RESEARCH ARTICLE

Etiopathological Study of Colisepticemia with Coinfection of Low Pathogenic Avian Influenza and *Mycoplasma* in Broilers

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Abstract

The present study was conducted to detect Low Pathogenic Avian Influenza (LPAI) and *Mycoplasma* in 30 broiler flocks (5 birds per flock) showing gross lesions of colisepticemia (Fibrinous pericarditis and perihepatitis) without any significant gross lesion in trachea and bronchi. The study comprised of flock wise mortality, gross and histopathological examination of colisepticemia and molecular identification from pooled sample of trachea for the presence of *E. coli*, LPAI, *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. The mortality among 30 flocks varied between 0.32 and 6.75% with an average of 1.89%. Gross and histopathological lesions were predominantly found in liver, heart, spleen and lungs. All the 30 flocks were found positive for *phoA* gene of *E. coli,* whereas 5 (16.60%), 9 (30%) and 4 (13.30%) flocks were positive for H9N2 subtype of *Avian influenza virus, i.e.* LPAI, *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, respectively. Findings of the present study confirmed the concurrent infection of colisepticemia with other primary pathogens such as LPAI, *M. gallisepticum* and *M. synoviae,* which indicate that *Escherichia coli* is a major secondary opportunistic pathogen in a respiratory disease complex of chicken.

Key words: Broiler, Colisepticemia, LPAI, Mycoplasma, PCR, RT-PCR.

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Introduction

The avian respiratory system has a wide range of commensal and potential pathogenic bacteria and/or viruses that interact with each other either synergistic or antagonistic, thus determining the severity of the complex disease. *Avian Influenza Virus* (*AIV*), *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Newcastle Disease Virus* (*NDV*), *Infectious Laryngotracheitis Virus* (*ILTV*), Avian Pathogenic *Escherichia coli* (APEC) and *Infectious Bronchitis Virus* (*IBV*) are the most significant respiratory pathogens in poultry. Among these *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are well-known pathogens for its ability to produce airsacculitis, especially in conjunction with *Escherichia coli* (*E. coli*) infection in chickens. Due to its vertical transmission through the eggs the disease caused by *M. gallisepticum* and *M. synoviae* is difficult to eradicate. In addition, early detection of infection with *Mycoplasma* is of paramount importance to prevent contamination of other flocks (Kleven, 2008).

Avian Influenza Viruses (*AIV*) has also been circulated widely in poultry all over the world. Although the *AIV* serotype H9N2 is pathotyped as a low pathogenic virus and appears to persist in chicks and spread to unaffected flocks through fecal-oral route without showing much of serious clinical signs. Under field conditions, asymptomatic infection of LPAI makes it extremely difficult to diagnose H9N2 infection. Due to simultaneous infection of *LPAIV* with other bacterial or viral diseases, the possibility of disease deterioration is presumed. Diagnosis and control of simultaneous infections would therefore be of great help in reducing economic loss due to both *Avian Influenza Virus* (H9N2) and avian mycoplasma (Dadras *et al*., 2014). The main objective of this study was to confirm the concomitant infection of *LPAIV* and avian mycoplasma in broiler flocks affected with colisepticemia.

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Materials and Methods

Sample Collection

The broiler birds from 30 broiler flocks (5 birds per flock) showing gross lesions of colisepticemia (Fibrinous pericarditis and perihepatitis) without any appreciable gross lesions in trachea and bronchi during necropsy examinations were selected for the study. The composite tracheal tissue (consisting of homogenized mixture of tracheal bifurcation from all 5 birds of a flock) were collected in cryovials containing RNA*later*® solution for molecular identification of LPAI (H9N2), whereas, for *E. coli* and *Mycoplasma* composite tracheal tissue were collected in cryovials and preserved at -80°C until nucleic acid extraction.

Pathological Studies

The pathological study comprised information related to strength of the flocks, age, and mortality pattern, gross and microscopic lesions. During necropsy, detailed post-mortem examination was carried out and gross pathological lesions were recorded and the tissue pieces of organs including liver, heart, spleen and lung were collected in 10% formal saline for histopathology.

