DETECTION OF MYCOPLASMOSIS IN POULTRY BY CONVENTIONAL AND MOLECULAR TECHNIQUE

T H E S I S

Submitted

in partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY MICROBIOLOGY

BY

BUKTE SWATI RAMRAO

Enrolment No : V/08/078

BOMBAY VETERINARY COLLEGE, MUMBAI

MAHARASHTRA ANIMAL AND FISHERY SCIENCES UNIVERSITY,

NAGPUR- 440 001

(INDIA)

2015

PGR - ANNEXURE – XIII

(See Rule No. 26.7)

DECLARATION BY THE STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled, **"DETECTION OF MYCOPLASMOSIS IN POULTRY BY CONVENTIONAL AND MOLECULAR TECHNIQUE"** or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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Ms. BUKTE SWATI RAMRAO has satisfactorily prosecuted her course of research for a period of not less than one semester and that the thesis entitled**, "DETECTION OF MYCOPLASMOSIS IN POULTRY BY CONVENTIONAL AND MOLECULAR TECHNIQUE"** submitted by her is the result of research work sufficient to warrant its presentation to the examination in the 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 her for a degree of any other University.

Place: Mumbai.

Date : / / 2015

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PGR - ANNEXURE – XV
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CERTIFICATE

This is to certify that the thesis entitled, **"DETECTION OF MYCOPLASMOSIS IN POULTRY BY CONVENTIONAL AND MOLECULAR TECHNIQUE"** submitted by **Ms. Bukte Swati Ramrao** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **MASTER OF VETERINARY SCIENCE** in **VETERINARY MICROBIOLOGY** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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Dedicated To
My beloved Parents,
My Loved ones
and My Family

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ACKNOWLEDGEMENTS

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Finally, I again thank all those whom I have mentioned and whom I could not, for helping me in completing this work, as it is customary, the errors that remain are entirely mine.

Place: Mumbai

Date: ***Dr. Swati Ramrao Bukte***

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LIST OF ABBREVIATIONS

AGE	: agarose gel electrophoresis
ATCC	: American type culture collection
BGSA	: Brilliant green salmonella agar
bp	: Base pair
DNA	: Deoxyribonucleoside Acid
dNTP	: Deoxynucleoside triphosphate
EDTA	: Ethylene diamine tetra acetic acid
EMB	: Eosin methylene blue
et al	: Et alia (and others)
MG	: *Mycoplasma gallisepticum*
MR	: Methyl red
MS	: *Mycoplasma synoviae*
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
TAE	: Tris-acetate-EDTA
Taq	: Thermus aquaticus
TSI	: Triple sugar iron
VP	: Voges-proskauer
w/v	: Weight /volume

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

INTRODUCTION

1. INTRODUCTION

The name *Mycoplasma* originates from the Greek words *mykes* and *plasma*, meaning fungus and formed, respectively. Mycoplasma is a trivial name for a group of microorganisms that belongs to the class *Mollicutes*. The name *Mollicutes*, from Latin *mollis* meaning “soft” and *cutis* meaning “skin” indicating their characteristic lack of a cell wall (Razin *et al*., 1998). *M. gallisepticum* (MG) and *M. synoviae* (MS) are the most important; they are the only ones listed by the World Organization for Animal Health (2014) as major pathogens of poultry.

Infections in poultry with *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are a major concern for the poultry industry (Kleven, 2003; Ley, 2003). Horizontal transmission occurs within a flock and the progeny of breeder birds can be infected by vertical transmission (Bradbury, 2005). *Mycoplasma* causes respiratory system disease in poultry which is often sub clinical or mild in nature. It is also present in multifactorial disease complex with other pathogens including *Escherichia coli, Avibacterium(Haemophilus) paragallinarum*, Newcastle disease virus and infectious bronchitis virus (Jordan, 1972; Naylor *et al.,* 1992; Kleven, 1998). MG is worldwide in distribution and in turkey causes infectious sinusitis (Levisohn and Kleven, 2000). MS infection has become systemic and results in infectious synovitis, an acute to chronic disease of chickens and turkeys. Lameness and respiratory disorder caused by this infection results in decreased growth rate and loss of egg production and causing economic loss in intensive production (Lockaby *et al*., 1998; Kleven and Ferguson-Noel, 2008).

The losses caused by *Mycoplasma* infections are due to increase in embryo and early chick mortality which may be upto 10-20% in breeders, poor feed conversion ratio and reduction in weight gain in broilers and egg production upto 10-20% together with a high medication cost in layers (Kleven, 1990; Yoder, 1991; Ley, 2008). *Mycoplasma gallisepticum* is a bacterial pathogen of poultry that is estimated to cause annual losses exceeding $780 million. MG is a pathogenic cause of Chronic Respiratory Disease (CRD) in poultry causing heavy economic losses due to decreased egg production in layers and meat production in broilers.

In developed countries, where complete eradication of the pathogen is difficult to attain, the outbreaks of poultry mycoplasmosis are usually controlled with the use of attenuated vaccines. The commercially available live *M. gallisepticum* vaccines include the F strain (Schering Plough), Ts-11(Bio-properties, Australia) and 6/85 strain from Intervet America, Millsboro (Liu *et al.*, 2001 and Lysnyansky *et al.*, 2005). Although, vaccination is effective mean to control the disease; it isn't the routine practice in India. Moreover, the use of antibiotics is observed to cause decrease in clinical signs, however, it does not eliminate the infection. Therefore, timely, accurate, sensitive method of detection of infection and management is important for prevention and control of poultry mycoplasmosis.

Various diagnostic techniques including serological test and isolation procedures have been used for the detection of pathogenic *Mycoplasma*. Swabs from the choanal cleft, oropharynx, oesophagus or trachea of the chickens can be used for isolation. Trachea, lung and airsac can be collected and used for isolation in dead birds. For sero-diagnosis of *Mycoplasma* species, methods such as rapid serum agglutination (RSA), Enzyme Linked Immuno Sorbent Assay (ELISA), and Hemagglutination Inhibition (HI) are available (Kleven, 1998). These are rapid, easy and require little expertise but can be hampered by interspecies cross reaction and non-specific reaction (Sahu and Olson, 1981), that's why it has low specificity.

The isolation and identification of *Mycoplasma* is being the gold standard test for confirmation of diagnosis (Ley, 2008). Specific diagnosis of *Mycoplasma* is not always easy due to the limitations of diagnostic tests together with the similarities in the disease they cause. It has become increasingly important to develop methods to characterize and identify *M. gallisepticum* strains and strain variability. Reliable methods for the differentiation of *M. gallisepticum* strains play a pivotal role in understanding the epizootiology and spread of the disease. Also, the increased use of live *M. gallisepticum* vaccines requires more powerful tools to differentiate vaccine strains from circulating field isolates, pathogenic and non-pathogenic (Serena *et al.,* 2012).

Molecular techniques such as PCR have become a valuable tool in the diagnosis of mycoplasmas, not only for its sensitivity but for its increasing specificity (Garcia *et al.,* 2005). PCR has allowed the study of microbial genes, directly amplified from samples, without the need for cultivation. The specificity of the method is highly dependent on the target chosen. Although capable of distinguishing isolates to the species level, extreme specificity is needed when live vaccine strains are used in the poultry industry. It is often critical that non-pathogenic strains such as vaccine strains can be distinguished from pathogenic strains. For this reason, membrane surface proteins such as Cytadhesin are often targeted and offer more variability and therefore better discrimination between strains. A highly specific and sensitive PCR was developed by Lysnyansky *et al*., (2005) for the detection of MG by targeting a partial region of the mgc2 cytadhesin gene. The PCR was tested by Garcia *et al*., (2005) amongst various other PCRs, including those targeting the 16S rRNA gene and the 16S-23S ITS region, for the detection of MG. This mgc2-PCR was shown to be the most specific and sensitive of the PCRs.

The mgc2 gene is fairly conserved in *M. gallisepticum* and has been used for the molecular identification of isolates (Lysnyansky *et al.,* 2005; Garcia *et al.,* 2005). The advantage of using the mgc2 gene-based method is the ability to differentiate between pathogenic field strains and vaccine strains by combining it with RFLP or sequencing of the DNA amplicons. Lysnyansky *et al*., (2005) used the mgc2-PCR-RFLP method, stating it would take few days to obtain an identification starting from when DNA is extracted from the choanal swabs. The primers used by the above authors to target the mgc2 gene, were tested by Garcia *et al*.,

(2005) against a wide variety of organisms. The results showed that the assay to be both specific and sensitive for *M. gallisepticum* strains.

The primers of study selectively amplify a 207 base pair DNA fragment within the 16S rRNA gene of MS. *Mycoplasma synoviae* (MS) is an important avian pathogen that can cause both respiratory disease and joint inflammation synovitis in poultry, inducing economic losses to the chicken industry. The PCR method reduces the time consuming, an effectiveness and efficient for detection of *M. synoviae* infection of poultry. It is then suggested that the PCR method could be an alternative method for detection of MS (Pourbakhsh *et al.,* 2010).

Several molecular techniques have been developed for differentiation of *Mycoplasma spp*. strains, including protein profile analysis (Khan *et al.,* 1987), restriction fragment length polymorphism (RFLP) (Kleven *et al.,* 1988), ribotyping (Yogev *et al.,* 1988), strain-specific DNA probes (Khan *et al*., 1989) and PCR with strain-specific primers (Nascimento*,* 1993) and sequencing (Lysnyansky *et al*., 2005). Using these techniques the difficulties in conventional methods can be avoided and more sensitive and specific detection of mycoplasma can be achieved.

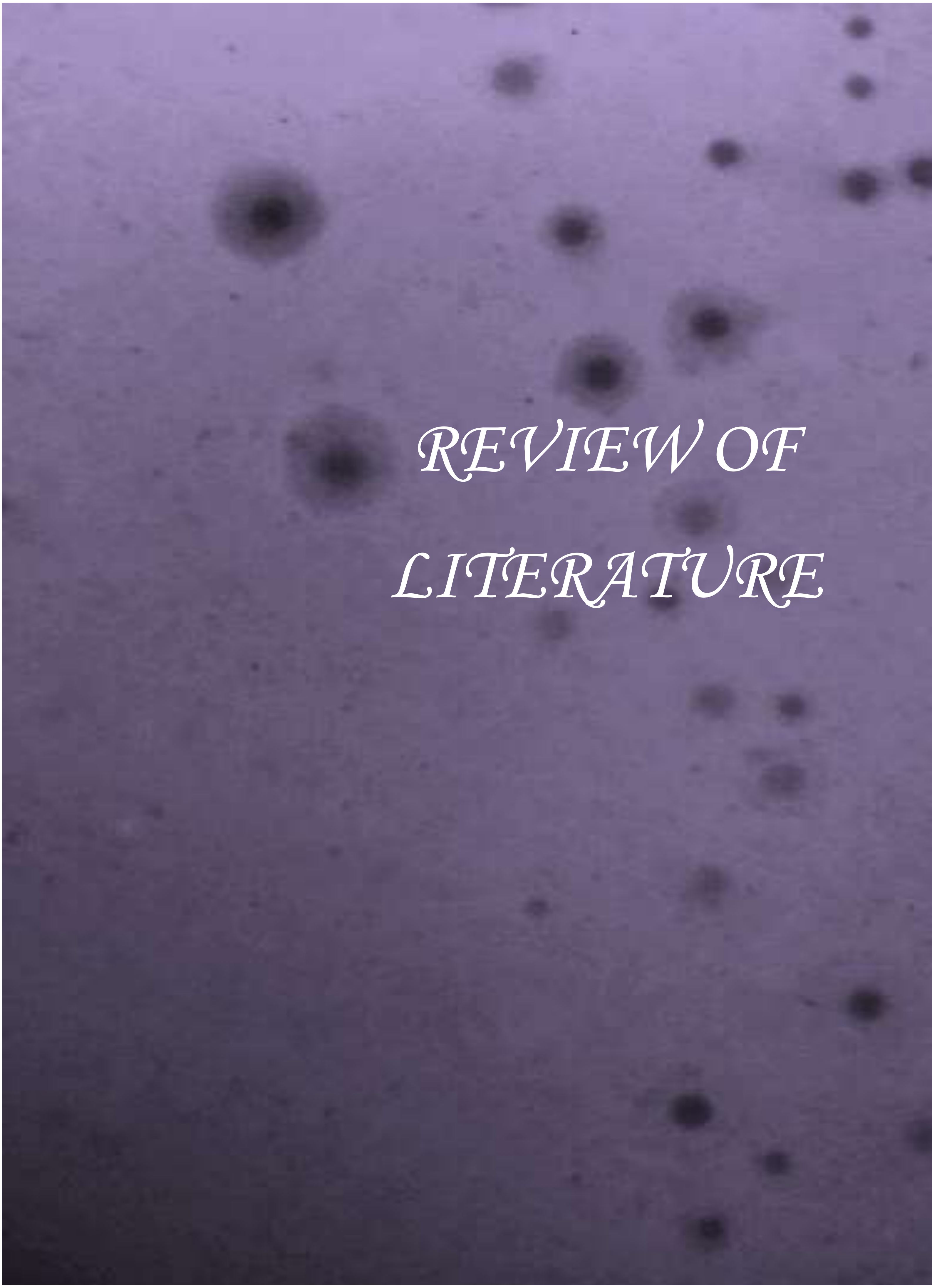
Prevention of infection and precise diagnosis of poultry mycoplasmosis is an important element in control strategies. Reliable and rapid diagnosis is needed to prevent dissemination of infection. Taking into account the present research is designed with following objectives.

Objectives:

i. Conventional diagnosis of poultry mycoplasmosis by Rapid Serum Agglutination Test and or cultural method.

ii. Detection of poultry mycoplasmosis directly in clinical specimens by PCR.

iii. Sequencing of PCR products and data analysis by BLAST.



REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

There are 22 named species of mycoplasma recovered from avian sources but only four of them are established pathogens for domestic poultry as *Mycoplasma gallisepticum* (MG), *M. synoviae* (MS), *M. meleagridis* (MM) and *M. iowae (MI)* (Jordon, 1996). Mainly *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS) are pathogenic for chickens and turkeys listed by World Organization for Animal Health (2014), whereas *M. meleagridis* (MM) and *M. iowae* (MI) are pathogens for turkeys (Ley and Yoder, 1997).

2.1 Conventional diagnosis of poultry mycoplasmosis by rapid serum agglutination test

Sarkar *et al*. (2005) examined 382 sera samples by Rapid Serum Plate Agglutination (SPA) test commercial MG antigen (Nobilis® MG) to detect the presence of antibodies against MG. The overall sero-prevalence of MG infection was 58.90% in Bangladesh.

Barua *et al*. (2006) carried serological test (SPA) on 400 samples which revealed 53% prevalence of *Mycoplasma gallisepticum* in broiler and 73% in layer at Lohagara, whereas 46% in broiler and 60% in layer at Satkania Upazilla, Bangladesh.

