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Mycoplasma synoviae Based Molecular, Phylogenic and Pathological Studies in Chickens

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ABSTRACT

Background: Given that *Mycoplasma synoviae* (MS) has the potential to cause serious respiratory illness in chicken and cause significant financial loss to farmers, the present work was designed to investigate the MS-derived pathology along with detailed molecular study of the MS organism, isolated from natural cases of MS infection.

Methods: The work was carried out in the Department of Veterinary Pathology, College of Veterinary Sciences and Animal Husbandry, Durg, Chhattisgarh, for the period of two years which was ended on October 2021. Total 428 birds were included in this study. Tissue samples from the suspected cases of MS infection were collected and stored at -20°C and 10% formalin for molecular analysis and histopathological study respectively. Tissues were collected from respiratory organs along with liver, kidney and spleen.

Result: Overall prevalence of MS in the Chhattisgarh state was recorded as 6.30% under present investigation. Genome sequence of the PCR positive amplicon from MS confirmed case isolated from Chhattisgarh showed 99% homology with the other genome sequences available in NCBI website. Phylogenic analysis revealed highest similarities of Chhattisgarh isolate with the isolates of China and Iran that were imported from the GenBank. There were various degenerative, inflammatory and necrotic lesions was found in the respiratory system along with liver, kidney and spleen of MS positive birds.

Key words: Chickens, *Mycoplasma synoviae*, Partial gene sequencing, Pathology, PCR, Phylogenic analysis.

INTRODUCTION

Poultry respiratory infections are a substantial cause of economic loss for the poultry industry around the world. Each year, respiratory disease outbreaks kills nearly 30% of chickens, making it one of the most significant hurdles to the poultry industry's progress. Avian Mycoplasmosis is a serious systemic disease of chickens that can be transmitted both vertically and horizontal. Infectivity, tissue tropism and pathogenicity all are characteristics of Mycoplasma infection strains (Buim *et al*., 2009; Majumder and Silbart, 2016).

The most dangerous strains in poultry are *Mycoplasma synoviae* (MS) and *Mycoplasma gallisepticum* (MG), which are commonly reported from hens and are responsible for chronic respiratory illnesses. Avian Mycoplasma infection primarily affects the respiratory system of broiler and layer poultry birds, causing 100% morbidity and roughly 30% mortality, making it a severe danger to the poultry industry (Saif, 2013; Tomar *et al*., 2017; Gupta, 2019).

Despite their small size and limited metabolic capabilities, mycoplasmas have attracted a lot of attention in pathology and microbiology as diseases to a wide range of hosts. Because they lack a hard peptidoglycan cell wall, they are classified as 'Mollicutes' (in Latin: mollis, soft; cutis, skin). The absence of a cell wall confers a set of distinct characteristics: They are resistant to medicines that target cell of the organism, such as beta-lactams and staining with the Gram stain is ineffective (Dudek *et al*., 2016; Razin and Hayflick, 2010).

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Mycoplasma is known to be a causative agent for chronic respiratory disease (CRD). MS is associated with aetiology of CRD. Most commonly, MS infections are usually sub-clinical. While in the case of combination with secondary infections, MS becomes systemic and affects the synovial membrane of joints and tendon causing acute and chronic infectious synovitis. Both MS and MG are transmitted laterally via direct contact with infected carrier birds and fomites (Stanley *et al*., 2001; Rouf *et al*., 2014). Keeping in view the above facts, the present work was undertaken to study the prevalence of MS in Chhattisgarh along with detailed molecular and pathological analysis.

MATERIALS AND METHODS

Location and place of work

The present study was carried out in the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Anjora, Durg, Chhattisgarh. Duration of the work was two years, which was ended on October 2021. The work has been done under three major categories *i.e*., to study the prevalence of *Mycoplasma synoviae* (MS) infection in Chhattisgarh, Molecular characterization and phylogenic analysis of MS and to study of MS correlated pathology in the natural cases of infection. For prevalence study of MS, samples were collected from different districts of Chhattisgarh State.

