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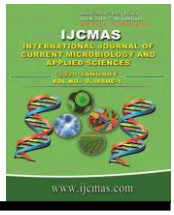

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Molecular and Phylogenetic Analysis of *Mycoplasma gallisepticum* in Haryana, India

Vaishali[1*], Davinder Singh[2] and Renu Gupta[1]

[1]Department of Veterinary Public Health and Epidemiology, LUVAS, Hisar, India
[2]Extension Specialist, Directorate of Extension Education, LUVAS, Hisar, India
**Corresponding author*

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A B S T R A C T

Avian mycoplasmosis is responsible for heavy economic loses to the poultry industry in India. It results from a complex interaction of various factors like viral, bacterial and housing conditions. *Mycoplasma gallisepticum* (MG) belongs to class Mollicutes is small in size, lacks bacterial cell wall with minimum genetic information. It is the principle pathogenic agent causing Chronic Respiratory disease (CRD) in chickens and infectious sinusitis in Turkeys and has a worldwide distribution. The objective of this study was molecular detection of *Mycoplasma gallisepticum* in poultry with respiratory affections in Haryana (India). 100 tissue samples including trachea, lungs and air sacs were collected and pooled from 100 different poultry flocks in different districts of Haryana. The samples were screened by Polymerase chain reaction (PCR) for *Mycoplasma gallisepticum.* A prevalence of 16% (16/100) was seen for *Mycoplasma gallisepticum* by using 16S to 23 S rRNA primer. PCR can be used for a simple and quick way to identify *Mycoplasma gallisepticum* from field samples.

Introduction

The most common causes of high mortality in poultry birds is due to respiratory distress, heat stress and *E.coli* infections. Avian mycoplasmosis is one of the most prevalent poultry problem affecting the industry economically. The Respiratory disease complex has contributed to heavy economic loses and it comprises cluster of factors responsible for the spread of disease and affecting mortality among the birds comprising of bird handling, viral, mycoplasma and various environmental factors like temperature, high ammonia concentration and others. *Mycoplasma gallisepticum* is the principle pathogenic agent causing Chronic Respiratory diseases and causes heavy mortality. Dodd in 1995 gave the first accurate description of *Mycoplasma gallisepticum* in turkey and it was known as epizootic penumoenteritis (Dodd 1995). Existence of disease related with *M. gallisepticum* were depicted in both

chicken and turkey and the harmful impacts were decreased with proper and synchronised management on the farms. Within intensive poultry farming, infection by CRD is found in association with avian influenza, Newcastle disease, colibacillosis and infectious bronchitis and further leads to more severe problems (Stipkovits *et al*., 2012).It is the principle pathogenic agent causing Chronic Respiratory disease (CRD) in chickens and infectious sinusitis in Turkeys and has a worldwide distribution (Li *et al*., 2010).

Mycoplasma gallisepticum infections vary from asymptomatic to severe symptoms like reduced feed efficiency, reduced egg production and decreased growth rate (Sarkar *et al.*, 2005). It is characterized by coughing, nasal discharge, rales and severe lesionson air sacs commonly air sacculitis (Tomar *et al*., 2017). Grossly, thickened air sacs with caseous or mucous exudate, catarrhal inflammation in bronchi, trachea and sinuses, fibrinous perihepatitis, interstitial pneumonia and adhesive pericarditis (Yamamoto, 1991; Charlton *et al*., 1996). There has been a horizontal and vertical transmission documentation (Kumaragurubaran *et al*., 2018).

Material and Methods

Sample Collection

A total of 100 samples were collected from seven different districts of Haryana. Bhiwani (n=2), Hisar (n=9), Jhajjar (n=19), Jind (n=20), Karnal (n=20), Panipat (n=23) and Sonepat (n=7). SonepatThe samples were collected from October 2018 to Feburary 2019. Trachea, lungs and air sacs were collected from poultry flocks and were pooled, and together known as "sample".

DNA Extraction

DNA was extracted from directly from tissues collected from various poultry farms using DNA extraction mini kit (QIAmp mini kit) as recommended by the manufacturer.

Gene specific PCR

PCR was carried out on 100 pooled tissue samples from various poultry flocks. 16 to 23 S rRNA PCR specific to *Mycoplasma gallisepticum* was per the protocols described by Ravi*et al*., 2007). The primer selected for *M. gallisepticum* for 16 to 23 S rRNA was; F-5' GTAGGGCCGGT GATTGGAGTTA3' and R-5' CCCGTAGCATTTCGCAGGTTTG 3' and the size of the amplified product was 810bp. The positive control used for *Mycoplasma gallisepticum* was S-6 serotype antigen (Salsbury laboratories, U.K.).

The protocol of Ravi*et al*. (2007) was used with certain modifications. The initial denaturation was achieved at 94 °C for 10 min with Sapphire fast PCR- hot start master mix. It was further followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 62°C for 30 sec and extension at 72 °C for 45 sec. The final extension was achieved at 72°C for 10 min.

