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 isomayl 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 mycoplasmosis infection.

Key words: Avian mycoplasmosis, Mycoplasma gallinarae, 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 (Kempt et al. 1993, Tettelin 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 (HiMedia Laboratories, India), from palatine cleft of live birds exhibiting clinical signs of mycoplasmosis. A total of 165 chonoal 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 WOAH (2008). Whereas, certain modifications were done in published protocols of phenol chloroform isomayl (P.C.I) method to get more concentration and good quality of extracted DNA. Briefly, the chonoal cleft swabs 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
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°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 μl distilled water and kept for 15 min for proper dissolution of DNA. The sample was then vortexed, spun and stored at −20°C until further use. The same modified protocol of P:C:1 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:1 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-ACC-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 μl volume, consisting of 0.6 μl DNA template, 2.3 μl PCR buffer, 2 μl MgCl₂, 0.8 μl dNTPs, 0.5 μl forward and reverse primers each, 0.4 μl Taq polymerase and 17.7 μl distilled water. The cycling conditions used were initial denaturation @ 95°C for 5 min, 30 cycles of denaturation @ 95°C for 1 min, annealing @ 54°C for 1 min., extension @ 72°C for 1 min, final extension @ 72°C for 5 min and hold @ 4°C. The amplified products of species specific 16S rRNA PCR assays were differentiated into M. gallisepticum and M. synoviae at species level was carried out using MG-13R 5’-GCT-TCC-TTG-CCG-TTA-GCA-AC-3’; MG-14 F 5’-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3’ and MS–F 5’-GAG-AA-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 μl consisting of 1 μl DNA template, 5.0 μl PCR buffer, 5.0 μl MgCl₂, 1.6 μl dNTPs, 1.2 μl MG primers (F and R each), 1.2 μl MS primers (F and R each), 0.8 μl Taq polymerase and 31.8 μl distilled water. The reaction mixtures prepared were spun quickly and set into thermal cycler (mastercycler nexus gradient, ependorf). The cycling conditions used were hot start @ 94°C for 5 min (Add Taq polymerase), 38 cycles of denaturation @ 94°C for 30 sec, annealing @ 52°C for 30 sec, extension @ 72°C for 45 sec, final extension @ 72°C for 5 min and hold @ 4°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 Aavani 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:1 was better than by heat freeze method; therefore P:C:1 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:1) 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:1 method

| Isolate code | Rapid heat freeze | P:C:1 |
|--------------|-------------------|-------|
|              | Conc. ng/μl (OD 260/280) | Purity | Conc. ng/μl (OD 260/280) | Purity |
| 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   |

In another study, Bagheri (2003) processed MG+MS+Other processed MG-8 384.5 1.70 1074.4 1.88 mg and MG-5 235.1 1.33 696.6 1.90 mg using the optimized conditions. A product of ~710 bp annealing G P:C:I; therefore this method is also recommended. In follow b P:C:I protocol aided in extraction of DNA in the purest form, ot requiring use of any elaborate reagents as compared to

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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           |
| Raigarh (Pen)              | 14                       | 03                     | 03                      | 00           |
| Raigarh (Karjat)           | 26                       | 00                     | 00                      | 00           |
| Delhi                      | 14                       | 03                     | 02                      | 01           |
| Pune (Lohagaon)            | 24                       | 07                     | 05                      | 00           |
| Nashik                     | 10                       | 00                     | 00                      | 00           |
| Total                      | 165                      | 25                     | 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 (Tettelin 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 Blum 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 (Mycogal®), strain MG-70, Biovet) and MS (Myovax®, 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.

Khalda 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 mycoplasmic infection.

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