MOLECULAR DETECTION OF *Mycoplasma synoviae* IN POULTRY

THESIS

Submitted
in partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY MICROBIOLOGY

BY
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Enrolment No : V/14/302

BOMBAY VETERINARY COLLEGE, MUMBAI

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(INDIA)
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DECLARATION OF THE STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled **“MOLECULAR DETECTION OF *Mycoplasma synoviae* IN POULTRY”** or part thereof has not been submitted for any of the other degree or diploma of any university, nor the data have been derived from any thesis or publications of any university or scientific organization. The sources of material used and all assistance received during the course of investigation have been duly acknowledged.

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Place: Mumbai

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Mr. PADAM LAL LUITEL has satisfactorily prosecuted his course of research work for a period of not less than one semester and that the thesis entitled **"MOLECULAR DETECTION OF *Mycoplasma synoviae* IN POULTRY"** submitted by him is the result of research work is sufficient to warrant its presentation to the examination in subject of **VETERINARY MICROBIOLOGY** for the award of **MASTER OF VETERINARY SCIENCE** (**M.V.Sc)** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur .

We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any other university.

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CERTIFICATE

This is to certify that the thesis entitled **"MOLECULAR DETECTION OF *Mycoplasma synoviae* IN POULTRY"** submitted by **Mr**. **PADAM LAL LUITEL** to the Maharashtra Animal Sciences University, Nagpur, in partial fulfillment of the requirement for the degree of **MASTER OF VETERINARY SCIENCES (M.V.Sc)** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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Date: *Padam Lal Luitel*

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LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
ATCC	American type culture collection
bp	Base pair
DNA	Deoxyribonucleoside Acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
et al	Et alia (and others)
HI	Hemagglutination-Inhibition Test (Hi)
ISRs	Intergenic Spacer Regions
MG	*Mycoplasma gallisepticum*
MR	Methyl red
MS	*Mycoplasma synoviae*
MSPA	*Mycoplasma synoviae* Protein *A*
MSPB	*Mycoplasma synoviae* Protein *B*
ND	Newcastle Disease
NAD	Nicotinamideadenine dinucleotide
MW	Molecular weight
OIE	Office International des Epizooties
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Log of hydrogen ion concentration
PPLO	Pleuropneumonia like organism
RNA	Ribonucleic acid
RAPD	Random Amplication Of Polymorphic DNA
SPA	Serum Plate Agglutination
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
vlhA	Variable lipoprotein Hemagglutinin A

UNITS OF MEASUREMENT

% : Per cent

°c : Degree Celsius

µg : Microgram (s)

µl : Microliter

µm : Micrometer

gm : Gram

h : Hour

I.U. : International unit

M : Molar

Mb : Mega base pairs

mg : Milligram

min : Minute (s)

ml : Millilitre

mm : Millimeters

mM : Millimolar

nm : Nanometers

pmole : Picomole (s)

rpm : Revolutions per minute

Sec : Second (s)

Taq : *Thermus aquaticus*

U : Unit

1. INTRODUCTION

Mycoplasmas are the smallest, wall less bacteria that are capable of replicating outside the cells. They belong to class Mollicutes (division Tenericutes) of order I, Mycoplasmatales. Mycoplasmas have small genome of 580-1350 kb with G+C content of 23-40%, require cholesterol for growth and have usual optimum growth temperature of 37^0C. To keep the parasitic mode of life, mycoplasmas have developed rather sophisticated mechanisms to colonize their hosts and resist the host immune system (Razin *et al.,* 1998). They infect humans and animals as well and there are more than 100 species (Razin *et al*., 1998; Brown *et al.,* 2007). *Mycoplasma gallisepticum* and *M. synoviae* are pathogenic species for chickens and turkeys (Ley and Yoder., 1997).

Mycoplasma synoviae belongs to the Mycoplasmataceae family and is a facultative anaerobic bacterium (Salisch *et al.,* 1998). MS requires Nicotinamide Adenine Dinucleotide (NAD) and cysteine hydrochloride as additional requirement due to complex nutritional requirements unlike other avian mycoplasmas. Chickens, turkeys and guinea fowls are the natural hosts of MS but ducks, geese, pigeons, house sparrows, pheasants and Japanese quail may also be susceptible. MS is prevalent in 35 poultry producing countries and infection occurs frequently in multi-age commercial layer complexes (Opitz *et al*., 1983; Mohammed *et al.,* 1986.).

M. synoviae (MS) is an important pathogen of domestic poultry, causing economic losses to the poultry industry. The economic losses are due to decreased egg production, growth and hatchability rates and significant downgrading of carcasses due to airsacculitis and arthritis lesions (Kleven *et al.,* 2003). MS was first described (Olson *et al*., 1956) as a major pathogen of chickens and turkeys and causes respiratory tract infection and synovitis worldwide (Kleven, 1997). Most of the damages resulting from Mycoplasma species infections in humans and animals are due to host immune and inflammatory responses rather than to direct deadly effects of Mycoplasma virulence factors. Different MS strains are characterized by differences in infectivity, tissue tropism and pathogenicity (Razin *et al*., 1998). Infection most frequently occurs as a subclinical upper respiratory infection, which can progress to respiratory tract disease with air sac lesions when impaired by other respiratory pathogens (e.g., Newcastle disease virus, Infectious Bronchitis virus) or when more virulent MS strains are involved (Kleven *et al.,* 1975; Lockaby *et al.,* 1999). The infection can also become systemic and result in acute to chronic infectious synovitis (Kleven *et al.,* 2003). MS is mostly involved in asymptomatic infections (Stipkovits and Kempf, 1996). Besides, MS has also been proven to affect the humoral response of chicks vaccinated with a La-Sota strain of Newcastle disease (ND) virus. Protection of MS- infected and ND-vaccinated broilers is induced only after a second dose of ND-vaccine (Nascimento *et al*., 2003; Silva, 2003).

Due to the expansion of poultry production and concentration of large, multiage production complexes in restricted geographic area, it is becoming more and more difficult to maintain flocks free of MS. The role of MS in egg production of commercial layers is questionable. Previous studies have revealed that MS infection is rather common in commercial layers (Mohammed *et al*., 1986; Hagan *et al*., 2004; Fabienne *et al*., 2006). In the US table egg industry, most pullets are MS free in the rearing phase but become infected with MS after their introduction into the multiage laying groups. In poultry, avian mycoplasmosis is spread vertically through infected eggs and horizontally by close contact.

The pathogenicity mechanisms of MS involve attachment and colonization of the upper respiratory tract plus additional unidentified factors associated with systemic invasion and lesion production. MS has two major surface antigens, MSPA and MSPB and it has shown that MSPA is a haemagglutinin and MSPB is a lipoprotein (Noormohammadi *et al.,* 1997). These antigens are both encoded by a single gene, *vlhA* (*variable lipoprotein and haemagglutinin*), possibly with post-translational cleavage generating the two proteins. The coding sequence of the *vlhA* gene is homologous to those for the *vlhA* genes of MG, even though MS is phylogenically distant from MG (Noormohammadi *et al*., 1998).

As the vertical transmission plays a major role in the spread of *M. synoviae* in chickens, the most effective control method is to monitor the flocks regularly and also eliminate the positive breeder flocks. Reliable and rapid diagnoses are needed to prevent infection dissemination (Seifi *et al*., 2012). Positive diagnosis may be made by isolation and identification of *M. synoviae*

(Kleven, 2003) but isolation and cultivation of *M. synoviae* is very tedious, difficult because of its fastidious nature and complex nutritional requirement and may not give the accurate prevalence (Bayatzadeh *et al*. 2011). Polymerase chain reaction is a simple, rapid and highly sensitive method of detection of MS-DNA in tissues or culture medium; moreover PCR procedure is comparable in sensitivity to isolation and identification (Salisch *et al*., 1998; Feberwee *et al.,* 2005). The Polymerase Chain Reaction (PCR) with Mycoplasma *16S ribosomal RNA* has been applied to detect a variety of *Mycoplasma* species (Kojima, 1997; Zendulkova, 2007). The rRNA is naturally present in high copy numbers (up to 10'000 molecules per cell) (Waters *et al.,* 1990). Ehtisham-ul-Haque *et al.,* (2011) compared conventional isolation technique with PCR for identification of MS infecting broiler birds. Recently several PCR assays have been used for the amplification of the conserved *vlhA gene* of MS (Amy *et al*., 2010).

Diagnostics of *Mycoplasma synoviae* (MS) is generally based on serological, culturing and molecular methods. Rapid diagnosis and identification of infections is very important in the poultry industry. The use of PCR makes it possible to shorten the time for obtaining result from research and the effective detection of genetic material of MS. There are many variations of the PCR method; few of them are nested, simplex, multiplex etc.

High number of false positive result in ELISA and SPA may occur due to cross reactions, lack of inactivation, age of the flock and use of inactivated vaccines. (Feberwee *et al.,* 2011).The diagnostic methods (SPA, HI and ELISA) should be only used as screening tools in monitoring programs to detect avian mycoplasmosis in poultry breeder flocks. Positive results should be confirmed by PCR or isolation using traditional microbiological methods (Luciano., 2011)

PCR based techniques are now routinely used for detecting pathogenic avian mycoplasmas. DNA target for PCR can be extracted from cultures or directly from swabs (Hammond *et al.,* 2009). PCR based assays are based on *16S rRNA* gene (Garcia *et al.,* 1996; Jarquin *et al.,* 2009). *16S-23SrRNA* Intergenic Spacer Region (ISRs) of most mycoplasmas are relatively small, species specific and intra specific conserved and can serve as an ideal genomic target for PCR diagnosis Raviv *et al.,* (2009). PCR targeting the *vlhA* gene can be also used to type strains of MS due to sequence variability in the N-terminal region of the gene among strains (Hammond *et al.,* 2009; Bencina *et al.,* 2001;

Jeffery *et al.,* 2007). Raviv *et al.,* (2009) described the development of a complete array of specific species real time PCR for four pathogenic Avian Mycoplasmas including MS and MG and have proposed the incorporation of these assays in routine diagnosis.

An early and accurate detection of etiological agent and diagnosis of disease can help in prevention and control of the disease in order to lower economic burden on livestock/poultry farmers and to increase GDP of the country. Therefore, present research is designed with the following objectives.

1. Molecular detection of *Mycoplasma synoviae* directly in clinical specimens by PCR.
2. Attempt for isolation of *Mycoplasma synoviae* from different specimens of poultry.

2. REVIEW OF LITERATURE

2.1 ***Mycoplasma synoviae* (MS)**

Infectious synovitis was first described and associated with mycoplasma infection by Olson *et al.* during the early 1950's. The causative organism was designated as Avian Mycoplasma Serotype S by Dierks *et al.,* in 1967 and subsequently confirmed *as a* separate species*, Mycoplasma synoviae* (MS) by Jordan *et al.*, (1982). Soon after its identification MS appeared to have worldwide distribution. During 1950’s and 1960’s the synovitis form of the disease was observed primarily in growing broilers, while since the 1970’s the respiratory form of the disease has been seen more frequently (Yoder and Olson, 1983). Flocks of laying chickens are commonly infected with MS with mild or subclinical signs. The outcome of infection is significantly affected by management factors and other respiratory pathogens. MS is the classical cause of infectious synovitis of chickens and turkeys. MS, more frequently produces a persistent infection of the upper respiratory tract which sometimes is involved with airsacculitis (Kleven *et al.,* 1972).

Positive diagnosis may be made by isolation and identification of *M. synoviae* (Kleven, 2003). Diagnosis can be made through serological tests as well. Polymerase chain reaction is a simple, rapid and highly sensitive method of detection of MS-DNA in tissues or culture medium and PCR procedures are comparable in sensitivity to isolation and identification (Salisch *et al.,* 1998; Feberwee *et al*., 2005).

In one survey, the cumulative mortality in twenty affected layer flocks ranged from 0.26% to 1.71% per week, while mortality in 20 unaffected flocks ranged from 0.07% to 0.30% per week (Vandekerchove *et al.,* 2004). In Brazil, the prevalence of MS in chicken flocks was reported to increase and even overcome that of MG in breeding flocks (Balen and Fiorentin, 1990).

Molecular detection was found to be significantly higher (98.82%) than culture isolation (42.35%) by Vandekerchove *et al*. (2004). PCR detection was primarily developed for MG (Nascimento and Yamamoto, 1991; Nascimento *et al.,* 1991) and was accepted worldwide for detection of all avian Mycoplasmas, either in specific DNA amplification for diagnosis (Lauerman, 1998; Nascimento *et al.,* 1998) or in nonspecific DNA banding pattern (RAPD) for strain identification (Fan *et al.,* 1995). An alternative to the nonspecific PCR procedure for strain identification is the direct sequencing of the amplified products (amplicons) from a specific PCR with primers directed to the *16S rRNA* gene (Ward *et al.,* 1990). Sequencing is more accessible nowadays and mycoplasma sequencing, including MG, has been facilitated because it has a small genome (Papazisi *et al*, 2003).

Different specimens have been suggested for detection and isolation of *M. synoviae* which include samples from live birds eg. swabs from the choanal cleft, oropharynx, oesophagus, trachea, eyes, cloaca and phallus. From dead birds, samples may be taken from the nasal cavity, infra-orbital sinus, trachea or air sacs.

2.2 Detection of *M. synoviae* directly in clinical specimens by PCR

2.2.1 *16S rRNA* PCR for *Mycoplasma synoviae*

Lauerman *et al.* (1993) evaluated *Mycoplasma synoviae* (MS) species-specific primers selected from the *16S rRNA* sequence by PCR. Analysis of MS PCR indicated 100% specificity and sensitivity. Fifty five individual isolates of MS tested PCR-positive and 44 isolates of 8 other species of avian mycoplasmas tested PCR-negative. Analysis of 122 flock data sets indicated sensitivity for the MS PCR test of 82% and a specificity of 100% as determined by comparison with cultures.

Kiss *et al.* (1997) attempted the Polymerase Chain Reaction (PCR) with primers complementary to the *16S rRNA* genes to detect avian Mycoplasmas. A primer pair designed for the detection of human and rodent Mycoplasma species was examined for its ability to detect the most important avian Mycoplasmas. After testing the respective reference strains it was found that *M. iowae, M. meleagridis and M. synoviae* could be detected by PCR with this primer pair. The results of the PCR- and RFLP-based identification procedures of 17 different field isolates agreed with those obtained by conventional methods.

Salisch *et al.* (1999) blotted the amplified fragments of the rDNA coding for *16S rRNA* for MG and MS on the nylon membranes, followed by dot-blot detection with two species-specific digoxigenin-(DIG)-labeled oligonucleotide probes.

Ramadass *et al.* (2006) isolated *Mycoplasma synoviae* among chickens in different parts of Tamil Nadu and performed polymerase chain reaction (PCR) for amplification 207bp regions from *16S rRNA* of MS. Among 1039 samples analyzed by PCR, 67 were positive for MS infections. A total of 47 culture negative samples were PCR positive for MS.

