RESEARCH ARTICLE

Isolation and Molecular Characterization of *Pasteurella multocida* from Ducks

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Abstract

Duck pasteurellosis also called as Duck cholera is a highly contagious disease of ducks caused by *Pasteurella multocida*. It has been found to be most commonly occurring bacterial disease among semi-mature and mature birds with 5 to 100 % morbidity and 0.5 to 80 % mortality rates. The present study was conducted to investigate the suspected outbreak of pasteurellosis in a duck farm. The samples were collected from morbid and dead birds after conducting post-mortem examinations and processed for further isolation, identification and molecular characterization. The samples were found to be positive for *Pasteurella multocida* by cultural and biochemical tests and confirmed as *Pasteurella multocida* by using *Pasteurella multocida* species specific KMT1 primers and capsular type A specific primers. It was further processed for virulence-associated *ompH* gene. The purified *Pasteurella multocida* species specific KMT1 product was sequenced and based on nucleotides homology and phylogenetic analysis, the sample was having 99.76 % homology to *Pasteurella multocida* strain VP161 chromosome. Based on nucleotides homology and phylogenetic analysis of the capsular type-specific *hyaD-hyaC* gene partial nucleotide sequences, the sample was having 98.60% homology to *Pasteurella multocida* strain PMISC1 *HyaD* (*hyaD*) gene specific for capsular type A.

Key words: Bipolar, Duck cholera, *Hya D* gene, *Pasteurella multocida*, Polymerase chain reaction

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Introduction

Ducks are considered as second widespread species among poultry following the chickens all over the world. Duck farming is getting popularity day by day as ducks are found relatively resistant to some of the diseases. But there are many constraints in large scale duck farming due to inadequate attention in management, diagnosis, control and prevention of various infectious diseases which play a vital role in high morbidity and mortality of ducks. Fowl cholera is a commonly occurring contagious avian bacterial disease caused by *Pasteurella multocida* often causing high mortality, thus resulting in large financial losses in poultry industry. *Pasteurella multocida* which belongs to Pasteurellaceae family is a Gram negative, non-motile, coccobacillus, capsulated, non-spore forming bacterium occurring singly, in pairs or occasionally as chains or filaments (Ievy *et al*., 2013; Akhtar, 2013). It infects a wide range of animal species, causing diseases such as haemorrhagic septicemia and shipping fever in cattle, atrophic rhinitis in pigs and fowl cholera in poultry (Glisson *et al*., 2003).

Fowl cholera, which is generally caused by serotypes A:1, A:3 or A:4, is a severe systemic disease which occurs in domestic poultry and wild birds and results in significant economic losses to poultry industries worldwide. Currently, incidences of fowl cholera along with other bacterial diseases are on the increase despite vaccination and proper medication as a sequel of various incriminating factors (Jonas *et al*., 2001). The diagnosis of fowl cholera is based on clinical signs, pathological findings and the isolation and identification of *Pasteurella multocida* by cultural and biochemical characteristics (Rimler and Glisson, 1997) and further by molecular characterization. The present study emphasized on isolation, identification and molecular characterization of *Pasteurella multocida* from heart blood, spleen, liver and lungs collected from affected ducks, and this study was undertaken as a preliminary investigation in order to produce vaccine for control of duck pasteurellosis.

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Materials and Methods

Cultural and Biochemical Identification of *Pasteurella multocida*

The affected and dead birds suspected for duck pasteurellosis outbreak from a duck farm were presented to the Institute of Animal Health and Veterinary Biologicals, Bangalore. The affected birds were examined for clinical signs and the blood samples were collected for further study. The post mortem examination was conducted on dead birds. Impression smears from the affected organs mainly heart, liver, spleen and lungs were taken for staining. The identification of etiological agent was done as per the standard techniques of bacteriology (Markey *et al*., 2013). Isolation of causative organism was attempted from heart blood and liver tissues by inoculating them on to nutrient broth, nutrient agar and blood agar. The plates were incubated at 37 °C for 24 h. Pure culture of the obtained isolate was subjected to Gram's staining, Loeffler's staining and various biochemical tests for further confirmation.

