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ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF FIELD ISOLATES OF MYCOPLASMA GALLISEPTICUM

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Abstract: In the present study, samples were collected from 28 poultry farms in and around Namakkal district of Tamil Nadu, which had respiratory problems like respiratory rales, sneezing, coughing with loss of weight and loss of egg production. Samples after collection were immediately inoculated in to sterile glass tubes containing 3.5 ml of Frey's medium without supplementation of NAD. Tubes were incubated at 37^{o}C with 90 per cent relative humidity until the phenol red indicator changed from red to yellow (5-7 days). Cultures were plated on Frey's agar medium and incubated at 37^{o}C with 90 per cent relative humidity for 4 to 5 days. Colonies were detected by microscopic examination at low power for characteristic "Fried egg" colonies and stained with Diene's stain revealed the denser centre stained with dark blue and the peripheral zone stained with less dense, representing surface growth, stained light blue. Out of 28 farm samples tested, only one sample (3.57 per cent) yielded the isolate of MG.

Keywords: *Mycoplasma gallisepticum*, Layer, Identification, Molecular Chareecterization.

Introduction

Mycoplasma gallisepticum (MG) infections are commonly known as chronic respiratory disease (CRD) of chickens and infectious sinusitis of turkeys. *Mycoplasma gallisepticum* infections are characterized by respiratory rales, coughing, nasal discharge and conjunctivitis. Mycoplasma infection is wide spread in poultry farms in Tamil Nadu (Ramadass *et al*., 2006).

Tracheal and choanal cleft swabs after collection were immediately inoculated in to sterile glass tubes containing 3.5 ml of Frey's medium without supplementation of Nicotinamide adenine dinucleotide. Tubes were incubated anaerobically at 37^{o}C with 90 per cent relative humidity until the phenol red indicator changed from red to yellow (5-7 days). Cultures were plated on Frey's agar medium and incubated anaerobically at 37^{o}C with 90 per cent relative humidity for 4 to 5 days. Colonies of organism were stained with Diene's stain and identified by microscopic examination at low power for characteristic "Fried egg" appearance. Out of

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28 farm samples tested, one sample (3.57 per cent) resulted in the isolation of *Mycoplasma gallisepticum*. Field isolate was identified by biochemical tests; fermented glucose, reduced tetrazolium and negative for arginine hydrolysis. The field isolate was confirmed by polymerase chain reaction for specific nucleic acid amplification of 16s rRNA *Mycoplasma gallisepticum* genome. The field and vaccine isolates produced predicted size of 530 bp amplicons in the PCR.

Materials and Methods

Isolation of MG

Materials

Frey's Medium

Mycoplasma broth base	22.5 g
Glucose	30.0 g
Horse serum	100.0 ml
Cysteine hydrochloride	0.1 g
Nicotinamide Adenine Dinucleotide (NAD)	0.1 g
Phenol red (1 per cent)	2.5 ml
Thallium acetate (10 per cent)-[A]	5 ml
Yeast extract (25 per cent)	100 ml
Penicillin G potassium-[B]	1,000,000 units
Distilled water	1000 ml

pH was adjusted to 7.8 with 20 per cent NaOH and sterilized by filtration using 0.22 µm filters.

[A]-For potentially contaminated specimens an extra 20 ml of 1 per cent thallium acetate per liter of medium was added to bring total concentration to 1:1500.

[B]-For potentially contaminated material, an extra 2 million units was added per liter of medium.

Frey's agar medium

All other components of Frey's medium were sterilized by filtration and warmed to 50°C. One per cent agar was sterilized by autoclaving at 121°C for 15 min and cooled to 50°C and these were mixed and poured to the plates to a depth of approximately 5 mm (15 ml for 100 x 15 mm plate).

Isolation of MG from field samples

Tracheal and choanal cleft swabs from live birds and trachea, liver, sinus, air sac lesions, or joint lesions at necropsy were collected from 21 poultry farms in and around Namakkal district of Tamil Nadu, which had problems like respiratory rales, sneezing, coughing with loss of weight and loss of egg production. Samples after collection were immediately inoculated in to sterile glass tubes containing 3.5 ml of Frey's medium without supplementation of NAD. Tubes were incubated anaerobically at 37°C with 90 per cent relative humidity until the phenol red indicator changed from red to yellow. The cultures were tested for the presence of MG by PCR and simultaneously plated on Frey's agar medium and incubated anaerobically at 37°C with 90 per cent relative humidity for 5 to 7 days. Colonies were detected by microscopic examination at low power for characteristic "fried egg" appearance (Frey *et al.,* 1968; Lauerman *et al*., 1993; Kiss *et al*., 1997).

Identification of MG

Glucose fermentation

Frey's broth was inoculated with the isolate and incubated at 37°C for one week.

Arginine hydrolysis

Frey's broth was inoculated with the isolate and incubated at 37°C for one week.

Tetrazolium reduction

Frey's broth containing 5.5 mg of 2,3,5-triphenyltetrazolium chloride was inoculated with the isolate and incubated at 37°C and observed daily for 14 days.

