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PRODUCTION AND EVALUATION OF AUTOGENOUS VACCINE AGAINST AVIAN COLIBACILLOSIS

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ABSTRACT

The present investigation aimed to prepare a potent *Escherichia coli* vaccine to control colibacillosis in chickens. Firstly a total of 7 *Escherichia coli* isolates: O125: K 70, O1: K-, O146: K-, O26: K: –, O 78: K 80, O126: K58 and O128: K 67 were collected from diseased chickens. The collected strains were examined for virulent genes (*stx1, stx2, eaeA* and *hylA*) using PCR and analyzed by SDS-PAGE. Inactivated whole culture and outer membrane protein (OMP) vaccines were developed from *Escherichia coli* serogroups O1 and O78 and determined their potency to induce antibody response against colibacillosis in comparison to Nobilis® vaccine using Micro-agglutination test (MAT) and Enzyme Liked Immunosorbent assay (ELISA). The prepared inactivated and *Escherichia coli* outer membrane protein vaccines were free from bacterial and fungal contamination and both of the vaccines were found safe with no clinical symptoms when inoculated subcutaneously with double field dose into chicks. The protection rate of the inactivated vaccine was 84%, the protection rate in Nobilis® *E. coli* inactivated vaccine was 80% and the protection rate in OMP vaccine was 92%. Meanwhile, the mortality rate was 28% among the unvaccinated chicken. The results of this study indicate the higher protection rate of OMP and suggesting further development of OMP as a vaccine for protection of chickens against *E. coli* infections.

Keywords: *Escherichia coli*, Vaccine, Outer membrane proteins (OMP), ELISA, Virulent genes.

INTRODUCTION

E. coli is the most ubiquitous microorganisms on Earth, existing as part of the commensally intestinal flora, where it is responsible for the production of many beneficial metabolic products. It is also capable of causing different disease syndromes in animal, poultry and humans**.** Multiple drug resistant *E. coli* are the causative agent of the majority infections of urinary tract in human (Sabir *et al.*, 2014). Avian pathogenic *E. coli* (APEC) strains are responsible for diverse diseases, leading to extreme losses in the poultry industry. Among these diseases is colisepticaemia, which is characterized by bacteriaemia and organs colonization of *E. coli* (Barnes *et al.*, 2003 and Nakazato *et al.*, 2009). APEC strains frequently belong to serotypes O5, O1, O8, O78, O18 and O2 were detected (Blanco *et al.*, 1997). Serogroups O2, O21, and O78 are commonly existing in poultry which deeply infested by colibacillosis (Ewers *et al*., 2004**);** nevertheless, consequently, the other representative similar strains from those serogroups provide the gross focus for unraveling APEC virulence mechanisms and for the development and evaluation of vaccine recommended candidates. Control of colibacillosis is problematic, due to restricted availability of relevant antimicrobial agents and frequent failure of vaccines to protect against the diverse range of ExPEC causing disease in birds (Kariyawasam *et al*., 2007). The clinical *E. coli* isolates exhibited high rates of multidrug resistance, extended-spectrum-β-lactamase production as well as metallo-β-lactamase production (Hussain *et al.*, 2014). This study aimed to evaluate the efficacy of purified *E. coli* outer membrane proteins against colibacillosis infections in comparison with the *E. coli* inactivated vaccine in chickens.

MATERIALS AND METHODS

Standard *Escherichia coli*: Seven *Escherichia coli* recovered from chickens suffering from colibacillosis where kindly obtained from Veterinary Serum, Veterinary biologics, Abbasia, Cairo, Egypt.

Identification of *E. coli* isolates: All isolates were identified biochemically using API 20E kit (Biomerieux-France cat # 20-100) (Collier *et al.*, 1998). The isolates were then identified serologically (Edwards and Ewing,

1986) by using standard monovalent and polyvalent antisera specific for different somatic and flagella antigens of *E. coli*.

