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Original Research

Etiopathological Study on Low Pathogenic Avian Influenza Complicated by Escherichia coli Infection in Layer Birds

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Abstract

Seven commercial layer flocks with lesions suggestive of respiratory and reproductive problems were investigated during the study period (one year) of January to December 2016, to evaluate the pathology of Low Pathogenic Avian Influenza (LPAI) virus with concurrent presence of secondary E. coli infection in laying chickens. The study comprised of necropsy, incidence, farm wise mortality, gross and histopathological lesions, detection of LPAI (H9N2) virus by RT-PCR, isolation of E. coli and detection of virulence genes associated with E. coli by PCR. Studies on the farm incidence and mortality due to LPAI among seven-layer farms showed an average mortality rate of 3.03% ranging between 1.04 to 18.75%. Gross and microscopic lesions were predominantly found in organs of respiratory and reproductive tracts. All the 52 samples from seven flocks tested for the presence of LPAI (H9N2) virus were found positive. Out of the 52 tissue samples processed for bacterial isolation, 40 samples (76.92%) were found positive for secondary E. coli infection.

Keywords: Avian influenza, *E. coli*, Layer, LPAI (H9N2), PCR

Introduction

Avian influenza is a highly infectious and dynamically evolving disease of birds causing high morbidity and mortality. The Low Pathogenic Avian Influenza (LPAI) H9N2 viruses generally cause decreased egg production and mild to moderate infections, but they can also cause heavy morbidity and mortality when the birds are co-infected with other agents (Nili and Asasi, 2003). In layer, the most common noticeable gross lesions in the necropsy finding were salphingitis and egg peritonitis. Many organs may also reveal small to large hemorrhages, but changes in the respiratory tract are much less prominent than in broiler (Vegad, 2013). Pathogenic *E. coli* is one of the most important pathogenic agents causing disease in fowls and mammalians either as primary pathogen or secondary invader (Ewers *et al*., 2004; kwon *et al*., 2008). During present days many commercial layer farms in and around Anand, Gujarat are reported to have mortality due to respiratory disease complex and drop in egg production with postmortem lesions suggestive of LPAI with concurrent *E. coli* infection. Layer flocks having such lesions are at increasing trend and prompted to study in detail about diagnosis of LPAI by RT PCR and isolation of *E. coli* organisms.

Material and Methods

Sample Collection

Field samples were collected between, January and December, 2016. Tracheal and oviduct tissue samples were collected from dead layer birds affected with LPAI presenting increased mortality and acute respiratory disease. The study was carried out in 52 samples (28 trachea and 24 oviduct tissue samples) from seven flocks, received for post mortem diagnosis at Department of Pathology, Veterinary College, Anand, Gujarat. The pathological study comprised information related to strength of the farm, clinical signs, mortality pattern and gross as well as microscopic lesions. During necropsy, the tissue pieces of organs including trachea, lung, oviduct, liver, kidney, proventriculus, pancreas and brain were collected in 10% neutral buffer formalin and preserved for further processing, staining and recording of histopathological lesions.

RNA and DNA Isolation from Tracheal and Oviduct Tissues

For molecular identification of LPAI (H9N2), the tracheal and oviduct tissues were collected from each flock in cryovials and stored at -80ºC after adding RNA*later*® solutions for RNA stabilization and storage till processing. A total of 52 samples from different layer flocks were collected and stored. Frozen tissues were thawed and processed for extraction of the RNA required for PCR identification. Viral RNA was extracted from all the different samples of trachea and oviduct tissues by TRIZOL-chloroform method. After extraction of viral RNA, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was carried out following two steps method i. e. formation of cDNA from RNA and then PCR with H9 and N2 specific primers with amplicon size of 287 and 310 bp, respectively.

For isolation, identification and molecular characterization of *E. coli*, a total of 52 swab samples from trachea and oviduct were collected and immediately cultured over MacConkey agar, pink colored colonies identified which were sub cultured on Eosin Methylene Blue (EMB) agar plates. Typical colonies of positive *E. coli* isolates from EMB agar were confirmed by staining procedure. The DNA of isolates of *E. coli* was extracted by UltraClean® Tissue & Cells DNA Isolation kit. *E. coli* were further characterized by PCR for virulence marker genes especially *iss, vat, papC, and tsh* using forward and reverse primer sequences of 174, 168, 201 and 153 bp, respectively as described by earlier worker (Ewers *et al*., 2004).

