IJAS Iranian Journal of Applied Animal Science

Polymerase Chain Reaction of Mgc2 and 16S rRNA Genes for Detection of *Mycoplasma gallisepticum*

Research Article

A. Zakeri [1*]

[1] Department of Animal Science, Tabriz Branch, Islamic Azad University, Tabriz, Iran

Received on: 27 Apr 2015
Revised on: 8 Jun 2015
Accepted on: 15 Jun 2015
Online Published on: Mar 2016

*Correspondence E-mail: zakeri@iaut.ac.ir

Online version is available on: www.ijas.ir

ABSTRACT

Mycoplasmas are very small bacteria lacking cell walls that belong to various genera within the class Mollicutes, and also the smallest organisms that can live independently. They are able to cause serious and chronic disease because of some unique characteristics. *Mycoplasma gallisepticum* (MG) is an important avian pathogen causing significant economical losses within the poultry industry. The aim of the present study was to investigate the prevalence of M. gallisepticum in poultry by PCR of mgc2 and 16 s rRNA. We examined the differentiating potential of diagnostic polymerase chain reaction (PCR) primers targeted to the 16S rRNA and mgc2 genes present in MG. For serological screaming test, we selected 26 farms and took blood samples for RSAT assay. For 16S rRNA and mgc2 PCR assay, we took 109 samples from 10 rapid slide agglutination test (RSAT) positive farms, including: lung, air sacs and tracheal swabs. The 16S rRNA and mgc2 PCR diagnostic primers are specific for MG in tests of all avian Mycoplasmas or bacteria present in the chicken trachea and are sensitive enough to readily detect MG in tracheal swabs from field outbreaks. 530 bp and 300 bp PCR products on electrophoresis gel appeared respectively with 16S rRNA and mgc2 PCR diagnostic primers, specific for MG. The test was successfully applied *in vivo* for detection of MG in clinical samples.

KEY WORDS 16S rRna, mgc2, PCR, Mycoplasma gallisepticum.

INTRODUCTION

Mycoplasmas are widespread in nature as parasites of human, mammals, reptile's fish, arthropods and plants (Kleven, 2008a). *Mycoplasmas* differ from other bacteria in their very small size and total absence of a cell wall; these characteristics account for their ' fried egg' type of colonial morphology, complete resistance to antibiotics that affect cell wall synthesis and their complex nutritional requirements. They tend to be host-specific, so that avian *Mycopalamas* are not generally known to infect mammalian or other species, but some non-avian *Mycoplasmas*, such as *Mycoplasma bovis*, can cross the species barrier and have been found in avian species. The avian *Mycoplasma* species that is pathogenic to commercial poultry, namely *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in chickens and turkeys and *Mycoplasma meleagridis* and *Mycoplasma iowae* in turkey, have been associated with significant economic losses (Kleven, 2008b; Lysnyansky *et al*. 2005). *Mycoplasma iowae* has more antigenic diversity, can survive longer in the environment and is relatively more resistant to many antimicrobials than the other avian *Mycoplasmas*. The diagnosis of avian pathogenic *Mycoplasmas* has typically been carried out by culture, serology and molecular assays such as polymerase chain reaction (PCR) and DNA probe. These tests each have advantages and disadvantages. Traditional culture isolation of *Mycoplasmas* is time-consuming and complicated, requir-

