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# ASSESSMENT OF IMMUNITY AGAINST AVIAN COLIBACILLOSIS INDUCED BY AN *AROA* MUTANT CONTAINING INCREASED SERUM SURVIVAL GENE IN BROILERS

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## ABSTRACT

Colibacillosis is an important disease in the poultry industry which causes serious economic damages. As it is suggested that vaccination is one of the means to control colibacillosis, we tried to investigate the vaccine potential of a Δ*aroA* derivative of an O78:K80 avian pathogenic *Escherichia coli* containing increased serum survival gene. 490 chicks were selected as follows: For assessment of virulence of *ΔaroA* mutant, 30 chicks were divided into three groups and injected with 0.5ml of PBS or bacterial suspension containing either$10^7$colony forming units (CFU) of mutant or parent strains via subcutaneous route. Macroscopic lesions and mortality rate were recorded in different groups during the week after challenge. For assessment of safety and immunogenicity of the *ΔaroA* mutant, three groups of 20 chicks were vaccinated by aerosol administration of 250 ml of suspension containing $10^8$ CFU of mutant strain at days 1 and 14, while the two other groups received PBS or wild type strain. Macroscopic lesions and mortality rate were recorded in different groups until day 21. To determine whether the vaccination is protective against challenges or not, the chickens were vaccinated at days 1 and 14 and challenged intramuscularly with either a homologous or heterologous strains at day 21. Macroscopic lesions and mortality rate were recorded in different groups during the week after challenge. The results revealed that the *ΔaroA* mutant was slightly virulent, however it was safe and did not cause mortality, lesions or weight loss after vaccination. Antibody responses were similar in the control and mutant groups and vaccination did not induce a significant humoral immunity. The mutant could not protect chickens against both homologous and heterologous challenges. This could be due to several factors such as the high amount of maternal antibodies in the first two weeks of life, and the vaccination procedure.

**Key words:** chickens, avian pathogenic *Escherichia coli, ΔaroA* mutant, vaccine

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## INTRODUCTION

Colibacillosis is one of the most important diseases of the poultry industry around the world. It causes considerable economical damage every year, due directly to increased mortalities and indirectly because of reduced weight gain, increased feed conversion rate and carcass condemnation (30). A wide range of *E. coli* serogroups, including O2 and O78 which are among most frequent, have been isolated from poultry lesions due to colibacillosis (7, 10, 13, 30).

It has been suggested that resistance to complement-mediated lysis and opsonophagocytosis are key factors in avian pathogenic *E. coli* (APEC) virulence (22, 23, 32). Iss is a plasmid-encoded outer membrane protein (OMP) which plays a subtle role in the resistance of APEC to serum (3, 19). The *iss* gene occurs more frequently in APEC than in strains isolated from apparently healthy birds, and has been reported in different serotypes (18, 27, 29). The strong association of Iss protein among different *E. coli* strains suggests that it could be a good antigen to control and detect APEC. In this case, Lynn *et al.* (17) have used recombinant *Escherichia coli* Iss protein for immunization of chickens against APEC. They showed that there were lower lesion scores in vaccinated chickens that had developed a humoral response to Iss, after both homologous and heterologous challenges in comparison to unimmunized birds. However, they placed emphasis on further investigations into the vaccine potential of Iss protein for protection against APEC.

Vaccination is one of the various methods which has been applied to control colibacillosis. Live, killed and subunit are different types of vaccines that have been used (14, 30). The live vaccines would be considered the most appropriate for immunization against colibacillosis in view of the fact that their construction is more economical than subunit vaccines and they are also suitable for mass administration via drinking water or aerosol (26). Moreover, in the production process of inactive vaccines, it is possible that antigenic determinants were changed or eliminated, while in live vaccines they will remain unchanged, which is essential for a proper immune response (15). By the advent of genetically defined mutants the risk of reversion to wild-type strain, which is always a challenge for the production of live vaccines, was removed. There has been some effort to construct genetically defined mutants of avian pathogenic *E. coli* (15, 16, 25). Evidence in literature showed that antibody responses did not differ significantly in chickens vaccinated with *Δcya Δcrp* mutants of either an O2 or an O78 APEC strain and control group. Although, the mutant of O2 strain induced only moderate protection against air sac infection (26). Kariyawasam *et al.* (15) revealed that *ΔgalE, ΔpurA* and *ΔaroA* mutants of an O78 APEC strain were safe and produced a stronger immune response in vaccinated chickens than in the control groups. However, they only provided protection against homologous challenge and failed to protect against heterologous challenge.