Results

Farm Incidence and Mortality

Studies on the flock wise mortality in seven-layer flocks revealed overall mortality of 3.03%. The mortality due to LPAI among these layer flocks varied from minimum of 1.04% to maximum of 18.75% (Table 1).

Table 1: Flocks-wise mortality due to LPAI in 7-layer flocks

Flock No.	Age of affected flock (week)	Strength of flock	Number of birds died during outbreak	Mortality percent
1	21	600	50	8.33
2	22	1286	73	5.67
3	31	549	99	18.03
4	32	864	162	18.75
5	34	7700	108	1.4
6	39	5000	93	1.86
7	86	5000	52	1.04
Gross Total		**20999**	**637**	**3.03**

Pathological Lesions

Gross pathological lesions were predominantly found in organs of respiratory and reproductive tract in almost all the birds examined from the affected flocks. Lesions in respiratory system were noticed in trachea. Trachea showed severe congestion and hemorrhages of tracheal mucosa with presence of mucus in lumen (Fig. 1) indicating acute stage of infection. In later stage trachea revealed presence of fibronecrotic caseous material in the lumen (Fig. 2).

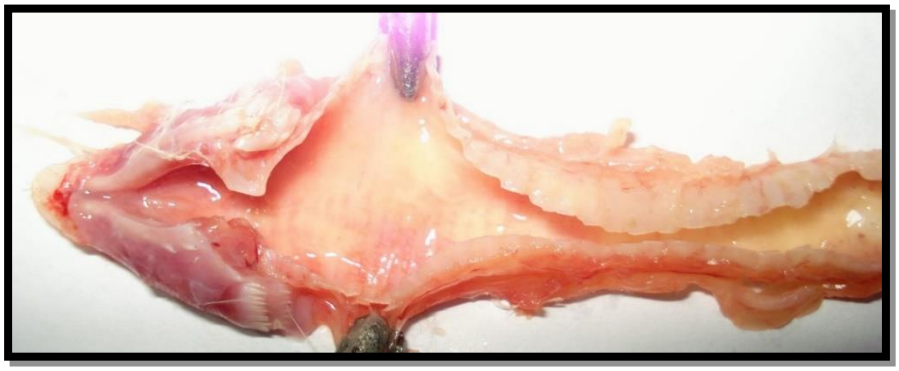

Figure 1: Layer bird affected with LPAI showing excessive watery mucous in lumen of trachea

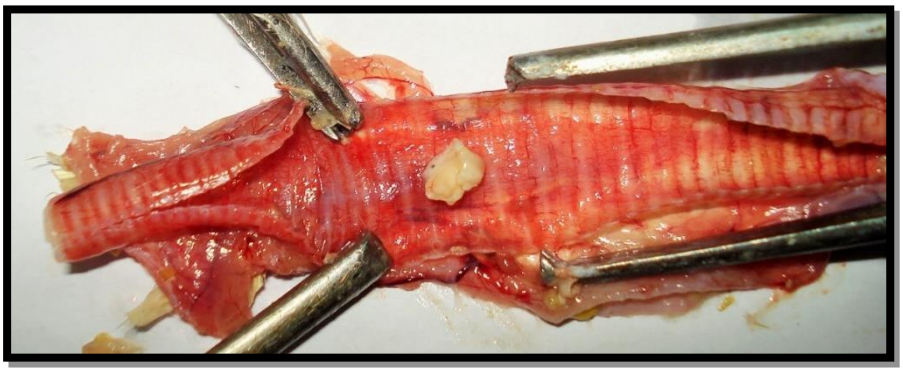

Figure 2: Layer bird affected with LPAI showing severe congestion, hemorrhages & fibronecrotic mass present in tracheal lumen

The abdominal cavity revealed fibrinous exudates and relatively large quantity of thick, white-yellow coloured fluid and serofibrinous peritonitis and ruptured ova intermixed with egg yolk material (Fig. 3). The serosal surface of oviduct was covered by excessive gelatinous clear exudate and lumen revealed clear excessive fluidy albumin with or without white to grey, flocculent material (Fig. 4).

