Evaluation of the Efficacy of Humoral Immunity Response of Killed Oil Adjuvant *Escherichia coli* Vaccine in Layer Chicken against Avian *E. coli* Serotype O_{78} Infection

Abstract

Colibacillosis is one of the most important bacterial diseases of chickens and turkeys which caused by avian pathogenic *Escherichia coli* (APEC). Mortality, low body weight, and high FCR in colibacillosis affected poultry farms and higher carcass condemnation at slaughterhouse caused great economic impacts on the poultry industry. In recent years, production of homologous and heterologous APEC vaccines has been evaluated. In this study, mineral oil as an adjuvant for inactivated *E. coli* used and inoculated via injection route to layer chicken. At 28 days of age, 60 birds were subsequently divided into six experimental groups of 10 chickens per group. Chickens in control group did not receive *E. coli* vaccines; whereas five treatment groups were vaccinated subcutaneously with a formalin inactivated, mineral-oil adjuvant *E. coli* vaccine containing isolate of *E. coli* serotype O_{78}; T_1, T_2, T_3, T_4, and T_5 groups were vaccinated at 28 days of age with 0.2 ml (8×10^6, 16×10^6, 33×10^6, 66×10^6, and 133×10^6 cfu/ml) per dose of *E. coli* O_{78} respectively. IgG antibody titers against *E. coli* was evaluated 10 weeks after inoculation with ELISA method. Results showed a significant rise in IgG antibodies titer in the immunized birds compared to the unimmunized control group ($P < 0.05$), anti IgG antibodies increased weekly after injection in most vaccinated groups up to four weeks. Overall, prepared *E. coli* vaccine in Razi institute, Shiraz branch induced high levels of immune responses in the vaccinated group as revealed by ELISA. Although, in order to make considerable immunological stimulus it is suggested that all the chickens in the experimental group receive a booster dose four weeks after the first immunization.

Keywords: APEC, Colibacillosis, Immunization, Vaccine

1. Introduction

Colibacillosis is caused by infection with a strain of *Escherichia coli* (1); *Escherichia coli* is a gram-negative rod-shaped bacteria. It is normally found in the intestine of poultry and other vertebrates. Though many *E coli* are not pathogenic, some have acquired virulence factors, greatly increasing their capacity to cause disease (2). Colibacillosis results in a localized or systemic infection caused by avian pathogenic *Escherichia coli* (APEC) (3).

Syndromes associated with colibacillosis can vary and include acute fatal septicemia, airsacculitis, pericarditis, perihepatitis, peritonitis, and lymphocytic depletion of the bursa and thymus (4); salpingitis and cellulitis (5), in laying hens, peritonitis and salpingitis are common, whereas disease in young chicks may include omphalitis (yolk sac infection) or swollen head syndrome (4). Previously, most APEC isolates were assigned to three main serogroups: O_1, O_2, and O_{78}; however, it has been shown that there is a great diversity in serogroups of APEC causing colibacillosis (6). Colibacillosis is one of the most commonly occurring and economically devastating bacterial diseases of poultry worldwide (7) resulting in multimillion dollar losses annually that affect many facets of poultry production. Control of colibacillosis is problematic due to widespread antimicrobial resistance among APEC isolates (8), restrictions on use of antimicrobial agents in poultry, and the lack of vaccines to provide protection against all types of APEC isolates causing colibacillosis. There are reports on *Escherichia coli* autogenous vaccines which mostly used in breeder flocks; however, evidences on the efficacy of such vaccines in terms of rising *E. coli* infections is rare. Therefore, the aim of the current study was to evaluate the efficacy of humoral immunity response in layer chicken vaccinated with *Escherichia coli* vaccine which developed in Shiraz, Razi Institute.

2. Material and Methods

2.1. Animals

In total, sixty unvaccinated layer chickens (Hy-line) of mixed sex were obtained on the day of hatching from a commercial hatchery of the Razi Vaccine and Serum Research Institute (Shiraz branch). These birds were kept in controlled area with free access to food and water at the poultry department of the Razi Vaccine and Serum Research Institute (Shiraz branch).

