Development of Duplex PCR Assay for the Detection of Mycoplasma Gallisepticum and Mycoplasma Synoviae Prevalence in Pakistan

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Abstract: Mycoplasma infections are of great concern in avian medicine, because they cause economic losses in commercial poultry production of Pakistan. Timely and efficient diagnosis of the mycoplasma is needed in order to practice prevention and control strategies. A duplex Polymerase Chain Reaction (PCR) was optimized for simultaneous detection of two pathogenic species of avian mycoplasmas. Two sets of oligonucleotide primers specific for *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, were used in the test. The developed assay exhibited high sensitivity and 100% specificity for the simultaneous detection of mycoplasma species prevalence in Pakistan.

Keywords: Mycoplasma, Pakistan, pathogenic species, poultry

INTRODUCTION

In Pakistan, the production of poultry has made awesome development in recent years. Due to increased demand and growing supply of poultry meat, it has become one of the most important growth industries in the commercial scenario of the country. Although poultry industry of Pakistan is well established and well structured, still it is confronted to many acute and fatal diseases. Among these, most mycoplasmal diseases are of key importance, such as chronic respiratory disease. There are more than a hundred species in the genus *Mycoplasma*, with a G+C (Guanine Cytosine) content of 23-40% (Kleven, 2003).

*Mycoplasma gallisepticum* is the member of the division Firmicutes, class Mollicutes, order Mycoplasma tales and the family Mycoplasmataceae (Ley, 2003). *M. gallisepticum*, an avian pathogen, among the pathogens belongs to this group *M. gallisepticum* is considered the most pathogenic of all avian *Mycoplasma* pathogens (Kleven, 2003). The most remarkable characteristics of the members of the genus are their lack of cell wall and utilization of UGA (Hnatow et al., 1998). *M. gallisepticum* infection leads to decreased egg production, growth and hatchability rates thus causing huge damage to the economy (Ferguson et al., 2004; Liu et al., 2001; Marois et al., 2001). *M. gallisepticum*is the primary causative agent of Chronic Respiratory Disease (CRD) and cause avian mycoplasmosis specifically under conditions of management stresses and/or other avian respiratory pathogens (Papazisi et al., 2002). The pathological condition observed during *M. gallisepticum* infection including chronic air sac infection. In some birds *M. gallisepticum* causing upper respiratory tract infections (Hong et al., 2005; Liu et al., 2001; Marois et al., 2001).

The first reports of *Mycoplasma synoviae* infection with arthritic involvement was declared in the decades of 50 and 60 in broiler flocks, but later in the decade of 70’s that the respiratory disease caused by *M. synoviae* was revealed (Rosales, 1991). *M. synoviae* is the member of class Mollicutes, order Mycoplasma tales and family Mycoplasmataceae. *M. gallisepticum* and *M. synoviae* are regarded to be the most important of the pathogenic mycoplasms and both are prevalent worldwide. *M. synoviae* belong to group of wall-less Gram-positive bacteria, genomes size is from 1358 kb to as little as 580 kb (Calderon-copete et al., 2009). *M. synoviae* infection in the meat producing birds leads to...
decrease growth rate and disapproval at processing. In turkey, *M. synoviae* infection may cause sinusitis, air sacculitis, synovitis and meningitis (Ghazv Khanian et al., 1973; Rhoadesr, 1977; Chin et al., 1991). *M. synoviae* caused Infectious synovitis (Kleven et al., 1991; Kleven, 1997). In chickens, turkeys and other birds, a less severe form of some of these signs and symptoms can be observed in *M. synoviae* infections, along with lameness, pale comb and head, swollen hocks and footpad. Extremely affected birds may show green faeces, but respiratory infection caused by *M. synoviae* is often without any symptoms. Similarly, most of the symptoms of *M. synoviae* infection are less severe and less visible and are characterized by impaired hatchability and embryo pepping, high embryo mortality, poor weight gain and mostly the same symptoms are studied in chickens affected by *M. synoviae* (Charlton et al., 1996). *M. synoviae* is one of the most important causes of the disease that may be presented as joint and/or respiratory condition (Lockaby et al., 1998; Yoder, 1991). In poultry birds and turkey, *M. synoviae* usually causing respiratory diseases in birds and thus leads to enormous loses of the valuable economy across the globe (Kleven, 1997). In addition to these *M. synoviae* infected symptomatic poultry show respiratory problems, cough, wheezing, aerosacculitis, impaired growth, sinusitis and synovitis, chronic and asymptomatic infections are both more common and more important, because of the damage and destruction they cause (Mohammad et al., 1987; Lockaby et al., 1998).