Luciano *et al*. (2011) tested serum from commercial poultry breeders (n = 2781) MS by serum plate agglutination (SPA), Hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) in Brazil. From 2,781 samples tested, 736 (26.46%) were positive in SPA. From 712 SPA-positive sera, 30 samples (4.21%) were positive in HI, and 150 samples (21.06%) were positive in ELISA. Hence, concluded that SPA test is easy and effective method for rapid diagnosis.

Mukhtar *et al.* (2012) collected a total of 640 sera samples from 81 commercial layer flocks with complaint of respiratory distress in Pakistan.

On the basis of serum plate agglutination test, 40 flocks were found positive for *Mycoplasma gallisepticum* indicating a share of 49.01% among the respiratory diseases. Among 40 positive flocks for *Mycoplasma gallisepticum* the highest prevalence (54.84 %) was found in pullets, followed by 46.34 %, and 44.44 % in adult and old laying flocks, respectively.

Mahfuzul *et al.* (2014) collected a total of 480 blood samples of chickens from different upazilas, Bangladesh. On the basis of the serum plate agglutination test, 55.83% (n=268/480) chickens were found positive for MG. The *Mycoplasma gallisepticum* infection was higher (62.5%) in backyard chickens as compared to those being reared in commercial farming systems (53.61%). With respect to age groups, the prevalence was highest in pullets (60.63%) followed by adults (55.63%) and old chickens (51.25%). Moreover, chickens reared in winter showed higher prevalence of MG (60.42%) as compared to those reared in summer (51.25%).

2.2 Conventional diagnosis of poultry mycoplasmosis by cultural method

Tiong *et al.* (1979) screened 240 batches of chickens with chronic respiratory syndrome for Mycoplasmas, 105 batches (43.8%) were positive for Mycoplasmosis. A total of 110 isolates of Mycoplasma was cultured, of which 9 (3.75%) isolates were identified as *M. gallisepticum.* Identification of the Mycoplasma isolate was carried out by biochemical and serological tests (disc growth inhibition and agar gel diffusion tests).

Branton *et al.* (1984) isolated MG from 15 (14.85%) of 101 tracheal swab samples and from 51 (47.23%) of 108 choanal cleft swab samples. This study indicates that swabs taken from the choanal cleft region yielded higher isolation rates and are more easily obtained than tracheal swabs.

Bayatzadeh *et al.* (2011) cultured 43 swab samples of trachea, lung and air sac taken from commercial broiler chicken farms in 3 main provinces of Iran (Tehran, Markazi and Qazvin), with clinical signs of the disease in PPLO broth media supplemented for *M. synoviae.* Isolation yielded 28 (65.1%) *M. synoviae* cultures using PPLO agar culture diagnostic method.

Nouzha *et al.* (2013) incubated 18 trachea swab from broiler chickens in Mycoplasma broth at 37°C under 5% of CO2, after the change in colour of broth, further inoculated on selective agar, culture gave a sensitivity rate of 72.72% .

Rauf *et al. (*2013) collected tracheal swabs, lung and airsac from birds suffering from respiratory diseases, isolated *M. gallisepticum* using Frey's medium found from white leghorn laying birds, 27.6% (104 out of 380) samples were positive.

Mahfuzul *et al.* (2014) collected a total of 480 blood samples of chickens from different upazilas, Bangladesh. On the basis of the serum plate agglutination test, 55.83% (n=268/480) chickens were found positive for MG.

2.3. Molecular diagnosis of Avian Mycoplasmosis by PCR

Garcia *et al*. (2005) accessed sensitivity and specificity of different PCR methods (16S rRNA, *mgc*2, LP, *gap*A and licensed DNA test kit) for the detection of *M. gallisepticum* from tracheal swabs samples of commercial poultry and fowl. 16S rRNA detected DNA of *M. gallisepticum* and *M. imitans* while others only amplified *M. gallisepticum.* The sensitivity of all PCR was ranged from 4 to 400 color-changing units/ amplification while 16S rRNA and *mgc*2 was recorded as 40 CCU/reaction and 4CCU/reaction for *ngap*A.

Bagheri *et al.* (2011) extracted DNA from 324 poultry samples, then 16 S rRNA region was amplified by PCR using specific primers. The result showed one amplified fragments, about 366 bp for positive cases. From 324 samples collected, 96 (29.63%) were found MG positive on the basis of PCR analysis.

Aziz *et al*. (2014) applied conventional and real time PCR were to detect the most important virulence genes i. e. mgc2 of for *M. gallisepticum .*

2.4 Comparative efficacy of RSA, cultural method and PCR in detection of Avian Mycoplasmosis

Evans *et al.* (2009) detected *Mycoplasma gallisepticum* infection in breeder flock by serum plate agglutination (SPA), PCR and culture. 30 choanal cleft/palatine fissure swabs were processed for MG-specific PCR and the positivity of PCR was found 36.7% (11/30). MG antibodies were detected in 100% (30/30) of the clinical samples.

Kaboli *et al*. (2013) investigated rural broiler breeder farms located around East Azarbaijan of Iran during 2011-2012 for MG for its prevalence by ELISA test, and then all positive cases were further examined by 16S rRNA methods. The results indicated that out of 73 samples, 23 (31.50%) of samples were positive in ELISA. All seropositive samples were positive in PCR.

Rauf *et al*. (2013) compared molecular diagnostic technique (PCR) with conventional isolation techniques for *Mycoplasma gallisepticum* (MG). DNA extracted from organs of infected birds was amplified by PCR using species specific primers of MG targeting 16S rRNA gene and visualized by agarose gel electrophoresis. Out of 104 culture positive isolates, 83 (79.8%) were confirmed as MG through Growth inhibition test (GIT) with 21.84 (83/380) overall percentage.

Percent proportion for MG in organs of infected birds was higher (68.94%) as determined by PCR. Confirmed presence of 185 base pairs DNA band was considered positive for MG. Thus concluded PCR as efficient method.

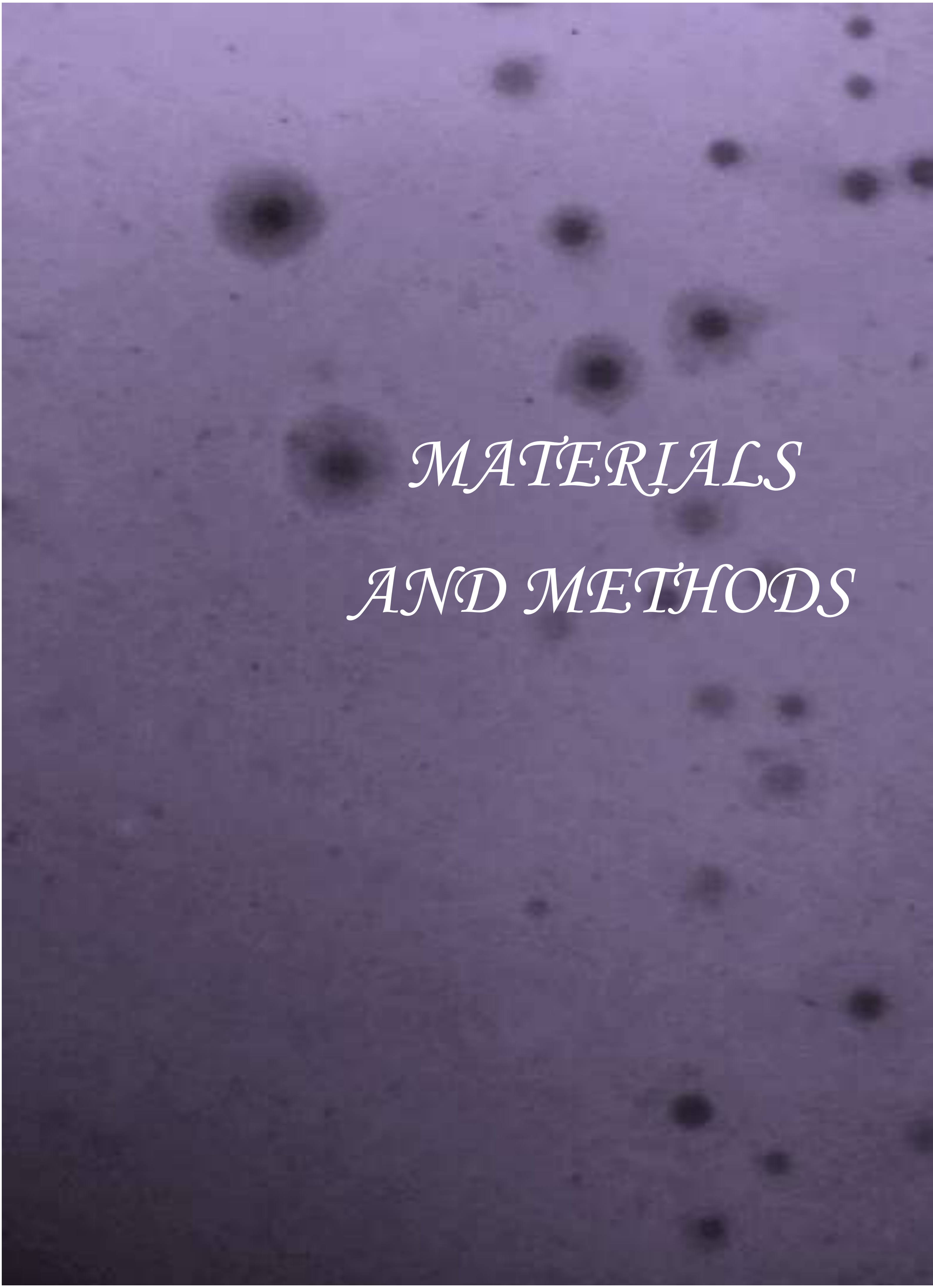
2.5 Gene specific PCR and Gene-targeted sequencing (GTS) for *M. gallisepticum*

Ralf *et al*. (1996) sequenced the entire genome of the bacterium *Mycoplasma pneumoniae* M129. It has a size of 816 394 base pairs with an average G+C content of 40.0 mol% and 75.9% showed significant similarity to genes/proteins of other organisms while only 9.9% did not reveal any significant similarity to gene sequences in databases.

Ferguson *et al.* (2005) analysed GTS of individual genes, gapA, MGA_0319, mgc2 and pvpA, identified 17, 16, 20 and 22 sequence types, respectively. GTS analysis using multiple gene sequences mgc2/pvpa and gapA/MGA_0319/mgc2/pvpA identified 38 and 40 sequence types, respectively. These results are believed to provide the first evidence that typing of M. gallisepticum isolates by GTS analysis of surface-protein genes is a sensitive and reproducible typing method and will allow rapid global comparisons between laboratories.

Serena (2013) characterized portions of the surface protein encoding pvpA, gapA and mgc2 genes and the uncharacterised surface lipoprotein gene designated MGA_0319 were sequenced and analysed. Nucleotide sequences were compared to vaccine and reference strains as well as to strains from different countries.

The South African genotype contained unique mgc2 and pvpA gene regions, while the Zimbabwean genotype proved to be even more distinct with unique gapA, mgc2 and pvpA gene regions. These results also allow for improved control strategies for southern Africa, and the use of more effective vaccine strains.



MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Glassware and Plastic wares

All glassware used were made up of neutral glass from M/s Borosil, India. All the glassware were treated and washed as per standard procedures before use. The plastic wares required for the present research viz. Petri plates, centrifuge tubes, micro-centrifuge tubes, PCR tubes, pipette tips,sterile dry swab with tube from Himedia, etc. were procured from M/s Tarsons India, Kolkata and pre-sterilized or got sterilized by autoclaving at 15 lbs. for 15 min. before their use in the experiment.

3.1.2 Media, Chemicals and reagents

The chemicals and reagents used during present investigation were obtained from different manufacturing companies. Pig serum, Penicillin G, PPLO Agar, PPLO Broth, yeast extract, Glucose solution, and other chemicals and reagents,etc. purchased from M/s Hi Media Laboratories Private Limited, Mumbai (India). Thallium acetate used was from M/s Sigma Aldrich Chemicals Private Limited (USA). Primers for MG and MS procured were M/s Bioserve Biotechnologies (I) Pvt Ltd, Hyderabad, India. All molecular grade reagents for PCR were obtained from M/s Merck Specialities Private Limited, Mumbai (India). Antigen used for RSA was supplied by x – OvO Limited, IZS Venezie, Italy.

3.1.3 Bacterial reference strains

Mycoplasma gallisepticum (ATCC 25204) and *Mycoplasma synoviae* (ATCC19610) were used as standard reference during present investigation.

3.1.4 Clinical specimens

A clinical sample (choanal swabs and sera samples) from total of 150 birds were collected and processed for detection of suspected live birds for the investigation in the present study. The specimens were collected from Pen (Raigad districts) and Narayangaon (Pune districts) of Maharashtra State (Table 3.1).

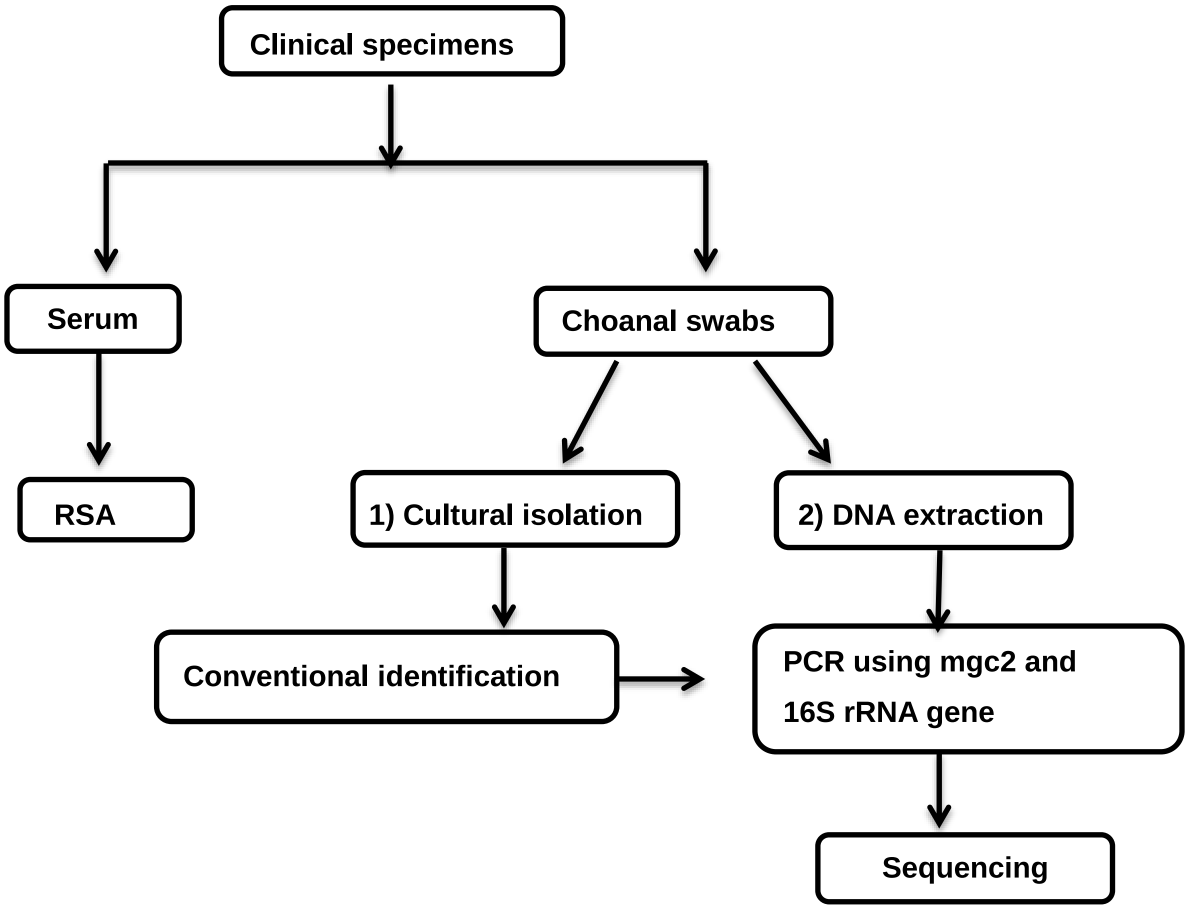
Table 3.1 Details of specimens collected for detection of mycoplasmosis in Poultry