Characterization of *E. coli* isolates using PCR: The whole genomic DNA of the recovered *E. coli* isolates was extracted by boiling methods previously reported by Srithan and Barker (1991) and Sambrook *et al*., (1989). The reaction mixtures were carried out in PCR tubes according to the methods previously explained by (Rey *et al.*, 2003), by mixing 5µl of the extracted DNA by boiling method, 1µ of each oligonucleotide primers, 0.2mM dNTPs (40µM), 1µl Ampli *Tag* DNA polymerase, 2.5 µl 10x buffer and the volume was completed by DDW. The time temperatures were adjusted to be: initial denaturation 94°C/2 followed by 35 cycle of denaturation at 94°C/60 sec., annealing at 55°C/45 sec. and extension at 72°C/sec. Final extension: 72°C/10 minute. The PCR products were kept overnight in the PCR machine at 4°C.

Preparation of bivalent *E. coli* inactivated vaccine (Charles *et al.*, 1994): *E. coli* serotype O1 and O78 were grown separately onto brain heart agar (Difco) in Roux bottles and incubated at 37ºC for 48hr. The colonies were collected using normal saline. The bacterial suspension was adjusted to contain $3x10^9$ cells /ml and inactivated by adding 0.5% formalin with agitation. Then Montanide ISA 206 VG (Seppic) was mixed as a ratio (1:1) as an adjuvant.

Preparation of outer membrane protein (OMP) vaccine: The OMP of *E. coli* serotypes were prepared by the method of Chart (1994) with modification**.** Each *E. coli* serotypes (O1 and O78) was grown separately onto brain heart agar (Difco) in Roux bottles and incubated at 37ºC for 48 hr., HEPES buffer (Sigma) adjusted at pH 7.4 was used to harvest the cells by centrifugation of bacteria at 6,708 *x g* for 10 min at 4 °C, the number of the cells were adjusted to be 3x109 cells/ ml. Cells were disrupted using sonication for 30 seconds. (sonication should be repeated till all cells are disrupted and gave no growth on agar medium). Centrifugation were carried out at 1700 xg for 30 minutes to remove the intact cells and the large debris, while the total protein was harvested by from the supernatant by centrifugation at 100, 000 xg for 60 minutes at 4ºC. The pellet of the total protein was resuspended in10um HEPES buffer. Then an equal volume of sarcosyl 2% (Sigma) was added and kept overnight at 4ºC. The protein concentration was adjusted to be 2mg/ml using Bradford protein assay method (Bradford, 1976). The prepared outer membrane proteins were tested with SDS-PAGE (Chart, 1994). The Montanide ISA 206 VG (Seppic) was mixed with the prepared OMP of *Escherichia coli* serotypes (O1 & O78) as a ratio (1:1) as an immunostimulant.

Evaluation of the prepared vaccine: The standard protocols previously described the American federal regulation (1985) were carried out to evaluate the sterility, purity and safety of the vaccines.

Experiment design: Four groups of 25 Acre breed chickens (one month old) were kept in separate house and all of them were vaccinated by subcutaneous injection. Each bird of the first group was vaccinated with 0.5 ml/chicken of the prepared inactivated vaccine, each bird of the second group was vaccinated with 0.5 ml/chicken of the prepared OMP vaccine and each bird of the third group was vaccinated with 0.5 ml/chicken of Nobilis®*E. coli* inactivated vaccine while, each bird of 4th served as unvaccinated control group (25 chickens). Groups 1 and 3 received booster dose 3 weeks after vaccination, while group 2 received booster dose 10 days after vaccination.

Challenge test: Each group was challenged by both virulent *E. coli* O1 and O78 strains through division of the chicken in each group. The chickens vaccinated with the prepared inactivated *E. coli* O1 and O78 vaccine (group1) and Nobilis®*E. coli* inactivated vaccine (group 3) were challenged at 3 weeks after second dose by intramuscular (I/M) injection of 0.2 ml broth containing $1x10^8$ CFU of virulent *E. coli* organism. The chickens vaccinated with outer membrane protein vaccine were challenged after 10 days from second dose by intramuscular injection of 0.2 ml broth containing $1x10^8$ CFU of the same organisms. The control unvaccinated group was infected I/M with 0.2 ml of *E. coli* O1 and O78 ($1x\ 10^8$ CFU).

