The Journal of Animal & Plant Sciences, 23(2): 2013, Page: 393-397
ISSN: 1018-7081

IDENTIFICATION OF *MYCOPLASMA GALLISEPTICUM* BY POLYMERASE CHAIN REACTION AND CONVENTIONAL DIAGNOSTICS FROM WHITE LEGHORN LAYER FLOCKS

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ABSTRACT

Molecular diagnostic technique (PCR) was compared with conventional culture isolation for *Mycoplasma gallisepticum* (MG) identification from field cases of chronic respiratory disease (CRD). White leghorn laying birds (n=380) suffering from respiratory diseases were screened by *in vitro* cultivation of MG on selective media. Growth inhibition test (GIT) was carried out by adding known antibodies against MG. DNA extracted from organs of infected birds was amplified by PCR using species specific primers of MG targeting 16SrRNA gene and visualized by agarose gel electrophoresis. Overall 27.6 percent (104/380) samples from field birds were positive for MG conventional cultivation methods. Out of 104 culture positive isolates, 83 (79.8%) were confirmed as MG through GIT with 21.84 (83/380) overall percentage. Percent proportion for MG in organs of infected birds was higher (68.94%) as determined by PCR. Confirmed presence of 185 base pairs DNA band was considered positive for MG. There was a significant difference between results of PCR and conventional isolation technique determined by Chi square test ($P<0.0001$). Results recorded by PCR were comparatively higher and technique found better for identification of MG. PCR can efficiently be used for early detection of MG in different poultry flocks. An early diagnosis can help to lower economic burden of poultry layer farmers due to this drastic malady.

Key words: *Mycoplasma gallisepticum*, isolation, antibodies, PCR and 16SrRNA gene.

INTRODUCTION

Pakistan's commercial poultry sector offers enormous opportunities to millions of people and alleviates poverty. Valuable food items like meat, eggs and by products are the prime components. Poultry shared 25.8 percent of total meat production and 13144 million eggs (Anonymous, 2012). This huge industry is facing multiple challenges and economic loss due to infectious diseases is major one (OIE, 2009). Morbidity and mortality caused by respiratory tract infections is much higher than others. *Mycoplasma gallisepticum* (MG) is a top ranking wall less bacteria isolated from cases of Chronic Respiratory Disease (CRD) in white leghorn layers (Marois *et al*., 2001), ducks and geese (Stipkovits and Szathmary, 2012).

Major respiratory signs of CRD include gasping, respiratory rales, coughing, nasal discharge and rhinitis. Occasionally, MG infections are associated with arthritis, salpingitis, conjunctivitis and fatal encephalopathy (Much *et al*., 2002). Differential diagnosis based on clinical and necropsy findings is confused with other infectious respiratory diseases. *In vitro* isolation and identification of MG is reliable but results are not accurate due to least tolerance in adverse environment (Levisohn and Kleven, 1984).

Accurate and reliable diagnosis on the basis of culture characters, biochemical profile (Kleven, 2003) and serological tests (Ley, 2003) are in routine practice but are time consuming. Among serological tests Haemagglutination Inhibition test and ELISA are frequently applied for the detection of MG (Purswell *et al.,* 2012). In serological techniques, chances of non-specific (false positive) results are higher because of *Mycoplasma synoviae* (MS) and MG, cross reactivity (Kleven *et al.,* 2000). Siddique *et al.* (2012) confirmed the presence of MG with other viral and bacterial pathogens in respiratory cases of different poultry birds by multiplex PCR. Ehtisham-ul-Haque *et al.* (2011) compared conventional isolation technique with PCR for identification of MS infecting broiler birds. Molecular detection was significantly higher (98.82%) than culture isolation (42.35%).

Appropriate and early diagnosis of MG becomes indispensable for timely treatment and eradication of this disease. It is the need of time to diagnose disease as early as possible in prevailing conditions of Pakistan to minimize losses due to MG. Keeping in view PCR; a molecular technique, was compared with conventional

diagnostic technique for the diagnosis of MG from birds having respiratory infections.

MATERIALS AND METHODS

Samples (n=380) were collected from thirty eight commercial layer farms in triplicate suffering from respiratory diseases in and around district Lahore, while visiting different layer farms. All birds were at production stage selected on the basis of respiratory problems like coughing, sneezing, lacrimation and respiratory sounds.