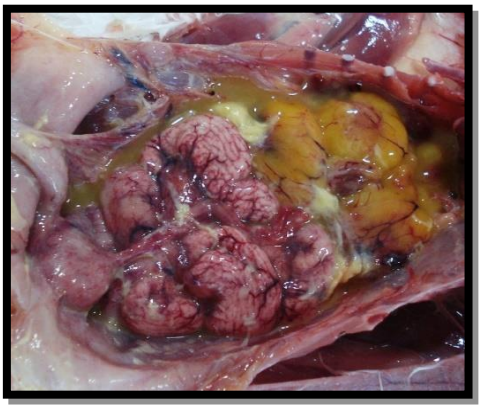

Figure 3: Layer bird affected with LPAI showing accumulation of fibrinous exudate with large quantity of thick white yellow color fluid in abdominal cavity.

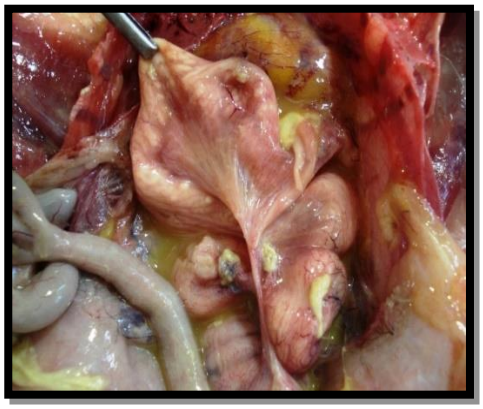

Figure 4: Layer bird affected with LPAI showing excessive gelatinous clear exudate on serosal surface of oviduct.

Ovary revealed fibrin flakes on the surface of follicles and ruptured ova intermixed with white-yellow coloured fluid (Fig. 5) in the abdominal cavity. The proventriculus showed petechial hemorrhages on mucosal surface in few birds affected with LPAI (Fig. 6).

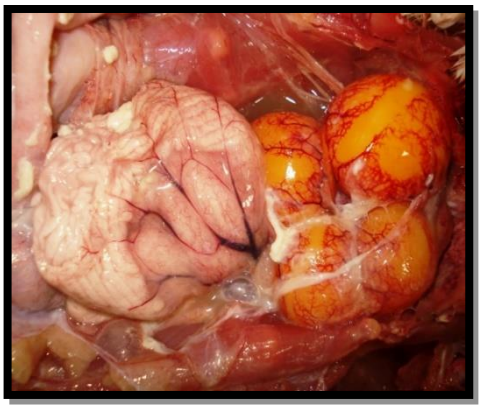

Figure 5: Layer bird affected with LPAI showing fibrin flakes on the ovaries.

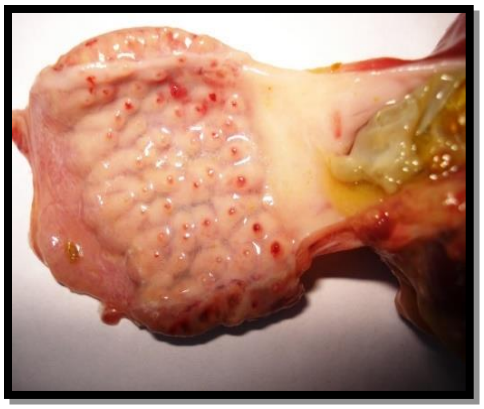

Figure 6: Layer bird affected with LPAI showing petechial hemorrhage on mucosal surface of proventiculus.

Microscopic changes were most consistently seen in the trachea and oviduct variable from mild to severe in nature. In many cases trachea showed congestion along with infiltration of mononuclear cells and presence of fibrinonecrotic and/or caseous masses in the lumen (Fig. 7). The mucosa of trachea also showed focal area of necrosis and deciliation of epithelial lining (Fig. 8).

