



## Genus specific PCR–RFLP and multiplex PCR for diagnosis of avian mycoplasmosis

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### ABSTRACT

Diagnosis of avian mycoplasmosis remains a serious problem, due to unusual nature of etiological agent. Although, many protocols for PCR are set with the published primers; they do not always yield *vis-à-vis* results under different existing conditions of laboratories. Therefore, the present research was aimed towards the optimization and evaluation of genus specific and multiplex PCR for molecular diagnosis of avian mycoplasmosis. The research was carried out systematically where in first DNA extraction protocol was standardized followed by optimization of PCR. Using optimized protocols, screening of 165 clinical specimens from mycoplasmosis suspected birds was carried out employing genus specific PCR-RFLP, followed by the confirmation of positive samples by multiplex PCR assay and sequencing of unidentified PCR products. Phenol chloroform isoamyl method was found superior to rapid heat freeze method for extraction of better quality and quantity of DNA. Genus specific *16S rRNA* based PCR-RFLP was found sensitive and convenient method for screening large number of clinical specimens for detection of mycoplasma infection.

**Key words:** Avian mycoplasmosis, *Mycoplasma gallinarum*, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, PCR, RFLP

The United States Department of Agriculture (USDA) and Organization for Economic Co-operation and Development (OECD) indicated that both India's and global per capita consumption of poultry meat is growing and chicken is India's preferred non-vegetarian protein source (Augustine and Shukla 2015). However, one of the significant reasons for decreased production and increase in mortality of chickens is bacterial infections, which mainly affect the respiratory system of the birds. Among the bacteria infecting respiratory system; *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are the most important etiologies in causing economic losses (Gharibi *et al* 2018). Therefore, rapid and early diagnosis of Mycoplasmosis is important in order to prevent spread of disease and to limit economic losses.

The amplification of genus and species specific genes by PCR, PCR-RFLP (for differential diagnosis) and multiplex PCR are used for rapid and accurate detection of *Mycoplasma* infection in poultry (Kempf *et al.* 1993, Tettlin *et al.* 1999). Although many protocols of PCR are set with the published primers; they do not always give *vis-à-vis* results in different existing conditions. Therefore, before implementing the molecular techniques for diagnosis; the in-house optimization of laboratory protocols is essential

to arrive to accurate diagnosis of disease. Taking into account the importance of PCR in diagnosis, the present research dealt with rapid, sensitive and specific diagnosis of poultry mycoplasmosis using optimized protocols of PCR, whose conventional diagnosis is difficult and often unsuccessful.

### MATERIALS AND METHODS

**Collection of clinical specimens:** Proper collection of specimens, transportation and accurate processing are the preliminary critical steps in proper diagnosis (WOAH 2008). The specimen were collected using sterile swabs dipped in pleuropneumonia like organism medium (HiMedia Laboratories, India), from palatine cleft of live birds exhibiting clinical signs of mycoplasmosis. A total of 165 choanal cleft swabs collected in sterile tubes were transported to the laboratory on ice and processed within 24–48 h.

**Extraction and quantification of DNA:** DNA from clinical specimens and cultures was extracted by rapid heat-freeze method as per the protocol described by WOA (2008). Whereas, certain modifications were done in published protocols of phenol chloroform isoamyl (P:C:I) method to get more concentration and good quality of extracted DNA. Briefly, the choanal cleft swabs collected were dipped in lysis buffer and placed at 4°C overnight (Nhu Van Thu *et al.* 2003) followed by incubation in water bath at 70°C for 2 h. In the next step, due to uncertain results

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of using only equilibrated phenol; this step was modified by adding equal volume of phenol: chloroform (1:1) followed by treatment with chloroform: isoamyl alcohol (24:1). The DNA was then treated with sodium acetate and absolute ethanol and kept at  $-20^{\circ}\text{C}$  for 2 h instead of keeping it overnight. The samples were then centrifuged and washed with 70% ethanol and air dried. The pellets were dissolved in 50  $\mu\text{l}$  distilled water and kept for 15 min for proper dissolution of DNA. The sample was then vortexed, spun and stored at  $-20^{\circ}\text{C}$  until further use. The same modified protocol of P:C:I was applied for extraction of DNA from cultures. The results were finally analyzed for the quality and purity of the extracted DNA using Nanodrop (ND 2000, Thermo Scientific, USA). The integrity and concentration of the DNA was checked by agarose gel electrophoresis and using gel documentation system (Gel Doc EZ Imager, Bio-Rad). The comparative evaluation of rapid heat-freeze and P:C:I method was carried out based on quality, purity and concentration of the DNA obtained by both the methods.