Sampling procedure: Trachea (154), lungs (113) and air sacs (113) were collected from each bird at necropsy and divided in two halves, one for immediate inoculation in Fray's broth on spot and other preserved for DNA extraction. Samples were transported to the laboratory of Pathology, UVAS Lahore in a refrigerated container (OIE, 2008; David *et al*., 2006). In laboratory, inoculated broths were incubated at 37^0C, while samples collected for DNA extraction were stored at -20^0C till further use.

In-vitro cultivation and identification: Fray's medium was prepared according to manufacturer's instructions. Component A, BBL broth base dissolved in distilled water, was sterilized by autoclaving at 121^0C, 15 lb/sq inch pressures for 15 minutes and B, glucose, horse serum, fresh yeast extract and thallium acetate, by filtration through sterile Seitz filtration system under negative pressure using 0.45µm filter paper. Ingredients of both components along with penicillin and phenol red were mixed under sterilized conditions and poured in labeled screw capped test tubes. Agar was prepared by adding 1% autoclaved Agar agar in prepared broth, kept at 37^0C in an incubator for 48 hours to check sterility (OIE, 2008). Tubes containing media inoculated with samples were incubated at 37^0C in humid and 5% carbon dioxide atmosphere. Cultured test tubes positive for growth were used for subculture on Frey's agar (Ley, 2003). Agar plates were observed for fifteen days under stereo-microscope for growth of MG and results were recorded.

Suspected MG isolates were confirmed through growth inhibition test (Clyde 1983; Fan *et al.*, 1995 and Lauerman *et al.*, 1995) by transferring the individual colony on Fray's agar containing hyper immune serum raised in rabbits against reference strain (ts-11 vaccine strain, Merial international). Agar plates were incubated and observations recorded.

Molecular characterization: Polymerase chain reaction (PCR) was used for detection of MG in organs (trachea, lungs and air sacs) of infected birds as described by Santha *et al*. (1990). Physical method was adopted for DNA extraction from tissue swabs by heat (95^0C in water bath for 10 minutes) and cold (-20^0C for 10 minutes) shocks. Swabs were dipped in PBS, centrifuged at 13,000 rpm for 20 minutes and pellet washed in PBS twice. Supernatant containing DNA was collected in 1.5ml screw capped eppendorf tube. Forward (MG -14F: 5'–GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3') and reverse (MG-13R: 5'–GCT-TCC-TTG-CGC-TTA-GCG-AC-3') primers targeting 16SrRNA gene of MG were used in the presence of reaction mixture (Taq Buffer=2.5µl, dNTPs Mix=2.5µl, $MgCl_2$=2µl, Taq Polymerase=0.3µl, DNA=4µl, DNAase-free deionized water=11.7µl, Primer F=1µl and Primer R=1µl) for amplification of DNA (Somsak and Sasipreeyajan, 2007). Briefly, initial denaturation at 94^0C for 4 minutes followed by 35 cycles of denaturation at 94^0C, annealing at 58^0C and extension at 72^0C each for 45 seconds and finally extension at 72^0C for 10 minutes in thermal cycler (Ependorf master cycler). Amplified DNA fragments were analyzed by Agarose gel (1.2%) electrophoresis as described by Santha *et al*. (1990) using DNA ladder (1kbp, Viventes) as standard. Agarose gel was placed in electrophoresis tank containing 1X TAE running buffer by providing 110 volts for 45 minutes. Gel was observed under UV trans-illuminator and image captured by gel documentation system. Results of molecular technique (PCR) and conventional diagnostic of MG were statistically analyzed by Chi square test with SAS version 9.1.3 portable software.

RESULTS

Percentage of positive samples revealed by culture isolation of *Mycoplasma gallisepticum* (MG) from white leghorn laying birds suffering from respiratory infection was 27.6 only (Table 1). Highest detection was in trachea (39.2%) followed by air sac (27.4%) and lowest in lungs (15.92%). Frey's broth inoculated with field samples exhibiting swirling growth with color change were sub cultured on agar plates. These results are based on visualization of typical fried egg appearance of individual colonies (Fig. 1) by observing agar plates under 10X objective lens of stereo microscope. Initially color of the colonies was yellow and light brown after 7 days. Suspected colonies (n=104) were identified as MG by growth inhibition test (GIT) and confirmed isolates were 79.8 percent. MG isolates confirmed from trachea were highest (30.51%) followed by air sacs (20.35%) and least from lungs (11.5%). Absence of growth in the presence of specific antibodies in medium was recorded as positive. Percentage of MG by culture isolation and confirmed by GIT was 21.84 (Table 1).