Molecular Detection of *LPAIV* and *Mycoplasma* in Colisepticemia affected Broiler Flocks

Total RNA and DNA was extracted from all the 30 composite samples collected from different farms by using RNeasy Mini Kit and QIAamp® Fast DNA Tissue Kit (Qiagen, Germany), respectively, as per the manufacturer's protocol. To confirm the involvement of *LPAIV* in colisepticemia affected broiler flocks, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed using QuantiTect Reverse Transcription kit (Qiagen, Germany) for formation of cDNA from RNA and then PCR by using H9 and N2 specific primers with amplicon size of 287 and 310 bp. In addition for *Mycoplasma* and *E. coli*, PCR was carried out to amplify *mec2* gene of *M. gallisepticum*, *16Sr RNA* gene of *M. synoviae* and *phoA* gene of *Escherichia coli* by using specific primers with amplicon size of 302, 207 and 720 bp, respectively. The details of primers used for identification of *LPAIV*, avian mycoplasma and *E. coli* as well as their thermal cyclic conditions have been given in Table 1 and 2, respectively.

Results and Discussion

The mortality due to colisepticemia among 30 broiler flocks comprising a total population of 1,71,317 birds aged between two and five weeks ranged from 0.32 to 6.75%, with an overall mortality of 1.89%. Age-wise mortality was 1.65, 1.97, 2.21 and 1.71% during the second, third, fourth and fifth weeks of age, respectively. The comparatively higher mortality during the third and fourth weeks indicates that this period may be particularly critical for the development of colisepticemia and associated respiratory disease complex in broilers.

Gross pathological lesions suggestive of colisepticemia were observed in all affected birds, although their severity varied among flocks and individual birds. The most prominent lesions were observed in the liver, heart, spleen, lungs and air sacs. Fibrinous perihepatitis, characterized by a white to yellowish fibrinous layer covering the hepatic surface, and fibrinous pericarditis, characterized by a thick fibrinous covering over the pericardium, were consistently observed (Fig. 1). Multifocal necrotic lesions on the splenic surface and, in a few cases, yellowish fibrinous flakes within the peritoneal cavity were also recorded (Fig. 2). These findings were consistent with the characteristic lesions described in colibacillosis and colisepticemia in broiler chickens (Nakamura *et al.,* 1985; Awad *et al.,* 2020).

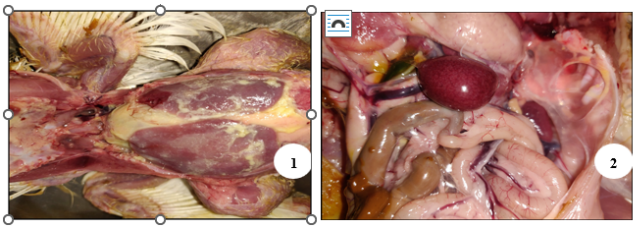


Fig. 1: Gross photograph showing fibrinous covering over the liver (fibrinous perihepatitis) and thick fibrinous covering over the pericardium (fibrinous pericarditis) in broiler bird affected with colisepticemia. **Fig. 2:** Gross photograph showing multifocal necrosis on the surface of spleen in broiler bird affected with colisepticemia.

Microscopic changes were more consistently seen in the liver and heart. Most of the sections showed deposition of fibrin on the outer surface of hepatic parenchyma along with infiltration of mononuclear inflammatory cells and congestion (Fig. 3). The characteristic microscopic lesion in the heart was mainly restricted to pericardium. The pericardium lesions consisted of variable degrees of fibrin covering the surface, *i.e.,* fibrinous pericarditis along with the infiltration of mononuclear cells and congestion (Fig. 4). In addition, myocardium also showed infiltration of mononuclear cells (Fig. 5). Though during gross examination, the lung lesions were not characteristic. The lung lesions were of the nature of haemorrhage, edema and congestion along with the presence of fibrinous exudate and infiltration of mononuclear cells in the alveolar parenchyma in most of the flocks (Fig. 6). Spleen showed multifocal areas of necrosis with mild to moderate congestion (Fig. 7) and some sections revealed presence of bacterial colony in splenic parenchyma (Fig. 8).

Molecular detection revealed all the composite samples positive for *phoA* gene which confirmed the presence of *E. coli* (Fig. 9a) whereas, H9N2 subtype of avian influenza virus,

Table 1: The details of primers used for identification of *LPAIV*, avian mycoplasma and *E. coli*

Targeted genes		Sequences (5'-3')	Expected product size (bp)	References
H9	F	ATCTAATCGCTCCATGGTATGG	287	Sarwar *et al.* (2013)
	R	TGACCAACCTCCCTCTATGA		
N2	F	CCAGCTCAAGTTGCCATGA	310	
	R	GGRTAACARGAGCATTCCTC		
mec2	F	CGCAATTTGGTCCTAATCCCCAACA	302	Garcia *et al.* (2005)
	R	TAAACCCACCTCCAGCTTTATTTCC		
16Sr RNA	F	GAGAAGCAAAATAGTGATATCA	207	Lauerman *et al.* (1993)
	R	CAGTCGTCTCCGAAGTTAACAA		
phoA	F	CGATTCTGGAAATGGCAAAAG	720	Alnahass *et al.* (2016)
	R	CGTGATCAGCGGTGACTATGAC		

