

PREPARATION AND EVALUATION OF AVIAN PATHOGENIC *E. COLI* LIPOPOLYSACCHARIDE AND RIBOSOMAL VACCINES AGAINST AVIAN COLIBACILLOSIS IN BROILER CHICKEN IN AL-SULAIMANIA PROVINCE

NAHLA M.S.*; SLIM S.A.** and YASSIN B.R.**

*Department of Microbiology, College of Veterinary Medicine, University of Sulaimania. Suliamania, Iraq.

**Department of Microbiology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.

Email: salar_nahla@yahoo.com

ABSTRACT

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This study was conducted to evaluate the effectiveness of Lipopolysaccharide (LPS) and ribosome vaccines on *E. coli* that isolated from broiler farms in AL-Sulaimania province. Seventy five broiler chicks were used for this experiment, and they were divided into three groups. Antibodies were evaluated by using enzyme linked immune sorbent assay (ELISA) and passive hemagglutination (PHA) test at age of 17th and 29th day, the results showed high antibody titers in vaccinated groups in comparison with control group. The results of the ELISA at the age of 17th day for LPS, ribosome, and control groups were the group 1.275 ± 0.095 , 1.592 ± 0.046 and 0.046 ± 0.009 respectively, while at the age of 29th day the results were 1.849 ± 0.038 , 2.048 ± 0.048 and 0.985 ± 0.010 in LPS, ribosome and control groups respectively. The results of PHA at 17th day were 192 ± 24.189 , 272 ± 56.406 , 1 ± 0.7003 in LPS, ribosome and control groups respectively, while at 29th day the results were 1344 ± 215.003 , 1792 ± 374.746 and 256 ± 112.813 in LPS, ribosome and control groups respectively.

Keyword: *E.coli*, Lipopolysaccharide, ribosomal vaccines, avian colibacillosis

INTRODUCTION

Avian Pathogenic *Escherichia coli* (APEC) is the causative agent of avian colibacillosis, an illness associated with systemic infection of internal organs and a diversity of symptoms (Moulin *et al.*, 2007).

Colibacillosis refers to any localized or systemic infection caused entirely or partly by avian pathogenic *Escherichia coli* (Barnes *et al.*, 2003). It is a globally spread infectious disease that represents a main concern in the poultry industry. It is not only a welfare issue but also an economic problem because it causes respiratory problems, reduced feed intake, growth retardation, uniformity reduction, and mortality (Ask *et al.*, 2006).

Colibacillosis, caused by avian pathogenic *E. coli* APEC, is also named air sac disease, chronic respiratory disease (CRD), or multi-causal respiratory disease, APEC can also cause infections in the upper respiratory tract, often referred to as the "swollen head syndrome" characterized by airsacculitis, pericarditis, peritonitis, salpingitis, polyserositis, septicaemia, synovitis, osteomyelitis and yolk sac infection (McPeake *et al.*, 2005).

The role of *E. coli* in chronic respiratory diseases in broiler is well documented and its pathogenicity has been correlated with numerous extrinsic and intrinsic bird related factors and conditions. The extrinsic factors include environment, exposure to other infectious agents, virulence and level of exposure duration, active and passive immune status and breed of chicken (Gross, 1990).

Since intensive breeding has been widely applied in the poultry industry, outbreaks of acute mortality in flocks due to avian colibacillosis have frequently been observed, and are responsible for the significant economic losses (Vandekerchove *et al.*, 2004).

Currently, the control of APEC has become not only an urgent issue but also a great challenge. Vaccination is an effective method for controlling infectious diseases (Yang, 2003). To date, a number of experimental vaccines have been developed to prevent colibacillosis (Lynne *et al.*, 2012 and Nagano *et al.*, 2012).

Infections caused by *Escherichia coli* have an economically significant impact on the poultry industry and a non-serotype-specific vaccine appears to be the most logical method of controlling them. The potential of controlling *Escherichia coli*

infections in commercial poultry through vaccination has been explored widely over the past several decades. In these attempts, live or killed bacteria and their various cellular components have been used to immunize chickens (Deb and Harry, 1976; Arp, 1980 and Kariyawasam *et al.*, 2002). Therefore, the aim of this study was to evaluate the effectiveness of ribosome and LPS vaccines on *E. coli* isolates from colibacillosis cases in broiler farms.