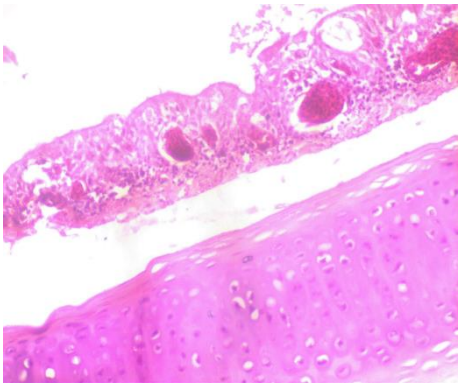

Figure 7: Section of trachea showing congestion and infiltration of mononuclear cells in layer bird affected with LPAI (H & E stain, 120X)

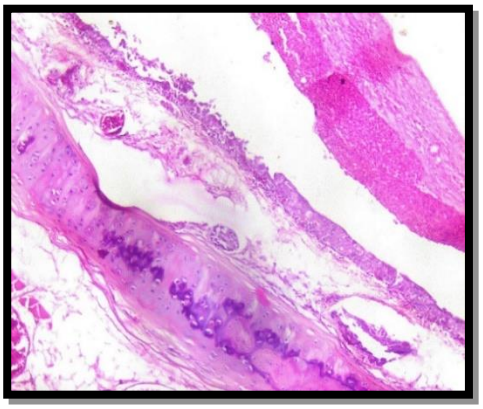

Figure 8: Layer bird affected with LPAI showing fibro-necrotic mass in lumen, necrosis and deciliation of epithelial lining of trachea (H & E stain, 120X).

The microscopic lesions in lung were characterized by congestion, haemorrhages and edema of parabronchi. Oviduct showed congestion of mucosa and separation of muscle fibers in the muscular layer with mild focal infiltration of inflammatory cells (Fig. 9).

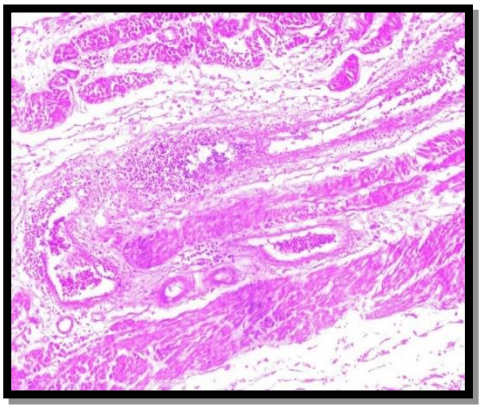

Figure 9: Layer bird affected with LPAI showing congestion, infiltration of inflammatory cells and separation of muscle fibers in the muscular layer of the oviduct (H & E stain, 120X).

The sections of spleen, kidney, liver, proventiculus and brain revealed mild congestion and hemorrhage in few birds. Section of pancreas in few cases showed focal areas of necrosis.

Molecular Detection of LPAI (H9N2) Virus by RT-PCR

All the 52 samples of trachea and oviduct were tested for the presence of H9 and N2 genes using primers of amplified products of band size 287 bp and 310 bp (Table 2). The amplification of expected size was observed in positive control and test samples (Fig. 10).

Table 2: Details of primers used for RT-PCR for detection of LPAI (H9N2) virus

Primer name			Primer sequences	Product size	Gene bank Accession No.	References
LPAI	H9	F	ATCTAATCGCTCCATGGTATGG	287 bp	KP865927.1	Sarwar *et al.*, 2013
		R	TGACCAACCTCCCTCTATGA			
	N2	F	CCAGCTCAAGTTGCCATGA	310 bp	HM370058.1	
		R	GGRTAACARGAGCATTCCTC			