**Genus specific PCR-RFLP for *Mycoplasma* spp.:** The amplification of *16S rRNA* gene containing variable regions for detection of *Mycoplasma* genus was carried out using published oligonucleotide primer sequences (Van Kuppeveld *et al.* 1992), Myco F (GPO-1): 5'-ACT-CCT-ACG-GGA-GGC-AGC-AGT-A-3'; Myco R (MGSO) 5'-TGC-ACC-ATC-TGT-CAC-TCT-GTT-AAC-CTC-3' (Sigma-Aldrich). Optimization of PCR assay in order to generate best amplification product was carried out by following the standard procedure of Sambrook and Russell (2008). Briefly, this was accomplished by testing different combinations of annealing temperatures selected based on melting temperature of the primers, annealing time, number of cycles, template DNA concentration and variation in different quantities of PCR mixture. The PCR was set in 25  $\mu\text{l}$  volume, consisting of 0.6  $\mu\text{l}$  DNA template, 2.5  $\mu\text{l}$  PCR buffer, 2  $\mu\text{l}$   $\text{MgCl}_2$ , 0.8  $\mu\text{l}$  dNTPs, 0.5  $\mu\text{l}$  forward and reverse primers each, 0.4  $\mu\text{l}$  taq polymerase and 17.7  $\mu\text{l}$  distilled water. The cycling conditions used were initial denaturation @  $95^{\circ}\text{C}$  for 5 min, 30 cycles of denaturation @  $95^{\circ}\text{C}$  for 1 min, annealing @  $54^{\circ}\text{C}$  for 1 min., extension @  $72^{\circ}\text{C}$  for 1 min, final extension @  $72^{\circ}\text{C}$  for 5 min and hold @  $4^{\circ}\text{C}$ . The amplified products of genus specific *16S rRNA* PCR assays were evaluated by agarose gel (2%) electrophoresis, stained with 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide (Bangalore Genei, India).

The PCR products (~710 bp) obtained from genus specific PCR were further subjected to RE digestion using *Hae* III enzyme (Fermentas, USA). The reaction was set in a final volume of 30  $\mu\text{l}$  consisting of 10  $\mu\text{l}$  PCR product, 2.0  $\mu\text{l}$  buffer, 1.0  $\mu\text{l}$  enzyme and 17  $\mu\text{l}$  distilled water. The contents were mixed well and kept at  $37^{\circ}\text{C}$  for 2 h in a water bath. The restriction enzyme selected using NEBcutter digested the PCR products at a specific site to split them into fragments of different sizes, specific for species under genus mycoplasma.

**Multiplex PCR assay for *M. gallisepticum* and *M. synoviae*:** The amplification of *16S rRNA* gene for detection

of *M. gallisepticum* and *M. synoviae* at species level was carried out using MG-13R 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3'; MG-14 F 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3' and MS-F 5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3'; MS-R 5'-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3' primers (WOAH 2008) manufactured and supplied by Sigma (India). Optimization of PCR assay was carried out in order to generate best amplification product (Sambrook and Russell 2008). The standardization for annealing time, number of cycles, template DNA concentration and PCR mixture quantities was done.

