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DETECTION OF *Mycoplasma gallisepticum* FROM FIELD SAMPLES OF LAYING CHICKEN USING PCR

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Abstract: *Mycoplasma gallisepticum* is the most important and infectious mycoplasmosis. It causes severe economic losses to poultry farmers in Namakkal district of Tamil Nadu. Sixty five commercial layer flocks with lesions suggestive of *Mycoplasma gallisepticum* were investigated during the study period of May 2014 to April 2015. The samples such as choanal cleft swab, tracheal swab, trachea, lungs and air sacs were inoculated into frey's broth medium and these were subjected to polymerase chain reaction and was confirmed using species specific primer. Out of these 65 farms, 36 farms were positive for MG. it is concluded that, PCR assay for the detection of *Mycoplasma gallisepticum* infection is good and rapid technique for diagnosis. So it is way to decrease the economic losses to poultry industry.
Keywords: PCR; *Mycoplasma gallisepticum*; Laying chicken.

Introduction

Poultry industry is facing a number of infectious and non infectious problems. Varieties of infections including bacteria, protozoa, fungi and virus result in devastating losses. Among bacterial infections chronic respiratory disease (CRD) caused by *Mycoplasma gallisepticum* (MG) is a serious threat to industry (OIE, 2008). Mycoplasma is the simplest and smallest bacterial cell. This organism can infect and grow in plant, animal, human and insect host. The Mycoplasmosis of poultry is one of the most important diseases which makes problem in our country (Kleven, 2003).

Mycoplasma gallisepticum can cause considerable economic losses in chicken due to reduced weight gain and meat quality, increased feed conversion ration and mortality in broilers, tremendous drop in egg production in layers and increase in embryonic mortality in breeders (Ley, 2008). Kempf *et al*. (1993) reported that PCR provided the basis of a sensitive, specific, rapid and non–radioactive method for detecting MG and was a valuable tool in early MG infection compared to serological and cultural procedure. Levinsohn and Kleven (2000) reported that PCR represents a rapid and sensitive alternative to traditional culture methods, which require time-consuming specialized technique.

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Serologic tests were used for *Mycoplasma gallisepticum* testing, but it needs at least of 1 week after infection for antibodies production. This test can be resulted by agglutination and hemagglutination inhibition. Hemagglutination inhibition test requires 3 week (Stipkovits and Burch, 1994).

Isolation of *Mycoplasma* is very difficult and cumbersome to perform. Diagnosis of *Mycoplasma* infections by serological procedures is sometimes hampered by interspecies cross–reactions and nonspecific reactions (Hagan *et al*., 2004). Therefore, molecular methods for diagnosing the disease may be more effective in comparison to cultural and serological methods. The technique has been proven to be very specific and sensitive method even for amplifying low amounts of nucleic acid to a level that cannot be easily detected by other methods. Therefore, present study was undertaken to culture MG by using specific broth medium and to confirm by Polymerase chain reaction using MG specific primers.

Materials and Methods

Sixty five commercial layer farms with lesions suggestive of *Mycoplasma gallisepticum* was investigated during the study period of May 2014 to April 2015. Samples were collected from layer farms from Poultry disease diagnosis and surveillance laboratory and during disease outbreaks in Namakkal district of Tamilnadu.

Out of these, 36 farms were positive for *Mycoplasma gallisepticum.* Samples such as choanal cleft swab and tracheal swab were collected from suspected ailing birds and necropsy was carried out in dead birds. Tissues samples like trachea, lungs, airsacs and liver were collected from dead birds.

These samples were inoculated into tubes containing 3-5 ml of Frey's broth medium with phenol red indicator, supplemented with 3 g of glucose, 100 ml of filtered horse serum, 0.1 g of cysteine hydrochloride, 5 ml of 10 % thallium acetate, 1,000,000 units of penicillin G potassium each for 1000 ml of Frey's broth medium. pH was adjusted to 7.8 with 20 % NaOH and these were sterilized by filtration. The inoculated samples were incubated at 37°C with 90 % relative humidity until the phenol red indicator changed from red to orange or yellow colour (3 –7 days).