No.	Source	Sample code	Specimens		No. of sample	Total
1	Pen (P)	No. P1 to P50	Choanal swab	Serum	50 each	100
2	Narayangaon(N)	No. N51 to N150	Choanal swab	Serum	100 each	200
Grand Total						**300**

3.2 Methods

The present investigation dealt with diagnosis of mycoplasmosis in poultry by conventional and molecular techniques. During investigation, the sera samples subjected to RSA for detection of *Mycoplasma* antibodies. The researches also included isolation of field isolates using conventional bacteriological methods and further their confirmation by PCR. Molecular detection of mycoplasmosis was carried out using species specific PCR assays targeting mgc2 gene of *M. gallisepticum* and 16SrRNAgene of *M. synoviae*. The broad strategic investigation steps are shown in Fig.3.1

Fig. 3.1 Investigations carried out for detection of mycoplasmosis



3.2.1 Collection of specimens

Success in recovery of *Mycoplasma* organisms after culturing specimens depends on careful collection of suitable specimens, proper transportation and accurate processing of clinical sample (World Organization for Animal Health, 2014). In the present study choanal swabs and blood samples were collected aseptically using suitable materials Choanal swab placed in sterile leak-proof Himedia cotton swabs and transported with care as early as possible and samples were processed immediately after reaching to laboratory. Blood samples kept undisturbed till serum got separated and separated sera samples transferred in other sterile eppendorf tube and transported on ice.

3.2.1.1 Collection of Choanal swab

The choanal (palatine cleft) swabs were collected for isolation of *Mycoplasma* spp. and for performing PCR directly. The choanal swabs were collected in duplicates from live birds exhibiting clinical signs viz. rales and sneezing, sticky material from nostrils, swollen sinuses, foamy material from the eyes etc. (World Organization for Animal Health, 2014). The specimens were collected by opening the beak of birds followed by inserting and gently rubbing the sterile swab in choanal cleft region (Plate 3.1). The swabs used for isolation of *Mycoplasma* spp. were dipped in sterile PPLO broth before collection in order to create optimal conditions for target organism during transport of the samples to the laboratory. All swabs taken in sterile tubes were transported on ice and processed within 24 hrs. after collection.

3.2.1.2 Collection of blood samples

Blood samples were collected from the wing vein of birds(Plate 3.2) and transferredin sterile eppendorf and kept undisturbed till serum got separated. These separated sera samples were transferred in another sterile eppendorf, transported in containers on ice and RSA was carried out within 24 hrs.

3.2.2 Rapid Serum Agglutination Test (RSA) for detection of antibodies against MG and MS in poultry sera samples

Antibodies against MG and MS in mycoplasmosis suspected poultry sera samples were detected by Rapid Serum Agglutination Test. The test was carried out at room temperature (20–25°C) within 24 hours of collection of sera samples. The RSA test was carried out as per method described by Arefin *et al*. (2011).

Drop approximately 0.02 ml of serum on two separate clean glass slide

add 0.02 ml of stained MG and MS antigen separately on above sera samples

Plate 3.1: Collection of choanal swabs from poultry

Plate 3.2: Collection of blood samples from wing vein

↓

Spread mixture over a circular area of approximately 1.5 cm diameter

Observe within 2 min.

↓

Positive reaction is indicated by the reaction agglutination/flocculation within 2 minutes.

↓

Reading of results carried out and negative samples do not show any changes and uniform mixture is observed

Known positive and negative controls included in the test.

3.2.3 Isolation of *Mycoplasma* spp.

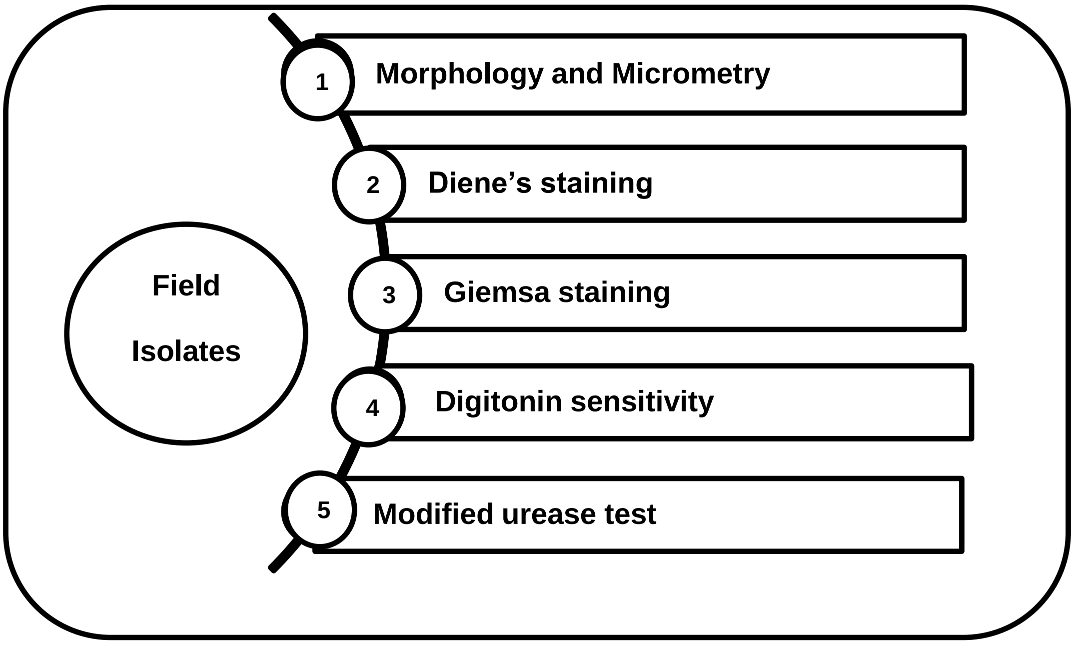
All the work involving handling of *Mycoplasma* organism was performed by taking appropriate precautions and following the strict aseptic procedures ad sterile conditions.

Choanal swabs were inoculated directly in PPLO broth and incubated at 37°C in presence of 5 % CO_2. On observing the growth i.e. yellow color of broth, immediately PPLO broth culture was inoculated on solid PPLO agar without and with NAD and cysteine hydrochloride for *Mycoplasma gallisepticum and Mycoplasma synoviae* respectively. The inoculated plates were incubated at 37°C in presence of 5 % CO_2 until the growth in the form of fried egg colonies was observed under stereo-zoom microscope. Incubation of broth cultures showing no color change was confirmed upto 21 days before discarding as negative. Positive cultures were processed further for identification.

3.2.4 Identification of *Mycoplasma* spp. by conventional methods

The colonies showing typical fried egg appearances suggestive of *Mycoplasma spp*. under stereo-zoom microscope were further studied by Micrometry of colony, Diene's staining, Giemsa staining, Digitonin disc diffusion assay and Modified urease test. Standard ATCC strains i.e. *Mycoplasma* *gallisepticum* (ATCC25204) and *Mycoplasma synoviae* (ATCC19610), were included as known positive controls in above test (fig 3.2).

Fig. 3.2 identification of *Mycoplasma* spp. by conventional bacteriological techniques



3.2.4.1 Micrometry of colony

The sizes of micro colonies were measured by using micrometry technique under low power (10X) of compound microscope. Before measuring the colony size the calibration factor was calculated as 80.78 using following formula.

$$\text{Calibration factor} = \frac{\text{Average occular micrometer reading}}{\text{Average stage micrometer reading}} \text{X10}$$

The size of micro colonies were measured with scale on ocular micrometer and the values were multiplied by calibration factor to get the different sizes of *Mycoplasma* colonies. The observed sizes of micro colonies showing size between 0.1-0.6 mm were considered as suggestive of *Mycoplasma* spp.

3.2.4.2 Diene's staining

Diene's staining of colonies suggestive of *Mycoplasma* spp. was performed as per method described by as below Quinn *et al*., 2004.

A block of agar containing microcolonies was placed with colony side facing upwards on a glass slide

↓

Thin film of Diene's stain was placed on a coverslip and allowed to dry

↓

The coverslip with stain was placed facing down word side on microcolonies

↓

The preparation was examined under the stereo-zoom microscope.

The denser centre of the microcolonies embedded in to the agar, stained dark blue. The less dense peripheral zone representing surface growth stained light blue.

3.2.4.3 Giemsa staining

All the isolates of *Mycoplasma* spp. were subjected to microscopic examination of morphology using Giemsa stain as per Quinn *et al.* (2004) with some modifications. The methanol-fixed smear was stained with Giemsa stain for a period of 30 to 40 min. in a coplin jar. The stained smear was air dried by gentle blotting between absorbent filter papers. The dried smear was observed under 1000 X power under oil immersion.

Annular forms i.e. circular and elliptical pleuropneumonia like organism 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.

3.2.4.4 Digitonin sensitivity

This test carried out to differentiates genus *Mycoplasma* from *Acholeplasma* based on requirement of cholesterol for growth. The test was carried out as per the protocol described by Quinn *et al.* (2004). The procedure carried was as follows described by Quinn *et al*. (2004).

Preparation of stock solution of 1.5 % (w/v) digitonin in 95% ethanol and storage at 4°C until used.

↓

Apply 25 µl of the stock solution of digitonin to 6-mm blank paper discs.

↓

Discs were dried overnight at room temperature and stored at 4°C until use.

↓

PPLO broth inoculated with test culture and incubated in presence of 10% CO_2 for 4 days.

↓

Test culture was spreaded evenly in 200 µl quantity on PPLO agar plate. Digitonin discs were pressed gently on the surface of the agar plates after the inoculum had dried.

↓

Plates were incubated for 4–7 days at 37°C in a 10% CO_2 incubator.

After incubation, all plates were examined under the stereo-zoom microscope, and the zones of inhibition were measured from the edge of the disc to the edge of clear zone of no growth (mm). Digitonin tests were considered positive when the clear zone observed was >5 mm and negative when the zone was <3 mm.

3.2.4.5 Modified Urease test

The genus *Ureaplasma* have the ability to produce the enzyme urease while *Mycoplasma* don't, thus for differentiation between these two organisms modified urease test was performed as per procedure described by Quinn *et al* (1999). The reaction showing color change from light to dark brown and finally to black due to deposition of manganese on the surface of the colony is considered as urease positive test which is seen in *Ureaplasma,* whereas *Mycoplasma* are negative for urease test.

3.2.5 Molecular Detection of *Mycoplasma* spp. by Polymerase Chain Reaction (PCR)

3.2.5.1 Extraction of DNA from cultures

DNA was extracted from cultures as per the protocol described by Ley *et al.* (1997) with slight modification i.e. increasing centrifugation speed from 13,000 rpm to 14,000 rpm. Briefly, the procedure followed was as follows:

A small piece of PPLO agar containing micro colonies of *Mycoplasma* was taken in 2-ml quantity of PPLO broth and incubated overnight at 37^0C in CO_2 incubator

↓

The broth culture was centrifuged at 14,000 x *g* for 30 min at 4°C

↓

Followed by washing of pellet two times with PBS

↓

The pellet was resuspended in 25 µl PBS

↓

The suspension was boiled at 95°C for 10 min, cooled on ice for 10 min, and centrifuged at 14,000 x *g* for 5 min

↓

The supernatant was removed and stored at 4° C for further use in PCR

3.2.5.2 Extraction of DNA from choanal swab

DNA was extracted from swab samples as per the protocol described by OIE, (2008) with slight modifications. Briefly, the procedure was as follows:

Swab samples were suspended in 1 ml of PCR-grade PBS in a 1.5 ml Snap-cap eppendorf tube.

↓

The suspension was centrifuged for 30 minutes at 14,000 rpmat 4°C.

↓

The supernatant was carefully removed with a micro pipette and the pellet was suspended in 25 µl nuclease free water.

↓

The tube with contents were boiled at 95°C for 10 minutes and then placed on ice for 10 minutes before centrifugation at 14,000 rpmfor 5 minutes. The DNA was in the supernatant. The DNA extracted was stored at - 20^0 C until further use.

3.2.5.3 Quantification of DNA

Quantification of DNA extracted from bacterial cultures and clinical specimens was done spectrophotometrically at 260 nm and 280 nm using Nanodrop spectro-photometer (Thermo Scientific, USA) for determination of sample concentration and purity. To determine quantity of DNA, 1 µl of DNA sample was loaded in the Nanodrop Spectrophotometer. With the help of Nano 2000 software the quantity and purity of DNA was evaluated.

3.2.5.4 Assessment of integrity of genomic DNA

The isolated genomic DNA samples were subjected to agarose gel electrophoresis followed by staining with ethidium bromide for assessing it's integrity. Agarose gel (2%) prepared in 1X TAE buffer was used for this purpose. The agarose was dissolved in 1X TAE by heating in microwave oven, Ethidium bromide was added to a final concentration of 0.5 µg/ml and mixed by gentle swirling. On cooling the lukewarm solution thus prepared was poured in the gel casting tray and comb was placed to form wells and the gel kept undisturbed and allowed to solidify. Subsequently, comb and slabs were removed and the gel with tray was transferred to electrophoresis apparatus. 1X TAE buffer was added in electrophoresis tank covering the agarose gel to a depth of about 10 mm. 2 µl of DNA sample was mixed with 0.5 µl 6X loading dye. The mixture was loaded in the wells and electrophoresis was carried out at a constant voltage of 90 V, till dye migrated about three-fourth distance of gel. The integrity of the DNA was checked by visualization of the DNA using gel documentation system (Gel Doc EZ Imager, Bio-Rad).

3.2.6 Avian *Mycoplasma* species specific PCR assay

3.2.6.1 Oligonucleotide primers

The amplification of 300 bp region of mgc2 gene of *Mycoplasma gallisepticum* and 207 bp region of 16S rRNA gene of *Mycoplasma synoviae* was carried out using published oligonucleotide primer sequences (Table 3.2 and 3.3), as per Lysnyansky *et al*. (2005) **and OIE, (2008) respectively**. The primers were manufactured and supplied by M/s Sigma and Bioserve Biotechnologies, Mumbai (India).