Collection of samples

Total of 428 necropsies included in the present investigation. Poultry farms located in the different districts of Chhattisgarh *i.e*., Durg, Rajnandgaon, Raipur, Balod, Bemetara, Surguja and Koriya were visited in order to collect the samples. All the dead birds were subjected for detailed necropsy examination and tissue samples were collected from the nasal sinus, trachea, lungs, air sacs, liver, kidney and spleen from the dead birds with suggestive lesions of MS infection like swollen head, sinusitis, air sacculitis, pneumonic lungs and tracheitis. Collected tissue samples were stored in -20°C and in 10% formalin for molecular and pathological study respectively.

Extraction of DNA

The genomic DNA from the collected tissue samples of trachea, lungs and air sacs were isolated by using UltraClean® Tissue and Cells DNA Isolation Kit. Quantity of DNA was measured by Qubit® Flurometer, Invitrogen life technologies as per manufacture's protocol and concentration was expressed in ng/µl.

Polymerase chain reaction (PCR)

For amplifying the target gene, 16S rRNA piece (214 bp), sense primer F (5′ CAG TCG TCT CCG AAG TTA ACA A 3′) and antisense R (5′ GAG AAG CAA AAT AGT GAT ACT A3′) of published primers were used. The positive control (extracted DNA of MS) was ordered from Poultry Diagnostic and Research Centre, Pune. The PCR reaction was conducted in thermocycler (Applied Biosystems, Thermo Fisher) using the following criteria: Initial denaturation 94°C for 2 minutes followed by 30 cycles of (Denaturation 94°C for 20 seconds, Annealing 50°C for 30 seconds, Extension 68°C for 40 seconds) and final Extension at 68°C for 5 minutes. PCR mixture was made in 20 µL containing 4 µL of extracted DNA (DNA template), 0.8 µL of each oligonucleotide primer, 10 µL 2X PCR TaqMixture (Himedia MBT061) and 4.4 µL PCR-grade water. For electrophoresis a 1.5% agarose gel was recruited for running a PCR product screening which was done by taking images of gel bands by using a gel documentary system (Bio Rad, UK).

Genomic sequencing and phylogenic analysis

The representative PCR positive samples of 16S ribosomal RNA gene were commercially sequenced at PDRC (Poultry Diagnostic and Research Centre), Pune, to get both forward and reverse sequences to generate consensus sequences. The sequences were initially analyzed using NCBI online BLAST server to identify the sequence specificity. Based on the BLAST results sequences were further compared with other nucleotide sequences of MG and MS available in the GenBank database.

The comparative sequence analysis of partial sequence of 16S ribosomal RNA gene of MS was carried out along with those of selected sequences available in the GenBank database. Sequence identities of nucleotides were analyzed and aligned using the Clustal W of DNASTAR-Lasergene v6 Software (Morla *et al*., 2016) and Neighbour-joining (NJ) method. Based on maximum identity score, sequences were selected and aligned, sequence distances were generated and the phylogenetic tree was constructed from derived nucleotide sequences.

Histopathology

Tissue samples fixed in 10% formalin were processed for histopathological examination. The small tissue pieces (approximately 0.5×0.5 cm) were washed in running water, of dehydrated in three changes of acetone, followed by three changes clearing agent *i.e*., benzene. Wax impregnation, blocking, section cutting and Hematoxylin and eosin staining procedures were done method described by Mohamed and Laurence (2011).

RESULTS AND DISCUSSION

Prevalence of MS

Over all prevalence of MS was recorded as 6.30% in the present work. Out of 428 postmortem cases, 198 were found to be suspected for MS infection in the present investigation. Out of them, 27 cases were confirmed as MS by PCR during the period of study. Highest prevalence of MS infection was found in Balod district with 16.92% prevalence rate, followed by Durg district with prevalence rate of 14.81%. Moreover, none of the samples were found positive for MS in Bemetra Rajnandgaon, Raipur, Koriya and Surguja districts of Chhattisgarh.

Our results showed close corroboration with Vaishali *et al*. (2020) recorded 19% prevalence of MS in Haryana by using 16S rRNA primers through Polymerase Chain Reaction (PCR). Overall prevalence of MS and MG combinedly observe as 72.72% with predominancy of MS by Buim *et al*. (2009).

