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Molecular Typing of Indian *Mycoplasma synoviae* Isolates

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ABSTRACT

Background: *Mycoplasma synoviae* is an economically significant pathogen in poultry with reported increased prevalence and virulence in recent years. The pathogen causes subclinical upper respiratory tract infection, air sacculitis and infectious synovitis.

Methods: This study aimed to characterize the field isolates of *M. synoviae*, from healthy and diseased birds of 13 poultry flocks of five states of India by DNA sequence analysis of the conserved 52 region of variable lipoprotein and haemagglutinin (*vlhA*) gene.

Result: Phylogenetic analysis revealed 7 sequence types. Single sequence type was found in three major states for commercial poultry production, which could be due to interstate transport of birds or chicks.Typing based on nucleotide insertion/deletion in the proline-rich repeat (PRR) region and the nucleotide polymorphisms of the RIII region of the 52 region of the *vlh*A identified field isolates as Type C (n=8), E (n=6) and L (n=5). Subtypes identified were C1 (n=3) and C3 (n=1) and others were novel. Most of the isolates were from birds having respiratory lesions or symptoms. Synovitis cases were very rare and the isolate from synovitis was found to be of subtype C3. Further, the presence of multiple types (C1, E and L) was found in one farm even though most of the farms were affected by only single type. Molecular typing of Indian *M. synoviae* isolateswas conducted for the first time to map the diversity among different Indian isolates from different regions and from different clinical conditions.

Key words: *M. synoviae,* Nucleotide polymorphism, Phylogenetic analysis, Poultry, Proline rich repeat, *vlhA* gene.

INTRODUCTION

Mycoplasma synoviae is an economically significant pathogen of poultry reported worldwide. It is mostly associated with subclinical respiratory infection in poultry. Co-infection with respiratory bacteria and viruses causes air sacculitis (Kleven *et al.* 1972). Respiratory form of infection is highly common as compared to other forms and its severity is affected by concurrent infection with other respiratory pathogens (Dijkman *et al.* 2014). Systemic infection results in infectious synovitis in poultry and turkey. Another condition caused by this pathogen is eggshell apex abnormality.

The prevalence of *M. synoviae* in the Indian poultry industry is well documented (Baksi *et al.* 2016; Sumitha and Sukumar, 2017; Rajkumar *et al.* 2018). Previous study shows a higher prevalence of *M. synoviae* than *M. gallisepticum* in poultry flocks in India (Rajkumar *et al.* 2018). Maintenance of Mycoplasma free flock is highly difficult in multi-aged farms and mortality with lesions of airsacculitis are common during adverse weather conditions like summer and winter. Antibiotic treatment is generally used for control of disease due to Mycoplasma in India. Despite the wide prevalence of this pathogen in India, not much has been done for the genetic data analysis and molecular typing of the Indian isolates.

The immune dominant variable lipoprotein haemagglutinin encoded by the *vlhA* gene which shows antigenic variation which helps in binding with host cell receptors and colonisation (Razin *et al.* 1998; Bercic *et al.* 2008). The *vlhA* gene has a conserved and variable region. The conserved 5' end of the *vlhA* gene (nucleotides 1-410) encoding for major surface protein B (MSPB), occurs in a single chromosomal copy, where as the remaining coding sequence occurs as multiple copies (Noormohammadi *et al.* 2000). Antigenic variation of vlhA protein occurs due to recombination of 3′ end of the expressed *vlhA* gene with one of the multiple pseudogenes. The conserved 5′ end region is a highly polymorphic region encoding the Proline Rich Repeats (PRR) in the N-terminal part of MSPB protein. Isolates varies in the length of the PRR. Studies have suggested length of the PRR is associated with invasiveness (Bencina *et al.* 2001).

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MATERIALS AND METHODS

Isolation and identification of *M. synoviae*

Tracheal/choanal cleft/oviduct/Hock joint swabs were collected from carcasses showing respiratory lesions or arthritis during routine postmortem examination at an organized poultry farm at Hyderabad, Telangana State. Also, tracheal/choanal cleft swabs from live birds were collected from poultry flocks from 7 Indian states as per the guidelines

of the Institutional Animal Ethics Committee (IAEC) during the period from March 2013 to February 2014. None of the flocks were vaccinated against *M. synoviae.* The activities were carried out at Avian Health Lab, ICAR-Directorate of Poultry Research, Hyderabad. *M. synoviae* was isolated in Frey's medium as described by Kleven (2003) and DNA was isolated using QIAamp, DNA mini kit (QIAGEN, Germany) and were screened for the presence of *M. synoviae* using MSF-MSR primer pair (International Office of Epizootics and Committee2008). A total of 817 swab samples were screened by PCR and from positive samples, 19 field isolates (details are given in Table 1) from 5 states and from carcasses showing respiratory lesions, salpingitis and arthritis were selected for gene targeted sequence analysis.