MATERIALS and METHODS

1. Experimental Chicks:

Seventy five (75) one day old broiler chicks (Breed: Ross, Belgium Origin) were brought in good condition from Al- jazera Hatchery-Amman-Jordon.

Table1: Primers were used for the detection of the *E. coli*.

Primer	Primer sequence (5_-3_)	Amplification products (bp)
ECO-f	GACCTCGGTTTAGTTCACAGA	585
ECO-r	CACACGCTGACGCTGACCA	

DNA extraction of *E. coli*

DNA was extracted from *E. coli* isolate using DNeasy Blood and Tissue Kit (Qigen, Germany).

DNA amplification

For PCR amplification, 5µl of DNA extract was added to 45 µl of PCR mixture containing 27.5 µL of nuclease-free water, 5 µL of each primer, 1.5 µL of nucleotide (dNTP) mix, 5 µL of PCR buffer, and 1 µL of Taq polymerase. The amplification conditions were: 1 cycle of 94°C for 3 min· 35 cycles of 94°C for 30 s, 60°C for 1 minutes and 68°C for 2 minutes, with a final extension at 68°C for 7 minutes and hold for 4°C. Amplified products were separated by electrophoreses on 1% agarose gel containing ethidium bromide. The agarose gel was prepared using 1gm agarose powder, 100 ml 1X Tris acetate EDTA (TAE) buffer and 5µl ethidium bromide. Agarose gel electrophoresis was conducted in 1X TAE buffer at 100 V for 35 minutes. 100 bp ladder (Gibco BRL), were used for electrophoresis. Bands were visualised under UV light on a transilluminator and photographed by image documentation system (Labortechnic, Germany).

4. Preparation of the vaccines:

A. Ribosome vaccine

The pure culture of *E.coli* isolates were sub-cultured on nutrient broth for 16 hour at 37 °C and harvested by centrifugation (4000 xg for 10 minute at 4 °C). The pellet was re-suspended in phosphate-buffered saline (PBS), washed three times and re-suspended in 100 ml PBS at a concentration of 2×10^9 CFU/ml.

2. Isolation and Identification of *E.coli* (Vaccine Strain)

The *E.coli* (vaccine strain) was isolated from the clinically affected broiler farms with colibacillosis in AL-Sulaimania Province. The isolates were culture on MacConkeys, and cosin, methylene blue (EMB) agar, and different biochemical tests were carried out for identification, the isolates were confirmed by using API E20 test and PCR technique.

3. Detection of *E. coli* by PCR

Oligonucleotide primers

For PCR analysis of locally isolated *E. coli*, oligonucleotide primers targeting 16S ribosomal DNA gene (Candrian *et al.*, 1991; Amit-Romach *et al.*, 2004), ECO-f and ECO-r were used (Table 1).

Suspended *E.coli* were broken by ultrasonication at 18kc force for a period of 25 minutes, then centrifuged at 27000 x g for 10 minutes at 4°C, the supernatant fluid were separated and re-centrifuged at 45000 x g for 30 minutes to precipitate the wreckage of the remaining cells.

Supernatant fluid (4/5) were separated carefully, re-centrifuged at 105,000 x g for 3 hours to get the parts containing ribosomes. Ribosomal vaccine (20 mg/ml) was prepared according to (Venneman and Bigley, 1969), then the total protein was determined by Biuret method and used for immunization.

B. Lipopolysaccharide vaccine

Lipopolysaccharide vaccine was prepared by using hot phenol according to (Westphal and Jann, 1965). Fifteen liter of special media which was previously mentioned are cultured by *E.coli* and incubated at 37 °C for 3 days then harvested by PBS and centrifuged at 5000×g for 30 minutes. The supernatant was discarded and precipitates were re-suspended in PBS using 88 ml distilled water, heated to 68 °C and mixed well. Eighty eight ml of phenol was added and heated to 68C° and mixed well using magnatic sterror at maximum speed for 10 minutes, and after concerning the efficiency of mixing put the flask containing the mixture in water bath at 68 °C for 30 minutes. The flask was then placed directly in the ice bath at 10 °C for 10 minutes. Then the emulsion was formed and centrifuged at 5000×g for 20 minutes, and it was noted that three phases was formed, the aqueous phase, the phenolic phase, and other phase represent proteins and other waste product (Figure 3-

1). Then the aqueous phase gently sucking to sterile tube by pipette. The phenolic phase and protein phase are re-extracted by adding 88 ml of distilled water heated to 65 °C and mix well, where the previous operation returned again.