Pathogenicity Studies

Following approval of IAEC (No. IAH/IAEC/QCS/HSV/2023-24/II/03 dated 22.09.2023), the pure cultures of the *Pasteurella multocida* isolates were subjected to pathogenicity studies. Two albino mice were inoculated intraperitoneally with 100 µL of 24 h broth culture and observed. The dead mice were subjected to post- mortem examination and heart blood, lung, spleen and liver were collected and subjected to reisolation of *Pasteurella multocida*. Impression smears prepared from heart, liver, spleen and lung from the dead mice were stained with Loeffler's methylene blue and examined microscopically (Gokben Ozbey and Adile Muz, 2006).

Molecular Characterization of *Pasteurella multocida* Isolates

Genomic DNA of bacterial isolate was extracted using the DNeasy Ultraclean Microbial Kit (Qiagen GmbH, Germany) following the manufacturer's instructions. The purity and concentration of the DNA were estimated by spectrophotometry at 260 and 280 nm. The culture was subjected for identification of *Pasteurella multocida* at species level (OIE, 2021) and for capsular type using oligonucleotide primers (Abbas *et al*., 2018). The confirmed *Pasteurella multocida* capsular type A culture was further subjected to molecular screening of virulence-associated *ompH* gene using oligonucleotide primers (Luo *et al*., 1997). The details of oligonucleotide primers used are listed in Table 1.

The PCR was carried out using 200 µL capacity thin wall PCR tubes with a final reaction volume of 25 µL. The reaction mixture contained Taq master mix red - (2X) (12.50 µL), 1.00 µL of each forward and reverse primer, 2.00 µL of Template DNA and 8.50 µL of Nuclease free water. The PCR reaction conditions of all the genes used are shown in Table 2. The PCR products were analyzed by agarose gel electrophoresis using ethidium bromide staining and UV transillumination, and documented using gel documentation system.

Sequencing and Phylogenetic Analysis

The amplified products of *Pasteurella multocida* species specific gene and capsular type A specific gene were sequenced in the forward and/or reverse directions by Sanger method (Sanger *et al*., 1977) of sequencing. A comparative analysis of sequences was performed using the Clustal W multiple sequence alignment program and Mega align module. Phylogenetic analyses were done using maximum likelihood neighbor-joining and maximum parsimony in

Table 1: Primers used for identification of *Pasteurella multocida*

Target gene	Primer sequence	Amplicon size	Reference
Pasteurella multocida universal primers KMT1	T7 5'- ATC-CGC-TAT-TTA-CCC-AGT-GG-3' SP6 5'- GCT-GTA-AAC-GAA-CTC-GCC-AC-3'	460 bp	OIE (2021)
Pasteurella multocida Type A specific primers	F: 5'- GC-CAA-AAT-CGC-AGT-GAG-3' R: 5'- TTG-CCA-TCA-TTG-TCA-GTG-3'	1044 bp	Abbas *et al*. (2018)
ompH	*F:* 5'-GCG TTT CAT TCA AAG CAT CTC-3' R: 5'-ATG ACC GCG TAA CGA CTT TC-3'	1000 bp	Luo *et al*. (1997)

Table 2: PCR conditions for different target genes

Sl. No.	Gene target	Initial denaturation	Denaturation	Annealing	Extension	Final extension
1	*Pasteurella multocida* universal primers KMT1	94 °C, 10 min	94 °C, 1 min	55 °C, 1 min	72 °C, 1 min	72 °C, 10 min
			35 cycles each of denaturation, annealing and extension for all 3 genes			
2	*Pasteurella multocida* Type A	94 °C, 10 min	94 °C, 1 min	55 °C, 1 min	72 °C, 1 min	72 °C, 10 min
3	*ompH* gene	94 °C, 5 min	94 °C, 15 s	56 °C, 60 s	72 °C, 1 min	72 °C, 10 min

MEGA11 to identify homology between different strains. Homology searches were performed using NCBI database and BLAST (Basic Local Alignment Search Tool) programme.