Marois *et al.* (2000) successfully detected *MS* by PCR from samples collected in the environment of experimentally infected chickens and turkeys. Results showed that in the experimental infection, 10 of 96 and 46 out of 96 samples of food, drinking water, feathers, droppings or dust were positive by culture and *Mycoplasma*-PCR, respectively. Under field conditions, 7/28 samples from the surrounding were found to be positive by cultural isolation, while PCR detected 17 positive samples out of 28.

Mardassi *et al.* (2005) processed 40 tracheal swabs for DNA extraction subjected for *16S rRNA* based PCR. Of the 40 cultures, 32 were positive. A total of 19 samples turned out to be PCR positive for *M. synoviae.*

Buim *et al.* (2009) carried out PCR for 1,046 samples of tracheal swabs and piped embryos collected from 33 farms with laying hens, breeders, broilers or hatchery, where respiratory problems or drops in egg production had occurred. The MG and MS prevalence on the farms was 72.7%. These results indicated high dissemination of mycoplasmas in the evaluated farms, with predominance of MS, either as single infectious agent or associated with other mycoplasmas in 20 farms (60.6%), and an increase of MS and decrease of MG infection in Brazilian commercial poultry.

Pourbakhsh *et al.* (2010) proposed the use of *Mycoplasma* specific PCR methods in order to detect *Mycoplasma* spp. from breeder farms. A total of 475 samples including choanal cleft, trachea, ovary and joint cavities from 23 broiler breeder farms were collected. The bacterial DNAs were extracted by phenol/chloroform method. Specific published primers that amplified a 207 bp region of the *16S rRNA* gene of MS were used for PCR.

Bayatzadeh *et al*. (2011) detected *M. synoviae* through PCR assay to demonstrate its presence in trachea and the lung/air sacs from commercial broiler chicken, with clinical signs of the disease. DNA was extracted from 43 samples by phenol/chloroform method and then PCR assay was used to amplify the conserved region of *16S rRNA* gene.

Yilmaz *et al.* (2011) determined pathological findings observed in serologically MG and MS positive chickens and compared immune histochemistry, PCR and culture methods for the diagnosis of mycoplasmosis. All 120 broiler chickens from commercial poultry farms with clinical symptoms of the disease were found to be MG (n = 27) and MS (n = 93) with the rapid slide agglutination test. The PCR amplification showed positive in 14 out of the 16 IHC positive cases of MS (211 bp band) and in all IHC positive cases of MG (302 bp band), leading to high agreement scores (97.8% and 100% respectively), MS and MG could be isolated from 12 and 3 cases, respectively.

Lobova *et al*. (2012) performed extraction of bacterial DNA directly from the tracheal smears, lung tissues and synovial fluid. From 45 samples collected (tracheal smears-32, lungs-11 and synovial fluids-2) and examined for detection of mycoplasma, only 2 samples of synovial fluid were found to be positive for *M. synoviae*.

Siddique (2012) studied cultural positivity of *Mycoplasma synoviae* in the sero-positive flocks and recorded as 3.9% and 9.2% from layer and boiler flocks, respectively while detection through PCR was as 7.1% and 13.1% respectively. The breeder flocks were negative for MS through cultural as well PCR based detection. The overall detection of MS from sero-positive flocks was recorded (5.6%) through culture and (8.8%) using PCR for amplification of *16S rRNA.* In case of sero-negative flocks the culture recovery percentage was recorded as 2.4%, 3.6% and 3.3% from breeder, layer and broiler flocks, whereas PCR based positivity was recorded in breeder (4.8%), layer (5.5%) and broiler (4.9%). The overall culture and PCR positivity from sero-negative flocks was recorded as 3.1% and 4.9%, respectively. The prevalence recorded for Mycoplasma synoviae through cultural isolation and identification was 5.0% and through PCR 7.9%.

Khalifa *et al*. (2013) described the isolation and molecular detection of *Mycoplasma synoviae* from tracheal swabs of diseased birds showing signs of respiratory distress in selected commercial (layer and broiler) farms. A number of 45 Mycoplasma isolates were recovered. Of these, 3 MS isolates were identified using growth inhibition and rapid serum agglutination (RSA) tests. The conventional PCR technique was applied for amplification 720 bp DNA fragment in *16S rRNA* for MS and was confirmed.

Senthilnathan (2015) processed 144 samples which were collected from suspected breeder birds and *16S rRNA* PCR assay was subjected. 57 out of 116 samples (49.1%) showed positivity to 207 bp *(16S rRNA*) product of MS.

2.2.2 ***VlhA* gene PCR for *Mycoplasma synoviae***

The *vlhA* (variable lipoprotein and hemagglutinin) gene encodes hemagglutinin, an abundant immunodominant surface lipoprotein of *Mycoplasma synoviae* (Bencina *et al*., 2001; Jeffery *et al*., 2006). The N-terminal end of the *vlhA* gene is present as a single chromosomal copy and owns hyper variable DNA sequences among different strains of *M. synoviae* (Noormohammadi *et al*, 2000). Cleavage of the VlhA protein generates the amino terminal (N-terminal) lipoprotein fragment MSPB and the carboxy (C-terminal) fragment MSPA, which are directly involved in binding *M. synoviae* to erythrocytes (Noormohammadi *et al*., 1997; Bencina *et al*., 1999). Validation of PCR and DNA sequence analysis of the N- terminal end of *vlhA* gene was done as an alternative for the detection and initial typing of field strains of *M. synoviae* in commercial poultry. A GenBank BLAST search did not find any other bacterial species sharing any significant homologous sequence similar to *vlhA*. The test was evaluated in clinical specimens for its usefulness to detect and discriminate *M. synoviae* strains without need for cultural isolation thereby giving early diagnosis of the infection (Hong *et al.,* 2004).

Bencina *et al.* (2001) described that VlhA protein is encoded by *vlhA* gene of which the 5'-end is present in the genome as a single copy which does not change its sequence during recombination of the *vlhA* gene with pseudogenes. 11 different types of *vlhA* sequences were shown in a comparison of the 5'-end *vlhA* sequences of 30 *M. synoviae* strains. This indicated that the analysis *vlhA* part was useful in strain differentiation. These distinct sequence motifs based on *vlhA* gene specific for individual *M. synoviae* strains can be used as markers for tracing their spreading between poultry farms.

Hong *et al.* (2004) validated and applied PCR and DNA sequence analysis on the N-terminal end of the *vlhA* as an alternative for the detection and initial typing of field strains of *M. synoviae* in commercial poultry. The PCR procedure was found to have the sensitivity and specificity required to be useful as diagnostic PCR with the added advantage of preliminary strain identification based on the DNA sequence analysis of the PCR product.

Allen *et al.* (2005) described that the *Mycoplasma synoviae*, contains a single expressed, full-length *vlhA* gene encoding its haemagglutinin protein along with a large number of *vlhA* pseudogenes. It was known that in *Mycoplasma gallisepticum* the *vlhA* genes are located in several loci around the chromosome and antigenic variation were generated by alternating transcription of more than 40 translationally competent genes.

Jeffery *et al.* (2007) used oligonucleotide primers complementary to the single copy conserved 5' end of the *vlhA* and could generate amplicons of approximately of 400bp from 35 different MS isolates and subjected to mutation. Sequencing of the amplicons represented that each single profile was related to unique sequence, some differing from each other just by one base-pair substitution. The result presented in this study showed that the PCR based single-strand conformation polymorphism (SSCP) analysis of *vlhA* gene high resolution detection tool for the detection and identification of MS strains.

Hammond *et al.* (2009) used published primers to detect *Mycoplasma synoviae* and identified the strain using the *vlhA* gene sequence. However, of 21 *M. synoviae* strains examined, 3 could not be amplified, so a new reverse primer was designed with a target in the conserved region of the *vlhA* gene. This allowed all 21 *M. synoviae* strains, a further 9 strains and also material from 11 swab samples from *M. synoviae*-positive birds, to produce a PCR product, suggesting that the method could also be suitable for clinical specimens. The protocol was then tested on the type strains of *M. synoviae* and the other 22 recognised avian Mycoplasma species, with amplification of *M. synoviae* only. He finally demonstrated that this PCR was equally more sensitive than other PCR tests.

Harada *et al.* (2009) applied pulsed-field gel electrophoresis (PFGE) and *vlhA* gene sequence analysis for typing the *Mycoplasma synoviae* live vaccine MS-H strain and field isolates from diseased chickens in Japan. The digestion protocol using restriction enzymes *BlnI* and *BamHI* as well as the *vlhA* sequence analysis allowed the discrimination of all 11 MS field isolates from the vaccine strain and allowed the generation of unique fragment pattern in epidemiologically unrelated isolates. It is found to be useful tool to determine whether MS clinical isolates are derived from the vaccine strain or clinical isolates.

Ehtisham-Ul-Haque (2010) studied and found sero-prevalence of MS (79.55%), MG (72.89%) and MG/MS combined infection (19.20%) through rapid serum agglutination (RSA) test. The sero-positive cases further resolved the culture recovery of MG (71.67%), MS (49.69%) and MG/MS combined (32.35%) on modified Frey's medium. Conventional duplex PCR was efficiently optimized for MG/MS detection at 288bp/373bp respectively with a sensitivity of detecting less than 1pg of DNA. Samples from sero-positive cases successfully accomplished the prevalence of MS (98.14%) followed by MG (93.34%) and MG/MS combined (82.35%) through duplex PCR. All the culture isolates were further confirmed with PCR identification and the sensitivity of PCR was calculated as 100% for both MG and MS PCR amplification was done for both the genes *vlhA* and *16S rRNA.*

Ramirez *et al.* (2008*)* performed polymerase chain reaction with one primer based on the intergenic spacer region (ISR) and validated for detection of MS. The primer was paired with a general primer from within the *23S rRNA* gene. The PCR primers were tested with the 22 other recognised avian *Mycoplasma* species to check the specificity and with 21 field isolates of MS from various hosts and countries, and with several swab samples. The PCR appeared to be considerably specific and sensitive. Four different sample preparation methods were compared for use in this PCR, and the amplification protocol was compared with three others, confirming the comparative sensitivity of the new PCR.

2.3 Isolation and identification *of Mycoplasma synoviae*

Saritha (2010) incubated 425 samples from both live and dead birds in Frey's Medium, out of which 153 (36%) samples were found to be positive with the characteristic signs of growth in the broth. Out of these 46 could be cultured in Frey's Agar Medium and 27 (58.6%) were found positive with typical colonies. Confirmation was done by amplifying *16S rRNA* gene of MS with a set of published primer sequences through PCR.

Bayatzadeh *et al.* (2011) detected *M. synoviae* through cultural isolation and polymerase chain reaction (PCR) assay to demonstrate the involvement of *M. synoviae* infection in trachea and the lung/air sac samples. Total of 43 samples were inoculated in PPLO broth media supplemented for *M. synoviae* isolation out of which 28 (65.1%) yielded the organism.

Tan *et al*. (2011) cultured swabs onto PPLO agar and broth and incubated at 37°C with 5 – 10% carbon dioxide. Presumptive Mycoplasma colonies were passaged onto PPLO agar without antibiotic three to four times for purity. The organisms were then subjected to biochemical tests and growth inhibition test for species confirmation. Identification of Mycoplasma species was made based on available specific antisera which focus on the pathogenic Mycoplasma of veterinary importance. From avian (poultry and bird), *M. synoviae, M. gallisepticum, M. gallinarum* and *M. columborale* were isolated.

Khalifa *et al*. (2013) recovered successfully 7 (4.11%) isolates out of 170 tracheal swabs samples from diseased birds of which 3 were *M. synoviae*. However PCR for MS was found 6.7 % positive.

Nagalaxmi *et al*. (2013) could not recover *Mycoplasma* species from 262 trachea and air sac samples by cultural isolation. However, *16S rRNA* PCR detected 33.7% sample as *Mycoplasma synoviae* using the same sample.

Tebyanian *et al.* (2014) used the *M. synoviae* specific Polymerase Chain Reaction (PCR) and microbiological methods in order to isolate and identify *M. synoviae* from suspected ostriches. Fifty three samples of different parts of lung and trachea were cultured in the same conditions in PPLO broth and to isolate and identify *M. synoviae*, PCR and microbiological methods were conducted. The identified isolates were confirmed by specific amplification of *16S rRNA* gene (163 and 207 base pair). Twenty five (47%) and seventeen (32%) out of 53 samples were found to be *M. synoviae* by PCR and microbiological methods respectively.

Senthilnathan (2015) processed144 samples from suspected breeder birds and examined by culture, only 18 samples (15.5%) on day 5 after inoculation.

2.4 Comparative efficacy of cultural isolation and PCR assay in detection of *Mycoplasma synoviae.*

Lauerman *et al.* (1993) evaluated *Mycoplasma synoviae* (MS) species-specific primers selected from the *16S rRNA* sequence by PCR. Analysis of cultures of avian mycoplasmas using the MS PCR indicated 100% specificity and sensitivity. Fifty five individual isolates of MS tested PCR-positive and 44 isolates of eight other species of avian mycoplasmas tested PCR-negative. Analysis of 122 flock data sets indicated sensitivity for the MS PCR test of 82% and a specificity of 100% as determined by comparison with culture.

Bayatzadeh *et al*. (2011) screened 43 swabs of trachea /lungs /airsacs collected from broiler chicken farms and achieved isolation rate of 26 (65.1%) for *M. synoviae*, while 24 (55.9%) specimens were found to be *M. synoviae* by species specific PCR assay.

Khalifa *et al*. (2013) recovered successfully 7 (4.11%) isolates out of 170 tracheal swabs samples from diseased birds of which 3 were *M. synoviae*. However PCR for MS was found 6.7 % positive.

Nagalaxmi *et al*. (2013) could not recover *Mycoplasma* species from 262 trachea and air sac samples by cultural isolation. However, *16S rRNA* PCR detected 33.7% sample as *Mycoplasma synoviae* using the same sample.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Glassware and Plasticware

All the glassware and plasticware used during present investigation were made of neutral glass, Borosil (India). All the glassware used during the course of research was prepared by following the standard procedures including sterilization at 160^0C in hot air oven for an hour. Plastic wares required for the present work were obtained from M/s Tarsons Products Pvt. Ltd. Kolkata (India) and Genaxy Scientific Pvt. Ltd., (India). The plastic wares used were either disposable or autoclaved at 15 lbs for 15 minutes at 121^0C prior to use.

3.1.2 Chemicals and reagents

The chemicals and reagents used during present investigation were obtained from following manufacturing companies. The chemicals and reagents used for PCR were of molecular grade.

Table 3.1 Media, Chemical and Reagents used

Sr. no	Manufacturer	Reagents/Equipment
1.	M/s Hi Media Laboratories Private Limited, Mumbai (India)	PPLO broth/agar, Glucose-D , NAD, Cysteine, Pig/Horse serum, Yeast Extract, Penicillin, Phenol Red
2.	M/s Sigma Aldrich Chemicals Private Limited (USA)	Taq Polymerase, Ethidium Bromide, dNTP, 10x buffer , $MgCl_2$, Primers, Thallium Acetate
3.	M/s Merck Specialities Private Limited, Mumbai (India)	Agarose
4.	M/s Bioinnovation	Primers

3.1.3 Clinical specimens

A total of 165 choanal swabs from live birds suspected for *Mycoplasma synoviae* were collected in duplicates for PCR and cultural isolation formed the material for investigation in the present study (Table 3.2).

