The protective role of outer membrane protein against Avian pathogenic *E.coli* in broilers

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Abstract

Colibacillosis considered a serious and economically important disease that affect the respiratory and digestive tract of chickens. It is well established that the serotypes of Escherichia coli commonly associated with colibacillosis in poultry, are O78, O1 and O2. Although previous attempts to develop a vaccine have not been very successful, vaccination is still the most effective and promising way of controlling the disease. Therefore, our study has been designed for the development of a vaccine composed of different antigens (LPS, OMP, LPS+OMP and Bacterin) of avian pathogenic E. coli O78, the most common isolates, in a field trial to protect of chicken against colibacillosis. A total of 100 clinical samples from ill broiler chicks with colibacillosis were collected from different geographical regions of AL-Qadisiya province. All subjected to isolation, identification, biotyping, serotyping and antimicrobial agent sensitivity for avian pathogenic E. coli by use biochemical, Api – 20E, and biotyping use patterns of different fermentation and sertyping by monospecific antisera for O and H antigen during the period extended from May, 2010 to December, 2010.

This study showed that the prevalence of resistant APEC O78 isolates is very high in broiler chicks farms in AL- Qadisiya province. Of a battery of antibiotics used , these isolates were sensitive for only few antibiotics, those include; Colistin sulphate and Gentamycin. The present study have carried out in two stages; FIRST STAGE: the cultivation of identified isolates, extraction and purification of target antigens, and analysis for carbohydrate and protein contents. In addition, gel filtration and SDS-PAGE were performed for extraction of antigens. The SDS-PAGE revealed OMP (400 mcg/ml) and showed 9 bands having molecular weight between 14.413- 77.263 D.

is the field trial, where 300 of 1 day aged broiler chicks were divided into 3 equal groups. of Three challenged groups (inoculated with 1x109 cells/ml) were immunized subcutaneously, OMP (2 mcg/bird), The fifth group (control positive) was inoculated with E. coli challenge dose only, while the Three (the placebo) was inoculated with 0.7 ml of PBS only. The first initial dose has been given at 5th day old of broiler chicks followed by a booster dose after 9 days. A clinical follow-up, morbidity and mortality were monitored daily. The challenge dose has been given at 29

days old for all of studied groups. At 33rd day old, the immunopathological parameters were assessed. They includes histopathological examination of different vital groups, CFU/g and cytokins assay for selected interleukins (IL-1B, IL-6, and TNF-α; TLiA) and immunoglobulines (IgG and IgM) assessment was performed. All using ELISA. Results shown the incidence of mortality rate ranged between 2.2% and 5.4% for the 4 vaccination groups. This mortality rate was significantly lower than that of positive control group (56.7%) after bacterial challenge. Also there was significant difference in median serum IgG concentration throughout the experimental groups. It was significantly higher in the vaccinated groups compared with positive control. Serum IL-6, IL-1B, and TNF-α concentrations. The infected chicks showed clear gross lesions characterized by developed colibacillosis expressed as lesions were found in the prevalence of pericarditis, perihepatitis and airsacculitis, whereas, all vaccinated groups appeared no significant gross lesions were reported in the organs (air sacs, heart and liver).

INTRODUCTION

Outer Membrane Proteins: Outer membrane protein (OMP) is the main structure and half of cell wall of gram negative bacteria. OMP is consisted of major OMP and trace of protein weight of subunits of major OMP is about 30- 40 KD and the descending order to molecular weight is as follows: microporous protein, k protein, outer membrane protein A and plasmid coded protein (PCP). Microprous protein contains OMPF and OMPC which both have good penetration for most of water soluble antibiotic drugs (Nikaldo *et al.,* 1992). Therefore, the OMP of *E. coli* plays an important role in drug resistant process (Nikaldo *et al.,* 1985; Lu Qq *et al.,* 2002). The difference between major OMP and its mobility can indirectly reveals the difference of OMP encoding gene in chromosome of isolated strains. The results showed that *E. coli* OMP achieve their pathogenic effects mainly by helping bacteria escape the immune defense and promote adsorption of host cells. Fantinatti *et al* (1994) found that the 40700 and 28800 of major OMP were absent in non-pathogenic strains through analyzing the OMP types with SDS-PAGE , these 2 proteins could play a role in the pathogenic process of sepsis caused by avian *E. coli* . *E. coli* OMP antibodies also have a passive immune protection effect. Gao *et al.* (2001) used purified OMP as coating antigen and detected OMP antibodies produced by chicken suffered from colibacillosis with enzyme linked immunosrbent assay (Gao *et al.,* 2001). Yu (2008) amplified pil A gene and OMPc gene of avian pathogenic *E. coli* strain, the expressed fimbraie and OMPC were trans formed into vaccine and the protective immune response was proved after the mice were immunized (Yu, 2008). Chen *et al.* (2005) analyzed the OMP of *E. coli* O2, O78 and resistant fusion strain isolated from poultry through SDS-page, the results showed that the major OMP bands were the same and had across protective immunity. Through protoplast fusion of avian *E. coli* to construct *E. coli* OMP fusion