DNA extraction

DNA was extracted by using Genomic DNA purification kit (Thermoscientific). PCR was carried out by using species specific primer pair for MG (Kiss *et al*., 1997) (Table 1).

Table 1: Primers and PCR conditions applied for detection of *Mycoplasma gallisepticum* at 530 bp

	Primer	Sequence (5'- 3')	PCR conditions			Reference
			Denaturation	Annealing	Extension	
MG	MG-F	AAC ACC AGA GGC GAA GGC GAG G	94 °C /30sec	62°C /30 sec	72°C /30sec	Kiss *et al*., 1997
	MG-R	ACG GAT TTG CAA CTG TTT GTA TTG G				

PCR was carried out in 0.5 ml of PCR tubes in a final reaction of 25 µl volume. Each reaction mixture contained 2X PCR Master mix (12.5 µl), 0.75 µl of 10 Pmol/ µl each of MG-F and MG-R primers and 1 µl of DNA template. The reaction mixture were adjusted to the final volume by adding DNAse free water.

All DNA amplification were performed in a thermal cycler (Master cycle Eppendorff) with initial denaturation at 95 °C for 4 minutes, followed by 35 cycles at different temperature segments (Table 1) corresponding to the target DNA denaturation, primer annealing and primer extension respectively. The final extension step was 72 °C for 10 minutes. The PCR amplified products were separated by agarose gel electrophoresis (1.5 % agarose) in 1X TAE buffer. DNA fragments were observed by ultraviolet transilluminator and photographed in a gel documentation system. The species specific primers used in the study successfully amplified the portion 530 bp of 16S rRNA gene.

Result and Discussion

Polymerase chain reaction is more sensitive and requires lesser time to offer result when compared to the tedious culture method (Marois *et al.,* 2002). Out of these 65 commercial layer farms, 36 farms were positive for MG. In the present study, MG positive samples produced 530 bp product corresponding to the 16S rRNA (Fig 1). These results are in agreement with earlier reports of Sivaseelan *et al.* (2015). The change of Frey's broth colour from red to orange or yellow with clear solution after 3 to 7 days of incubation indicated that the Frey's medium is suitable for isolation of mycoplasma. The changing of colour from red to yellow with turbidity within 2 days of incubation indicated the medium was contaminated. This finding is in accordance with Jalaladdini *et al.* (2014), who observed turbidity in PPLO culture, indicating bacterial growth.

Samples collected from suspected ailing birds yielded more positivity by PCR than compared to samples from dead birds. This might be due to contamination of tissues easily by secondary bacteria after death. Samples taken from choanal cleft swab which could be easily

obtained than the trachea yielded high positivity in PCR. These findings are in agreement with Branton *et al.* (1984). These might be due to anatomical relationship of the choanal cleft to the lacrimal duct system and the nasal cavity provides an understanding of why culture of this site should be considered to isolate MG from live birds (Varley and Jordan, 1978). Nickel *et al.* (1977) stated that the choanal cleft represents a permanent communication between the oral and nasal cavities, which might act as a reservoir of MG.

Mycoplasma galliseticum delayed culture after 7th day did not yield good result when compared to samples taken from 3 to 7 days. This indicated that the PCR should be carried out immediately after the colour change of Frey's medium to orange or yellow which usually occurs after incubation of 3 - 7 days. Otherwise positivity would not be appreciated through PCR. These findings are in agreement with Bradbury (1998) and Jalaladdini *et al*. (2014), who reported that in PPLO culture, mycoplasma survive for 15 days only. Sub-culturing of Frey's medium yielded more positivity of MG by PCR than the first isolation. This might be due to sub-culturing of Frey's medium will yield pure culture.

Conclusion

In the present study, species specific PCR assay could be used for confirmation of *Mycoplasma gallisepticum* from field samples when it was performed on Frey's broth medium. Result from this study indicates that *Mycoplasma gallisepticum* infection is widespread in poultry farms of Namakkal district of Tamil Nadu and PCR method could be used for rapid and sensitive detection of *Mycoplasma gallisepticum.*

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Fig 1: **MG:** 530 bp PCR products on agar gel electrophoresis