Evaluation of the humoral immune response of the vaccinated chickens: Micro-agglutination test (MAT) (Brown *et al.*, 1981) and ELISA (Voller *et al.*, 1976) were carried to measure the humoral immune response of the vaccinated birds. Coating of the ELISA plates were carried out by sonicated O1 antigen (4mg/dl) and O78 (0.9 mg/dl) antigen (Barrow *et al*., 1992) in a separated plate. The results were calculated according to formula described by Briggs and Skeels (1984).

Statistical Analysis: The statistical procedures used were according to Snedecor (1985). The student t-test was used in addition to the Analysis of Variance Fisher (F-test).

RESULTS

Confirmatory tests for identification of the collected isolates: All recovered isolates were *E. coli* strains when confirmed by standard microbiological techniques. The isolates were belonging to serogroupes: O 125: K 70, O1: K-, O146: K-, O26: K: –, O 78: K 80, O126: K58 and O128: K 67 as confirmed by using *E. coli* polyvalent and monovalent antisera.

Characterization of *E. coli* isolates using PCR: Amplification of 890 bp fragment of intimin gene was detected in O125: K70, O146: K- and O78: K80 strains. *stx2* gene was observed in O1: K^- , O126:K58 and O146:K- strains (Table 2 and Figures 1 and 2). Meanwhile, *stx1* and *hly* genes could not be detected among the examined strains.

Characterization of outer membrane protein of *E. coli* strains by SDS-PAGE:-SDS-PAGE of *E. coli* isolates revealed common electrophoresis pattern of outer membrane proteins especially among the molecular weight 20.83-28.5 and 15.74-19.79 kDa shared in all serotype (Figure 3). It clear that O1 have 7 bands range from 116 kDa to 14 kDa, O126 had 4 bands range from 95.91 kDa to 15.74 kDa, O125 have 5 bands range from 36.39 kDa to 13.7 kDa and O78 had 3 bands range from 33.55 kDa to 18.24 kDa. The rest of serotypes (O26, O128 and O146) had 2 bands range from 17.24 kDa to 23.24 kDa.

Results of quality control of prepared vaccines: The prepared inactivated and *E. coli* outer membrane protein vaccines prepared for immunization of birds were free from bacterial and fungal contamination. The vaccines were found safe and no clinical symptoms were appeared when inoculated subcutaneously with double field dose into chicks.

Evaluation of the prepared vaccines:

Micro agglutination test: The micro agglutination test (MAT) was conducted to measure the antibody titers against *E. coli* in broilers vaccinated with inactivated or Nobilis ®*E. coli* inactivated or OMP vaccines and challenged with virulent *E. coli* O1 strain (Figure 4). It is clear that the geometric mean of MAT in inactivated vaccine was increased from 80 at 1st week after vaccination to 100.8 at 3rd week and to160 at 3rd week after booster dose, while the titer after challenge decreased to 47.6 at 1st week then increased to 80 in 2ndweek. In Nobilis ® *E. coli* inactivated vaccine ,the geometric mean of MAT was increased from100.8 at 1st week then decreased to 80 at 2nd,3rdand 4th week then increased to 160 at 2nd and 3rd week after booster dose, while the titer after challenge reached to 160. In OMP vaccine, the geometric mean titer was 160 at 1st and 2nd week after vaccination, while after challenge it decreased to 80 at 1st week then increased to 160 at 2ndweek.

Among birds challenged with *E. coli* O78 strain (Figure 5), it is clear that the geometric mean of MAT in inactivated vaccine was increased from 127 at 1st week after vaccination to 201at 3rd week and decreased to160 at 1st to 3rd week after booster dose while the titer after challenge decreased to 80 at 1st week and 2ndweek. In Nobilis ®*E. coli* inactivated vaccine, the geometric mean of MAT was increased from 67.27 at 1st week to 160 at 3rd week then decreased to80 at 2nd week post booster dose, then increased to 160 at 3rd week after booster dose, while the titer after challenge became 80. In OMP vaccine, the geometric mean titer was increased from 254 to 320 after vaccination, while the titer after challenge decreased by 40 at 1st week then increased to 160 in 2ndweek, respectively.

