The Journal of Animal & Plant Sciences, 25(1): 2015, Page: 108-113
ISSN: 1018-7081

CHARACTERIZATION OF *MYCOPLASMA GALLISEPTICUM* ISOLATED FROM COMMERCIAL POULTRY FLOCKS

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ABSTRACT

Chronic Respiratory Disease (CRD) is caused by *Mycoplasmas gallisepticum* (MG) and is prevailing in layer, broiler and breeder poultry flocks. The infected birds showed sneezing, rales, coughing and exudates from nostrils and eyes. Mycoplasma species were recovered 27.3% overall, 39.3 per cent of the tracheal swabs, 15.9 per cent of tracheal tissues, 27.4 per cent of lung tissues and 25 per cent of air sacs. Polymerase chain reaction confirmed the nucleic acid of the MG from tracheal swabs (68.18%), tracheal tissues (42.47%), lung tissues (31.85%) and air sacs (50%) using universal and MG specific primer. Higher percentage of MG confirmation through PCR is due to detection of nucleic acid of MG from medicated birds. It is concluded that culture method was laborious and time consuming and failed to detect mycoplasma species from medicated birds and is less sensitive than that of PCR. PCR is rapid, sensitive and accurate method for diagnosis of MG from suspected cases.

Key words: *Mycoplasma gallisepticum*, CRD, PCR, Frey's broth**.**

INTRODUCTION

Mycoplasmas are common in poultry, amongst which *Mycoplasma gallisepticum* (MG) is the most pathogenic and is causing Chronic Respiratory Disease (CRD). It is characterized by rales, coughing, nasal discharge, air sacculitis, and conjunctivitis with low mortality in commercial poultry in chickens (Ley *et al.*, 1997; Ley, 2003; Saif *et al.*, 2003; Bradbury, 2005; Nascimento *et al.*, 2005). Postmortem examination reveals mucupurrulent exudates in nasal cavities, trachea, bronchi and air sacs (Klevin *et al.*, 1984). The disease causes mitigation in growth, feed conversion, egg production, hatchability and chick quality (Klevin *et al.*, 1984), increased chick mortality, medication cost, condemnation and costly monitoring program involving serology and PCR (Klevin *et al.*, 1984; Ley *et al.*, 1997; Bradbury, 2005; Gaunson *et al.*, 2006).

Mycoplasma gallisepticum isolated from samples collected from morbid birds is characterized on the basis of cultural, morphological, biochemical, serological (rapid spot plate agglutination- RSPA, enzyme linked immunosorbent assay-ELISA and haemagglutination inhibition-HI) and molecular characteristics-PCR (Ley and Yoder, 1997; Soeripto *et al*., 1989; Kleven, 2008). Pertinent literature regarding its isolation, molecular characterization and control, is scanty. Present project is therefore designed to isolate and characterize causative agent of the disease on the basis cultural, morphological and molecular features (PCR).

MATERIALS AND METHODS

Isolation of causative agents: A total of 20 poultry flocks (Layer, broiler and breeder) in Khyber Pakhtoon khwa and Punjab provinces of Pakistan were visited. Signs and symptoms of birds suspected for MG were recorded. Postmortem examination of morbid birds was conducted. Tracheal swabs and tissue samples (n=388) were collected from MG suspected poultry birds and each of the samples was isolated on Frey's medium (OIE, 2004; Osman *et al*., 2009). For having pure culture, the growth was further passaged thrice on fresh Frey's broth by filtering the growth each time through 0.2um pore size syringe filter (Timms, 1967). Positive broth tubes showing uniform growth turbidity were blindly streaked onto Frey's agar medium and incubated at 37^0C for 21 days and observed under stereomicroscope at every three days intervals, whereas, negative plates, showing no growth up to 15 days of humid incubation, were discarded. The medium showing fried egg colonies under microscope were harvested, put in 15 per cent glycerol buffer and slowly frozen at -80 ^{0}C. The fried egg colonies were collected in PCR clean microfuge tubes for further genomic extraction followed by confirmatory PCR test. Agar plates showing mycoplasma colonies under microscope where each colony was looking like fried egg with a dark central raised area and a translucent flat periphery.

Polymerase Chain Reaction: All the sources, including mycoplasma suspected Frey's broth, egg fried colonies from Frey's agar medium, swabs and morbid organ portions directly collected from MG suspected poultry flocks, were used for DNA extraction using DNEasy blood and tissue extraction kit (QIAGEN, USA) as per manufacturer's instructions. For ascertaining the success of DNA extraction process, the DNA suspension was run through agarose gel electrophoresis to visualize the presence or absence of any DNA bands. For confirming the presence of MG, the DNA extracted samples were first processed for polymerase chain reaction (PCR) using species specific primer pairs and then processed for MG specific primer pairs (OIE, 2004) using method as described by Osman *et al*. (2009). Briefly: the PCR reaction (50ul) consists of 5ul of 10X buffer, DNTP 250µM concentrations (Fermentas, EU), 50 pmole of each primer, 2.25 mM $MgCl_2$, 2 U of Taq DNA polymerase, 10 µl of DNA template were put in Swift Mini Thermocycler (Esco Micro Pte Ltd, Singapore) under standard PCR conditions for 35 cycles.