Table 3.2 Details of specimens collected for detection of MS in poultry

<table>
<tr><th>SR. NO.</th><th>SOURCE</th><th>TYPE OF BIRDS</th><th colspan="2">NO. OF SAMPLES</th></tr>
<tr><td>1</td><td>Kolkata</td><td>Layer</td><td colspan="2">37</td></tr>
<tr><td>4</td><td>Khopoli</td><td>Broiler</td><td colspan="2">14</td></tr>
<tr><td rowspan="2">6</td><td rowspan="2">Karjat</td><td>Layer</td><td colspan="2">26</td></tr>
<tr><td>Broiler</td><td colspan="2">14</td></tr>
<tr><td>7</td><td>Delhi</td><td>Layer</td><td colspan="2">20</td></tr>
<tr><td>8</td><td>Lohagaon</td><td>Grower</td><td colspan="2">24</td></tr>
<tr><td rowspan="2">10</td><td rowspan="2">Nashik</td><td>Layer</td><td colspan="2">10</td></tr>
<tr><td>Broiler</td><td colspan="2">20</td></tr>
<tr><td colspan="2" rowspan="2">Total</td><td>Broiler</td><td>48</td><td rowspan="2">165</td></tr>
<tr><td>Layer</td><td>117</td></tr>
</table>

3.1.4 Reference strain

Mycoplasma synoviae (ATCC® 25204™) was used as the reference strain used during the present research. It was revived and propagated in the medium recommended by ATCC.

3.1.5 Media

The media used during the isolation of *Mycoplasma synoviae* were selected and followed from different researchers (Ehtisham, 2010; Raviv and Ley, 2011). PPLO broth/agar (modified Frey's medium) was prepared (Frey *et al*., 1968; Kleven, 1997) by substituting some ingredients whereas ATCC medium was used for cultivation of ATCC strain.

3.2 Methods

3.2.1. Collection of Choanal Swabs

The clinical specimens were collected in duplicate for performing PCR and for isolation of *Mycoplasma synoviae*. The choanal (palatine cleft) swabs were collected from live birds exhibiting respiratory symptoms, sometimes in association with swollen hock joint/synovial joint etc. as per the protocol described by World Organization for Animal Health (2008). The swabs were collected by opening the beak of birds followed by inserting and rubbing the sterile swab in choanal cleft which is located at inner side of mouth cavity along midline of upper beak followed by inserting it up to the pharynx and rolled it once/twice in trachea. The readymade guarded sterile swabs (Hi Media Laboratories Pvt.Ltd.) were used. Before sampling the swabs were dipped in PPLO broth supplemented with NAD and Cysteine in order to create optimal conditions for the organism during transport to the laboratory. Both sets of the swabs taken in sterile tubes were transported on ice and processed within 24-48 hours after collection.

3.2.2 Molecular detection of *Mycoplasma synoviae* directly in clinical specimens by PCR.

3.2.2.1 Extraction of DNA

3.2.2.1.1 Extraction of DNA from clinical samples

DNA was extracted from swabs as per the protocols described by Behbahan *et al*. (2008)/WOAH (2008)/ Silveria *et al*. (1996) with slight modifications. Briefly, the procedure was as follows:

Swab samples were placed in 2ml snap-cap eppendorf tube and chopped off one end; 500µl lysis buffer was added and kept overnight at 4^0C

⇩

Proteinase K (20µg/ml) was added in 5µl and the samples were incubated at 70^0C for one and half hour

⇩

To the lysed cells, equal volume of Phenol: Chloroform (1:1) was added and vortexed for 15 minutes and centrifuged at 12000/rpm for 15 minutes at 4^0C

⇩

The supernatant was taken carefully in fresh tube (2ml) and the equal volume of Choloroform: Isoamyl (24: 1) was added

⇩

The tubes were vortexed and centrifuged at 4^0C for 15 minutes each

⇩

The supernatant was transferred in fresh tubes (2ml) and 1/10 volume of Sodium Acetate and double volume of ethanol were added and mixed thoroughly

⇩

The tubes were incubated at -20^0C for 2 hours/ overnight

⇩

The tubes were centrifuge at 12000 rpm for 20 minutes at 4^0C

⇩

The supernatant was discarded and 500µl of 70% ethanol was added and mixed well followed by centrifugation at 12000 rpm for 15 minutes at 4^0C

⇩

The supernatant was discarded and the pellet was air dried.

⇩

To the dried pellet 50µl of autoclaved distilled water was added and kept at 55^0C for proper reconstitution of the DNA

⇩

The extracted DNA was stored at – 20^0C until further use

3.2.2.1.2 Extraction and purification of DNA from Reference strain

DNA was extracted from reference strain as per the protocol described by Ley *et al.* (1997) with slight modifications. Briefly, the procedure was as follows:

ATCC broth culture of *M. synoviae* ((ATCC® 25204™) in 0.5ml quantity was taken in 2-ml eppendorf tube

⇩

The suspension was centrifuged at 13,000 rpm for 30 min at 4°C

⇩

Followed by washing two times with PBS

The remaining steps of extraction of DNA were followed as of **3.2.2.1.1**

3.2.2.2 Quantification of DNA

Nanodrop Spectro-Photometer (Thermo Scientific, USA), set at optical density of 260 nm and 280 nm was used for quantification and determination of purity of extracted DNA. DNA concentrations and purity were calculated using the software - ND2000 (Thermo Scientific, USA) available with the instrument

3.2.2.3 Assessment of integrity of genomic DNA

Agarose gel (0.8%) was prepared in 1X TAE buffer by melting it and adding Ethidium Bromide @ 0.5µg/ml. The solidified gel was placed in 1X TAE buffer in electrophoresis tank such that the buffer covered the agarose gel to a depth of about 10 mm. 5 µl of DNA sample was mixed with 0.5 µl of 6X loading dye and loaded in the wells. Electrophoresis was carried out at a constant voltage of 100 V, until the dye migrated about three-fourth distance of the gel. The integrity of the DNA was checked by visualization of the DNA using gel documentation system (Gel Doc EZ Imager, Bio-Rad).

3.2.2.4 Detection of *M.synoviae* by Polymerase Chain Reaction

3.2.2.4.1 *M.synoviae* species specific *16S rRNA* PCR Assay

The oligonucleotide primers used for PCR of *16S rRNA* are mentioned in table 3.3. The primers were procured from M/s Sigma Aldrich Chemicals Pvt. Ltd. (USA).

Table 3.3: Oligonucleotide primers used for PCR of *16S rRNA* gene

Primer	Oligonucleotide sequence	Reference
MS–F	5’-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3’	WOAH (2008)
MS–R	5’-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3’	

3.2.2.4.1a Reaction Mixture:

The composition of the constituents used for preparing the reaction mixture is mentioned in table 3.4

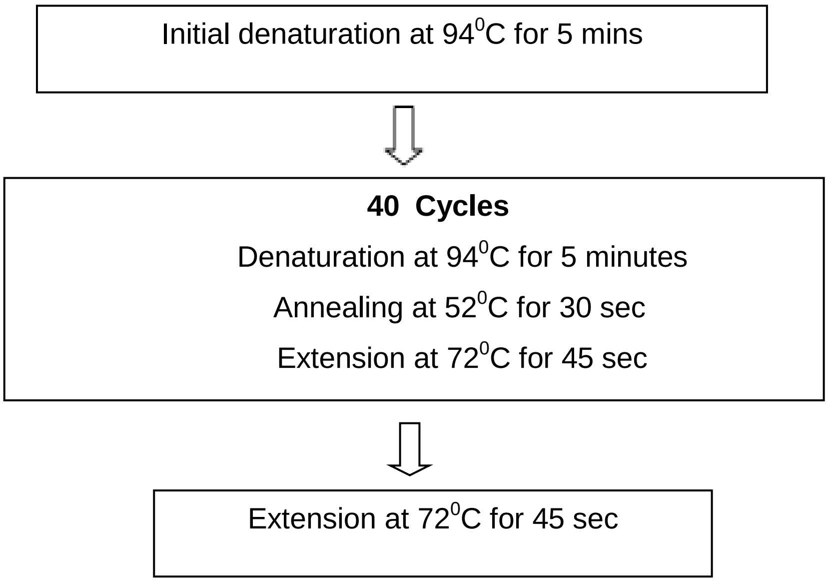
Table 3.4: Reaction mixture for *16S rRNA* PCR for MS

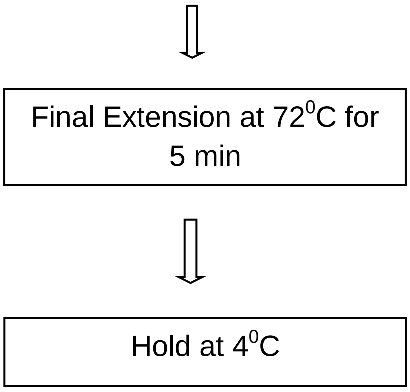
Particulars	**Concentration (µl)**
H_2O Ultra-pure	17.1
10X PCR Buffer	2.5
dNTPs (l0 mM)	0.8
F Primer (20 pmole/µl)	0.6
R Primer (20 pmole/µl)	0.6
Taq Polymerase (5 U/µl)	0.4
$MgCl_2$ (50 mM)	2.5
DNA	0.5
Total	25.00

3.2.2.4.1b <u>Cycling conditions</u>

The reaction mixtures prepared as in table 3.4 were spun briefly and set into thermal cycler (Master Cycler Nexus Gradient, Eppendorf). The cycling conditions used are as shown in fig. 3.1

Fig. 3.1: Cycling conditions for *16S rRNA* gene PCR of MS





3.2.2.4.2 *vlhA* gene based PCR Assay

The oligonucleotide primers used for PCR of *vlhA* gene are mentioned in table 3.5. The primers were procured from M/s Sigma Aldrich Chemicals Pvt. Ltd. (USA).

Table 3.5: Oligonucleotide primers used for PCR of *vlhA* gene

Primer	**Oligonucleotide sequence**	**Reference**
vlhA–F	5’-GATGCGTAAAATAAAAGGAT-3’	Hong *et al.* (2004)
vlhA–R	5’-GCTTCTGTTGTAGTTGCTTC-3’	

3.2.2.4.2a Reaction Mixture

The composition of the constituents used for preparing the reaction mixture is mentioned in Table 3.6

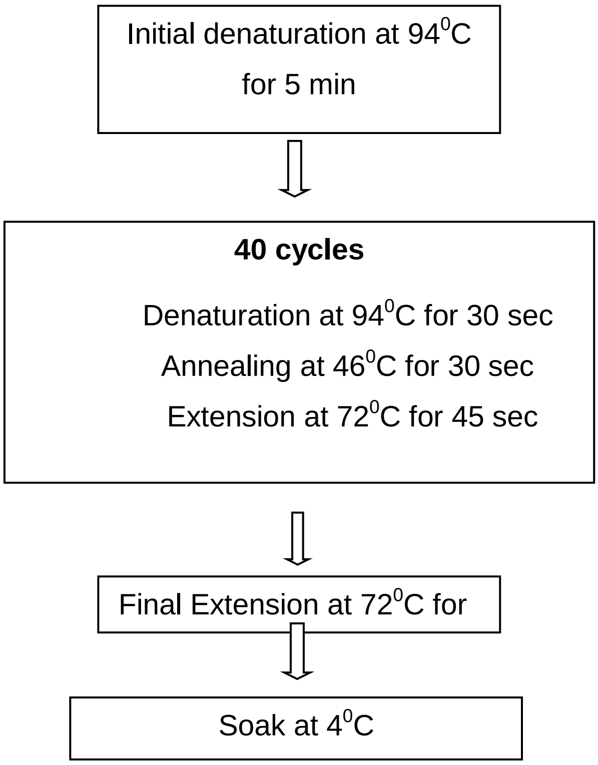
Table 3.6: Reaction mixture for *vlhA* gene PCR *for* MS

Particulars	Concentration (µl)
H_2O Ultra-pure	18.0
10X PCR Buffer	2.5
dNTPs (l0 mM)	1.0
F Primer (20 pmole/µl)	0.5
R Primer (20 pmole/µl)	0.5
Taq (5 U/µl)	0.4
$MgCl_2$ (50 mM)	1.5
DNA	0.6
Total	25

3.2.2.4.2b Cycling Conditions

The reaction mixtures were spun briefly and set into thermal cycler (Master Cycler Nexus Gradient, Eppendorf). The cycling conditions used were as shown in fig. 3.2

Fig. 3.2: Cycling conditions for *vlhA* gene PCR *of* MS



3.2.2.5 Evaluation of PCR products

The amplification products generated from the MS *16S rRNA* and *vlhA* gene PCR assays were evaluated by agarose gel (1.5%--2%) electrophoresis stained with Ethidium Bromide (0.5µg/ml). A 100bp DNA ladder was electrophoresed simultaneously in order to compare the molecular weights of the amplicons generated by the PCR assay. The products were visualized and documented using Gel Documentation and Analysis System (Gel Doc EZ Imager, Bio-Rad) with UV light source. The size of PCR products was estimated with the help of Image Lab (Version 4.1) software available with the gel documentation system.

3.2.3 Isolation and identification of *M. synoviae* from clinical specimens

Collection of swabs was carried out following all the possible aseptic conditions using PPLO media as the transportation medium for isolation of *M. synoviae.* The swabs were inoculated directly in PPLO broth and incubated at 37°C in presence of 5% CO_2 tension. Different combinations of media were prepared in the form of broth and agar plates which were additionally supplemented with NAD and cysteine (Appendix III).

The inoculated broth was constantly observed until the change of the red color of the broth to pale yellow was evident. The samples showing no change in color were further incubated up to 20 days before declaring them as negative .The broth tubes which exhibited color change from red to yellow were transferred on the agar plates and incubated at 37^{O}C at 5% CO_2. Out of these, the samples in which growth was observed on the agar plates were again transferred in the broth and sub cultured.

3.2.3.1 Diene’s staining of Mycoplasma colonies

For staining of colonies Diene’s staining was performed as per method described by Dienes & Weinberger, 1951. Briefly, the procedure was as follows:

A block of agar containing micro colonies was placed with colony side upwards on a glass slide

⇩

A light film of Diene’s stain was placed on a coverslip and allowed to dry

⇩

The coverslip was then placed with the stain side downwards on micro colonies of the agar block

⇩

The preparation was examined under the stereo-zoom or light microscope.

The denser center of the micro colony which grew deep into the agar stained dark blue. The less dense peripheral region micro colony surface grew on the agar surface stained light blue in color.