Using ELISA: The ELISA test was conducted to measure the antibody titers against *E. coli* in broilers vaccinated with inactivated or Nobilis® *E. coli* inactivated or OMP vaccines and challenged with virulent *E. coli* O1 strain as shown in Figure (6). It is clear that the geometric mean of ELISA in inactivated vaccine was increased from 14576 at 1st week to 26724 at 3rd week after vaccination and to 39421 at 3rd week after booster dose, while the titer after challenge decreased to 27268 at 1st week and increased to 39355 at 2ndweek. In Nobilis® *E. coli* inactivated vaccine, the geometric mean of ELISA was increased from 21120 at 1st week after vaccination to27784 at 3rd week then decreased to 19907 at 1st week after booster dose then increased to 40179 at 3rd week after booster while the titer after challenge decreased to 15171 at 1st week and increased to 37068 at 2nd week. In OMP vaccine, the geometric mean titer reached to 11975 and 12268 at1st and 2nd weeks post vaccination respectively, while the titer after challenge increased to 16866 and 111171 in 1st and 2ndweek respectively.

Among birds challenged with *E. coli* O78 strain, it is clear that the geometric mean of ELISA in inactivated vaccine was increased from 17205 at 1st week after vaccination to 32434 at 3rd week and to57930 at 2nd week then decreased to 22253 at 3rd week after booster dose,, while the titer after challenge decreased to 10760 at 1st week and increased to 36199 in 2nd week (Figure 7). In Nobilis ® *E. coli* inactivated vaccine, the geometric mean of ELISA was increased from 27353 at 1st week after vaccination to 33708 at 3rd week then increased to 64466 at 1st week after booster dose, then decreased to 11246 in 3rd week after booster dose, while the titer after challenge increased to 23496 at 1st week and increased to 24378 in 2ndweek. In OMP vaccine, the geometric mean titere increased from 20787 to 31915 after vaccination, while the titer after challenge decreased to 27861 at 1st week then increased by 98175 in 2ndweek, respectively

Challenge test: The protection rate of inactivated vaccine was 84%, the protection rate in Nobilis® *E. coli* inactivated vaccine was 80% and the protection rate in OMP vaccine was 92%. Meanwhile, the protection rate was 28% among the unvaccinated chicken group (Table 3).

Table 1. Oligonucleotide primers used (Rey *et al*., 2003).

Primer designation	Specificity	Length (mer)	Sequence (5'- 3')	Amplified product size (bp)
***eaeA*- F**	Intimin	20	GTG GCG AAT ACT GGC GAG AGA CT	890
***eaeA*-R**		20	CCCCATTCTTTTTCACCGTCG	
***stx1*-F**	Shiga toxin1	21	ACA CTG GAT GAT CTC AGT GG	614
***stx1*-R**		21	CTG AAT CCC CCT CCA TTA TG	
***stx2*-F**	Shiga toxin2	21	GAGCGAAATAATTTATATGT	320
***stx2*-R**		21	CGAAATCCCCTCTGTATTTGCC	
***hylA*-F**	hemolysin	19	ACG ATG TGG TTT ATT CTG GA	165
***hylA*-R**		19	CTT CAC GTG ACC ATA CAT AT	

Table 2. Characterization of *E. coli* strains by PCR.

***E. coli* serotypes**	**PCR positive form**			
	***Stx1* gene**	***Stx2* gene**	***eaeA* gene**	***hylA* gene**
O 125: K 70	-	-	+	-
O146: K-	-	+	+	-
O 78: K 80	-	_	+	-
O128: K 67	-	-	_	-
O1: K-	-	+	-	-
O26: K: –	-	-	-	-
O126: K58	-	+	-	-

Table 3. Results of challenge test among chickens vaccinated with inactivated, Nobilis ®*E. coli* inactivated and OMP vaccines.

Groups	Total No. of challenged birds	No. of dead birds / Total No.	Protection rate
Group 1	25	4/25	84 %
Group 2	25	5/25	80 %
Group 3	25	2/25	92 %
Control	25	18/25	28 %

Group 1: chickens vaccinated with formalized inactivated *E. coli* vaccine.
Group 2: chickens vaccinated with Nobilis inactivated *E. coli* vaccine.
Group 3: chickens vaccinated with outer membrane protein *E. coli* vaccine.
Control: unvaccinated group.