Table 3.2: Oligonucleotide primers for *Mycoplasma gallisepticum* PCR (mgc2 gene)

Primer	Oligonucleotide sequence	Reference
Mgc2 2F	5’-CGC-AAT-TTG-GTC-CTA-ATC-CCC-AAC-A-3’	Lysnyansky *et al*. (2005)
Mgc2 2R	5’-TAA-ACC-CAC-CTC-CAG-CTT-TAT-TTC-C-3’	

Table 3.3: Oligonucleotide primers for *Mycoplasma synoviae* PCR (16S rRNA gene)

Primer	Oligonucleotide sequence	Reference
MS–F	5’-GAA-GCA-AAA-TAG-TGA-TAT-CA-3’	OIE (2008)
MS–R	5’-GTC-GTC-TCC-GAA-GTT-AAC-AA-3’	

3.2.6.2 Reaction Mixture

The PCR was set in a final volume of 50 µl as shown in table 3.4

Table 3.4: Reaction mixture for *Mycoplasma* specific *mgc2* PCR and 16S rRNA MS PCR of direct specimens

Particulars	Concentration (µl)
Nuclease free H_2O	35.75
10 × PCR Buffer	5.00
$MgCl_2$ (50 mM)	2.00
dNTP(l0 mM)	1.00
F Primer (0.1 mM)	0.50
R Primer (0.1 mM)	0.50
Taq (5 U/µl)	0.25
Total	50.00

Table 3.5: Reaction mixture for *Mycoplasma gallisepticum mgc2* specific PCR of isolates

Particulars	Concentration (µl)
Nuclease free H_2O	31.6
10 × PCR Buffer	5
$MgCl_2$ (50 mM)	4
dNTP(l0 mM)	1
F Primer (0.1 mM)	2
R Primer (0.1 mM)	2
Taq (5 U/µl)	0.4
Total	50.00

3.2.6.3 Cycling conditions for *Mycoplasma gallisepticum*

The above reaction mixtures prepared in 50 µl quantity were spinned briefly and set into thermal cycler, Master cycler nexus gradient (eppendorf). The cycling conditions used are shown below (fig. 3.3 – fig. 3.5)

Fig. 3.3 Cycling conditions for mgc2 gene PCR of direct specimens

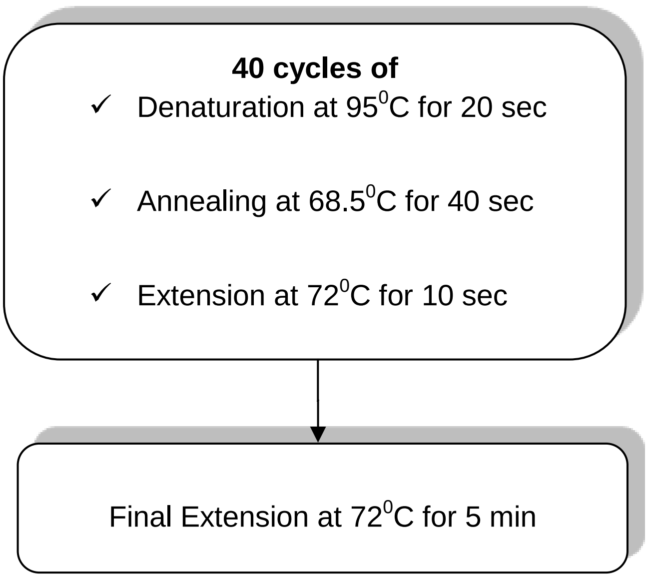


Fig. 3.4 Cycling conditions for mgc2 PCR of isolates

40 cycles of

- ✓ Denaturation at 95^{0}C for 20 sec
- ✓ Annealing at 56.6^{0}C for 40 sec
- ✓ Extension at 72^{0}C for 10 sec

↓

Final Extension at 72^{0}C for 1 min

Fig. 3.5 Cycling conditions for MS specific 16S rRNA PCR

40 cycles of

- ✓ Denaturation at 94^{0}C for 30 sec
- ✓ Annealing at 55^{0}C for 30 sec
- ✓ Extension at 72^{0}C for 60 sec

↓

Final Extension at 72^{0}C for 5 min

3.2.6.4 Evaluation of PCR products

The amplification products of both *M. gallisepticum* and *M. synoviae* PCR assays were evaluated by agarose gel (2%) electrophoresis stained with ethidium bromide. A 100 bp DNA ladder was electrophoresed simultaneously in order to compare the molecular weights of the amplicons generated in test samples in PCR assay. The products were visualized and documented using Automatic Computerized Gel Documentation and Analysis System (Gel Doc EZ Imager, Bio-Rad) using UV light source. The size of PCR product was estimated with the help of Image Lab (Version 4.1) software available with the gel documentation system.

3.2 Alignment of nucleotide Sequence of mgc2-PCR amplicon

The sequencing of PCR products was carried out at Bioserve Biotechnologies (I) Pvt Ltd, Hyderabad, India. The sequences obtained from the ABI files were analysed and curated using Chroma's light software Version 2.01. The curated sequences were submitted to look for their similarity in the NCBI database. To search for the similarity, BLAST tool at NCBI was used.

RESULTS

4. RESULTS

The present research was planned to detect mycoplasmosis in poultry by conventional and molecular techniques. Samples of total 150 birds were collected from Raigad (Pen) and Pune / Sangli (Narayangaon) districts of Maharashtra. A total of 150 birds were investigated by Rapid Serum Agglutination test, cultural isolation and direct PCR of clinical specimens for detection of mycoplasmosis using conventional and molecular methods.

4.1 Conventional diagnosis of poultry mycoplasmosis by RSA

A total of 150 sera samples collected from mycoplasmosis suspected birds were subjected to rapid slide agglutination test using commercial MG and MS antigens x–OvO Limited, IZS Venezie, Italy. (Plate 4.1). Out of 150 sera samples tested, a total of 93 (62%) found positive for mycoplasma antibodies in RSA. Out of 93 RSA positive, 73 (48.67%) were MG and 20 (13.33%) were MS antibodies positive. Six (4%) sera samples were found positive in RSA for both MG and MS antibodies due to cross reaction.

Out of 93 RSA positive, 22 (23.65%) and 41 (44.08%) were also positive in cultural isolation and PCR respectively. Out of 93 RSA positive sera samples, 18 (19.35%) were from Pen (Raigad) and 41(44.08%) were from Narayangaon (Pune) respectively.

4.2 Conventional diagnosis of poultry mycoplasmosis by cultural method

Choanal swabs of live birds exhibiting symptoms of respiratory system suggestive mycoplasmosis were processed for isolation of *Mycoplasma* spp. using Pleuropneumonia like Organism (PPLO) medium. PPLO broth media changed colour due pH change (red to yellow) indicated growth of Mycoplasma (Plate 4.2). Isolation was successful only from 24 choanal swabs (16%) while from remaining 126 choanal swabs (84%) none of isolates could be recovered in cultural isolation.

The isolates grown on PPLO agar and showing typical ‘fried egg’ appearance under stereo-zoom microscope (Plate 4.3) were subjected for

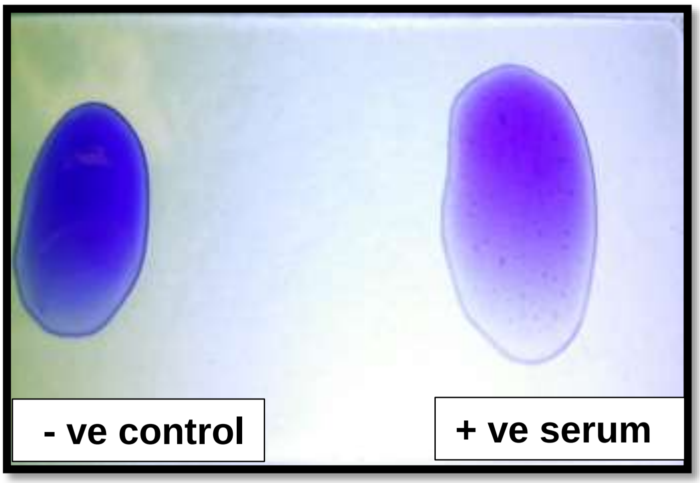


Plate4.1: Rapid Serum Agglutination test

Plate 4.2: PPLO media showing pH change (red to yellow) indicates growth of *Mycoplasma*

micrometry, wherein colony sizes laid between 0.1-0.6 mm in diameter (Plate 4.4). The colonies were further stained with Diene's stain appeared dark blue in center and towards periphery light blue colored (Plate 4.5). The isolates stained with Giemsa staining exhibited characteristic morphology of genus *Mycoplasma*, showing pleomorphic and ring forms (Plate 4.6). All the isolates were found to be Digitonin sensitive (Plate 4.7) and urease negative (Plate 4.8). Thus all 24 isolates were identified as *Mycoplasma* spp. based on above results and differential features from genus *Acholeplasma* and *Ureaplasma* (Table 4.1). The numbers of isolates recovered from Narayangaon (17) were greater than from birds of Pen (7) as shown in Table 4.2.

Table 4.1 Conventional Differentiation of Genus *Mycoplasma* from *Ureaplasma* and *Acholeplasma*

Sr. No.	Particulars	*Mycoplasma*	*Ureaplasma*	*Acholeplasma*
1	Sensitivity to Digitonin	Sensitive	Sensitive	Resistant
2	Modified urease test	Negative	Positive	-
3	Colony size (mm)	0.1-0.6	0.01-0.05	up to 3

Table 4.2 Details of *Mycoplasma* spp. Isolates recovered

Sr. No.	Isolate	Source	Specimens
1	P 1	Pen	Choanal swabs
2	P 8		
3	P 15		
4	P 17		
5	P 21		
6	P 24		
7	P 37		
8	N 67	Narayangaon	
9	N 68		
10	N 69		
11	N 70		
12	N 77		

Plate 4.3: ***Mycoplasma*** **colonies showing Fried egg appearance under stereo zoom microscope**

