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Isolation and characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* from poultry

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Avian mycoplasmosis caused by *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) is a frequently reported infectious disease in poultry. The flock screening is done by serological assays and the confirmation can be done by cultivation or PCR. *Mycoplasma* infections are usually diagnosed by serological procedures that are sometimes hampered by interspecies cross-reactions and nonspecific reactions (Hagan *et al.* 2004).

Several workers have described use of PCR (Nascimento *et al.* 1991) and nucleic acid probes (Khan and Kleven 1993) for detection of mycoplasma in culture or clinical samples. The technique has been proven to be a very specific and sensitive method for amplifying low amounts of nucleic acid to a level that cannot be easily detected by other methods.

The study was undertaken to isolate MG and MS in culture using specific media from samples collected from different poultry farms, and to characterize MG and MS by polymerase chain reaction using MG- and MS-specific primers and DNA sequence analysis.

Mycoplasma gallisepticum PG31 and *M. synoviae* WVC 1853 were obtained from Dr. Janet M. Bradbury, University of Liverpool, Department of Veterinary Pathology, Lehurst, Neston CH647TE, UK, for using as reference strains. Samples were collected from commercial and government poultry farms in different parts of Tamil Nadu. In live birds, nasal secretions were obtained and from dead birds, samples of trachea, lungs and air sac were obtained for culture. Samples were immediately inoculated into tubes containing 5 ml Frey's broth medium. *M. gallisepticum* reference cultures were grown in Frey's broth media (DIFCO) with phenol red indicator, supplemented with 0.15 mg of thallium acetate, 1000 IU penicillin-G/ml, 0.3% dextrose and 1.5% swine serum. *M. synoviae* cultures were grown in similarly supplemented Frey's broth media with the additional supplementation of 0.1 mg NAD/ml, 0.0125% cysteine hydrochloride. The cultures were incubated at 37°C with 90% relative humidity until the phenol red indicator changed from red to yellow (5–7 days). Cultures were plated onto Frey's agar medium when the colour changed and all samples were tested for the presence of MG and MS at the same time by PCR. Broth cultures in 50 µl of Frey's medium suspended in 500 µl of PBS were centrifuged at 10 000 rpm for 10 min. The supernatant was removed and pellet was suspended in 20 µl of sterile water, boiled for 5 min and then chilled for 10 min on ice before being processed for PCR. The processed sample (2 µl) was used for PCR (Kiss *et al.* 1997).

For the detection of MG, a species-specific primer pair (Kiss *et al.* 1997) was used. The sequences of the forward primer was 5'-AAC ACC AGA GGC GAA GGC GAG G–3' and the reverse primer was 5'-ACG GAT TTG CAA CTG TTT GTA TTG G–3'. The forward and reverse primers for MS were 5'-GAA GCA AAA TAG TGA TAT CA–3' and 5'– GTC GTC TCC GAA GTT AAC AA –3', respectively (Lauerman *et al.* 1993).

PCR procedure

PCR for MG: The PCR was carried out in 50 µl volumes. Each reaction mixture contained 10×PCR buffer (500 mM KCl, 200 mM Tris HCl, pH 8.4, 10 mM of dNTPs, 35 pmol/µl each of MG-1 and MG-2 primers, 5U Taq DNA polymerase and 2 µl of the sample. The reaction mixtures were adjusted to the total volume by adding distilled water. All DNA amplifications were performed in a thermal cycler that was programmed to heat the DNA at 94°C for 5 min, followed by 35 cycles at 3 different temperature segments, (94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec) that corresponded to target DNA denaturation, primer annealing and primer extension, respectively. The final extension step was 72°C for 10 min (Kiss *et al.* 1997).

PCR for MS: The PCR mix was made for 50 µl total volume sample by adding 10×PCR buffer, 10 mM dNTPs, 20 pmoles each of MS-1 and MS-2 primers, 5U Taq polymerase. PCR mix (48 µl) was dispensed into a 500 µl microcentrifuge tube and 2 µl of sample was added. The PCR was performed in a thermal cycler using the following

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temperatures for the respective number of cycles: 94°C for 5 min for 1 cycle; followed by 94°C for 1 min, 50°C for 1 min and 72°C for 2 min for 35 cycles and final extension at 72°C for 7 min. The PCR product was held at 4°C until processed. The amplified DNA product was visualized by electrophoresing 10 µl of the PCR product mixed with 3 µl tracing dye in a 2% agarose gel with ethidium bromide (0.5 µg/ml) and DNA bands were observed using UV transilluminator (Lauerman *et al.* 1993).

Nucleotide sequence analysis

The 530 bp products of MG and 207 bp product of MS were purified by kit. Purified PCR products were sequenced by fluorescent – labelled dideoxynucleotides in an automated nucleic acid sequencer. Sequence data were compared with other sequences from Gene Bank by BLAST analysis.