ing tow to three weeks to be completed, because of the slow- growing nature of some mycoplasmas such as *Mycoplasma iowae*. Birds infected with *Mycoplasma iowae* do not consistently produce a humoral response that can be detected by the conventional serological tests that are used with the other avian *Mycoplasma* pathogens. 16S ribosomal RNA gene is a component of the 30S subunit of prokaryotic ribosome and it is 1542 nucleotides in length and widely use to molecular detection of microorganism such as mycoplasmas. The avian pathogen *Mycoplasma gallisepticum* induces severe chronic respiratory disease in chickens as well as sinusitis in turkeys. These diseases are globally prevalent and economically damaging to the Iranian poultry industry due to their effects on feed efficiency. Although *Mycoplasma gallisepticum* infections mainly affect the respiratory tract, they may occasionally cause keratoconjuctivitis, salpingitis, arthritis and fatal encephalopathy. The avian pathogen can be transmitted from infected birds via aerosol or via the egg. Recently, PVPA gene and mgc2 genes have been used in high accuracy to recognize *Mycoplasma gallisepticum* (Lysnyansky *et al.* 2005). One of the proteins that are effective in MG connection is mgc2. A part of this protein is located on the surface and end of organelle. The antibody of this protein inhibits the process of extra cellular fluid cells connection. Mgc2 protein has 40.9% and 31.4% of homology with P30 protein of *Mycoplasma pneumonia* and P32 MG respectively. *Mycoplsma* mutants, who have lost their viscidity, are unable to cause infection and regaining this ability goes in parallel with gaining their infectious ability. Mgc2 protein is effective in connection with MG to the host cell and its lack causes it to lose this ability (Gharaibeh and Roussan, 2008). In recent years, mgc2 gene has been considered for designing exclusive primers for this gene. To recognize MG because of its high stability in different passages and specialty for *Mycoplasma gallisepticum* (Lysnyansky *et al.* 2005; Feberwee *et al.* 2005).

This sentence does not have any meaning as it is written: the author should reconsider it and re-write accordingly. The primers like mgc2-2F/2R for mgc2 gene were first used (Lysnyansky *et al.* 2005) for recognition of *Mycoplasma gallisepticum*. The aim of this research was to determine by PCR the prevalence of *Mycoplasma gallisepticum* among poultry using 16S rRNA and mgc2 genes in clinical samples.

MATERIALS AND METHODS

Sampling for rapid serum agglutination test (RSAT)

372 serum samples from 26 farms, including 8 broiler breeders' farms and 18 layer farms were taken and sent to serology lab to RSAT test (Bradbury and Levisohn, 1996).

Preparation of culture media

The Frey culture media and PPLO cultures media were used in this study (Hosseini, 2006).

Sampling for culture

Sampling was only done from flocks which were RSA positive. In this study 510 samples from 17 broiler breeders and laying farms were taken.

Sampling of live birds

Samples were taken by using sterile cotton swabs from Chonal cleft and after inoculation in lab tube containing *Mycoplasma* culture media. Each sample was transferred into ice full flasks. Then the flask containing samples were transferred to the laboratory and incubated in 37 ˚C.

Sampling of dead birds

Samplings of necropsies carried out in birds were taken from their trachea, air sacs and lungs by cotton swabs.

Sampling for PCR

Ten out of 17 farms were chosen because positive in RSAT test and sterile swabs from Chonal cleft, trachea, air sacs and lungs were prepared from each farm. Three swabs from 3 birds were dropped into a lab tube containing 1 cc PPLO broth and transferred to laboratory.

DNA extraction

Bacterial DNA was extracted by phenol/chlorophorm method. One mL of each PPLO broth culture was centrifuged in a microcentrifuge at 14000 rpm for 5 minutes. Cell pellets were re-suspended in phosphate buffered saline (PBS). 100 µL of harvested bacterial suspension was added to 100 µL lysis buffer and the tube was placed in a 56 ˚C bath for 4 hours.

Then 200 µL saturated phenol was added and the tube was centrifuged (13000 rpm or 15700 g) for 20 min. The upper phase was transferred to the next tube and an equal volume of mixed phenol/chlorophorm (1:1) was added. After centrifugation at 13000 rpm for 20 min the aqueous phase was transferred and added to an equal volume of pure chloroform and was centrifuged (13000 rpm) for 5 min. The upper phase was mixed with 1/10 volume of acetate sodium and precipitated with a 2 fold volume of cool and absolute ethanol. After final precipitation using by 70% ethanol, the DNA was dried and re-suspended in 50 µL TE buffer and stored at 4 ˚C.

PCR of 16S rRNA gene and the determination of the MG species

The used primers were specific for detecting 16S rRNA gene in MG.