Collection the water phase resulting from the processes of extraction and adding to it 1 mg/ml of DNase, RNase then incubate at 37 °C for 24 hours, and then held for the solution process of dialysis against distilled water for 4 days. Then the liquid is maintained collected.



Figure 1: Showed 3 main phases. Aqueous phase, phenolic phase, and other phase represent proteins and other waste product.

5. Experimental design:

Seventy five (75) broiler one day old chicks were divided into 3 groups; Group 1 were injected with 0.5 ml of LPS antigen. Group 2 were injected with 0.5 ml of ribosome antigen at a concentration of 2×10^9 CFU/ml. Group 3 were injected with 0.5 ml of physiological saline solution. At seven days of age groups, 1, 2, and 3 were vaccinated with the prepared vaccines subcutaneously. Blood samples were collected at 10 days post vaccination to determine the antibody titers by ELISA and PHA test.

At day 19th of age challenging test (1×10^8 CFU/ml S/C) were conducted. Blood samples were collected at 10 days post challenging to determine the antibody titers by ELISA and PHA test.

6. Passive Hemagglutination test (PHA)

PHA test were carried out according to Herbert, (1987).

A. Sheep RBCs

Five ml of sheep blood was collected by sterile syringe, then mixed with 5 ml of Alsevers solution, which may be kept at 4°C for 24-72 hours. Blood sample was centrifuged at 1500 rpm for 10 minutes to discard Alsevers solution. Blood sample was washed three times with PBS (pH=7.2) and centrifugation at 1500 rpm/5 minutes. RBCs were re-suspended in PBS for final concentration of 2.5%.

B. Tanned RBCs

Ten ml of (2.5%) RBCs was mixed with equal volume of tannic acid solution (1:20000), incubated at 37°C for 20 minutes and shaken gently every 5 minutes, then the mixtures was centrifuged at 2000 rpm for 10 minutes to remove tannic acid solution, then RBCs washed three times with PBS (pH=7.2) and centrifugation at 1500 rpm per 5 minutes.

C. Sensitization of washed tanned RBCs

Serial dilutions of particular antigen were prepared to sensitize washed tanned RBCs and to detect the optimal antigen concentration which gave apposite reaction with higher antibody dilution.

One ml of sonicated APEC antigen was mixed with one ml washed tanned RBCs then incubated at 37 °C for 30 minutes, and gently shaken every 5 minutes, then the mixture was centrifuged at 2000 rpm per 10 minutes to remove the excessive antigens, finally the sensitized RBCs were washed with PBS (pH=7.2) and centrifuged at 1300 rpm per 5 minutes three times.

D. Serum inactivation

The serum samples were incubated at 56 °C for 30 minutes in water bath to impair the complement activity.

Procedure

Add 50 µl of normal rabbit serum to wells of microtiterplate (96 U shape wells), then added 50µl of serum sample to first well in rows, then made serial dilutions of serum by pipetting 50 µl of mixture and transferred to next wells until last well, from which 50 µl was discarded, then to each well 50 µl from sensitized tanned RBCs was added, and the plate covered with aluminum paper and incubated at room temperature for at least 2 hrs, after that, the reactions was read, reading was repeated after 18 hours /4 °C.

The positive results was appeared when RBCs agglutinate and form carpet shape. The negative reaction was appeared when RBCs precipitate at bottom as dot like without agglutination.

7. ELISA test:

ELISA test was done according to (Leitner *et al.*, 1990), the kit of ELISA including *E.coli* Ab Conjugate, Substrate A, Substrate B, Stop solution *E.coli* antibody Negative control and *E.coli* antibody positive control.