2.2. Isolation, identification, and serotyping

Poultry pathogenic *Escherichia coli* serotype O_{78} was obtained from the microbiology department of the Razi Vaccine and Serum Research Institute (Shiraz branch), this bacterium selected for this study was isolated in the laboratory from the hearts and livers of 4–8-day-old broiler chickens suffering from colibacillosis infection with perihepatitis and pericarditis. For isolation of *E. coli*, tryptic soy broth (Merck, Germany), MacConkey agar (MCA) and Eosin-methylene blue (EMB) agar were used as enrichment, differential and selective medium respectively. The enrichment, the MCA, and EMB agar were incubated at 37°C for 24 hr. The smooth, moist colonies having metallic sheen on EMB agar were randomly sub-cultured. The isolates were identified on the basis of their cultural, morphological and biochemical characteristics (9).

Identification of *Escherichia coli* O_{78} was done by polymerase chain reaction (PCR) in experimentally infected specimens (10). The PCR amplifications were conducted in a 25 µL reaction volume containing 12.5 µL of Master Mix 2X AMPLIQON (Denmark), 1.5 U Taq polymerase, 1.5 Mm $MgCl_2$,1 µL of each primer, PCR buffer, and RNase-free water. To confirm *Escherichia coli* strain O_{78} from PCR test using forward and reverse primers OG_{78} related to wzx gene was used based on the presence of 992 bp fragment (Table 1; Figure 1). Polymerase chain reaction was done in an Eppendorf thermocycler (Germany) during 30 cycles with denaturing temperature of 94°C for one minute, annealing temperature of 55°C for 40 seconds, and an

extension temperature of 72°C for 1 minute was done. The polymerase chain reaction product was electrophoresed on a 1% agarose gel (Figure 1).

Table 1. The exclusive primer sequences used for molecular confirmation of Escherichia coli bacteria by PCR

Gene	Primer sequence (5′-3′)	Size (bp)	Reference
wzx	Forward: GGTATGGGTTTGGTGGTA Reverse: AGAATCACAACTCTCGGCA	992	Liu B. et al. Vet Microbiol.2010