The aim of present study was to evaluate the safety, immunogenicity and protective ability of an *aroA* mutant of an O78:K80 APEC containing the *iss* gene against avian colibacillosis in broilers.

## MATERIALS AND METHODS

### Chickens

Four hundred and ninety commercial day-old broiler chickens vaccinated against Marek's disease were obtained from a local hatchery and maintained in pens at the animal husbandry facilities of the University of Tehran Faculty of Veterinary Medicine in Karaj, Iran. The chickens were fed *ad libitum* with no antibacterial or anticoccidial components in their feed. The study was conducted in accordance with guidelines established by the "Guide for the Care and Use of Laboratory Animals" by the Institute of Laboratory Animal Resources (12).

### Bacterial strains

The mutant strain was constructed by deletion of the *aroA* gene from a wild type strain belonging to O78:K80 serotype

(20). The virulent wild-type avian *E. coli* containing the *iss* gene (laboratory designation *E. coli* strain χ1378) was isolated from a chicken with systemic colibacillosis in Iran (6). A virulent O2:K12 serotype of *E. coli* containing the *iss* gene was used for heterologous challenge. For preparation of bacterial suspensions, the bacterial strains were grown for 18 h at 37 ºC in flasks containing tryptic soy broth (TSB). Then, bacterial cells were harvested by centrifugation at 3000 rpm for 15 min and washed three times with a sterile solution of 0.9% w/v of NaCl. Bacterial suspensions were initially adjusted to an absorbance ($A_{600}$) of 0.8 and 1 to reach estimated concentrations of $10^7$ CFU/ml and $10^8$ CFU/ml, respectively (1). Finally, the exact amount of bacteria was determined by a surface plate bacterial count method. All media used in the current study were purchased from Merck, Germany.

**Characterization of mutant strain**

Mutant strain was cultured in minimal medium and aromatic-mix medium for confirmation of its phenotypic characteristic as previously described by Nayeri Fasaei *et al.* (20). Moreover, to ensure deletion of the *aroA* gene, DNA from the mutant strain was extracted by boiling method and amplified with following primers: $E_1$ (GAT AAC AGC GAT TTC TAC CG) and $E_2$ (TTC TTC ATG TGA ATC ATC CG). Each 25 µl of reaction mixture contained 2.5 µl of 10X PCR buffer (CinnaGen, Iran), 1.5 µl of 50 mM $MgCl_2$ (CinnaGen, Iran), 2 µl of 10 mM deoxynucleoside triphosphates (CinnaGen, Iran), 1 µl of each of $E_1$ and $E_2$ primers (10 pmol), 1.5 U of *Taq* DNA polymerase (CinnaGen, Iran), and 5 µl of DNA template. PCR amplification program involved denaturation at 94°C for 2 min followed by 32 cycles of 94°C for 30 s, 57.8°C for 30 s, and 72°C for 37 s, with a final extension step at 72°C for 13 min in a TC-512 thermocycler (Techne, Cambridge, UK). The PCR products were analyzed by electrophoresis on 1.5% agarose gel at 80 V for 75 minutes. Gels were stained in a 0.5 µg/ml ethidium bromide solution for 15 minutes and photographed by CCD Video Camera.

**Assessment of attenuation of mutant strain**

In this experiment, the attenuation of mutant was evaluated by infecting day old chickens as described previously (25). 30 chickens were divided into three groups of 10 and injected with 0.5 ml of PBS or bacterial suspension containing either$10^7$ CFU/ml of mutant strain or $10^7$ CFU/ml of wild type χ 1378 strain via subcutaneous route. Chickens were monitored every 6 hours for the first day and then every 12 hours until day 7. Mortalities were recorded and surviving chickens were euthanized at day 7. Macroscopic lesions were scored according to data from Peighambari *et al.* (26). Samples were taken from bone marrow, blood, liver and air sacs and cultured on MacConkey agar.