(F) = Forward primer; (R) = Reverse primer

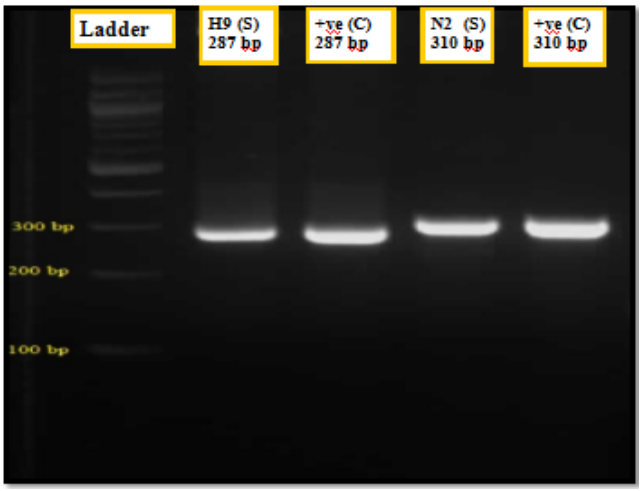

Figure 10: Gel image showing amplified product with H9 and N2 specific primers.
(Ladder: 100 bp, H9 (S): Test sample, H9 (C): Positive control for H9, N2 (S): Test sample, N2 (C): Positive control for N2)

All the 52 samples tested for detection of LPAI (H9N2) virus were found positive by RT-PCR.

Isolation of *E. coli* and Molecular Detection of Virulence Genes

Out of the 52 tissue samples processed for bacterial isolation, 40 samples (76.92%) were found positive for secondary *E. coli* infection by Gram's staining. Molecular characterization of *E. coli* isolates was carried out for detection of presence of four virulence genes i.e. *iss*, *vat*, *papC*, and *tsh* using forward and reverse primer sequences of 174, 168, 201 and 153 bp, respectively (Fig. 11).

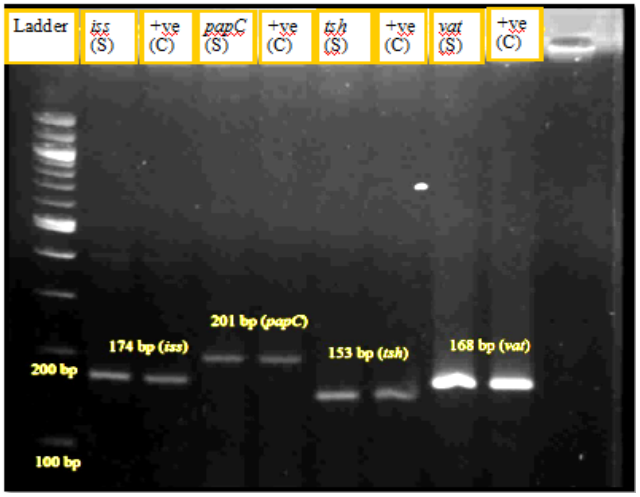

Figure 11: Agarose gel image showing amplified product of *iss* (174 bp), *vat* (168 bp), *papC* (201 bp) and *tsh* (153 bp) genes.
(Ladder: 100 bp; *iss* (S)*:* test sample; +ve (C): positive control of *iss* gene; *vat* (S)*:* test sample; +ve (C): positive control of *vat* gene; *papC* (S)*:* test sample; +ve (C): positive control of *papC* gene; *tsh* (S)*:* test sample; +ve (C): positive control of *tsh* gene.)

DNA was extracted from tissue samples and subjected to PCR with *E. coli* gene specific primer pairs; details were presented in Table 3.

Table 3: List of virulence associated gene primers of *E. coli*

Primer name	Primer Sequence		Product Size	Gene bank Accession No.
Iss	**F**	CCCCAATTGGACAGAGAAAA	174 bp	X52665
	R	ATCGATGGGCCTATTGTGAG		
papC	**F**	AATAAAAACGTGGCGGACTG	201 bp	Y00529
	R	ACGCAGGTAAGCAGAATCGT		
Tsh	**F**	TCTCAATGCGTCGTAACAGC	153 bp	AF218073
	R	CCTTCAGATGAACGTCAGCA		
Vat	**F**	CACGCTACTGAATGCCTGAA	168 bp	AY151282
	R	TGGCAGGTTAATGGTGTGAA		

(F) = Forward primer; (R) = Reverse primer

Out of the 52 *E. coli* affected tissue samples tested, the genes *iss, vat, papC* and *tsh* were found positive in 38, 36, 17 and 2 samples, respectively.

