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Article

Isolation and molecular detection of avian mycoplasmosis in selected areas of Mymensingh district in Bangladesh

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Abstract: Avian mycoplasmosis caused by several species of *Mycoplasma* including *Mycoplasma gallisepticum, M synoviae, M. meleagridis and M. iowae.* Among these *Mycoplasma gallisepticum* is the most important poultry pathogen in Bangladesh. For effective control of Mycoplasmosis, proper early diagnosis is the corner stone. The research work was designed, a total of 20 samples, lung exudates, swabs from trachea and air sacs were collected from dead birds of different poultry farms in Mymensingh district during October-December, 2007. Samples were collected in 10% buffered formalin for histopathological study. Swabs were collected in mycoplasma broth supplemented with supplement-G. Additionally Kanamycin solution was added to prevent the growth of gram–Ve bacteria and then the organisms were transferred into mycoplasma agar for isolation. Histopathological studies were conducted using routine procedure in Hematoxylin and Eosin stain. Isolated Mycoplasma were subjected to DNA extraction, Nested PCR was done using a commercial PCR kit. The histopathological study revealed the presence of mycoplasmal related tissue changes, such as severe congestion and infiltration of mononuclear cells in different organs. The extracted DNA accumulated at the upper position of DNA ladder as band without any smear formation. The DNA from avian mycoplasmas was amplified and gave amplified product of 975 bp by outer primer and 395 bp by inner primer which was much smaller than the expected size. In this study, preliminary results from field samples suggest that culture using mycoplasma agar and broth supplement with Supplement-G and Kanamycin solution could be useful for the isolation of pathogenic avian mycoplasmas.

Keywords: mycoplasmosis; *Mycoplasma gallisepticum*; culture; polymerase chain reaction

1. Introduction

Mycoplasma gallisepticum is an avian pathogen that causes the chronic respiratory disease of chickens and results in significant economic losses to the poultry industry (Jenkins *et al*., 2007). The first Mycoplasma identified in 1890 was *Mycoplasma mycoides* subspecies mycoides, the cause of contagious bovine pleuropneumonia. Similar types of Mycoplasmas which were subsequently identified, called pleuropneumonia-like organisms (PPLO).

Avian Mycoplasmosis can be caused by several species of *Mycoplasma* including *Mycoplasma gallisepticum, M synoviae, M. meleagridis and M. iowae.* Among these *Mycoplasma gallisepticum* is the most important poultry pathogen in Bangladesh. It causes a continuing problem in commercial poultry industry and can cause significant economic loss from chronic respiratory disease, reduced feed efficiency, decreased growth and decreased egg production (Butcher, 2002).

M. gallisepticum infections are also known as chronic respiratory disease (CRD) of chickens (Butcher, 2002). They only remain viable in the environment, outside the chicken, for typically up to 3 days. For this reason, MG

is fairly easy to eliminate on single-age, all-in all-out poultry farm (Gary, 2007). In Bangladesh the prevalence of Mycoplasmosis markedly increased in the winter season and may reach upto 61.45% which is a threat for our poultry sector (Sikder *et al.,* 2005). The prevalence of *Mycoplasma synoviae* in layers occur in the age older than 60 weeks (Dufour-Gesbert *et al.,* 2006). The morbidity of the disease is variable and may be as high as 100%. Mortality in broiler is usually less than 1% but may increase up to 10% (Kleven, 1997).

The natural hosts of MG are Chickens, turkeys, quails, parrots, pheasants, pigeons, and peacocks (Ley, 2003). *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are pathogenic for chickens and turkeys, and *Mycoplasma jowae* affects primarily turkeys, and *Mycoplasma meleagridis* infects turkeys only (Ley and Yoder, 1997*), Mycoplasma synoviae* may cause either respiratory disease or synovitis (Kleven, 1997). The mode of Transmission of avian Mycoplasma is very wide and transmission occurs both vertically and horizontally (Fabricant and Levine, 1963; Yoder and Hofstad, 1965; Glisson and Kleven, 1984). The severity of clinical manifestations of *M. gallisepticum* varies widely. In chickens, infection may be in apparent or result in varying degrees of respiratory distress, with slight to marked rales, difficult breathing, coughing, and/or sneezing.