Tomer *et al*. (2017) recorded high prevalence of MG over MS in the broiler chickens of Haryana state. They

observed prevalence of MS as 2.1% by RPA method of detection. On the contrary, Rajkumar *et al*. (2018) recorded 25.98% incidence of MS and 9.45% incidence of MG in the suspected dead birds. In their study, they found higher incidence of MS than that of MG.

Molecular characterization of *Mycoplasma synoviae* (MS) PCR

In the present study 16S rRNA PCR or Species-specific PCR (encoding region of 16S rRNA) was performed using

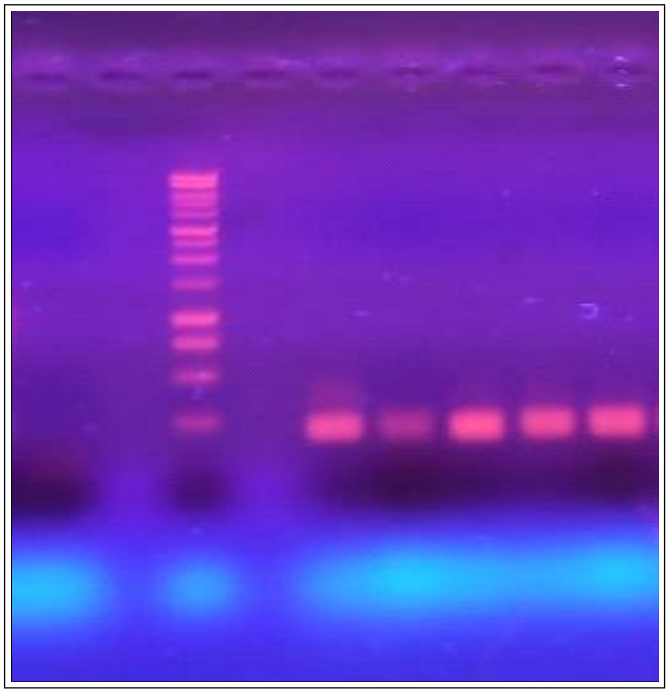

Fig 1: Electrophoresis of *Mycoplasma synoviae* PCR amplicon (214bp).
Lane 3: 200 bp DNA ladder; Lane 4: Negative control; Lane 5: Positive control; Lane 6-9: Positive samples of MS.

Table 1: Homology sequence identity between Chhattisgarh MS isolate and NCBI strain.

Strain MS	Accession number	Origin	Identity (%)
Mycoplasma synoviae strain MSR-1030 16S ribosomal RNA gene, partial sequence	GQ871762.1	Iran	99.42%
Mycoplasmopsis synoviae strain HN01 chromosome, complete genome	CP034544.1	China	99.42%
Mycoplasma synoviae strain B464-15-3-2 16S ribosomal RNA gene, partial sequence	MH539126.1	South Africa	99.42%
Mycoplasma synoviae strain pal12 16S ribosomal RNA gene, partial sequence	KX259335.1	Gujrat, India	99.42%
Mycoplasma synoviae strain CK.MS.UDL.KRC.PK.2014.18 16S ribosomal RNA gene, partial sequence	KJ130525.1	Pakistan	99.42%
Mycoplasma synoviae strain MSR-1031 16S ribosomal RNA gene, partial sequence	GQ871768.1	Iran, Tehran	99.42%
Mycoplasma synoviae strain VC&RI-NKL-SV3 16S ribosomal RNA gene, partial sequence	MK907908.1	Tamil Nadu, India	99.42%
Mycoplasma synoviae strain HMS-12192B 16S ribosomal RNA gene, partial sequence	KF181196.1	Egypt	99.42%
Mycoplasma synoviae strain MSR-862 16S ribosomal RNA gene, partial sequence	JF832935.1	Iran, Karaj	99.42%

published primer sequences to identify the genomic DNA of all the field isolates. All the field isolates (N=27) of MS produced an amplicon of 214 bp using combination of forward and reverse primers confirming MS (Fig 1).

Bagal *et al*. (2019) studied the pathology and molecular diagnosis of MS infections in broiler chickens from Western Maharashtra. They used 16S rRNA gene specific to MS to diagnose the infection. Positive samples produced an amplicon of 207 bp confirming the MS in given samples. Marouf *et al*. (2020) worked on molecular study of MS in Egypt. They used 16S rRNA gene for the detection of MS by PCR. The MS positive samples produced an amplicon containing 210 bp in their work.