PCR products were run on agarose gel electrophoresis (Osman *et al*. 2009). Briefly, Gel was prepared by dissolving 1.5gm of agarose in 100 ml of TBE buffer into a glass flask and after proper mixing suspension was boiled in a microwave until it is melted and dissolved to form a clear homogenous solution. The solution was cooled down to 60-70° C and 4ul/100ml of Ethidium bromide was added and poured on the electrophoretic tray. The comb was positioned in molten gel and allowed to harden by storing at room temperature for half hour. A 5µl of 250 bp and 100 bp DNA ladder was loaded to the first two well of agarrose gel. 5ul from each of PCR products and 2ul of loading dye was mixed and load in remaining wells of the gel. Lid was closed on chamber and electrodes were attached. The gel was run and electrophoresis was carried out at 140 V approximately for 45 minutes until the loading dye has migrated 2/3 of the way down the gel. Gel was removed and the presence of marker and PCR product bands was observed on UV illuminator and photographed using gel documentation system (Osman *et al.,* 2009). Specificity and sensitivity of cultural and PCR techniques for characterization of *Mycoplasma gallisepticum* were compared.

RESULTS AND DISCUSSION

Mycoplasma gallisepticum (MG) causes chronic respiratory disease (CRD) in poultry (broilers, layers and breeders) and is prevailing in Pakistan (Evans *et al.,* 2005; Rabbani *et al.,* 2012). The infected birds show sneezing, rales, coughing, and exudates from nostrils and eyes. The infected birds show swollen sinuses, suborbital swelling, high morbidity, low mortality (5%), weight gain (22-30 %), feed conversion ratio (11-20%), egg production and hatchability and increased condemnation of carcass at processing plants (10-20%) (OIE, 1996; Kleven, 1997; Hasan *et al.,* 2002; Steinlage *et al.,* 2003; Spencer *et al.,* 2002; Evans *et al.* 2005; Nascimento *et al.,* 2005; Shankar, 2008). On necropsy of the diseased birds, catarrhal exudates in nasal passage and trachea, accumulation of caseous material in lungs and cheesy material in air sacs were common lesions. Grossly, lesions in joints were not observed in any of the dead bird. In most of the cases, mycoplasmosis is complicated with other microorganisms and environmental factors to enhance its severity (Kleven, 2008). The bacterium multiplies in lungs, trachea, and air sacs and rarely in sinuses, and causes chronic macroscopic lesions such as catarrhal exudates in nasal and paranasal passages and in trachea. Massive air-sacculitis and cheesy materials stick to the respiratory surface of the air sacs. In complicated cases, *E. coli* causes fibrino-purulent pericarditis, peri-hepatitis and turbid exudates in pericardial sacs (Chanie *et al.,* 2009). *Mycoplasma gallisepticum* is small prokaryotes that lack a cell wall and are bound by a plasma membrane (Razin *et al.,* 1998). In Pakistan, 10 per cent flocks are MG positive through PCR (Rabbani *et al.,* 2012) and require heavy investment for medication of the birds to mitigate active dissemination and progression of the organisms in poultry. Mycoplasma species grew well and showed turbidity with yellow coloration in Frey's broth and showed pure colonies like fried egg appearance on Frey's agar medium on day 10 of incubation at 37^0C and 10% CO_2. Pathogenic avian mycoplasma species show similar cultural characteristics in mycoplasma broth and agar medium (Frey's *et al.,* 1968; Chanie *et al.,* 2009).

On repeated culture on the enrichment broth, Mycoplasma species showed turbidity in the broth within three days post incubation at 37^0C. The bacterium ferments glucose of the medium and produces acid metabolites, which decreased pH of the medium and changed the color of phenol red to yellow. Mycoplasma species isolates were recovered from 39.3 per cent of the tracheal swabs, 15.9 per cent of tracheal tissues, 27.4 per cent of lung tissues and 25 per cent of air sacs. Mycoplasma infection is prevailing in 2.1 per cent of the layers and 19.5 per cent of the broilers (Heleili *et al.,* 2011) 38.1 per cent of layers, 31.3 per cent of broilers, 14.3 per cent of breeders (Saad-Gharaibeh and Al-Roussan, 2008) and 33.3 per cent of layers, 4.9 per cent of broilers and 30.5 per cent of broiler breeder (Osman *et al.,* 2009).

