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ORIGINAL ARTICLE

Comparative evaluation of outer membrane protein and whole cell antigen vaccine against avian pathogen *Escherichia coli* infection in broiler chicken

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ABSTRACT

Background and Objectives: Present study was aimed at assessing protective efficacy of outer membrane protein (OMP) vaccine in comparison to inactivated whole cell antigen vaccine after challenge with homologous serogroup (O2) of avian pathogenic *Escherichia coli* in broiler chickens.

Materials and Methods: The outer membrane proteins were extracted by sarcosyl method and protein concentration was determined by nanodrop spectrophotometer. The study comprised of 120 birds divided into 6 groups. The birds were subcutaneously immunized twice with primary vaccine in the first week followed by booster vaccine in second week.

Results: The protection rate of 82% was found in whole cell inactivated antigen, 91% in OMP vaccine and 27% among the unvaccinated group. The antibody (IgG) response was found significantly higher in OMP vaccine group than whole cell antigen group. In unvaccinated groups chicks, the antibody titer never reached to the protective level till the termination of experiment. The bacteria were re-isolated from the infected broiler chickens for the confirmation of induced infection and were characterized using standard cultural and biochemical tests belonging to O2 serogroup.

Conclusion: Our study demonstrated that the outer membrane protein (OMP) vaccine provided significantly higher protection (91%) and antibody response compared to the inactivated whole cell antigen vaccine (82%) against *Escherichia coli*

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O2 infection in broiler chickens. Birds vaccinated with OMP exhibited fewer pathological lesions and a stronger immune response. The findings underscore the potential of OMP-based vaccines as a safer, more immunogenic alternative for controlling colibacillosis.

Keywords: *Escherichia coli*; Colibacillosis; Chickens; Bacterial outer membrane proteins; Vaccines; Immune response

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) is responsible for causing severe infections in poultry such as coli septicemia, coligranuloma, air sacculitis, fibrinous pericarditis, perihepatitis and cellulitis (1, 2). High incidence of this disease has led to severe economic losses to the poultry industry worldwide (3). Also several studies have shown that APEC may also act as a human pathogen due to sharing of homologous virulence genes with extra intestinal pathogenic *E. coli* (4, 5). Administration of antimicrobials has been the primary approach for disease control, however, the indiscriminate use of antibiotics has led to severe complications including emergence of multidrug resistance (6). As a result, vaccines have become the effective choice for prevention and control of this disease. Currently, killed *E. coli* vaccines, including autogenous vaccines, have generally failed to meet the expected results because of the diverse characteristics of APEC strains (7). While the protection by the inactivated whole cell vaccines is of short duration owing to rapid destruction and elimination of organisms from the host, live attenuated vaccines can be hazardous due to residual virulence caused by insufficient attenuation. Additionally, in live attenuated vaccines there remains a danger of reversal of virulence of organisms. Furthermore, these vaccines are unable to offer effective cross-protection against infections caused by multi-serogroup APEC strains (8). Thus development of an effectively cross protective vaccine based on defined non-toxic components of the bacteria seems desirable. Vaccines made from the outer membrane proteins (OMPs) against economically important poultry pathogens such as *Pseudomonas* and *Salmonella* have been studied in the past with considerable success against both homologous and heterologous serotypes (9, 10). In general, in *E. coli*, 0.2%-0.5% of the outer membrane and periplasm proteins are packaged into OMVs (Outer membrane vesicles). Over recent years, the atomic structures of several outer membrane proteins, belonging to six families, have been determined. They include the OmpA membrane domain, the OmpX protein, phospholipase A, general porins (OmpF, PhoE), substrate-specific porins (LamB, ScrY) and the TonB-dependent iron siderophore transporters FhuA and FepA (11). Outer membrane proteins (OMPs), expressed on the surface of Gram-negative bacteria, are quickly recognized as extracellular foreign particles by the host immune system, thereby generating an immune response against bacterial pathogens (12). Several attempts have been made in the past to evaluate the OMP immunogenicity of Gram-negative bacteria (13, 14). Present study aimed to evaluate and compare the protective effect of outer membrane proteins and whole cell antigen in broiler chicken challenged with *E. coli* infection. While traditional vaccines often fail to provide broad and lasting immunity due to the antigenic variability of APEC and limitations in safety and efficacy, this study demonstrated that OMPs, extracted via the sarcosyl method and formulated with Montanide adjuvant, elicit a significantly stronger and more sustained immune response. With a higher protection rate (91%) and antibody titer compared to the WCAg vaccine (82%), the OMP-based formulation showed promise as a safer and more immunogenic alternative. This research highlights the potential of OMPs as defined, non-toxic components capable of stimulating protective immunity without the drawbacks of live or whole-cell vaccines, and thus contributes valuable insights towards the design of next-generation vaccines for controlling colibacillosis in poultry.