Genome sequencing

Partial Nucleotide sequencing of PCR product of one isolate of MS from Chhattisgarh were carried out. This particular isolate was further designated as MS/Avian/ Durg/13/2020. The nucleotide sequence revealed final consensus of 175 bp sequence which was further used for alignment. BLAST (Basic Local Alignment Search Tool) analysis revealed that nucleotide sequence of PCR product of 16S rRNA gene of MS/Avian/ Durg/13/2020 isolate showed 99.42% homology with other nucleotide sequences of MS available in GenBank which is shown in Table 1. Sequence alignment report is shown in Fig 2.

Phytogenic analysis of MS

The phylogenetic tree was constructed from the nucleotide sequences recovered from GenBank for genome sequence of MS isolate. A total of 09 genome sequences of 16S rRNA retrieved from NCBI database and were used to construct the phylogenetic tree. The accession numbers for 16S rRNA genomic nucleotide sequences for MS obtained from GenBank were from Iran, China, South Africa, Gujarat, India, Pakistan, Iran, Tehran, Tamil Nadu, Egypt and Iran (Accession Number: AGQ871762.1, CP034544.1, MH539126.1, KX259335.1, KJ130525.1, GQ871768.1, MK907908.1, KF181196.1 and JF832935.1 respectively). All the above isolates were used for analysis along with MS isolate recovered from Chhattisgarh *i.e*., MS/Avian/ Durg/13/2020.

Phylogenetic tree was made using the DNASTAR-Lasergene v.6 software package and MEGA-X (Molecular Evolutionary Genetics Analysis) software tool. The distance matrices and phylogenetic tree were constructed using the Meg-align and Editseq algorithm. Phylogenetic analysis showed one clade (Fig 3). The tree showed highest similarities of MS/Avian/ Durg/13/2020 isolate with the isolates of China and Iran that were imported from the GenBank. Furthermore, it can be concluded that MS/Avian/ Durg/13/2020 isolate along with isolates of China and Iran might be sharing common ancestors.

Kursa *et al*. (2019) studied molecular and phylogenetic evaluation of MS in the Polish chicken layer flocks. They reported molecular epidemiological data on MS infection in

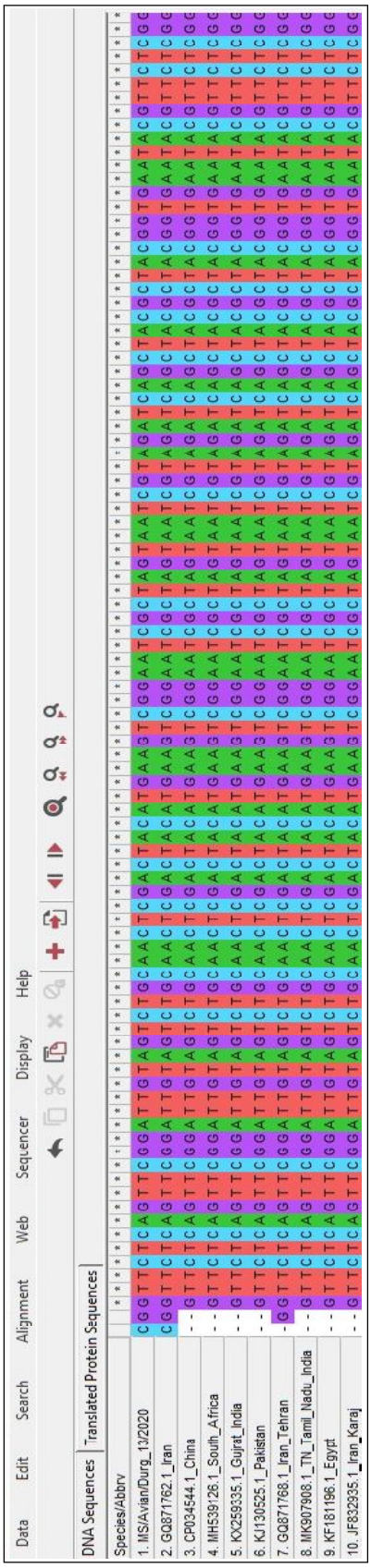

Fig 2: Alignment report of MS.

layer chickens first time in Poland. They detected PCR positive cases of MS in 202 (22%) chickens. They observed the infection rate as 34% in 2010, rose to 44% in 2012 and declined to 29% in 2016. Phylogenetic analysis of Polish MS strains using a partial sequence of the *vlhA* gene showed nine genotypes (A-I), out of which, F and C were found as most occurred genotype. Ali *et al*. (2020) found that their strain of MS *i.e*., Iraq strain IS was 100% similar with the isolates of USA, UK, Australia and Brazil.