Results and Discussion

Cultural and Biochemical Identification of *Pasteurella multocida* from Ducks

Clinically affected ducks exhibited anorexia, depression, lameness and respiratory manifestation. On postmortem examination of dead birds, petechial and ecchymotic haemorrhages were common, particularly in subepicardial and subserosal layers of liver. The liver was swollen with multiple, small, necrotic foci. Pneumonia and air sacculitis were also seen (Mohan and Pradeep Kumar, 2008; Selim-Amany *et al.,* 2017). Mucoid exudate in nasal tract and subcutaneous tissue with haemorrhagic consolidation of the lungs were observed (Fig. 1). Miliary necrotic foci on liver and spleen, pin point and ecchymotic haemorrhages on epicardium, liver and intestine with increased amount of serous fluid in the pericardial sac were seen as described by Mohan and Pradeep Kumar (2008), Eldin and Reda (2016) and Selim-Amany *et al*. (2017).

Impression smears from the affected organs mainly heart blood, heart, liver and lungs were taken, air dried methanol fixed and stained with Loeffler's methylene blue and Gram's staining. Microscopic examination of methylene blue stained impression smears of liver, heart and heart blood revealed the presence of bipolar organisms (Fig. 2a). The blood samples from live birds, liver and heart samples from dead birds showed growth in nutrient broth, nutrient agar and blood agar plates. The colonies on nutrient agar were small, glistening, mucoid, dew drop like with considerable variation in colony size, ranging from rounded, convex, discrete colonies with circular edges to large watery colonies with flowing margins. Small mucoid dew drop, non-haemolytic colonies were observed on blood agar medium (Fig. 3). The microscopic examination of fresh broth culture by hanging drop method revealed that the organisms were non-motile.

The organisms when Gram stained, appeared as Gram-negative coccobacilli (Fig. 2b) and revealed bipolarity when stained with Leishman's stain. These features corroborate with many workers (Akhtar, 2013; Ievy *et al.,* 2013; Selim-Amany *et al*. 2017; Ponnusamy *et al*., 2018). The culture showing typical Gram-negative coccobacilli were further processed through biochemical characterization, which

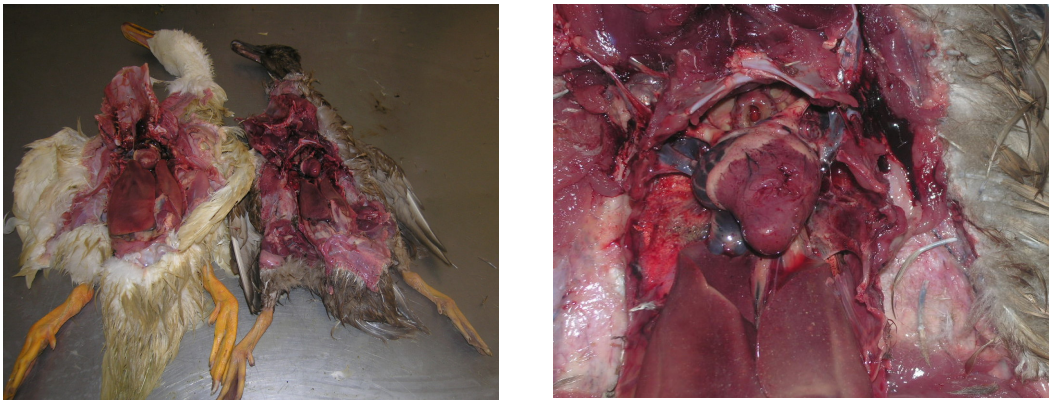
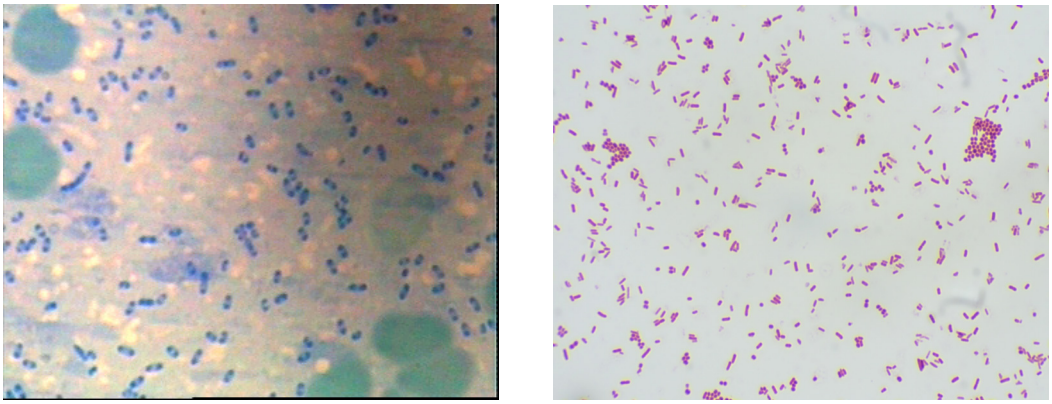