Diene's Stain (for Mycoplasmal microcolonies)

Agar block containing MG microcolonies facing upwards were placed on a microscope slide. Light film of Diene's stain were placed on a cover slip and allowed to dry. Then it was put stain-side downwards, on the microcolonies on the agar block. The preparation was examined under the low-power object of a light microscope (Quinn *et al*., 1994).

Polymerase chain reaction for identification of MG

Polymerase Chain Reaction (PCR) for detection of MG was done by amplifying a 530 bp fragment corresponding to 16s rRNA, as per the method of Kiss *et al*. (1997) with minor modifications. An aliquot (1.0 ml) of suspected cultures was centrifuged; pellet washed with PBS, resuspended in 20 µl nuclease free water and boiled for 5-10 minutes and then kept in ice. The PCR was carried out in a 50 µl reaction. The DNA amplifications were performed in the thermal cycler with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30

sec. The final extension step was 72°C for 10 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 2 µl tracking dye in a two per cent Agarose gel with ethidium bromide (0.5 µg/ml) and DNA bands were observed using transilluminator.

Results

Isolation and identification of MG from field outbreaks: Out of 28 farm samples collected from layer farms in and around Namakkal, samples from one farm developed yellow color change of Frey's broth after 3 to 4 days of incubation at 37°C with 90 per cent relative humidity. These cultures when plated on Frey's agar medium and incubated at 37°C with 90 per cent relative humidity for 4 to 5 days developed characteristic "fried egg" appearance colonies (Plate 1.1).

Identification Of Mg Isolates

Colonial morphology: Colonies of MG isolated on Frey's agar stained with Diene's stain revealed denser centre stained with dark blue and the peripheral zone less dense, representing surface growth, stained light blue (Plate 1.2).

Glucose Fermentation: Glucose fermentation pattern of MG isolate is presented in Table 1 and Plate 2.1. The isolate fermented glucose and a change of colour from pink to yellow was taken as positive reaction.

Arginine Hydrolysis: Arginine hydrolysis pattern of MG isolate is presented in Table 1 and Plate 2.2. The isolate was negative for arginine hydrolysis. The medium remained pink after four days of incubation.

Tetrazolium Reduction: Tetrazolium reduction pattern of MG isolate is presented in Table 1 and Plate 2.3. An insoluble precipitate formation in the medium was taken as positive reaction. The isolate produced insoluble precipitate.

Table -1: Biochemical Characters Of *Mycoplasma Gallisepticum* Isolate

S.NO	BIOCHEMICAL TESTS	MG ISOLATE
1	Glucose fermentation	+
2	Arginine hydrolysis	-
3	Tetrazolium reduction	+

Polymerase Chain Reaction: Five suspected cultures and one vaccine strain 6/85 were subjected to nucleic acid amplification of 16s rRNA MG genome by polymerase chain

reaction (PCR). Out of the five suspected cultures one culture and the vaccine strain produced predicted size of 530 bp amplicons in the PCR (Fig-1).

Fig-1: PCR amplification of MG showing predicted size of 530 bp from clinical samples

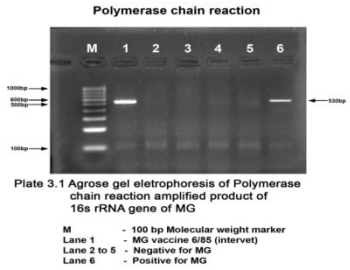

Plate 3.1 Agrose gel eletrophoresis of Polymerase chain reaction amplified product of 16s rRNA gene of MG

M - 100 bp Molecular weight marker
Lane 1 - MG vaccine 6/85 (intervet)
Lane 2 to 5 - Negative for MG
Lane 6 - Positive for MG

Chronic respiratory disease affected birds manifested respiratory rales, dyspnoea, sneezing, mucoid nasal discharge and swelling of lower eyelid (Plate 4.1). On post mortem examinations congested tracheal mucosa, slightly pneumonic lungs and air sac abnormalities varying from mild cloudiness to accumulation of yellowish inspissated caseated exudates were observed (Plate 4.2).

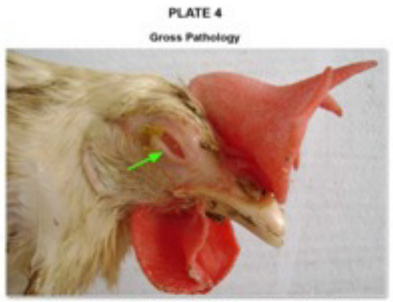

Plate 4.1 Severe edema in the facial subcutis and eyelids (arrow) in a CRD affected layer

Plate 4.2 Yellowish caseated mass (arrow) in the thorasic air sac of a CRD affected layer

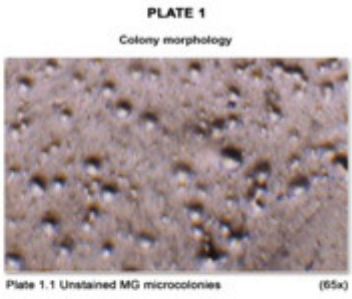

Plate 1.1 Unstained MG microcolonies (65x)