This study showed that cultivable *M. gallisepticum* and *M. synoviae* can be detected in a naturally infected poultry

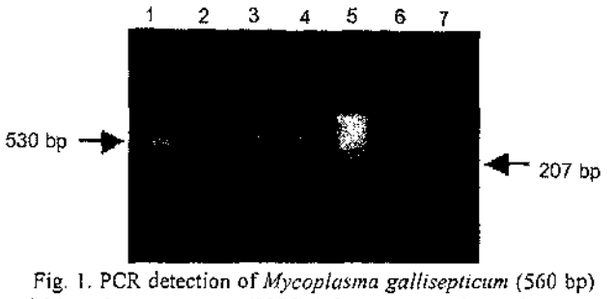

Fig. 1. PCR detection of *Mycoplasma gallisepticum* (560 bp) and *Mycoplasma synoviae* (207 bp) from suspected samples.

219 air sacs tested by PCR. Similarly lung samples also yielded less positive reaction. Tracheal samples gave more isolation of both MG and MS, when compared to other samples.

The higher sensitivity of PCR than culture found in this study agrees with the results of other workers thus, demonstrating the capacity of PCR to detect MG and MS infections before culture results are obtained (Lauerman *et al.* 1993). Mycoplasmas are fastidious organism with unique medium requirements. It requires 4 to 5 days for growth and initial cultures commonly contain other contaminants also (Kleven and Yoder 1989). In the present study, PCR provided rapid diagnosis and identification of organisms when it was performed on broth or colonise from agar media inoculated in the traditional manner. Results from this study indicated that species-specific PCR assay could be used for confirmation of *M. gallisepticum* and *M. synoviae* infections from field samples following brief culture in Frey's medium.

The nucleotide sequences of the PCR products from 16S rRNA gene of the isolates were obtained. The BLAST analysis showed that the homology ranged from 96–99.6% among MG strains. The isolate TNCG225 showed high homology with the strain R(AEO16969.1). The nucleotide sequence comparison with the A5969 (L35043.3) resulted in homology ranging from 96.0% to 99.6%. The isolates TNNG876, TNTG811 and TNCG225 showed 99.6, 97.9 and 96.6% homology with the strain R (M22441.1) respectively. Nucleotide homology data indicated that all the MS isolates, TNCS427, TNNS815, TNTS880 showed 100% homology

Table 1. Comparison of PCR and culture for identification of MG and MS isolates from 4 types of samples from poultry

	Trachea	Air sac	Lungs	Nasal swab	Total
Mycoplasma synoviae					
Total no. of samples tested	636	219	158	26	1,039
Samples positive by PCR	49	9	5	4	67
Samples positive by culture	21	2	–	1	25
Mycoplasma gallisepticum					
Total no. of samples tested	636	219	158	26	1,039
Samples positive by PCR	29	3	4	–	36
Samples positive by culture	10	1	–	–	11

by culture and by use of MG-PCR and MS-PCR, respectively. Of 138 samples tested using the MG- and MS-PCR, 36 samples were positive for MG, and 67 samples were positive for MS infection. A typical PCR banding pattern is given in Fig. 1, showing 530 bp amplicon for MG and 207 bp amplicon for MS. The specificity of PCR was checked with positive control MG and MS reference cultures. MG and MS isolations were achieved in 11 and 28 of the 1 039 samples tested, respectively (Table 1). Of 636 tracheal samples tested by PCR, 49 were positive of MG and 29 to MS, whereas only 9 samples were positive for MG and 3 for MS out of

with *Mycoplasma synoviae* WVU1853 strain.

Results from this study indicated that mycoplasma infection is widespread in poultry farms in Tamil Nadu, and PCR method could be used for rapid and sensitive detection of avian mycoplasmosis. Among different samples collected for diagnosis, trachea appears to be the best sample for diagnosis.

SUMMARY

Avian mycoplasma infection is one of the major health problems facing the poultry industry. Prevalence of

Mycoplasma gallisepticum and *Mycoplasma synoviae* among chickens in different parts of Tamil Nadu, were studied by isolation and by performing polymerase chain reaction (PCR) for amplification of a specific products of 530 bp and 207 bp regions from rRNA of MG and MS, respectively. Among 1039 samples analysed by PCR, 36 samples were positive for MG and 67 were positive for MS infections. A total of 26 and 47 culture negative samples were PCR positive for MG and MS respectively. The amplified products were subjected to nucleotide sequencing and the obtained sequences were compared with those of standard strains. Nucleotide homology data indicated that all the MS isolates showed 100% homology with *Mycoplasma synoviae* 16rRNA strain from Australia and MG isolates showed 96–99% homology with that of reference strains. Results indicated that majority of the isolates are very virulent, which is evident from heavy mortality that have been reported in poultry farms.

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