Both *M. gallisepticum* and *M. synoviae* have been implicated to cause serious economic losses to the poultry industry of Pakistan (Rehma et al., 2011). Diagnosis of both the *M. gallisepticum* and *M. synoviae* are relying on epidemiological data, clinical signs and symptoms of the disease, analyzing the macro- and microscopic lesions, mycoplasma serology and/or isolation and identification. The causing agent may be detected in fragments of infected organs (trachea, air sacs and lungs), as well as can also be identified in the infraorbital and ocular sinus and synovial exudates. Immunological detection methods can also be used to identify mycoplasmal isolates. These include the Direct immunofluorescence, Indirect Fluorescent Antibody (IFA) test, Immunoperoxidase (IP) tests, Growth inhibition (GI) test, Metabolism inhibition (MI) tests, IP (OIE), Hem agglutination inhibition (HI) test, Enzyme-Linked Immunosorbent Assays (ELISA) and Serum Plate Agglutination (SPA) test and immunoblot assays to detect antigenic differences among vaccine and wild *M. gallisepticum* strains (Kelven, 1994, 1995). Majority of the existing methods used for the detection of the pathogenic mycoplasma species are either highly expensive, time consuming or less reliable for the qualitative detection of different pathogenic mycoplasmas prevalent in Pakistan. Due to limitations of the Multiplex approach for the efficient diagnosis of the bacterial genotypes prevalent in Pakistan, we conducted this study in order to develop an efficient, less time consuming and cost effective duplex PCR assay for the detection of pathogenic mycoplasma species in poultry birds which is capable to detect the prevalent viral types in Pakistan. Moreover, we also investigated broiler, breeders and Layer birds from various poultry farms of the country for the presence of *M. gallisepticum* or *M. synoviae* infection.

**MATERIALS AND METHODS**

**Field samples and experimental birds:** The current study was carried out at Grand Parents Laboratory (GP Lab) Lahore after having approved by the Board of study of the Institute of Biotechnology and Genetic Engineering, Peshawar. The study was conducted in accordance with internationally accepted guidelines. Experimental birds and field samples were provided by various poultry farms from across the country. Chicks were artificially infected with various strains of *M. gallisepticum* and *M. synoviae* and slaughtered after the signs and symptoms of the disease appeared. Tracheas, air sacs, ovaries, joints and lungs were collected from the infected birds for the isolation of *M. gallisepticum* and *M. synoviae* DNA, at GP Lab Lahore, where chickens and other birds are brought for the diagnosis of various diseases from different commercial farms from all over the country. Apparently healthy and effected Broiler, Layer and breeder stocks in various poultry farms across the country were also investigated for the presence of AAV and CAV infections by appearance of the clinical signs and symptoms of the diseases or post mortem examination. The morbidity and mortality rates in the case of AAV and CAV were also recorded. Determination of any co-infection was carried out microbiologically using standard procedures.

**DNA extraction:** Tracheas, air sacs, ovaries, joints and lungs were collected from the infected birds for the isolation of *M. gallisepticum* and *M. synoviae* DNA. DNA was isolated from the samples using QIAMP® DNA Mini Kit (Qiagen GmbH, D-40724, Hilden, Germany) according to the manufacturer’s instruction. The DNA was then stored at -20 °C until used.

**Oligonucleotide primers:** Two sets of primers for *M. gallisepticum* and for *M. synoviae* were synthesized by Invitrogen (Carlsbad, California) which were used to amplify 723 bp and 207 bp from their respective viral genomes (Nascimento et al., 1991; Lauerman et al., 1993).

