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Pathological Investigation and Molecular Detection of Avian Pathogenic *E. coli* Serogroups in Broiler Birds

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Abstract

The objective of the present study the *rfb* gene clusters in avian pathogenic *E. coli* cardinal serotypes O1, O2 and O78 strains and to develop a multiplex polymerase chain reaction method for serotyping of the O-antigens. The multiplex polymerase chain reaction method was used for the identification of serotypes of APEC. The second part of the study was to study the pathological lesions caused by most prevalent sero group in experimentally infected broiler chicks. A total of 100 tissue samples (50 lungs and livers each) were collected from colibacillosis suspected broiler birds and subjected to isolation and identification of *E. coli* by conventional methods. Multiplex PCR was used for confirmation of three serogroups i.e., O1, O2 and O78. We found more O2 33% than O1 8% and O78 zero percent. These results suggested the prevalence of O2 sero group in our study. The prevalent sero group (O2) was experimentally inoculated in broiler birds at day 7 of their age. Lungs and liver samples from these experimentally infected birds were taken at days 14 and 21 of their age and subjected to histopathology. We found that there was hepatomegaly, coagulative necrosis, congestion and infiltration of inflammatory cells in infected livers. The lungs were congested and there were macrophages, lymphocytes and heterophils too. There was mostly hepatic form of colibacillosis with this infective strain.

Keywords: Colibacillosis; *E. coli* sero groups; Pathogenic *E. coli*; Multiplex PCR; Histopathology; Broiler birds

Introduction

Escherichia coli is a gram negative bacterium, uniform staining, non-acid fast, non-spore forming bacillus usually 2-3 × 10^6 µm. Most of *E. coli* is non-pathogenic but some strains which can establish themselves outside of the intestine they lead to disease. *E. coli* serotypes which cause systemic diseases in birds are called avian pathogenic *E. coli* (APEC).

APEC is the causative agent of colibacillosis, distinguished by multiple organ lesions like pericarditis, airsacculitis, peritonitis, perihepatitis, salpingitis, osteomyelitis, synovitis, or yolk sac infection. One of the principal causes of morbidity and mortality in poultry worldwide is colibacillosis. Infection of the respiratory tract causes high economic losses followed by septicemia [1]. *Escherichia coli* are the normal intestinal inhabitant in poultry. It is opportunistic bacteria which attack when the immunity of the bird is compromised. It is not only pathogenic to avian species but recent studies have revealed that it has the zoonotic potential for human beings too as recent studies show the possibility of avian pathogenic.

Escherichia coli being incriminated in extra intestinal diseases in humans as well [2]. The present study was conducted to study the *rfb* gene clusters in avian pathogenic *E. coli* cardinal serotypes O1, O2 and O78 strains and to develop a multiplex polymerase chain reaction method for serotyping of the O-antigens. The multiplex polymerase chain reaction method was used for the identification of serotypes of APEC. The second part of the study was to study the pathological lesions caused by most prevalent serogroup in experimentally infected broiler chicks [3,4].

Materials and Methods

Isolation and identification

A total of 100 tissue samples (50 lungs and 50 livers) were collected from colibacillosis suspected broiler birds. Tissue samples were used for streaking on different growth media. MacConkey agar was used as primary culture media. Swabs from Colibacillosis suspected lungs and liver were taken and swabbing was performed on MacConkey agar. Colonial morphology and pink color colonies were observed. A single colony from positive MacConkey plates was taken and streaked on to EMB agar. This medium is selective for *E. coli*. Green metallic sheen was observed on EMB agar. Congo red media was used for differentiation between pathogenic and non-pathogenic bacteria. Pink colored colonies were considered as pathogenic [5].

DNA extraction

A Gene-Jet Genomic DNA Purification Kit (Thermo Fischer scientific catalog No. K0722-250) was used for the extraction of DNA from tissues samples. Purity and concentration of DNA was tested by using Nano Drop spectrophotometer (ND-2000 UV-Vis Nano Drop Technologies Wilmington, DE). For the present study, reported primers were used (Tables 1 and 2; Chart 1).