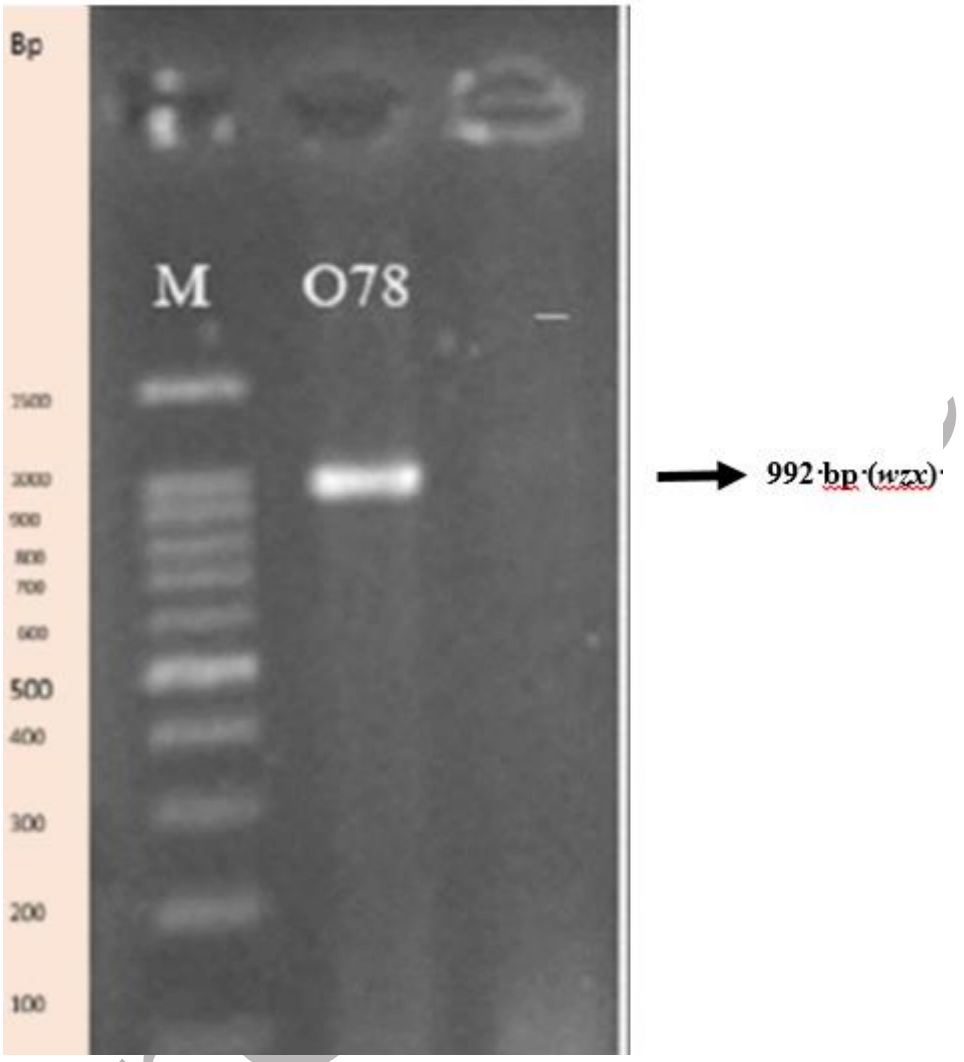

Figure 1. Molecular PCR test to confirm *Escherichia coli* O_{78}.

2.3. Antigens

The PCR confirmed isolate was proliferated in tryptic soy broth (Merck, Germany) (24 h/37°C). After incubation, cell suspension was inactivated with 0.3% formaldehyde at 37°C for 24 h. The cells were separated through centrifuging (5,000 g/20 min), and the pellet was resuspended in phosphate-buffered saline (PBS, pH 7.2). A suspension was adjusted to the turbidity standard 2 $x10^9$ bacteria / ml.

2.4. Adjuvants and Vaccines

To produce the oil adjuvanted vaccines , standard W/O emulsions, including Montanide™ ISA 70, shacked softly on the mixer at room temperature, and the aqueous phase was combined at a 70:30 ratio (w/w, adjuvant:antigen or PBS) as suggested by the adjuvant manufacturer (Seppic, France).

2.5. Immunization of Chickens

60 chickens were randomly allotted to six treatment groups (ten birds each), (control, T_1, T_2, T_3, T_4, and T_5). Birds in the control group did not receive *E. coli* vaccines; T_1, T_2, T_3, T_4, and T_5 groups were vaccinated at 28 days of age with 0.2 ml (8×10^6, 16×10^6, 33×10^6, 66×10^6, and 133×10^6 cfu/ml per dose of *E. coli* O_{78} respectively) formalin inactivated, mineral-oil adjuvanted vaccine subcutaneously containing one isolate of *E. coli* (serotype O_{78}).

2.6. Serum Titer of Anti- *E. coli* O_{78} Antibody

Blood samples were randomly collected from brachial vein of 60 chickens prior to immunization, and then from 10 chickens/group/weekly up to 10 weeks after immunization. The sera were separated through centrifuging (3500 g/10 min), followed by storing at -20°C until analysis of the antibody responses against *E. coli* O_{78} using an indirect enzyme-linked immunosorbent assay (ELISA).