strains by both parents OMP antigen and O antigen, laid the foundation for the development of new *E. coli* bivalent vaccine (Ding *et al.*, 2004). These studies showed that OMP plays an important role in occurrence and immune protection of avian colibacillosis (Franco *et al* ., 2000 and Zhang *et al.*, 2010).

The outer membrane of *E. coli* contains 60% of the envelope protein. Using polyacrylasmide gel electrophoresis, six protein bands were found in the outer membrane fraction of which one band (mol. wt 44000) accounted for 70% of the total outer membrane protein this protein this protein referred to as the major outer membrane protein (Molly *et al*., 2000). The outer membrane protein is a continuous structure on the surface of gram negative bacteria and, in bacterial pathogens has particular significance as a potential target for protection against a number of organisms (Burnens *et al.*, 1995).

The correlation between OMP patterns and clonal identity of O2 and O78 strains can be exploited for the rapid identification of pathogenic clones and in the development of control measures against outbreaks of colisepticemia in the poultry industry. In conjunction with 0 serotyping, characterizing the OMP patterns of isolates through comparison with reference strains provides a reliable method for discriminating among the major O2 and O78 clones at a fraction of the effort of multilocus enzyme electorphoresis. The clone specific OMP patterns may help in the selection of specific strains for developing vaccines that protect against the major O2 and O78 clones associated with avian diseases. One intriguing possibility is that any isolate from electrophoresis, which includes both O2 and O78 strains, may afford cross protection against all O2 and O78 strains of this clone. Bolin and Jensen (1987) have demonstrated that antibodies to iron regulated OMP can passively immunize against and protect turkeys from *E. coli* septicemia. In addition, the cross protective attributes of *P. multocida* serotypes, when grown in vivo, have been ascribed to OMP (Snipes *et al.*, 1988). In chickens, cell lysates of ultrasonicated O2: K1 strains have protected against infection by O78: K80 strains (Melamed *et al.*, 1991). These findings indicate that common antigens can protect chickens against infection by strains of the two major serotypes recovered from poultry. The development of successful vaccines against avian colisepticemia will be aided by the ability to identify and target the common pathogenic clones of the O2 and O78 serogroups (Kapur *et al.*, 1992).

3.2. Methods:

3.2.1. Collection of samples, and identification and isolation of *E. coli*.

A – Clinical observations: In most cases, broiler chicks were listless with ruffled feathers and indications of fever, labored breathing, occasional coughing and rales. Bacteria were recovered

originally from a variety of tissues, with most isolates being collected from air sac, pericardium, heart, liver, gall bladdar and spleen.

B – Samples: A total of 100 specimens were collected randomly from broiler chicks with colibacillosis, durind the period extended from may, 2010 to December, 2010. Their geographic distribution is elucidated in table (3-1). Collection was carried out under aseptic technique with care must be take to avoid faecal contamination of samples, the swabs collected from airsac, liver, heart, gall bladder and spleen after that placed in 2ml of transport media (Brian–heart infusion broth), immediately and following collection the samples were transported to laboratory by cool box.

Table (3-4) :Districts of sample collection from broiler chicks.

Region	No – of birds
Adiwania	20
Afak	20
Dagarha	20
Shameya	20
Hamza	20
Total	100

Specimens were streaked onto Blood agar plates and MacConkey agar and Incubated at 37 Cº for 24 hrs. Diagnosis depend on morphological character (shape, color and size) of colony. Typical lactose ferment colonies were picked randomly and transferred to MacConkey agar plates and Eosin methylene blue agar plates and incubated for additionally over night, then the colonies were individually identified using gram stain and traditional biochemical tests according to Colle *et al*. (1996).

C– Gram stain: single colony from Eosin methylene blue agar by loop was spread on clean slide, fixed and stainend with gram stain according to (Quinn *et al*., 1998), then examined under oil immersion.