Amplification of bacterial nucleic acid

Amplification of *E. coli* DNA in the sample of broiler birds was conducted and then amplicons were confirmed with the help of agarose gel electrophoresis. It is a process in which various things of different charges and molecular weight are divided by an electric field. These substances undergo traveling various distances through agarose gels.

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Primers	Sequences (5' to 3')	Product size
ECO-F	5'CGATGTTGAGCGCAAGGTTG 3'	
ECO1-R	5'CATTAGGTGTCTCTGGCACG 3'	263 bp
ECO2-R	5'GATAAGGAATGCACATCGCC 3'	355 bp
ECO78-R	5'TAGGTATTCCTGTTGCGGAG 3'	623 bp

Table 1: Primer sequence and their details.

Stages	PCR Conditions	Cycles
Initial Denaturation	95°C for 5 min	1
Denaturation	95°C for 2 min	
Annealing	57°C for 30 seconds	30
Extension	72°C for 40 seconds	
Final Extension	72°C for 10 min	1

Table 2: Thermocycler Conditions for PCR.

Reagents	Volume (µL)			
DNA (50 ng/uL)	1.0			
PCR master mix (Fermentas, USA)	12.5			
Water	7.5			
Primers	ECO-F	ECO1-R	ECO2-R	ECO78-R
	1	1	1	1

Chart 1: PCR reaction mixture composition.

1.2% gel was prepared to check the PCR product by boiling 1.2 g of agarose powder in 100 mL of TAE Buffer (Tris, Glacial Acetic Acid and EDTA) in the microwave till the agarose was completely dissolved.

Then 5 µL of Ethidium bromide (Cons.10 mg/mL) was added into the gel solution. Ethidium bromide results in the staining of DNA by allowing the line to be look at under ultra-violet (UV) light. Gel was poured in a dual comb caster and stay for half-an-hour [6-8]. Then the gel was put in the electrophoresis tank and PCR products were suffused with it. Before loading the PCR products to the wells of the gel plate, tracking dye Bromophenol blue was added so that the distance traveled on the agarose plate could be seen more easily. For each row of wells, 4 µL PCR products with 3 µL loading dye were loaded. 100 volts charge was passed through the gel for 30 minutes. This resulted in the travelling of negatively charged DNA towards the positively charge electrode. The gel was then observed under UV light in Gel documentation system (Bio-Rad) and photographed.

Inoculum preparation

For Colony Forming Unit count (CFU), the organisms were grown in nutrient broth with yeast extract for overnight. Then 10 fold dilutions were made and 0.5 ml of each dilution will be transferred to the nutrient agar aseptically. The diluted samples were spread on the petri plate with sterile L-shaped glass spreader. The plates were then incubated at 37°C for 24-48 hours. Only those plates displaying 30-300 colonies were counted following incubation. The number of bacteria per ml of original sample was obtained by multiplying the diluting factor with the number of colonies. The results of CFU were expressed as number of organisms per ml of sample. Each bird was injected with 1 ml suspension of *E. coli* (4.5×10^7).

Experimental infection and pathological investigation

After confirmation of avian pathogenic *E. coli* by PCR, 2 groups (20 chicks in each group) of broiler chicks were made. One group was infected with most prevalent serogroup of avian pathogenic *E. coli* via intra-tracheal route and second group was the control group which was not infected. Birds were inspected for any gross pathological lesions. After that postmortem study was performed on each group and difference of pathological lesions were noted. Histopathology of the organs affected was done by following schedule (Table 3) [3,9-12].

Histopathology

Tissue samples were collected for histopathological examinations. These were processed with standard techniques for fixation, dehydration, clearing, embedding, sectioning and staining.

Statistical design

Statistical analysis was conducted with the Statistical Package for Social Science, (SPSS for Windows version 20, SPSS Inc., Chicago, IL, USA). The data was analyzed by statistical analysis using chi square. $P<0.05$ was considered as level of significance [13,14].