MATERIALS AND METHODS

Bacterial strains. Microbial Type Culture Collection and Gene Bank (MTCC) culture of serogroup O2 of APEC that had already been maintained in Division of Veterinary Pathology, SKUAST- Kashmir after confirmation by National Salmonella and Escherichia Centre (NSEC), Kasauli were used for the study.

Extraction and purification of outer membrane protein from bacterial culture. The pure culture of O2 serogroup of *E. coli* was inoculated on selective

enriched media. This isolate was further confirmed on the basis of morphology, cultural characteristics and biochemical testing.

Preparation of sonicated extract (SE). For harvesting sonicated extracts (SE) of *E. coli*, the already described method was employed (15). Briefly the bacterial culture was harvested by spinning at 8000 RPM for 10 minutes at 4ºC. After three washings with PBS (pH 7.4), the cells were finally suspended in 10mM HEPES buffer, pH 7.4 (Sigma, USA), placed on ice and then subjected to 30 cycles of sonification wherein each cycle was of 30s with 30 % amplitude of sonication (QSONICA Sonicator, Model: Q150, USA), with 30s interval for cooling between each cycle. SE was collected by removing intact cells, which are the cell debris by centrifugation at 10000 RPM for 20 min at 4ºC. The supernatant was stored at -20ºC till further use.

Preparation and analysis of OMPs. The outer membrane proteins were harvested from sonicated extracts (SE) by centrifugation at 1,00,000×g for 60 minutes at 4ºC. The clear gel like pellet was resuspended in 2% w/v sodium lauryl sarcosinate detergent (Pharmacia, UK) in 10mM HEPES buffer and left for 2 hrs at 4ºC. The detergent insoluble fraction was harvested by centrifugation again at 1,00,000×g for 60 minutes at 4ºC and the pellet was washed thrice in distilled water. The OMP pellet was finally dissolved in 200 µl of PBS and stored at -80ºC until further use (15). The protein concentration of the preparation was estimated using a nanodrop spectrophotometer.

The analysis of OMPs was done by SDS-PAGE (4.8% concentrated gel and 10% separation gel). The 10 µl OMP was added in the 10 µl 2 × Tris-HCl buffer and heated in boiling water for 5 min and then cooled to room temperature. The low molecular weight standard proteins and above OMP samples were infused to the sample tanks by a mircosyringe. Then electrophoresis was conducted with 20 mA constant current and stopped until the indicator reached a distance of 1 cm from the silicone rubber frame bottom. After electrophoresis, the gel was immersed in 0.25% coomassie brilliant blue stain for 1 h. Then the staining fluid was discarded and the gel was rinsed thoroughly with distilled water for several times. Finally, the gel was kept for bleaching in bleaching solution with shaking until the protein bands were visualized clearly (16).

Before immunization, frozen samples of the extracted OMPs were thawed and mixed with sterile phosphate buffer saline (PBS) to form an emulsion with a concentration of 50µg/0.5ml (1 Dose) (17). Then Montanide ISA 206 VG (Seppic) was mixed at a ratio (1:1) as an adjuvant (18).

The OMP preparation was tested for its sterility as per the standard microbiological procedure (19). The sterility of the vaccine was checked by inoculating 1 ml of vaccine into 50 ml of nutrient broth and incubating at 37ºC for 2-3 days.

Preparation of *E. coli* whole cell vaccine. *E. coli* serotype O2 was grown in nutrient broth on a shaking incubator at 37ºC for 20 hours at 150 rpm till the culture was saturated. The bacterial growth was harvested by pelleting at 12000 rpm for 15 min and the pellet was washed twice in NSS. The bacterial suspension was adjusted to contain 3×10^9 cells/ml (20) and was inactivated by adding 0.5% formalin with agitation and incubated at 37ºC for 24 hours. Then Montanide ISA 206 VG (Seppic) was mixed at a ratio (1:1) as an adjuvant (18).

Determination of *E. coli* challenge dose for chickens. For determination of challenge dose of *E. coli*, the standard method of McFarland (20) was used. *E. coli* serotype O2 was grown in nutrient broth in an incubator at 37ºC for 48 hours. The bacterial growth was harvested by pelleting at 8000 rpm for 20 minutes and the pellet was washed twice in NSS. The bacterial suspension was adjusted to 1×10^8 bacterial cells in 0.5 ml (20). Then the birds were injected I/P with 0.5 ml suspension of *E. coli* (Serotype O2) containing 1×10^8 bacterial cells (21).

A total of 120 (day old) broiler chickens were procured commercially. All birds were reared under standard conditions of management in experimental animal house at FVSc and AH, Shuhama, Srinagar. Prior to the arrival of day old chicks, the experimental rooms, utensils etc. were thoroughly cleaned, sterilized with 2.5% phenol and 2% formaldehyde solution. Birds were maintained on pre-starter ration for the first 12 days, starter feed up to the 25th day and later, and on finisher ration for the rest of the time. Birds were given access to antibiotic free feed and water *ad libitum*.