These primers were used to determine MG in pure fertilizing or clinical samples (Feberwee *et al*. 2005; Fan, 1995; Garcia *et al*. 2005).

These primers were MG-10F (5'-AACACCAGAGGCGAAGGCGAGG-3') and MG-11R (5'-ACGGATTTGCAACTGTTTGTATTGG-3'). The PCR reaction was done in 25 mL volume and in all aspects of positive and negative controls. Its compounds were water (17.36 µL), PCR buffer (10X)(2.50 µL), dNTP (10 m*M*)(0.75 µL), F primer (10 µm)(0.15 µL), R primer (10 µm)(0.15 µL), Tag DNA polymerase (50 U/µL) (0.10 µL (1unit)), $MgC1_2$ (50 m*M*)(2.00 µL) and Template DNA (1.94 µL). All reactions were done in Thermocycler and its program was primary isolation of DNA (5 min, 94 ˚C), 30 cycles including: isolation, coupling and elongation of DNA (30 sec in 94 ˚C, 60 sec in 58 ˚C and 30 sec in 72 ˚C) and terminal elongation (10 min, 72 ˚C).

Mgc2 PCR and the determination of the *Mycoplasma gallisepticum* species

In order to confirm the samples, the polymerase chain reaction method was used. The used primers were specific for mgc2 gene in *Mycoplasma gallicepticum* and these primers have been used for recognizing mg in pure fertilizing or clinical sample (Charlton *et al*. 1999; Collett, 2005).

These primers were mgc2-2F (5'-CGCAATTTGGTCCTAATCCCCAACA-3') and mgc2-2R (5'-TAAACCCACCTCCAGCTTTATTTCC-3'). The PCR reaction was done in 25 mL volume and in all aspects of positive and negative controls, was used simultaneously. Its compounds contained: water (19.00 µL), PCR buffer (10X)(2.50 µL), dNTP (10 m*M*)(0.20 µL), F primer (10 µm)(0.10 µL), R primer (10 µm)(0.10 µL), Tag DNA polymerase (50 U/µL)(0.10 µL (1 unit)), $MgC1_2$ (50 m*M*)(2.00 µL) and template DNA (1.00 µL). All reactions were done in Thermocycler and its program was primary isolation of DNA (5 min, 94 ˚C), 30 cycles including: isolation, coupling and elongation of DNA (30 sec in 94 ˚C, 60 sec in 58 ˚C and 30 sec in 72 ˚C) and terminal elongation (10 min, 72 ˚C). All the above reactions were done in Thermocycler, Gradient Mastercycler (Eppendroff, Germany).

Electrophoresis

Electrophoresis was run on agarose 2% gel (TAE Buffer), and then placed for one hour under 90 volt of electrical potential difference.

The gel was then extracted from the buffer and washed with water, and placed in UV (BioRAD, Bio-RAD lab, California, USA) set under irradiation. Once the picture was seen on monitor, a print out was taken (Kempf *et al*. 1993; Khan, 2002).

RESULTS AND DISCUSSION

Results of RSA test

From 372 serum samples taken from 26 farms, 17 farms were positive in RSA test. These 17 farms included 3 broiler breeders and 14 commercial laying hen. From all 372 given samples, 137 samples were positive in RSA test, 202 samples were negative, and from 33 suspicious serum samples, 10 samples were reported positive in 1/8.

Result of culture

510 samples were collected from 17 farms with 30 samples from each farm; 254 positive samples for *Mycoplasma gallisepticum* was isolated (49.8%). Usually, the first signs of growth and color change in liquid are seen at the 5th day. In some cases evidence of growth in samples was not seen until the 22nd day.

Following the color change in the medium from red to yellow, which is indicative of pH change from glucose fermentation, the sample was assumed to be positive and the following steps were carried out for confirming and purifying: 1) positive sample to the new liquid broth and solid environment and 2) storage with glycerol 5% in the freezer at -70 ˚C to verify the presence of MG in the medium broth. If the presence of MG is then confirmed in the broth, the solid environment is placed in CO_2 and high humidity until the colony is evident.