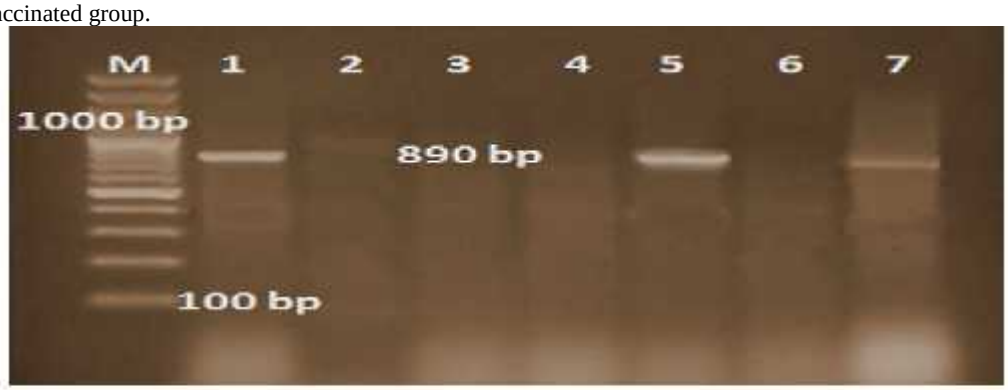

Figure (1): Agarose gel electrophoresis PCR assay showing amplification of 890 bp fragment of *eaeA* gene. Lane M: 100 bp-1500 bp DNA marker (Bio Basic Incorparation), Lane 1: O78:K80 strain, Lane 2: O1: K strain, Lane 3: O26: K- strain, Lane 4: O128:K67 strain, Lane 5: O146: K- strain, Lane 6: O126:K58 strain and Lane 7: O125:K70 strain.

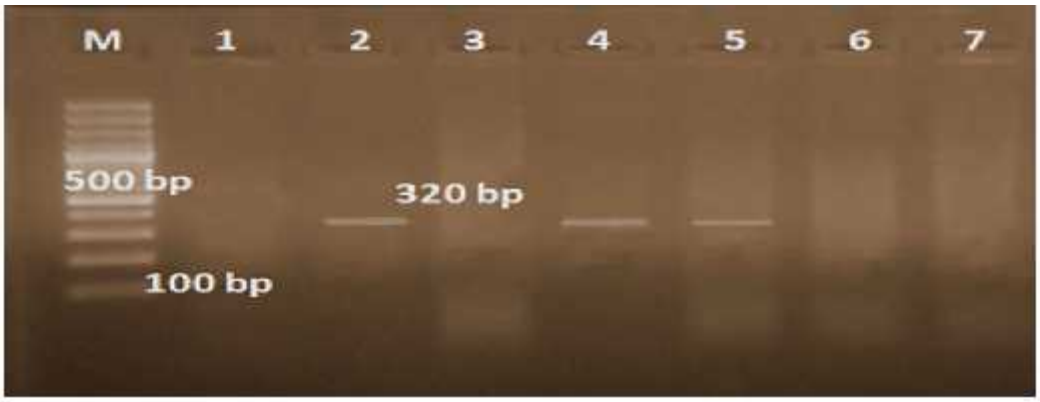

Figure (2): Agarose gel electrophoresis PCR assay showing amplification of 320bp fragment of *stx2* gene. Lane M: 100 bp-1500bp DNA Marker (Bio Basic Incorporation), Lane 1: O26: K- strain, Lane 2: O1: K- strain, Lane 3: O128:K67 strain, Lane 4: O146: K- strain, Lane 5: O126:K58 strain, Lane 6: O78:K80 strain and lane 7: O125:K70 strain.