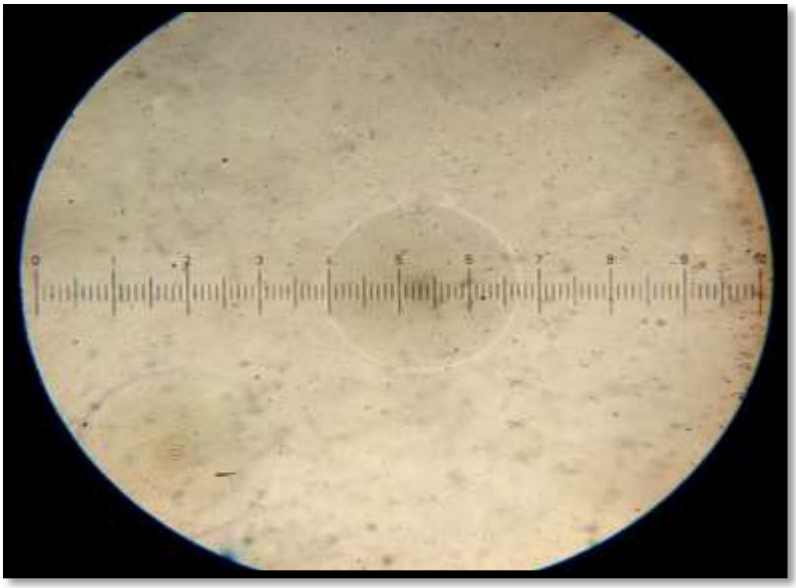


Plate 4.4: Micrometry of ***Mycoplasma*** **colony**

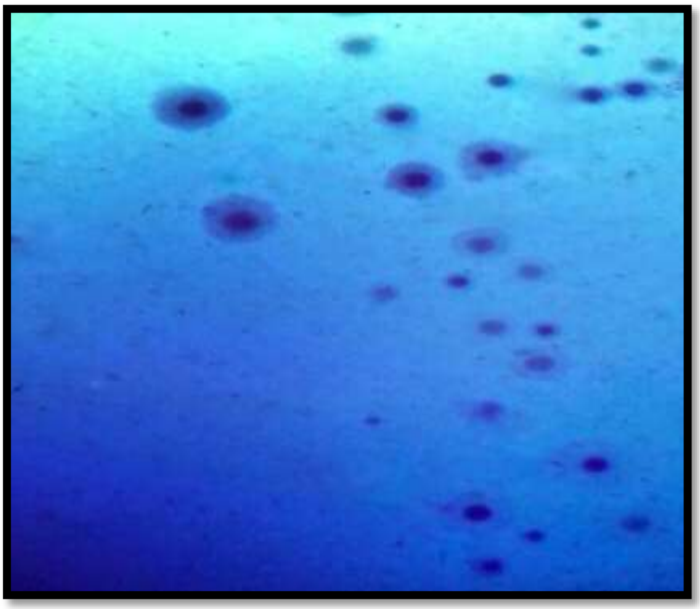


Plate 4.5: Diene’s staining of *Mycoplasma* colonies

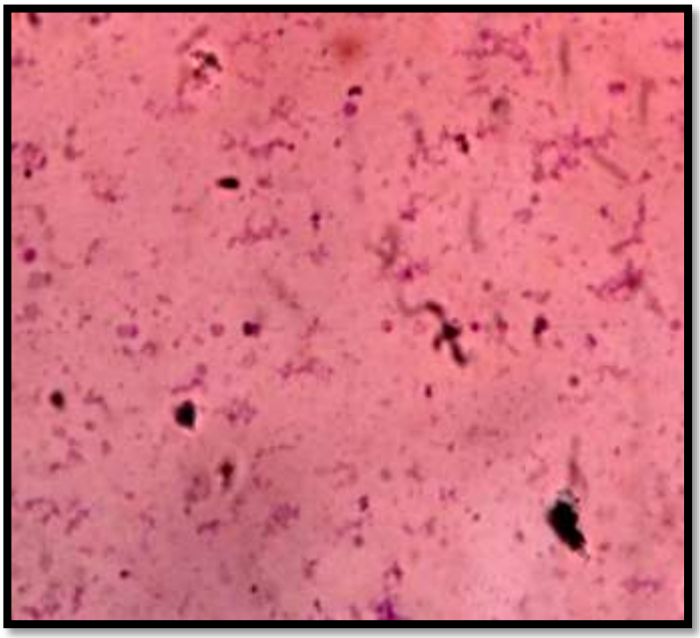


Plate 4.6: Giemsa staining of *Mycoplasma* culture

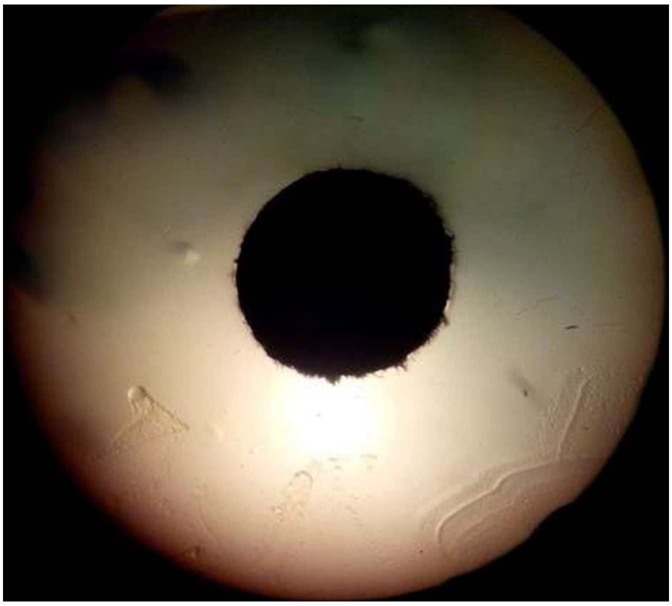


Plate 4.7: Digitonin sensitivity of *Mycoplasma*

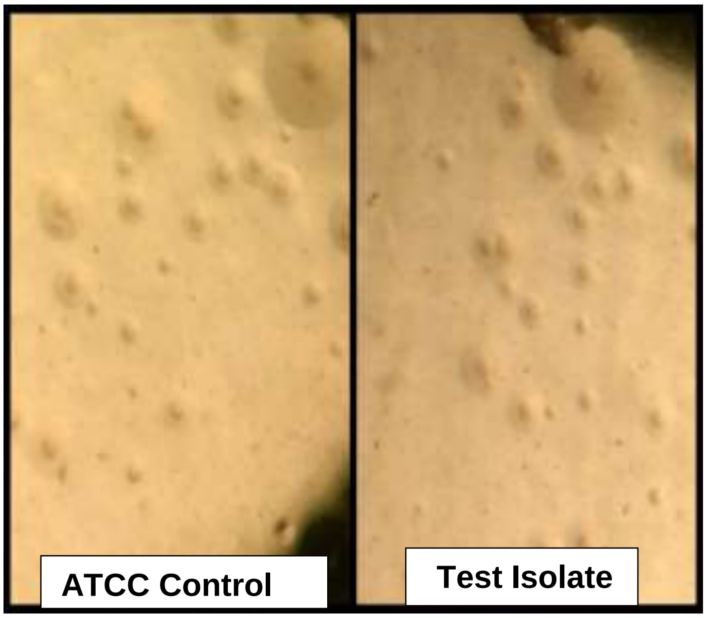


Plate 4.8: Modified Urease test of *Mycoplasma* Colonies showing negative results

13	N 80		
14	N 84		
15	N 87		
16	N 88		
17	N 90		
18	N 96		
19	N 100		
20	N 101		
21	N 103		
22	N 104		
23	N 106		
24	N 108		

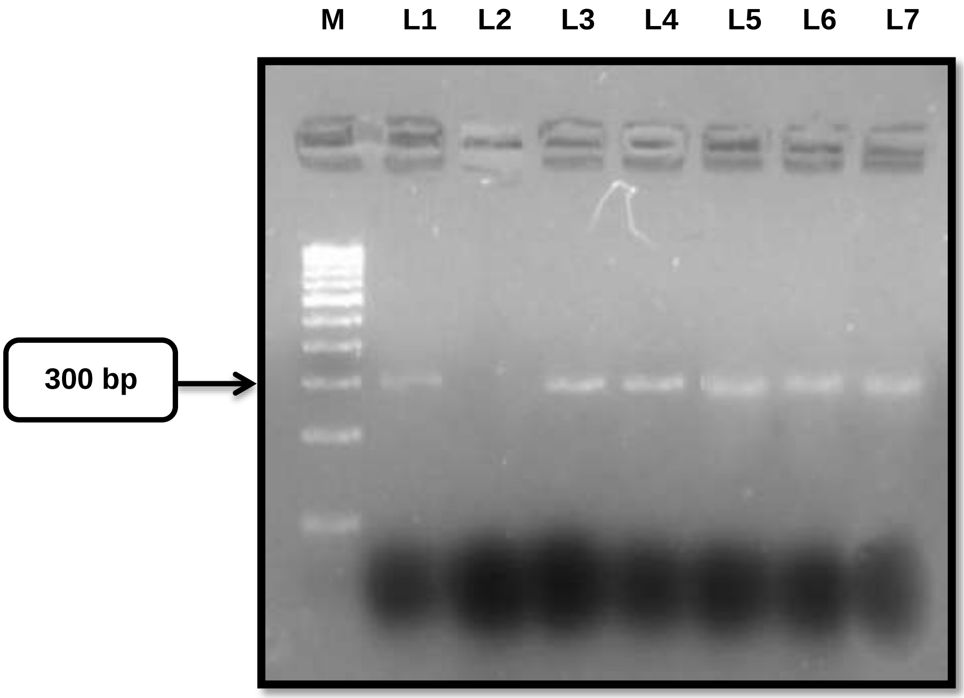
All 24 isolates conventionally identified *Mycoplasma* spp. isolates subjected to PCR amplification of *Mycoplasma gallisepticum* specific mgc2 gene and *Mycoplasma synoviae* species specific 16S rRNA PCR. All 24 *Mycoplasma* spp. isolates along with reference strains of *Mycoplasma gallisepticum* (ATCC 25204) produced *Mycoplasma gallisepticum* specific amplification product of 300 bp (Plate 4.9) Whereas, in species specific 16S rRNA PCR of *Mycoplasma synoviae*, only reference strain of *Mycoplasma synoviae* (ATCC 19610) yielded *Mycoplasma synoviae* specific PCR amplification product of 207 bp and none of isolates were showed any amplification product (Plate 4.10).

A total of 24 *Mycoplasma* spp. isolates were recovered with isolation rate of 16 %. All 24 conventionally identified isolates were confirmed as *Mycoplasma gallisepticum* by mgc2 PCR assay. Out of 24 birds positive for isolation of *Mycoplasma gallisepticum* obtained, 22 (91.67%) were positive in RSA and 23 (95.83%) were positive by direct PCR.

4.3 Molecular diagnosis of Mycoplasmosis by PCR

A total of 150 choanal swabs subjected for direct detection of mycoplasmosis by PCR targeting *Mycoplasma gallisepticum* specific mgc2 gene and 16S rRNA gene for *Mycoplasma synoviae.* The amplified PCR products were evaluated by agarose gel electrophoresis in comparison with the known positive

Plate 4.9: *M. gallisepticum* species specific mgc2 PCR assay of *Mycoplasma* spp. Isolates



M – 100bp of DNA ladder, L1 – ATCC control for *M.gallisepticum*, L2- MG negative isolate, L3- L7 MG positive isolates

ATCC controls i.e. *Mycoplasma gallisepticum* (ATCC 25204) and *Mycoplasma synoviae* (ATCC 19610).

Out of 150 choanal swabs subjected to PCR, targeting species specific mgc2 gene for *Mycoplasma gallisepticum* and 16S rRNA gene for *Mycoplasma synoviae, a* total of 85 (56.67%) samples were found positive and remaining 65 (43.35%) were found negative in PCR. All 85 PCR positive specimens yielded an amplification product of 300 bp specific for MG (Plate 4.11). Whereas, none of 150 specimen produced MS specific amplicon of 207 bp except reference strain (Plate 4.12), thus indicating no incidence of MS infection.

Out of 85 (56.67%) specimens positive for direct PCR, 41 (48.23%) were positive in RSA and 23(27.05%) were positive by cultural method.

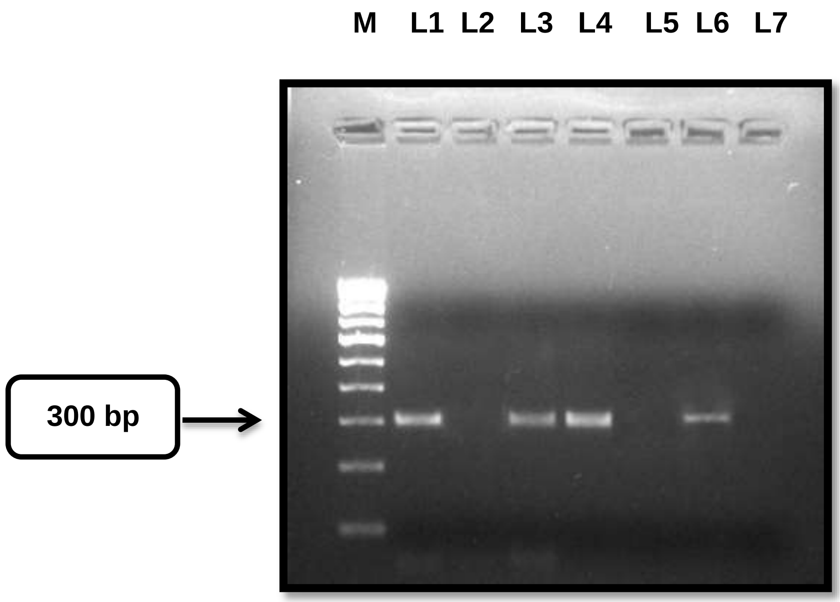
4.5 Comparative efficacy of RSA, cultural method and PCR for detection of Mycoplasmosis

Comparative efficacy of RSA, cultural method and PCR was studied by investigating specimens from a total of 150 birds (Table 4.4). Rapid Serum Agglutination, cultural isolation and direct PCR. In Rapid Serum Agglutination test, out of 150 sera samples, 93 (62%) were positive for mycoplasma antibodies i.e. 73 (48.67%) MG and 20 (13.33%) were positive for MS antibodies respectively.

In cultural isolation, only 24 (16%) isolates were recovered and found to be positive for *Mycoplasma gallisepticum* and none of *Mycoplasma synoviae* isolate could be recovered from choanal swabs. Direct PCR yielded *a* total of 85 (56.67%) samples positive, all of which belonged to MG and none of the sample could be found positive for the MS. In both cultural and PCR methods, only MG was found to be involved in poultry mycoplasmosis. PCR was found to be superior technique for specific and sensitive detection of poultry mycoplasmosis.

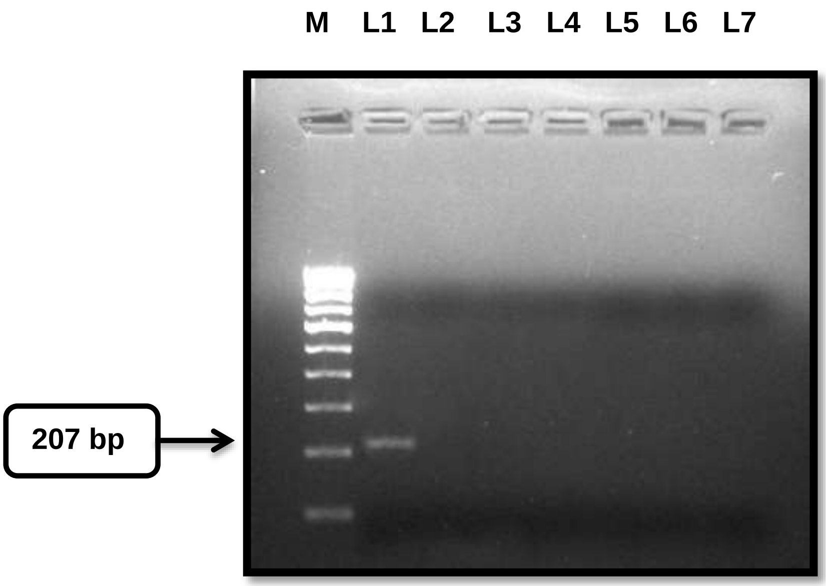
Out of 150 swabs, only 24 (16%) proved to be positive in cultural isolation and conventionally identified as *Mycoplasma gallisepticum* spp. and in PCR they were confirmed as MG spp., whereas 85 (56.67%) choanal swabs were found positive in MG species specific mgc2 PCR and none of samples could be positive for the MS.

Plate 4.10: *M. gallisepticum* species specific mgc2 PCR assay of clinical specimens



M – 100bp of DNA ladder, L1 – ATCC control for *M.gallisepticum*; L2- negative control, L3-L7 – PCR product of MG in clinical specimens

Plate 4.11: *M. synoviae* species specific 16S rRNA PCR assay of clinical specimens



M – 100bp of DNA ladder, L1 – ATCC control for *M. synoviae;* L2-negative, L3- L7 – PCR product of MS clinical specimens

Table 4.3 Comparative efficacy of RSA, cultural isolation and PCR assay in detection of mycoplasmosis

Sr. no.	Sample no	RSA	Culture	PCR		Sr. no.	Sample no	RSA	Culture	PCR
1	P 1	+	+	+		22	P 22	-	-	-
2	P 2	+	-	+		23	P 23	-	-	+
3	P 3	+	-	+		24	P 24	+	+	+
4	P 4	+	-	+		25	P 25	-	-	-
5	P 5	+	-	-		26	P 26	-	-	+
6	P 6	+	-	+		27	P 27	-	-	+
7	P 7	+	-	+		28	P 28	-	-	-
8	P 8	+	+	+		29	P 29	-	-	-
9	P 9	-	-	-		30	P 30	-	-	+
10	P 10	-	-	+		31	P 31	-	-	-
11	P 11	-	-	+		32	P 32	-	-	+
12	P 12	-	-	+		33	P 33	-	-	+
13	P 13	+	-	+		34	P 34	-	-	+
14	P 14	-	-	-		35	P 35	-	-	-
15	P 15	+	+	+		36	P 36	-	-	+
16	P 16	-	-	-		37	P 37	+	-	+
17	P 17	+	+	+		38	P 38	-	-	+
18	P 18	-	-	-		39	P 39	-	-	-
19	P 19	-	-	-		40	P 40	-	-	-
20	P 20	-	-	+		41	P 41	-	-	+
21	P 21	+	+	+		42	P 42	+	-	-

Sr. no.	Sample no	RSA	Culture	PCR		Sr. no.	Sample no	RSA	Culture	PCR
43	P 43	-	-	+		64	N 64	+	-	-
44	P 44	+	-	-		65	N 65	-	-	-
45	P 45	-	-	-		66	N 66	+	-	-
46	P 46	-	-	+		67	N 67	+	+	+
47	P 47	-	-	+		68	N 68	-	+	+
48	P 48	-	-	-		69	N 69	-	+	+
49	P 49	+	-	-		70	N 70	-	+	+
50	P 50	+	-	+		71	N 71	-	-	+
51	N 51	-	-	+		72	N 72	-	-	+
52	N 52	-	-	+		73	N 73	-	-	-
53	N 53	-	-	+		74	N 74	-	-	-
54	N 54	-	-	+		75	N 75	-	-	+
55	N 55	-	-	+		76	N 76	+	-	-
56	N 56	-	-	+		77	N 77	+	+	+
57	N 57	+	+	+		78	N 78	-	-	-
58	N 58	+	-	+		79	N 79	-	-	-
59	N 59	-	-	+		80	N 80	+	+	+
60	N 60	-	-	+		81	N 81	+	-	-
61	N 61	-	-	-		82	N 82	+	-	+
62	N 62	-	-	-		83	N 83	+	-	-
63	N 63	-	-	-		84	N 84	+	+	+

