Differential Diagnosis of Avian Mycoplasmosis*

K. Shrine Nagalakshmi, T.M.A. Senthilkumar¹, M. Parthiban and P. Ramadass

Department of Animal Biotechnology, Madras Veterinary College, Chennai – 600 007

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Chronic respiratory disease (CRD) affecting poultry may be caused by multiple etiology including *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Escherichia coli*, *Pasteurella multocida*, *Haemophilus paragallinarum* and viral pathogens like Newcastle disease virus (NDV) and infectious Bronchitis Virus (IBV). Though severe infection is caused by MG and MS, mixed infections involving respiratory viral pathogens are commonly recognized in chicken and turkey flocks. Isolation and identification of mycoplasmas based on culture, biochemical and serological assays and other etiological agents take substantially long time for diagnosis. Recently, polymerase chain reaction (PCR) method (Slavik, et al., 1993; Pang, et al., 2002) and DNA probes (Hyman, et al., 1989) have been adapted for rapid detection of these respiratory pathogens of poultry. Multiplex PCR was also used for detection of avian respiratory pathogens by Wang, et al. (1997). In the present study, PCR assay was used for detection of these respira-

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¹Corresponding author : Email : tmaskumar@yahoo.com

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Fig 1. PCR amplification of the 16S rRNA gene of *Mycoplasma synoviae*. Lanes 1, 5 & 6: Positive samples Lanes 2, 3, 4 & 7: Negative samples Lane 8: DNA molecular weight marker (100 bp)

Fig 2. PCR amplification of the *Haemophilus paragallinarum*. Lane 1: DNA molecular weight marker (100 bp) Lanes 2 & 4: Positive samples Lanes 3 & 5: Negative samples

...atory pathogens from poultry tissues and swab samples and the results were compared with the cultural and biochemical methods of identification.

**Materials and Methods**

PPLO Mycoplasma broth (Frey’s medium, DIFCO) was used with penicillin and thallium acetate for the isolation of MG. For isolation of MS from tissue samples, L-Cysteine hydrochloride, 0.1% and β-Nicotinamide adenine dinucleotide (NAD), 0.1% were added as supplements into Frey’s medium with antibiotics. Brain Heart Infusion Broth (BHI) was used for isolation of *Haemophilus paragallinarum*. *E. coli* isolation was carried out with Nutrient broth and Eosin Methylene Blue agar (EMB). *E. coli* isolates were confirmed by growing them in Triple sugar iron agar (TSI) and further confirmed by Simon’s citrate agar, MR-VP and Indole’s test. *Pasteurella multocida* was isolated by inoculating the samples into Brain Heart Infusion Broth, Dextrose Starch Agar, and Mac Conkey agar. They were confirmed by TSI agar slants and carbohydrate fermentation tests.

282 trachea and air sac samples were collected from different poultry farms in Tamil Nadu. The samples collected were cut into small pieces and inoculated into Frey’s media, BHI broth and Nutrient Broth cultures. For identification and characterization of virus etiology samples were collected in glycerol saline.

PCR was carried out as described by Kiss et al. (1997) for *Mycoplasma gallisepticum* using the primers MG-F 5’ AAC ACC AGA GGC GAA GGC GAG G-3’ and MG-R 5’ ACG GAT TTG CAA CTG TTT GTA TTG G 3’ targeting 16S rRNA gene. Reaction consisted of 5 min of initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 30 sec annealing at 55°C for 30 sec and extension at 72°C for 30 sec with final extension at 72°C for 10 min.

For *Mycoplasma synoviae*, PCR described by Lauerman et al. (1993) was carried out using the primers MS-F 5’ GAA GCA AAA TAG TGA TAT CA-3’ and MS-R 5’ GTC TGC TCC GAA GAC AAC AAC AA-3 targeting 16S rRNA gene. PCR Reaction of 5 min of initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 1 min; annealing at 50°C for 1 min and extension at 72°C for 2 min with final extension at 72°C for 10 min was carried out.

For *Haemophilus paragallinarum* PCR was done as per Chen et al. (1996) using the primers N1-F 5’ TGA GGG TAG TCT TGC ACG CGA AT -3’ and R1-R5’ CAA GGT ATC GAT CGT CTC TCT ACT-3’. PCR cycle consisted of 5 min of initial denaturation at 94°C followed by 25 cycles
of denaturation at 94°C for 1 min annealing at 65°C for 1 min and extension at 72°C for 2 min with final extension at 72°C for 10 min.