Polymerase chain reaction confirmed the nucleic acid of the *Mycoplasma gallisepticum* from tracheal swabs (68.18%), tracheal tissues (42.47%), lung tissues (31.85 %) and air sacs (50%) using universal and MG specific primers. Cumulatively PCR confirmed MG from (49.74%) field samples collected during the study. PCR is a good technique for confirmation of MG from infected

birds (Liu *et al.,* 2001; Pharr *et al.,* 2002; Kleven *et al.,* 2004; Barbour *et al.,* 2005; Lysnyansky *et al.,* 2005; Lierz *et al.,* 2007; Raviv *et al.* 2007; Ghorashi *et al.,* 2010; Nazarpak 2010). PCR based nucleic acid detection is considered as an alternative method to that of conventional isolation technique (Thu *et al.,* 2000; Feberwee *et al.,* 2005; Ferguson *et al.* 2005; Raviv *et al.,* 2007; Evans and Leigh, 2008; Callison *et al.,* 2006; Hess *et al.,* 2007). MG is prevailing in 82.4 per cent of layers, 64.8 per cent of broiler breeders, and 17.1 per cent of broilers are positive (Osman *et al.,* 2009) and 25.8 per cent of the commercial farms are positive as confirmed through PCR (Faisal *et al.,* 2011).

Cultural methods of MG are laborious and time consuming, and could not isolate the organism and it is difficult in chronic cases of disease and medicated birds. New technique such as PCR is used for diagnosis of MG (Nascimento *et al.,* 1991; Chin *et al.,* 1993; Kemf *et al.,* 1993; Ley *et al.,* 1993). PCR proved more specific than culture method for identification of *Mycoplasma gallisepticum* from field samples. Samples collected from medicated birds were PCR positive, whereas, the same samples were culture positive on specific medium (Stanely *et al.,* 2001; Finklin and Kleven, 2006). PCR and culture methods detect MG from 97 and 67 per cent of the suspected samples, respectively. Culture method detects MG on day 26 PI, whereas PCR show positive results on day 54 PI (Kemf *et al.,* 1993, 1994). This difference of detection percentage could be due to fastidious nature of the organism and high sensitivity of PCR (Frey *et al.,* 1968; Chanie *et al.,* 2009). Culturing of Mycoplasma is a gold standard technique but it could not isolate organism from chronic cases and medicated birds as MG concentration low in those conditions (Hyman *et al.,* 1989) and anti-mycoplasmal substances, antiserum and different types of inhibitors also decreased chances of isolation and time of isolation increases (Jordan, 1979). Viable organism needed for success in isolation, alternatively, PCR detect the nucleic acid of MG even from medicated birds and frequent than culture (Kemf *et al.,* 1993). PCR amplifies DNA from dead or live pathogen (Josephson *et al.,* 1993). Higher percentage of positive samples is obtained by PCR (97 per cent) than that of culture method (67 per cent; Kemf *et al.,* 1993).

High prevalence of MG in these poultry flocks may be due to its horizontal transmission from the infected chickens, eggs, wild birds, vehicles or fomites to the healthy susceptible chicken flocks (Jordan, 1985). Poor management, cold air currents during winter, vaccination, high-density poultry farming and rearing of multi age group chickens in the same premises, may act as potential causative factors for immunity break down against MG infection in chickens (Pradhan, 2002). Moreover, inadequate ventilation, contaminated litter, frequent movement of rodents, wild birds, pets, technical persons, professionals, visitors amounting to poor biosecurity measures are some of the contributing factors to outbreaks of MG infection (Dulali, 2003). Replication of vaccinal virus in chicken body post-vaccination, formaldehyde poisoning during spray, accumulation of ammonia gas due to poor ventilation and flow of dust particles in air due to very dry litter cause potential damage to tracheal epithelium of the chickens during inhalation which in turn support the multiplication of contaminating Mycoplasma infection (Anderson *et al.,* 1965; Carlile, 1984).

It is concluded that *Mycoplasma gallisepticum* is prevailing in layer, broiler and breeder in Pakistan. Culturing method is laborious and time consuming and failed to detect Mycoplasma species from medicated birds and is less sensitive than that of PCR. PCR is rapid, sensitive and accurate method for diagnosis of MG from suspected cases.

Acknowledgements: Thankful for the financial support of Higher Education Commission (HEC), Islamabad, through its IRSIP program, Animal Diagnostic Lab, PennStat University, State College, PA, USA; PAK-USA Science and Technology Cooperation program-International Collaborative Research Project # 335 "Characterization of *Mycoplasma gallisepticum* isolates in Pakistan and their use in production of indigenous diagnostic antigen and bacterin" funded by National Science Academies, USA through HEC, Islamabad, and Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT, USA.

Table- 1: Comparison of PCR and Culturing Methods

Sr#	Sample type (n)	PCR positive sample (%)	Culture[+] positive sample (%)
1	Tracheal Swab (154)	105 (68.18)	55 (39.3)
2	Trachea (113)	48 (42.47)	18 (15.9)
3	Lungs (113)	36 (31.85)	31 (27.4)
4	Air sac (8)	4 (50.0)	2 (25)
Overall	**388**	**193 (49.74)**	**106 (27.3)**

[+] Frey's medium

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