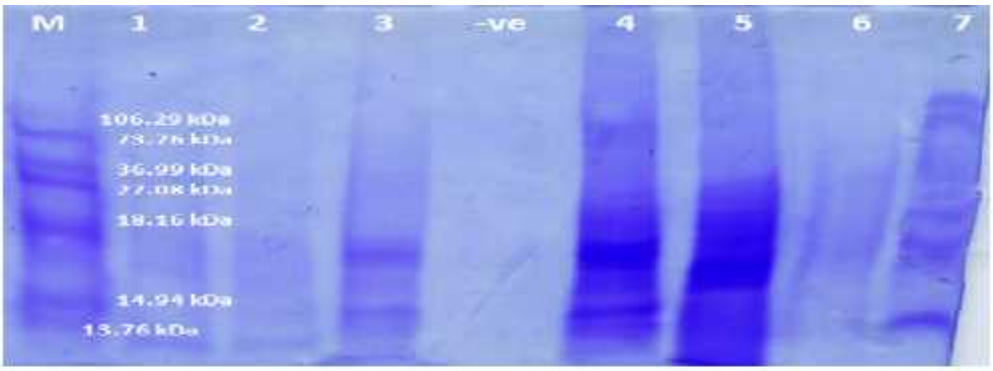

Figure (3): SDS-PAGE profile analyses of the prepared outer membrane protein of *E. coli* strains. Lane M: Spectra™ multicolor broad range protein ladder (Fermentas), Lane 2: O26: K: – strain, Lane 8: O126:K58 strain, Lane 3: O146:K- strain, Lane 4: O78:K80 strain, Lane 5: O128:K67 strain, Lane 6: O125:K70 strain and Lane 7: O1:K- strain.

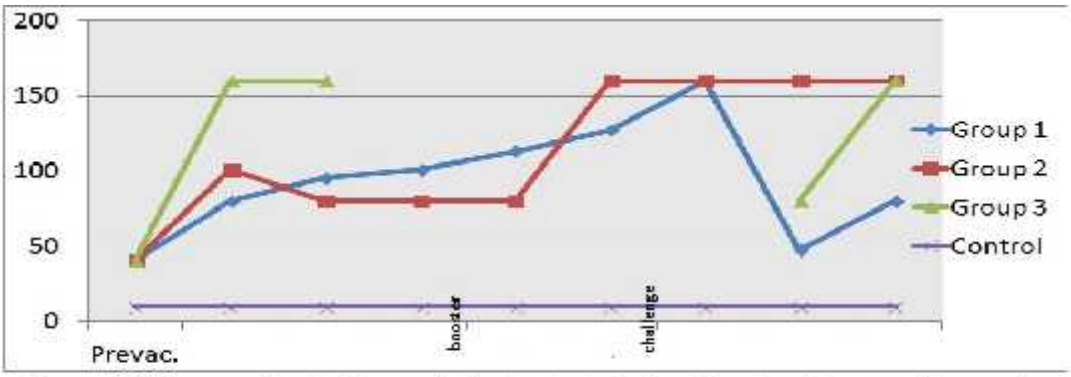

Figure (4): The results of microagglutination test (using O1 antigen) among the vaccinated chicken challenged with virulent *E. coli* (O1) strain. Group 1: chickens vaccinated with formalized inactivated *E. coli* vaccine, Group 2: chickens vaccinated with Nobilis inactivated *E. coli* vaccine, Group 3: chickens vaccinated with outer membrane protein *E. coli* vaccine, Control: unvaccinated group and Prevac.: pre vaccination. Challenge with virulent *E. coli* O1 strain.

