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Early Detection of *Mycoplasma Gallisepticum* using PCR from Field Samples in Layer Chicken

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Abstract:

Mycoplasma gallisepticum (MG) is an important avian pathogen which causes significant economic losses within layer poultry industry. To control of this economic losses, early and specific detection is necessary. A total of 48 (tracheal swabs, lungs and air sacs) samples were collected from different infected/suspected flocks causing respiratory distress. DNA was extracted and then, 16sRNA gene was amplified by PCR using species specific primers and identified 6 positive samples (12.5%). MG exhibited from tracheal swab, lung and air sac were 25, 6.25 and 6.25% respectively. Based on result, it is concluded that PCR using tracheal swab can be efficient for rapid screening and early detection of MG in layer chicken.

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1. Introduction

In Bangladesh, poultry is the most widely held livestock species by people. The expansion of poultry farming has led to the establishment of many allied industries.This sector offers enormous opportunities of people and alleviates poverty.Valuable food items like meat, eggs and by products are the prime components. But these industry facing multiple challenges and huge economic losses due to infectious diseases is major one. Morbidity and mortality rate of poultry is caused by respiratory tract infections is much higher than others. *Mycoplasma gallisepticum* (MG) is a top ranking a cell wall less prokaryotic bacteria belongs to the class Mollicutes, isolated from cases of chronic respiratory disease (Hafez, 2011).The MG outbreaks have caused significant losses to the poultry industry (Ley, 2008).It causes reduced weight gain and meat quality and decreased feed efficiency, increase in embryonic mortality in breeders (Carpenter et al., 1981; Hossain et al., 2007; Kleven and Noel, 2008; Ley, 2008) and tremendous drop in egg production in layers (Mohammed et al., 1987 ; Bradbury, 2007; Levisohn and Kleven, 2000a) and the clinical signs associated with MG infection in chickens include respiratory rales, nasal discharge, coughing and sinusitis (Saif et al., 2003).Occasionally, MG infections are associated with arthritis, salpingitis, conjunctivitis and fetal encephalopathy.This pathogen transmitted from infected birds via eggs or inhalation of contaminated airborne droplets, resulting in rapid disease transmission throughout the flocks.

Programs for control and eradication of the pathogen from flocks are diagnosed by different traditional methods such as microbial culture and serological (Ley et al., 2008). Serology is the only reliable tools for detecting the subclinical infection in the flock (Barua et al., 2006) but it needs at least of 1week 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).There are two major serological methods, which were used for screening of farms which are Rapid Serum Plate Agglutination (RSA) and Enzyme Linked Immunosorbent Assay (ELISA) test; however, there were differences in sensitivity and specificity of these methods. Diagnosis of MG infections by serological procedures is sometimes hampered by interspecies cross-reactions and nonspecific reactions (Klaven et al., 2000; Hagan et al., 2004). Culture isolation of its is time consuming and complicated, requiring two to three weeks to complete growth because of the slow-growing in nature and contamination with other organism. Recently, for rapid diagnosis of MG, developing nucleic acid-based molecular biological techniques have been employed and for rapid and effective identification of Mycoplasma strains, PCR based methods is a excellent tools have been proved (Han Wang et al., 1997; Ben et al., 2005; Feberwee et al., 2005).This technique also has been proven to be very specific and sensitive method even for amplifying low amounts of nucleic acid to a level that can not be easily detected by other methods. Therefore, present study was aimed to test feasibility of using tissues directly PCR based identification of *Mycoplasma galliseptcum* in layer poultry flocks without any need of enrichment or culture.

2. MATERIALS AND METHODS

2.1. Sample collection and preparation: A total of 48 (Tracheal swabs, lungs and air sacs) tissue samples were collected from ten layer poultry farm of five northern districts of Bangladesh. Tracheal swabs were collected using sterile cotton in PPLO broth containing 15% inactivated horse serum and lung and air sac in buffered glycerol (50%) from sacrificed and post mortem bird which was suffering from respiratory problems like sneezing, coughing,

lacrimation. The collected samples were carried out in cool box under chilling conditions to the laboratory of Hajee Mohammad Danesh science and Technology (HSTU) University. Tissues were triturated directly without any enrichment in pestle and mortar. After filtering by 0.45 filter membrane DNA was extracted from tissue.

2.2. DNA extraction: Genomic DNA (Deoxyribonucleic acid) was extracted using DNA isolation kit (Genomic DNA mini kit) according to manufacturer's instructions. The extracted DNA was collected in 1.5ml screw capped eppendorf tube and kept at -20^{0}C till further use.

2.3. Quantification of DNA: Quantification of extracted DNA was done using Nanodrop spectro-photometer (Thermo Scientific, USA) for determination of DNA concentration and purity.