**For *M. gallisepticum*:** *M. gallisepticum*-F (5’-GGAT CCCATCTGACCCGAGAAAA-3’) *M. gallisepticum*-R (5’-CTTCTCA ATCAGTGTAACCTGA-3’),

**For *M. synoviae*:** *M. synoviae*-F (5’-GGAAGCAA AATAGTGATATCA-3’) *M. synoviae*-R (5’-CAGTCG CCTCCGAAGTTTACAA-3’)
**PCR amplification:** Duplex PCR was conducted in a 25 µL reaction volume, containing 12.5 µL 2 × PCR Master Mix (Fermentas, Canada), 1 µL (20 µM) each of the Primer (M. gallisepticum-F, M. gallisepticum-R, M. synoviae-F and M. synoviae-R), 1 µL each template DNA (from both AAV and CAV) and 6.5 µL nuclease free water. PCR was performed in an automatic DNA thermal cycler (Techne® Endurance TC-312). The cycling protocol consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 45 sec, 55 °C for 1 min and 72 °C for 2 min. Final extension was carried out at 72 °C for 10 min.

**Detection of PCR products:** PCR products were detected on 1% agarose gel prestained with Ethidium Bromide at 110 V for 20 min. 100 bp ladder (Gibco BRL) was used as a DNA size marker.

**Specificity and sensitivity of the duplex assay:** Specificity of the duplex PCR was assessed by examining its ability to amplify only M. gallisepticum and M. synoviae genomes. Each duplex assay performed included two simplex reactions in which M. synoviae primers were used to amplify the M. gallisepticum DNA while the M. synoviae primers were mixed with M. gallisepticum DNA in another reaction tube to check if there was any non-specific amplification. The AAV and CAV-specific primers were also used to amplify other viral and bacterial samples available at GP Lab. The sensitivity of the duplex assay was investigated by serially diluting bacterial DNA (100-1 pg) stock of both mycoplasmal DNA.

**RESULTS AND DISCUSSION**

Respiratory pathogens are responsible for huge economical losses in the poultry industry of Pakistan. The ethology of respiratory disease is always very complicated, involving more than one pathogen at the same time. M. gallisepticum and M. synoviae are considered important respiratory pathogens of poultry in our country and worldwide (Table 1). Regarding the mycoplasmosis prevalence, inadequate amount of data is available in Pakistan. Conventionally, mycoplasmosis identification is achieved via serological, cultural and immunological methods. Poultry industry is the main sector of the economy of Pakistan that produces an employment (direct/indirect) and income for about 1.5 million people. The current investment in this industry is about Rs. 200 billion, with an annual growth of 8-10%. Poultry industry in Pakistan, during the year 2004-05, had suffered an enormous loss of about Rs. 5.4 billion due to these respiratory viral infections (Government of Pakistan, 2007). Especially for M. synoviae, losses have been occurred due to transient immune depression and increase of 1 to 4% in the mortality rate of broilers in the final phase of production (Kleven, 1994). In this study, available isolates of M. gallisepticum and M. synoviae were experimentally inoculated in birds. The various organs of these birds were used for DNA isolation after the signs and symptoms typical of the above-mentioned infections had appeared. DNA extraction was carried out from various organ and PCR protocols for the detection of M. gallisepticum and M. synoviae were optimized. The optimized PCR protocols were then used to investigate the prevalence of Mycoplasmas infection in birds collected from various poultry farm of Pakistan.

Throughout the development of the duplex PCR, assay for M. gallisepticum and M. synoviae different changes to the temperature of annealing and concentrations of primers were used. The duplex PCR products consist of 732 bp for M. gallisepticum and 207 bp for M. synoviae, which were visualized by gel electrophoresis (Fig. 1). Results of the duplex PCR assay in the case of both the experimental birds and field samples indicated that the assay is highly specific for the detection of both mycoplasma species. Total 250 samples taken from the experimental birds, in the case of M. synoviae and were used for DNA extraction and subsequently subjected to the duplex assay. Among the experimentally inoculated birds, 100% samples turned out to be positive for M. gallisepticum/M. synoviae. The specificity of the duplex assay was 100% for the detection of both the mycoplasma species (Table 2 and 3). Field samples collected from poultry farms across the country were also used for the detection of mycoplasma using the duplex assay. All the birds samples used for DNA extraction had clinical manifestations of M. gallisepticum or M. synoviae infections. Analysis of the field samples (100) by duplex PCR assay indicated that 92% of samples turned

