Preparation and Evaluation of Avian Pathogenic E. coli Lypopolysaccharide and ribosomal vaccines against avian colibacillosis in broiler chicken in AL-Sulaimania province

Nahla M.S¹, SLIM S.A², Yassin B.R²

1. Department of Microbiology, College of Veterinary Medicine, University of Sulaimania. Sulaimania, Iraq.
2. Department of Microbiology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.

Corresponding Author email: salar_nahla@yahoo.com

ABSTRACT

This study was conducted to evaluate and study the effectiveness of ribosome and LPS vaccines that of locally isolated strain of E. coli bacteria in poultry in AL-Sulaimania province. 75 broiler chicks was used for the purpose of the experiment were divided to three groups and were given the vaccine by subcutaneous. Antibodies evaluated by ELISA and PHA test at the age of 17th and 29th days results showed high antibody titers in vaccinated groups in compared with control group, where the results of the ELISA at the age of 17th day for the group LPS 1.275 ± 0.095, in ribosome group 1.592 ± 0.046 and in control group 0.046 ± 0.009 at the age of 29th day the results was in group of LPS 1.849 ± 0.038 in group ribosome 2.048 ± 0.048 and in control group 0.985 ± 0.010. For testing the PHA results were the age of 17th day as follows in the Group of LPS 192 ± 24.189 in group ribosome 272 ± 56.406, in control group ± 0.7003. At the age of 29th day was in the LPS 1344 ± 215.003 In the group ribosome 1792 ± 374.746 In group control was 256 ± 112.813. Has been able to vaccines produced locally to reduce the mortality rate after an examination of the challenge, reaching the deaths of the LPS was 2 in ribosome group 1 and in the control group.

Key words: E.coli, Lypopolysaccharide, 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 multicausal 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, septicemia, synovitis, osteomyelitis and yolk sac infection (McPeake et al., 2005)

The role of E. coli in chronic respiratory diseases in meat-type chickens 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 duration of exposure, 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 (Nagano et al 2012; Lynne 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).

Materials and method
E.coli / vaccine strain
The E.coli vaccinated strain was isolated from the clinically affected broiler farms with colibacillosis in AL-Sulaimania Province. Then the isolate was confirmed by culturing on selective culture media and the biochemical tests were carried out using the API 20 system to be sure that it was avian pathogenic Escherichia coli.

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

Culture in different media
All samples were placed on nutrient agar plates and incubated for overnight at 37°C for the growth of the organisms. After primary culture of the organisms, a small amount of inoculum from nutrient agar was sub-cultured in the nutrient agar and Macconkey agar to observe the colony morphology. Characteristic colony morphology of the organisms indicating the features of E. coli was selected for subculture on EMB agar (Carter, 1986).

Preparation cultures media
All media were sterilized by autoclaved at 121°C and 15, pound/inch for 15 min. (Quinn et al., 2000).

Preparation of the vaccines
A. Ribosome vaccine
The bacteria were grown in Nutrient Broth for 16h at 37°C and harvested by centrifugation (4080 xg for 10 min. 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 x 10^9 CFU/ml.

Bacteria were suspended with PBS and broken by ultra sonication at 18kc force for a period of 25 minutes using a sonicator. Then non-broken cells and other cell debris are deposited in cooled centrifuge at 27000 x g for 10 minutes, then the supernatant fluid was separated and re-centrifuged at 45000 x g for 30 minutes to precipitate the wreckage of the remaining cells.