To ensure that the chicks were free from *E. coli* infection, fecal swabs were taken from the chicks, and examined bacteriologically for detecting carri-

er state. The swabs were inoculated in freshly prepared nutrient broth, Mac Conkey's agar and EMB and the growth was examined after 24-36 hours of incubation at 37ºC. Chicks found *E. coli* negative after performing culture and biochemical studies were used for further studies. Birds were divided into 6 groups with each consisting of 20 birds. The birds were immunized and challenged as shown in Table 1.

Sample collection. Three Birds from each group were sacrificed on days 4, 13, 19, 27 and 35. Samples (blood, liver, heart, lung and spleen) were collected. Blood samples were collected from chickens of all the experimental groups including the unimmunized control group at different time intervals in sterile 1.5 ml Eppendorf tubes. Blood was allowed to clot at room temperature for 2 hours. After breaking the clot with a pasture pipette, sera were collected following centrifugation at 4000 rpm for 10 min to allow erythrocytes to settle down and the tubes were kept in refrigerator overnight for further recovery of the sera. In the whole process, caution was taken to avoid hemolysis and later on, sera samples were stored at -20ºC for further use.

Patho- anatomical studies. The carcasses of dead and sacrificed birds were subjected to a thorough and systematic necropsy for examining and recording of the lesions characteristic of colibacillosis which included perihepatitis, pericarditis, omphalitis, tenosynovitis, airsacculitis and cellulitis. Representative samples of liver, heart, lung and spleen from all the groups of chickens were collected in 10% buffered formalin for comparative histopathological examination and processed by routine paraffin embedding technique.

Re-isolation. The isolation and identification of *E. coli* was carried as per the standard microbiological procedure. Representative samples from (heart, spleen, lung, liver and intestine) were inoculated in nutrient broth and incubated at 37ºC for 24 hours following re-inoculation on MacConkey agar and again incubated at 37ºC for 24 hours. The lactose fermenting colonies were re-inoculated on Eosin Methylene Blue agar and colonies producing metallic sheen were transferred to nutrient agar slants and incubated at 37ºC for 24 hours and stored at 4°C for further identification. Identification of isolates as *E. coli* was carried out using standard morphological and biochemical tests as stated earlier (19). The *E. coli* isolates were characterized by standard morphological and biochemical tests were sent to National Salmonella and Escherichia Centre, Central Research Institute, Kasauli—173204 (H.P), for Serogrouping.

ELISA. Sera samples collected from different experimental groups were subjected to ELISA for detecting antibodies against OMP and whole cell antigen. Indirect ELISA was performed to detect the antibody titer (IgG) in serum as per the standard method (22) with slight modifications.

Antigen preparation. *E. coli* colonies from McConkey agar plates were inoculated in 5ml nutrient broth and incubated overnight at 37ºC. The overnight nutrient broth was subcultured in nutrient broth at a dilution of 1:100 and incubated overnight in shaking incubator at 37ºC. The broth culture of the organism was dispensed in fresh 50 ml tubes, centrifuged at 7500 rpm and supernatant was discarded. The pellet was washed in10ml sterile phosphate buffer saline (PBS) and centrifuged at 7500 rpm twice. The pellet was sonicated (QSONICA Sonicator, Model: Q150,

Table 1. Vaccination and challenge schedule for broiler chickens in different experimental groups

Group	Day 5	Day 14	Day 20
1	Distilled Water	Distilled Water	Distilled Water
2	OMP	OMP	-
3	Whole Cell Antigen	Whole Cell Antigen	-
4	-	-	Infection/Challenge
5	OMP	OMP	Infection/Challenge
6	Whole Cell Antigen	Whole Cell Antigen	Whole Cell Antigen

*Dosage: - OMP (50µg) 0.5ml s/c; Infection 0.2ml I/p (1×10^8 bacterial cells); Distilled water 0.5ml s/c; whole cell vaccine 0.5 ml s/c (3×10^9 cells/ml).

USA) in sterile PBS buffer for one minute followed by cooling for 30 seconds (15 times). A protease inhibitor (PMSF 1mM) was added during sonication. Centrifugation was done at 13000 rpm and supernatant was transferred to fresh tubes. The concentration of supernatant was checked. 20 ng of antigen was coated per well.