D– Culture on MacConkey agar: loopful of the suspected culture was streaked on the prepared MacConkey agar and incubated at 37 Cº for 24 hrs. Avian pathogenic *E. coli* are lactose fermenter (pink colony).

E– Culture on Eosin methylene blue agar: loopful of the suspected culture was streaked on the prepared Eosin methylene blue agar and incubated at 37 Cº for 24 hrs. Avian pathogenic *E. coli* colonies appear greenish metallic sheen.

F– Biochemical tests:

1 – Oxidase test: A strip of filter paper which soaked previously with oxidase reagent (1 % solution of tetramethyl–p–phenylene–diamine dihydro chloride), then the colony was picked up with a sterile wooden stick and smeared over the filler paper. A positive result was indicated by an intense dark purple during 5 – 10 seconds (MacFaddin, 2000).

2 – Catalase: It done by spreading single colony of bacteria from *E. coli* on a clean slide, then mixed with a drop of hydrogen peroxide (3%). The production of O_2 bubbles as a positive reaction (Quinn *et al*., 1998).

3 – Ureace test: It was made by culturing the isolate on slant of urea agar and incubated at 37 Cº for 24 hrs, and if color of media converging from yellow to pink color, indicate as a positive reaction (Colle *et al*., 1996).

4 – Indol test: This test is used to detect indol rings from the destruction of the amino acid tryptophan and carried out by addition of Kovac's reagent into 5 ml of peptone water culture placed in tubes and mixed gently , and the red ring was appeared as appositive reaction (Macfaddin, 2000).

5 – Methyl red test: This test was carried out by inoculating 4 ml of MR–VP media with 0.5 ml of bacterial suspension and incubated at 37Cº for 24 hrs, then 3 drops of MR added to the media and mixed gently. The development of clear red color is indicative of positive reaction (Quinn *et al*., 1998).

6 – Voges – Proskaur Test: MR-VP broth was inoculated with 0.5 ml of bacterial suspension and incubated at 37 Cº for 24 hrs. One ml of 40% KOH and 3 ml of 95% solution of α–naphthol were added. A positive reaction was indicated by the development of a pink color in 2–5 min. (Macfaddin, 2000).

7 – Citrate Utilization test: Simmon's citrate slant was inoculated with bacterial colony and incubated at 37Cº for 24 hrs. A blue color of media is an indication of positive reaction (Colle *et al*., 1996).

8 – Gelatin liquefaction test: Bacterial colonies were cultured on gelatin media and incubated at 37Cº for 24 hrs. Non solidifying of gelatin after stored at 4º C for 10 minutes indicated positive reaction (Macfaddin, 2000).

9 – Triple sugar iron (TSI) test: The bacterial colony was grew on slant of TSI agar by stripping and streaking, then incubated at 37Cº for 24 hrs. Gas bubbles was appeared as indication of CO_2 formation, and black precipitate was appeared as indication of H_2S production (Quinn *et al*., 1998).

10 – Phenylalanine deaminase test: Slant of phenylalanine agar inoculated and incubated at 37 Cº for 24 hrs, then 4 -5 drops of ferric chloride reagent were added and mixed in order to cover the colonies with reagent. Green color was appeared as a positive reaction (Macfaddin, 2000).

11-Api–20E system (Analytical profile index for Enterobacteriaece test): According to Atlas (1995), this test (Api–20E system, BioMerieux, France) was used for diagnosis the bacterial isolates. This test have 25 strips, each strip contain 20 microtubes with contained dried material. According to the manufacturer instructions, the following steps have been done:

1. Preparation of the strip: 5 ml of distilled water added in to a strip container to from a humid condition.
2. Preparation of bacterial suspension: pure colonies were transferred from culture by a loop into a sterile 5 ml distal water and mixed well to from a suspension.
3. Inoculation of strip: tube of each strip was filled with bacterial suspension by a sterile pasture pipette except tubes GEL, VP, and CIT may be filled the cupule of them.
4. The upper part of tubes LDC, ADH, H_2S, URE and ODC were covered with sterile mineral oil in order to ensure anaerobic condition.
5. The strip container covered and incubated at 37Cº for 24 hrs.
6. Reading of strip.
7. After incubation, the following reagents was added directly into VP tube and waited 10 minutes.
    - **a.** Drop of covax reagent into IND tube.
    - **b.** Drop of TAD reagent into TAD tube.
    - **c.** Drop of VP1 reagent then drop of VP2.