Results

Results of isolation and identification

A total of 100 tissue samples (Lungs and Livers) were collected and streaked on different growth media. First of all, samples were streaked directly on MacConkey agar which was used as primary growth media. A total of 80 samples were found positive for *E. coli* on MacConkey agar out of 100 samples (80%). These were further streaked on EMB agar (Eosin Methylene Blue) out of which 60 petri plates (75%) were found positive which gave confirmatory metallic green sheen [15]. Congo red media was used for differentiation between pathogenic and non-pathogenic *E. coli*. Twenty four petri plates out of 60 EMB growth plates were found Pathogenic which were observed as pink colonies on Congo red media (40%) (Figure 1).

Results of PCR

DNA from 24 Congo red positive colonies was extracted and was confirmed by gel electrophoresis of 1.2% gel. Polaroid photo of the gel was taken by using Gel documentation system was used for recording the band of obtained. Twenty four samples were found pathogenic on the basis of pink colonies on Congo red media. These samples were further processed for confirmation of three serogroups of *E. coli* i.e., O1, O78 and O2 by PCR. Out of twenty four pathogenic isolates, 8 isolates were found to be of O2 serogroup on the basis of PCR. So out of total 100 tissue samples 8 samples were found pathogenic (8%). Two DNA samples were found to be of O1 serogroup on the basis of 263 bp bands on gel electrophoresis (2%) [16,17]. So, in our present study O2 serogroup was found in most of the extracted DNA samples i.e., 33% of 24 pathogenic isolates. O1 serogroup was only found to be 8% i.e., 2 samples out of 24 samples. No DNA sample of O78 serogroup was found in our study samples (Figure 2).

Results of histopathology

It was noted that there was mononuclear cells infiltration and thin

Days of Trial	Group 1	Group 2
Day 7	Infected by intra-tracheal route	Not infected
Day 14	Lungs and liver collection	Lungs and liver collection
Day 21	Lungs and liver collection	Lungs and liver collection

Table 3: Experimental Design.

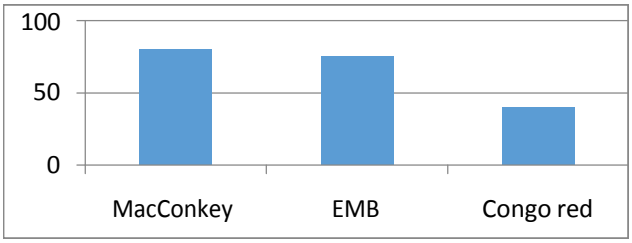


Figure 1: Results of isolation and identification.

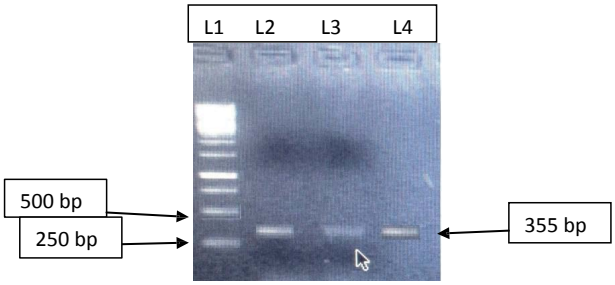
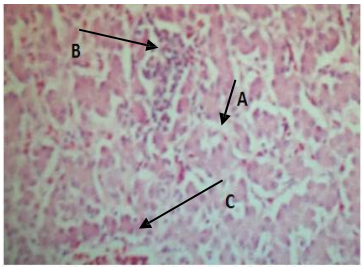


Figure 2: Gel electrophoresis of PCR product showing amplification of 355bp fragment of *E. coli serogroup O2*. Where in Lane; L1=Gene Ruler 250 bp DNA Ladder (Thermo scientific); L2=Sample with O_2 serogroup; L3=Sample with O_2; Serogroup L4=Sample with O_2 serogroup.