| Table 1: Typical signs and symptoms of M. gallisepticum, M. synoviae |
|--------------------------|-----------------------------------|
| **Respiratory pathogen** | **Signs and lesions**               |
| M. gallisepticum         | Chronic respiratory disease, Dyspnea, rales, Airsacculitisn, Infectious sinusitis, Conjunctivitis, Coughing, Sneezing Nasal exudates and low meat and egg production. |
| M. synoviae              | Respiratory disease, Joint exudates, low meat an egg production and poor weight gain |
out positive for either M. gallisepticum or M. synoviae (Table 2 and 3) while 8% turned to be negative by the duplex PCR which manifest signs and symptoms of mycoplasma. Negative samples were also analyzed by singleplex M. gallisepticum & M. synoviae PCR as well, which gave the same results. The duplex PCR assay developed in this study indicated a high prevalence (92%) of the mycoplasma in the tested samples (Table 2 and 3). In this study, we observed that M. synoviae was predominant mostly in the layers and breeder with respiratory problem. The high prevalence of mycoplasma may be due to the endemic nature of the infection in Pakistan caused by lack of any control strategy. Mycoplasma prevalence in chickens has been noticed to be high in many countries lacking the control strategy. For instance, M. gallisepticum prevalence was 73% in chickens in America in 1984 (Mohammad et al., 1986).

In layer, the prevalence of mycoplasma may be due to their concentration in some localities and the absence of proper sanitary barriers that may enable isolation of farms, pre-disposing farms for disease spread. Other important factors include resistance to antimicrobial treatment and immunogenic system escape. Mycoplasma can remain active for extended periods and thus can infect the new flock introduced in to sheds. Layers and breeder remain on the sheds for the extended periods and during these periods; they go through various production stages. Longer duration at sheds causes many pathogenic attacks in poultry. One of the features of the mycoplasmosis, mostly caused by the M. synoviae, is the asymptomatic manifestation, though it cause tremendous damage to the host’s health and sometime may even cause’s suppression of the host immune system (Kelven et al., 1991; Stipkovits and Kempf, 1996).

The duplex PCR assay developed is capable to detect DNA from both M. gallisepticum and M. synoviae in one single reaction. Although several studies showed that the individual PCR technique can be used for each avian respiratory pathogen (Zhao and Yamamoto, 1993; Slavik et al., 1993), however no one had the potential to detect both avian mycoplasmas in a single reaction. The duplex PCR assay developed during this study was specific and sensitive (Fig. 1). Duplex PCR developed for the simultaneous detection of M. gallisepticum and M. synoviae is very cost-effective and saves time as compared to simplex PCR for the detection M. gallisepticum and M. synoviae separately. Result of the duplex PCR showed that this optimized duplex PCR was able to detect and differentiate the avian respiratory mycoplasmas in the experimentally infected as well as in the field samples. Immunological and serological detection methods can also be used to identify mycoplasmal isolates. These include the Direct immunofluorescence, Indirect Fluorescent Antibody (IFA) test, Immuno Peroxidase (IP) tests, Growth Inhibition (GI) test, Metabolism inhibition (MI) tests, IP (OIE), Hemagglutination Inhibition (HI) test, enzyme-linked immunosorbent assays (ELISA), Serum Plate Agglutination (SPA) test and immunoblot assays. These are difficult to interpret because antibodies are present against the mycoplasmal isolates in both healthy and in infected birds. These tests are highly laborious, time consuming and confer low specificity and sensitivity (Ley, 2003; Yoder, 1991; Kleven et al., 1988). According to previous data, the sensitivity and specificity of ELISA for the detection of M. gallisepticum was 74.60% (Ahmad et al., 2008). Screening programs that are only based on seroconversion may be inadequate for mycoplasmosis diagnosis and control. These authors suggest the adoption of other techniques to confirm the presence of the agent (M. synoviae), such as DNA detection by molecular assays (PCR) because antibodies based tests are not informative about the active infection (Ewing et al., 1998). PCR represents a rapid and sensitive alternative to traditional mycoplasma culture methods, which require specialized media, reagents for serotyping isolates and are time-consuming (Kempf, 1998; Levisohn and Kleven, 2000).