Sr. no.	Sample no	RSA	Culture	PCR		Sr. no.	Sample no	RSA	Culture	PCR
85	N 85	+	-	-		106	N 106	+	+	+
86	N 86	-	-	-		107	N 107	+	-	-
87	N 87	+	+	+		108	N 108	+	+	+
88	N 88	-	+	+		109	N 109	-	-	+
89	N 89	+	-	-		110	N 110	+	-	+
90	N 90	+	+	+		111	N 111	+	-	-
91	N 91	-	-	+		112	N 112	-	-	-
92	N 92	-	-	-		113	N 113	+	-	+
93	N 93	-	-	-		114	N 114	+	-	+
94	N 94	-	-	+		115	N 115	+	-	-
95	N 95	+	-	-		116	N 116	+	-	+
96	N 96	+	+	+		117	N 117	+	-	+
97	N 97	-	-	-		118	N 118	+	-	-
98	N 98	-	-	-		119	N 119	+	-	-
99	N 99	-	-	-		120	N 120	-	-	-
100	N 100	+	+	-		121	N 121	+	-	-
101	N 101	+	+	+		122	N 122	-	-	-
102	N 102	+	-	-		123	N 123	+	-	-
103	N 103	+	+	+		124	N 124	-	-	+
104	N 104	+	+	+		125	N 125	-	-	+
105	N 105	+	-	-		126	N 126	-	-	+

Sr. no.	Sample no	RSA	Culture	PCR		Sr. no.	Sample no	RSA	Culture	PCR
127	N 127	+	-	+		139	N 139	+	-	+
128	N 128	-	-	+		140	N 140	-	-	+
129	N 129	-	-	-		141	N 141	+	-	+
130	N 130	-	-	+		142	N 142	+	-	
131	N 131	+	-	+		143	N 143	+	-	+
132	N 132	+	-	-		144	N 144	+	-	+
133	N133	+	-	-		145	N 145	+	-	
134	N 134	+	-	+		146	N 146	-	-	+
135	N 135	-	-	-		147	N 147	+	-	+
136	N 136	+	-	-		148	N 148	+	-	
137	N 137	-	-	-		149	N 149	+	-	
138	N 138	+	-	-		150	N 150	+	-	+

4.5 Gene specific PCR and Gene-targeted sequencing (GTS) for *M. gallisepticum*

A total 150 choanal swabs were collected from birds showing typical signs of mycoplasmosis, 24 (16%) choanal swabs were found positive for isolation for *Mycoplasma gallisepticum* specific mgc2 gene PCR. All the amplified products obtained were at 300 bp when compared with 100 bp DNA ladder on agarose gel electrophoresis.

A representative PCR product of MG isolate was subjected to nucleotide sequencing. Further, the BLAST analysis of obtained sequences revealed that the amplicon was corresponding to Poultry mgc2 gene without any polymorphism or mutation (Plate 4.13). The curated sequences were submitted to look for their similarity in the NCBI database. To search for the similarity, by BLAST at NCBI

Plate 4.12: BLAST analysis of mgc2 gene PCR product sequence of *Mycoplasma* isolate at NCBI

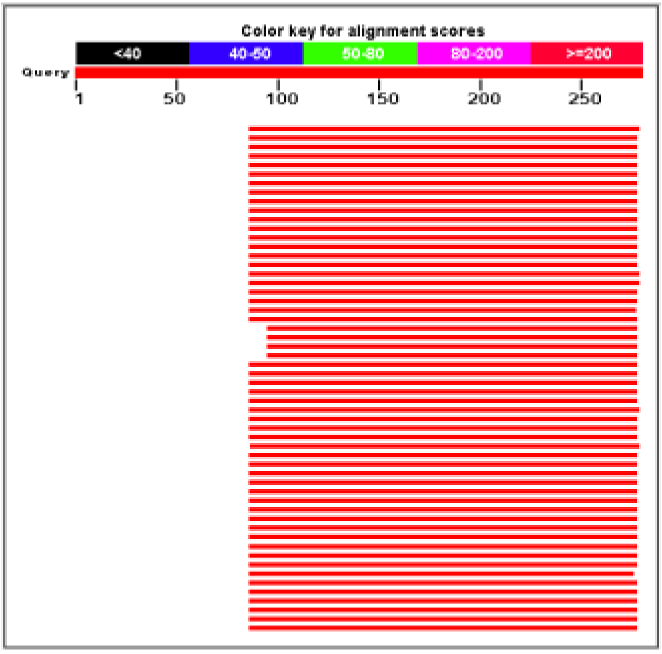
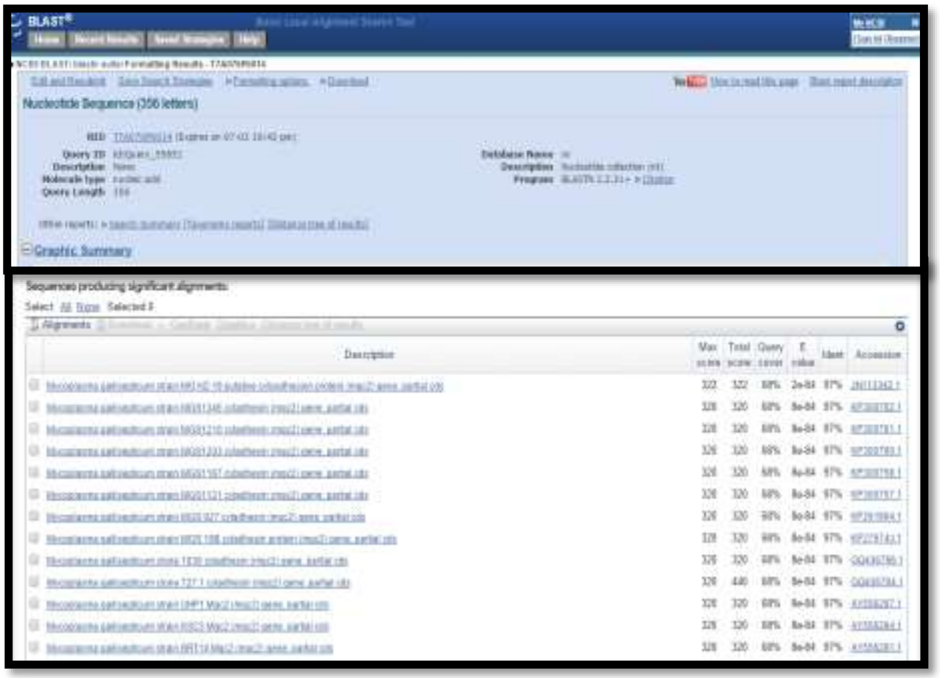


Plate 4.13: % Similarity with other strains of *mycoplasma gallisepticum*



4.14: Sequence analysis of mgc2 gene PCR of *Mycoplasma gallisepticum*

was used. The submitted sequence was showing similarity above 95 % with *Mycoplasma gallisepticum* (strain MG HZ- 19) Putative Cytoadhesion Protein (mgc2) gene (Plate 4.14).The sequence of *Mycoplasma gallisepticum* mgc2 gene PCR of isolate N67 obtained from the ABI files were analysed and curated using Chroma's lite software Version 2.01 (Plate 4.15).

DISCUSSION

5. DISCUSSION

Poultry industry is one of the organized and vibrant sectors of India with current investment of Rs. 200 billion and with 8-10% annual robust and 15 to 20% annual growth rate (Anonymous, 2011). It's a source of employment for about 1.5 million people. Every family in rural areas and every fifth family in urban areas are associated with poultry production in one way or the other (Rashid *et al*., 2009). Despite its high growth rate, poultry sector is facing a lot of problems in terms of infectious diseases; among them respiratory tract infections has its major role (Ali and Reynolds, 2000).

Therefore, rapid, accurate, precise and sensitive detection of such respiratory pathogens which include *Mycoplasma* spp. as one of the important pathogen is essential for control of the outbreaks. Therefore, the present investigation was planned for detection of mycoplasmosis in poultry by conventional and molecular methods.

5.1 Rapid Serum Agglutination Test (RSA) for detection of antibodies

In the present study serological evidence of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in the respiratory distress cases was recorded using Serum Plate Agglutination (SPA) test. A total of 150 sera samples were collected, 93 (62%) found positive for RSA out of which 73 (48.67%) and 20 (13.33%) were positive for MG and MS respectively. Six (4%) sera samples were found positive in RSA for both MG and MS antibodies due to cross reaction.

Mukhtar *et al.* (2012) collected a total of 640 sera samples from 81 commercial layer flocks with complaint of respiratory distress. On the basis of serum plate agglutination test, 40 flocks were found positive for MG indicating a share of 49.01% among the respiratory diseases. These results are found in accordance to the present research.

Similar findings as of present results for serological evidence of *mycoplasma* antibodies recorded by Mahfuzul *et al.* (2014) who collected a total of 480 blood samples of chickens from different upazilas, Bangladesh. On the basis of the serum plate agglutination test, 55.83% (n=268/480) chickens were found positive for MG. The MG infection was higher (62.5%) in backyard chickens as compared to those being reared in commercial farming systems (53.61%). Moreover, Luciano *et al*. (2011) tested serum from commercial poultry breeders (n = 2781) MS by serum plate agglutination (SPA), Hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA). From 2,781 samples tested, 736 (26.46%) were positive in SPA.

However, the lower serological rate than the present study was observed by Luciano *et al*. (2011) whereas higher serological rate have been observed, 83.10% by Heleili *et al.* (2011) for MG.

5.2 Isolation and identification of *Mycoplasma* spp.

In the present investigation cultural isolation of *Mycoplasma* spp., was carried out from choanal swabs of birds suspected for mycoplasmosis (OIE, 2008). Similar specimens were used for attempting isolation of *Mycoplasma* spp. by Gharaibeh and Roussan (2008), Evans *et al.* (2009) and Bayatzadeh *et al.* (2011) who achieved better diagnosis of Mycoplasmosis from the same specimens.

Isolation of *Mycoplasma* spp. was attempted using Pleuropneumonia Like Organism (PPLO) medium and suitable incubation conditions. Several selective media have been employed for isolation and evaluated for their suitability for growth of *Mycoplasma* spp. viz. Frey's medium, modified Hayflick medium, Pleuropneumonia like organism (PPLO) medium (OIE, 2008; Hanif and Najeeb, 2007; Ongor *et al.*, 2009; Heleil *et al*.,2011; Rouf *et al*., 2013).

In the present investigation, a total of 150 choanal swabs were processed. Isolation was successful only from 24 choanal swabs (16%) while remaining 126 choanal swabs (84%) were found to be negative for cultural isolation. Isolates recovered on PPLO agar were differentiated from genus *Acholeplasma* and *Ureaplasma* (Quinn *et al.* 2004) and confirmed as *Mycoplasma* spp. based on results of conventional methods. The isolation rate of *Mycoplasma* spp. i.e. 8.18% and 9% was observed by Tiong *et al.* (1979) and Hanif and Najeeb (2007) respectively. Behbahan *et al*. (2005) investigated the cases of avian mycoplasmosis and could recover 100 (81.31%) *Mycoplasma* isolates from 123 tracheal and airsac samples. Behbahan *et al*. (2008) prospered in isolating 28 field isolates of *Mycoplasma gallisepticum* and 4 field isolates of *M. synoviae* on enriched Mycoplasma broth and agar. Gharaibeh and Roussan (2008) conveyed 31.6% prevalence of MG in the 76 flocks by culturing tracheal swabs. Evans *et al.* (2009) succeeded in isolating 14 (46.67%) *Mycoplasma gallisepticum* cultures from 30 choanal cleft/palatine fissure swabs by using modified Frey's media. Heleili *et al.* (2011) isolated 143 Mycoplasmas strains from 237 organ samples of laying hens and broiler chicken representing a positivity rate of 60.33%. Khalda *et al.* (2013) recovered 7 (4.11%) isolates from 170 tracheal swabs. Nouzha *et al.* (2013) achieved 72.72% (7/18) isolation rate of *Mycoplasma* spp. using tracheal swab. Rauf *et al.* (2013) reported isolation rate of 27.6% (104 /380) of *M. gallisepticum* from tracheal swabs, lung and airsac.

Direct detection of the organism by cultivation and isolation is difficult because *M. gallisepticum* and other avian pathogenic Mycoplasma species are slow-growing, relatively fastidious organisms that require one or more weeks for its growth and identification (Garcia *et al.,* 2005). Their isolation is often impaired by the overgrowth of non-pathogenic Mycoplasma species or other faster growing bacteria and fungi (Garcia *et al.,* 1995). Another factor to consider is the selective pressures on populations of mycoplasmas, which would differ substantially in vivo and in vitro.

In this present study all 24 isolates confirmed as Mycoplasma spp. with isolation rate of 16%. However, a great difference in rate has been observed among the reported values due to above mentioned causes. The result of isolation rate of present study fall in the range reported by above authors.

5.3 Molecular Detection of Mycoplasmosis from clinical specimens

PCR has become a valuable tool in the diagnosis of *Mycoplasma* species, not only for its sensitivity but for its increased specificity (Kempf *et al*., 1993). In order to overcome the difficulties encountered during conventional and serological techniques, several molecular techniques have been developed for differentiation of *Mycoplasma* spp. strains. As in present investigation, several authors found PCR as sensitive and specific for detection of poultry mycoplasmosis.

In the present study, *Mycoplasma gallisepticum* specific mgc2 gene PCR and *Mycoplasma synoviae* specific 16S rRNA PCR assays were employed for detection of poultry mycoplasmosis directly in clinical specimens.

Lauerman, (1998) used 16S rRNA PCR method for confirmation of *M. gallisepticum* and *M. synoviae* infection in chickens. Also, Lauerman (1998) published a validated PCR assay for MG, MS and other avian mycoplasmas based on unique sequences (OIE, 2008).

Out of 150 choanal swabs subjected to PCR, targeting species specific mgc2 gene for *Mycoplasma gallisepticum* and 16S rRNA gene for *Mycoplasma synoviae, a* total of 85 (56.67%) samples were found positive and remaining 65 (43.35%) were found negative in PCR. All 85 PCR positive specimens yielded an amplification product of 300 bp specific for MG. Whereas, none of 150 specimen produced MS specific amplicon of 207 bp except reference strain, thus indicating no incidence of MS infection.

The similar range of sensitivity of PCR in detection of mycoplasmosis was observed by Bayatzadeh *et al.* (2011) and Rauf *et al*. (2013) who reported sensitivity of PCR in 55.9% and 68.94% cases respectively. However, lower sensitivity of PCR than in present investigation is reported by Evans *et al.* (2009), Khalda *et al.* (2013), Bagheri *et al.* (2011) and Kaboli *et al*. (2013) i.e. 36.7%, 17.8%, 29.63% and 31.50% respectively.

The results of resent study for *Mycoplasma synoviae* are in contrast to Nagalakshmi *et al.* (2013), Bayatzadeh *et al.* (2011), Khalda *et al.* (2013) who reported 33.7%, 55.9%, 6.7% incidence of MS by employing 16S rRNA species specific PCR assays.