8. Challenge test:-

Ten fold dilutions from *E.coli* were prepared from 24 hours culture on peptone water to obtain 1×10^8 CFU/ml to be used for experimental infection of chick according to (Sambrook *et al.*, 1989).

RESULT

Escherichia coli/ vaccine strains were obtained from chickens died with colibacillosis (Fig.2), the results



Figure 2: Shows typical perihepatitis in broiler chicks caused by avian colibacillosis

of gross colony morphology on McConkey's agar was as a convex shape, dark pink color and, on EMB agar was as a characteristic of metallic green sheen, and then the *E.coli* isolates were confirmed by using API E 20 test see (Fig.3).

Instead of biochemical API 20E tests, PCR and its related method have been reported to identify *E. coli*. In this study, the isolated *E. coli* organisms from colibacillosis diseased birds were grown in nutrient broth, DNA was extracted and amplified by PCR using ECO-f and ECO-r primer targeting *E. coli* 16S ribosomal DNA. On agarose gel electrophoresis, single band was visualized at molecular weight of 585 bp, (Fig. 4).



Figure 3: The results of biochemical tests in API 20E system by isolated strain of *Escherichia coli*.

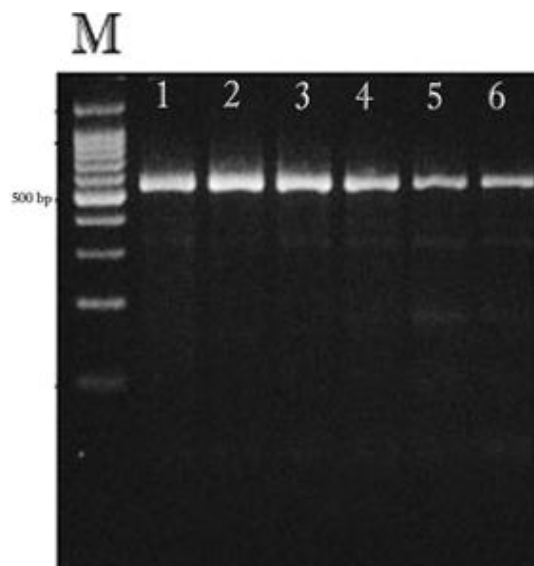


Figure 4: Agarose gel electrophoresis showing the PCR amplified products of *E. coli* 16S ribosomal DNA. Lanes M: DNA molecular weight marker (100 bp ladder marker with molecular weight range from 00 - 3000 bp); Lanes 1 to 6: positive with 585 base pair.

Antibody titers to LPS, ribosome, and control vaccine were measured by using ELISA and PHA tests at 17th and 29th day of age, it was found that there were significant differences ($P < 0.05$) among all groups as showing in the Table (1), after vaccination and challenging. Immune response of vaccination measured by ELISA showed a significant increase ($P < 0.05$) in ELISA titer of all groups as compared with the control group. The vaccinated

group with ribosomal vaccine showed a significant increase ($P < 0.05$) more than that of LPS vaccine, while immune response measured by (PHA) showed a significant increase in immune response ($P < 0.05$) in all vaccinated groups as compared with the control one as show in Table (2). The group vaccinated with ribosome vaccine showed a significant increase ($P < 0.05$) more than that of LPS group.

Table 1: Antibody titers to LPS and ribosome, control vaccine by using ELISA at at 17th and 29th day of age.

Groups	Antibody titers (Mean±SE) at 17 th days	Antibody titers (Mean±SE) at 29 th days
LPS	1.275±0.095 B	1.849±0.038 B
Ribosome	1.592±0.046 A	2.048±0.048 A
Control vaccine	0.046±0.009 C	0.985±0.010 C

The different capital letters refer to significant difference ($P < 0.05$) among groups Means±SE (n=8).

Table 2: Antibody titers of chickens after immunization with LPS and ribosome vaccines at 17th and 29th day of age.

Groups	Antibody titers (Mean±SE) at 17th days	Antibody titers (Mean±SE) at 29th days
LPS	192±24.189 B	1344±215.003 B
Ribosome	272±56.406 AB	1792±374.746 A
Control	3.75±0.700 D	256±112.813 C

The different capital letters refer to significant difference ($P < 0.05$) among groups Means±SE (n=8).