100 µl (1:200 in coating buffer) of sonicated *E. coli* and OMP as antigen (20 ng/100 µl) was added to each well of a flat bottomed 96 well plate (Nunc/Tarson) and incubated overnight at 4°C. The plates were washed thrice with PBS Tween-20 (0.05%) and then incubated with 200 µl of 5% blocking buffer (Skimmed milk in PBS-T) at 37°C for 2 hours. The plates were again washed thrice with PBS Tween-20 (0.05%). Sera samples (1:200) diluted in PBS-T were added to each well and plate was incubated at 37°C for 2 hours followed by three times washing with PBS Tween-20 (0.05%). 100 µl of goat anti-chicken IgG alkaline phosphate (*Sigma* 1:15000) diluted in PBS-T was added to each well and plate was incubated at 37°C for 2 hours. Plates were washed with PBS Tween-20 (0.05%) and incubated with 100 µl o-Phenylenediamine dihydrochloride (OPD) in the dark at room temperature for 15 minutes. The reaction was stopped by adding 50 µl of 2N H_2SO_4. Absorbance was determined using multimode ELISA reader (CYTATION 3, Biotech) at 492 nm.

Statistical analysis. Data pertaining to antibody titer was analyzed through two factorial CRD experiment. One of the factor being age group in weeks at five levels and another factor representing the group (whether vaccinated or unvaccinated broiler chicken) at six levels.

RESULTS

Cultural and morphological characterization. MTCC culture of serogroup O2 of APEC appeared pink on MacConkey agar and greenish with metallic sheen on Eosin Methylene Blue agar. Gram staining revealed short pink rods arranged singly or in pairs. The pure cultures from MacConkey agar plates were characterized biochemically and gave the positive results typical for *E. coli.*

Extraction, purification and quantification of APEC (serogroup O2) outer membrane protein (OMP). OMPs were extracted from serogroup O2 of *E. coli* by sarcosyl method and upon quantification, the protein concentration as determined by nanodrop spectrophotometer was found to be 19.88 mg/ml. The purified OMPs were analyzed by SDS-PAGE which revealed a clear thick intense polypeptide band (Fig. 1) after Coomassie brilliant blue staining. The approximate molecular weight of outer membrane proteins extracted from serogroup O2 of *E. coli* was found to be between 40-50 kDa.

Immunization and challenge study. The inactivated whole cell antigen (vaccine) and outer membrane protein vaccine prepared from serogroup O2 of *E. coli* were found sterile after 48 hrs of incubation at 37°C on MacConkey/ and or EMB and Sabrouds Dextrose agar. Safety testing of the preparations in experimental chicks revealed no untoward clinical symptoms or mortality on subcutaneous injection of both the vaccines. The birds were observed for a week. The results of immunization and challenge of chickens with homologous serogroup O2 of *E. coli* have been summarized in Table 2. The protection rate of whole cell inactivated antigen (vaccine) was 82%, while as that of OMP vaccine was 91%. Meanwhile, the protection efficacy was 27% among the unvaccinated chicken group.

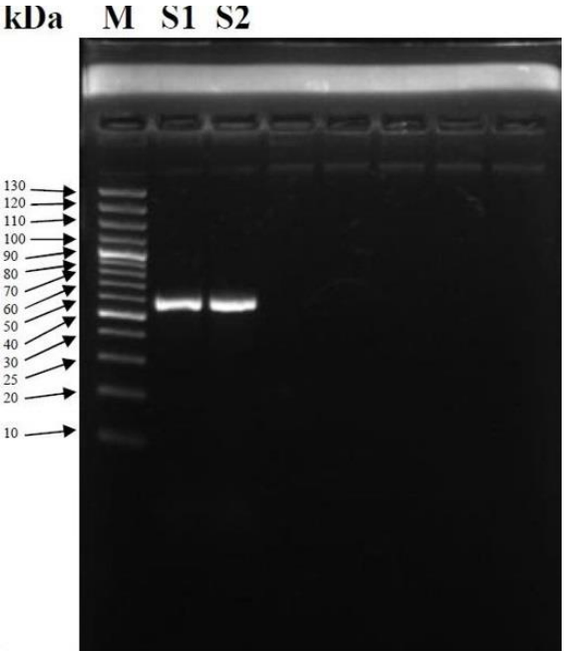

Fig. 1. SDS-PAGE analysis of purified OMP of serogroup O2 of *Escherichia coli.*
Lane M: Protein MW marker Lane S1 and S2: Purified OMP

Immune response studies. The immunogenicity of the OMP vaccine prepared from serotype O2 of APEC was evaluated by monitoring immune responses at regular intervals. The OMP vaccine inoculated chickens did not show any untoward reaction or mortality and the birds remained active throughout the post vaccination period. The collected sera samples were analyzed for antibody IgG development against *E. coli* infection by employing an Indirect ELISA and the results are presented in Table 3.