PCR amplification, sequencing, phylogenetic analysis and typing

The *vlhA* gene was amplified by PCR as per El Gazzar *et al.* (2012) and was sequenced. The sequences of the single copy conserved region at the 5' end of *vlhA* gene from all the isolates and reference strains were trimmed from nucleotide 24 to 376 of the sequence of reference strain WVU1853 (Genbank accession number KC832824) and was used for phylogenetic analysis. The details of the reference strains used are given in the Table 2. The nucleotide sequence editing was accomplished using DNASTAR (Madison, WI). Evolutionary analyses were conducted in MEGA X (Kumar *et al.* 2018). Molecular Phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Isolates were typed and subtyped based on nucleotide and corresponding amino acid insertion/deletion in the proline-rich repeat (PRR) region and the nucleotide polymorphisms of the RIII region of the 52 region of the *vlh*A (Bencina *et al.* 2001; Hammond *et al.* 2009; Limpavithayakul *et al.* 2016).

RESULTS AND DISCUSSION

Partial sequence analysis of *vlhA* genes of field isolates showed the presence of variations and the grouping based on 100% similarity placed the isolates into 7 sequence types (Table 1). Nucleotide sequence similarity between these sequence types varied from 92.8% to 98.3%. One reason for the occurrence of variation can be that Mycoplasma species have a high percentage of mutations when compared to other bacteria (Woese *et al.*1985; Heldtander *et al.* 2001). Phylogenetic tree was constructed using nucleotide sequences of 19 field isolates and 12 reference strains of *M. synoviae*. Phylogenetic analysis showed clustering of field isolates in 4 groups (Fig 1). The cluster I included sequence types 1 and 2 (Hyderabad) and one reference strain SP 267 from Spain. The cluster II consisted of sequence types 3 and 4 from Kolkata and Bengaluru respectively. Cluster III included one novel field isolate MGS 482, which had only 95.2-98.3% similarity with other 18 field isolates, but had 99.1% similarity with MS H vaccine strain. The cluster IV had sequence types5 (MGS 996 from Hyderabad, 1336 and 1342 from Namakkal) and 6 (MGS 13B from Bengaluru). They had 100% identity with that of reference strain ULB 925KF (Slovenia). By performing

Table 1: Sample details and molecular characteristics of *M. synoviae* field isolates used in this study.

Isolate Id	Place of isolation	Clinical condition/PM lesion	*vlhA* gene PRR (nt)	Size of PRR (AA)	*vlhA* Genotype andsubtype	Sequence type based on similarity	GenBank accession
MGS 211	Hyderabad	Airsacculitis, tracheitis	105	35	L	1	KR232800
MGS 543	Hyderabad	Healthy bird	105	35	L	1	KR232817
MGS 931	Hyderabad	Airsacculitis, salpingitis	105	35	L	1	KR232806
MGS 949	Hyderabad	Airsacculitis, infraorbital sinus swelling	105	35	L	1	KR232807
MGS 996	Hyderabad	Airsacculitis, hock joint swelling	96	32	C1	5	KR232801
MGS 1225	Hyderabad	Airsacculitis, tracheitis	105	35	L	1	KR232802
MGS 1330	Hyderabad	Airsacculitis, pneumonia	57	19	E	2	KR232808
MGS 1333	Hyderabad	Airsacculitis, tracheitis	57	19	E	2	KR232809
MGS 482	Palampur	Healthy bird	96	32	C3	7	KR232811
MGS 248	Kolkatha	Healthy bird	96	32	C	3	KR232803
MGS 250	Kolkatha	Healthy bird	96	32	C	3	KR232818
MGS 254	Kolkatha	Healthy bird	96	32	C	3	KR232804
MGS 264	Kolkatha	Healthy bird	96	32	C	3	KR232812
MGS 2B	Bengaluru	Respiratory signs	57	19	E	4	KR232813
MGS 4B	Bengaluru	Respiratory signs	57	19	E	4	KR232814
MGS 5B	Bengaluru	Respiratory signs	57	19	E	4	KR232815
MGS 13B	Bengaluru	Respiratory signs	57	19	E	6	KR232816
MGS1336	Namakkal	Healthy bird	96	32	C1	5	KR232805
MGS1342	Namakkal	Healthy bird	96	32	C1	5	KR232810

phylogenetic analysis of the *vlhA* gene, we observed that most of the field isolates clustered independently from foreign strains except for sequence type 5 which was 100% identical to ULB 925KF. Hundred percent sequence similarity with foreign strains suggest that they may have originated from a common global strain. Identical types 5 and 6 were obtained from poultry farms of Telangana, Tamil Nadu and Karnataka, three important states for commercial poultry production in India. The culling and replacement of poultry flocks and transport of birds for meat purpose may be an important reason for the prevalence of the same strain in the southern states as suggested by Buim *et al.* (2010).

