation, cultivated vero cells in the DMEM medium were grown as a monolayer.

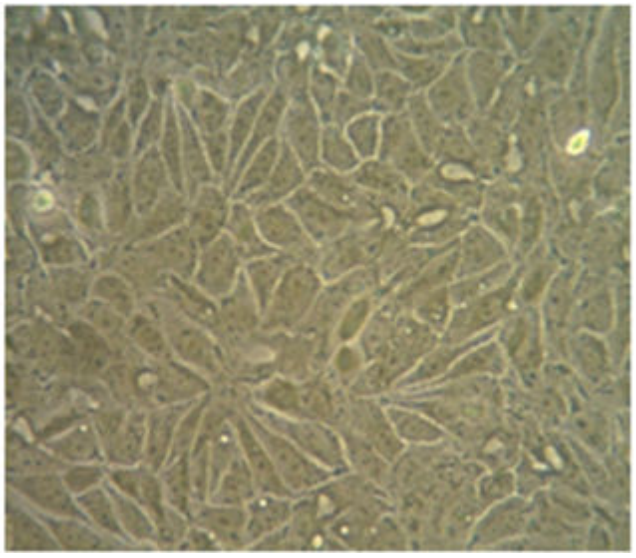

Figure 1: Monolayer growth of vero cell in DMEM medium.

Inoculated Vero cell with *E. coli O157:H7*

During the 24 hr of exposure to the *E. coli O157:H7*, the viability of the Vero cells had reduced substantially in a dose dependent manner. The incubation of Vero cell with *E. coli O157:H7* (104-107 CFU/ml) for 24 hr intimately adhere to host cell and produced cytopathic effects.

Safety assessment of the vaccines

The injection places were monitored after injection during the trial. Any modification of animal behavior per group could be mentioned. Local and general responses in mice were not observed. The general status of the animals remained stable and there was no observation of abnormal temperature rise in any of the tested groups.

ELISA test for the antibody detection

As shown in **Figure 2**, VO (bacterial inoculated in vero cells) group have the highest antibody titer among the studied groups. The AO (bacteria with alum adjuvant) and VO groups show the increment in the antibody until 6 weeks after immunization. These groups have the meaningful differences other than TSB (control) group (p<0/05(, while V (vero cell) group did not show significantly differences with control group (p>0/05(.

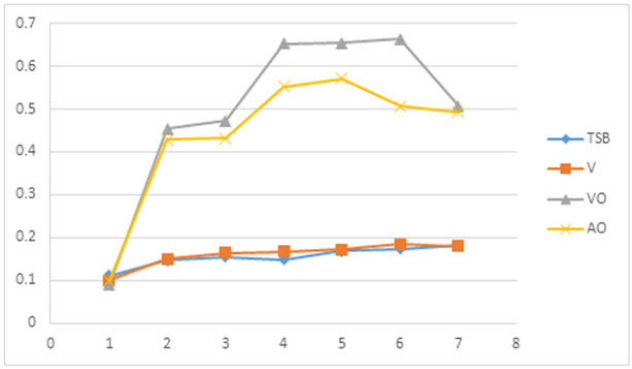

Figure 2: Immune response measured by indirect ELISA on sera from mice.

Immune responses induced by vaccines

TNF-y and IL-1a cytokine titers measured by ELISA in serum and splenocyte cultures from mice immunized with different vaccine formulations at 28th day post-immunization are shown in **Figures 3 and 4**. Cytokine measurement of serum and spleen culture supernatants demonstrated that animal vaccinated with vaccine formulation containing vero cells (VO group) significantly enhanced cytokine titers, in comparison to animals vaccinated with vaccine formulations containing formalin inactivated bacteria along with alum gel (AO group) and control groups **(Figure 3)**.