It is evident from Table 3 that while comparing the antibody titers among all the groups throughout the sampling week intervals and until the termination of experiment, the antibody IgG titer was found to be at a significantly ($p<0.05$) higher level in group 5 (OMP + challenge) and reached a peak of 9.385 at 4th week post vaccination (PV). In group 6 (WCAg+ C) chicks vaccinated with whole cell antigen, the antibody IgG titer reached a peak of 8.7 at 4th week PV which was significantly ($p<0.05$) lower than that of group vaccinated with OMP vaccine. In chicks belonging to group 2 and 5 which were inoculated with OMP, the antibody IgG titer was significantly higher when compared to other groups and the titer was maintained at an elevated level till the termination of experiment. In group 3 and 6 wherein chicks were vaccinated with whole cell antigen (vaccine), the antibody IgG titer at 2 week PV was found to be 7.2, which was significantly ($p<0.05$) lower as compared to the corresponding values of titer in chicks belonging to group 2 and group 5, however, from third week PV the antibody titers were raised to safe protective levels of 7.8 and 8.7 respectively in these groups till the termination of experiment. The antibody IgG titer in unvaccinated groups i.e; group 1 (control group) and group 4 (Challenge group) chicks, failed to reach to the protective level till termination of the experiment. The comparison of the antibody IgG response among different week revealed to be the highest at 4th week PV (Fig. 2), while comparing the IgG titer among the various groups, it was recorded that the antibody response was the highest in group 5 broiler chickens throughout the experiment (Fig. 3).

Clinical signs. Most of the experimental birds other than those belonging to group 4 (unvaccinated challenge group) did not exhibit any significant clinical signs. The most striking clinical signs (inappetence, ruffled feathers, inability to move, sitting on their hocks and huddling around the heat source) were observed in birds belonging to group 4 (unvaccinated challenge group). In addition, diarrhea, severe lameness and respiratory signs (snicking, labored breathing, gasping and respiratory rales) were recorded in few of these affected birds. In some birds, heads were

Table 2. Challenge test among broiler chickens immunized with inactivated whole cell antigen and OMPs of *E. coli.*

Groups	Number of challenged birds	Number of dead birds / Total No.	Protection rate
OMP vaccine	11	1/11	91%
Inactivated *E. coli* Whole cell antigen	11	2/11	82%
Unvaccinated group	11	8/11	27%

Table 3. ELISA antibody IgG response of broiler chickens immunized with inactivated whole cell antigen and OMPs of *E. coli.*

Groups Weeks	Group 1 (control)	Group 2 (OMP)	Group 3 (WCAg)	Group 4 (CG)	Group 5 (OMP + C)	Group 6 (WCAg+ C)	Mean
Pre- vaccination	5.366^{p}	5.351^{p}	5.350^{p}	5.371^{p}	5.367^{p}	5.370^{p}	5.363^{e}
1 week PV	5.650^{o}	6.945^{i}	6.781^{j}	5.655^{o}	6.952^{i}	6.791^{j}	6.462^{d}
2 week PV	5.932^{n}	7.408^{g}	7.274^{h}	5.943^{n}	7.411^{g}	7.281^{h}	6.875^{c}
3 week PV	6.241^{l}	7.916^{e}	7.453^{g}	6.508^{k}	8.520^{c}	7.801^{f}	7.407^{b}
4 week PV	6.170^{m}	8.685^{b}	8.291^{d}	6.825^{j}	9.385^{a}	8.736^{b}	8.015^{a}
Mean	5.872^{f}	7.261^{b}	7.030^{d}	6.060^{e}	7.527^{a}	7.196^{c}	
CD ($p<0.05$)	Weeks (W) = 0.028		Groups (G) = 0.030		Weeks × Groups (W*G) = 0.068		

*Means with different superscripts differ significantly ($p<0.05$)

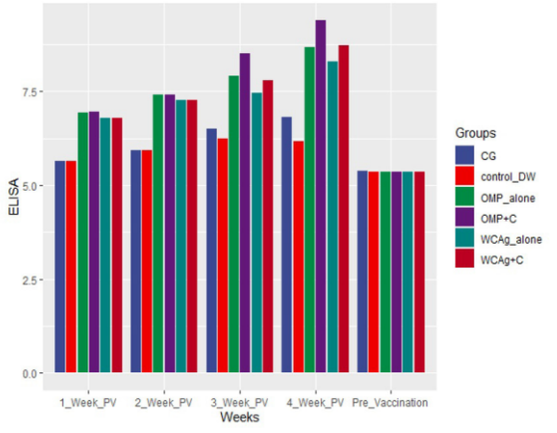

Fig. 2. Comparison of antibody (IgG) response of broiler chickens immunized with
inactivated whole cell antigen and OMP's of *Escherichia coli* throughout the weeks.