Results from 16S rRNA PCR and determination of *Mycoplasma gallisepticum* species

To confirm samples, the PCR method was used. Of all the 109 samples obtained from 10 farms, 46 samples were similar (42.2%) in PCR, showing 530 base pair bands on agarose gel. The used primers are completely specific for *Mycoplasma gallisepticum* species, creating a 530 base pairs band (Figure 1).

Results of mgc2 PCR and determination of *Mycoplasma gallisepticum* species

For confirmation of samples from 10 farms where the *Mycoplasma gallisepticum* was isolated, PCR method was used.

From 109 samples that were taken from 10 farms, 46 samples were similar to 42.2% in PCR, showing a 300 base pairs band on agarose gel. The used primers were completely specific for *Mycoplasma gallisepticum* species. In this method, mgc2-2F (Gen accession # U34842) and mgc2-2R (Gen accession # U34842) primers were used that were specific for *Mycoplasma gallisepticum* species. The PCR product produced in this reaction in all these samples and ts-11 vaccine strain was equal to 300 base pairs. The results are shown in the Figure 2.

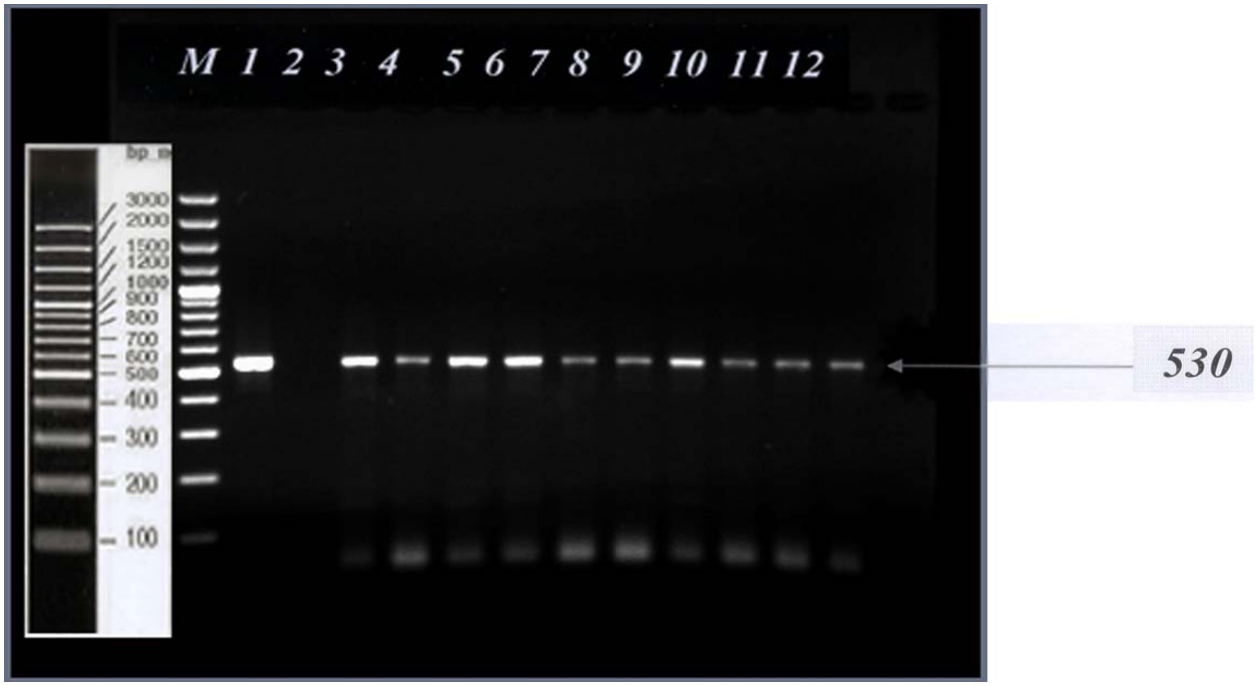

Figure 1 PCR product of 16S rRNA gene
PCR product of 16S rRNA gene with mgc2-2F and mgc2-2R primers
M: gene ruler 100 bp plus DNA ladder
Lane 1: ts-11; Lane 2: negative control and Lane 3-12: field isolated