**Evaluation of immunogenicity and safety of mutant strain**

In this experiment, three groups of 20 chickens were vaccinated by aerosol administration of 250 ml of suspension containing $10^8$ CFU/ml of mutant strain at days 1 and 14 while two other groups received the same amount of PBS or wild type χ 1378 strain suspension. The size of the droplets was adjusted to 50-100 µm (30). The chickens were monitored on a daily basis for signs of illness and death. At day 21, surviving chickens were euthanatized, macroscopic lesions were scored and bacterial culture was performed. For serum preparation, three ml of blood were taken from each chick at day 21 and serum was separated by centrifugation at 3000 rpm for 15 min and stored at –20°C. To prepare air sacs washing, after opening the internal cavity of the chickens, 1 ml of sterile solution of 0.9% w/v of NaCl was directed to each of the left and right air sacs. By pippeting, the air sacs were washed and the collected liquid was kept at –20°C. To examine the ability of mutant strain to develop humoral immune response, the amount of anti-*E.coli* antibodies was determined in the sera and air sacs washings of chickens. Serological evaluation was performed by two methods as follow:

**Rapid agglutination test:** One drop of serum and also one sterile drop of a solution of 0.9% w/v of NaCl were placed

on a clean slide. Then, a drop containing whole cell antigen was added to each of them and after mixing, agglutination time was determined. For whole cell antigen preparation, *E. coli* strain χ 1378 was cultured on brain heart agar. After 24h of incubation at 37°C, the culture media were washed by a sterile solution of 0.9% w/v of NaCl and bacterial suspension was mixed with the same amount of sterile alcohol. This suspension was then adjusted to the estimated concentration of $9\times10^8$ CFU/ml, equivalent to the third McFarland tube.

**Tube agglutination test:** For each serum sample, nine clean tubes were considered. In the first tube 0.1 ml of serum was mixed with a 0.9 ml of sterile solution of 0.9% w/v of NaCl and then 0.5 ml of diluted serum was transferred to a second tube containing 0.5 ml of a sterile solution of 0.9% w/v of NaCl. Two- fold serial dilution of serum was continued till the 1:640 dilution of serum was obtained. Finally, 0.5 ml of whole cell antigen suspension was added to each tube. Two tubes, one containing only antigen suspension and the other containing only a sterile solution of 0.9% w/v of NaCl, were used as controls. The last dilution with visible agglutination was recorded.

**Assessment of protection against homologous challenge**

To determine whether vaccination is protective against homologous challenge or not, the chickens were vaccinated at days 1 and 14. For this reason, 100 chickens were divided into five groups as follow: two groups of 26 chickens received 250 ml of suspension containing $10^8$ CFU/ml of mutant or wild type χ1378 strain by aerosol route and another group of 26 chickens were vaccinated orally by administration of one ml of $10^8$ CFU/ml of mutant strain suspension. Two groups of 11chickens, one received PBS and the other received no treatment, were also considered as negative control groups. At day 21, the chickens were challenged with wild type χ1378 strain intramuscularly (33). The chickens were monitored daily for signs of illness and death and all chickens were weighed at days 1,7,14 and 21. Surviving chickens were euthanatized at day 28, macroscopic lesions were scored and bacterial culture was performed as described before. This experiment was done in duplicate.

**Assessment of protection against heterologous challenge**

The experiment was designed similarly to the protection study against homologous challenge, but a virulent *E. coli* strain, which belonged to O2:K12 serotype, was used for challenge. This experiment was done in duplicate.

**Statistical analysis**

For comparison of the macroscopic lesions and antibody titer in the different groups, non-parametric test was used followed by U-Mann-Whitleng test for two by two comparisons of groups. The variation in the weight gain of chickens was analyzed by repeated measure analysis of variance. Analysis of mortalities in different groups was performed by Fisher exact test. $P<0.05$ was considered significant.

## RESULTS

**Characterization of mutant strain**

In contrast to wild type χ1378 strain, mutant strain had no growth in the minimal medium, but it was spread in the aromatic-mix medium. A 575 bp band corresponding to the deleted *aroA* gene was observed in the mutant strain (Figure 1).

**Attenuation of mutant strain**

All chickens treated with wild type χ1378 strain died during the 72 hours after inoculation with signs of acute colisepticemia such as dark and congested internal organs. In contrast, only one chicken was found dead in the mutant group, with progressive signs of air sacs infection, perihepatitis and pericarditis. The O78:K80 *E. coli* strain was recovered from the lesions of dead chickens. No mortality in the control group was recorded.

**Immunogenicity and safety of mutant strain**

No mortalities were observed in the group which received the mutant strain or sterile solution of 0.9% w/v of NaCl, while eight chickens died in the group treated with wild type χ1378 strain, and this difference was significant($P<0.05$). In the case of macroscopic lesions, significant differences were found between the wild type group and either the mutant or control groups ($P<0.05$) (Table 1). The results of rapid and tube agglutination tests showed that there was no significant difference in the antibody response between the mutant group and the control group ($P> 0.05$).