The results were converted into numerical profile and compared with the company index. Each strip divided into seven parts, each part have three test numbered with (1, 2, 3) and each positive test is given specific number, but negative test not given any number, then all three number of each group which about 0–7 (7 digit profile), therefore finally we had seven number which compared with index to give genus and species of bacterial isolates.

3.2.2. Serotping of Avian pathogenic *E. coli* by agglutination test.

The serotyping of APEC isolates were performed by kit for veterinary laboratories Agency New How Addlestone. Surrey K Tis 3NB, UK for *E. coli* O2: K1 typing serum OK "F21" (Avian strain), *E. coli* O1: K1 typing serum OK "F1" (Avian strain) and *E. coli* O78: K80 typing serum OK "F103" (Avian strain). All are manufactured by VLA, UK. After diagnosis of bacterial isolates by gram stain, biochemical test and Api –20E system, the serotyping was performed as follow:

1. The APEC isolates were grown on brain heart infusion agar over night at 37 C° for 24 hrs.

2. Twenty microlitter of antiserum was applied onto each glass slide.
3. Ten colonies was transferred to each drop of antiserum and mixed well, the amount of culture should be sufficient to give a distinct milky turbidity.
4. The slide were tilted for 5 – 10 seconds.
5. The reactions were read with by naked eye by holding the slide in front of a light source a gainst a black back ground.
6. A positive reaction was seen as a visible agglutination.

3.2.3. Preservation of bacterial isolates:

The tentative isolates were cultured on sterile brain heart infusion broth and glycerol 20% and incubated at 37 C for 24 hrs, after turbidity appeared, they stored in a deep freezing (Bopp *et al*., 2005).

3.2.4. Fermentation of carbohydrates by *E. coli* isolates:

The *E. coli* isolates were characterized by their ability of utilize the following sugars: maltose, lactose, sucrose, dulcitol, adonitol, salicin, raffinose, dextrin, xylose, rhamnose and mannitol. The indicator used for sugar fermentation (Bromothymol blue broth) base was prepared by dissolving peptone (10g), sodium chloride (5g) and bromothymol blue (0.018 g) in 1 litter of distilled water. Each carbohydrate solution was prepared by dissolving 1% of corresponding sugar in the broth base medium, and only salicin was prepared at 0.5%. Each *E. coli* isolate was inoculated into prepared sugar medium and incubated at 37 C for 24 hrs. The test was recorded as positive when the medium turned from bluish colour to yellow (Gross, 1994; Bopp *et al*., 2005).

3.2.5. Antimicrobial drug sensitivity test:

In vitro antimicrobial drug sensitivity test was performed on confirmed *E. coli* isolates presenting as greenish metallic sheen colonies on Eosin methylene blue agar.

The antimicrobial sensitivity test was proceeded according to Bauer *et al.* (1996). The Mueller Hinton agar used in antimicrobial testing using a sterile wire loop, the surfaces of 3-4 isolated colonies were transferred to a tube containing 4 ml of nutrient broth medium. The broth was inocubated at 37 C^{o} until it′s turbidity exceeded that of the Mcfarland 0.5 barium sulphate standard. A sterile cotton swab was used to streak the dried surface of Muller-Hinton plates. The inoculated plates were allowed to remain on flat surfaces. The selection of antibiotic disk concentration and interpretation of the zone size were done as recommended by the manufacturers (Oxoid, UK) and the National Committee for Clinical Laboratory Standards (NCCLS, 1990). The following antibiotics concentration per disk were used: ciprofloxacin (5 mcg), sulphamethazole–trimethoprim (25 mcg), tetracycline (30 mcg), chloramphenicol (30 mcg),

ampicillin (10), amoxicillin (25 mcg), cephalothin (30mcg), cefotaxine(30 mcg), streptomycin (10 mcg), Gentamycin (10 mcg) and Colisitn (10 mcg).

3.2.8. Extraction and Purification of Outer membrane proteins of Avian pathogenic *E. coli* according to Nurminen (1978).