The PCR was set in a volume of 50  $\mu\text{l}$  consisting of 1  $\mu\text{l}$  DNA template, 5.0  $\mu\text{l}$  PCR buffer, 5.0  $\mu\text{l}$   $\text{MgCl}_2$ , 1.6  $\mu\text{l}$  dNTPs, 1.2  $\mu\text{l}$  MG primers (F and R each), 1.2  $\mu\text{l}$  MS primers (F and R each), 0.8  $\mu\text{l}$  Taq polymerase and 31.8  $\mu\text{l}$  distilled water. The reaction mixtures prepared were spun quickly and set into thermal cycler (mastercycler nexus gradient, eppendorf). The cycling conditions used were hot start @  $94^{\circ}\text{C}$  for 5 min (Add Taq polymerase), 38 cycles of denaturation @  $94^{\circ}\text{C}$  for 30 sec, annealing @  $52^{\circ}\text{C}$  for 30 sec, extension @  $72^{\circ}\text{C}$  for 45 sec, final extension @  $72^{\circ}\text{C}$  for 5 min and hold @  $4^{\circ}\text{C}$ . The amplified products of species specific *16S rRNA* PCR assays were differentiated into MG and MS based on the amplicon sizes in agarose gel electrophoresis.

**Gene-targeted sequencing (GTS) of *Mycoplasma* spp.:** The sequencing of PCR products of ~710 bp region of *16S rRNA* gene of *Mycoplasma* spp. was carried out with ABI Big Dye Terminator Kit version 3.1 using automated genetic analyser at Aavanira Biotech Pvt. Ltd., India. The chromatogram were visualized with finch TV application and forward and reverse sequences assembled manually. The sequences were subjected to BLAST analysis with GenBank using BLASTn algorithm. One of the sequence was submitted to NCBI GenBank database through online submission system Bankit and accession number was obtained.

## RESULTS AND DISCUSSION

**Extraction and quantification of DNA:** The isolates and reference strains [*Mycoplasma gallisepticum* (ATCC® 19610™) and *Mycoplasma synoviae* (ATCC® 25204™)] processed by rapid heat-freeze technique yielded lesser concentration of DNA with more impurities as compared to phenol/ chloroform method (Table 1). The overall concentration, quality and purity of DNA extracted by P:C:I was better than by heat freeze method; therefore P:C:I method was used for extraction of DNA from choanal swabs for performing further PCR.

Silveria *et al.* (1996) observed similar results in a comparative experiment of a non-phenolic (rapid heat freeze) and a phenolic method (P:C:I) of DNA extraction. The phenolic method yielded more concentration and better purity of DNA as compared to the non-phenolic method. Few of the other researchers also followed only Phenol/ chloroform method for DNA extraction as seen in the present

Table 1. Comparative values of DNA extracted by rapid heat freeze and P:C:I method

| Isolate code | Rapid heat freeze |                     | P:C:I       |                     |
|--------------|-------------------|---------------------|-------------|---------------------|
|              | Conc ng/μl        | Purity (OD 260/280) | Conc. ng/μl | Purity (OD 260/280) |
| MG-1         | 76.4              | 1.63                | 283.1       | 1.72                |
| MG-2         | 155.7             | 1.16                | 566.8       | 1.76                |
| MG-3         | 286.3             | 1.20                | 485.0       | 1.84                |
| MG-4         | 114.6             | 1.63                | 478.8       | 1.88                |
| MG-5         | 235.1             | 1.33                | 696.6       | 1.90                |
| MG-6         | 93.8              | 1.40                | 249.6       | 1.77                |
| MG-7         | 84.2              | 1.97                | 432.7       | 1.86                |
| MG-8         | 384.5             | 1.70                | 1074.4      | 1.87                |
| MG-9         | 1074.8            | 1.51                | 1315.1      | 1.73                |
| MG-10        | 103.6             | 1.32                | 259.4       | 1.82                |
| MG ATCC      | 65.3              | 1.72                | 283.0       | 1.96                |
| MS ATCC      | 386.9             | 1.76                | 733.4       | 1.81                |

study. The bacterial DNA extracted by phenol/chloroform method was used in the PCR assay to amplify the conserved region of *16S rRNA* gene for detection of *Mycoplasma* spp. in clinical samples (Buim *et al.* 2009, Jamshidi *et al.* 2014). The similar findings of P:C:I method being better was observed by Psifidi *et al.* (2015) in a comparative analysis of 11 different techniques of DNA extraction. Moreover, P:C:I protocol aided in extraction of DNA in the purest form, followed by rapid heat freeze method. However, being rapid heat freeze method, less time consuming and laborious and not requiring use of any elaborate reagents as compared to P:C:I; therefore this method is also recommended (WOAH 2008, Buim *et al.* 2009).