Table 2: Steps and cycling conditions for PCR

Organism	Initial Denaturation	35 cycles			Final Extension
		Denaturation	Annealing	Extension	
LPAIV (H9N2)	95°C/3 min	98°C/10 sec	54°C/20 sec	72°C/25 sec	72°C/5 min
MG and MS	94°C/5 min	94°C/30 sec	56°C/20 sec	72°C/30 sec	72°C/5 min
E. coli	94°C/5 min	94°C/45 sec	56°C/45 sec	72°C/60 sec	72°C/8 min

i.e. LPAI (Fig. 9b), *M. gallisepticum* (Fig. 9c) and *M. synoviae* (Fig. 9d) were detected from 5 (16.60%), 9 (30%) and 4 (13.30%) flocks, respectively, in colisepticemia affected flocks without appreciable gross lesions in trachea and bronchi. In addition to this it was also seen that 8 (26.60%) out of 30 broiler flocks were positive for APEC+MG without any complications of LPAI+MS, 3 (10.00%) were positive for APEC+LPAI, 2 (6.66%) were positive for APEC+MS, 1 (3.33%) was positive for APEC+LPAI+MS and 1 (3.33%) flock was positive for APEC+LPAI+MG+MS.

The precise cause of respiratory diseases is very difficult to determine since more than one pathogen are involved at a time. As we know *AIV*, MG, MS, *NDV*, *ILTV*, APEC and *IBV* are the major respiratory pathogens which are responsible for heavy mortality and economic losses in poultry. The aim of present study was to detect presence of *LPAIV* and *Mycoplasma* in flocks showing lesions of colisepticemia without appreciable gross lesion in trachea and bronchi. However, the presence of caseous plug in bronchi is characteristic lesion of LPAI infection. Concurrent infection of LPAI with other respiratory pathogens such as, *E. coli* and *Mycoplasma* are responsible for production of severe clinical disease with subsequent high mortality (Kaore *et al*., 2018). The mortality among selected broiler flocks (0.32 to 6.75%) was in agreement with that of earlier authors (Delicato, *et al.,* 2003; Chaudhari, 2017; Awad, *et al.,* 2020), which reflect that *E. coli* infection can cause mortality between 2 to 4% among broiler flocks. In addition, the mortality observed mainly during 3rd, 4th and 5th weeks of age in broiler birds indicates that it was due to concomitant infection of various respiratory pathogens.

The gross lesions including fibrinous perihepatitis, fibrinous pericarditis and air sacculitis observed presently in all the affected birds were in concordance with those reported by earlier workers (Delicato *et al.,* 2003; Chaudhari, 2017; Awad *et al.,* 2020). The diffuse splenomegaly and hepatomegaly with multifocal greyish areas on their surfaces were also reported by Chaudhari (2017) and Awad *et al.* (2020).

The histopathological lesions like fibrinous perihepatitis and fibrinous pericarditis along with infiltration of mononuclear inflammatory cells in parenchyma and congestion were consistently observed in all the liver and heart tissues examined. Similar lesions were also reported by earlier workers (Chaudhari, 2017; Awad *et al*., 2020). Moreover, multifocal areas of necrosis and presence of bacterial colony in splenic parenchyma were similar to those reported earlier. Lungs showed congestion, edema and haemorrhage along with the presence of fibrinous exudate and infiltration of mononuclear cells in the alveolar parenchyma confirmed earlier reports (Chaudhari, 2017; Awad *et al*., 2020).

During the study, colisepticemia affected flocks having normal respiratory tract were selected to know whether respiratory pathogens like LPAI, MG and MS were present in concomitant with *E. coli*. A result showed that all flocks were found positive for presence of *E. coli*. However, 5/30, 9/30 and 4/30 flocks were positive for LPAIV, MG and MS, respectively. In addition, combinations of more than one respiratory pathogen along with *E. coli* infection were detected in majority of flocks which was in agreement with earlier findings (Delicato *et al.,* 2003; Chaudhari, 2017; Awad