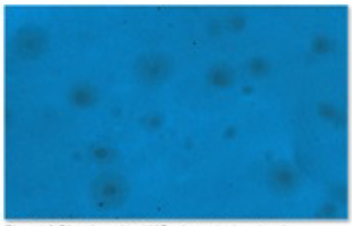

Plate 1.2 Diene's stained MG microcolonies showing the characteristic "fried egg" appearance (100x)

Discussion

Avian mycoplasmosis is widespread and is one of the most common respiratory diseases of poultry. *Mycoplasma gallisepticum* is the most pathogenic avian *Mycoplasma* species and leads to great economic losses worldwide (Mohammed *et al.,* 1987). The disease occurs when birds infected with MG are under stressful conditions. Subsequent invasion by secondary bacteria causes major damage to the bird. Avian mycoplasmosis is considered to be an important problem in broilers, breeders and commercial layers. In layers and breeders the

disease causes a 10 to 20 per cent decrease in egg production and 5 to 10 per cent increase in embryo mortality respectively. As the organisms are transmitted through egg, the MG infected breeder flocks should usually be depopulated (Stipkovits and Kempf, 1996).

Isolation and identification of MG: Frey *et al*. (1968) used Frey's broth supplemented with an additional 0.15 g of thallium acetate and 10, 00,000 IU of penicillin G per 1000 ml. In the present study Frey's broth without NAD was used for collection of samples for primary enrichment of MG from dead and live birds since addition of NAD favours the growth of *Mycoplasma synoviae*. Subsequently the broth cultures were subcultured on Frey's agar with NAD and cystein hydrochloride where as Kleven (1998a) used cystein hydrochloride with out NAD. Supplementation of NAD will support the growth of both MG and MS (Kleven, 1998a).

Mycoplasma gallisepticum could be isolated from one farm out of samples collected from 28 farms. The lower isolation rate (3.57 per cent) might be due to advancement in the age of the bird and the antimycoplasmal drugs which are being used in the field to control the disease. This is in agreement with the findings of Jordan (1979), Branton and Deaton (1985) and Ley and Yoder (1997). Ghaleh Golab Behbahan *et al*. (2008) reported that aivlosin, lincomycin-spectinomycin (1:2), tylosin, tiamulin, enrofloxacin and also lincomycin were commonly more effective against the field isolates.

Identification of MG: The field isolate developed tiny, smooth, circular, translucent masses with dense central area (fried egg appearance) on Frey's agar after 3 to 4 days of incubation at 37°C with 90 per cent relative humidity, which is in accordance with the observation of Kleven (1998b). Colonies of MG isolated on Frey's agar were stained with Diene's stain. The staining revealed dense centre with dark blue and the peripheral zone with less dense light blue, representing surface growth. This finding is in agreement with that of Quinn *et al*. (1994). The isolate was positive to fermentation of glucose without formation of gas, negative to arginine hydrolysis and positive to tetrazolium reduction. These findings are correlated well with that of Holt *et al*. (1994).

Polymerase chain reaction: Polymerase Chain Reaction has been proved to be very specific and sensitive method for amplifying low amounts of nucleic acid to a level that can be easily detected (Saiki *et al*., 1985; Mullis and Faloona, 1987; Innis *et al*., 1990). This method of detection was primarily developed for MG (Nascimento and Yamamoto, 1991; Nascimento *et al*., 1993) and was accepted worldwide for detection of all avian mycoplasmas, either for

specific DNA amplification for diagnosis (Lauerman, 1998; Nascimento *et al*., 1998) or for nonspecific DNA banding pattern (RAPD) for strain identification (Fan *et al*., 1995).

A major advantage of the use of the 16S rRNA gene vs. random cloned genes as the basis for PCR amplification is that all strains of a species are homogeneous for this gene (Nascimento *et al*., 1993). In the present study, species specific primers were used to amplify the 16s rRNA MG gene which amplified one field and one vaccine isolate and produced a 530 bp product. The other four cultures were not amplified, which indicates that they were not MG and might be some other Mycoplasma sp. The results are in accordance with the findings of Kiss *et al*. (1997) and Ramadass *et al*. (2006).

Symptoms and Gross lesions: The symptoms associated with MG infection in chickens include respiratory rales, nasal discharge, coughing, severe edema in the facial subcutis and eyelids, keratoconjunctivitis, and occasional corneal opacity (Carpenter *et al.,* 1981; Branton and Deaton, The gross lesions observed in the present study were congested tracheal mucosa, slightly pneumonic lungs and air sac abnormalities varying from mild cloudiness to accumulation of yellowish caseated exudates. The gross lesions observed were indistinguishable from the earlier reports of Nunoya *et al*.,1985; Mohammed *et al*., 1987; Nunoya *et al*., 1995; Ley, 2003).

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