Taking two samples of the supernatant fluid one makes as smear and stained by gram stain and the other cultured on the macconkey agar to make sure there were no whole bacterial cells or other pollutants. Then 4/5 from the supernatant fluid separated carefully, and centrifuged at 105,000 x g for 3 hours to get the parts containing ribosomes. This sludge suspended in a solution of 0.01M phosphate buffer saline containing 10^4 M of magnesium chloride with a pH (7.1) and the temperature of 4°C to become the focus 20 mg / ml. Then added to the ribosomes solution equal size of a substance sodium didiocyil sulphate (SDS) a concentration of 0.25% in a solution of 0.01 M from the buffer phosphate containing 10 M of magnesium chloride and the mixture are mixed well continuously for one hour at room temperature and then saved in the fridge at a temperature 4c for the next day. Then the SDS were separated by centrifuged at 37000 x g for 15 minutes and the upper part of the supernatant fluid were discarded and centrifuged at 105000 x g for a period of 2 hours where was obtained precipitate is ribosomes part which dissolve in buffer phosphate 0.01M containing 10^-3M from magnesium chloride and keeping at the temperature (-20°C) until use (Venneman and Bigley, 1969). Total protein was determined by Biuret method and used for immunization.

B. Lipopolysaccharide vaccine
Adopted the method of hot phenol described by (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 &centrifuged at 5000xg for 30 minutes. The supernatant was discarded and resuspension precipitate in PBS using 88ml distilled water heated to 68°C and mixed well. 88 ml of phenol was added and heated to 68°C and mixed well using magnetic 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 5000xg 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 88ml 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 1mg/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.

Experimental design

Seventy five (75) broiler chicks one day old were divided in to 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 $2 \times 10^9$ CFU/ml.
Group 3: were injected with 0.5 ml of physiological salt 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 ($1 \times 10^8$ 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.

Passive Hemagglutination test (PHA) (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 hrs. Blood sample was times with PBS (PH=7.2) and centrifuged 1500 rpm/5 minutes. Then centrifuged 1500 rpm/10 minutes to discard Alsevers solution and RBCs washed three times 2.5% of sheep RBCs suspension was prepared.

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 shaked gently every 5 minutes, then the mixtures was centrifuged 2000 rpm for 10 minutes to remove tannic acid solution, then RBCs washed three times with PBS (PH=7.2) and centrifuged 1500 rpm per 5 minutes.

C. Sensitization of washed tanned RBCs

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

One ml of sonicated APEC antigen was mixed with one ml washed tRBCs then incubated at 37°C for 30 minutes, and gently saked every 5 minutes, then the mixture was centrifuged 2000 rpm per10 minutes to remove the excessive antigens, finally the sensitized RBCs were washed with PBS (PH=7.2) and centrifuged 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

Added 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 tRBCs was added, and the plate covered with aluminum paper and incubated room temperature for at least 2 hrs, after that, the reactions was read and reading repeated after 18hr/4c.

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

Control

50 μl non-sensitized RBCs+50 μl NRS.
50 μl of non-sensitized RBCs+50 μl first dilution of positive serum.
50 μl of non-sensitized RBCs+50 μl of antigens.
50 μl of sensitized RBCs+ 50 μl of PBS.
50 ml of sensitized RBCs + 50 ml of NRS. RBCs.
50 μl of non-sensitized RBCs +0.05 μl of PBS.

ELISA test
ELISA test was done according to (Leitner et al., 1990).

A. Kit content
Microwell plate : microwell plate coated with recombinant E.coli Ag.
E.coli Ab Conjugate : purified E.coli Ag bound to peroxidase
Concetrated Wash Buffer : Tri-Hcl buffer containing 0.1% Tween 20.
Substrate A: Citrate phosphate buffer containing hydrogen peroxidase.
Substrate B: Buffer containing tetramethylbenzidine (TMB)
Stop solution: 0.5M Sulfuric acid
E.coli Ab Negative control: Normal serum of non-reactive for E.coli Ab.
E.coli Ab positive control : Inactivated serum containing E.coli Ab