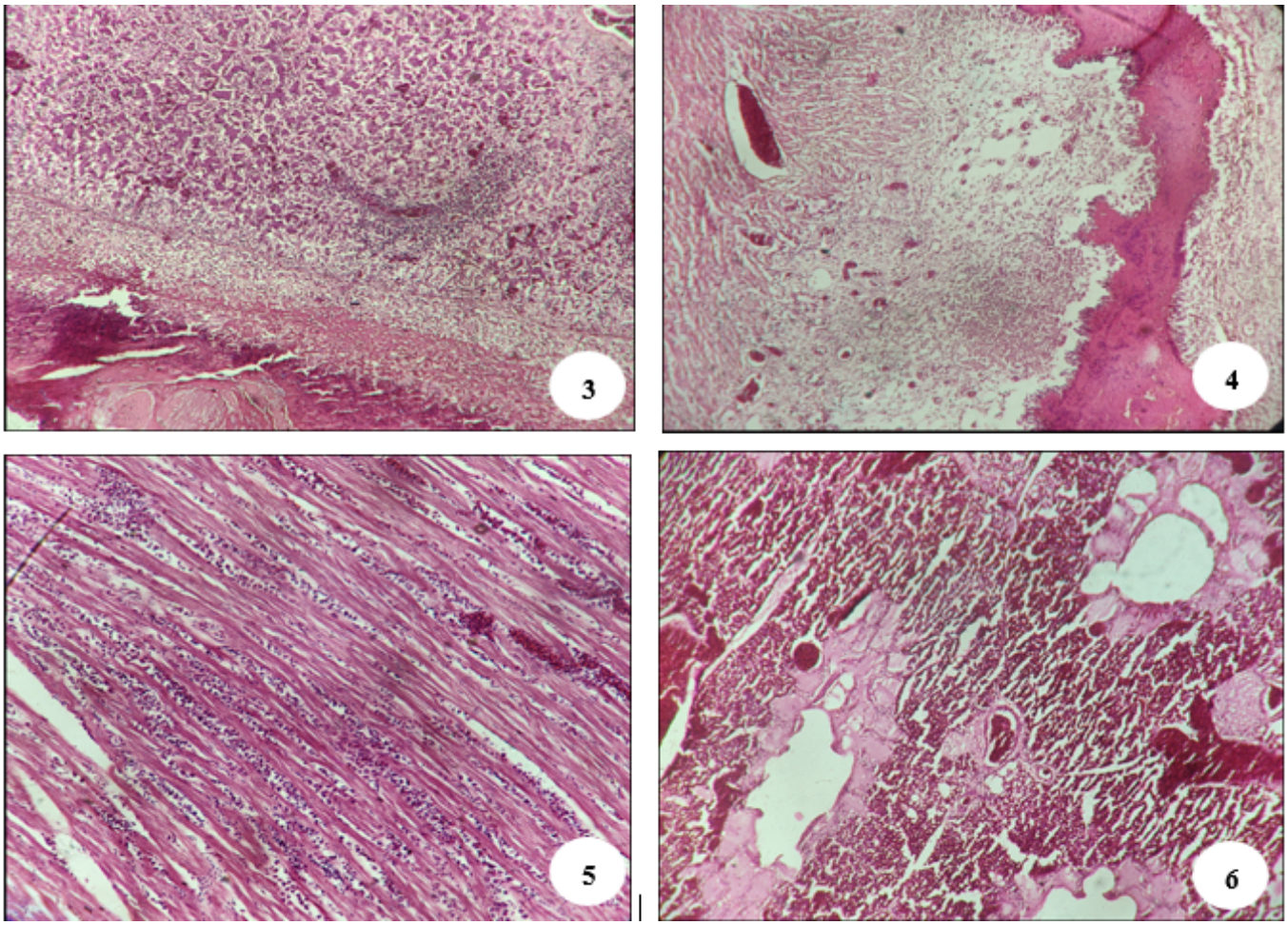


Fig. 3: Section of liver showing infiltration of mononuclear inflammatory cells and congestion in the hepatic parenchyma with fibrinous perihepatitis in *E. coli* affected broiler birds. H&E x120; **Fig. 4:** Section of heart showing pericardium with thick fibrin covering the surface i.e. fibrinous pericarditis along with the infiltration of mononuclear cells and congestion. H&E x120; **Fig. 5:** Section of heart showing mononuclear cells infiltration in the myocardium. H&E x240; **Fig. 6:** Section of lung showing peri bronchial and peri vascular oedema and severe congestion in the alveolar parenchyma H&E x120.