1) Outer membrane proteins extraction:

a – Bacterial growth: avian pathogenic *E. coli* was activated by inoculating into 500 ml of brain heart infusion broth, incubated at 37 Cº foe 24 hrs, then the bacterial suspension was examined by gram stain for any contamination. Bacterial suspension was cultured on brain heart infusion agar (100 Petri dishes), and incubated at 37 Cº for 24 hrs, then harvested with PBS (pH=7.2) and centrifuged by cooling centrifuge 3000 rpm for 30 min at 4Cº, then bacterial cells were washed via PBS (pH=7.2) three times and centrifuged at 2000 rpm for 10 minutes.

b – Extraction of OMP: Bacterial cells were washed twice with solution (a). To prepare outer membrane, bacterial cells (1 g wet weight) was suspended in 10 ml of solution (b), After 10 minutes, 0.4 ml of solution (c) was added and after 5 minutes the mixture was centrifuged 3000 rpm/ 20 minutes, then the pellet was washed once with 10 ml of solution (d) and once by solution (e). The cell envelope prepared from (1 g) of bacteria were extracted with 10 ml of TX buffer (solution g) followed by centrifuged 3000 rpm/ 20 minutes at room temp., and the pellet washed once with 20 ml of TX buffer, but the supernatant was discard. The pellet was then extracted with 10 ml of solution (g) followed by centrifugation at 3000 rpm for 20 min. at room temp, then the pellet was treated with 20 ml of solution (g).

3.2.9. Purification of Outer membrane proteins:

One gram of outer membrane was extracted twice with 10 ml of 2% TX buffer in presence of 0.01 M $MgCl_2$, then centrifuged at 300 rpm for 15 min. and the sediment (one gram of TX treated envelope) was suspended in 12 ml of 0.01 M EDTA containing 0.2 % TX, then the suspension was heated for 5 min. at 100 Cº and then incubated at 37 Cº over night after receiving trypsin (0.5 mg). thereafter the mixture was centrifuged at 8000 rpm for 20 min., and the supernatant was collected and the sediment was digested once more with trypsin and centrifuged. All supernatants were collected and ultrafillterated by an amicon apparatus with an XM50 filter and 2 litter of distilled water with further filtration. The precipitate (the protein fraction) was suspended in 100 ml of distilled water and centrifuged at 8000 rpm for 20 min. Finally, the precipitate was suspended in 10 ml of distilled water.

Chemical analysis of outer membrane proteins of APEC:

1) Carbhydrate estimation according to Dubois et al (1956).
2) Protein estimation by using Biuret method as in LPS.

3) SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970).

Sample and standard proteins were diluted (1:10) with buffer which composed of 1 ml of 5M tris – HCI (pH=6.8), 0.8 ml glycerin, and 1.6 ml of 2–mercaptoethanol and heated for 3-5 minutes, then prepared the upper layer of 4 % acrylamide gel and the lower layer of 12% acrylamide gel. Electrophoresis was started by switching on 20 miliamperes to gel layers until the tracking dye entered the separating gel and then the voltage increased until the tracking dye reached near the bottom of gel (8 cm). Scan gel, side down at 595 nm by Epson scanner and results are analyzed automatically by photo capt molecular weight software, Results were measured depended on kemtrol serum control that provided by Helena company (Europe).

3.3. Experimental birds and vaccination experiment:

Six hundreds unvaccinated broiler chicks (Ross) of mixed sex (aged 1d.) were obtained from a commercial Babel Hatchery in Adiwania province, and randomly divided into Three groups (100 per each). All groups were housed together in the suitable hall, the concrete floor was covered with wood shavings, the temperature and relative humidity accorded with the requirements of broiler chicks. Commercial pullet feed and clean water were available *ad libitum* and staff decontaminated their footwear and put on clean hall coat. Birds were vaccinated as follow:

Group I (OMP group; T1): inoculated with OMP as 2 mcg/ g of broiler chick (0.2 ml/ chick, sc) on 5^{th} day, then repeated after 9 days.

Group II (negative control group; C-): inoculated with PBS (0.2 ml/ chick, sc) on 5^{th} day, then repeated after 9 days.

Group VI (positive control group; C+): inoculated with APEC (O78), were made using Macferlands tube and spectrophotometer to justify the challenge does (0.2 ml/ broiler chick, sc).

Blood samples were collected from five broiler chicks of each group to separate serum for immunological assay. Body weights were measured on 5, 20, 30, and 35 days of age. Feed and water consumption for each group between 1- 35 days should be measured. Mortality was defined as the number of broiler chicks that had died before the end of the study. Morbidity of the number of broiler chicks sick with or without lesion in either air sac, pericardium or perihepatic should be estimated.

