

## Research Article

### 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 Mycoplasmaales 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 Mycoplasmaales and family Mycoplasmataceae. *M. gallisepticum* and *M. synoviae* are regarded to be the most important of the pathogenic mycoplasmas and both are prevalent

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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 causes sinusitis, air sacculitis, synovitis and meningitis (Ghazi khania *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, aerosaculitis, 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 it 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, ovary, 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, ovary, 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 QIAampR 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 CCCATCTCGACCACGAGAAAA-3') *M. gallisepticum*-m-R (5'-CTTTCA ATCAGTGAGTAACTGATGA-3'),

**For *M. synoviae*:** *M. synoviae*-F (5'-GAGAAGCAA AATAGTGATATCA-3') *M. synoviae*-R (5'-CAGTCG TCTCCCGAAGTTTAACAA-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

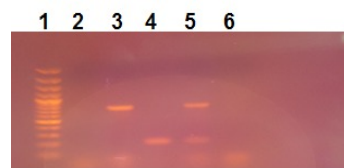


Fig. 1: Agarose gel electrophoresis of duplex PCR-amplified products from purified DNAs of known avian respiratory pathogens; Lane 1: DNA marker; Lane 2: MI; Lane 3: *M. gallisepticum*; Lane 4: *M. synoviae*; Lane 5: *M. gallisepticum*; *M. synoviae*; Lane 6: Negative control

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
<i>M. gallisepticum</i>	Chronic respiratory disease, Dyspnoea, rales, Airsacculitis, Infectious sinusitis, Conjunctivitis, Coughing, Sneezing Nasal exudates and low meat and egg production.
<i>M. synoviae</i>	Respiratory disease, Joint exudates, low meat and egg production and poor weight gain

Table 2: Poultry samples tested for *M. Gallisepticum* by duplex PCR

S. No	Source	No. of samples	PCR positive	Organ used for DNA Isolation
1	Field samples	100	92 (92%)	Tracheal swabs
2	Experimental samples	250	250 (100%)	Tracheal swabs
3	Total	350	342 (97.7%)	

Table 3: Poultry samples tested for *M. Synoviae* by duplex PCR assay

S. No	Source	No. of samples	PCR positive	Organ used for (DNA Isolation)
1	Field samples	100	92 (92%)	Tracheal swabs
2	Experimental samples	250	250 (100%)	Tracheal swabs
3	Total	350	343 (98%)	

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 single plex *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 imititis*, *Escherichia coli* and *Mycoplasma meleagridis*) 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* 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 of 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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#### REFERENCES

- Ahmad, A., M. Rabbani, T. Yaqoob, A. Ahmad, M.Z. Shabbir and F. Akhtar, 2008. Status of Igg antibodies against Mycoplasma Gallisepticum in non-vaccinated commercial poultry breeder flocks. J. Anim. Pl. Sci., 18: 2-3.
- Calderon-Copete, S.P., G. Wigger, C. Wunderlin, T. Schmidheini, J. Frey, M. A. Quail and L. Falquet, 2009. The Mycoplasma conjunctivae genome sequencing annotation and analysis. B.M.C. Bioinform., 10: S7.
- Charlton, B.R., A.J. Bermudez, M. Boulianne, R.J. Eckroade, J.S. Jeffrey, L.J. Newman, J.E. Sander and P.S. Wakenell, 1996. Avian Disease Manual. In: Charlton, B.R. (Ed.), American Association of Avian Pathologists, Kennett Square, Pennsylvania, USA, pp: 115-125.
- Chin, R.P., C.U. Meteyer, R. Yamamoto, H.L. Shivaprasad and P.N. Klein, 1991. Isolation of Mycoplasma synoviae from the brains of commercial meat turkey with meningeal vasculitis. Avian. Dis., 35: 631-637.
- Ewing, M., L.K.C. Cookson, R.A. Phillips, K.R. Turner and S.H. Kleven, 1998. Experimental infection and transmissibility of *Mycoplasma synoviae* with delayed serologic response in chickens. Avian. Dis., 42: 230-238.
- Ferguson, N.M., V.A. Leiting and S.H. Kleven, 2004. Safety and efficacy of the virulent Mycoplasma