**Protection against homologous challenge**

No significant difference regarding mortality and macroscopic lesion scores was seen between the studied groups ($P> 0.05$). However, the groups which were vaccinated by mutant strain had significantly higher weight gain in comparison to the group treated with wild type χ1378 strain ($P<0.05$) (Table 2). Similar results were obtained for heterologous challenge.

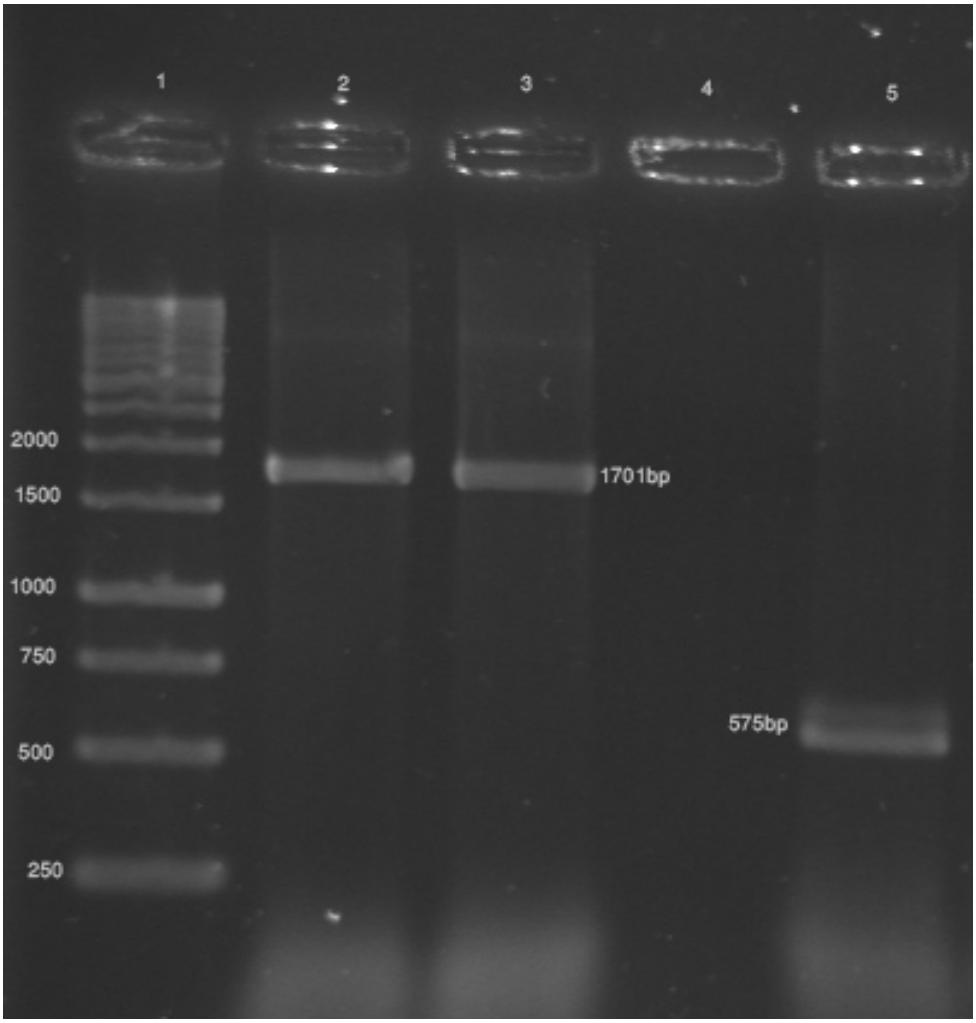


**Figure 1.** Two different sizes of *aroA* gene in wild type χ1378 strain and mutant strain with $E_1$ and $E_2$ primers. 1kb DNA ladder (lane 1); *aroA* gene in wild type strain before deletion (lanes 2 and 3); lane 4 is empty; *aroA* gene in mutant strain after deletion (lane 5).