2.7. Serology

The antigen response following challenge with *E. coli* O_{78}, was defined by Enzyme-Linked Immunosorbent Assay (ELISA). As mentioned before, the samples were centrifuged at 3500×g for 10 minutes. The serum fraction was delivered to separate tubes and kept at -20 °C until they were followed for ELISA evaluation of antibody content. For ELISA, the 96-well plates were coated overnight at 4 °C with 0.5 µg whole cell sonicates of *E. coli* O_{78}. Carbonate-bicarbonate

buffer (pH 9.6) was used for diluting sonicates. Each well was then washed; this and all subsequent washing steps consisted of one wash in 300 µL washing buffer (PBS + 0.05% Tween 20). After that, 200 µL bovine serum albumin (BSA) added as blocking solution for 2 h at room temperature and then washed. 100 µL of serum samples were added to each well, after incubation and washing, 100 µL polyclonal goat anti-chicken IgG diluted 1:4000 in diluting buffer, were added to each well and the plates incubated for 1 h at 37 °C and then washed. To find the binding, 100 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to each well and incubated for 15 minutes and then the reaction was stopped by addition of 100 µL 1 M H_2SO_4. The optical density was read at 450 nm, using a spectrophotometer (BioTek Instruments).

3. Results

Results of serological tests are shown in table 2. Data showed the significant titer in IgG antibodies in the immunized birds compared to the unimmunized control group (P <0.05; Figure 2), *E. coli* vaccine which developed in Shiraz, Razi Institute raised higher IgG titers in most of the weeks (Figures 3 and 4). No differences in antibody titers against *Escherichia coli* between experimental and control groups were found at wk 5 to 10 (Figures 4 and 5). Mean titre of OD of sera of vaccinated groups was generally higher than their control counterparts (Figure 2); however, there were no differences between the T_5 and T_1 groups, but the significant differences found in T_5 group compared to the corresponding values for T_2, T_3, and T_4 groups.

Table 2. LS mean±SE of antibody titer against *Escherichia coli* (SP%) obtained in Hyline selected laying chickens at different weeks post-immunization

Traits	Treatment						*P*-value		
	Control	T_1	T_2	T_3	T_4	T_5	Treatment	Time	Treatment × time

1.0499 ±0.043^{c}	1.5800± 0.046^{b}	1.6226± 0.043^{b}	1.6189± 0.044^{b}	1.5900± 0.043^{b}	1.7488 ±0.045^{a}		<.00 01	<.0 001	0.029 3

T_1, T_2, T_3, T_4, and T_5 groups were vaccinated at 28 days of age with 0.2 ml (8 x10^6, 16 x10^6, 33 x10^6, 66 x10^6, and 133 x10^6 cfu/ml) per dose of *E. coli* O_{78} respectively.
Means with different letters differ significantly ($P \leq 0.05$).