**Genus specific PCR-RFLP for *Mycoplasma* spp.:** Genus specific PCR targeting *16S rRNA* gene was optimized using the reference strains of MG and MS as positive controls. Initially a gradient PCR was employed with different annealing temperatures to obtain the best amplification at ~710 bp, as visualized by agarose gel electrophoresis. The DNA extracted from a total of 165 clinical specimens and reference strains were subjected to PCR for *Mycoplasma* spp. using the optimized conditions. A product of ~710 bp could be amplified from all the reference strains and isolates.

Out of 165 clinical samples processed, 25 samples (15.15%) were positive through genus specific PCR. The PCR showed high sensitivity and specificity for the samples with the concentration ranging 50–100 ng/μl of DNA.

The amplified genus specific PCR products from clinical specimens and reference strains of MG and MS were then digested with restriction enzyme *HaeIII*. The enzyme split *M. gallisepticum* amplicon into two fragments of ~500 bp and 200 bp, whereas *M. synoviae* amplicon was split in two fragments of ~650 bp and 50 bp. Out of the 25 samples positive in genus specific PCR, 16 (64%) showed a restriction pattern specific for MG, while 7 (28%) exhibited a pattern for MS. Whereas, the two undigested samples in RFLP indicated an infection of mycoplasma other than from MG and MS species. Thus, the overall incidence of avian mycoplasmosis observed was 15.15%, *M. gallisepticum* (9.69%) being more prevalent than *M. synoviae* (4.24%) in the study area.

PCR is the most sensitive and reliable tool for the diagnosis of avian mycoplasmosis in field samples (Muhammad *et al.* 2018). In the present study, the genus-specific primers were found to react with mycoplasmal species. Genus specific PCR-RFLP showed good sensitivity and specificity towards a variety of mycoplasmal species and differentiation at species level (Van Kuppeveld *et al.* 1992). Fan *et al.* (1995) also observed similar results after using PCR and RFLP to detect and differentiate four pathogenic avian *Mycoplasma* species and 10 non-pathogenic species of avian mycoplasmas. Kiss *et al.* (1997) also attempted PCR with primers targeting *16S rRNA* genes, to detect avian mycoplasmas. After screening the respective reference strains, it was found that *Mycoplasma* could be detected by PCR and distinction could be made among the species by digestion of the product with restriction enzymes. Kempf (1998) described that most of the avian *Mycoplasma* species can be differentiated, according to their unique RFLP patterns generated with different restriction endonucleases. In a similar study, Behbahan *et al.* (2005) showed that the primer sequences selected on the genus level successfully amplified 780 bp fragment of the target DNA and restriction digestion detected 55 samples as *M. gallisepticum* and *M. synoviae*. In another study, Bagheri

Table 2. Genus PCR-RFLP and multiplex PCR analysis of clinical specimens

| Source of samples collected | No. of samples processed | Genus PCR MG+MS+Others | No. of samples positive |    |               |    |
|-----------------------------|--------------------------|------------------------|-------------------------|----|---------------|----|
|                             |                          |                        | RFLP                    |    | Multiplex PCR |    |
|                             |                          |                        | MG                      | MS | MG            | MS |
| Kolkata                     | 37                       | 05                     | 03                      | 02 | 03            | 02 |
| Raigarh (Pen)               | 14                       | 03                     | 03                      | 00 | 03            | 00 |
| Raigarh (Karjat)            | 26                       | 00                     | 00                      | 00 | 00            | 00 |
|                             | 14                       | 03                     | 02                      | 01 | 02            | 01 |
| Delhi                       | 20                       | 05                     | 03                      | 02 | 03            | 02 |
| Pune (Lohagaon)             | 24                       | 07                     | 05                      | 00 | 05            | 00 |
| Nashik                      | 10                       | 00                     | 00                      | 00 | 00            | 00 |
|                             | 20                       | 02                     | 00                      | 02 | 00            | 02 |
| Total                       | 165                      | 25                     | 16                      | 07 | 16            | 07 |

*et al.* (2011) amplified *16S rRNA* region by PCR using specific primers for *Mycoplasma* spp.