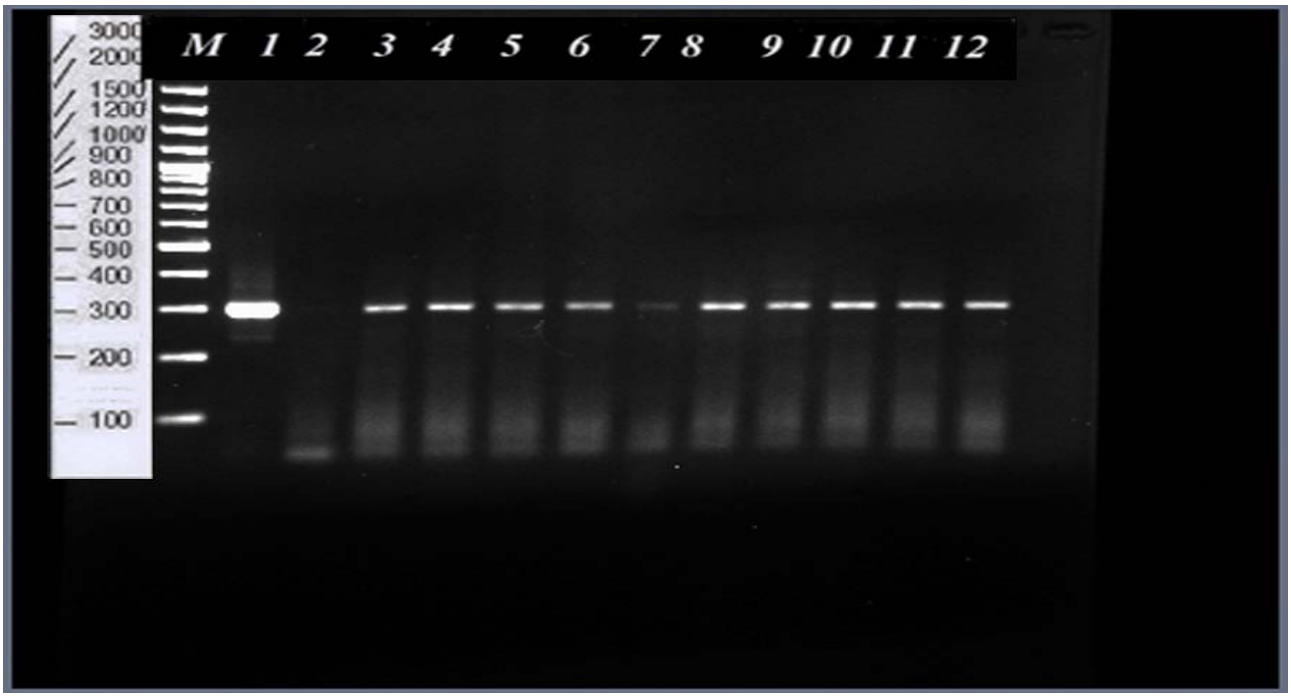

Figure 2 PCR product of mgc2 gene
PCR product of mgc2 gene with MG-10F and MG-11R primers
M: gene ruler 100bp plus DNA ladder
Lane 1: ts-11; Lane 2: negative control and Lane 3-12: field isolated

The wide occurrence of *Mycoplasmas* has frequently led researchers with little or no expertise in mycoplasmology to suggest that structures resembling *Mycoplasmas* in tissues of oysters, bryozoans and Giardia are *Mycoplasmas*. In 1958 five serotypes were described, in 1960 eight serotypes, in 1964 twelve serotypes and in 1967 nineteen serotypes of *Mycoplasma* (Gharaibeh and Roussan, 2008).