Only early diagnosis of the disease could be the corner stone of the control programme. The organisms can be diagnosed by serological tests, culture and nucleic acid based technologies (Nascimento *et al.,* 2005). The laboratory method to culture and identification of Mycoplasma takes 5-10 days and these methods are tedious, expensive and confer little guarantee of sensitivity and species specificity. Most *Mycoplasma* species grow best at 37 °C, increased humidity and CO2 tension in the atmosphere to enhance growth of Mycoplasma (OIE, 2005). Several suitable media are required for isolation of the organisms such as-Mycoplasma agar base, Mycoplasma broth base, Mycoplasma supplement-G.

To overcome these problems a more rapid and sensitive method is required for diagnosis of Mycoplasmosis. The organisms are usually diagnosed by isolation and serological tests. Several serological tests are used to detect *Mycoplasma gallisepticum* (MG) antibodies, the most commonly used tests are rapid serum agglutination (RSA) test, enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition (HI) test, radioimmunoassay, microimmuno- fluorescence and immuno passive (IP) assay (OIE, 2008). The use of serological tests has been limited due to low sensitivity or specificity and specific hyper immune sera are required which are not commercially available. On the other hand isolation of these organisms is very much difficult because these fastidious organisms require specific growth medium and the process is time consuming. Recently, nucleic acid based technologies such as in vitro amplification of DNA by the polymerase chain reaction (PCR), DNA probes, and restriction fragment length polymorphism (RFLP) are used successfully for the identification of mycoplasmosis. Polymerase chain reaction (PCR) has been proven as a rapid, sensitive and specific diagnostic test for identification of these fastidious organisms. Polymerase chain reactions based on 16S rRNA gene used to differentiate two major avian mycoplasmas such as *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). The objectives of the present study were (i) to develop histopathology, culture and PCR protocol for the rapid and sensitive diagnosis of avian Mycoplasma; (ii) to develop a good cultural protocol for the isolation and identification of avian Mycoplasma.

2. Materials and Methods

The research work was performed in the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh. Histopathological studies from Mycoplasma infected chickens, isolation and identification of organisms from collected samples, extraction of DNA, PCR, agar gel electrophoresis were performed.

2.1. Sources of samples

A total of 20 samples (lung exudates, and swabs from trachea, larynx, and air sacs) were collected from dead birds of 5 different poultry farms in Mymensingh district during post mortem examination. Packed sterilized cotton swabs were taken and tubes were used for collection of swabs from air sacs, trachea, lungs, nasal cavity, and infra-orbital sinus.

2.2. Histopathology

Larynx, trachea, lungs were collected for histopathological study by hematoxylin and Eosin stain (Nunoya, *et al.,* 1995; Gaunson *et al.,* 2000; Rodrigues *et al.,* 2001; Lam, 2003; Saha *et al.,* 2006).

2.3. Media for culture

Media used for this research work were Mycoplasma broth base on Oxiod Ltd. Bahingstoke, Hampshire England, Mycoplasma agar base on Mycoplasma selective suppliment-G on Oxoid Ltd (Bridson, 1998), Kanamycin solution (Sigma Chemical Co. ST. Loui, USA).

2.4. Isolation and identification of Mycoplasma Organism

Swabs were collected from air sacs, trachea and lungs in Mycoplasma broth in sterile condition (Vance *et al.*, 2004). Swabs containing test tubes were incubated at 37 °C for 5 days for growth of the organisms. From primary Culture, organisms were inoculated in mycoplasma agar in candle jar at 37 °C for 5-7 days (OIE, 2005). After growing of the organisms, ten fold dilusion was performed (Timenetsky *et al.*, 2006). Then Subculture was performed in Mycoplasma agar for second and third time (for 5-7 days). The organisms formed egg fried appearance colony (Del *et al.*, 1995) and stock culture were made in mycoplasma broth.

2.5. Inoculation of different inoculums on media

The mycoplasmal broth medium was used for the primary growth of organisms from collected samples. Then these were streaked on mycoplasma agar and subculture also was performed on these medium for obtaining pure culture of the organisms. Inoculated plates were incubated at 37 °C in candle jar for 5-7 days. Increased humidity and CO2 tension in the atmosphere were provided (Harasawa *et al.*, 2004; Laura *et al.*, 2005; OIE, 2008; Gharaibeh *et al.*, 2008).

2.6. Identification of isolated Mycoplasma by using Nested-PCR

Nested PCR was used to detect the organisms. PCR was done as per standard procedure (Mardassi *et al*., 2004) using a commercial PCR kit (PCR Master Mixture Kit, FINNZYMES-PhusionTM Flash, Keilaranta 16 A, 02150 Espoo, Finland). Primers used in this study are summarized in Table 1.