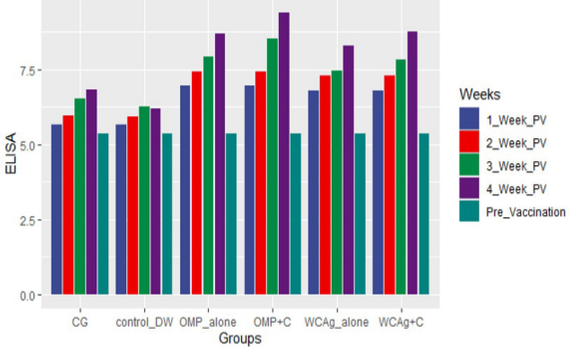

Fig. 3. Comparison of antibody (IgG) response of broiler chickens immunized with inactivated whole cell antigen and OMP's of *E. coli* all over the groups.

swollen due to swelling of infraorbital sinuses and conjunctivitis.

Gross pathology. The birds belonging to the experimental group revealed lesions typical of colibacillosis which varied in intensity among vaccinated and unvaccinated groups. No significant gross pathological lesions were observed in the birds belonging to groups 1, 2 and 3 till the end of the experiment. Necropsy of birds from group 4 (unvaccinated challenge) before challenge revealed no significant gross changes. However, the organs like liver, lung, spleen, intestine and heart of dead birds after challenge revealed lesions typical of colibacillosis. Grossly, the liver was mostly congested with areas of focal necrosis, covered by a thin to thick layer of fibrin adherent to hepatic capsule (Fig. 4A). Heart was congested, covered by a layer of

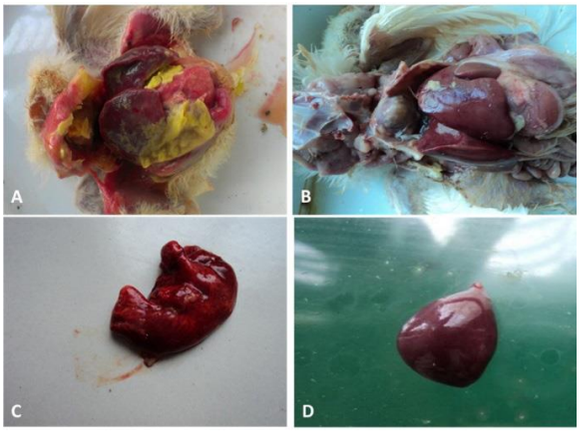

Fig. 4. Gross pathological lesion in unvaccinated challenge group. (A) Thick fibrin layer on liver. (B) Heart showing thickening of pericardium and adhesions within chest cavity. (C) Congested, edematous and consolidated lung. (D) Congested and enlarged spleen.

thin to thick fibrin adherent to the heart giving a characteristic "bread and butter" appearance (Fig. 4B). The lesions in lungs of challenged birds varied from mild congestion, oedema to consolidation (Fig. 4C). Grossly spleen was slightly enlarged and congested with some cases revealing presence of necrotic foci speckled on its surface (Fig. 4D).

The postmortem examination of sacrificed birds belonging to group 5 before challenge revealed no gross pathological changes. However, 6 days post challenge, one bird was found dead and postmortem examination revealed changes in air sacs and pericardium. From group 6, no gross pathological changes were observed in any organ in birds sacrificed before the challenge. After four days of challenge infection, two birds were found dead and the gross pathological changes were observed in liver and heart and were similar to those observed in group 5 but were more severe in intensity. However, necropsy of birds sacrificed two weeks post challenge, revealed mild pericarditis.

Histopathology. No significant microscopic changes were observed in any organ of the birds during the entire course of the experiment in groups 1, 2 and 3. Liver parenchyma showed well organized lobules consisting of sinusoids and hepatocytes radially organized around the central vein (Fig. 5a). The cardiac muscle showed normal histomorphology with characteristic branching pattern and cross striations (Fig. 5b). The structures like bronchiole and parabronchi were well appreciated in the histological sections of

lungs. The presence of numerous well developed cup shaped atria in the parabronchi was observed in the lung sections of sacrificed birds (Fig. 5c). Spleen also appeared normal with the presence of well differentiated intermixed red and white pulp. The central artery and its associated structures like PALS (Peri arterial lymphatic sheath) and peninsular artery were appreciated in the histological sections of spleen. Lymphoid follicles were well developed and no lymphoid cell depletion or cellular infiltration was observed in splenic parenchyma (Fig. 5d).