Total RNA extracted by Trizol reagent (Invitrogen) from the samples was subjected to reverse transcriptase-PCR for IBV as described by Kwon et al. (1993) using the primers F 5’ CAT AAC TAA CAT AAG GGC A-3’ and R 5’ TGA AAA CTG AAC AAA AGA CA-3’. cDNA synthesis was carried out a 47°C for 30 min and 5 min of initial denaturation at 94oC followed by 35 cycles of denaturation at 94°C for 1 min; annealing at 50°C for 1 min and extension at 72°C for 2 min with final extension at 72°C for 10 min.

The extracted RNA samples were screened for NDV using RT-PCR as described by Stauber et al. (1995) with the primers F 5’ GGA GGA TGT TGG CAG CAT T-3’ and R 5’ GTC AAC ATA TAC ACC TCA TC-3’ spanning the cleavage site of F0 fusion protein coding sequence. cDNA synthesis was carried out as earlier and followed by 35 cycles of denaturation at 94°C for 1 min; annealing at 60°C for 3 min and extension at 60°C for 2 min with final extension at 72°C for 10 min. The PCR amplicons were analysed on 1.5% agarose gel.

**Results and Discussion**

Totally, 184 samples were found positive for *E.coli* out of 282 samples tested (65%) by culture inoculation. The colonies of *E.coli* yielded classical metallic sheen on the EMB plate. They were further characterized by biochemical tests using IMVIC tests and growth on Triple sugar iron agar (Triple sugar iron +ve, Simmon’s citrate agar –ve, Indole test +ve, Methyl red test +ve and Voges-Proskauer –ve).

Out of 282 samples tested, 169 samples were obtained in pure culture of *Pasteurella multocida*. They were further characterized by biochemical tests like triple sugar iron agar test, indole production test and carbohydrate fermentation tests (Triple sugar iron +ve, Sucrose +ve, Sorbitol –ve, Arabinose +ve, Mannose+ve, Maltose—ve, Inulin –ve, inositol –ve, Fructose +ve and Indole +ve).

All the samples tested were negative for *Mycoplasma gallisepticum*. Ninety-five samples out of 282 samples yielded positive results for *Mycoplasma synoviae* (33.7%), giving 207 bp amplicon. Three samples out of 282 samples tested were found positive for *Haemophilus paragallinarum* by PCR. Of 100 samples tested by RT-PCR for NDV, seven samples were positive. All the samples were found negative for IBV.

Florentin et al. (2003) used PCR for detection of MS and concluded that PCR was most sensitive test for early detection of MS infection. MG infection could not be detected in this study. In recent years, the parent stocks are being vaccinated against MG. This could be the reason for low incidence of MG infection in the field and the inability to detect positive infection in the study.

Mixed infections of NDV, IBV, ILTV, AIV along with MG and MS had been reported by Cavanaugh et al. (1997). Similar infections had also been reported by Georgiades et al. (2001). None of the hundred samples tested were found to be positive for IBV. Though, in this study...
IBV infections were not detected few workers reported the incidence of Mycoplasma infection along with IBV infection. (Pang et al., loc. cit; Landman and Feberwee, 2004).

Summary

In the present study, differential diagnosis of avian mycoplasmosis was conducted with 282 samples collected from different poultry farms in Tamil Nadu. The samples were cultured in Frey's Mycoplasma broth and agar. When MG- and MS-specific primers were used for the detection of Mycoplasma infections, 95 samples showed positive results for MS (33.7%) and none of the samples were positive for MG by PCR assay. For differential diagnosis of respiratory infections, isolations with selective media were attempted for organisms such as *E. coli*, *Pasteurella multocida* and *Haemophilus paragallinarum*. PCR assay was used for detection of *Haemophilus paragallinarum* as well as Newcastle Disease Virus and infectious Bronchitis Virus.

Pure cultures of *E. coli* were obtained in 184 samples out of 282 samples tested (65.2%), which were further confirmed by biochemical tests. *Pasteurella multocida* isolates were obtained in 169 samples out of 282 samples (59.9%) and these isolates were also confirmed by biochemical tests. The samples were also screened by PCR using specific primers and only 3 samples showed the presence of *Haemophilus paragallinarum* out of 282 samples tested. Of 100 samples tested by RT-PCR for detection of NDV and IBV, only seven samples were found to be positive for IBV.

References