B. Assay procedure
Reagent and specimens were allowed to reach room temperature (15-30 °C) prior to testing. Procedure must be strictly followed. Assay must be proceeding to completion within time. Arrange the controls so that well A1 is the blank well. From well A1, arrange the controls horizontal or vertical configuration. The procedure below assigns specific wells arranged vertical configuration. Configuration may depend upon software.
Prepare working wash Buffer by diluting the concentrated Wash Buffer 1:25.
Leave A1 as blank well.
Add 50 μl of negative control in wells B1 and C1 (Blue Reagent).
Add 50 μl of positive control in well D1 and well E1(Red Reagent).
Add 50 μl of specimen to assigned wells starting at F1.
Remove unused strips from the microwell plate, and store in original re-sealable pouch at 2-8°C.
Add 50 μl of conjugate to each well except for the Blank well.
Mix gently, Cover the microwell plate with the plate sealer and incubate at 37°C for 30 minutes.
Remove the plate Sealer, Wash each well 5 times with 350 μl of working Wash Buffer, Turn the microwell plate upside down on absorbent tissue.
Add 50μl of Substrate A to each well; add 50μl of Substrate B to each well.
Mix then cover micro well plate with plate Sealer and incubate at 37 C° for 15 minutes.
Remove the plate Sealer, Add 50μl of Stop Solution to each well.
Read at 450/630 nm within 30 minutes.

Challenge test
Ten fold dilutions from E.coli were prepared 24 hours culture on peptone water to obtain 1x 10⁸ CFU/ml to be used for experimental infection of chick according to (Sambrook et al., 1989).

Result and Discussion
ELISA
The antibody titers to, LPS and Ribosome, vaccines are listed in Table (1) they were measured at 17th days and 29th days of age.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Antibody titers (Mean±SE) at 17th days</th>
<th>Antibody titers (Mean±SE) at 29th days</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>1.275±0.095</td>
<td>1.849±0.038</td>
</tr>
<tr>
<td>Ribosome</td>
<td>1.592±0.046</td>
<td>2.048±0.048</td>
</tr>
<tr>
<td>Control</td>
<td>0.046±0.009</td>
<td>0.985±0.010</td>
</tr>
</tbody>
</table>

The different capital letters refer to significant difference (P< 0.05) among groups Means±SE (n=8).
There were significant differences (P < 0.05) among all groups as showed in the table (1) after vaccination and chllenging. Immune response of vacinnation 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 TLR4 by lipopolysaccharide (LPS) results in the expression of the proinflammatory cytokine IL-1β. 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 proinflammatory 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 (Andersson et al., 1972). LPS-activated B-lymphocytes 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 in fact, the eukaryotic ribosome is composed of four RNA molecules and more than 70 ribosomal proteins (Wool et al., 1990). There is increasing evidence that ribosomal proteins are capable of extra chromosomal functions. 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 heamagglutination test

<table>
<thead>
<tr>
<th>Groups</th>
<th>Antibody titers (Mean±SE) at 17th days</th>
<th>Antibody titers (Mean±SE) at 29th days</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>192±24.189</td>
<td>1344±215.003</td>
</tr>
<tr>
<td>Ribosome</td>
<td>272±56.406</td>
<td>1792±374.746</td>
</tr>
<tr>
<td>Control</td>
<td>3.75±0.700</td>
<td>256±112.813</td>
</tr>
</tbody>
</table>

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

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

Passive heamagglutination test is considered a routine method for the quantification of antibody titers (Stavitsky, 1954).

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) 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 and Venneman, 1972), whom noticed that serum antibodies against ribosomes were apparrent 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), 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, 2009) mentioned that the all chickens vaccinated with the LPS produced anti-lipopolysaccharide antibodies of the IgG subclass in their sera, rise in antibodies in the serum of chickens administered the LPS vaccine.

### Mortality rate

The number of mortality was recorded, in LPS group there was two chickens, in ribosome there was one chicken, and in the control group there were six chickens, see (Table 3)
Table 3. Number of mortality of broiler chickens vaccinated with different E.coli vaccines after challenging

<table>
<thead>
<tr>
<th>Group</th>
<th>LPS</th>
<th>Ribosome</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>2/25</td>
<td>1/25</td>
<td>6/25</td>
</tr>
</tbody>
</table>

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. Iss is regarded a specific genetic marker for avian pathogenic E. coli strains (Stordeur et al., 2004).

References