3.2.3.2 Giemsa staining for mycoplasma species

Microscopic examination using Giemsa staining was done for the isolates as per Maurice (1956). In a coplin jar, the smear on glass slide was covered with Methanol-fixed smear and was stained with Giemsa stain for a period of 3 to 6 hrs. The step was followed by air drying and gentle blotting between absorbent filter papers. The dried smear was observed under high power under oil immersion. Annular forms i.e. circular and elliptical PPLO which was characterized chiefly by the lack or thinness of stainable material in the central region of the organism. The protoplasm appeared to be concentrated at the periphery of the circular PPLO particle-evenly distributed in the form of a ring, or unevenly distributed, yielding lopsided rings, "signet rings," and monopolar forms.

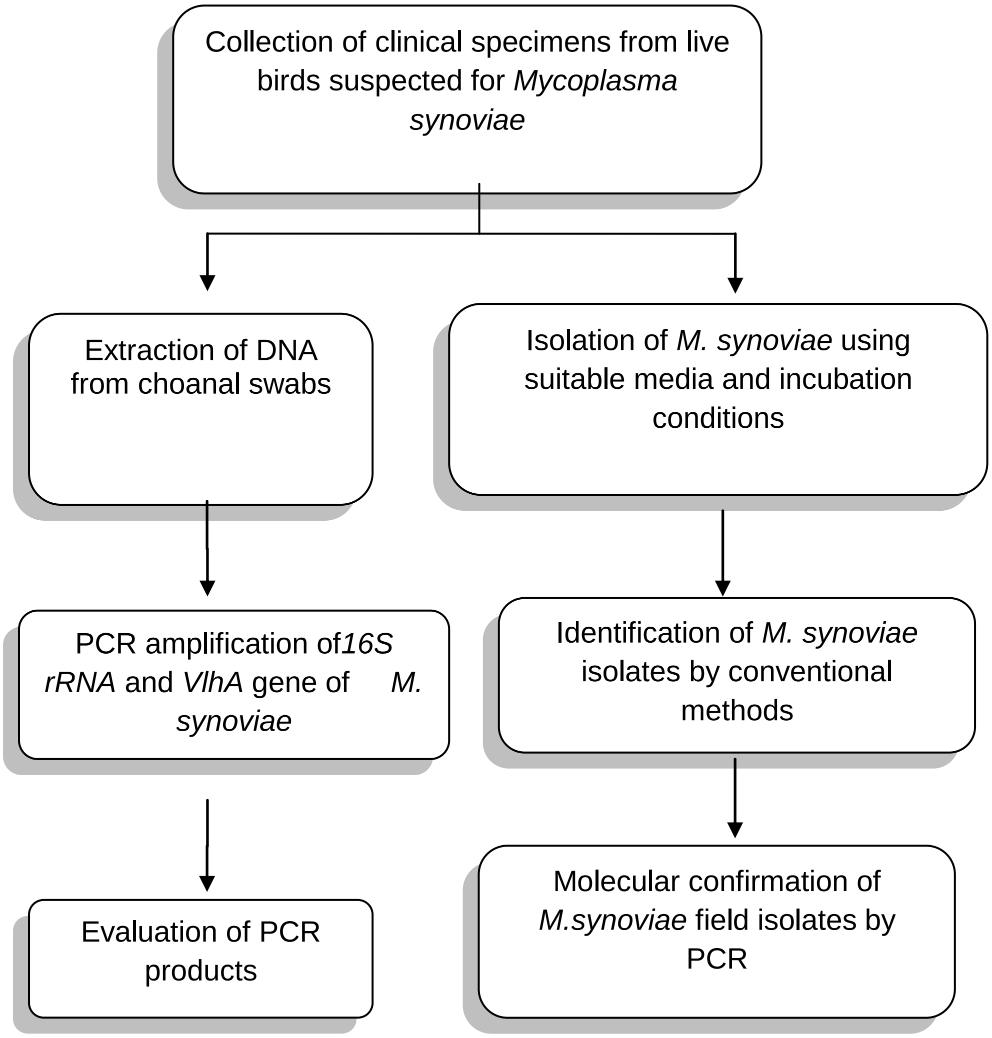
3.2.3.3 Molecular confirmation of *M.synoviae* isolates

Molecular confirmation was done by extraction of DNA **(3.2.2.1.1)** followed by PCR assay **(3.2.2.4)**

4. RESULTS

The present research was carried out for detection of *Mycoplasma synoviae* (MS) by molecular techniques and conventional methods. It was done in two phases; the first phase included molecular detection of *Mycoplasma synoviae* by PCR assays targeting *16S rRNA* gene and *vlhA* gene directly from clinical samples. The second phase included cultural isolation and identification of *Mycoplasma synoviae* using conventional and PCR assays.

Fig. 4.1 Plan of experiment



In the present study, choanal swabs were collected from 165 birds (from 48 broilers + 117 layers) suspected for *M. synoviae* infection based on the clinical signs (respiratory symptoms and synovitis). The swabs were collected in duplicate for direct detection of MS and cultural isolation (one set each). ATCC® 25204™ was used as a reference strain during the research.

4.1. Molecular Detection of *M. synoviae* directly from clinical specimens

4.1.1 *16S rRNA* based PCR for detection of *M. synoviae*

Extraction of DNA was carried out from total 165 clinical specimens and reference strain. All extracted DNA were subjected for *16SrRNA* based *M. synoviae* species specific PCR assay. Out of 165 samples processed, 7 (4.2%) were found to be positive for *M. synoviae* yielding the amplicon of approximately 207bp (Plate 4.1). Out of 117 samples from layers, 4 (3.4%) and of 48 from broilers, 3 (6.25%) yielded positive results in *16S rRNA* MS specific PCR (Table 4.1). Thus, the overall incidence of MS observed was 4.2% with an incidence rate of 3.4% and 6.25% in layers and broilers respectively.

Table 4.1 Result of *16S rRNA* gene PCR of *M. synoviae*

Sr. No.	**Source**	**Type of Birds**	**Specimens**	**No. of Sample**	***PCR* Positive (MS)**
1	Kolkata	Layer	Choanal swabs	37	02
2	Khopoli	Broiler	Choanal swabs	14	00
3	Karjat	Layer	Choanal swabs	26	00
		Broiler		14	01
4	Delhi	Layer	Choanal swabs	20	02
5	Lohagaon	Layer	Choanal swabs	24	00
6	Nashik	Layer	Choanal swabs	10	00
		Broiler		20	02
TOTAL				**165**	**07**

Out of 20 samples collected from Delhi region, 2 (10%) were found to be positive for MS whereas 2 (5.4%) out of 37 were positive from Kolkata region. Among three positive from Maharashtra region, 2 (6.6%) and 1 (2.5%) were from Nashik and Karjat regions respectively (Table 4.1).

4.1.2 *vlhA* gene based PCR for *M. synoviae*

A total of seven clinical specimens (DNA) which were found to be positive in *16S rRNA* were subjected to surface variable lipoprotein-haemagglutinin (*vlhA*) gene PCR of *M. synoviae* along with the reference strains, to detect strain variations if any among them.

All seven samples positive for *16S rRNA* of MS were also found to be positive for *vlhA* gene PCR. The PCR products of all seven samples and reference strain observed in an average range of 316bp to 396bp (Hong *et. al.*, 2004) (Table 4.2; Plate 4.3).

Table 4.2 Result of *vlhA* gene PCR of *M. synoviae*.

.

Sr. No.	**Source**	**Type of Birds**	***vlhA* Gene Positive (MS)**	**Amplicon Size**
1	Kolkata	Layer	02	~ 380bp
2	Karjat	Layer	00	
		Broiler	01	~360bp
3	Delhi	Layer	02	~ 380bp
4	Nashik	Layer	00	
		Broiler	02	~360bp
Total			**07**	

However, the amplicon size of specimens originating from different geographical regions varied in the sizes. The strains from Maharashtra region yielded PCR product of ~360bp, whereas Delhi and Kolkata regions produced

~380bp size amplicon. Thus indicating strains from Maharashtra region, Delhi and Kolkata regions were distinct.

4.2. Isolation and identification of *Mycoplasma synoviae*

The birds suspected for *Mycoplasma synoviae* infection exhibiting respiratory distress were selected and the samples from the upper cleft palate were collected. A total of 165 clinical specimens (choanal swabs) from suspected live birds were processed for isolation of *Mycoplasma synoviae,* primarily using enrichment media Pleuropneumonia Like Organism (PPLO) broth followed by inoculation on PPLO agar with varying percentage of different ingredients additionally supplemented with NAD and cysteine (appendix III). Different percentages and combinations of ingredients were also tried in the media for isolation of *Mycoplasma synoviae.* The inoculated media were incubated under suitable incubation conditions.

In cultural isolation, the color change of PPLO broth was observed in only two samples, which were further, cultivated on solid media suitable incubation conditions. On solid medium, both showed colonies with typical “fried egg” appearance. Further staining with Diene’s stain and Geimsa also revealed organisms with a typical morphology of Mycoplasma. However, subsequent confirmation by PCR could not yield MS specific PCR product, thus indicating that these isolates belonged to other species of genus Mycoplasma. Being MG and MS commonly involved in poultry mycoplasmosis, these isolates were subjected to MG specific PCR which yielded MG specific amplicon of ~185bp confirming their identity as *M. gallisepticum*. This indicated that *M. gallisepticum* can grow in PPLO medium of *M. synoviae* interfering in specific diagnosis.

In the present study, *Mycoplasma synoviae* could not be isolated from any of the clinical samples, including from seven *16S rRNA* and *vlhA* positive PCR specimens, thus giving an isolation rate of 0% for MS.

4.3. Comparative efficacy of cultural isolation and PCR for detection of *M. synoviae* infection

Out of 165 clinical specimens (choanal swabs) processed for detection of *Mycoplasma synoviae* infection by direct PCR and cultural isolation, only PCR could detect seven (4.2%) positive cases of MS infection, whereas cultural isolation failed to detect the same. Moreover, PCR could detect the strain variation occurring in MS of different geographical area. Thus PCR was proved to be more sensitive, specific, rapid and easy method than cultural isolation for diagnosis of avian mycoplasmosis resulting due to *M. synoviae* infection.

In the present study, the overall incidence of mycoplasmosis due to *M.synoviae* was observed very low with comparatively higher incidence in broilers than layers.

5. DISCUSSION

Mycoplasma synoviae (MS) is one of the most important and pathogenic poultry Mycoplasmas. *M. synoviae* infection occurs as a subclinical upper respiratory infection. Less frequently, it becomes systemic and results in infectious synovitis. It leads to an acute to chronic infectious disease of chickens and turkeys. Lameness and respiratory disorder caused by this infection result in decreased growth rate and loss of egg production. There is considerable variation among isolates in their ability to cause disease and many isolates may cause little or no clinical disease (Lockaby, 1999).

5.1 Molecular detection of *M. synoviae* by *16S rRNA* PCR

In the present study, a total of 165 choanal swabs were collected from live birds (117 layers and 48 broilers) suspected for MS infection and subjected to PCR. Out of 165 samples screened, MS could be detected from 7 (4.2%) samples by *16S rRNA* PCR showing an amplicon of ~ 207bp product. Out of 117 samples from layers, PCR could detect MS infection in 4 samples with a rate of 3.4 % in layers and in 3 (6.2%) of 48 samples from broilers.

Lauerman *et al*. (1993) screened 122 flocks by PCR. Analysis of cultures of avian mycoplasmas using the MS PCR indicated 100% specificity and sensitivity. Analysis data sets indicated sensitivity of 82% for the MS PCR and a specificity of 100% as determined by comparison with culture. Thus, *16S rRNA* gene based species specific PCR for MS proved to be a useful tool in detection of infection directly from clinical samples.

Various researchers have performed the identification of *M. synoviae* directly from clinical samples by targeting the *16S rRNA* gene. (Lauerman *et al*., 1993; Ramadass *et al.,* 2006; Bayatzadeh *et al*., 2011; Lobova *et al*., 2012; Siddique, 2012; Nagalaxmi *et al*., 2013; Khalda *et al*., 2013; Kamble, 2014).Lauerman *et al*. (1993) evaluated similar *M. synoviae* (MS) species-specific primers, selected from the *16S rRNA* sequence, by PCR in 122 flocks. In another study, Ramadass *et al.* (2006) detected *M. synoviae* from chickens in different parts of Tamil Nadu by performing PCR for amplification of 207 bp region from *16S rRNA* of MS. Similarly, Lobova *et al*. (2012) and Khalifa *et al*. (2013) also targeted *16S rRNA* gene for identification of MS directly from clinical samples. Bayatzadeh *et al*. (2011) and Nagalaxmi *et al*. (2013) attempted *16S rRNA* based species-specific PCR to detect MS from trachea /lungs /air sacs of suspected birds.

5.2 **Incidence rate of *M. synoviae***

In the present study, the overall incidence of MS was 4.2% with incidence rate of 3.4% and 6.25% in layers and broilers respectively. Various workers, who targeted *16S rRNA* gene for direct identification of MS, reported similar lower incidence rate as observed in the present study by PCR. Ramadass *et al.* (2006) screened 1039 samples by PCR, out of which 67 (6.44%) were found to be positive for MS infections. Similarly, Khalifa *et al*. (2013) could detect only 11 (6.7%) out of 170 tracheal swab samples as MS positive by MS specific PCR. In a study similar to the present research, Lobova *et al*. (2012) performed extraction of bacterial DNA directly from a total of 45 samples (tracheal swabs-32, lungs-11and synovial fluids-2) for detection of mycoplasma by *16S rRNA* based PCR. Only 2 (4.4%) samples of synovial fluid were found to be positive for *M. synoviae*. Siddique (2012) detected *M. synoviae* in the sero-positive layer and broiler flocks. PCR detected 7.1% and 13.1% of the layers and broilers, respectively, positive for MS. The breeder flocks did not yield any results by cultural as well as PCR based detection. The detection of MS from sero-positive and sero-negative flocks was recorded to be 8.8%and 4.9%, respectively, using PCR. The overall prevalence recorded for *M. synovial* through PCR was 7.9%. Kamble (2014) performed PCR of 159 samples collected from suspected birds and found 3 (1.8%) positive by *M. synoviae* species specific PCR assay, whereas Bukte (2015) found 0% incidence of MS in 150 samples by *16S rRNA* PCR.

The higher incidence of MS than in present study is reported by various authors. Bayatzadeh *et al*. (2011) screened 43 swabs of trachea /lungs /air sacs collected from broiler chicken farms and found 24 (55.9%) specimens to be *M. synoviae* by species specific PCR assay. Nagalaxmi *et al*. (2013) detected MS infection in 88 (33.7%) out of 262 trachea and air sac samples. Senthilnathan *et al.* (2015) reported a comparatively higher prevalence rate of *M. synoviae.* The PCR assay of 116 samples with species specific primer of *M. synoviae* was carried out and 57 (49.1%) cases were found to be positive for MS.