2.7. Quantification of DNA concentration and Primers used in the study

For quantification of DNA concentration, the spectrophotometer's wavelength was set at 260 nm after the spectrophotometer UV lamp was warmed up.

Table 1. Primers used in this study.

Primer			Sequence	Reference	bp
Outer	F	pMGAFo	GTGAAGAAAAAAAACATATTAAAGTTT	Mardassi *et al*. 2004	1900
	R	pMGARo	CTAAGATGGATTTGAAACATTAGT		
Inner	F	pMGAFi	CTAGTTAATACTAGTGATCAAGTGAAACTA		500
	R	pMGARi	TTGAACATTGTTCTTTGGAACCATCAT		

3. Results

3.1. Clinical History

A total of 20 samples, which include lung exudates, swabs from trachea and air sacs were collected from five different poultry flocks with suspected mycoplasmosis. The clinical histories of the outbreaks are summarized in Table 2.

Table 2. Clinical history of the suspected *Mycoplasma* outbreaks.

Sample no.	Type of bird	Age	Flock Size	Clinical signs	Morbidity	Mortality
1	Broiler	16 days	1000	Nasal secretions, tracheal rales, decrease body weight.	50%	9%
2	Layer	32 weeks	170	Drowsiness, whitish to greenish feces, decrease egg production	30%	0%
3	Broiler	7 days	500	Marked depression,	20%	5%
4	Broiler	20 days	900	Dullness, nasal secretions, tracheal rales, whitish faces	15%	6.5%
5	Broiler	21 days	500	Gasping, nasal secrtion, tracheal rales, less intake of food.	20%	6%

Clinical signs, morbidity and mortality reported by the farmers at the time of submission of samples were recorded. Nasal secretion, tracheal rales, decrease egg production, decrease weight were the common clinical signs in the affected blocks. At the time of submission of the samples, the morbidity and mortality in the flocks ranged from 15% to 50% and 0% to 9%, respectively.

3.2. Pathological studies

Gross and histopathological findings of poultry confirmed for Mycoplasmas by culture and molecular study.

3.3. Gross lesions

Postmortem findings included catarrhal exudates in the nasal passages and trachea and also cheesy exudates around the eye and in trachea in few of the affected chickens. In some cases, hemorrhages were also present in trachea. Lungs were severely consolidated with fibrin deposition (Figure 1a). The pleura became thickened and fibrosed. Fibrinous perihepatitis with necrotic foci (Figure 1b) and pericarditis were observed in some chickens. The thoracic air sac contained caseous mass, and abdominal air sacs became cloudy and thickened.

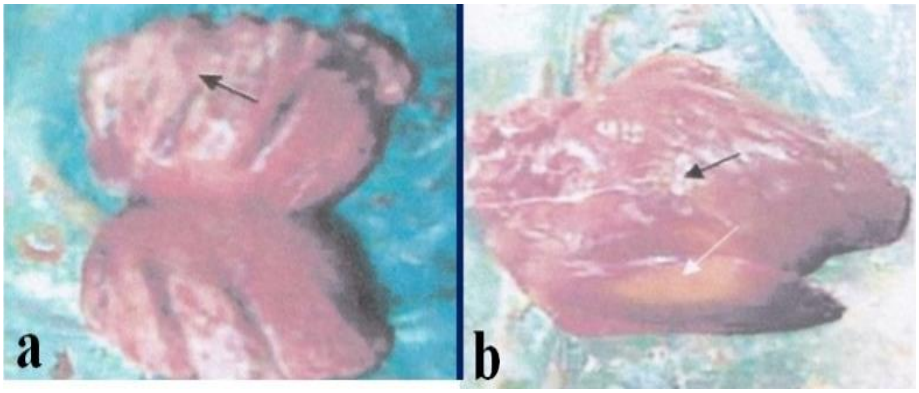

Figure 1. Consolidated lungs (black arrow) with fibrin deposition in Mycoplasma infected chicken (a) and Perihepatitis (black arrow) with necrotic foci (white arrow) in liver of Mycoplasma infected chicken (b).

3.4. Histopathological findings from positive cases

Severe congestion and infiltration of mononuclear cells were found in the section of Larynx (Figure 2a). In trachea, mononuclear cellular infiltration in the mucosa and sloughing of mucosal lining were found (Figure 2a).In the lung, chronic interstitial pneumonia with loss of bronchiolar epithelium was found (Figure 2b). Pneumonia was characterized by severe congestion and accumulation of fibrin in the lung parenchyma (Figure 2c).