fibrinous layer over liver. Thickening of the liver capsule was noted due to invasion of mononuclear cells and there was marked congestion in hepatic portal areas and the central vein. There was atrophy of adjoining hepatic cords due to greatly distended and congested sinusoids. Besides these changes, hepatic cells in various phases of degeneration along with hemorrhages, areas of congestion and fatty changes in a few areas could be seen [18]. There was total demolition of hepatic cord settlement at some places. Necrotic areas were invaded predominantly by mononuclear cells and usual foci of necrosis were also observed. In chicks, the changes in liver were noticed at 14 and 21 days post infection. At first there was slight invasion of mononuclear cells in the portal areas, which was average to severe at subsequent intervals. Additionally, the deleterious changes and vacuolation in the hepatocyte were also observed in a few places [19-22]. There was infiltration of heterophils, severe congestion, lymphocytes and macrophages in the peribronchial alveoli as well as the wall of the bronchus. There was marked presence of granuloma in lungs. Some birds displayed thickening of the pleura and consolidated areas covered with yellowish fibrin in lungs (Figures 3a-3d).

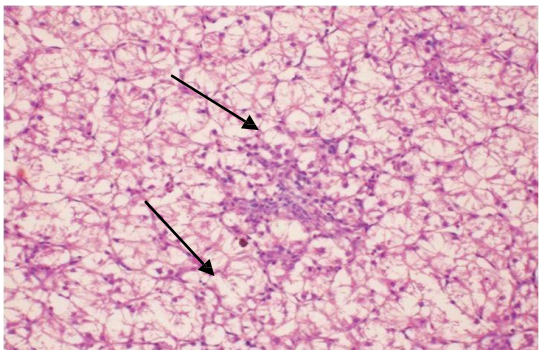
Discussion

E. coli causes drastic types of ailments such as coli granuloma (Hjarre's disease), pericarditis, avian cellulites (inflammatory process), salpingitis, osteomyelitis, colisepticemia, swollen head, syndrome air sacculitis, panophthalmitis, peritonitis, enteritis, omphalitis / yolk sac infection and synovitis [1]. In this current study, all the described forms of colibacillosis were not observed [23]. However, the observed forms of colibacillosis could be classified as a fibrinous layer over the liver surface. *E. coli* alone does not fabricate typical gross lesions. The lesions are most eminent when concurrently affected with other organisms such as *Mycoplasma*. *E. coli* can cause disease by attaching with the mucosal epithelia and another form by incursion to the mucosal epithelia. The observed lesions of liver were in the form of necrosis, degeneration, and severe inflammation associated with desquamation of mucosal epithelia [24]. Based on the observed lesions, the form of colibacillosis in the current study could be classified into hepatic form of colibacillosis. These types of histopathological lesions were reinforced by different



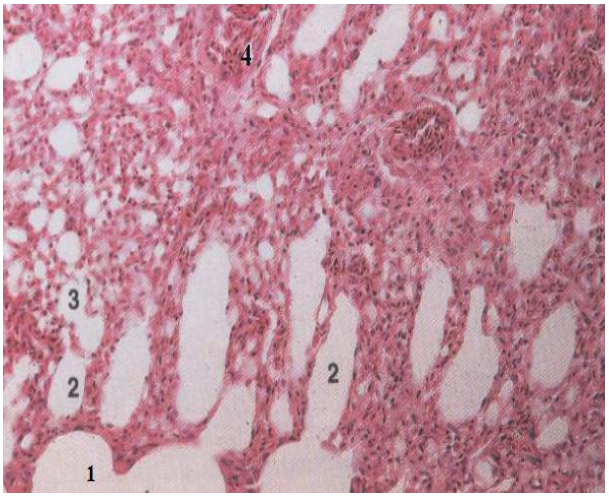
A: Coagulated necrosis; B: Infiltration of inflammatory cells; C: Congestion

Figure 3a: Histopathological view of diseased liver.