**Table 1.** Mortality and macroscopic lesions score of different groups in the study of immunogenicity and safety of mutant strain.

| Group | Mortality | Macroscopic lesions | | | |
|---|---|---|---|---|---|
| | | Air sacs infection | Perihepatitis | Pericarditis | total |
| PBS | 0[a*] | 0.19±0.4[a] | 0.05±0.21[a] | 0.095±0.3[a] | 0.11±0.31[a] |
| Mutant strain | 0[a] | 0.23±0.53[a] | 0.1±0.3[a] | 0.14±0.35[a] | 0.15±0.4[a] |
| Wild type strain | 8[b] | 1.33±1.42[b] | 0.85±0.96[b] | 0.95±0.92[b] | 1.04±1.12[b] |

* Values with different letters in the same column differ significantly (P<0.05)

**Table 2.** Weight gain of chickens in different groups in protection studies.

| Group | Weight (in gram) | | | |
|---|---|---|---|---|
| | Day 1 | Day 7 | Day 14 | Day 21 |
| Aerosol vaccination with wild strain | 46.06±1.65[a*] | 93.37±1.72[a] | 223.43±25.72[a] | 433.25±57.73[a] |
| Aerosol vaccination with aroA mutant | 46.06±1.74[a] | 97.51±2.10[a] | 223.98±20.09[a] | 492.01±59.86[b] |
| Oral vaccination with aroA mutant | 46.13±1.74[a] | 98.80±2.07[a] | 233.98±16.87[a] | 519.38±67.17[b] |
| Treatment with PBS | 47.22±1.23[a] | 100.31±1.69[a] | 237.17±22.85[a] | 493.84±66.91[b] |
| No treatment | 46.05±1.3[a] | 94.48±1.8[a] | 228.77±22.33[a] | 527.72±63.23[b] |

* Values with different letters in the same column differ significantly (P<0.05)

## DISCUSSION

Mutation of certain auxotrophic genes such as *aroA* has been employed to construct attenuated strains of various bacteria including APEC, *Salmonella typhimurium, Salmonella choleraesuis, Bordetella pertussis* (11, 15, 21, 28). In this study, we evaluated the attenuation, immunogenecity and protective ability of the *aroA* mutant from a native *E. coli* O78: K80 strain in broilers. The results of the attenuation study revealed the safety of the mutant strain, as vaccination with mutant had no impact on mortalities and lesions, which is in agreement with previous data (15, 26). The attenuation is due to the inability of *aroA* mutants to synthesize chorismic acid, which is an intermediate product of p-aminobenzoic acid, aromatic amino acids, and folate biosynthesis rather than indirect effects on the expression of virulence factors (28, 31). Additionally, the weight gain of the chickens during the first three weeks of life showed that there was no negative effect because of vaccination (Table 2). Similarly, Panigrahy *et al.* (24) have found that the body weights of chickens vaccinated with an oil-emulsified E.coli (O1:K1) bacterin were comparable to those of unvaccinated chickens. Thus, it appears that vaccination with live genetically defined *aroA* mutants of APEC could be as safe as vaccination with APEC killed vaccines.

In contrast to the findings of Kariyawasam *et al.* (15), vaccination failed to protect chickens against homologous challenge and there were high mortalities in the vaccinated groups after the challenge. One possible explanation is that in the present study, a systemic route of challenge which has severe outcome regarding mortalities and lesions was used, but in that study an aerosol challenge was applied. In addition, in the current study droplets were delivered with a size of about 50µm, which was larger than those in the study of Kariyawasam *et al.* (15). Whereas large droplets can not penetrate deeply in the respiratory tract, a small one can pass

through the lower parts of the respiratory tract. Consequently, small droplets would stimulate a higher proportion of the respiratory tract and may induce a stronger immune response. However, similar to the results of the current study, it is indicated in several studies that vaccination against colibacillosis is not successful in the first two weeks of a chicken's life (1, 2, 4, 26). This could be due to, at least in part, the presence of a maternally derived antibody which gradually decreases after 10 days post hatching. It has been shown by Elazab *et al.* (9) that passively-introduced antibodies can react with the vaccine antigen or suppress immune response to vaccination not only during early life, but also when they are present in low levels. Lynne *et al.* (17) observed that immunization with recombinant Iss protein induced protection against colibacillosis in chickens, while in our experiment the *aroA* mutant containing the *iss* gene did not protect chickens against colibacillosis. It is possible that mutant did not survive long enough in the tissues of chickens to express an adequate amount of Iss protein. While it seems that there is a delicate line between attenuation and immunogenecity, the use of a more virulent strain for the construction of mutants could be considered as an approach to improve the vaccine potential of the mutants (15, 26). In case of inability of oral vaccination to protect chickens, there is evidence that it is necessity to vaccinate chickens with $10^{10}$ CFU/ml of bacteria (1, 8).

The failure in protection against heterologous challenge was in accordance with previous studies (5, 15, 26). This serogroup associated protection highlights the possible role of LPS as a crucial factor in immunization with live *E. coli* vaccine. Based on the results of the current study, further investigations on other serogroups of APEC may be required to determine whether live vaccine can be effective against avian colibacillosis or not.

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