3.4. Immunological assessment (ELISA method):

Qualitative detection of chicken IgM and IgG classes antibodies, and IL-1β, IL-6, and TNF-α have been performed according to the manufacturer instructions (ABO, Switzerland) as in the following steps:

c) Microscopic examination: Specimens (1 cm^3) were taken from liver, spleen, lung, bursa of fabricia and air sacs. The tissues were kept immediately in 10 % formalin buffer. After 72 hrs of

the fixation the specimens were washed with tap water and then processing was routinely done with asset of up grading alcohols concentration from 70 % to absolute 100% for two hrs in each concentration to remove water from the tissue, then clearance was done by xylol. Thereafter, specimens were embedded with paraffin wax at 58 C on two stages, then blocks of specimens were made with paraffin wax and sectioned by rotary microtome at 5 mm for all tissue. All tissue were stained with hematoxylin and eosin stain and the histopathological changes were observed under light microscope (Luna, 1968).

3.6. Statistical analysis:

Data were translated into a computerized database structure. An expert statistical advice was sought for statistical analyses where computer assisted using SPSS version 13 (Statistical Package for Social Sciences). Frequency distribution for selected variables was done first. Compliance of quantitative random variables with Gaussian curve was analyzed using the Kolmogorov Smirnov test.

RESULTS

4.1. Incidence of collibacillosis:

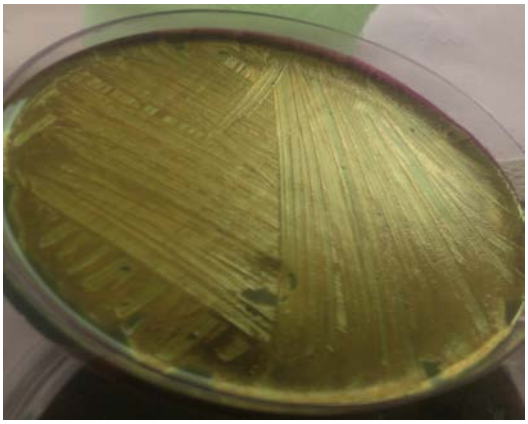
Based on clinical manifestations and postmortem examination, the overall infection rate in the epidemiological study was 65%. A total of 100 chicks were examined for evidence of infection, the infection rate ranged between 50% in Hamza district to 80% in Afak district. The differences observed in infection rate by source of chicks were statistically not significant (table 4-1).

Table (4-1): The infection rate by source of chicks in the prevalence study.

	Total	Positive evidence for infection	
Source of chicks	**N**	**N**	**%**
AL–diwanyia	20	11	55
Afak	20	16	80
Dagara	20	15	75
Shmeyia	20	13	65
Hamaza	20	10	50
Total	**100**	**65**	**65**

P (Chi-square) = 0.22[NS]

A total of 65 cases with infection were examined. The most frequently observed organ involved with infection was the air sac and liver (44.6% and 41.5% respectively). The heart came next in frequency (12.3%). Finally only one case (1.5%) had the lung as the predominant organ infected, table (4-3) and figure (4-2).



Figure(4-3) control positive culture of EMB

4.2. Results of biochemical test :

E. coli strain was diagnosed and the results were elucidated in appendix (1).

4.3. Definitive diagnosis:

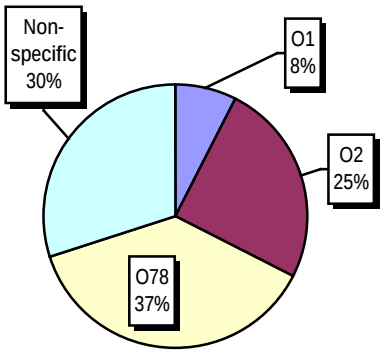
4.3.1. Api–20E test Confirmed the diagnosis of *E. coli* isolates.

4.3.2. Serotyping:

Serotyping was performed on 40 cases with diagnosed infection. Serotype O78 was the most frequent (37.5%) followed by serotype O2 (25%) and O1 (7.5%). About a third (30%) had no-specific serotype (non-typable), table (4-4) and figure (4-3).

Table (4-1): Frequency distribution of cases with infection (n=40) by serotype.

Serotype	N	%
O1	3	7.5
O2	10	25
O78	15	37.5
Non-typable	12	30
Total	**40**	**100**



Figure(4-4): Pie chart showing the frequency distribution of cases with infection (n=40) by

serotype.

4.3.4. Antimicrobial drugs sensitivity test:

As shown in table (4-5) and figure (4-4), Colistin sulphate was the antibiotic with highest sensitivity rate (93.8%) for culture positive specimens, followed by Gentamycin (84.6%). Antibiotics with least reported bacterial sensitivity (9.2%) were Ampicillin, Amoxicillin, Cephalexin and Sulphamethazole–trimethoprim.

Table (4-2): Antibiotic sensitivity of cultured specimens (n=65).