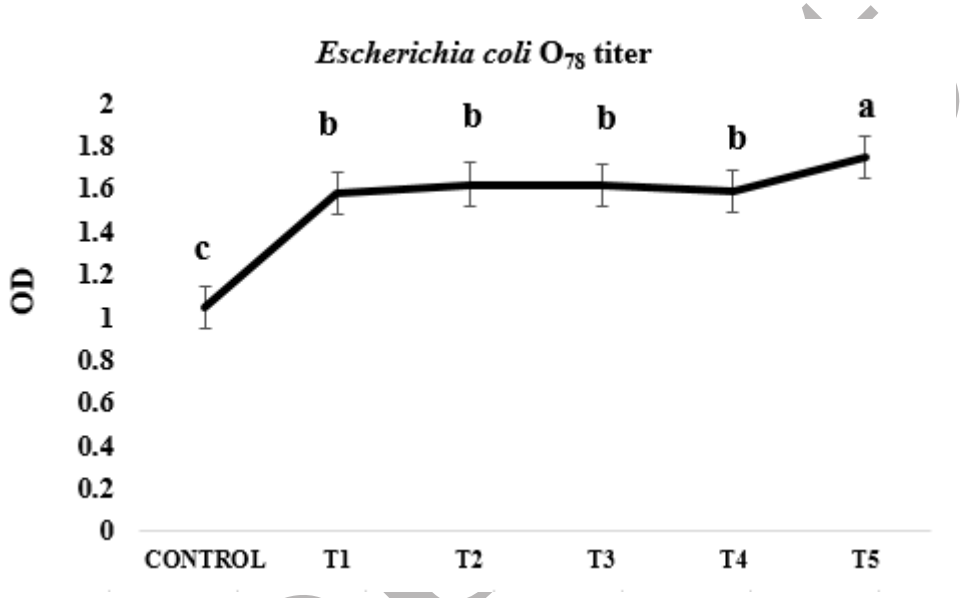

Figure 2. Mean titre of OD of sera of laying chickens following vaccination with *Escherichia coli* O_{78} which produced in Shiraz, Razi Institute. T_1, T_2, T_3, T_4, and T_5 groups were vaccinated at 28 days of age with 0.2 ml (8 x10^6, 16 x10^6, 33 x10^6, 66 x10^6, and 133 x10^6 cfu/ml) per dose of *E. coli* O_{78} respectively. Means with different letters differ significantly ($P \leq 0.05$).

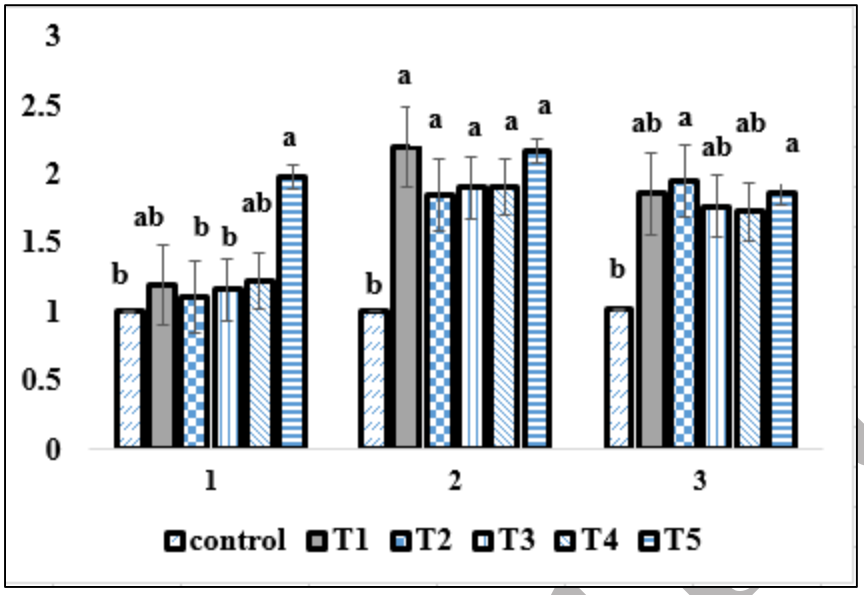

Figure 3. Effect of treat × time (wk) interaction on antibody titers against *E. Coli* (SP%) in Hyline selected laying chickens at different weeks post-immunization. T_1, T_2, T_3, T_4, and T_5 groups were vaccinated at 28 days of age with 0.2 ml (8×10^6, 16×10^6, 33×10^6, 66×10^6, and 133×10^6 cfu/ml) per dose of *E. coli* O_{78} respectively. [a,b]Within each week, least squares means with different letters differ significantly ($P \leq 0.05$).