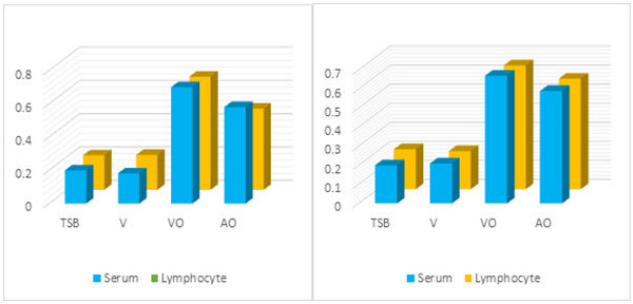

Figure 3: Serum and supernatant titers of splenocyte cultures in immunized and control (TSB) mice measured by indirect ELISA at 28th day post-immunization, IL-1a (left) and TNF-y (right).

As shown in **Figure 3**, the maximum IL-1a titers were observed among the groups injected with VO vaccine in both the serum and spleen culture supernatants samples. Their optical density was 0.67 and 0.65 for serum and splenocyte suspensions, respectively. The serum TNF-y levels for the VO group were significantly higher than those in all other vaccine and control groups (P=0.0133).

Also, for the VO groups, serum cytokine levels were significantly ($p<0.0001$) higher than those of the control mice. The spleen culture supernatants of the VO group have the highest titer for the IL-1a and TNF-y. Control groups showed lower cytokine levels than the immunized groups of the vaccines (VO and AO groups) for both serum and splenocyte suspensions. The results showed that, for the VO group both serum and supernatant of splenocyte content of more TNFy than IL-1a ($p>0.05$) (**Figure 4**).

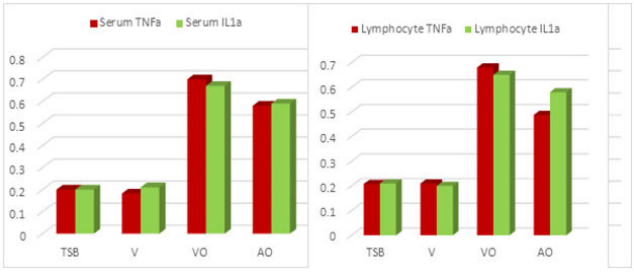

Figure 4: Serum and Splenocyte suspension IL-1 a and TNF-y responses in immunized and control mice at 28th day post-immunization.

Protective efficacy of vaccines after a lethal challenge

Half mice of each group were challenged two weeks after the final immunization with *E. coli O157:H7* at a lethal dose of 1 × 104 CFU/ml. Calpan Meyer survival curve **Figure 5** shows the percentage of protection of mice after immunization against the lethal dose of bacteria. The mice of TSB (control) and V groups showed lethargy, sluggish activity and eventually died within 3 and 5 days, respectively.

The results showed that VO immunized group conferred 100% protection in the challenge period, which had better survival rate than AO group. For the AO group 20% of mice died on day 6 and 80% of mice survived up to the end of the challenge time.

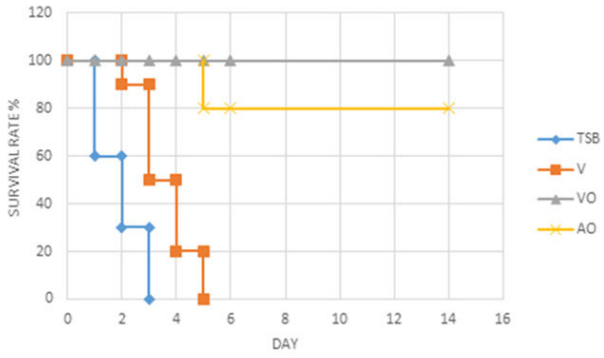

Figure 5: Survival curves mice groups (TSB, V, VO and AO). Mice were challenged i/p with a lethal dose of *E. coli O157:H7* on 14 days post-immunization.

Discussion

Unlike normal mammalian cells, vero cells are defective in its production of interferon and do not secreted interferon alpha or beta when infected by a pathogen but is sensitive to the action of interferon [33].

The past two decades have seen a wonderful progress towards the development of innovative vaccine adjuvants which have now entered clinical practice [34,35].The addition of adjuvants to vaccines improves, sustains and directs the immunogenicity of antigens, reducing the amount of antigen, effectively modulating appropriate immune responses and improving the efficacy of vaccines [36]. Also, improvement of

new generation vaccine systems to prevent infection is important in avoiding the disadvantages of currently used vaccines.