2.4. Polymerase chain reaction (PCR): The PCR protocols adapted targeting 16SrRNA gene of *M. gallisepticum*. The PCR primers were obtained from Macrogen, Singapore (Table 1). Positive sample was used 6/85 strain vaccine (Mycovac, Intervet).

2.5. Gel electrophoresis: A total of 10 µl PCR products with 2 µl of 6X Gel loading dye were detected by gel electrophoresis in 1.5% agarose gel in 1X TAE buffer. Gel was run for 45 minutes at 100 V, stained with 3µl ethidium bromide (0.5 µg/mL of gel) and exposed to ultraviolet light and photographed in a gel documentation system. Commercial 2µl of 50 bp DNA ladder (Fermentas, USA) were used as molecular-weight markers in gel running.

3. Results

Polymerase chain reaction is more sensitive and requires lesser time to offer result when compared to the tedious culture method (Marois et al., 2002). In the present study, MG specific 16S rRNA gene was amplified 732bp for detection of MG in clinical specimens. Several studies was to investigate the prevalence of *M. gallisepticum* in poultry by PCR of 16 s rRNA gene of (Rasoulinezhad et al., 2017; Zakeri, 2016; Hassan et al., 2014; Rauf et al., 2013; Hess et al., 2007; Garcie et al., 2005 and Lauerman, 1998).

The species specific primers (Table 1) used in the study successfully. Out of 48 samples, 6 samples (12.5%) were found PCR positive for the MG. This study showed that MG can be detected from field samples directly within 1-2 days without need of culturing of this fastidious organism and supported by McAuliffe et al., (2005). 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 tracheal swab which could be easily. From PCR result, 6 (12.5%) positive bands showed successfully in different tissues (Fig. 1) and 25% from tracheal swab and 6.25% from lung and air sacs (Table 3).

Table 1: Primers applied for detection of MG

	Primer	Sequence (5-3)	Product size	Referrence
MG	MGGha_F	5'-GGA-TCC-CAT-CTC- GAC-CAC-GAG-AAA-A-3'	732bp	Nascimento *et al*., 1991
	MGGha_R	R5'-CCT-TCA-ATC-AGT-GAG-TAA-CTG-ATG-A-3'		

Table 2: Composition of PCR reaction mixture and thermal profile (Rauf *et al*., 2013)

Composition	Total volume (25µl)	Thermal profile				
		1 cycle	35 cycle			1 cycle
		Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
Master Mix	12.5	94°C 5 min.	94°C 1 min.	55°C 1 min.	72°C 2 min.	72°C 10 min.
Forward primer (100 pmol/µl)	0.5					
Reverse primer (100 pmol/µl)	0.5					
Template DNA	2					
Nuclease-free Water	9.5					

Table 3: Tissue distribution pattern of MG by PCR in different tissues

Tissue used for cultural test	Culture			P value
	Tested Samples	Positive Samples	Percent of Positive Samples	
Tracheal swabs	16	4	25	0.18 (NS)
Lung	16	1	6.25	
Air sacs	16	1	6.25	
Total	48	6	12.5	