PCR amplicon product of 183 base pairs visualized in agarose gel revealed that MG primers had successfully targeted the respective gene (Fig 2). Detection of MG from trachea was relatively higher (80.51%) by PCR than in case of lungs and air sacs which

was 72.2 and 74.19 percent, respectively (Table 2). Statistically significant difference was there between detection of MG from field samples by molecular technique (PCR) and conventional culture isolation tools as determined by Chi square test (P<0.0001). PCR may be a reliable tool for early detection of infectious agents in field cases.

Table 1: *In vitro* cultivation of *Mycoplasma gallisepticum* and confirmation through growth inhibition test

S/N	Organ	Samples	Culture isolation		Growth inhibition Test	
			Positive samples	Percentage	Positive samples	Percentage
1	Trachea	154	55	39.28%	47	30.51%
2	Lungs	113	18	15.92%	13	11.5%
3	Air sac	113	31	27.43%	23	20.35%
	Total	380	104	27.6%	83	21.84%

Table 2: Molecular identification of *Mycoplasma gallisepticum*;

S/N	Organ	Samples	PCR results		Culture results	
			Positive	Percentage	Positive	Percentage
1	Trachea	154	124	80.51%	55	39.28%
2	Lungs	113	62	54.86%	18	15.92%
3	Air sac	113	76	67.25%	31	27.43%
	Total	380	262	68.94%	104	27.6%

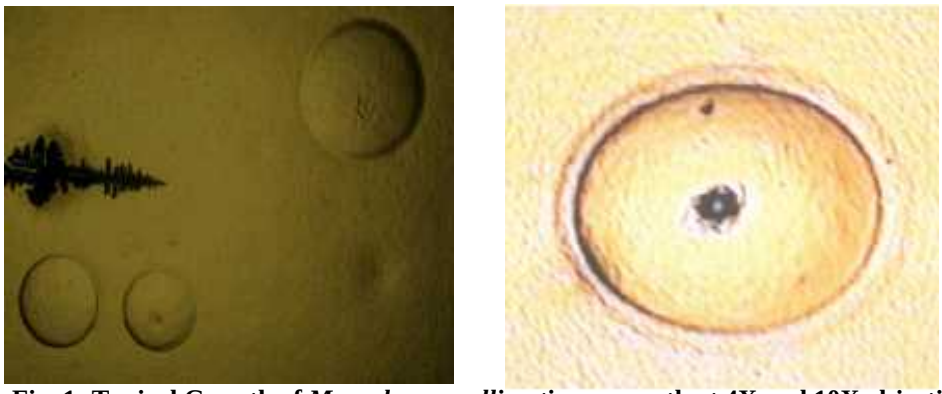

Fig. 1: Typical Growth of *Mycoplasma gallisepticum* growth at 4X and 10X objective lens

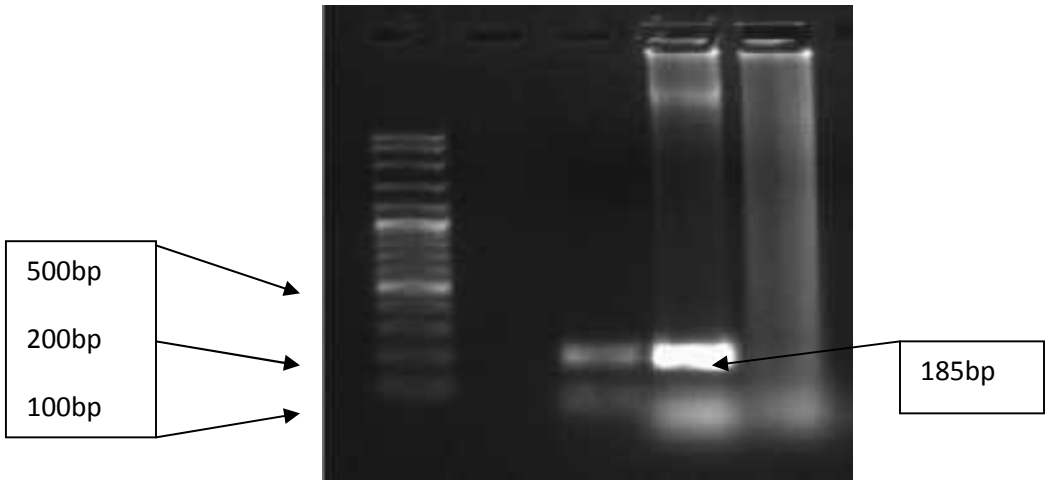

Fig. 2: DNA based detection of *Mycoplasma gallisepticum*
Lane M=1kbp DNA ladder, Lane 1=Control negative, Lane 2-4=PCR product