Analysis of PCR product

The amplified PCR products were analysed by agarose gel electrophoresis using 2% agarose containing 0.5 µg/ml ethidium bromide in tris-borate EDTA (TBE) buffer and visualized under UV trans illuminator, as per the method of Sambrooke*et al*., (1989).

The amplified DNA product was examined by comparison with standard DNA marker (100 bp DNA ladder, Takara dye plus). The image of gel was obtained using gel documentation system (Alpha Imager). PCR amplicons of the positive samples were purified as per the protocols recommended by MACHEREY-NAGEL NucleoSpin Gel and PCR Clean-UP.

Nucleotide Sequencing, Phylogenetic Analysis and Accession number

The PCR products of *M. gallisepticum* were purified and sent for sequencing at DNA sequencing facility of Department of Animal Biotechnology, College of Veterinary Sciences, LUVAS, Hisar. The nucleotide sequencing was done by Automated DNA sequencer, Applied Biosystem 3130 XL Genetic analyser in both directions.

The sequences were trimmed and analysed in MeAlign program (Lasergene, DNASTAR). The sequences were aligned by ClustalW software. The nucleotide sequences obtained for *Mycoplasma gallisepticum* (16 to 23 S rRNA) by using Mega 7.0.26 (Molecular Evolutionary Genetic Analysis). They were aligned by ClustalW. The phylogenetic tree was made by maximum likelihood tree using 1000 bootstrap value. The sequences reported in this article have been deposited in the GenBank database under accession number MK922122.

Results and Discussion

16/100 (16%) samples were found to be positive for *Mycoplasma gallisepticum* by Polymerase Chain Reaction.

Mycoplasma gallisepticum 16S-23S rRNA intergenic spacer region

Phylogenetic analysis carried out by using the sequence of the present study with 19 other sequences (Table 2) and is depicted in Figure 2.

The sequences were in the same clad along with other sequence reported from India (KX759108) and other countries Japan (AB098504), USA (KC247863, AY744942, JN935873). The sequences from other countries were present in different clads including Brazil (KT824823, KJ019166), Egypt (GU357708), Germany (KP710079), South Africa (MF196171) and USA (KJ468424, KJ468432). It was very divergent from South Africa (KY362223).

Fig.1 Agarose gel showing *Mycoplasma gallisepticum* from poultry field samples. M- 100 bp ladder; 1-8- samples; 9- negative control.

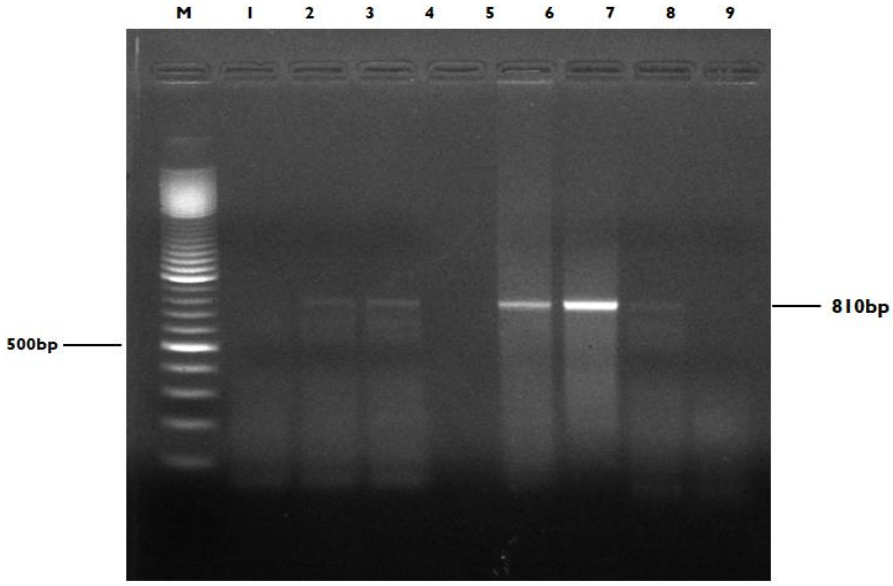

Fig.2 Phylogenetic tree based on partial nucleotide sequences of 16S-23S rRNA IGSR gene of *Mycoplasma gallisepticum*. Phylogenetic tree constructed by the maximum likelihood tree method using 1000 bootstrap replicates value in Mega7.0 software.