Antibiotic sensitivity (n=65)	N sensitive for specific antibiotic	Percent sensitive (%)
Colistin sulphate	61	93.8
Gentamycin	55	84.6
Ciprofloxacin	39	60
Streptomycin	36	55.4
Tetracycline	15	23.1
Cefotaxime	15	23.1
Chloramphenicol	15	23.1
Ampicillin	6	9.2
Amoxicillin	6	9.2
Cephalexin	6	9.2
Sulphamethazole–trimethoprim	6	9.2

4.4.4. Measurement of OMP of Avian pathogenic *E. coli* O78:

Outer membrane proteins; OMP (400 mcg/ ml) were extracted from 22 g (wet weight) of APEC O78 was 7 mg/ ml.

4.4.4.1. SDS-Polyacrylamide Gel Electrophoresis (SDS–PAGE):

1) **Standard proteins**: results showed 6 bands which have molecular weight between 14000 to 230000 D (table 4-6 and figure 4-7).
2) **APEC O78 proteins**: showed 9 bands having molecular weight between 14.413 to 77.263 D (table 4-7 and figure 4-8).

Table (4-6): show bands and molecular weight of standard proteins.

Protein No	Molecular weight Dalton
B1	230000
B2	150000
B3	80000
B4	67000
B5	20000
B6	14000

Table (4-7): show bands and Molecular weight of OMP prepared from APEC O78.

Protein No	Molecular weight Dalton
B1	77263
B2	75126
B3	67647
B4	61290
B5	44589
B6	34922
B7	29905
B8	17161
B9	14413

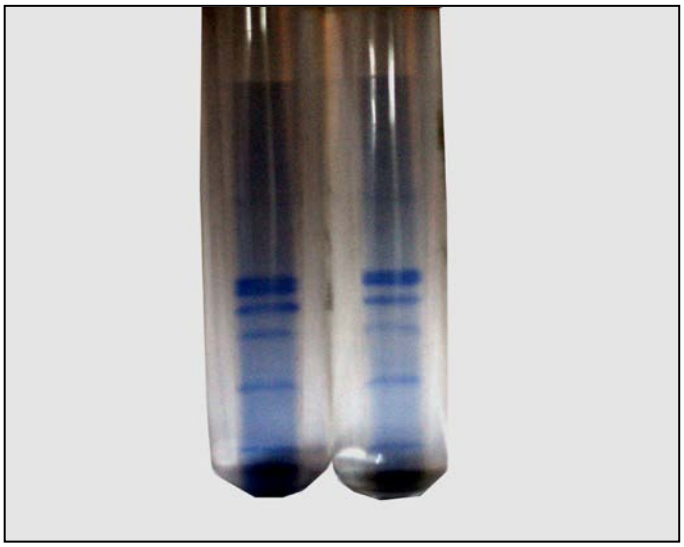
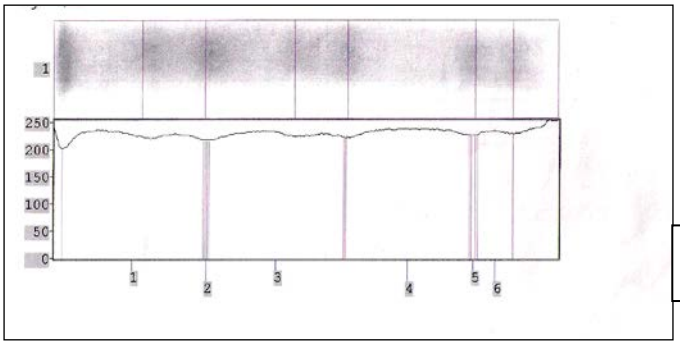
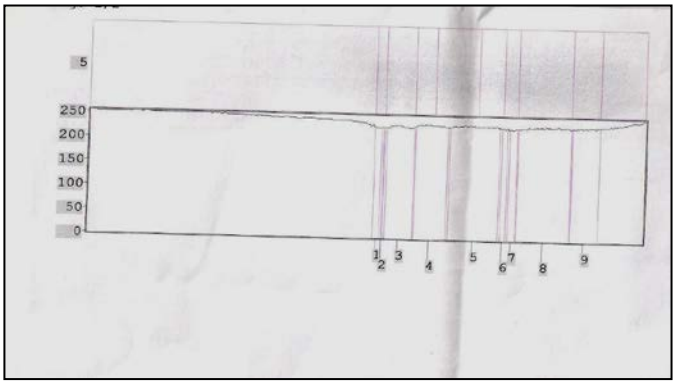


Fig (4-7): electrophoretic pattern of OMP prepared from APEC O78 by SDS Polyacrylamide Gel Electrophoresis (SDS–PAGE).