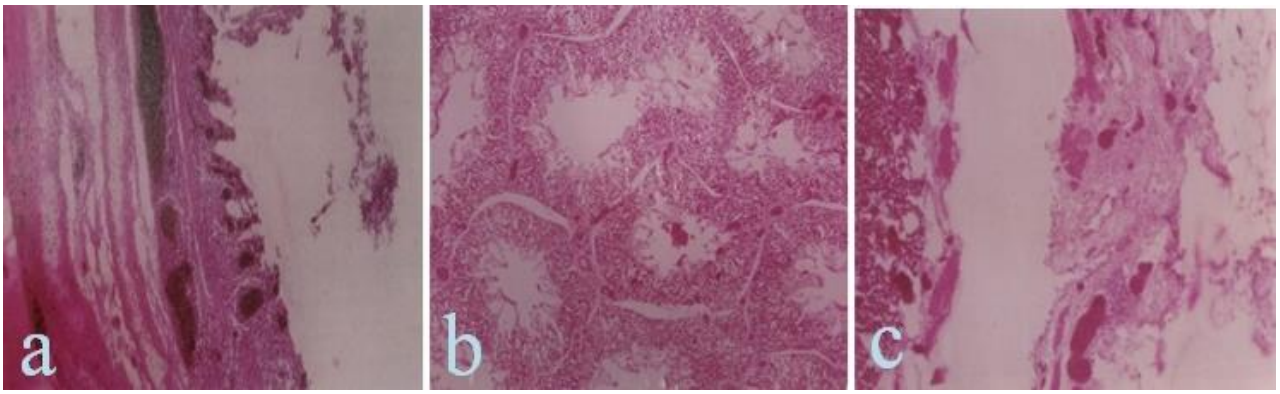

Figure 2. Section of trachea showing loss of mucosal epithelium, infiltration of mononuclear cells and congestion in mucosa (H & E staining, x 82.5) (a), Chronic interstitial pneumonia with loss of bronchiolar epithelia (H & E staining x 82.5) (b) and Pneumonia characterized by severe congestion and accumulation of fibrin in the lung parenchyma (H & E staining, x 330) (c).

3.5. Isolation and Characterization of Mycoplasma species by different methods

3.5.1. Cultural characteristics in Mycoplasına broth

Swabs containing test tubes were incubated at 37°C for 7 days for the growth of the organisms that was found from 5 days of inoculation. The growth of *Mycoplasma spp.* in mycoplasmal broth was characterized by diffused turbidity (Figure 3).

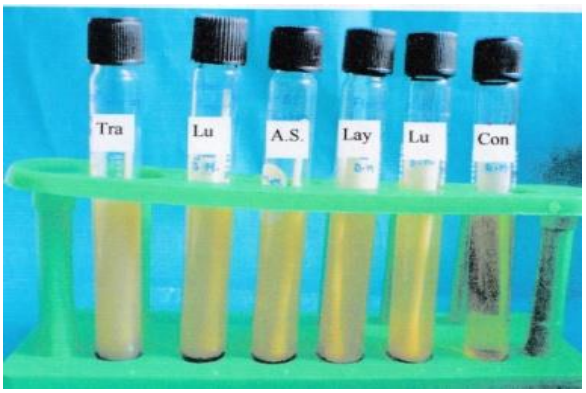

Figure 3. Mycoplasmal broth showed turbidity of the medium. (Tra=Trachea, Lu-Lung, A.S. =Air sac, Lay=Larynx, Con=control, no inoculation)

3.5.2. Cultural characteristics in Mycoplasma agar

Culture was performed in mycoplasma agar in candle jar at 37°C. After 7 days, the growth of the organisms was observed (Figure 4a).

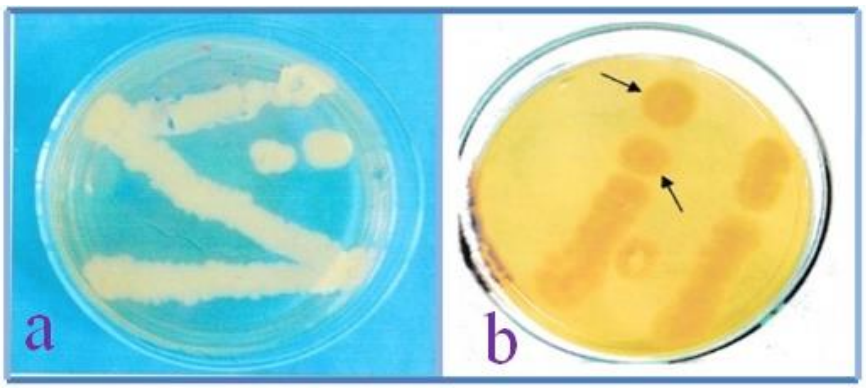

Figure 4. Mycoplasma agar showed growth of organisms but typical egg fried appearance colony could not be found after 7 days (a). Mycoplasma agar showing typical egg fried appearance colony (arrow) with a dense raised centre in colonies (arrow) (b).