Gross pathology

Gross lesions in MS positive cases were observed in nasal cavity, trachea, lungs, air sacs, liver and kidney specifically. In the present study any sort of swelling in joints or keel bursa were not found. External examination of MS positive bird revealed ruffled feather along with emaciated body condition. Chickens of MS positive cases showed viscous creamy to grey exudate, filled into the nasal cavity and secreted outside as nasal discharge. Nasal cavity was found congested and oedematous. There was marked swelling in the head of the birds also been recorded.

There was mild to moderate tracheitis detected along with catarrhal exudation that was filled in the tracheal lumen (Fig 4). Mild congestion was reported as another notifiable finding. Focal pneumonic consolidation of lungs was observed as major finding in the particular cases. Hepatosplenomegaly was noted as a major pathologic finding in the natural cases of MS. Liver was found to be moderately enlarged along with congestion in focal areas. Kidneys were enlarged and swollen. Oedema was also noted as an important finding. Some kidneys were observed mottled along with pale in appearance. Haemorrhagic kidney was found in few cases of MS.

Gross lesions observed in present work were found in close association with Kleven, (2003), Gabriel, *et al*. (2005), Saif (2013), Bagal *et al*. (2019).

Microscopic lesions

There was mild to moderate congestion observed in the nasal cavity of the birds with MS infection (Fig 5). There was mild sloughing of nasal mucosal epithelium recorded as another major finding. Nasal chamber of the bird was found to be filled with exudate composed of degenerated mucosal epithelial cells, mucous and inflammatory cells. Trachea of the positive birds showed mild to moderate thickening of mucosal epithelium due to deposition of mononuclear cells and heterophils. There was hyperplasia noted in the mucous secreting cells as they found filled and bulged due to filling of overproduced mucous inside them (Fig 6). Moderate sloughing was documented on the brush border epithelium of the trachea along with thin covering of mucous found to overlay the sloughed or denudated mucosa.

Congestion was noted as most observed finding in the lungs of MS positive cases. Pneumonia characterized by infiltration of mononuclear cells and oedema was noted in certain cases. Parabronchial lumen was found to be filled with necrotic debris and inflammatory exudate in marked amount in the lungs of MS affected chickens. Mild to moderate airsacculitis was evidenced in the cases of MS. Microscopically, there was oedema and inflammatory cell accumulation reported as major findings.