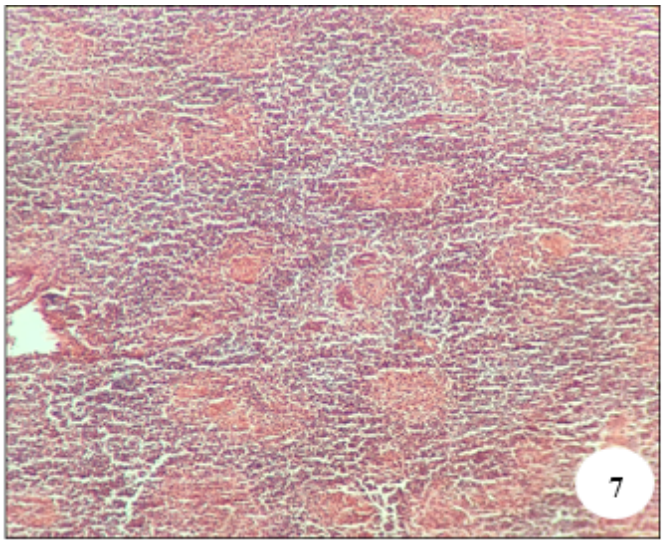
et al., 2020). The mixed infection of various respiratory pathogens might be responsible for high morbidity and mortality in colisepticemia affected birds.

Conclusion

The study demonstrated that *E. coli* was consistently associated with colisepticemia in the examined broiler flocks, with characteristic systemic lesions and variable mortality. Detection of LPAIV, *M. gallisepticum* and *M. synoviae* in several flocks, despite the absence of obvious respiratory lesions, indicates their possible role as predisposing or concurrent pathogens. Thus, molecular diagnosis along with effective biosecurity, management and control of respiratory pathogens is important for reducing colisepticemia-associated losses in broilers.

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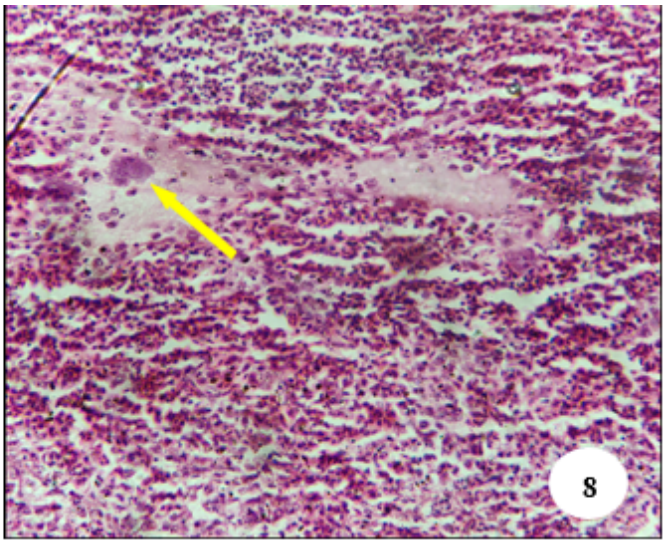


Fig. 7: Section of spleen showing multifocal areas of necrosis with mild to moderate congestion in a broiler bird affected with *E. coli* infection. H&E x120; **Fig. 8:** Section of spleen showing presence of bacterial colony in splenic parenchyma. H&E x480.

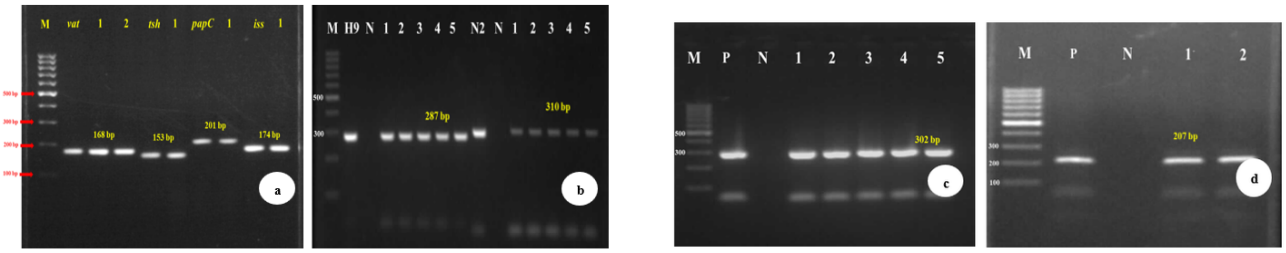


Fig. 9: Agarose gel showing amplified product of (a) *phoA* gene (720bp) (b) H9N2 gene (c) mec2 gene (302bp) (d) Gene encoding 16Sr RNA of *M. synoviae* (207bp); M: DNA ladder-100 bp; 1-5: Test sample; H9: Positive control for H9; N2: Positive control for N2; N: Negative control; P: Positive control.

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