**Multiplex PCR for *M. gallisepticum* and *M. synoviae*:** The optimization of multiplex PCR was carried out using standard strains of MG and MS. The PCR product for MG was amplified at ~185 bp and MS was amplified at ~207 bp. Out of 25 samples subjected to multiplex PCR, 16 (64%) were positive for MG and 7 (28%) were positive for MS. The samples which were not digested by RE in RFLP, also showed negative results in multiplex, indicating those being negative for MG or MS. Thus, the results of multiplex were correlated with genus PCR-RFLP (Table 2).

The sequencing of genus specific PCR products was done which did not digest in RFLP and could not be identified at species level by multiplex PCR. An accession number MF 180236 was obtained from NCBI for sequence submitted. BLAST analysis revealed that the sequences matched to *16S rRNA* gene of *Mycoplasma gallinarum* and showed 99% identity with published *M. gallinarum* strains of accession nos. MF 196180, JN935884, NR113687, FJ666137 and MF 196176.

Multiplex PCR is a technique used for genetic screening, disease screening and other applications where it is necessary to amplify several products in a single reaction. Although this technique often requires extensive optimization (Loffert *et al.* 1998), this is a pooling strategy to minimize the number of laboratory procedures (Tettlin *et al.* 1999). Pang *et al.* (2001) developed a multiplex PCR and optimized it to simultaneously detect six avian respiratory pathogens including MG and MS. The assay was able to detect and differentiate co-infections with two or more pathogens.

Species specific multiplex PCR targeting *16S rRNA* was carried out on 25 samples, 16 (64%) and 7 (28%) samples were found positive for MG and MS infections respectively. Thus, in multiplex PCR, *M. gallisepticum* and *M. synoviae* could be detected simultaneously with good specificity and sensitivity. A similar study was carried out by Behbahan *et al.* (2005) who extracted *Mycoplasma* from tracheal and air sac samples of birds suspected with mycoplasmosis. Feberwee *et al.* (2005) compared the detection of avian *Mycoplasma* by two commercially available polymerase chain reaction (PCR) tests, i.e. multiplex PCR and simplex PCR. Considering the single amplification tests as the gold standard, the Multiplex MG-MS showed 100% specificity and sensitivity. Lierz and Hafez (2008) performed species-specific polymerase chain reactions for the detection of poultry pathogens, viz. *M. gallisepticum*, *M. imitans*, *M. iowae*, *M. meleagridis* and *M. synoviae*. Multiplex PCR was also attempted by Bium *et al.* (2009) who screened 33 samples from commercial poultry farms, demonstrating a higher presence of the major avian mycoplasmal strains; *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). Whereas, Fraga *et al.* (2012) optimized multiplex MG-MS PCR with vaccine strain for MG (Myco-Galli®, strain MG-70, Biovet) and MS (Mycovax®, strain MS-H, Merial). The specificity of the Multiplex MG-MS was

verified by testing DNA extracts from different Mollicutes, including *M. gallisepticum* and *M. synoviae*. No other *Mycoplasma* species was detected by Multiplex MG-MS. Khaldia *et al.* (2013) used conventional PCR technique to amplify 140 bp and 720 bp DNA fragments specific for MG and MS, respectively. The study showed accuracy and sensitivity for both MG and MS as observed in present research. Mettifogo *et al.* (2015) performed the generic PCR for detection of many species of Mollicutes Class. The system (multiplex PCR) was demonstrated to be very rapid, sensitive and specific. However, the standardization of this technique is tedious and was not found as robust as genus PCR-RFLP.

It is concluded that, before implementing the molecular techniques for diagnosis of poultry mycoplasmosis; the in-house optimization is essential to get the accurate results. Phenol chloroform isoamyl method is superior to rapid heat freeze method for extraction of better quality and quantity of DNA. Genus specific *16S rRNA* based PCR-RFLP is sensitive and convenient for screening the large number of clinical specimens for mycoplasma infection.

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