5.3 *vlhA* gene based PCR for *M. synoviae*

Another assay targeting non-conserved gene region of *vlhA* gene was performed. A total of seven clinical specimens (DNA) which were found to be positive in *16S rRNA* were subjected *vlhA* gene PCR, gene fragment between ~350 to ~390bp was amplified from all seven samples. The amplicon size of specimens originating from different geographical regions varied in the sizes. The strains from Maharashtra region yielded PCR product of ~360bp, whereas Delhi and Kolkata regions produced ~380bp size amplicons, whereas amplicon size of the reference strain laid between the size of Maharashtra and Delhi strains. Thus indicating strain variations among the different geographical areas under study. However, all strains from Delhi and Kolkata were similar whereas all strains from Maharashtra were also similar.

Bencina *et al.* (2001) described that *VlhA* protein is encoded by *vlhA* gene of which the 5'-end is present in the genome as a single, conserved copy. Eleven different types of *vlhA* sequences of the 5'-end of *vlhA* were compared from 30 *M. synoviae* strains. This analysis of *vlhA* fragments indicated that it is useful in strain differentiation. They reported that, these distinct sequence motifs based on *vlhA* gene specific for individual *M. synoviae* strains can be used as markers for tracing their spread between poultry farms. Many other workers have used *vlhA* gene amplification for detection of *M. synoviae* and differentiation among the strains (Hong *et al.,* 2004; Jeffery *et al.,* 2007; Hammond *et al.,* 2009; Harada *et al.*, 2009; Syed, 2010).

Hong *et al.* (2004) extracted DNA directly from the tracheal swabs and carried out the amplification reactions for both *16S rRNA and vlhA* gene. From the 20 samples analysed, only two found to be *M. synoviae* by *16S rRNA* gene, showed negative for *vlhA* gene. *M. synoviae* was detected in 34 remaining samples by both PCR assays. The PCR procedure was found to have the sensitivity and specificity required to be useful as diagnostic PCR with the added advantage of preliminary strain identification based on the DNA sequence analysis of the PCR product. Similarly, Jeffery *et al.* (2007) used oligonucleotide primers complementary to the single copy conserved 5' end of the *vlhA* and could generate amplicons of approximately of 400bp from 35 MS positive samples. Sequencing of the amplicons represented that each single profile was related to unique sequence, some differing from each other just by one base-pair substitution.

In another finding, Hammond *et al.* (2009) used published primers to detect *M. synoviae* and identified the strain using the *vlhA* gene sequence. Around 32 samples, including *M. synoviae* strains and clinical samples from MS infected birds, were screened and found positive suggesting that the method could also be suitable for clinical specimens. Finally it was demonstrated that this PCR was equally sensitive to other PCR assays. Harada *et al.* (2009) applied *vlhA* gene sequence analysis for typing the *Mycoplasma synoviae* vaccine MS-H strain and field isolates from diseased chickens in Japan. The *vlhA* sequence analysis allowed the differentiation of all 11 MS field isolates from the vaccine strain. It was found to be useful tool to determine whether MS clinical isolates are derived from the vaccine strain or clinical isolates. In a study conducted by Syed (2010), sero-prevalence of MS was 79.55%. Conventional PCR was efficiently optimized for MS detection with a sensitivity of detecting less than 1pg of DNA. Isolation was carried out and all the isolates were confirmed with PCR identification of both the genes *vlhA* and *16S rRNA* andwas found to be very sensitive for MS.

5.4 Isolation and identification of *M. synoviae*

In the present study, *Mycoplasma synoviae* could not be isolated from any of the clinical samples. Moreover, all seven *16S rRNA* and *vlhA* gene PCR positive samples also could not yield any organism in *in-vitro* cultivation. Cultural isolation and cultivation of *M. synoviae* is very tedious, difficult because of its fastidious nature and complex nutritional requirement and may not give the accurate prevalence of the organisms in birds thereby increasing the chance of false negative results (Bayatzadeh *et al*. 2011)

Similar findings of unsuccessful isolation were observed by various workers (Ramadass *et al.* 2006; Saritha 2010; Bayatzadeh *et al*. 2011; Khalifa *et al*. 2013; Nagalaxmi *et al*. 2013; Kamble, 2014). Nagalaxmi *et al*. (2013) attempted the isolation of MS from 262 trachea and air sac samples but could not recover even single isolate of *M.synoviae*. Khalda *et al.* (2013) cultured

170 tracheal swabs samples from diseased birds and recovered only 3 isolates of *M. synoviae*. In another study, Ramadass *et al.* (2006) detected *Mycoplasma synoviae* among chickens in different parts of Tamil Nadu. Out of 1039 samples analysed, only 20 (1.9%) samples yielded pure isolates by conventional isolation. Kamble (2014) attempted isolation of MS from suspected birds. Out of 159, none could be isolated by conventional method of isolation. In a similar study, Saritha (2010) incubated 425 samples from both live and dead birds in Frey's Medium, out of which 27 (6.3%) were found positive with typical colonies. Confirmation was done by amplifying *16S rRNA* gene of MS with a set of published primer sequences through PCR.

On the contrary, Bayatzadeh *et al*. (2011) isolated 24 (55.9%) *M. synoviae* out of 43 swabs of trachea /lungs /air sacs collected from broiler chicken farms and later confirmed by species specific PCR assay. In a study, Khalifa *et al*. (2013) performed the isolation of *M. synoviae* from tracheal swabs of diseased birds showing clinical signs. Out of 45 Mycoplasma isolates recovered, only 3 were identified as *M.synoviae.*

5.5. Comparative efficacy of cultural isolation and PCR for detection of *M. synoviae* infection

Out of 165 clinical specimens (choanal swabs) processed for detection of *Mycoplasma synoviae* infection by direct PCR and cultural isolation, only PCR could detect seven (4.2%) positive cases of MS infection whereas cultural isolation failed to detect the same. Moreover, PCR could detect the strain variation occurring in MS of different geographical area. Thus PCR was found to be more sensitive, specific, rapid and easy method than cultural isolation for diagnosis of avian mycoplasmosis resulting due to MS infection.

Lauerman *et al*. (1993) screened 122 flocks by PCR. Analysis of cultures of avian mycoplasmas using the MS PCR indicated 100% specificity and sensitivity. Analysis data sets indicated sensitivity of 82% for the MS PCR and a specificity of 100% as determined by comparison with culture. Thus, *16S rRNA* gene based species specific PCR for MS proved to be a useful tool in detection of infection directly from clinical samples.

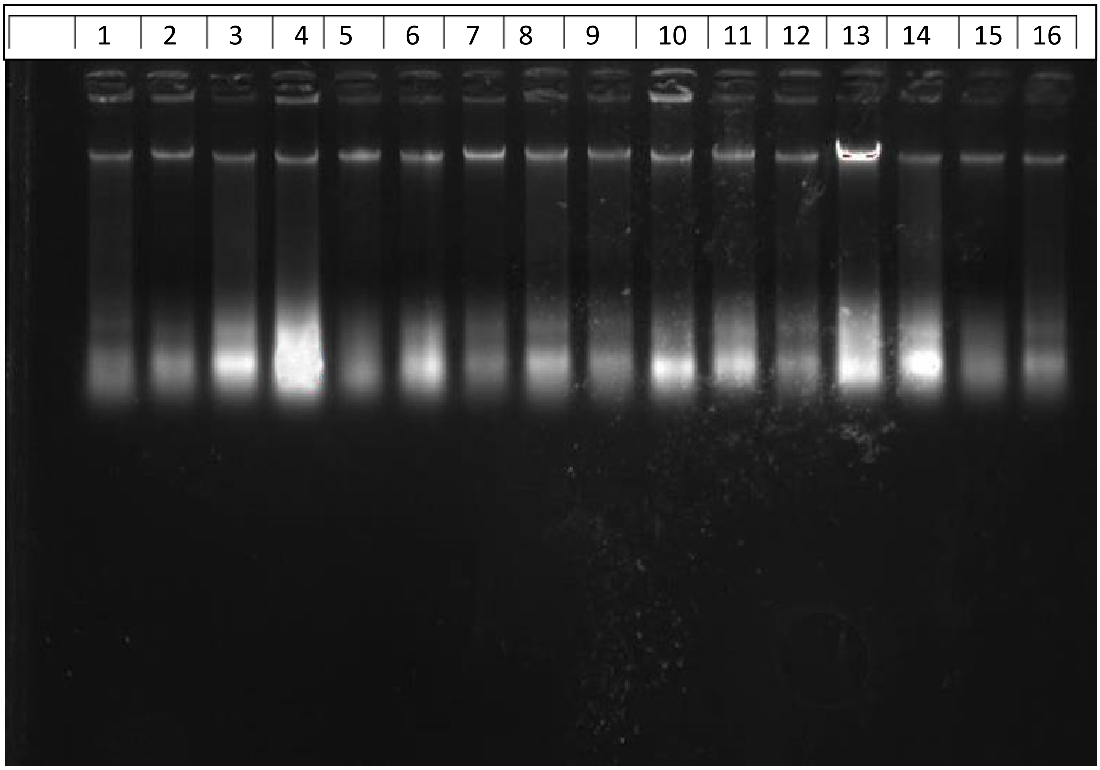
Lauerman *et al*. (1998) stated *16S rRNA* PCR method as commonly used for confirmation of *M. synoviae* infection in chickens and turkeys in the United States. It is fast, sensitive, specific and relatively inexpensive. It was concluded by Bayatzadeh *et al*. (2011) that PCR is a more rapid, effective, sensitive and inexpensive method than the standard culture technique. Therefore, PCR can be used as an alternative method for traditional culture toward the detection of *M. synoviae*. The true prevalence of *M. synoviae* may best be reflected by PCR results (Ehtisham-ul-haque., 2010). It was clear from the study conducted by Fan *et al*., (1995) that PCR can serve as a simple, rapid and easy technique in routine mycoplasma detection and identification procedure. Specific result can be achieved with samples taken by swabbing different organs. Best result could be achieved by combining PCR with culture than culture alone (Jefferey *et al*., 1995).Mardassi *et al.* (2004) developed a duplex PCR assay targeting the hemagglutinin multigene families, *vlhA* and *pMGA*, of *M. synoviae* and *M. gallisepticum*, respectively. The assay proved to be specific and sensitive enough to justify its use for the simultaneous detection of the two major avian mycoplasma species from field isolates. Because of difficulties involved in obtaining pure cultures of *M. synoviae* and the lack of ability to type cultures using the *16S rRNA* PCR method, Noormohammadi *et al*. (2000) attempted to develop a PCR procedure that would be useful as a diagnostic tool as well as offer the ability to do preliminary ‘‘fingerprinting’’ by sequencing the PCR product. The N-terminal end of the *vlhA* was shown to have potential as a target gene for identification of specific strains of *M. synoviae* (Bencina *et al*., 2001).

Plate 3.1: Collection of specimen from suspected bird

Bird showing nasal rale ; suspected for mycoplasma infection

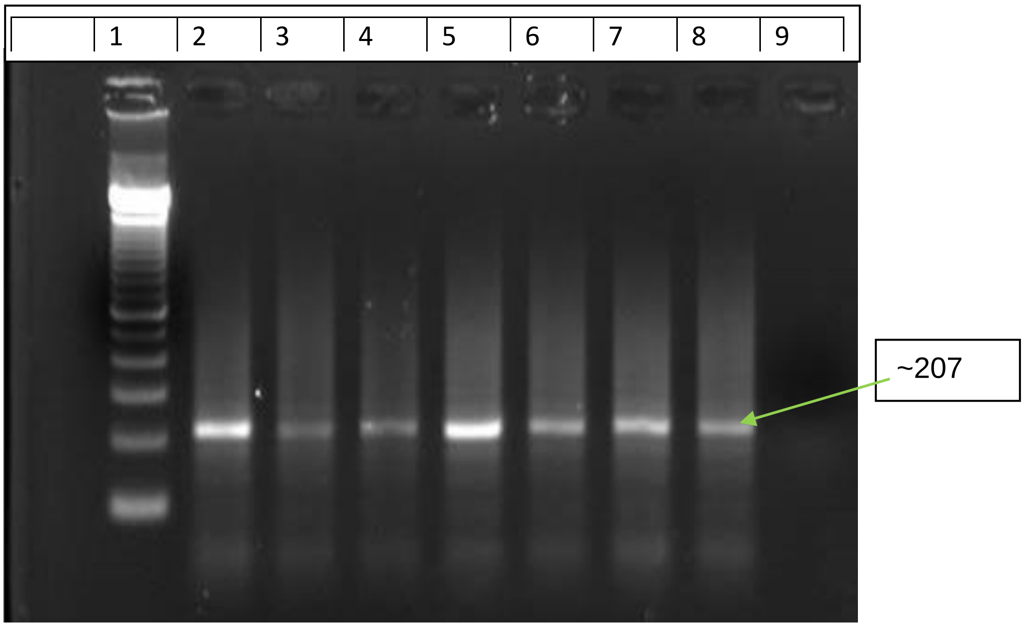
Plate 3.2: Media used during cultural isolation of *M. synoviae*