DISCUSSION

Diagnosis of *Mycoplasma gallisepticum* (MG) can be carried out in different ways using culture isolation techniques as well as PCR (Ley, 2003). Results regarding isolation of MG through traditional culture techniques depicts that upper respiratory tract is more prone to infection. The highest 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. Results of present study were substantiated by the finding of Gharibeh and Roussan (2008) that 38% of samples taken from trachea of layer birds were positive for *Mycoplasma* through culture technique. Similarly, Evans *et al*. (2009) strengthened the findings of present study that culture techniques may be reliable but in the meantime are laborious and time consuming. Reason for this is obvious as these procedures require up to 30 days for the confirmation of the target agent. Whereas Marois *et al.* (2002) reported 3.75 percent culture identification from environmental samples. The reasons for low percentage of MG on isolation might be due to low prevalence of Chronic Respiratory Disease and environmental conditions in that particular area. Another possible cause may be that samples were not taken directly from infected birds rather from soil which can affect the survival of MG.

In case of Growth inhibition test (GIT), for identification of isolates by using hyper immune sera raised in rabbit against reference strain, MG growth was not revealed. Occurrence pattern of bacteria was in accordance with Senterfit, (1983) and Clyde (1983) for confirmation of Mycoplasma species. Woode and McMartzn (1973) supported the reliability of GIT for identification of MG culture. There was not much difference among percent prevalence of MG in different organs by conventional diagnostics. Results of Janet *et al.* (1993) differ from the findings of present study. The reason may be due to different lab conditions and presence of other strains of MG. Immunity based diagnostic methods are frequently practiced for identification of MG and response to different used vaccines. Haemagglutination Inhibition test and ELISA are most common techniques for screening of MG and status of immunity (Purswell *et al.,* 2012). However, chances of non-specific results are more. Serological techniques have disadvantage of cross reactivity between MG and *Mycoplasma synoviae* (MS) isolates (Kleven *et al.,* 2000).

Due to lack of differentiation in colony characteristics of different Mycoplasma species and time consuming nature of previous techniques, PCR was applied for diagnosis of MG in accord with Pakpinyo *et al*. (2004). Results regarding detection of MG through PCR were in corroboration with Marois *et al.* (2002). Positive cases through culture identification were only 3.75 percent in comparison to molecular technique (42.4%). Results were higher (64.37%) on using MG specific primers. A possible reason for low number of positive cases by culture may be due to presence of non-viable *Mycoplasma*. These results attribute to the fact that PCR can detect DNA from both viable and non-viable bacteria and hence is more reliable diagnostic test in terms of sensitivity and specificity.

This study aided to investigate the comparative detection potential of two diagnostic techniques when applied to field isolates of MG. On statistical analysis a significant difference ($P<0.0001$) was found between PCR and culture. In contrast, Evans *et al*. (2009) declared 36.7% positive samples by PCR corresponding to size with that of MG control. Salisch *et al*. (1998) concluded that a commercial PCR based test kit for the detection of MG is specific and at least as sensitive as culture because of the few samples detected by culture. Ehtisham-ul-Haque *et al.* (2011) preferred PCR for early detection of MS from infected broilers. Significantly higher percentage (98.82) was reported in comparison to conventional diagnostics. In agreement to present findings PCR was declared more sensitive tool for early identification of infectious agents. Presence of MG along with other respiratory pathogens of poultry was confirmed by Siddique *et al.* (2012) using multiplex PCR. Technique was documented to be reliable for early detection of microbes in line with present study. Molecular diagnosis was more accurate, rapid and efficient for early identification of MG than conventional culturing techniques.

Acknowledgement: I acknowledge HEC Pakistan for providing funds for my higher studies. Especially to Mr. Zeeshan Haider finance manager for his cooperation.

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