There was apparent deletion of large DNA segments, so that the sequence types in same cluster were identical except for the region of deletion. For example, in cluster II, sequence type 3 and 4 are 100% identical, but type 4 has a deletion of 60 nucleotides and in cluster III as compared to type 3, sequence type 5 and 6 are 100% identical, but type 6 has a deletion of 48 nucleotides. It could be due to that both originated from a single strain and one had undergone deletions due to passage in field or both types entirely two different strains. Similar deletions were described by Hammond *et al.* (2009) in isolates from a single farm. These insertions or deletions in *vlhA* gene observed in the isolates may be also be related to pathogenicity (Bencina *et al.* 2001).

Insertions or deletions in the PRR and nucleotide polymorhysms in RIII region are highly useful for epidemiological analysis of *M. synoviae* isolates (Bencina *et al.* 2001; Limpavithayakul *et al.* 2016). In our study, the 19 field isolates were classified into 3 genotypes C (n=8), E (n=6) and L (n=5) based on the length of the PRR (Table 1). Based on the point mutations in the RIII region, the subtypes identified were C1 (n=3) and C3 (n=1) and other subtypes were novel. Alignment of the amino acid sequence of the PRR is given in Fig 2. All the types encoded a conserved signal peptide sequence (IAPAVIAIS). The first eight AAs of

Table 2: Details of the reference sequences used for phylogenetic analysis of *vlhA* gene of *M. synoviae.*

Name of strain	Country of isolation	Accession number
MSG 398	India	JX855829
MSG 508	India	JX855831
MSG 510	India	JX855832
K1968	USA	AF314230
WVU1853	USA	KC832824
MS H	Australia	AF464936
94046 W1B-17a	Australia	AY913827
ULB925KF	Slovenia	AF314228
B48/05	UK	FM164344
SP267	Spain	FN666088
AMS 8	Austria	KC832807
B47/06	Slovakia	FM164373
B3/98	Holland	FM164354
M2008.13	France	KJ606947
MZ3	Israel	KC832820
FMT	Israel	KC832825
MSR25	Iran	JX960390
EGY.Ras.joint.4	Egypt	KT957968
NZMSID 182	Japan	AB507392
WT 37	Japan	AB501278
WT60	Japan	AB501281
AHRU2015CU1303.1	Thailand	KX168683
AHRU2014CU5801.2	Thailand	KX168685
AHRU2015CU2807.1	Thailand	KX168693
CHN-FJCXZ2-2-2013	China	KU572366
CHN-QZ114-1-2013	China	KU572389
CK.MS.UDL.PK.2014.5	Pakistan	KP316021
CBU090710	South Korea	KM986004