5.4 Comparative efficacy of RSA, cultural isolation and direct PCR assay in detection of Poultry mycoplasmosis

In present research work a total of 150 choanal swabs were subjected for cultural isolation of *Mycoplasma* spp. and directly to PCR assay whereas 150 sera samples of same birds subjected to Rapid Serum Agglutination test. In Rapid Serum Agglutination test out of 150 sera samples, 93 (62%) sera samples found positive in RSA, out of which 73 (48.67%) and 20 (13.33%) were positive for MG and MS antibodies respectively. In cultural isolation only 24 (16%) isolates were recovered and found to be positive for *mycoplasma gallisepticum* and none of *Mycoplasma synoviae* isolates could be recovered from choanal swabs. Direct PCR yielded 85 (56.67%) MG positive cases and none of samples could be positive for the MS. In both cultural and PCR methods only MG was found to be involved in poultry mycoplasmosis.

Out of 24 isolates recovered, 22 (91.67%) were positive and 23(95.83%) were also positive in RSA and direct PCR. In RSA some false positive, false negative and cross reaction observed. However, RSA found equally sensitive but not specific since cross reaction also observed among MG and MS antibodies. Thus, Silveira *et al*. (1996) found PCR was superior technique for rapid, sensitive and specific detection of poultry mycoplasmosis.

5.5 Alignment of nucleotide Sequence of mgc2-PCR amplicon

The amplification of mgc2 gene of Mycoplasma isolates was carried out using specific primer sequences. All 24 the amplified products obtained were at 300 bp when compared with 100 bp DNA ladder on agarose gel electrophoresis. A representative PCR product of N67 isolate from Pune district was subjected to nucleotide sequencing.

The curated sequences were submitted to look for their similarity in the NCBI database. To search for the similarity, by BLAST at NCBI was used. The submitted sequence was showing similarity above 95 % with *Mycoplasma gallisepticum* (strain MG HZ- 19) Putative Cytoadhesion Protein (mgc2) gene .The sequence of *Mycoplasma gallisepticum* mgc2 gene PCR of isolate N67 obtained from the ABI files were analysed and curated using Chroma's lite software Version 2.01.

SUMMARY AND CONCLUSIONS

6. SUMMARY AND CONCLUSIONS

The common pathogens of avian respiratory tract infection are *Mycoplasma gallisepticum* and *Mycoplasma synoviae* which produce sub clinical disease. These organisms are difficult to detect with routinely used isolation and identification procedures. In view of this the present research was planned to detect mycoplasmosis in poultry by conventional and molecular techniques.

A total of 300 clinical specimens which included choanal swabs (150) and sera samples (150) collected from birds suspected for mycoplasmosis from Pune and Raigad districts of Maharashtra. Sera samples (150) collected for detection of antibodies against MG and MS employing Rapid Serum Agglutination test. Choanal swabs (150) collected in duplicates were subjected for isolation of *Mycoplasma* spp. and direct PCR.

A total of 150 sera samples collected from mycoplasmosis suspected birds were subjected to rapid slide agglutination test using commercial MG and MS antigens. Out of 150 sera samples tested, a total of 93 (62%) found positive for mycoplasma antibodies in RSA. Out of 93 RSA positive, 73 (48.67%) were MG and 20 (13.33%) were MS antibodies positive. Six (4%) sera samples were found positive in RSA for both MG and MS antibodies due to cross reaction.

Isolation of *Mycoplasma* spp. was carried out in Pleuropneumonia Like Organism (PPLO) medium and suitable incubation conditions. Out of 150 choanal swabs subjected for isolation, only 24 isolates (16%) were recovered while remaining 126 (84%) specimens were proved to be negative in cultural isolation. All 24 field isolates were identified as *Mycoplasma* spp. by differentiation from *Acholeplasma* and *Ureaplasma* based on conventional test results. Thus isolation rate is 16%.The confirmation of isolates at species level was done by PCR with 100%.

In direct molecular detection of *mycoplasma gallisepticum* specific mgc2 PCR assay, a total of 85 (56.67%) out of 150 choanal swabs were found to be PCR positive, whereas, 65 (43.35%) samples were proved to be negative and none of samples could be found positive for MS specific 16S rRNA PCR. MG specific amplification was observed in 85 (56.67%) samples with the amplification product of 300 bp.

In present research work a total of 150 choanal swabs were subjected for cultural isolation of *Mycoplasma* spp. and directly to PCR assay whereas 150 sera samples of same birds subjected to Rapid Serum Agglutination test. In Rapid Serum Agglutination test out of 150 sera samples, 93 (62%) sera samples found positive in RSA, out of which 73 (48.67%) and 20 (13.33%) were positive for MG and MS antibodies respectively. In cultural isolation only 24 (16%) isolates were recovered and found to be positive for *mycoplasma gallisepticum* and none of *Mycoplasma synoviae* isolates could be recovered from choanal swabs. Direct PCR yielded 85 (56.67%) MG positive cases and none of samples could be positive for the MS. In both cultural and PCR methods only MG was found to be involved in poultry mycoplasmosis.

The amplification of mgc2 gene by PCR yielded specific amplification products of 300 bp in all the samples. A representative PCR product was subjected to nucleotide sequencing. The result, of BLAST analysis of obtained sequences revealed that the amplicon was corresponding to Poultry mgc2 gene without any polymorphism or mutation. Further submission of sequence to NCBI for accession no. is under process.

6.2. Conclusion

1. Isolation of *Mycoplasma* species using PPLO medium can be achieved for direct evidence of aetiology for pathogenicity and epidemiological study.

2. Rapid Serum Agglutination technique can be used to screening birds for *Mycoplasma* antibodies.

3. Conventional tests were found to be useful in identification of genus *Mycoplasma* and differentiation from *Acholeplasma* and *Ureaplasma.*

4. Species specific mgc2 PCR proved to be useful in discrimination and confirmation of field isolates of *Mycoplasma* spp. at species level.

5. MG species specific mgc2 PCR assay was found to be sensitive and specific for diagnosis of poultry mycoplasmosis directly in clinical specimens.

6. *M. gallisepticum* was found to be the only species involved in poultry mycoplasmosis, detected by both cultural isolation and PCR technique.

7. Comparative results of RSA, cultural isolation and direct PCR indicated that PCR is superior to cultural isolation for crucial, rapid, specific and sensitive detection of poultry mycoplasmosis. However, RSA is proved to be comparable with the PCR.

8. Sequencing of mgc2 gene found that the sequences were stable and useful in typing and strain identification / differentiation.

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APPENDICES

APPENDICES

Appendix – I

Media, stains and solutions

1. **PPLO (Pleuropneumonia Like Organism) Broth Medium**

Part A

Ingredients	gm/ml
PPPLO broth base without crystal violet (HiMedia)	: 21
Distilled water	: 700

Part B

Ingredients	ml
Pig serum (heated at 56°C for 1 hour) 25% (w/v)	: 150
Fresh yeast extract 10%(w/v)	: 100
Glucose solution 5% (w/v)	: 10
Thallous acetate 1% (w/v)	: 10
Penicillin G (200,000 IU/ml)	: 05
Phenol red solution 0.1% (w/v)	: 20

PPLO Broth medium was prepared by autoclaving Part A at 121°C, at 1 atmospheric pressure for 15 minutes and, after cooling, was added to Part B, 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.

2. PPLO (Pleuropneumonea Like Organism) Agar Medium

For the corresponding solid medium, PPLO broth base in part A was replaced by 36 gm of PPLO agar base (HiMedia). The mixture was autoclaved as before and kept in a water bath at 56°C. The constituents of part B, omitting the phenol red. Parts A and B are mixed carefully to avoid the production of air bubbles, and were dispensed into sterile Petri plates.

For selective isolaton of *Mycoplasma synoviae*, 1% solution each of NAD and cysteine hydrochloride was mixed in equal parts and 2 ml was added per 100 ml of medium

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25%Yeast extract Solution

Yeast extract powder	: 25 gm
Distilled water	: 100 ml

The yeast extract powder was suspended in distilled water for preparation of yeast extract. This was heated to boiling point, cooled and then centrifuged for 20 minutes at 3000 rpm. 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 (0.45 µm). The extract was stored at –20°C until used.

10% Glucose solution

Glucose	: 10 gm
Distilled water	: 100 ml

After dissolving glucose in distilled water the pH was adjusted to 7.8–8.0 using 0.1 M NaOH and sterilized by filtration (0.45 µm).

0.1% Phenol red

Phenol red	: 0.1 gm
Distilled water	: 100 ml

Phenol red was prepared by grinding 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 min. It was stored at 4°C until used.

5% Thallium acetate

Thallous acetate	: 05 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 used upto 1 week.

Diene's stain

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

Giemsa stain (Himedia pvt. Ltd.)

Working Giemsa stain was prepared by adding 30 drops of stock solution in 30 ml distilled H_2O.

Modified urease test solution

a) Urea solution (10%)

Urea	: 10 gm
Distilled water	: 100 ml

b) Manganese Chloride solution (0.8%)

Manganese Chloride	: 08 gm
Distilled water	: 950 ml

The modified urease test solutions was prepared by adding and mixing equal quantity of above two solutions (a and b).

Digitonin stock solution

Digitonin	: 0.015 gm
95% ethanol	: 1 ml

Digitonin Stock solution was prepared in 1.5% (w/v) concentration using 95% ethanol. Digitonin discs were prepared by adding 25 µl of the stock solution to 6 mm diameter paper discs.

Appendix – II

Reagents used for DNA extraction and Agarose Gel Electrophoresis (AGE)

1. Phosphate buffer saline (PBS) pH 7.4

NaCl	: 8.0 gm
KCl	: 0.2 gm
Na_2HPO_4	: 1.13 gm
KH_2PO_4	: 0.2 gm
Distilled water	: 1000 ml

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

2. Ethidium bromide (10 mg / ml)

Ethidium bromide	: 20 mg
Distilled water	: 2 ml

3. 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.

4. 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)

5. 6X Loding dye (Type IV)

Sucrose	: 40 % w/v in DW
Bromophenol blue	: 0.25 % w/v in DW

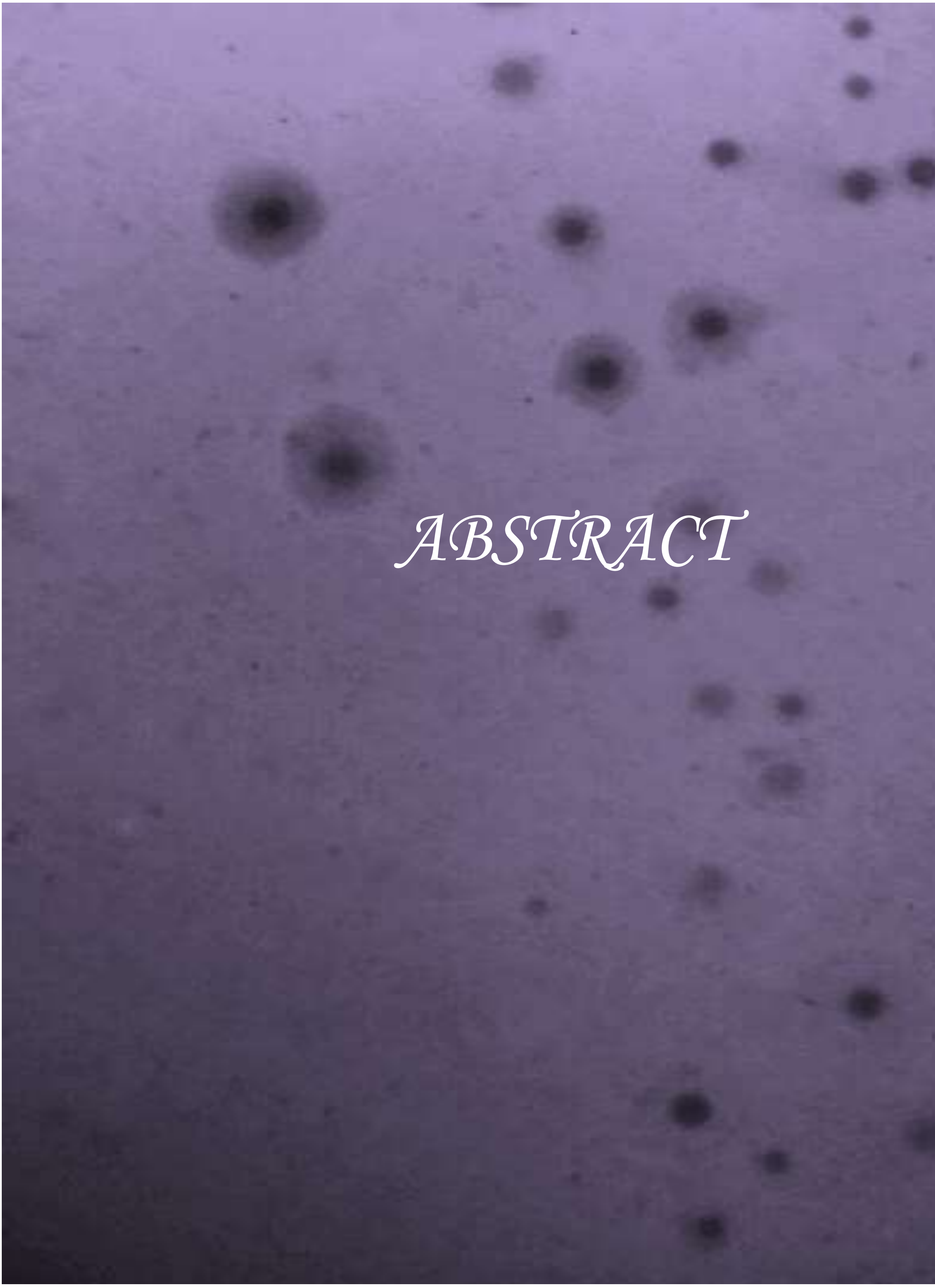
Store the solution at 4^0 C.

Appendix – III

List of equipments

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

Sr. No.	Name	Manufacture
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.



ABSTRACT

Appendix – G

THESIS ABSTRACT

a)	Title of the thesis (in Capital letters)	**:**	Detection of Mycoplasmosis in poultry by conventional and molecular technique
b)	Full name of student	**:**	BUKTE SWATI RAMRAO
c)	Name and address of Major Advisor	**:**	**Dr. (Mrs.) R. S. Gandge** Associate Professor, Department of Microbiology, Bombay Veterinary College, Parel, Mumbai-400 012.
d)	Degree to be awarded	**:**	M. V. Sc.
e)	Year of award of degree	**:**	2015
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)	**:**	
	Signature of the Associate Dean		

ABSTRACT

In present study, clinical samples (choanal swabs and serum) from live birds were processed for detection of poultry mycoplasmosis. The research was carried by detecting mycoplasma antibodies, isolation and identification of *Mycoplasma* species and molecular detection of mycoplasmosis by PCR assays directly in clinical specimens.