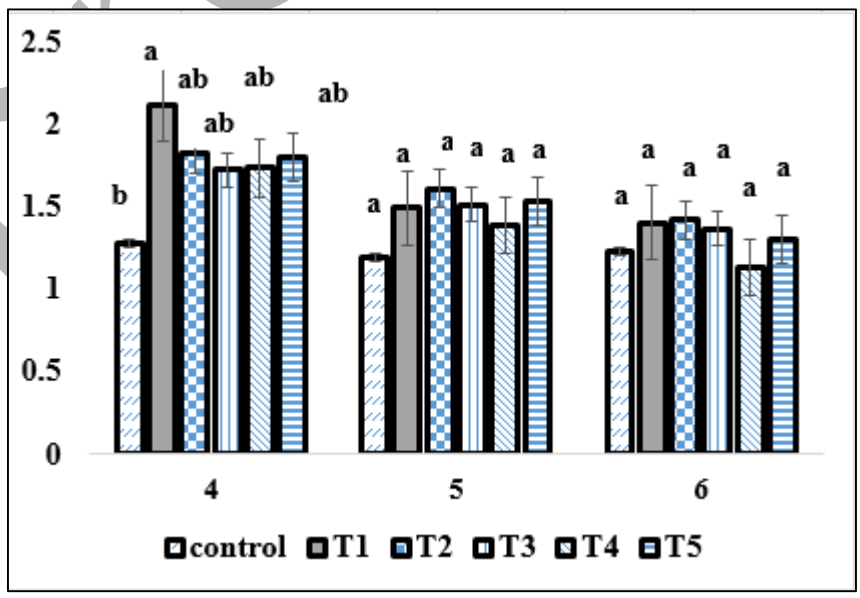

Figure 4. Effect of treat × time (wk) interaction on antibody titers against *E. Coli* (SP%) in Hyline selected laying chickens at different weeks post-immunization. T_1, T_2, T_3, T_4, and T_5 groups were vaccinated at 28 days of age with 0.2 ml (8 x10^6, 16 x10^6, 33 x10^6, 66 x10^6, and 133 x10^6 cfu/ml) per dose of *E. coli* O_{78} respectively. [a,b]Within each week, least squares means with different letters differ significantly ($P \leq 0.05$). No differences were found at wk 5 and 6.

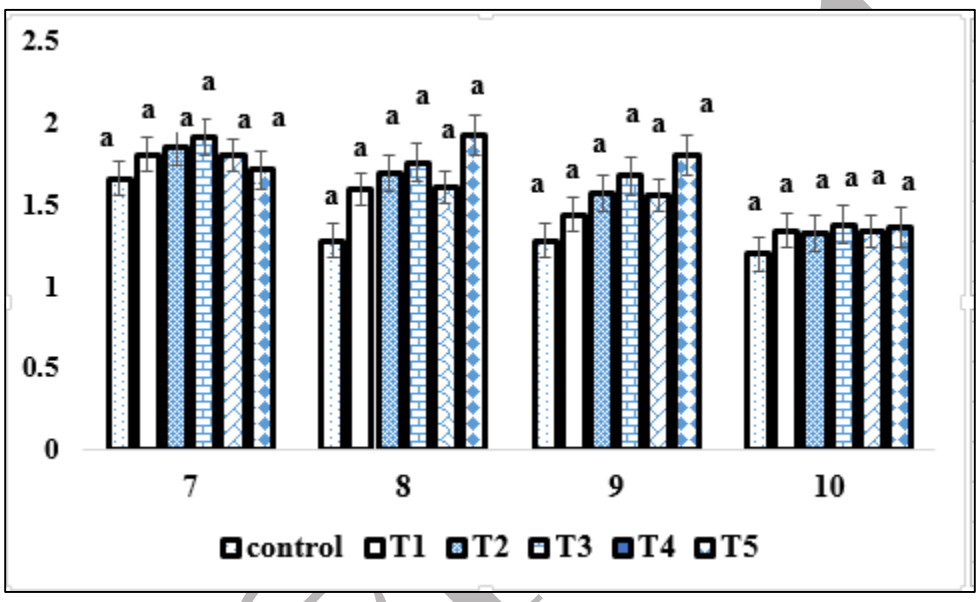

Figure 5. Effect of treat × time (wk) interaction on antibody titers against *E. Coli* (SP%) in Hyline selected laying chickens at different weeks post-immunization. T_1, T_2, T_3, T_4, and T_5 groups were vaccinated at 28 days of age with 0.2 ml (8 x10^6, 16 x10^6, 33 x10^6, 66 x10^6, and 133 x10^6 cfu/ml) per dose of *E. coli* O_{78} respectively. No differences were found at wk 7, 8, 9, and 10.