Fig. 1: Pin point and echymotic haemorrhages on epicardium and lung consolidation



(a) (b)

Fig. 2: (a) Bipolar organisms in methylene blue stained impression smears. (b) Gram stained smear from colonies, Gram-negative coccobacilli

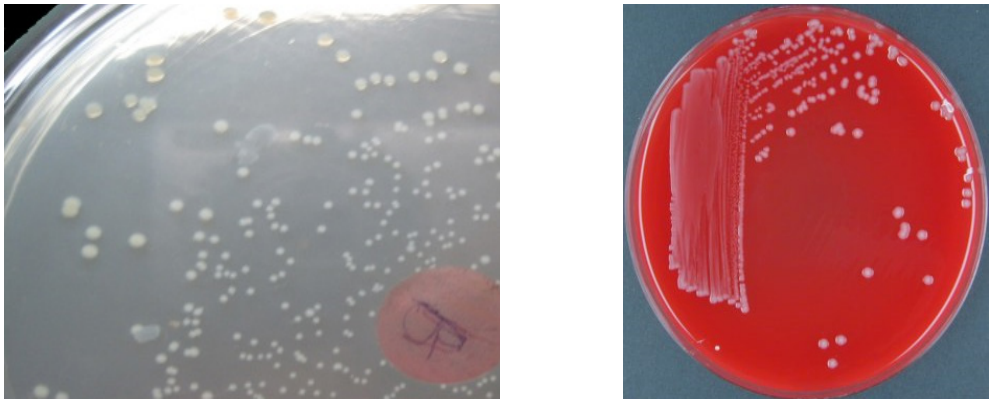


Fig. 3: Colonies on nutrient agar and Non-haemolytic colonies on blood agar

revealed the avian *Pasteurella* species +ve for Catalase, Oxidase, Indole production and Nitrate reduction, as well as Glucose, Fructose and Sucrose fermentation, while –ve for motility, maltose fermentation, haemolysis on blood agar and urease production. These results were consistent with the findings of Shivachandra *et al*. (2006), Ievy *et al*. (2013), and Panna *et al*. (2015) for *Pasteurella multocida*.

In a pathogenicity study the mice inoculated with the suspected *Pasteurella multocida* culture died within 24-48 h, showing generalized septicemia with highly congested trachea, lungs and enlarged spleen and proved to be toxigenic (Abd-Elsadek *et al*., 2021). Stained impression smears from heart blood, liver revealed characteristic bipolar organisms. Further the organisms were confirmed as *Pasteurella multocida* by biochemical tests. These findings were similar to other studies (Kapoor *et al*., 2004; Tirumurugaan *et al*., 2004). Panna *et al*. (2015) also observed pathogenicity in chicken after challenging them with the virulent *Pasteurella multocida* Type A and the chickens were died within 24 h, where they observed characteristics changes in heart, liver and spleen.

Molecular Characterization of *Pasteurella multocida* Isolates

The isolated culture was subjected to PCR using species specific KMT1SP6 and KMT1T7 primers which amplified a PCR amplicon of 460 bp and confirmed as *Pasteurella multocida* species (Fig. 4). Further, the PCR screening of the confirmed *Pasteurella multocida* isolates from clinically affected ducks for capsular type A, it amplified a capsular type-specific *hyaD-hyaC* gene of 1044 bp size. The confirmed *Pasteurella multocida* capsular type A culture was further subjected to molecular screening of virulence-associated *ompH* gene. The *ompH* gene which is a virulent gene that belongs to *Pasteurella multocida* was amplified which produced an amplicon of approximately 1,000 bp (Fig. 5). In the present study, *Pasteurella multocida* species specific PCR which amplified KTM1 gene confirmed the isolate as *Pasteurella multocida* thus helped in rapid identification of pasteurellosis in ducks. Ponnusamy *et al*. (2018) reported that *Pasteurella multocida* species specific PCR provides rapid identification of avian pasteurellosis irrespective of serotypes and can be used as rapid and highly specific diagnostic method for confirmatory detection of *Pasteurella multocida* infection. The PCR screening for capsular type A which amplified a capsular type-specific *hyaD-hyaC* gene, which was in agreement with Zhangcheng *et al*. (2018) and Abd-Elsadek *et al*. (2021). The capsular type A, followed by type D and type F have been established as the most prevalent capsular type of *Pasteurella multocida* in Indian duck (Borah *et al*., 2023), while previously Eldin and Reda (2016) reported the predominance of capsular type A, followed by type D in ducks.