Specificity of the duplex PCR assay for the M. gallisepticum and M. synoviae was determined by the ability of the M. gallisepticum and M. synoviae primers to specifically amplify M. gallisepticum and M. synoviae DNA in a PCR reaction. DNA from various viral and bacterial strains (Adeno virus, chicken anemia virus, avian pneumo virus, Mycoplasma imitit, Escherichia coli and Mycoplasma meleagris) was used in the PCR reaction along with M. gallisepticum and M. synoviae primers. In none of the reactions with other viral and bacterial types, even non-specific amplification was detected while only the M. gallisepticum and M. synoviae genomes were amplified. It reflected that these M. gallisepticum and M. synoviae

<table>
<thead>
<tr>
<th>S. No</th>
<th>Source</th>
<th>No. of samples</th>
<th>PCR positive</th>
<th>Organ used for DNA Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Field samples</td>
<td>100</td>
<td>92 (92%)</td>
<td>Tracheal swabs</td>
</tr>
<tr>
<td>2</td>
<td>Experimental samples</td>
<td>250</td>
<td>250 (100%)</td>
<td>Tracheal swabs</td>
</tr>
<tr>
<td>3</td>
<td>Total</td>
<td>350</td>
<td>342 (97.7%)</td>
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Table 2: Poultry samples tested for M. Gallisepticum by duplex PCR

<table>
<thead>
<tr>
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<td>3</td>
<td>Total</td>
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<td>343 (98%)</td>
<td></td>
</tr>
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Table 3: Poultry samples tested for M. Synoviae by duplex PCR assay
primers are 100% specific for the amplification of their respective genome. Apart from this, each time the duplex assay was carried out; two simplex reactions were performed in such a way that M. gallisepticum primers were used to amplify the M. synoviae DNA and the M. synoviae primers were mixed with M. gallisepticum DNA in another tube to check if there appeared any amplified product.

Sensitivity of the assay we determined by serially diluting the bacterial DNA. Initially, we started the PCR reaction for ILTV having 500 pg DNA per reaction, which did not give any amplification. Lowering the DNA concentration had a significant effect on PCR amplification of the viral DNA as DNA in the range of 1-100 pg could be detected. The duplex PCR was able to detect DNA from both M. gallisepticum and M. synoviae at levels as low as one pg.

In Pakistan, due to shortage of technological expertise in the modern diagnostics, avian respiratory infections are usually diagnosed based on clinical, microbiological and immunological techniques: which usually gave false or untimely diagnosis of serious infections making the management of bird not only uneconomical but also sometimes threatening for public health. Because of the economic importance of M. gallisepticum and M. synoviae for the poultry industries, we made attempts in this study to develop and optimize a cost effective duplex PCR assay and single plex PCR for the diagnostic set ups in our country. Serial dilutions of the M. gallisepticum and M. synoviae DNA extracted from the effected organ and swabs showed that as low as 1 pg DNA could be effectively detected. The specificity of the duplex assay was determined by using the primer specific for M. gallisepticum to amplify the M. synoviae and vice versa. The specificity of the duplex PCR assay was also determined by using the primers specific for M. gallisepticum and M. synoviae against the other viral and bacterial DNA isolated from birds in the GP Lab. We noticed that no non-specific amplification occurred when these above-mentioned primers were used against other viral and bacterial DNAs. The specificity and sensitivity of the duplex and single plex PCR assays developed during the study was high enough to be used for the diagnosis, screening and surveillance of the poultry. These assays were also less time consuming.

M. gallisepticum and M. synoviae infections are prevalent among the poultry birds of Pakistan, causing high mortality and morbidity. The duplex PCR assay for M. gallisepticum and M. synoviae is highly sensitive and 100% specific for the detection of respiratory pathogens. This could be used for screening of poultry birds and poultry products. It is also cost effective and will save precious foreign exchange spent on the diagnosis of these pathogens, from the other countries. The study will also help to develop potent vaccines against the local types.

Based on the above conclusions, it can be recommended that imported birds should be screened for M. gallisepticum and M. synoviae using the optimized protocol.

Further, it can also be recommended that characterization of novel strains should be carried out. Flocks should be monitored for the appearance of novel strains on regular basis to devise timely diagnostic strategies.

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