4. Discussion

As before mentioned, colibacillosis is an economically important for the avian industry, which causes multimillion-dollar losses annually (11). Preparing effectual colibacillosis control measures highly favorable. Colibacillosis control mostly concentrates on management methods made biosecurity plan for reduce preparing conditions among production birds, such as mycoplasma or

viral infections (11). Although, management methods that have reduced colibacillosis in the past may not be as efficient in the future. Moreover, use of antimicrobial factors in animal production is being given close investigation at this time with restriction being placed on the use of certain therapeutic factors in avian production (12). Finally, control of avian colibacillosis using vaccines in specified conditions may demonstrate favorable. Up to the present time, vaccines formulated to impede avian colibacillosis have been faced with mixed results. Vaccines against APEC of different serogroups have been generated. Killed bacterial vaccines, including autogenous vaccines, sub-unit vaccines, and live-attenuated vaccines are in use for prevention of APEC (13, 14, and 15). A great number of these vaccines have only been effective against homologous challenge. The present report is to address the efficacy of humoral immunity response of killed oil adjuvant *Escherichia coli* vaccine in layer chicken against avian *E. coli* infection. The titer in IgG antibodies in the experimental groups were higher compared to the control group. Increased titer in IgG antibodies was more pronounced in T_5 birds which receiving the highest number of bacteria per mL. Śmiałek et al. (16) showed that the use of live, attenuated, aroA gene-deleted vaccine against colibacillosis cause a reduction in the amount of *E. coli* in the population of avian. These results are in agreement with findings of El-Mawgoud et al. (17), who showed that live *E. coli* spray vaccination of broiler chickens decreased the APEC colonization in the liver and heart of the birds after *E. coli* infection. Also Roland et al. (18) reported the use of live, attenuated, *E. coli* vaccine derived O_{78} LPS, protected the white leghorn chicks against avian pathogenic *E coli* O_{78} strain. In the present study killed *Escherichia coli* vaccine was used, because the production process of live vaccine requires a lyophilizer and the facility of live vaccine preparation was not available in Shiraz, Razi Institute. Killed *E. coli* vaccines, including autogenous vaccines, protect only against homologous challenge (5, 19). Finally, the use of killed vaccines requires knowledge

of the serotype(s) of *E. coli*, which are included in the substantial outbreaks. Unfortunately, vaccination with killed vaccines may stress the birds, and the adjuvants may induce local reactions (20). Sub-unit vaccines may provide an extensive protection against more serotypes of APEC. However, the disadvantage of stress to birds during vaccination, and side effects of adjuvants have also been recorded for the sub-unit vaccine (20). Vaccination of broiler parents by the inactivated subunit Nobilis® *E. coli* was found to reduce the number of sequence types of *E. coli* isolated from diseased broiler parents in the vaccinated flock compared to the control group, which shows a potential for sub-unit vaccine to make less the outbreak of specific clones of APEC (21). The live Poulvac® *E. coli* vaccine includes an aroA mutant of a strain of serotype O78:K80 and ST23, but protection is not limited to this specific serotype and sequence type (22). Recent experimental studies have combined vaccination with live attenuated *E. coli* vaccine with autogenous vaccines and it seems possible to take a synergy of protection (23). The investigation of Kariyawasam et al. (24) revealed that collected IgY from eggs took from hens under different vaccination programs could cause passive maternal protection of day-old chicks when *E. coli* was used for challenge compared to the control groups, documenting that vaccination of parents may transfer the immunity to the chicks, under experimental conditions. Based on the results of this study the application of killed oil adjuvant *Escherichia coli* vaccine which produced in Shiraz, Razi Institute had greater efficacy in rising IgG titers in layer hens in comparison with unvaccinated group; however, to evoke immunological response, the second immunization is suggested four weeks after the first immunization.

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Authors' Contribution

Study concept and design: A.S; M.H.H; F.S

Acquisition of data: F.S; R.R; F.D; M.H; S.A; A.R

Analysis and interpretation of data: F.S; M.H

Drafting of the manuscript: F.S

Critical revision of the manuscript for important intellectual content: A.S; M.H; F.S

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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