The number of mortality in LPS vaccinated, ribosome vaccinated and control groups were two, one and six chickens respectively as shown in Table (3).

Table 3: Number of mortality of broiler chickens vaccinated with different E.coli vaccines after challenging.

Group	LPS	Ribosome	Control
Mortality	2/25	1/25	6/25

DISCUSSION

Escherichia coli isolates were collected from chickens with colibacillosis in broiler farms around Sulaimania Province, The clinical manifestations were observed in the affected birds with colibacillosis in addition to, inflammatory changes such as pericarditis, perihepatitis and air sacculitis were seen in the autopsied birds as show in (Fig.1), (Omer *et al.*, 2008).

The isolated *E.coli* were identified by using different biochemical tests, and it was found that these isolates

were gram negative bacilli, it's appearance on Macconkey's agar was as a convex shape, dark pink color colonies by fermenting lactose sugar, while its appearance on EMB agar was metallic green sheen colonies, and these isolates were confirmed by using API E20 test (Fig. 3), and by using specific base pair primer for *E. coli* 16S ribosomal DNA identification by PCR (Fig.4), this result was similar to other authors (Amith-Romach *et al.*, 2004).

Antibody titers to LPS vaccinated, ribosome vaccinated, and control are measured by using ELISA tests at 17th and 29th day of age, it was found

that there were significant differences ($P < 0.05$) among all groups as showed in the Table (1), after vaccination and challenging. Immune response of vaccination measured by ELISA showed a significant increase ($P < 0.05$) in ELISA titer of all groups as compared with the control group. The group vaccinated with ribosomal vaccine showed a significant increase ($P < 0.05$) more than that of LPS vaccine. These results are agreed with (Panigaraphy *et al.*, 1984), who mentioned that vaccinated chickens were protected against colibacillosis infection as compared to those unvaccinated. Gyimah *et al.* (1985), noted that vaccinated chicks showed high antibody titers, suffered low morbidity and mortality, mild gross lesions significantly lower than unvaccinated one.

Stimulation of Toll-like receptor - 4 (TLR-4) by lipopolysaccharide (LPS) results in the expression of the pro-inflammatory cytokine IL-1 β . Toll-like receptor 4 detects the presence of pathogen and this result coincides with (Akashi *et al.*, 2001), who stimulates bacterial killing mechanisms, and induces pro-inflammatory cytokines (Kogut *et al.*, 2005). LPS induce an immune response that mimics the immune response to a bacterial infection, LPS increased the release of cytokines and induced an inflammatory response, which is followed by the production of antibodies (Poxton, 1995; Leshchinsky and Klasing, 2001).

Lipopolysaccharide showed direct activation of B-lymphocytes in vitro, and produced antibodies of diverse specificities, mainly of the IgM type within a short of time period (Andersson *et al.*, 1978).

The ribosomal vaccine, showed to be recognized by the immune system of the host with high frequency and this result agrees with (Requena *et al.*, 2000). Ribosomal vaccine leading to the direct stimulation of B- lymphocytes and T-cell involvement through the release of cytokines which could modulate B-cell differentiation and immunoglobulin secretion. Uptake and presentation of these ribosomal protein antigens lead to strong immune responses against these antigens. The ribosomal vaccine leading to the immunoregulatory processes by their action on the B cells, T cells and cytokines secretion (Sven and Hofstad, 1990).

Passive hemagglutination test is considered a routine method for the quantification of antibody titers (Stavitsky, 1954). Immune response measured by (PHA) showed a significant increase in immune response ($P < 0.05$) in all vaccinated groups as compared with the control group. The group vaccinated with ribosome vaccine showed a significant increase ($P < 0.05$) more than that of LPS group.

It is clear that there was a great correlation between the results of ELISA and PHA tests. These results

agree with (Leitner *et al.*, 1990) and (Rosenberger *et al.*, 1985), who found that there was higher correlation between ELISA and PHA titer, and the titer in both tests were parallel.