From the unvaccinated challenge group (group 4), the organs of sacrificed birds collected before challenge revealed no histopathological changes. After challenge infection, liver, heart, lungs and spleen of dead birds revealed histopathological lesions typical of colibacillosis. Hepatic parenchyma showed degenerative changes in the form of cellular swelling along with congestion of blood vessels, individualization of hepatocytes and distortion of hepatic cords, fatty degeneration along with heterophilic infiltration, necrosis and oval cell hyperplasia and invariably thickened liver capsule (Fig. 5e). Heart revealed thickening of pericardium with fibrinous exudate along with mononuclear cell infiltration, congestion and hemorrhages in myocardium and disruption of myocardial fibres with minimal leucocytic infiltration consisting of both heterophils and mononuclear cells (Fig. 5f). The histopathological lesions in lungs were characterized by congestion of interlobular septa, hemorrhages in the para bronchi and mild infiltration of heterophils and mononuclear cells in the lumen of primary bronchioles and tertiary bronchioles (Fig. 5g). Histopathological examination of spleen revealed mild to severe congestion of blood vessels and hemorrhages in the splenic parenchyma, infiltration of heterophils and depletion of lymphoid elements together with multiple focal areas of necrosis (Fig. 5h). From the OMP vaccinated challenge group (Group 5), microscopic examination of organs collected from sacrificed birds before challenge, did not reveal any significant pathological changes. Mild lesions in liver (Fig. 5i), heart (Fig. 5j), lungs (Fig. 5k), and spleen (Fig. 5l), were noticed in some of the birds sacrificed after the chal-

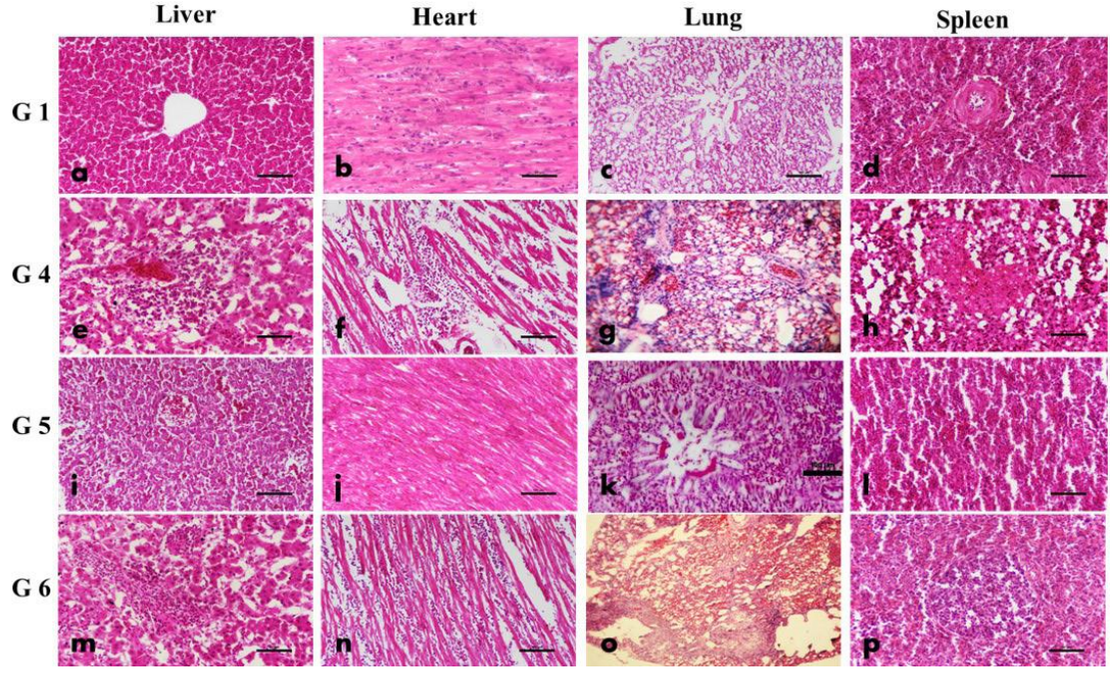

Fig. 5. Histopathological analysis of liver, heart, lung and spleen: G1: Normal histological features of liver (a), heart (b), lung (c) and spleen (d) observed in group 1 (control group). G4: (e) Liver from group 4 (unvaccinated challenge) showing congestion, individualization of hepatocytes along with distortion of hepatic cords. (f) Heart showing severe muscle degeneration along with cellular infiltration in myocardium. (g) Lung revealing vascular congestion. (h) Spleen showing severe degeneration of splenic parenchyma. G5: Near normal histology with mild changes in liver (i), heart (j), lung (k) and spleen (l) from birds sacrificed of group 5 (OMP vaccinated challenge group). G6: Moderate lesions observed in liver (m), heart (n), lung (o) and spleen (p) from birds sacrificed of group 6 (whole cell vaccinated challenge group).

lenge. Similarly, from whole cell antigen vaccinated group (Group 6), mild to moderate microscopic lesions were noticed in these organs (Fig. 5m, n, o and p) sacrificed after the challenge infection.

Re-isolation and identification. For confirmation of the induced infection, samples including liver, lung, heart and spleen collected from the infected broiler birds were analyzed for *E. coli.* Pink colonies on MacConkey agar and greenish colonies with metallic sheen on Eosin Methylene Blue agar after an overnight incubation were confirmed as *E. coli*. Microscopically, the organisms were Gram-negative, pink short rods, arranged singly or in pairs. All the *E. coli* isolates were positive for Indole and Methyl Red test and were negative for Voges- Proskauer and Citrate Utilization test. Serogroup confirmation of the bacterial isolates from experimentally infected chicken confirmed the isolates belonging to serogroup O2.