A typical fried egg apperance Mycoplasma colony was found after 3 passage at 7days. (Figure 4b)

3.5.3. DNA Extraction and Polymerase Chain Reaction

For DNA extraction bulk culture was done from a single colony of Mycoplasma agar in Mycoplsama broth for 3days at 37^0c in incubator. The extracted DNA revealed a good quality which accumulated at the upper position of DNA ladder as band without any smear formation (Figure 5a).

The 16S rRNA gene of avian mycoplasmas was amplified by using commercial PCR kit (PCR Master Mixture Kit, FINNZYMES OY, Keilaranta 16 A, 02150 Espoo, Finland) and genus specific primer. Samples (1, 2 and 3) were amplified and gave amplified product of 975 bp by outer primer (Figure 5b) and 395 bp by inner primer with annealing temperature 48°C-57°C but the better result yield from 48°C, 48.8°C, 50.6°C (Figure 5c).

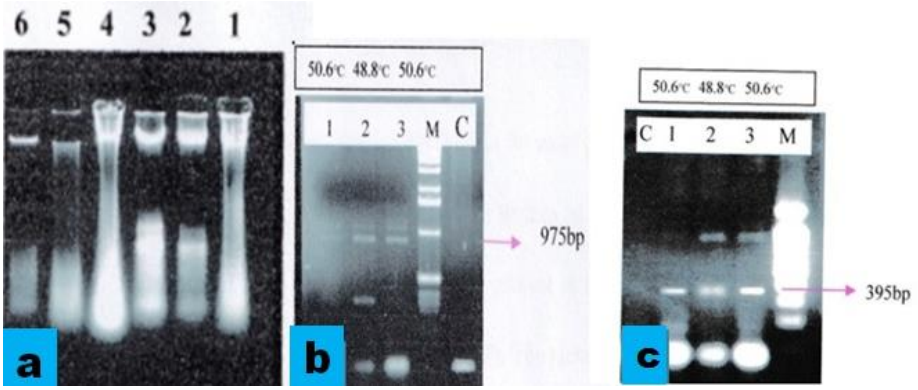

Figure 5. Electropherogram of ethidium bromide stained genomic non amplified DNA of *Mycoplasma.* Lane 1-6 different *Mycoplasma* isolates (Figure 5a). Agarose gel electrophoresis of PCR products from avian mycoplasmas using outer primers designed by Mardassi *et al,* (2004). M=Marker, 1, 2 and 3= Mycoplasma samples, C=Water control. Lane 1-3 showed positive band (975 bp) (Figure 5b). Agarose gel electrophoresis of PCR products from avian mycoplasmas using inner (primers designed by Mardassi et al., (2004). M=Marker, C=Water control; 1, 2 and 3 = Mycoplasma samples. Lane 1-3 showed positive band (395 bp) by inner primer (Figure 5c).

4. Discussion

Mycoplasma gallisepticum is an avian pathogen that causes the chronic respiratory disease of chickens (Wang *et al.,* 1997; Yang H. *et al.,* 2004; Jenkins *et al.,* 2007; Butcher, 2007). Like other poultry producing countries, mycoplasma infection is one of the important economic diseases for poultry industry in Bangladesh, both for commercial exotic breeds and indigenous local breeds. For effective control of the disease, proper early diagnosis is the corner-stone. The present study was conducted principally to describe the clinical, gross and histopathological findings in infected chickens, for the diagnosis of mycoplasma in chickens.

In pathological investigation, catarrhal exudates were found in the nasal passages and trachea and cheesy exudates around the eye and trachea in few of the affected chickens. In some cases, haemorrhages were also present in trachea. Lungs were severely consolidated with fibrin deposition. The pleura became thickened and fibrosed. Fibrinous perihepatitis and pericarditis were observed in some chickens. The thoracic air sac contained caseous mass, and abdominal air sacs became cloudy and thickened. These types of pathological lesions were supported by different authors for mycoplasmosis (Ley and Yoder, 1997; Nunoya *et al.,* 1995; Rodrigues *et al.,* 2001; Bradburry, 2001).