A

B



Figure(4-8): schematic diagrams of electrophoretic pattern of (A): standard protein (B): OMP of APEC O78. They Were determined according to the schematic diagrams obtained by photo Capt Molecular weight soft wa

Table (4-8): mortality rate among the 6 study groups for the period after vaccination until after bacterial challenge.

	First 5 days before vaccination			After the 1st vaccine dose			After the 2nd vaccine dose			After challenge with bacteria		
	Fatality			Fatality			Fatality			Fatality		
Groups	**Total at risk**	**N**	**rate (%)**	**Total at risk**	**N**	**rate (%)**	**Total at risk**	**N**	**rate (%)**	**Total at risk**	**N**	**rate (%)**
Control (not inoculated, PBL vehicle vaccine)	100	8	8	92	2	2.2	90	1	1.1	89	5	5.6
Positive control (inoculated but unvaccinated)	100	10	10	90	0	0	90	0	0	90	51	56.7
OMP vaccine	100	9	9	91	1	1.1	90	1	1.1	89	0	0

Note: **1**.Total at risk at the beginning of a specific time interval is equal to total at risk for the previous time interval – fatality during that interval. **2**. The fatality rate for positive control group was significantly higher (P value by Fisher Exact test <0.001) than that for each of the 4 vaccination groups.

Table (4-10): Incidence rate of positive culture in selected organs after 7 days of bacterial challenge by study group.

		Positive culture									
	Total	Positive evidence of infection-Air sac		Positive evidence of infection-Liver		Positive evidence of infection-Heart		Positive evidence of infection-Lung		Positive evidence of infection in any site	
Study group	N	N	%	N	%	N	%	N	%	N	%
Control (not inoculated, PBL vehicle vaccine)	5	5	100	5	100	5	100	2	40	5	100
Positive control (inoculated but unvaccinated)	5	5	100	5	100	5	100	0	0	5	100
OMP vaccine	5	5	100	3	60	2	40	0	0	5	100

5-7. SDS – Polyacrylamide Gel Electrophoresis (SDS – PAGE) of outer membrane protein of *E. coli* O78 .

The previous study of vaezzadeh *et al* ., (2004) who established 6 distinct bands with the same molecular weights is similar finding in this respect, but t Kshash (2008) have reported 3 to 4 distinct bands with the different molecular weights between 5000 to 49000 dalton .

An interesting thing is the presence of 4 bands with molecular weights 14.413 KD , 17.161 KD , 75.126 KD and 77.263 KD , which have not been observed by others seven clear polypeptide bands were observed using SDS – PAGE .

The outer membrane protein profile obtained by the method of Laemmli (1970) on slab gel were showed distinct protein bands with apparent molecular weights ranging from 14.413 KD – 77.263KD

The sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS – PAGE) . The poly peptide patterns between the strains O78 and O2 were also very similar and two major bands with molecular weights of 44KD and 25KD (Vaezzadeh *et al* ., 2004) .

Many scientists have used several methods for extraction of OMP , in this study we have used of EDTA and SDS , and Triton x – 100 , to cleave and breakdown of cell wall and liberate their compenent, Trypsin was used to degraded all proteins except outer membrane proteins (Malladi *et al* ., 2004).

DISCUSSION

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of outer membrane protein of E. coli O78

This result was in agreement with reports of Vaezzadeh et al. (2004) which established 6 distinct bands with the same molecular weights but disagreement with Kshash (2008) which reported 3 to 4 distinct bands with the different molecular weights between 5000 to 49000 Dalton. An interesting thing is the presence of 4 bands with molecular weights 14.413 KD, 17.161 KD, 75.126 KD and 77.263 KD, which have not been observed by others. Seven clear polypeptide bands were observed using SDS-PAGE.

The outer membrane protein profile obtained by the method of Laemmli (1970) on slab gel were showed distinct protein bands with apparent molecular weights ranging from 14.413 KD – 77.263 KD. The poly peptide patterns between the strains O78 and O2 were also very similar and two major bands with molecular weights of 44KD and 25KD were very distinctive (Vaez zadeh et al., 2004). Many scientists have used several methods for extraction of OMP, in this study using of EDTA and SDS, and Triton x-100, to cleavage and breakage cell wall and liberate their components and trypsin was used to degraded all proteins except outer membrane proteins (Mallad et al., 2004).

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