DISCUSSION

Colibacillosis causes significant economic losses in the poultry industry globally. Currently, there is no fully reliable method to control *E. coli* infections in commercial poultry flocks. Therefore, this study aimed to develop an effective OMP based vaccine from the pathogenic *E. coli* serogroup O2 to combat colibacillosis in chickens. O2, O78, O19, and O20 are the most common *E. coli* serogroups isolated from poultry in India (23, 24). Outer membrane protein based vaccines have advantages over live attenuated vaccines, including high immunogenicity, enhanced safety, and improved intrinsic adjuvant effects. As a result, they represent a promising new option for combating bacterial infections (25). In this study, OMPs were extracted from serogroup O2 of *E. coli* by sarcosyl method for the construction of potent vaccine in order to protect the broiler chickens against colibacillosis. For quantification, nanodrop spectrophotometer was used to determine the protein concentration which was found to be around 19.88 mg/ml. The use of OMPs against colibacillosis infection in this study corroborates with the research findings of earlier workers (17, 21). The OMPs form a continuous structure on the surface of Gram-negative bacteria and have particular significance as a potential target for protective immunity. OMP vaccines have been used with considerable success to induce protection against a number of organisms (26). The approximate molecular weight of OMPs extracted from serogroup O2 of *E. coli* was found to be around 40-50 kDa. These results were in agreement with the findings of earlier workers (27). Numerous studies in the past have also extracted and analyzed OMP's of *E. coli* from birds and humans by SDS-PAGE and found that polypeptide band with 44 kDa is the major OMP band accounting for 70% of the total OMPs (28, 29). In the present study, no clinical symptoms were observed in birds after vaccination with inactivated whole cell antigen (vaccine) and *E. coli* OMP vaccine and the vaccines were free from fungal and bacterial contamination. The broiler chickens after immunization were challenged with homologous serogroup of *E. coli* from which the vaccines were prepared, and the results obtained clearly depict that protection was conferred by these two vaccines used in this study. The protection rate of whole cell inactivated antigen (vaccine) was found to be around 82% while the protection rate of OMP vaccine was 91%. Meanwhile, the protection rate was only around 27% among the unvaccinated group of broiler chickens. The results of this study were found to be in concordance with the findings of earlier authors (21) who reported protection rate of 84% with inactivated vaccine, 80% in case of Nobilis® *E. coli* inactivated vaccine and the protection rate of 92% in OMP vaccine, with only 28% protection rate among the unvaccinated chicken group. Comparing the antibody titers among all the groups throughout the weeks and until the termination of experiment showed significantly higher rates in group 5 chicks (OMP+Challenge) and a peak of 9.3 at 4 week PV. The results of this study also corresponded with the previous findings (28) who reported higher antibody titer levels following the booster dose of OMP in chickens, and this titer thereafter remained constant following further boosters. They also reported that the purified *E. coli* OMP can induce significant protection immunity against colibacillosis in chickens. Antibody producing ability of OMP vaccine preparations has also been well documented by the previous workers (30).

Gross pathological lesions were severe in liver of affected birds which included congestion and areas of focal necrosis, but in some birds liver was covered by a thick layer of fibrin. The results are in concurrence with the earlier reports (31, 32) who reported deposition of fibrinous exudate on liver surface besides other changes in birds affected with colibacillo-

sis. Grossly heart was covered by a layer of fibrinous tissue in birds from unvaccinated challenge group. Similar type of lesions have been described earlier (33-36). On histopathological examination, the organs of sacrificed birds collected before the challenge in all the groups did not reveal any pathological changes. However, after the challenge, organs including liver, heart, lungs and spleen collected from dead birds, and few of the sacrificed birds from three infected groups revealed pathological lesions typical of colibacillosis in microscopic examination. Similar kind of microscopic lesions have been reported earlier (31, 36-38).

The findings of this study underscore the superior protective efficacy and immunogenic potential of the outer membrane protein (OMP) vaccine over the traditional inactivated whole cell antigen vaccine against *Escherichia coli* O2 infection in broiler chickens. The significantly higher antibody response and protection rate observed in the OMP-vaccinated group affirm its capability to induce robust and lasting immunity with minimal pathological lesions post-challenge. This establishes OMP-based vaccination as a promising and safer alternative to conventional vaccines, especially in light of increasing antimicrobial resistance and the need for targeted, non-toxic immune prophylactic strategies in poultry health management. The study thus provides a strong foundation for further development and potential commercialization of OMP vaccines for effective control of colibacillosis in poultry.

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