Melamed *et al.* (1991) recorded that the degree of protection conferred by the vaccine was positively correlated with the antibody titer against colibacillosis.

Chicks from the control non vaccinated showed poor immune response than that from vaccinated when challenged by *E. coli*. These results are agreed with (Panigaraphy *et al.*, 1984), who mentioned that vaccinated chickens were protected against active respiratory infection showed more immune response as compared with those unvaccinated.

Group vaccinated with Ribosome produce good immunity against challenge and this is due to ribosome elicits both humeral and cell mediated immunity and this result was agreement with that of (Youmans and Youmans, 1970; Venneman, 1972), whom noticed that serum antibodies against ribosomes were apparent 7 days after injection of the ribosomal vaccine as measured by PHA test. Whereas (Jonson, 1972; Swendsen and Johnson, 1976) demonstrated that ribosomal protein acts as the principle immunogen.

LPS vaccine showed high antibody titration measured by PHA test and this result was agreement with (Chen *et al.*, 1999), who found that vaccination with LPS were giving good protection and using the PHA test was much more sensitive in detecting *E.coli* antibodies. Also (Yaguchi *et al.*, 2009) mentioned that the all chickens vaccinated with the LPS produced anti-lipopolysaccharide antibodies of the IgG sub-class in their sera, rise in antibodies in the serum of chickens administered the LPS vaccine.

The difference in the mortality between groups may be due to the degree of protection conferred by the vaccine was positively correlated with the antibody titer against colibacillosis (Melamed *et al.*, 1991).

Mortality caused by APEC infection may be due to that after invading the bloodstream APEC causes a septicemia resulting in massive lesions in multiple internal organs and in sudden death of the birds (Ewers *et al.*, 2003).

The ability of the bacteria to acquire iron and the resistance to the bactericidal effects of serum, predominantly conferred by the increased serum survival (ISS)-protein, enables APEC to multiply quickly in their hosts. increased serum survival (ISS) is regarded a specific genetic marker for avian pathogenic *E. coli* strains (Stordeur *et al.*, 2004).

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إعداد وتقييم لقاح متعدد السكريد الشحمي والراببوسومات من الطيور المصابة بالعصيات القولونية الممرضة ضد داء العصيات القولونية في الدجاج اللحم في محافظة السلیمانة

نهلة محمد سعيد، سالم صباح عبد الله، ياسين بير رضا

Email: salar_nahla@yahoo.com

وقد أجريت هذه الدراسة لتقييم فعالية لقاح متعدد السكريد الشحمي ولقاح الريبوسوم على العصيات القولونية المعزولة من حقول فروج اللحم في محافظة السلیمانة. تم استخدام خمسة وسبعين فروج اللحم لهذه التجربة، وكانت مقسمة إلى ثلاث مجموعات. تم تقييم الأجسام المضادة باستخدام جهاز اليزا (ELISA) واختبار (PHA) passive hemagglutination في سن 17 و يوم 29 وظهرت النتائج بان عدد الاجسام المضادة عالية في مجموعات المحصنة بالمقارنة مع مجموعة المراقبة، وكانت نتائج ELISA في عمر 17 للمجموعة الملقحة ب متعدد السكريد الشحمي 1.275 ± 0.095 ، في مجموعة الريبوسوم 1.092 ± 0.066 ومجموعة المراقبة 0.046 ± 0.009 ، في حين كانت النتائج عند عمر 29 يوم في المجموعة الملقحة ب متعدد السكريد الشحمي 1.849 ± 0.038 ، في مجموعة الريبوسوم 2.048 ± 0.048 ومجموعة المراقبة 0.985 ± 0.010 . وكانت نتائج PHA في يوم 17 كالتالي 24.189 ± 192 ، 56.406 ± 272 ، 0.7003 ± 1 في المجموعة الملقحة بمتعدد السكريد الشحمي، ومجموعة الريبوسوم ومجموعة المراقبة على التوالي، بينما في يوم 29 كانت النتائج 210.003 ± 1344 ، 374.746 ± 1792 ، و 112.813 ± 256 في المجموعة الملقحة بمتعدد السكريد الشحمي، ومجموعة الريبوسوم ومجموعة المراقبة على التوالي.