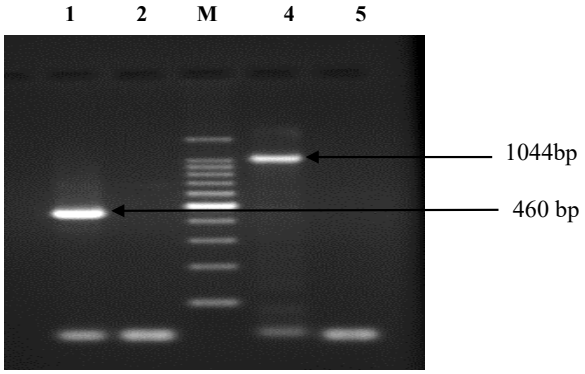


Fig.4: PCR amplification of KMT1gene and capsular type A specific HyaD gene of Pasteurella multocida. Lane 1: PCR Amplification of Pasteurella multocida specific KMT1gene, Lane 2: No template control, Lane M:100bp DNA ladder, Lane 4: PCR Amplification of Pasteurella multocida type A specific HyaD gene, Lane 5: Negative control

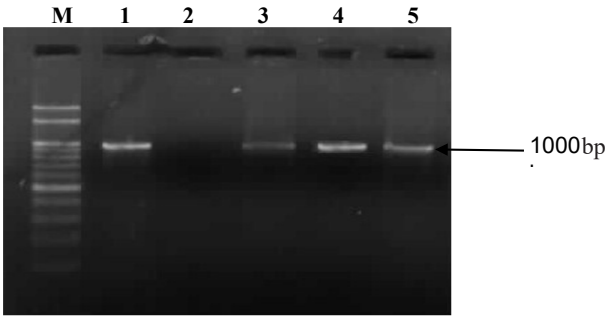


Fig. 5: PCR amplification of *ompH* gene of *Pasteurella multocida*. Lane M: 100bp DNA ladder, Lane 1: Positive control, Lane 2: No template control, Lane 3-5: Duck isolates

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. ident	Accession
Pasteurella multocida strain PMISC1 HyaD (hyaD) gene, partial cds	Pasteurella multocida	1279	1279	100%	0	99.29	JF922885.1
Pasteurella multocida serovar A3 Kmt1 (hyaD) gene, partial cds	Pasteurella multocida	1267	1267	100%	0	99.01	AY225345.1
Pasteurella multocida strain VP161 chromosome, complete genome	Pasteurella multocida	1258	1258	100%	0	98.73	CP048792.1
Pasteurella multocida strain CQ7 chromosome, complete genome	Pasteurella multocida	1258	1258	100%	0	98.73	CP033598.1
Pasteurella multocida strain Q chromosome, complete genome	Pasteurella multocida	1258	1258	100%	0	98.73	CP033597.1
Pasteurella multocida strain FCf83 chromosome, complete genome	Pasteurella multocida	1258	1258	100%	0	98.73	CP038875.1
Pasteurella multocida strain FCf45 chromosome, complete genome	Pasteurella multocida	1258	1258	100%	0	98.73	CP038873.1
Pasteurella multocida strain FCf76 chromosome, complete genome	Pasteurella multocida	1258	1258	100%	0	98.73	CP038874.1
Pasteurella multocida strain FCf71 chromosome, complete genome	Pasteurella multocida	1258	1258	100%	0	98.73	CP038872.1
Pasteurella multocida strain FCf15 chromosome, complete genome	Pasteurella multocida	1258	1258	100%	0	98.73	CP038871.1