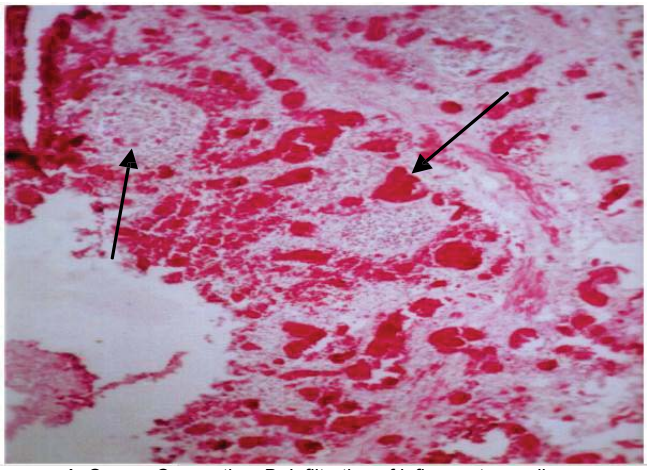
A: Mononuclear cells infiltration;
B: Fatty degeneration

Figure 3b: Histopathological view of diseased liver.



1: Lumen of the Parabronchus (Tertiary Bronchus); 2: Conical Ducts, Atria; 3: Air Capillaries; 4: Blood Vessels Filled with Nucleated Erythrocytes

Figure 3c: Histopathological view of normal lung.



A: Severe Congestion; B: Infiltration of inflammatory cells

Figure 3d: Histopathological view of *E. coli* infected lungs.

authors. There was infiltration of heterophils, severe congestion, lymphocytes and macrophages in the peribronchial alveoli as well as in the wall of the bronchus. There was marked presence of granuloma in lungs. In liver, severe infiltration of leukocytes mononuclear cells were observed. Hepatomegaly and enlarged lungs were also noted. Microscopically, all deceased birds did not displayed analogous gravity of lesions in all organs in the current study correlated with the findings of others [4-6]. Colibacillosis is a contagious ailment of birds caused by *E. coli* [7] which is classified as one of the main reasons of morbidity and mortality, connected with huge financial losses to the poultry sector by its alliance with different disease conditions, either as primary pathogen or as a secondary pathogen [8,25,26]. Localized or systemic colibacillosis can have a variety of symptoms. In this study, there were same findings with the previous studies. Coli-granuloma is an infrequent form of colibacillosis, which is characterized by granulomas in caecum, duodenum, liver and mesentery. Coli-granuloma was found in liver and lung in our cases. The histopathological changes observed in the present study had similarities to observations in earlier [7]. It has been noted in the previous studies that there has been pitiful flock performance correlated with increased early mortality rates [9]. Hatchery practices have been reported as the cause of early mortalities of layer and broiler breeders. Twenty four locally isolated isolates of *E. coli* were tested by ECO-F, ECO1-R, ECO2-R and ECO78-R primers and showed 355-bp products from eight field samples after 1.2% agarose gel electrophoresis. Traditionally, isolation and identification of *E. coli* was used as the main tool for the diagnosis of colibacillosis. The isolation and identification rely upon the culture of the organism using various selective media and biochemical tests. PCR and its comparable methods have been reported to identify *E. coli,* instead of biochemical and ELISA tests. The PCR is used as a highly sensitive and peculiar test for the presence of pathogenic bacteria in clinical specimens [10]. PCR is also reliable and swifter than traditionally culture methods [11]. We also found the same results in the current study [27,28]. In this study, the isolated *E. coli* organisms from all collected birds were cultured in MacConkey agar, EMB agar and Congo red media, DNA was extracted and amplified by PCR using ECO-F, ECO1-R, ECO2-R and ECO78-R primers targeting *E. coli* DNA and found 355 bp amplicon after 1.2% agarose gel electrophoresis. The similar result also found by other authors [12]. This base pair is specific for *E. coli* O2 serogroup not for others [29-32].

The recent study assisted similar findings to those observed in layer flocks, with *E. coli* infections being due to multiple strains of *E. coli* [13]. This study supports the Koch's postulates for APEC according to which wild type pathogen originally isolated in an outbreak of APEC infection in the field could cause disease in healthy chickens infected with the cultured strain [33].

Conclusion

It was concluded with the results of present study that there are different serogroups of avian pathogenic *E. coli* in field and PCR is best technique to recognize the different strains of *E. coli*. The results of histopathology also confirmed that experimental infection of pathogenic serogroup O_2 produce pronounced lesions in lungs and livers of broiler birds and there is early mortality and decreased weight gain.

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