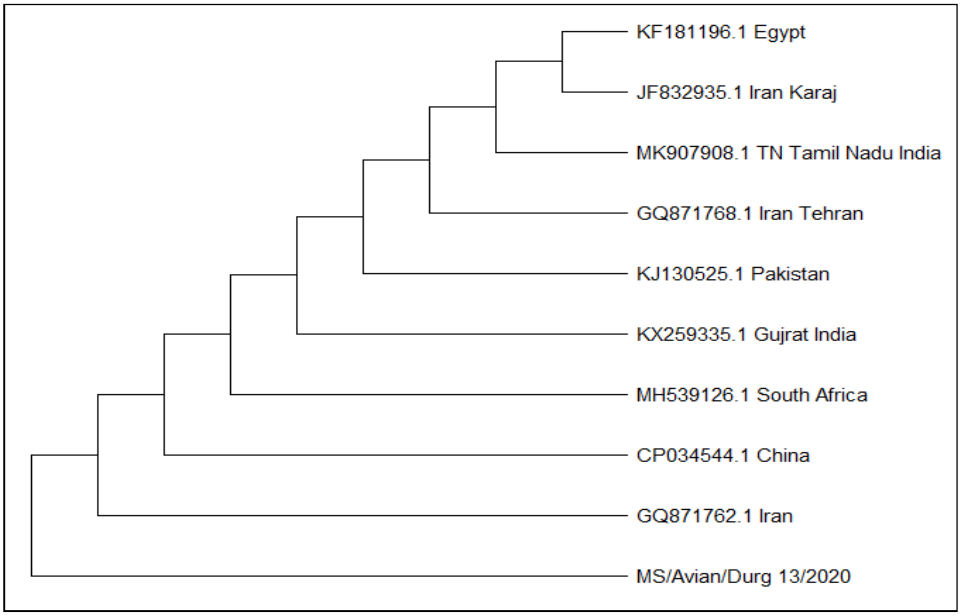

Fig 3: Phylogenetic tree showing relationship among MS/Avian/Durg 13/2020 along with other sequence retrieved from NCBI GenBank.

Liver showed congestion and mild haemorrhages. Mild degenerative changes along with leukocyte infiltration were also noted in positive cases. Kidney samples showed necrotic and degenerative changes in tubular as well as glomerular area of the kidneys along with varying degree of oedema (Fig 7). There was disruption and sloughing of tubular epithelium recorded which ultimately filled into the lumen of the tubules making them blocked. There was infiltration of mononuclear cells also been documented in certain cases. Fibrous tissue deposition was found at moderate amount in severe cases of MS in chicken's kidneys.

Our results showed observational similarities with the findings of Kleven, (2003), Ley, (2003), Saif (2013) and Bagal *et al*. (2019).

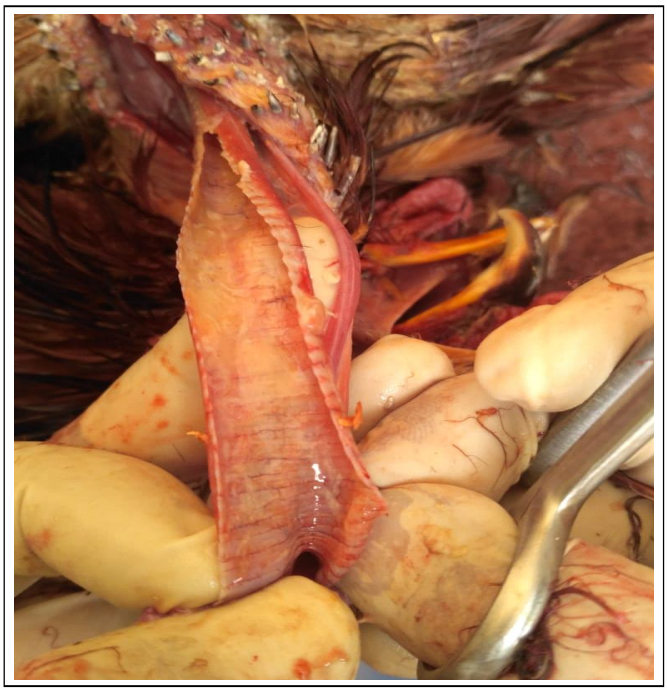

Fig 4: Trachea of a MS positive bird showing deposition of catarrhal exudate in the tracheal lumen.

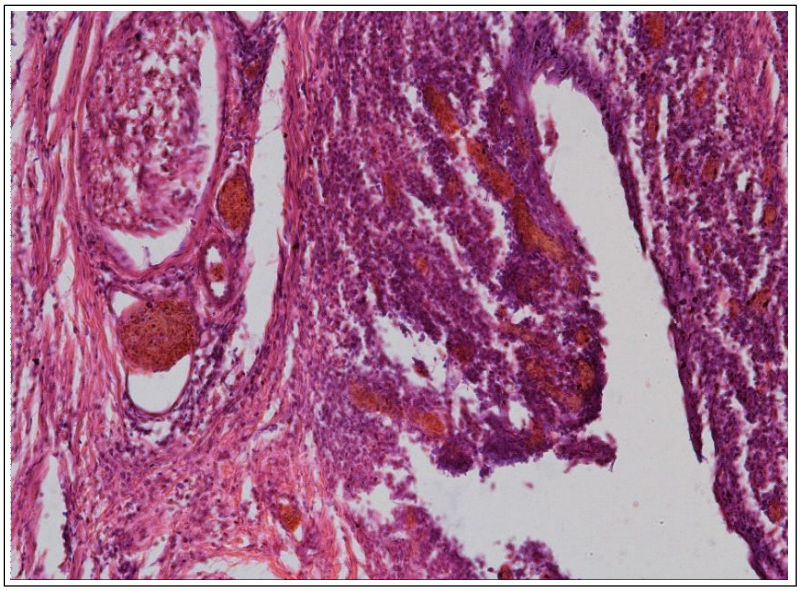

Fig 5: Microscopic image of nasal cavity from MS positive bird showing congestion (H&E X400).