A total of 300 clinical specimens (150 sera samples + 150 choanal swabs from same birds) suspected for poultry mycoplasmosis were collected from Pen (Raigad districts) and Narayangaon (Pune districts) of Maharashtra state. The sera samples collected were subjected to Rapid Serum Agglutination test for detection of *Mycoplasma* antibodies. Choanal swabs collected in duplicates were processed for *Mycoplasma* isolation and direct PCR targeting mgc2 gene of *M. gallisepticum* and 16S rRNA gene of *M. synoviae*. The sequencing of PCR products was carried out of representative PCR product and the sequences obtained from the ABI files were analysed and curated using Chroma's light software Version 2.01.

In the present study out of 150 sera samples were tested for mycoplasma antibodies, total 93 (62%) found positive for RSA out of which 73 (48.67%) and 20 (13.33%) were positive for MG and MS respectively. 6 (4%) sera samples were found RSA positive for both MG and MS antibodies due to cross reaction.

A total of 24 *Mycoplasma* spp. isolates were recovered with isolation rate of 16 %.All 24 isolates which were identified by conventional method were confirmed by MG species specific mgc2 PCR assay. All isolates were found to be *Mycoplasma gallisepticum*. Out of 24 birds positive for isolation of *Mycoplasma gallisepticum*, 22 (91.67%) and 23 (95.83%) were positive for RSA and direct PCR respectively.

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Out of 150 choanal swabs subjected to PCR, targeting species specific mgc2 gene for *Mycoplasma gallisepticum* and 16S rRNA gene for *Mycoplasma synoviae, a* total of 85 (56.67%) samples were found positive and remaining 65 (43.35%) were found negative in PCR. All 85 PCR positive specimens yielded

an amplification product of 300 bp specific for MG. Whereas, none of 150 specimen produced MS specific amplicon of 207 bp except reference strain, thus indicating no incidence of MS infection

The comparative results of Rapid Serum Agglutination test out of 150 sera samples, 93 (62%) i.e. 73 (48.67%) and 20 (13.33%) were positive for MG and MS respectively. In cultural isolation, only 24 (16%) isolates were recovered and found to be positive for *mycoplasma gallisepticum* and none of isolates could be recovered from choanal swabs for *Mycoplasma synoviae*. Direct PCR yielded 85 (56.67%) MG positive cases and none of samples could be positive for the MS. In both cultural and PCR methods, only MG was found to be involved in poultry mycoplasmosis.

A representative PCR product of mgc2 gene was subjected to nucleotide sequencing. The result of BLAST analysis of obtained sequences revealed that the amplicon was corresponding to mgc2 gene of *Mycoplasma gallisepticum* without any polymorphism or mutation. The curated sequences were submitted to look for their similarity in the NCBI database by BLAST at NCBI. The sequence obtained from isolate of present study showed similarity above 95 % with *Mycoplasma gallisepticum* (strain MG HZ- 19 and others) Putative Cytoadhesion Protein (mgc2) gene.

<u>प्रबंध सारांश</u>

1.	प्रबंधाचे नांव	:	**पारंपारीक आणि आण्विक तंत्राने पक्ष्यांच्या मायकोप्लाझ्मोसीस् रोगाचा तपास**
2.	विद्यार्थ्याचे नांव	:	बुकटे स्वाती रामराव
3.	मार्गदर्शकाचे नांव	:	**डॉ. (सौ.) आर.एस. गंदगे,** सहाय्यक प्राध्यापक, पशुवैद्यकीय सुक्ष्मजीवशास्त्र विभाग, मुंबई पशुवैद्यकीय महाविद्यालय, परळ, मुंबई–400 012
4.	पदवी	:	पदव्युत्तर पदवी
5.	पदवी प्रदान करण्याचे वर्ष	:	2015
6.	मुख्य विषय	:	पशुवैद्यकीय सुक्ष्मजीवशास्त्र
7.	प्रबंधाची एकूण पाने	:	
8.	सारांशाचे एकूण शब्द	:	
9.	विद्यार्थ्याची सही	:	
10.	विभाग प्रमुखाचे नाव, सही आणि पत्ता	:	
11.	सहयोगी अधिष्ठाता मुंबई पशुवैद्यकीय महाविद्यालय परळ, मुंबई–400 012	:	

प्रबंध सारांश

पारंपारीक आणि आण्विक तंत्राने पक्ष्यांच्या मायकोप्लाइम्मोसीस् रोगाचा तपास

कोंबडयांमधील मायकोप्लाइम्मोसीस् रोगाचा तपास करण्यासाठी कोएनल स्वाब आणि रक्तजल गोळा करण्यात आले. सध्याच्या अन्वेषणामध्ये मायकोप्लाइम्मा प्रतिपिंड निर्देशन, पारंपारीक पद्धतीने मायकोप्लाइम्मा जिवाणूचे अलगीकरण व पी सी आर पद्धतीने त्याची पुष्टी करण्यात आली. तसेच मायकोप्लाइम्मा आयसोलेटच्या रोगाचा तपास थेट नमुन्यामध्ये पी सी आर पद्धतीने करण्यात आला.

या अन्वेषणामध्ये महाराष्ट्र राज्यातील पेण (रायगड जिल्हा) आणि नारायणगाव (पुणे जिल्हा) येथील पक्ष्यांतील मायकोप्लाइम्मोसीस् रोगासाठी संशयित एकूण ३०० (१५० रक्तजल नमुने + १५० कोएनल स्वाब) नमुने गोळा करण्यात आले. मायकोप्लाइम्मोसीस् रोगासाठी द्विगुणन संशयित कोएनल स्वाब गोळा करण्यात आले आणि त्याचा उपयोग प्रत्येकी मायकोप्लाइम्मा जिवाणूचे अलगीकरण आणि मायकोप्लाइम्मा गॅलिसेप्टीकम जिवाणूच्या mgc2 आणि मायकोप्लाइम्मा सायनोव्हीच्या 16S rRNA जनुकसाठी थेट पी सी आर लक्षित करण्यात आले. त्याच पक्ष्यांतील रक्तजल नमुन्यांचा उपयोग रॅपिड रक्तजल प्रसमुहन (RSA) चाचणीसाठी करण्यात आला. प्राप्त झालेले थेट पी सी आर उत्पादने अनुक्रमासाठी पाठविण्यात आले आणि ABI फाईल्स पासून प्राप्त झालेल्या विश्लेषणांचा Croma’s Lite Software आवृत्ती २.०१ वापरून विश्लेषण करण्यात आले.

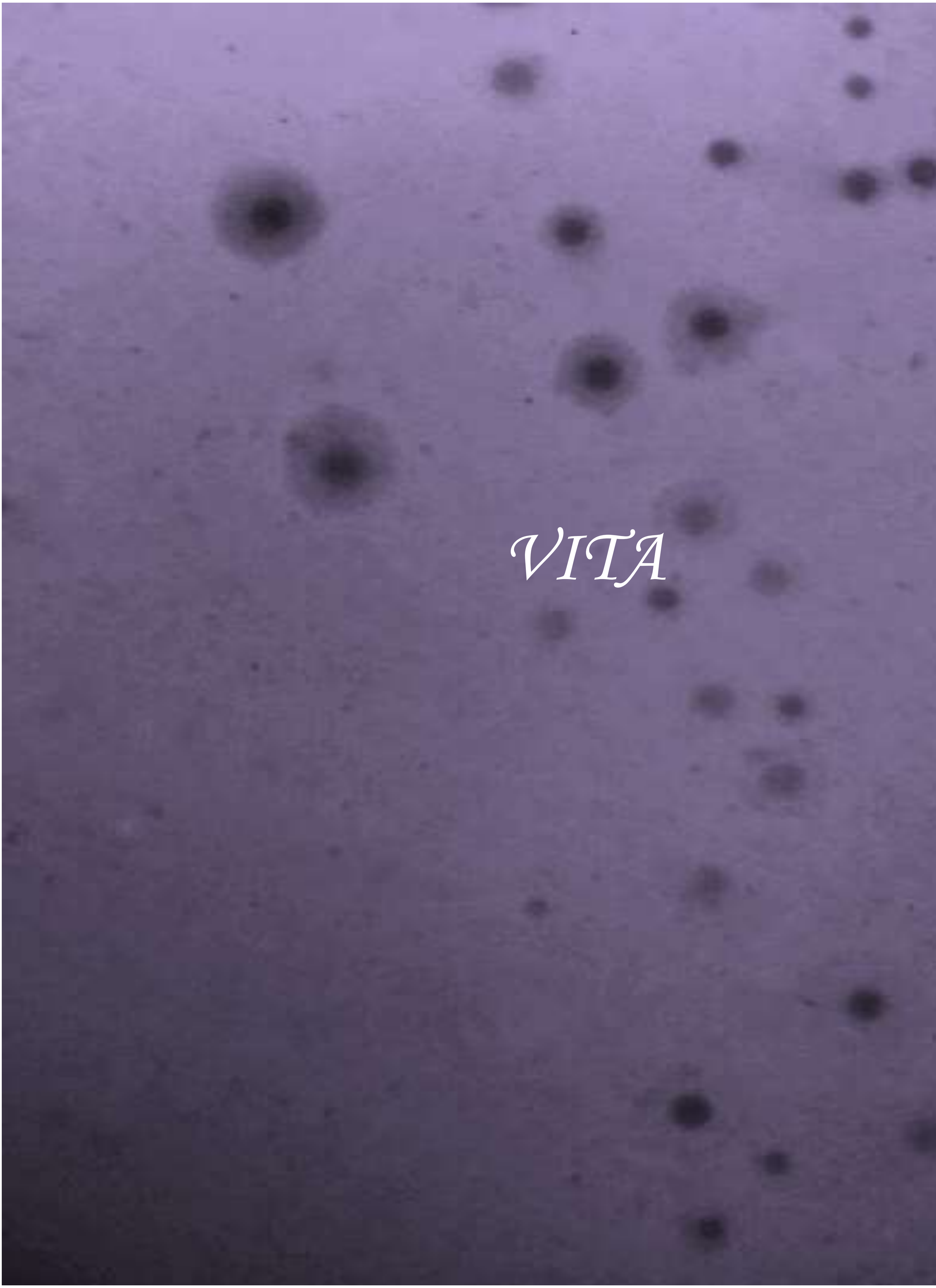
मायकोप्लाइम्मा प्रतिपिंडासाठी तपासलेल्या १५० नमुन्यापैकी एकूण ९३ (६२%) नमुने रॅपिड रक्तजल प्रसमुहन चाचणीसाठी सकारात्मक आढळले, त्यापैकी ७३ (४८.६७%) मायकोप्लाइम्मा गॅलीसेप्टीकम व २० (१३.३३%) मायकोप्लाइम्मा सायनोव्ही साठी सकारात्मक आढळले. ६ (४%) रक्तजल नमुने क्रॉस प्रतिक्रिया झाल्यामुळे, दोन्ही मायकोप्लाइम्मा गॅलीसेप्टीकम आणि मायकोप्लाइम्मा सायनोव्ही ॲन्टीबॉडीजसाठी सकारात्मक आढळले.

एकूण २४ मायकोप्लाइम्मा विलगीकृत आयसोलेटस् १६% च्या दराने प्राप्त झाले आणि सर्व २४ विलगीकृत आयसोलेटस् पारंपारीक पद्धतीने ओळखुन तसेच विशिष्ठ उपवर्गासाठी mgc2 पी सी आर पद्धतीने पुष्टी करण्यात आली आणि सर्व आयसोलेटस् मायकोप्लाइम्मा गॅलीसेप्टीकम प्रजातीचे असल्याचे आढळून आले. एकुण २४ अलगीकृत सकारात्मक नमुन्यापैकी, २२ (९१.६७%) रॅपिड रक्तजल प्रसमुहन चाचणीमध्ये आणि २३ (९५.८३%) थेट पी सी आर करीता सकारात्मक आढळून आले.

एकूण १५० कोएनल स्वाब, मायकोप्लाझ्मा गॅलीसेप्टीकम mgc2 उपवर्ग विशिष्ठ जनुक व मायकोप्लाझ्मा सायनोव्ही 16S rRNA जनुक साठी थेट पी सी आर च्या मदतीने तपासण्यात आले. त्यापैकी एकूण ८५ (५६.६७%) नमुने थेट पी सी आर मध्ये सकारात्मक आढळले ज्यामध्ये सर्व ८५ नमुने मायकोप्लाझ्मा गॅलीसेप्टीकम विशिष्ठ ३०० bp प्रवर्धन उत्पादनासाठी सकारात्मक आढळले, आणि शिल्लक ६५ (४३.३%) नमुने मायकोप्लाझ्मा गॅलीसेप्टीकम साठी नकारात्मक आढळले. सर्व १५० नमुने थेट पी सी आर पद्धतीमध्ये मायकोप्लाझ्मा सायनोव्ही साठी नकारात्मक आढळले.

तुलनात्मक परिणामामध्ये करण्यात आलेल्या रॅपिड रक्तजल प्रसमुहन चाचणी मध्ये १५० रक्तजल नमुन्यांपैकी एकुण ९३ (६२%) नमुने RSA सकारात्मक आढळली त्यापैकी ७३ (४८.६७%) मायकोप्लाझ्मा गॅलीसेप्टीकम साठी व २० (१३.३३%) मायकोप्लाझ्मा सायनोव्ही साठी सकारात्मक आढळले. मायकोप्लाझ्मा जिवाणूच्या अलगीकरणामध्ये एकूण २४ (१६%) नमुने मायकोप्लाझ्मा गॅलीसेप्टीकम साठी सकारात्मक आढळले आणि मायकोप्लाझ्मा सायनोव्ही च्या अलगीकरणास नकारात्मक आढळले. थेट नमुन्याच्या पी सी आर पध्दतीमध्ये ८५ (६५.६७%) नमुने मायकोप्लाझ्मा गॅलीसेप्टीकमसाठी सकारात्मक आढळले. दोन्ही अलगीकरण आणि थेट पी सी आर पद्धतीद्वारे पक्ष्यांच्या मायकोप्लाझ्मोसीस् रोगामध्ये फक्त मायकोप्लाझ्मा गॅलीसेप्टीकम जिवाणूंचा प्रादुर्भाव आढळून आला.

सर्व मायकोप्लाझ्मा जिवाणू संवर्ध उत्पादनापैकी एक mgc2 जनुक पी सी आर चे प्रतिनिधी उत्पादित Nucleotide अनुक्रमासाठी पाठवण्यात आले. परिणामामध्ये पक्ष्यांच्या mgc2 जनुक amplicon चे विश्लेषण BLAST पद्धतीने करण्यात आले. प्राप्त झालेल्या अनुक्रमाला NCBI database वर त्याची समांतरता पाहण्यासाठी सादर करण्यात आले. सादर केलेला अनुक्रम मायकोप्लाझ्मा गॅलीसेप्टीकम (Strain MG HZ-19) Putative Cytoadhesion Protein (mgc2) जणुकाशी ९५% पेक्षा अधिक टक्क्यांवर समांतरीत आढळला.


VITA

VITA

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