NS means statistically not significant

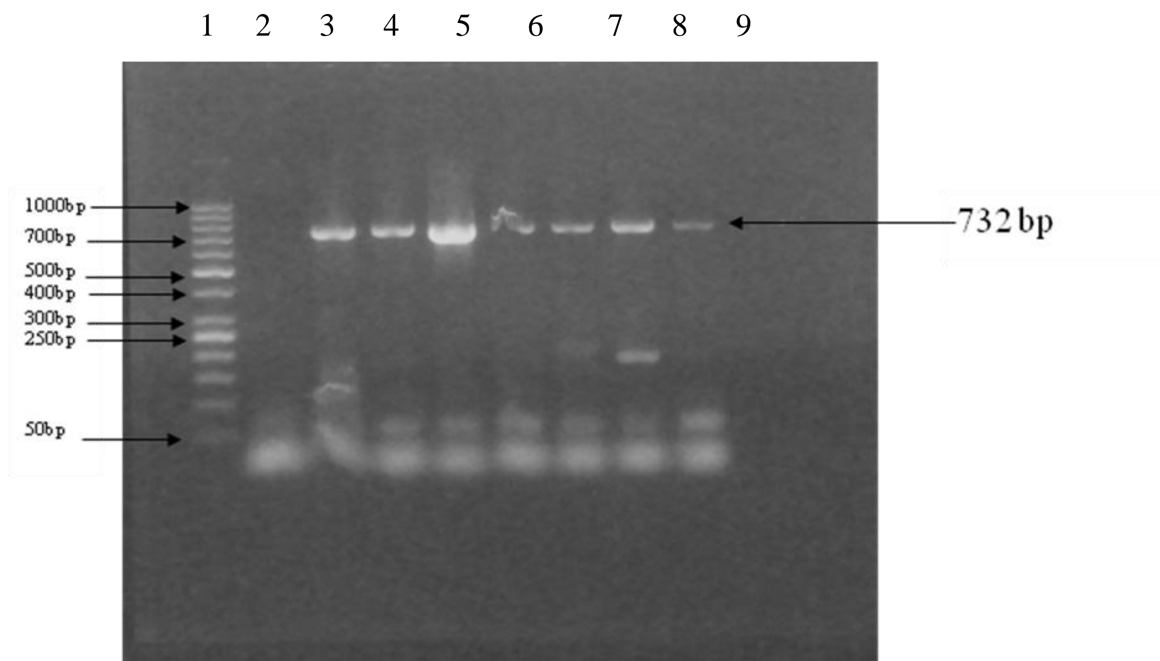

Figure 1: Electrophoresis analysis (1.5% agarose gels) of PCR products of 732bp, Lane 1 = 50 bp DNA marker, Lane 2 = Negative control, Lane 3 = Positive control, Lanes 4,5,6,7 (Tracheal swab) and 8, 9 (Air sac, lung).

4. DISCUSSION

Diagnosis of *M. gallisepticum* can be carried out by various procedures like necropsy to observe gross and microscopic lesions, immune response determination by serology like SPA, ELISA, HI tests (Dufour-Zavala *et al*., 2008; OIE, 2008; Qasem *et al*., 2015). Detection of *M.gallisepticum* to find either organisms or DNA by culture or PCR methods (Pakpinyo et al., 2006). Various workers stated that for the growth of this organism hard to culture, as they have unique medium requirments and requires 3-4 weeks contains other contaminents (McAuliffe et al., 2005; Kleven, 2008; Bagheri et al., 2011). Serological tests should be only used as screening tools in monitoring programs to detect avain mycoplasmosis in poultry flocks but have disadvantage of cross reactivity between MG and MS isolates (Purswell et al., 2012).

In the present study the tissue distribution for MG to some extent varied between different respiratory tissues. The highest (25%) prevalence of MG by PCR in 732bp was found from tracheal swabs followed by air sacs (6.25%) and lungs (6.25%). The results of resent study for MG are in contrast to Ramadass et al., (2006), Rauf et al., (2013), Gondal et al., (2015), Jafar et al., (2015) and Hossam et al., (2016) had documented that tracheal swab samples gave more isolations than from lung and air sac. Results regarding characterization of MG through PCR depicts that upper respiratory tract is more prone to infection. The highest 33.3% (17/51), occurrence of this bacterium in trachea might be attributed to a factor that it is the first organ of respiratory tract which is exposed to the infectious agent (Nascimento et al., 2005). Localization of this bacterium is supported by ciliated epithelium of trachea. Local environment supports its growth and propagation than any other organ of the respiratory system. McAuliffe et al., (2005) demonsrtated that without need of culturing, this fastidious orginsm from field sample can be detected by PCR. Ramadass et al., (2006); and Lauerman et al., (1993) also reported that for rapid screening and diagnosis of this organism from naturally infected bird species specific PCR may be applied before the culture.Various workers stated that for the growth of this organism hard to culture, as they have unique medium requirments and requires 3-4 weeks contains other contaminents (Bagheri et al., 2011; Kleven, 2008; McAuliffe et al., 2005). Present study illustrates to diagnose MG infection as compared to traditional culture of the organism the combined advantage of PCR having high sensitivity and specificity, simple and cheap.

PCR test lead to rapid results and relatively low costs compared with culture (Feberwee et al., 2005; Pourbakhsh et al., 2010). PCR method can be advantageous for rapid and sensitive molecular detection (Hyman et al., 1989). Feberwee et al., (2005) showed that diagnosis of *M.Gallisepticum* and *M.synoviae* (MS) with different diagnostics methods (culture, SPA, HI, ELISA and PCR) a high number of false positive results was found in ELISA and SPA.They also diagnosed in HI 4.21 % were positive from 712 sera positive in SPA. Current research of findings was confirmed with their. Evans et al. (2009) declared 36.7 % positive samples by PCR corresponding to size with that of MG control. Salisch et al., (1998) described for detection of MG a commercial PCR based test kit is specific and at least as sensitive as culture because of the few samples detected by culture.

5. Conclusion

Result from this study indicates that *Mycoplasma gallisepticum* infection is widespread in poultry farms of northern area of Bangladesh and species specific PCR assay could be used for rapid, sensitive detection and confirmation of *Mycoplasma gallisepticum* from field samples. PCR assay as molecular tests that can be completed in one day by using tracheal swab without culturing in poultry flocks suffering from respiratory disease. Future work may include the possibility of making autogenous vaccines from these isolates.

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