Plate 4.1: Agar Gel Electrophoresis of DNA extracted from clinical specimens



Lane 1-16; DNA bands shown in Agar Gel Electrophoresis

Plate 4.2: *M. synoviae* species specific *16S rRNA* PCR assay



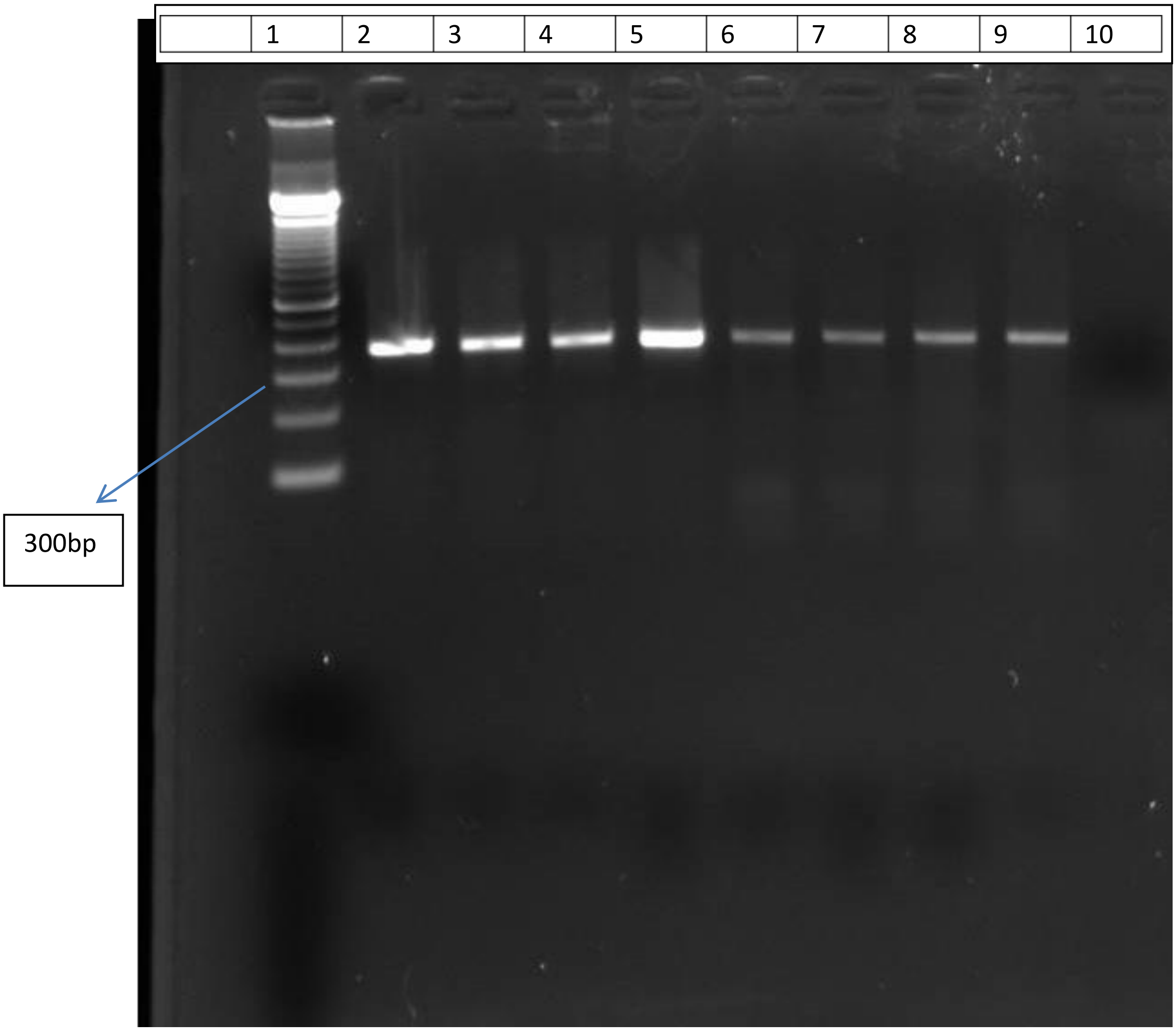
Lane 1: 100 bp ladder

Lanes 2: Reference strain as standard positive

Lanes 3-8: Positive clinical specimens showing bands at ~207 bp

Lane 9: Negative

Plate 4.3: *M. synoviae* species specific *vlhA* gene PCR assay

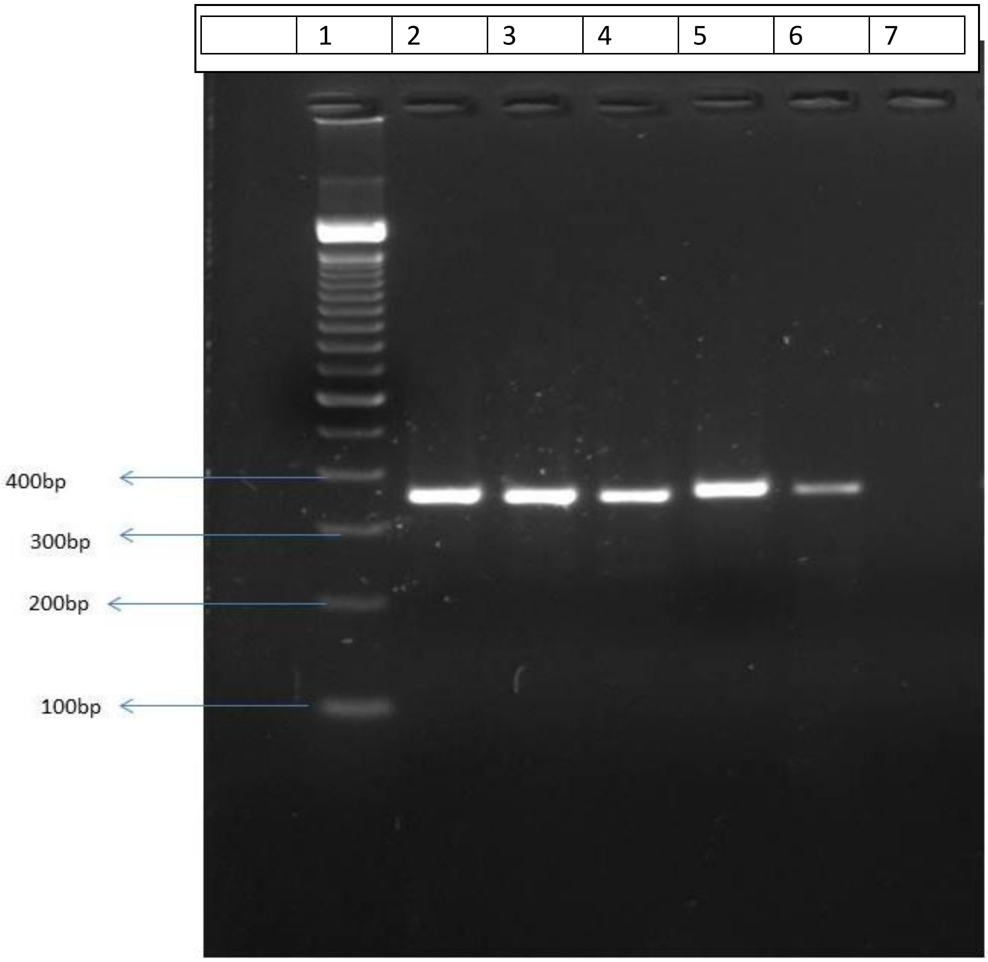


Lane 1: 100 bp DNA ladder

Lanes 2-9: Positive clinical specimens for *vlhA* gene

Lane 10: Negative

Plate 4.4: *M. synoviae* species specific *vlhA* gene PCR assay



Lane 1- 100bp ladder

Lane 2- 3: *vlhA* amplicons of MS samples from Maharashtra

Lane 4: *vlhA* amplicons of the reference strain.

Lane 4: *vlhA* amplicons of MS samples from Delhi

Lane 5: *vlhA* amplicons of MS samples from Kolkata

5. SUMMARY AND CONCLUSION

Mycoplasma synoviae (MS) belongs to the Mycoplasmataceae family and it is smallest, fastidious, facultative anaerobic wall-less bacterium. *M synoviae* infection is also known as infectious synovitis. It affects chickens and turkeys and was first recognized as an acute to chronic infection of chickens and turkeys affecting respiratory system and joints. Sub clinically; the infection may be prevalent in upper respiratory tract.

The present study focused on detection of *M*. *synoviae* infection by molecular technique viz. Polymerase Chain Reaction directly from clinical samples. Swabs were collected from choanal region of birds showing respiratory distress and synovitis; suspected for *M*. *synoviae* infection and subjected to PCR. The assay targeted the amplification of *16S rRNA* and *vlhA* genes. A total of 165 choanal swabs were collected from live birds suspected for MS and subjected to DNA extraction and further to PCR assays. Out of 165 specimens, MS could be detected from only seven (4.2%) by *16S rRNA* based PCR showing an amplification of ~ 207 bp product.

Another assay targeting non-conserved gene region of *vlhA* gene was also performed. The gene fragment of ~350bp was amplified from all the samples (7out of 165) which were found to be positive for MS by *16S rRNA* based PCR. The amplicon size of specimens originating from different geographical regions varied in the sizes. The strains from Maharashtra region yielded PCR product of ~360bp, whereas Delhi and Kolkata regions produced ~380bp size amplicon. Thus indicating strain variations among the different geographical areas under study.

The clinical specimens (choanal swabs) and reference strain (ATCC® 25204™) were also inoculated in PPLO (Pleuropneumonia Like Organism) media to attempt for propagation and isolation of *M. synoviae*. The organism could not be isolated from any of the clinical samples inoculated in the media for isolation.

The present study demonstrated the involvement of MS in respiratory distress cases of chickens using simplex PCR based amplification of conserved region of *16S rRNA* and non-conserved gene region *vlhA* gene of

M. synoviae. It was found to be a simple, sensitive, more specific and cost effective alternative to conventional methods.

Thus from the present study, it can be concluded that:

- The overall incidence of MS was 4.2% with incidence rate of 3.4% and 6.25% in layers and broilers respectively
- Species specific *16S rRNA* based PCR was found to be useful for detection of *M. synoviae* directly in the clinical specimens.
- Surface variable lipoprotein-haemagglutinin *vlhA gene* based PCR assay was found to be useful for detection *M. synoviae* strains variation originating from different geographical regions.
- Cultural isolation was found to be difficult and less sensitive, time consuming and costly.
- Thus, it is suggested that the true prevalence of *M. synoviae* may best be reflected by PCR results.
- Further studies on molecular detection of *M. synoviae 16S rRNA* and *vlhA gene* PCR will give the molecular epidemiology useful in prevention and control strategies.

BIBLIOGRAPHY

Allen, J. L., A. H. Noormohammadi and G. F. Browning (2005) The *vlhA* loci of *Mycoplasma synoviae* are confined to a restricted region of the genome. Microbiology., **151**(3): 935-40.

Amy, N., M. L. Kristina and R Ziv (2010) Revised *Mycoplasma synoviae vlhA* PCR. Avian Diseases, **54**: 1292-1297.

Balen, L., O. and L. O. Fiorentin (1990) *Mycoplasma synoviae* e seu impacto econômico sobre a avicultura. In: Conferência Apinco de Ciência e Tecnologia Avícolas; Campinas, São Paulo. Brasil. pp. 35-140.

Bayatzadeh, M., S. A Pourbakhsh, A. R Homayounimehr, A Ashtari and A. R Abtin (2011) Application of culture and polymerase chain reaction (PCR) methods for isolation and identification of *Mycoplasma synoviae* on broiler chicken farms, **66**:87-94.

Behbahan, G., K. Asasi, A. Afsharffar and S. A. Pourbakhsh (2005) Isolation and identification of *Mycoplasma gallisepticum* by PCR and RFLP. Iranian journal of vet research, **6**(2): 2.

Bencina, D., M. Drobnic-Valic, S. Horvat, M. Narat, S. H. Kleven and P. Dovc (2001) Molecular basis of the length variation in the N-terminal part of *Mycoplasma synoviae* hemagglutinin. FEMS Microbiol. Lett., **203**:115-123.

Bencina, D., M. Narat, P. Dovc, M. Dobnic-Valic, F. Habe and S.H. Kleven (1999) The characterization of *Mycoplasma synoviae* EF-Tu protein and proteins involved in hemadherence and their N-terminal amino acid sequences. FEMS Microbiol. Lett., **173**: 85-94.

Brown, D. R., R. F. Whitcomb and J. M. Bradbury (2007) Revised minimal standards for description of new species of the class Mollicutes (division Tenericutes). Int. J. Syst. Evol. Microbiol., **57**: 2703–2719.

Buim M. R., Mettifogo E., Timenetsky J., Kleven S. and Ferreira A. J. P (2009) Epidemiological survey on *Mycoplasma* and *Mycoplasma synoviae* by multiplex PCR in commercial poultry. Pesq. Vet. Bras. **29:**552-556.

Bukte, S. R., (2015) Detection of Mycoplasmosis in Poultry by conventional and molecular technique. M.V.Sc. Thesis Submitted to MAFSU, Nagpur. India.

Dienes L, H.J Weinberger (1951). The L form of bacteria. Bacteriol Rev., **15**(4):245-288.

Dierks R. E., J. A. Newman and B. S. Pomeroy (1967) Characterization of avian mycoplasma.Biology of mycoplasma.**143**:170-189.

Ehtisham, S. (2011) Isolation, identification and characterization of Mycoplasma species from respiratory problems in poultry. M.Sc. (Hons.), Thesis, Deptt. Vet. Microbiol., Univ. Agri (U.A.F), Faisalabad. Pakistan.

Ewing, M. L., L. H. Lauerman, S. H. Kleven and M. B. Brown (1996) Evaluation of diagnostic procedures to detect *Mycoplasma synoviae* in commercial multiplier breeder farms and commercial hatcheries in Florida. Avian Dis., **40** (4): 798-806.

Fabienne*,* D.G., Alexandra Dheilly., Corinne Marois., Isabelle Kempf (2006) Epidemiological study on Mycoplasma synoviae infection in layers. Vet Microbiol,**114** (1-2):148-154.

Fan, H. H., S. H. Kleven, M. W. Jackwood, K. E. Johansson, B. Pettersson and S. Levisohn (1995) Species identification of avian mycoplasmas by polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis., **39**(2): 398- 407.

Feberwee, A, T. S de Vries, W. J Landman (2011) Seroprevalence of *Mycoplasma synoviae* in Dutch commercial poultry farms. Avian Pathol. **37**:629–633.

Feberwee, A., D. R. Mekkes, J. J. de Wit, E.G. Hartman, and A. Pijpers (2005) Comparison of culture, PCR, and different serologic tests for detection of Mycoplasma gallisepticum and *Mycoplasma synoviae* infections. Avian Dis., **49**: 260-68.

Frey, M. L., R. P. Hanson and D. P. Anderson (1968) A medium for isolation of avian mycoplasmas. Am. J. Vet. Res., **29:** 2163-2171.

Garcia, M., I. Gerchman, R. Meir, M. W. Jackwood, S. H. Kleven, and S. Levisohn (1996) Detection of *Mycoplasma meleagridis* and *M. iowae* from dead-in-shell turkey embryos by polymerase chain reaction and culture. Avian Patho**l., 26**:765–778.

Hagan, Jennifer C.[1] Nicholas J. Ashton , Janet M. Bradbury and Kenton L. Morgan, (2004) Evaluation of an egg yolk enzyme-linked immunosorbent assay antibody test and its use to assess the prevalence of *Mycoplasma synoviae* in UK laying hens. Avian Pathology, **33**: 91-95

Hammond, P. P., A. S. Ramírez, C. J. Morrow and J. M. Bradbury (2009). Development and evaluation of an improved diagnostic PCR for *Mycoplasma synoviae* using primers located in the haemagglutinin encoding gene *vlhA* and its value for strain typing.Veterinary Microbiology**136:** 61–68.

Harada, K., M. Tanaka, M. Uchiyama, T. Yamamoto, K. Oishi, M. Arao, and T. Takahashi (2009) Molecular typing of Japanese field isolates and live commercial vaccine strain of *Mycoplasma synoviae* using improved Pulsed-Field Gel Electrophoresis and *vlhA* gene sequencing. Avian Dis., **53** (4): 538–543.

Hong, Y., M. Garcia, V. Leiting, D. Bencina, L. Dufour-Zavala, G. Zavala and S. H. Kleven (2004) Specific detection and typing of *Mycoplasma synoviae* strains in poultry with PCR and DNA sequence analysis targeting the hemagglutinin encoding gene *vlhA*. Avian Dis., **48**: 606-616.

Jarquin, R., J. Schultz, I. Hanning and S. C. Ricke (2009) Development of a Real-Time polymerase chain reaction assay for the simultaneous detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* under industry conditions. Avian Dis**, 53**: 73-77.

Jeffery, N., G. F. Browning and A. H. Noormohammadi (2006) Organization of the *Mycoplasma synoviae* WVU 1853T *vlhA* gene locus. Avian Pathol., **35(**1): 53-57.