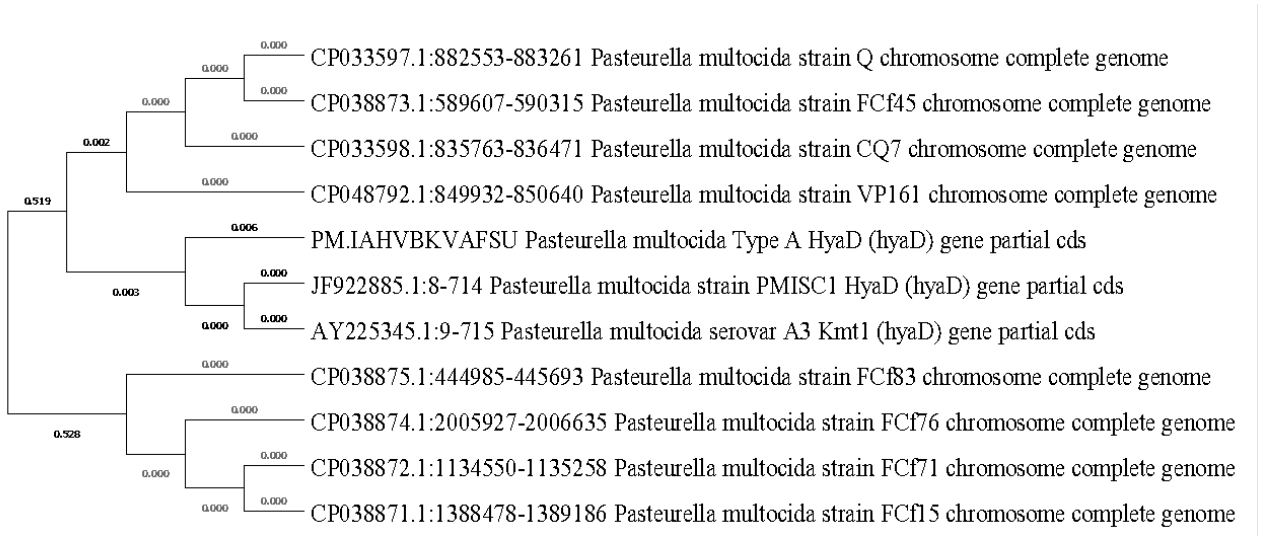


Fig. 6: Phylogenetic tree of nucleotide sequences of *Pasteurella multocida* isolate from ducks

Outer membrane proteins (OMPs) have been considered a virulence associated genes which involve in colonization and invasion of the organism and are considered as potential antigenic candidates (Lee *et al*., 2007). OMPs of *Pasteurella multocida* play an important role in host-pathogen interaction and disease processes making its identification critical to determine the protective antigens and to develop novel diagnostics (Priyadarshini *et al*., 2014). The available data on the prevalence of virulence-associated gene in *Pasteurella multocida* of avian origin reflected the consistent distribution of *ompH* in capsular types A and D of *Pasteurella multocida* (Deka *et al*., 2017)

Sequencing and Phylogenetic Analysis

The purified PCR products of *Pasteurella multocida* species specific gene and type A specific gene were sequenced using forward and reverse primers from both the directions. The comparison of our isolate from the present study with other published sequences was carried out using BLAST program. Based on nucleotides homology and phylogenetic analysis our isolate was closely related to the *Pasteurella multocida* strain PMISC 1 HyaD (*hyaD)* gene (Accession number JF922885.1:8-714) with 99.29% identity and *Pasteurella multocida* serovar A3 Kmt1 (*hyaD)* gene (Accession number AY225345.1:9-715) with 99.01% identity. The sequencing and phylogenetic characterization of isolate confirmed *Pasteurella multocida* type A in ducks. Thus, molecular characterization is highly specific and important in swift diagnosis of the disease.

Conclusion

In the present study the *Pasteurella multocida* was successfully isolated and further molecular characterization and sequencing helped in early diagnosis and confirmation of pasteurellosis in ducks caused by capsular type A organism. This underscored the importance of vigilant monitoring and diagnostic efforts in managing the disease in duck farms. The isolated strain provides a valuable resource for developing effective vaccines to control the disease and minimizing economic loss to the poultry industry along with good biosecurity. Continued research and collaboration among veterinarians, breeders and policy makers are imperative in advancing disease control strategies and safeguarding the poultry industry.

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