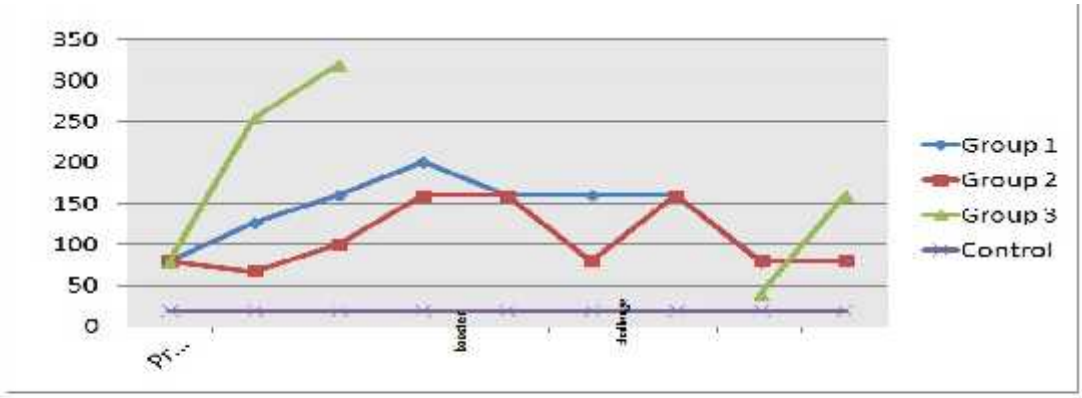

Figure (5). The Results of microagglutination test (using O78 antigen) among the vaccinated chicken challenged with virulent *E. coli* O78 strain. Group 1: chickens vaccinated with formalized inactivated *E. coli* vaccine, Group 2: chickens vaccinated with Nobilis inactivated *E. coli* vaccine, Group 3: chickens vaccinated with outer membrane protein *E. coli* vaccine, Control: unvaccinated group and pre vaccination. Challenge with virulent *E. coli* O78 strain.

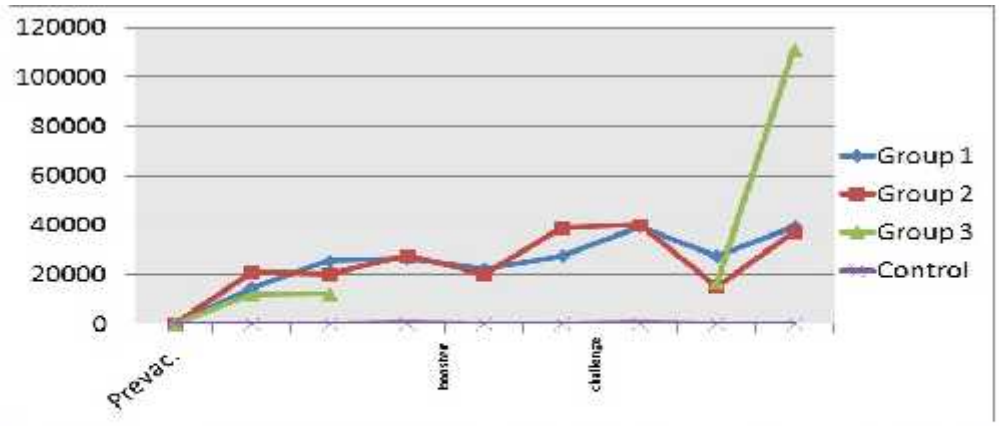

Figure (6): The results of ELISA test (using O1 antigen) among the vaccinated chicken challenged with virulent E. coli O1 strain. Group 1: chickens vaccinated with formalized inactivated *E. coli* vaccine, Group 2: chickens vaccinated with Nobilis inactivated *E. coli* vaccine, Group 3: chickens vaccinated with outer membrane protein *E. coli* vaccine, Control: unvaccinated group and Prevaccination. Challenge with virulent *E. coli* O1 strain.

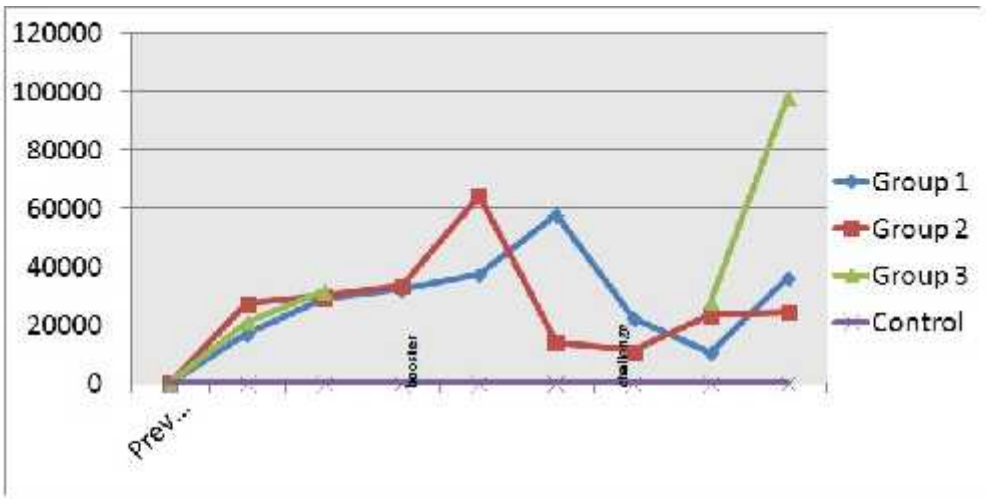

Figure (7): The results of ELISA test (using O78 antigen) among the vaccinated chicken challenged with virulent *E. coli* O78 strain. Group 1: chickens vaccinated with formalized inactivated *E. coli* vaccine, Group 2: chickens vaccinated with Nobilis inactivated *E. coli* vaccine, Group 3: chickens vaccinated with outer membrane protein *E. coli* vaccine, Control: unvaccinated group and Prevac.: pre vaccination. Challenge with virulent *E. coli* O78 strain.