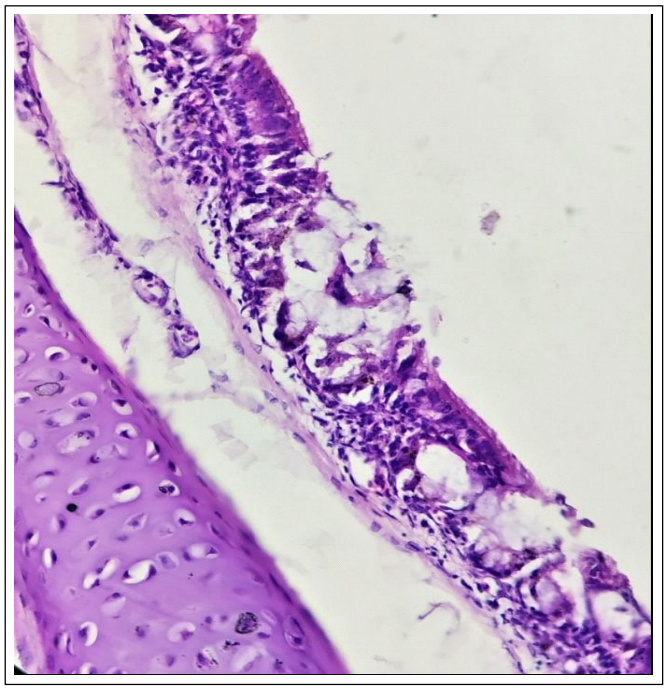

Fig 6: Microscopic image of trachea from MS positive bird showing thickening of mucous membrane along with over production of mucous (H&E X400).

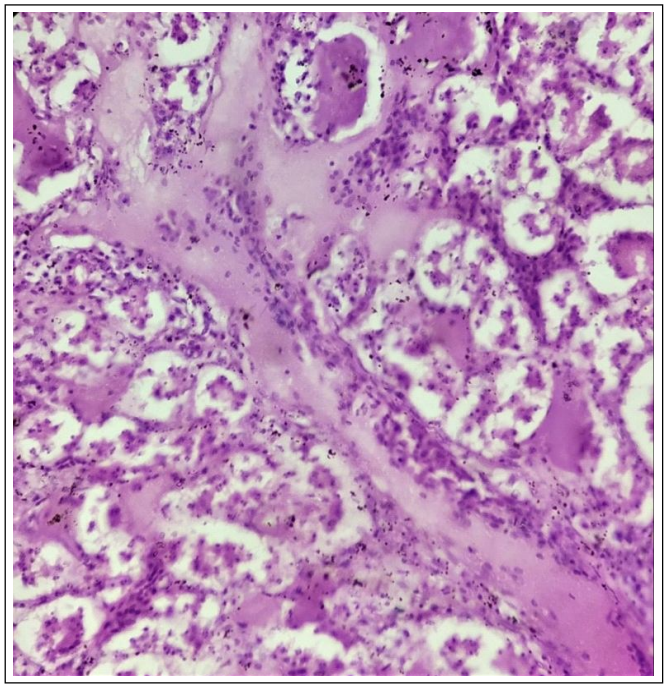

Fig 7: Microscopic image of MS positive bird showing mononuclear cell infiltration along with oedema in kidney tissue (H&E X400).

CONCLUSION

Molecular and phylogenic analysis revealed that the Chhattisgarh isolate of MS were closely aligned to the isolates of China and Iran. The migratory birds around the world may play a big role in spreading the infectious agent from one area to another and this was validated in Eastern North America in which wild birds have been found to carry MG infection. MS proven to cause various inflammatory, degenerative and necrotic changes in the various visceral organs of poultry.

Conflict of interest: None.

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