Discussion

The present study evaluated flock wise incidence of LPAI with respect to its age susceptibility and mortality pattern. Farm wise incidence of LPAI when viewed with the earlier reports (Halverson *et al.,* 1980; Naeem *et al.,* 1999; Davison *et al.,* 2003; Kinde *et al*., 2003) it reflected that the LPAI can cause mortality around 3.03% which is quite alarming and prevailing as one of the major disease conditions in layer birds.

Gross lesions of LPAI were mainly observed in the organs of respiratory and reproductive tracts. The tracheal lesions like severe congestion and hemorrhages with presence of mucus and fibrinous flakes in the lumen observed during present outbreaks were in accordance with earlier reports (Ahmad *et al.,* 2008; Jakhesara *et al.,* 2014; Savalia *et al*., 2006) and specific of LPAI**.** Lesions in the reproductive system involved abdominal cavity, oviduct and ovaries characterized by serofibrinous peritonitis, transmural oviduct edema and ruptured ova intermixed with egg yolk material respectively which were in agreement with the early reports (Davison *et al.,* 2003; Kinde *et al*., 2003; Jakhesara *et al.,* 2014) during field outbreaks as well as experimental infection with H9N2 virus among layer birds.

Histopathologically, trachea showed congestion along with infiltration of mononuclear cells and presence of fibrino necrotic and caseous masses in the lumen that corroborated the reports of earlier workers (Nili and Asasi, 2003). In lung, the lesions like congestion, haemorrhages and edema of parabronchi were also observed by earlier workers (Savalia *et al*., 2006). Oviduct showed congestion of mucosa and separation of muscle fibers in the muscular layer with mild focal infiltration of inflammatory cells. The sections of spleen, kidney, liver and proventiculus revealed mild congestion and hemorrhage in few birds. Section of pancreas in few cases showed focal areas of necrosis.

All the 52 samples tested for detection of LPAI (H9N2) virus were found positive by RT-PCR. Earlier workers (El Houadfi *et al.,* 2016; Pawar *et al*., 2012; Ahmed *et al*., 2009) also carried out molecular detection of LPAI (H9N2) by RT- PCR from tracheal and oviduct swabs/ tissues from respiratory infection of layer birds. Molecular detection of H9N2 avian influenza virus from all the seven flocks studied revealed that all the affected flocks were confirmed cases of H9N2 LPAI virus infection.

To analyze the risk of secondary *E. coli* infection in case of LPAI, isolation, identification and characterization of *E. coli* from tissues like trachea and oviduct was undertaken. Out of the 52 tissue samples processed for bacterial isolation, 40 samples (76.92%) were found positive for secondary *E. coli* infection. The findings when viewed in the context of earlier workers (Ahmad *et al*., 2008; Nagarajan *et al*., 2009) gave impression that invasion of *E. coli* is the major secondary complication once the respiratory tract damaged by primary avian influenza (H9N2) virus infection. Molecular characterization of *E. coli* isolates for presence of four virulence genes i.e. *iss, vat, papC,* and *tsh* revealed highest number of isolates 32/40 (80%) positive for presence of *iss* gene followed by *tsh* 22/40 (55%), *vat* 17/40 (42.5%) and *papC* 11/40 (27.5%) gene. The finding of present study to detect the presence of *iss* in *E. coli* isolates were similar with the findings (Ewers *et al*., 2004; Kwon, 2008; Arabi *et al.,* 2013) [who] found *E. coli* isolates

positive for the presence of *iss* to the tune of 82.7%, 96.4% and 73.8%, respectively from avian pathogenic *E. coli*. in layer chickens. The finding of the present study indicated that *iss* might be common virulence gene among pathogenic *E. coli* in layer birds and combined effect of more than one virulence gene might be responsible for pathogenicity of *E. coli* organism.

Conclusion

In conclusion, the study highlighted that mixed infections of LPAI and *E. coli* were consistent in tracheo-bronchitis with caseous plugs. Natural co-infection of layer with bacterial agent is common and often results in increased mortality. Invasion of *E. coli* is a major secondary complication once the respiratory tract especially trachea damaged by primary avian influenza (H9N2) infection.

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Conflict of Interests

There is no conflict of interest.

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