In this study, we demonstrated the ability of a subcutaneously delivered *E. coli O157:H7* vaccine with new adjuvant vero cell to induce Humoral and cellular responses by immunization of Balb/c mice. As well as, here we compare TNF-y and IL-1 a cytokine titers measured by ELISA in serum and splenocyte cultures. The study of the cytokine production is essential to recognize the cellular immune stimulatory, so the serum and splenocyte suspension cytokines after vaccination were studied.

For the first time, we have used vero cell as adjuvant system which approved by WHO for growing the high titer for some vaccine [37]. A versatile vero cell has been utilized to deliver an extensive range of candidate and licensed vaccines versus emerging viral diseases [38] but yet it has not been used as an adjuvant for bacterial vaccine.

Vaccine development has played a vastly essential role in combating infectious disease. One of the current challenges is development of vaccine against *E. coli O157:H7* infections. Several researchers explored vaccination to prevent intestinal colonization and subsequent *E. coli O157:H7* shedding [39]. Vaccinated mice against *E. coli O157:H7* with the chitosan nanoparticle as adjuvant. Also intimate bacterial adhesion, intimin (I), with its own receptor (Tir) and *E. coli* secreted protein a are highly immunogenic proteins for vaccine development against *E. coli O157:H7*. Numerous researchers evaluated type III secreted protein-based vaccines [23,40,41]. Finlay, Potter et al. [9] vaccinated cattle with type III secreted proteins by *E. coli O157:H7* which reduced the numbers of bacteria shed in faeces [9].

Furthermore cells containing IL-I and/or TNF-y were detected in pathologic conditions. Tumor necrosis factor (TNF-y) and interleukin1 (IL-1) are pleiotropic molecules that play a vital role in immune reactions [42]. IL-1 discovered in Inter Digitizing Reticulum Cells (IDRCs) and in epithelioid macrophages of granulomas. TNF-y demonstrated in epithelioid macrophages of sarcoid and tuberculous granulomas and in scattered cells of the lymphoid tissue [43].

On the other our works, we investigated the adjuvant capacity of DNA and alum for P. multocidaon Humoral and cellular immunity by the investigation of interleukin-6 (IL-6) and IL-12 titers with ELISA in serum and splenocyte cultures [44]. At the following, we evaluated the adjuvant capacity of vero cell for *E. coli O157:H7* on the cellular and Humoral immunity by the measurement of IL-1a and TNF-y cytokine titers in serum and splenocyte cultures from the immunized mice.

We found that vero cells inoculated with bacteria (VO group) were superior to the AO vaccine. Besides, the levels of serum IL-1a and TNF-y were higher than those of the other vaccinated groups post-immunization, VO vaccine increased the antibody IgG titer among the other studied groups and keep the titer at high level for the most time.

As regards lymphocyte proliferation assay, a significant difference was observed in the stimulation index of VO group compared to alum adjuvant group. Our results showed that addition of inactivated vero cell to the bacteria significantly increases in both IL-1a and TNF-y production. Although the exact mechanism of action is still unclear; it is possible that vero cell stimulates the production of cytokines, in particular, TNFy. In fact, there are some similarities between the levels of the serum and splenocyte suspension cytokines.

Even more important is the effectiveness of experimental vaccines to confer immunity against a lethal challenge given 14 days after a single dose to mice. The immunized VO group mice showed high rates of protection (100%) to the challenge with the live *E. coli O157:H7* bacteria in comparison to the negative control (TSB).Mortality rate was 100% in the TSB control group after 3 days, 100% in the V group after 5 days and 20% in the AO group after 5 days reported a morbidity of BALB/c mice of about 45% after challenge with *E. coli O157:H7*, in contrast to a morbidity of about 75% for the negative control [45,46].

Conclusion

Consequently, the present study has developed a protective effect of vero cell as a vaccines adjuvant against diarrheal diseases which induced safeguard against a lethal challenge in standard Balb/c mice, offered a candidate vaccine of bacterial ghosts against diarrheal diseases which might represent improved nonliving bacterial vaccines and also the our results showed that the vero cell adjuvant vaccine offered a good immunity against *E. coli O157:H7* which successfully induced long-term protection against infection in Balb/c mice.

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