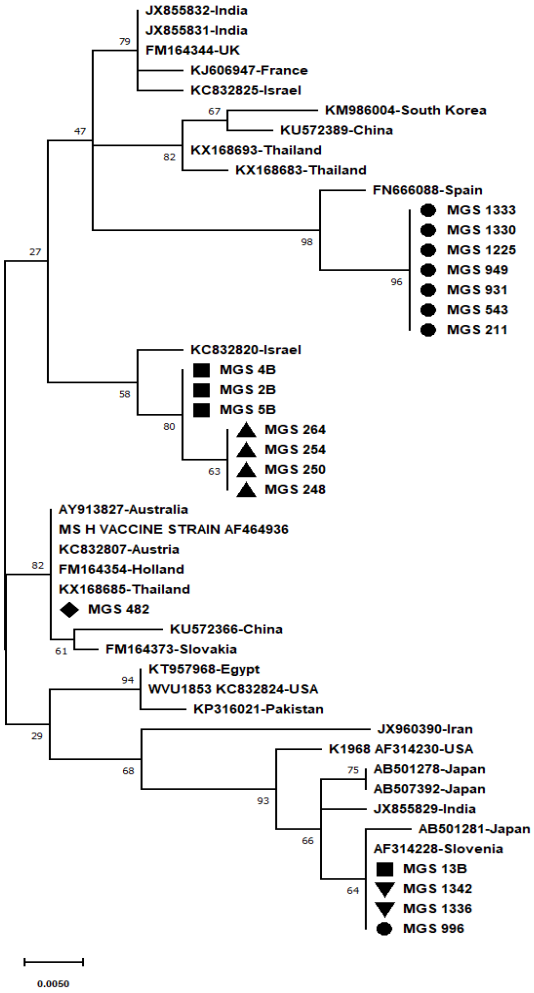

Fig 1: Dendrogram of field and reference strains of *M. synoviae* isolates constructed by clustal-W alignment of *vlhA* gene by the maximum likely hood method with 1000 bootstrap replicates using MEGA X (Kumar *et al.*, 2018). It includes 19 Indian field isolates and 12 reference strains. The solid labels shows field isolates of different place of isolation (square-Bengaluru, circle-Hyderabad, upward triangle-Kolkata, diamond-Palampur and downward triangle-Namakkal). Empty squares represent the foreign reference strains and empty triangles represent sequences from India.

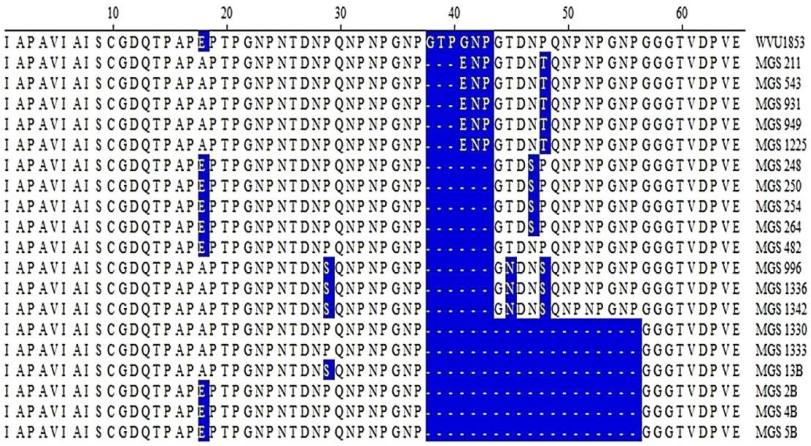

Fig 2: Alignment of amino acid sequence of field isolates representing the polymorphism of the PRR regionas compared to reference strain WVU 1853 corresponding to nt position 73 to 267 in the vlhA sequence (GenBank accession number AF035624). Types E, L and C had deletions of 19, 3 and 6 amino acids respectively.

their predicted MSPBs had an identical sequence (CGDQTPAP). Isolates from Hyderabad belonged to types L, C1 and E. Isolates from Bengaluru, Kolkata and Namakkal belonged to types E, C and C1 respectively. Types E, L and C had deletions of 19, 3 and 6 amino acids respectively as compared to the reference sequence of strain WVU 1853. Bencina *et al.* (2001) described that the highly invasive strain K1968 is of type B, having a long PRR and the length of PRR can be associated with invasiveness of the isolate. In the present study most of the isolates were from birds with only respiratory lesions or symptoms. Synovitis cases were very rare and the isolate from synovitis (MGS996) was found to be of subtype C1. Sequence analysis showed that this isolate was 100% identical to strain ULB925 (AF314228) which was also isolated from chicken joint (Bencina *et al.* 2001). Multiple types (C1, E and L) were found in one particular farm and all other farms were affected by only single type.

CONCLUSION

The frequency of *M. synoviae* infection has been found increasing worldwide as compared to *Mycoplasma gallisepticum,* the most important avian Mycoplasma (Landman, 2014). The differentiation of prevalent strains is necessary for tracing the spread of the pathogen. This study provides the first molecular typing and subtyping of Indian *M. synoviae* isolates. The genetic analysis showed that most of the farms had single sequence type, but one particular sequence type was found in three major states for commercial poultry production, which could be due to interstate transport of birds or chicks. Also there was 100% identity between few Indian isolates and a foreign strain. The isolates from different clinical conditions (salpingitis, arthritis and air sacculitis) did not show any variation in the sequences. Variation was also evident from the length of the *vlhA* gene region encoding PRR. The prevalence of *M. synoviae* in Indian poultry flocks along with the results of the present study insists the urgent need for implementation of effective prophylactic measures, such as application of proper biosecurity measures and vaccination to control the *M. synoviae* infection in Indian poultry flocks.

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Conflict of interest statement

There is no conflict of interest among authors and there is no financial or personal relationship between authors and other organizations which that might inappropriately influence or bias their work.

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