DISCUSSION

Colibacillosis is responsible for great economic losses in the poultry industry worldwide. At present no completely reliable method for controlling *E. coli* infections in commercial poultry flocks. So the present investigation aimed to prepare a potent vaccine from the pathogenic *E. coli* serogroups O1 and O78 to control colibacillosis in chickens. The most frequently serogroups amongst the *E. coli* isolated from poultry in India were O2, O78, O19 and O20 (Mishra and Chlabra, 2002) while, the two major serogroups incriminated in avian sepsis were O2 and O78 (Ron *et al.*, 1991).

Seven *E. coli* isolates were collected from diseased chicken and serotyped as O125: K 70, O1: K-, O146: K-, O26: K: –, O 78: K 80, O126: K58 and O128: K 67. Some APEC strains are able to produce toxins like labile temperature (LT) and stable temperature (ST) enterotoxins and verotoxins known as Shiga-toxins (Stx). Several putative virulence genes facilitate adhesion of APEC to the epithelium of the respiratory tract, their multiplication and spread within the host, the development of resistance to host defense mechanisms, manifestations in organs and the induction of cytopathic effects (Janben *et al*., 2001). With PCR, the *E. coli* isolates had at least one virulence gene. Our result is in an agreement with that of Parreira and Gyles (2002) who reported that 53 % of APEC with *stx* gene sequences; one isolate harbored *stx2* sequence, two have both *stx*1 and *stx2* sequences while 49 isolates harbored only *stx*1sequences. Farooq *et al.* (2009) reported that 19.81 % of *E. coli* isolates from different avian species containing at least one virulence gene.

SDS-PAGE of the prepared OMP of *E. coli* isolates revealed common electrophoresis pattern especially among the molecular weight 20.83-28.5 and 15.74-19.79 kDa. Wu *et al.* (2014) recorded that the lipid bilayer membrane of Gram-negative bacteria composed of the inner layer of phospholipids and lipopolysaccharides in the outer layer.

Vaccine immunization is considered as important means of controlling the colibacillosis in poultry. The main goal of the present investigation was to prepare an efficient *E. coli* vaccine to control colibacillosis in chickens. So inactivated and OMP vaccines were prepared from *E. coli* O1 and O78 strains. The OMP may be responsible for immunity of colibacillosis in poultry (Tie *et al*., 2010).

It is clear that *E. coli* OMP antibodies had an immune protection effect among the vaccinated group. Vaez Zadah *et al.* (2004) reported higher antibody titer following the booster dose of OMP in chickens, however; it remained constant following further boosters. The purified *E. coli* outer membrane protein can induce significant protection immunity against colibacillosis diseases in chickens, and probably in immunocompromised hosts with repeated urinary infections (Vaez Zadah *et al.*, 2004). It is evident that on challenging with *E. coli* virulent strain, the protection rate of the prepared vaccines was varied between 84% and 92% among the vaccinated groups. Chickens injected by Vaez Zadah *et al.* (2004) by OMP reported mortality in three out of fifteen following challenge with a LD,

however, in case of challenge with lethal dose (LD) 100 homologous strain of O2, mortality in the unvaccinated chickens was 12. A study conducted by Lu *et al*. (1991) showed a considerable success in using OMP vaccines against various organisms.

Therefore, achieving the best immunizing efficacy needs vaccine strains base on the O serotyping. Consequently, more researches are required to determine evaluate the immunization efficacy of the multivalent based on the OMP types. The results of this study recommended further development of OMP vaccine against *E. coli* infections.

Conclusions: The results of the present study indicate the higher protection rate of OMP and recommended further development of OMP vaccine against *E. coli* infections.

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