Jeffery, N., R. B. Gasser, P. A. Steer and A. H. Noormohammadi (2007) Classification of *Mycoplasma synoviae* strains using single-strand conformation polymorphism and high-resolution melting-curve analysis of the *vlhA* gene single-copy region. Microbiology, **153**: 2679-2688.

Jordan F. T. W., H. Erno, G. S. Cottew, K. H. Hinz and L. Stipkovits (1982) Characterization and taxonomic description of five mycoplasma serovars (serotypes) of avian origin and their elevation to species rank and further evaluation of the taxonomic status of Mycoplasma synoviae. Japanese Journal of Systematic Bacteriology; **32**:108-15.

Kamble, S. Y. (2014) Molecular Diagnosis of Mycoplasmosis in Poultry. M.V.Sc. Thesis Submitted to MAFSU, Nagpur. India.

Khalifa, Khalda A., Egbal Sidahmed Abdelrahim, Magdi Badwi Amalm.Mohamed (2013) *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in Chickens Sudan Veterinary Research Institute (VRI, Khartoum, Sudan.

Kiss, I., Katalin Matiz, Eva Kaszanyitzky, Yleana Chavez, K. E. Johanssonc (1997) Detection and identification of avian mycoplasmas by polymerase chain reaction and restriction fragment length polymorphism assay. Elsevier Veterinary Microbiology, **58**(1): 23–30.

Kleven, S., O. J. Fletcher and R. B. Davis (1975) Influence of strain of *Mycoplasma synoviae* and route of infection on development of synovitis or airsacculitis in broilers. Avian Diseases, **19**:126–135.

Kleven, Stanley., Dyarl D. King and P David (1972) Airsacculitis in Broilers from *Mycoplasma synoviae*: Effect on Air-Sac Lesions of Vaccinating with Infectious Bronchitis and Newcastle Virus Avian Diseases, **16** (4): 915-924. .

Kleven, S. H (1997) Mycoplasmosis. In: Swayne, D.E., J.R. Glisson, M.W. Jackwood, J.E. Pearson, and W.M. Reed. A Laboratory Manual for the Isolation and Identification of Avian Pathogens. 4rth Ed. Kennett Square, (PA): American Association of Avian Pathologists: 74-80.

Kleven, S. H (2003) *Mycoplasma synoviae* Infection. In: Saif, Y. M., H. J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, and D.E. Swayne. Diseases of Poultry, 11th ed. Iowa State Press, Ames, Iowa: 756-766.

Kleven, S. H. and M. Garcia (2003**)** Epidemiology of mycoplasmal infections based on analyses of gene polymorphisms. FEMS Microbiol. Lett., 222 S1:101. Kleven, S.H., 2008a. Control of Avian Mycoplasma Infections in Commercial Poultry. Avian Dis., **52:** 367-374.

Kojima, A., T Takahashi, M Kijima, Y Ogikubo, M Nishimura, S Nishimura (1997) Detection of Mycoplasma in avian live virus vaccines by polymerase chain reaction. Biologicals, **25**(4):365–71.

Lauerman, L H., F J Hoerr, A R Sharpton, S M Shah, V L van Santen (1993) Development and application of a polymerase chain reaction assay for *Mycoplasma synoviae*. Avian Dis, **3** (3):829–34.

Lauerman L. H (1998) Mycoplasma PCR assays. In: Nucleic acid amplification assays for diagnosis of animal diseases. Ed L. H. Lauerman, American Association of Veterinary Laboratory Diagnosticians, Turlock, Calif: 39-66.

Ley, D. H. and H. W. Yoder (1997) *Mycoplasma gallisepticum* infection. In: Diseases of Poultry. 9th ed B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif, ed. Iowa State Unversity Press, Ames, IA. 194-207.

Lobova, D., L. Kohoutova, D. Molinkova, K. Rosenbergova, O. Kubicek, V. Celer (2012) Prevalence of etiological agents of selected respiratory infections in chicken and turkey farms in the Czech Republic Veterinary Medicina, **57**(3): 125–132.

Lockaby, S. B., F. J. Hoerr, L. H. Lauerman, B. F. Smith, A. M. Samoylov, M. A. Toivio-Kinnucan and S. H. Kleven (1999) Factors associated with virulence of *Mycoplasma synoviae.* Avian Diseases, **43**:251– 261.

Luciano R. L., A. L. S. P. Cardoso., G. F. Z. Stoppa., A. M. I. Kanashiro, A. G. M. de Castro and E. N. C. Tessari (2011) Comparative study of serological tests for *Mycoplasma synoviae*, diagnosis in commercial poultry breeders. Veterinary Medicine International Volume 2011:5.http://dx.doi.org/10.4061/2011/304349

Mardassi, B. Ben Abdelmoumen., R. Ben Mohamed, I. Gueriri, S. Boughattas, and B. Mlik (2005) Duplex PCR To Differentiate between *Mycoplasma synoviae* and *Mycoplasma gallisepticum* on the Basis of Conserved Species-Specific Sequences of Their Hemagglutinin Genes. Journal Of Clinical Microbiology, 948–958

Marois, C., F. Oufour-Gesbert,and I. Kempf. (2000) Detection of *Mycoplasma synoviae* in poultry environment samples by culture and polymerase chain reaction. Veterinary Microbiology, **73**: 311-318.

Maurice C. Shepard.(1956) identification of “t” mycoplasmas in primary agar cultures. American journal. 174, Unusual Isolates from Clinical Material Pages 109–119

Mohammed, H. O., R. Yamamoto, T. E. Carpenter and H. B. Ortmayer (1986) Comparison of egg yolk and serum for the detection of *Mycoplasma gallisepticum* and *M. synoviae* antibodies by enzyme-linked immunosorbent assay. Avian Dis., **30** (2): 398-408.

Noormohammadi A. H., P. F. Markham, M. F Duffy , K. G. Whithear and G. F. Browning (1998) Multigene Families Encoding the Major Hemagglutinins in Phylogenetically Distinct Mycoplasmas. Infection Immunology., **66(**7):3470-5**.**

Nagalaxmi, K. S., T.M.A Senthilkumar, M. Parthiban and P. Ramadass (2013) Differential Diagnosis of Avian Mycoplasmosis. Indian Vet. J., **90** (6):12-15.

Nascimento, E. R., R. Yamamoto, K. R Herrick and R. C. Tait (1991) Polymerase chain reaction for detection of *Mycoplasma gallisepticum*. Avian Dis., **35**(1):62-69.

Nascimento, E R., R. C .F. Silva, M. O. Platenik, V. L. A. Pereira, M. L. Barreto and A. R. A. Correa (2003) Effect of *Mycoplasma synoviae* infection on serologic response to Newcastle La Sota vaccine. In: 13th Congress of the World Veterinary Poultry Association and American Association of Avian Pathologists/American Veterinary Medical Association . Annual Meeting; Denver, Colorado, USA, 65.

Nascimento, E. R., M. G. F. Nascimento, M. G. M. Danelli, S. L. Machado, G. B. Lignon and P. A. Polo (1998) Comparison of PCR kits for the detection of *Mycoplasma gallisepticum* and *M. synoviae* (MS) in MS infected and uninfected chickens. In: Proceedings of the 47th Western Poultry Disease Conference; Sacramento, California, USA: 84-86.

National Poultry and Improvement Plan (NPIP) and Auxiliary Provisions. Washington, DC.USDA (2002) Auxiliary Provisions on National Poultry Improvement Plan (NPIP), Ch. 01, 9 CFR-Part 147, Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture, USA.

Noormohammadi, A. H., P. F. Markham, A. Kanci, K. G. Whithear and G. F. Browning (2000) A novel mechanism for control of antigenic variation in the haemagglutinin gene family of *Mycoplasma synoviae.* Mol. Microbiol., **35:** 911-923.

Noormohammadi, A. H., P. F. Markham, K. G. Whithear, I. D. Walker, V. A. Gurevich, D. H. Ley and G. F. Browning (1997) *Mycoplasma synoviae* has two distinct phase- variable major membrane antigens, one of which is a putative hemagglutinin. Infect. Immun**., 65:** 2542-2547.

Olson, N. O., D. C. Shelton, J. K. Bletner, D. A. Munro, and G. C. Anderson (1956).Studies of infectious synovitis in chickens. Am. J. Vet. Resarch, **17**:747-754.

Opitz, H. M (1983) *Mycoplasma synoviae* infection in Maine's egg farms. Avian Diseases. **27**:324-326.

Papazisi, L., T. S. Gorton, G. Kutish, P. F. Markham, G. F. Browning, D. K. Nguyen, S. Swartzell, A. Madan, G. Mahairas and S. J. Geary (2003) The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain Rlow. Microbiology, **149**: 2307-2316.

Pourbakhsh, S. A., G. R Shokri, M Banani, F Elhamnia, Ashtari (2010) Detection of *Mycoplasma synoviae* infection in broiler breeder farms of Tehran province using PCR and culture methods. Archives of Razi Institute; **65**(2): 75-81.

Ramadass, P., R Ananthi, T M A Senthil Kumar, G Venkatesh, V Ramaswamy (2006) Isolation and characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* from poultry. The Indian Journal Of Animal Sciences.**76**:10.

Ramirez, A. S., Naylor, C. J., Pitcher, D. G., Bradbury, J. M. (2008). High inter-species and low intra-species variation in 16S–23S rDNA spacer sequences of pathogenic avian mycoplasmas offers potential use as a diagnostic tool. Veterinary Microbiology **128**: 279–287.

Raviv, Z. and S. H. Kleven (2009) The development of diagnostic Real-Time TaqMan PCRs for the four pathogenic avian mycoplasmas. Avian Dis., **53**: 103-107.

Raviv Ziv and David H. Ley (1997) *Mycoplasma synoviae* infection. Slide submitted to American Association of Avian Pathologists, Inc. 12627 San Jose Blvd., Suite 202 Jacksonville, FL 32223.

Razin, S., D. Yogev and Y. Naot (1998) Molecular biology and pathogenicity of mycoplasmas. Micro.Molec. Biol. Rev., **62**: 1094-1156.

Saritha, S., G R Veeregowda, D Rathanamma, G leena, Y I Vinay Kumar,Prem Sagar, CS Naraya (2010) Isolation and molecular characterization of MS from chicken. Diagnosis of mycoplasmosis, **80**: 142.

Salisch, H., K. H. Hinz, H. D. Graack and M. Ryll (1998) A comparison of a commercial PCR-based test to culture methods for detection of *Mycoplasma* *gallisepticum* and *Mycoplasma synoviae* in concurrently infected chickens. Avian Pathol., **27**: 142- 147.

Salisch, H., M. Ryll, K. H. Hinz and U. Neumann (1999) Experiences with multispecies polymerase chain reaction and specific oligonucleotide probes for the detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. Avian Pathol., **28**: 337- 344.

Seifi, S., M Shirzad (2012) Incidence and risk factors of *Mycoplasma synoviae* infection in broiler breeder farms of Iran. Veterinary World Journal, **5**(5):265.

Senthilnathan G., S. Shenbagam, T. Suryanarayana and M. Thiyageeswaran (2015) Isolation and molecular confirmation of *Mycoplasma synoviae* infection from broiler breeder farms in Tamilnadu. Indian J. Anim. Res., **49** (1) : 91-94.

Siddique Abu Baker. (2012) Molecular characterization of opportunistic pathogens from avian Mycoplasma cases in Punjab. Thesis submitted to Intitute Of Microbiology, Faculty Of Veterinary Science University Of Agriculture Faisalabad.

Silva, R. C. F (2003) Vacinação contra a doença de Newcastle em aves Gallus gallus SPF infectadas por *Mycoplasma synoviae* [dissertação]. Niterói (RJ): Universidade Federal Fluminense. Stipkovits. L. and I. Kempf, 1996. Mycoplasmoses in poultry. Rev. Sci. Tech, Off. Int. Epiz., **15**(4): 1495-1525.

Silveira, Ricardo M., Laurimar Fiorentin and Edmundo K. Marques (1996) Polymerase Chain Reaction Optimization for *Mycoplasma gallisepticum* and *M. synoviae* Diagnosis. Avian Diseases., **40**(1):218-22.

Stipkovits L. & Kempf I (1996) Mycoplasmoses in poultry. Rev. Sci. Technol. Int. Epizoot. **15**:1495-1525.

Tan, I. J., H Dahlia, Z Darrahimah, P Chandrawathani, M Ramlan (2011) Isolation of Mycoplasma species from various animal hosts in Malaysia Veterinary Research Institute, Department of Veterinary Services. **2**(1):53

Tebyanian, Hamid., Seyed Hanif Mirhosseiny, Babak Kheirkhah, Mehdi Hassanshahian, Hamze Farhadian (2014) Isolation and Identification of *Mycoplasma synoviae* From Suspected Ostriches by Polymerase Chain Reaction Jundishapur J Microbiol, **7**(9): e19262

Vandekerckhove, D., P. De Herdt, H. Laevens, P. Butaye, G. Meulemans, F. Pasmans (2004). Significance of interactions between Escherichia coli and respiratory pathogens in layer hen flocks suffering from colibacillosis-associated mortality. *Avian Pathol.* **33**:298–302.

Vardaman, T. H and J. H. Drott (1980) Comparison of *Mycoplasma synoviae* hemagglutinating antigens by the hemagglutination-inhibition test. Avian Dis., **24** (3): 637-640.

Ward D M., R. Weller, M.M Batson (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature; **345**:63-5.

Waters, A P., T. F Mccuthan (1990) Ribosomal RNA: Nature's own polymerase amplified target for diagnosis. Parasitology Today, **6**(2):56–9.

WOAH (2008) Terrestrial Manual Chapter 2.3.5:482-496.

Yilmaz, F., N. Timurkaan, A. Kiliç, H. Kalender, Ü. Kilinç (2011) Detection of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* in chickens by immunohistochemical, PCR and culture method. School Of Health Sciences, Veterinary Control and Research Institute Turkey. **162**(2): 79-86.

Zendulková D, A Madanat, P Lány, K Rosenbergová, Z Pospíšil (2007) Detection of *Mycoplasma agalactiae* by Polymerase Chain Reaction in Jordanian Sheep and Goat Herds. Acta Veterinarian Brno, **76**(1):71–7.

APPENDICES

Appendix – I

Reagents used for DNA extraction

1. Lysis buffer (100ml)

Tris HCl (50mM)	0.788gm
EDTA (50mM)	1.8gm
SDS (1%)	1gm
NaCl	0.58gm
Autoclaved Distilled Water	100ml

2. Proteinase K (20mg/ml)

Proteinase K	20mg
Water	1ml

3. Phenol: chloroform 1:1 (100ml)

Phenol	50ml
Chloroform	50ml

4. Chloroform: Isoamyl Alcohol (24:1) 100ml

Chloroform	96ml
Isoamyl alcohol	4ml

5. Sodium acetate (3M) 100ml

Sodium acetate	24.6gm
Water	100ml

6. Absolute Ethanol……………………100ml

7. Ethanol 70% (100)

Ethanol……………………………70ml
Water …………………………….. 30ml

8. Autoclaved distilled water ad libitum

Sterilize by autoclaving at 15 lbs pressure (121°c) for 15 minutes. Store at 4°c.