Histopathological methods were used for the detection of tissue alterations in Mycoplasma infected cases. Microscopically, in trachea, mononuclear cell infiltration in the mucosa and sloughing of mucosal lining were found. In the lung, chronic interstitial pneumonia with loss of bronchiolar epithelium was found. Severe congestion and infiltration of mononuclear cells in the lung parenchyma and in bronchioles were found. Pneumonia was characterized by severe congestion and accumulation of fibrin in the lung parenchyma. Severe congestion and infiltration of mononuclear cells were found in the section of larynx. These types of histopathological lesions were supported by different authors (Nunoya, *et al.,* 1995; Gaunson *et al.,* 2000; Rodrigues *et al.,* 2001; Lam, 2003; Saha *et al.,* 2006).

Mycoplasmal cultures in mycoplasma selective media with kanamycin were used for the isolation of Mycoplasma spp. Previously only Penicillin was used in media culture to prevent the growth of bacteria but it could only prevent the Gram positive bacteria, as a result typical colony in Mycoplasma agar could not be found. In this study, Kanamycin solution was used in broth media to overcome the problem because it prevents the growth of all Gram negative bacteria. Samples were collected from oropharynx, trachea, and air sacs in this study but lung sample yielded better result as previously described (Vance *et al.,* 2004; Kleven *et al.,* 1989; OIE, 2008). Mycoplasma agar base, Mycoplasma broth base, Mycoplasma supplement-G' were used to propagate the organisms (Bridson, 1998). After collection of sample, the swabs were inoculated into mycoplasma broths and incubated at 37°C for 7 days which were supported by different authors (Ley *et al.,* 1997; Harasawa *et al.,* 2004; Gharaibeh *et al.,* 2008). Turbidity was found in the mycoplasmal broth and the results are in agreement with a previous study (Del and Tully, 1995; Kleven, 1997; OIE, 2008).

The agar plates were incubated at 37°C for 28 days with 3 passages. CO2 tension and extra moisture was supplied in candle jar to enhance growth of organisms which were supported by different authors (Harasawa *et al.,* 2004; Laura *et al.,* 2005; OIE, 2008; Gharaibeh *et al.,* 2008). Growth of organisms was observed after 7

days but typical colonies were found after 28 days in mycoplasma agar which were smooth, circular, nipple or fried egg appearance colony and there were no growth of bacteria due to use of kanamycin solution in broth corresponded with the findings of other authors (Del and Tully, 1995; Yoder and Hofstad, 1964; OIE, 2008).

Polymerase Chain Reaction (PCR) is a molecular technique which is specific for identification and differentiation of *Mycoplasma* species (Slavik *et al.,* 1993; Ricardo *et al.,* 1996; Zhang *et al.,* 1999; Lia *et al.,* 2000). In this study, Nested PCR was used to detect avian mycoplasmas which were supported by several authors (Mardassi *et al.,* 2004; Vogl *et al.,* 2008). Gradient temperatures were used in this study, to amplify the target portion of DNA. The ranges of annealing temperature were 48°C-57°C but better results obtain from 48°C, 48.8°C, 50.6°C. Two sets of oligonucleotide primers PMGAF, PMGAR. (Outer) and PMGAFli, PMGAR1; (Inner) were selected for the diagnosis of avian mycoplasmas. PCR products of pathogenic avian mycoplasmas were amplified in agarose gel. It was expected that outer primer will produce an amplicon of 1900 bp and inner primer will produce an amplicon of 500 bp. However in this study the primers amplified the DNA but the expected size was much smaller, 975 bp with outer primer and 395 bp with inner peimer. Therefore, the primer needs to varisied again.

5. Conclusions

Typical Mycoplasma colonies were found after 3rd passages of 7 days inoculation period. Other bacterial growth was not demonstrated due to addition of kanamycin solution in the Mycoplasma broth. However bacterial contamination is a common problem encountered in Mycoplasma isolation. This method might be useful for many laboratories, because the antisera are not commercially available which is required for serological tests. In this study, preliminary results from field samples suggest that culture using kanamycin solution could be a useful diagnostic test for the isolation of pathogenic poultry Mycoplasmas.

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Conflict of interest

None to declare.

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