- gallisepticum strain K5054 as a live vaccine in poultry. Avian. Dis., 48: 91-99.
- Government of Pakistan, 2007. Economic Survey of Pakistan Government of Pakistan, Finance Division. Economic Advisors Wing, Islamabad, Pakistan pp: 33-34.
- Ghazi-Khanian, G., R. Yamamoto and D.R. Cordy, 1973. Response of turkeys to experimental infection with *Mycoplasma synoviae*. Avian. Dis., 17: 122-127.
- Hnatow, L.L.J., C.L. Keeler, L.I. Tessmer, K. Czymmek and J.E. Dohms, 1998. Characterisation of MGC2: A *Mycoplasma gallisepticum* cytoadhesin with homology to the *Mycoplasma pneumoniae* 30-kilodalton protein P30 and *Mycoplasma genitalium* P32. Infect. Immun., 66: 3436-3442.
- Hong, Y., M. Garcia, S. Levisohn, P. Savelkoul, V. Leiting, I. Lysnyansky, D.H. Ley and S.H. Kleven, 2005. Differentiation of *Mycoplasma gallisepticum* and other DNA-based typing methods. Avian. Dis., 49: 43-49.
- Kempf, I., 1998. DNA amplification methods for diagnosis and epidemiological investigations of avian mycoplasmosis. Avian. Patho., 27: 7-14.
- Kleven, S.H., 1994. Summary of discussions Avian *Mycoplasma* team, international research program on comparative Mycoplasmaology. Avian. Patho., 23: 587-594.
- Kleven, S.H., 1995. *Mycoplasma* research methods for lab and field explained. Poultry. Digest., 54: 40-41.
- Kleven, S.H., 1997. *Mycoplasma Synoviae* infection. In: Calnek, B.W., H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif (Eds.), Diseases of Poultry. Iowa State University Press, Iowa, Ames IA, USA, pp: 220-228.
- Kleven, S.H., 2003. Mycoplasmosis. In: Saif, Y.M., H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, D.E. Swayne (Eds.), Diseases of Poultry. 11th Edn., Iowa State Press, Blackwell, Ames, Iowa, pp: 719-774.
- Kleven, S.H., G.N. Rowland and N.O. Olson, 1991. *Mycoplasma Synoviae* Infection. In: Calnek, B.W., H.J. Barnes, C.W. Beard, H.W.J. Yoder (Eds.), Diseases of Poultry. Iowa State University Press, Ames, Iowa, pp: 223-231.
- Lauerma, L.H., F.J. Hoerr, A.R. Sharpton, S.M. Shah and V.L. Santen, 1993. Development and application of polymerase chain reaction assay for *Mycoplasma synoviae*. Avian. Dis., 37: 829-834.
- Liu, T., M. Garcia, S. Levisohn, D. Yoger and S.H. Kleven, 2001. Molecular variability of the adhesion-coding gene *pvpA* among *Mycoplasma gallisepticum* strains and its application in diagnosis. J. Clin. Microbiol., 39(5): 1882-1888.
- Levisohn, S. and S.H. Kleven, 2000. Avian mycoplasmosis (*Mycoplasma gallisepticum*). Rev. Sci. Tech. Off. Intern. Epizo., 19: 425-442.
- Ley, D.H., 2003. *Mycoplasma gallisepticum* infection. In: Saif, Y.M., H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, D.E. Swayne, (Eds.), Diseases of Poultry. Iowa State University Press, Ames, Iowa, USA, pp: 722-744.
- Lockaby, S.B., F.J. Hoerr, L.H. Lauerma and S.H. Kleven, 1998. Pathogenicity of *Mycoplasma synoviae* in Broiler chickens. Vet. Patho., 35: 178-190.
- Marois, C., F. Dufour-Gesbert and I. Kempf, 2001. Molecular differentiation of *Mycoplasma gallisepticum* and *Mycoplasma immitis* strains by pulse-field gel electrophoresis and random amplified polymorphic DNA. J. Vet. Med., 48: 695-703.
- Mohammed, M.O., T.E. Carpenter and R. Yamamoto, 1987. Economic impact of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in commercial layer flocks. Avian. Dis., 31: 477-482.
- Nascimento, E.R., R. Yamamoto, K. Herrick and R.C. Tait, 1991. Polymerase chain reaction for detection of *Mycoplasma gallisepticum*. Avian. Dis., 35: 62-69.
- Papazisi, L., L.K. Silbart, J.R.S. Frasca, D. Rood, X. Liao, M. Gladd, M.A. Javed and S.J. Geary, 2002. A modified live *Mycoplasma gallisepticum* vaccine to protect chickens from respiratory disease. Vaccine, 20: 3709-3719.
- Rehman, L.U., B. Sultan, I. Ali, M.A. Bhatti, S.U. Khan, K.U. Zaman, A.T. Jahangiri, N.U. Khan, A. Iqbal, Z.A. Swati and M.U. Rehman, 2011. Duplex PCR assay for the detection of avian adeno virus and chicken anemia virus prevalent in Pakistan. J. Viro., 8: 440.
- Rhoades, K.R., 1977. Turkey sinusitis: Synergism between *Mycoplasma synoviae* and *Mycoplasma meleagridis*. Avian. Dis., 21: 670-674.
- Rosales, A.G., 1991. Respiratory infection in saopaulo. Proceeding of APINCO 1991 Campinas Conference. Sao Paulo., Brasil, pp: 163-176.
- Stipkovits, L. and I. Kempf, 1996. Mycoplasmosis in poultry. Rev. Off. Int. Epizoot., 15: 1495-1525.
- Slavik, M.F., R.F. Wang and W.W. Cao, 1993. Development and evaluation of the polymerase chain reaction method for diagnosis of *Mycoplasma gallisepticum* infection in chickens. Mol. Cell. Probes., 7: 459-463.
- Yoder, H.W., 1991. Mycoplasmosis. In: Calnek, B.W., H.J. Barnes, C.W. Beard and Others (Eds.), Diseases of Poultry. Ames, Iowa, USA, pp: 196-198.
- Zhao, S. and R. Yamamoto, 1993. Detection of *Mycoplasma synoviae* by polymerase chain reaction. Avian. Patho., 22: 533-542.