Appendix – II

Reagents used for Agarose Gel Electrophoresis (AGE)

1) Ethidium bromide (10 mg / ml)

Ethidium bromide	: 20 mg
Distilled water	: 2 ml

2) 0.5 M EDTA (pH 8.0)

Dissolve 18.612 g EDTA $2H_2O$ in 80 ml distill water and adjust pH to 8.0 using NaOH pellets. Adjust the final volume to 100 ml. Filter through Whatman filter paper no. 1 and sterilize by autoclaving at 15 lbs for 15 min. Store at room temperature.

3) Tris – Acetate – EDTA (TAE) stock solution (50 X)

Tris base	: 24.2 g
Glacial acetic acid	: 5.7 ml
0.5 M EDTA, pH 8.0	: 10 ml
Distilled water to make	: 100 ml

(For preparing the working solution (1X), dilute the stock TAE in distilled water)

4) 6X Loding dye (Type IV)

Sucrose : 40 % w/v in DW
Bromophenol blue : 0.25 % w/v in DW
Store the solution at 4^0 C.

Appendix – III

Media, stains and solutions

a) Composition of PPLO broth medium (modified Frey's medium) described by Frey *et al.,* 1968; Kleven, 1998 and used by Ehtisham., 2010)

Ingredients	**Quantities**
PPLO broth base	2.55 g
Glucose	1g
Fresh Horse serum	15 ml
Yeast extract	10 ml
Cysteine hydrochloride (1%)	0.01g
β-Nicotinamide adenine dinucleotide (NAD) (1%)	0.01 g
Phenol red (1%)	0.25 ml
Thallium acetate (10%)	0.5 ml
Ampicillin	0.1 g
Distilled Water	100 ml

Composition of PPLO agar medium (modified Frey's medium)

Ingredients	**Quantities**
Mycoplasma agar base	3.5 g
Glucose	0.3 g
Fresh Horse serum (Heat inactivated)	12 ml
Yeast extract (25%)	10 ml
Cysteine hydrochloride (1%)	0.01 g
β-Nicotinamide adenine dinucleotide (1%)	0.01 g

Phenol red (1%)	0.25 ml
Thallium acetate (10%)	0.5 ml
Penicillin/Ampicillin	0.1 g
Distilled Water q.s.	100 ml

PPLO Broth medium was prepared by autoclaving PPLO broth base, yeast extracts at121°C, at 1 atmospheric pressure for 15 minutes and after cooling the remaining ingredients were added which had previously been sterilized by filtration. The pH was adjusted to 7.8. The medium was mixed thoroughly and poured into sterile Test tubes/plates.

b) Mycoplasma Modified Frey's Broth Medium (Yoder and Olson, 1983) used by Raviv and ley 2011.

Ingredients	**Quantities**
Deionized distilled water	88.0 ml
Mycoplasma Frey's broth base	2.5 g
Swine serum (heated 56 C for 30 min)	12.0 ml
Dextrose	0.3 g
Thallium acetate (10%)	0.5 ml
Potassium penicillin G (aqueous)	500,00 units
Phenol Red (1%)	0.25 ml
NAD (1%)	1.25 ml
Cysteine hydrochloride (l%)	1.25 ml

Adjust to pH 7.8 with 20% NaOH and filter Sterilize. For agar plates 1.5% agar is used.

Media for ATCC

Part A

PPLO Broth	0.35 g
Tryptone	1 g
Peptone	0.5 g
DI Water	64ml
DNA (fish sperm)	0.2 g

Part B

CMRL-1066 medium (10x concentrated;ATCC 20-2207)	5 ml
Yeastolate (2%, autoclaved)	10 ml
Fetal bovine serum (heat-inactivated)	.8.5 ml
Swine serum (heat-inactivated)	8.5 ml
Glucose (50% aqueous solution)	2.0 ml
L-cysteine solution	1.0 ml
NAD solution	1.0 ml

L-cysteine solution

L-cysteine	0.01 g
DI Water	10 ml

NAD solution

NAD	0.01 g
DI Water	10 ml

Part A was adjusted to pH 7.4 and autoclaved at 121ºC and aseptically added **Part B.**

25%Yeast extract Solution

Yeast extract powder : 25 gm
Distilled water : 100ml

The yeast extract was prepared by suspending yeast extract powder in distilled water. This was heated to boiling point, cooled and then centrifuged for 20 minutes at 3000 *g*. The supernatant fluid was decanted and adjusted to pH 8.0 with 0.1 M NaOH. This was clarified by centrifugation and then sterilized by filtration. The extract was stored at –20°C until used.

30% Glucose solution

Glucose : 30gm
Distilled water : 100ml

The weighed glucose dissolved in distilled and adjusted to pH 7.8–8.0 with 0.1 M NaOH. It was sterilised by filtration and stored at 4°C until used.

50% Glucose solution

Glucose : 50gm
Distilled water : 100ml

0.1% Phenol red

Phenol red : 0.1gm
Distilled water : 100ml

1% Phenol red

Phenol red : 1gm
Distilled water : 100ml

Phenol red was ground in 0.1 M NaOH (2.8 ml), and then made up to 100 ml in sterile distilled water and autoclaved at 115°C at 1 atmosphere for 30 minutes. It was stored at 4°C until used.

10% Thallous acetate

Thallous acetate	: 10 gm
Distilled water	: 100 ml

Thallous acetate was dissolved in distilled water, filter-sterilised and stored at–20°C until used.

Penicillin solution

Benzyl penicillin	: 200,000 IU
Distilled water	: 1 ml

Penicillin solution was stored at 4°C, and has a shelf life of 1 week.

1% Cysteine hydrochloride

Cysteine hydrochloride	: 0.1gm
Distilled water	: 10ml

1% β-Nicotinamide adenine dinucleotide

β-Nicotinamide adenine dinucleotide	: 0.1gm
Distilled water	: 10ml

Diene's stain solution

Ingredients	**gm/ml**
Methylene blue	: 2.50
Azure II	: 1.25
Maltose	: 10.00
Sodium carbonate	: 0.25
Distilled water	: 100

Appendix –IV

List of equipment

Some of the important equipment used during the study were as shown below.

Sr. No.	Name	Manufacturer
1	Micropipettes	Eppendorf research plus.
2	Cooling centrifuge	REMI.
3	Vortex	Genei.
4	Microwave oven	LG
5	Nanodrop spectrophotometer	Thermo Scientific.
6	Ice maker	Sanyo, Japan.
7	Deep freeze (– 20^{o}c)	Voltas TATA.
8	Power pack	Tarsons, Consort.
9	Water bath	Biotechnics India.
10	Mastercycler nexus gradient Thermal cycler	Eppendorf.
11	Submarine gel electrophoresis apparatus	Superfit.
12	Horizontal gel electrophoresis apparatus	Techno source.
12	Gel documentation system (Gel Doc EZ Imager)	Bio-Rad.
13	Mini centrifuge	Genei.
14	pH meter	Toshniwal Instruments, India.
15	Weighing balance	OHAUS.
16	Micrometer	Hp.

Appendix – G

THESIS ABSTRACT

a)	Title of the thesis (in Capital letters)	**:**	MOLECULAR DETECTION OF *mycoplasma synoviae* IN POULTRY
b)	Full name of student	**:**	Padam Lal Luitel
c)	Name and address of Major Advisor	**:**	Dr. (Mrs) R.S. Gandge, Professor and HOD, Department of Veterinary Microbiology.
d)	Degree to be awarded	**:**	MVSc.
e)	Year of award of degree	**:**	2016
f)	Major subject	**:**	Veterinary Microbiology
g)	Total number of pages in the thesis	**:**	
h)	Number of words in the abstract	**:**	
i)	Signature of Student	**:**	
j)	Signature, Name and address of forwarding authority (HOD / SH)	**:**	
i)	Signature of the Associate Dean		

ABSTRACT

Mycoplasma synoviae is an economically important pathogen of poultry worldwide, causing respiratory infection and synovitis in chickens and turkeys. Early detection of *M. synoviae* infection is of critical importance for subsequent chemotherapy.

The present research was carried out study the occurrence of *Mycoplasma synoviae* using Polymerase Chain Reaction directly from the clinical samples and conventional method of cultural isolation from commercial chicken. PCR with primers complementary to the *16S rRNA* gene generating amplicon size of ~207bp was used to detect *Mycoplasma synoviae*. *vlhA* gene PCR was used for further confirmation and detection of variations in field strains for tracing epidemiology of MS in different geographical areas. (Some districts of Maharashtra, Delhi and Kolkata)

A total of 165 choanal swabs were collected from live birds suspected for MS and subjected to species specific *16S rRNA* PCR. The overall incidence of MS detected was 4.2% with incidence rate of 3.4% and 6.25% in layers and broilers respectively

In *vlhA* gene PCR, gene fragment of ~350bp (ranging from 316 to 396) was amplified in all seven samples which were found to be positive for MS by *16S rRNA* based PCR. The amplicon size of specimens originating from different geographical regions varied in the sizes viz. in Maharashtra region ~360bp, Delhi and Kolkata regions ~380bp.

Cultural isolation of 165 samples was carried out using PPLO broth supplemented with NAD and cysteine and suitable incubation conditions. All 165 samples including seven PCR positive specimens subjected to cultural isolation were turned out to be negative for recovery of *Mycoplasma synoviae*.Thus, giving an isolation rate of 0% of *Mycoplasma synoviae* in clinical sample. PCR was found to be a simple, sensitive, more specific and cost effective alternative to conventional methods.

प्रबंध सारांश

१.	प्रबंधाचे नाव	:	कोंबडयामधल मायकोप्लासमा सायनोवीचे रेण्वीय पद्धतीचे निदान
२.	विद्यार्थ्याचे नाव	:	पदम लाल लुईटेल
३.	मार्गदर्शकाचे नाव व पत्ता	:	डॉ.सौ.र.स.गंदगे प्राध्यापक सूक्ष्मजीवशास्त्र विभाग, मुंबई पशुवैद्यकीय महाविद्यालय, परळ मुंबई - ४०००१२
४.	पदवी	:	एम.व्ही.एस्सी.
५.	पदवी प्रदान करण्याचे वर्ष	:	२०१६
६.	मुख्य विषय	:	सूक्ष्मजीवशास्त्र
७.	प्रबंधकाची एकूण पाने	:	
८.	सारांशाचे एकूण शब्द	:	
९.	विद्यार्थ्याची सही	:	
१०.	विभागप्रमुखाचा पत्ता व सही	:	
११.	सहयोगी अधिष्टयांची सहीः		

सारांश

गावठी कोंबडयामधील आणि इंग्लिश कोंबडयामधील श्वासोच्छश्वासाचे ाजार आणि सायनोवायटीस आजारास कारणीभूत असलेला जगभरातील मायकोप्लासमा सायनोटीव हा आर्थिकदृष्टया अत्यंत महत्वाचा जीवाणू आहे. प्रथमनिदानासाठी आणि नंतरच्या रसायन उपचार पद्धतीसाठी मायकोप्लासमा सायनोविए ओळखणे हे निर्णायकदृष्टया महत्वाचे आहे.

सदर संशोधन/प्रबंध मायकोप्लास्मा सायनोविएचा प्रादुर्भाव पाहण्यासाठी महाराष्ट्रातील वेगवेगळ्या भागातील व्यापारीदृष्टया उपलब्ध असलेल्या कोंबडयांच्या फार्ममधून घेतलेल्या नमुन्यांमधून पीसीआर या पद्धतीने व पारंपारिकपणे जीवाणुची कृत्रिमरित्या वाड करुन ओळखणे या पद्धतीने तपासण्यात आला. मायकोप्लासमा सायनोविएच्या तपासणीकरीता पीसीआर प्रायमर सोबत १६ एसआर अरएनए हे विथ चाचणीद्वारे अप्लिकेशन प्रोडक्ट २०७ बीपी वापरुन रोग परिस्थितीविज्ञानिक मायकोप्लासमा सायनोवीएच्या वेगवेगळ्या स्ट्रेन परिस्थितीविज्ञानिक पद्धतीने पुष्टी करण्यात आली.

मायकोप्लासमा सायनोवीए संशयित जीवंत पक्षांचे १६५ चोअनल स्लॅब गोळा करण्यात आले व ते १६ एस आर आर एनअ पीसीआर पद्धतीने तपासण्यात आले. सर्वसामान्य/सरासरी ४.२/मायकोप्लासमा सायनोवीएचा प्रादुर्भाव आढळून आला. त्यामध्ये ३.८द्व व ६.२५% हा अनुक्रमे अंडी घालणारे व मांसल कोंबडया आढळून आल्या.

VlhA जनुक हे पीसीआर तुकडे ३५० बीपी (३१६ ते ३९६ दरम्यान) सात नमुन्यांमधून १६ एस आर आरएनए पीसीआर वापरुन मायकोप्लासमा सायनोवीए बाधीत आढळून आले.

ॲम्लीकॉन नमुने हे वेगवेगळ्या भौगोलिक भागातून जसे की ३६० बीपी महाराष्ट्र विभाग, ३८० बीपी दिल्ली व कोलकाता विभाग यामधून गोळा करण्यात आले.

मायकोप्लासमा सायनोवीए जीवाणुंची कृत्रिमरित्या वाढ करण्यासाठी गोळा केलेले स्वॅब (गोळे) पुरक असलेल्या पीपीएलो आगार एनडीए आणि सिस्टिन हे वाढविण्यात आले व ते सर्व १६५ नमुने सात बाधीत पीसीआर नमुनेसहीत जे कृत्रिमरित्या वेगळे करण्यात आले होते ते सर्व मायकोप्लासमा सायनोवीए साठी अबाधित / नकारात्मक आढळून आले. म्हणून मायकोप्लासमा सायनोवीएन्ना या वैद्यकीय नमुन्यात शुन्य टक्के वेगळा दर देण्यात आले. तसेच या संपूर्ण तपासणीमध्ये वापरण्यात आलेले पीसीआर यंत्रप्रणाली ही साधी, सोपी व कमी खर्चिक अशी होती.

VITA

Dr. Padam Lal Luitel was born on 29th June 1990 in Pakyong, District East,Sikkim. He passed out his SSC with 80% and HSSC with 68% in the year 2007 and 2009 respectively from Central Board of Secondary Education.

With the interest of taking care of animals since childhood, he joined the College Of Veterinary Sciences and Animal Husbandry, Aizawl, Mizoram in the year 2009 to pursue Bachelor in Veterinary Sciences and Animal Husbandry and finally completed successfully in the year 2014. To pursue higher and specialized degree, he joined the studies in the discipline of Veterinary Microbiology, Bombay Veterinary College, Mumbai for his post-graduation, which he successfully completed in the year 2016.

He was an active student all throughout his schooling and college and had actively participated in extracurricular activities like quizzes, debate and other sports meets etc.

He was a member of NSS and NCC during his graduation and has obtained the certificates. He has successfully done his masters credit seminar on the topic “Mycobacteria Other Than Tuberculosis”.