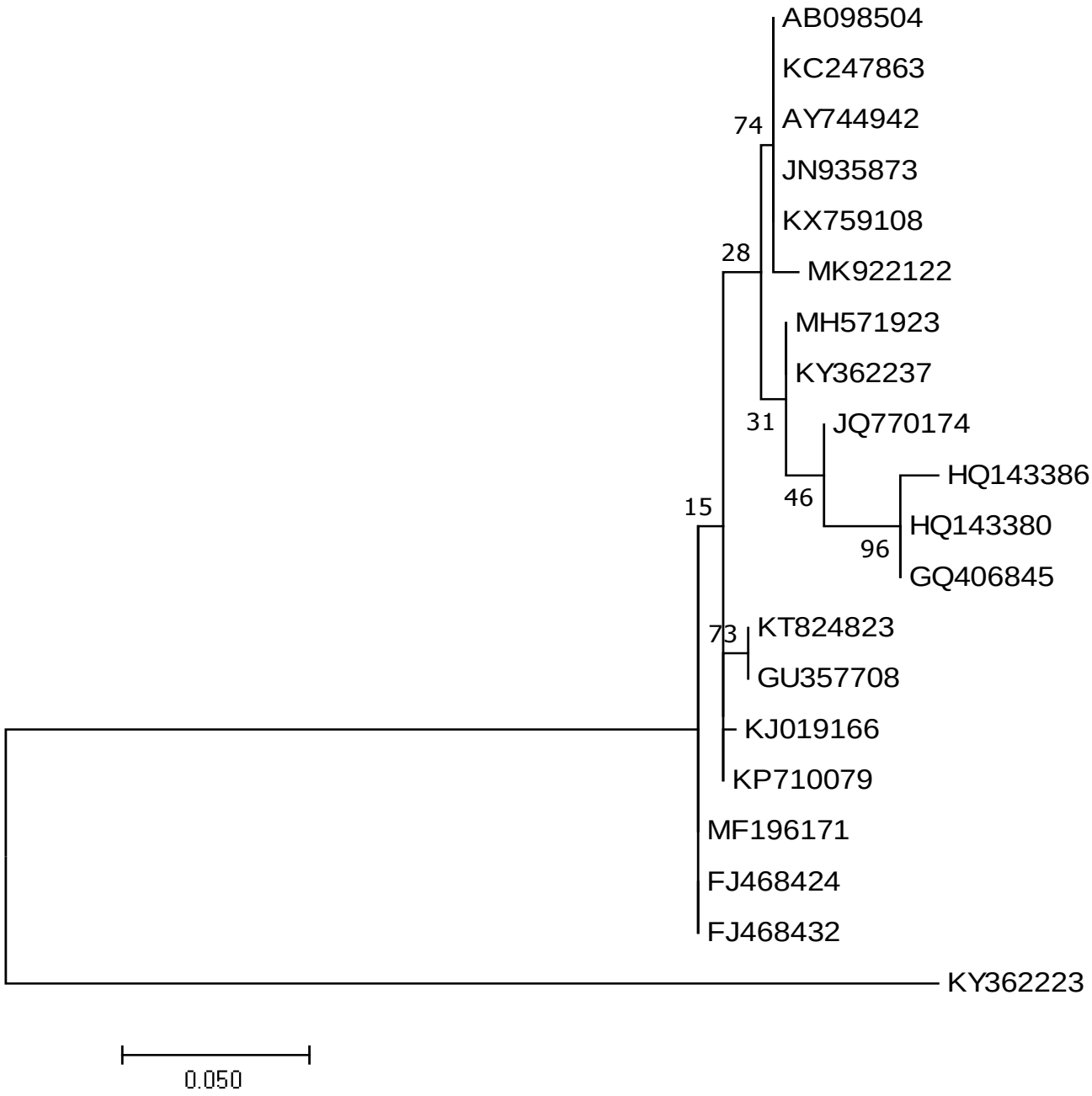

Table.1 Accession number of various isolates obtained for *Mycoplasma gallisepticum*

Accession number	Year	Country	Strain/Host
MK922122	2019	Haryana, India	Lungs, trachea, air sacs
AB098504	2002	Japan	PG31
KC247863	2012	USA	USA/R/CK60
AY744942	2004	USA	ATCC19610
JN935873	2011	Rockville, USA	PG31CX95
KX759108	2016	Haryana, India	PT-89
MH571923	2018	South Africa	B2777-15A-8
KY362237	2016	South Africa	ZA_MGB1932_15
JQ770174	2012	Australia	6-85
HQ143386	2010	Jordan	OR/83/CKB
HQ143380	2010	Egypt	EGY/67240/CK08
GQ406845	2009	Egypt	RabE2-09
KT824823	2015	Brazil	BRA/UFF/LSA020
GU357708	2008	Egypt	RabE4-08
KJ019166	2011	Brazil	2011/UFMG1
KP710079	2011	Germany	2038/11/CK
MF196171	2017	South Africa	B878-14-M2_157
KJ468424	2014	USA	LVT 9815
KJ468432	2014	USA	LVT 9815
KY362223	2016	South Africa	ZA_MG B642_08

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