Different species of *Mycoplasmas* are *Mycoplasma gallisepticum*, *Mycoplasma iowae*, *Mycoplasma synoviae*, *Mycoplasma meleagridis*, *Mycoplasma gallinarum*, *Mycoplasma gallinaceum*, *Mycoplasma cloacale*, *Mycoplasma iners*, *Mycoplasma gallopavonis* and *Mycoplasma glacophilum*.

Mycoplasma gallisepticum is responsible for chronic respiratory disease in chickens and infectious sinusitis in turkeys. Infections in commercial poultry flocks result in significant losses due to reduced egg production, poor feed conversion rate and carcass disposal in the course of processing. *Mycoplasma gallisepticum* can be spread out only for short distances by the air-born route (Hosseini, 2006).

Whenever excellent biosecurity is practiced, there have been many instances where infection has not spread to adjacent houses within a complex. *Mycoplasma gallisepticum* infection is a chronic respiratory disease in avian species. Chickens, turkeys, quails, parrots, pheasants, pigeons and peacocks are the natural hosts of *Mycoplasma gallisepticum* infection (Ongor *et al*. 2009).

The mortality rate is low unless a secondary microorganism infection is present. Infection with this bacterium causes sneezing, conjunctivitis, air sacculitis and decreased egg production in affected birds. The *Mycoplasma gallisepticum* organisms in infected birds can be transmitted to other birds via direct contact, that is, horizontal transmission.

Mycoplasma gallisepticum diagnosis can be carried out by various techniques using necropsy to observe gross and microscopic lesions, serology to determine the immune response, including SPA and elisa tests and *Mycoplasma gallisepticum* detection to find either the organisms or their DNA, using culture and isolation and PCR procedures (Luciano *et al*. 2011).

Polymerase chain reaction technique has increased the sensitivity of organism detection based on specific sequences of nucleotides. Many studies were performed on *Mycoplasma gallisepticum* in poultry and have linkedits presence with respiratory and locomotors illness as well as economic loses.

A study on *Mycoplasma gallisepticum* showed that culture was positive for 49/73 swabs while PCR detected 70/72 positive samples (Kempf *et al*. 1993).

The use of molecular technique for detection of *Mycoplasma gallisepticum* infection was same used in this study, confirming the efficacy of the molecular test itself in poultry samples.

Mycoplasma gallisepticum has been isolated from naturally occurring infections in chickens, turkeys, pheasants, chukar partridge, peafowl, bobwhite quail and Japanese quail. *Mycoplasma gallisepticum* has also been isolated from duck, from geese, from house finches, from a golden pheasant in Australia, and from a yellow-napped Amazon parrot.

The result of PCR is available in 1 or 2 days, however for culture of *Mycoplasma gallisepticum*, 1 to 3 weeks are required. PCR can also represent an excellent test for mixed *Mycoplasma* infections or with concurrent bacterial infections as well as growth hinderers like antibody, antibiotic.

For determination of *Mycoplasma gallisepticum*, different bands of 16S rRNA, PVPA, mgc2 genes with different methods of PCR are carried out (Garcia *et al*. 2005). They have used different special primers with different methods of PCR for determining MG species. In this study, determination of MG species has been done by using the 16S rRNA gene, by forming the 163 basic pair's bands with specific primers with a sensitivity of 100% (Feberwee *et al*. 2005).

Furthermore, OIE has also determined MG species by using specific primers (Garcia *et al*. 2005). The 16S rRNA, PCR examination is for determining *Mycoplasma* species with special primers MG-10F and MG-10R with PCR product of 350 basic pairs band which is used for all fields strain and the vaccine strain ts-11, which can be used with 100% sensitivity (Ghaleh Golab *et al*. 2005).

The mgc2-PCR with 300 base pair bands for determination of *Mycoplasma gallisepticum*, is specific for the mgc2 gene of *Mycoplasma gallisepticum* and it can be distinguished from all the other *Mycoplasma* and bacteria which are present in avian's trachea (Lysnyansky *et al*. 2005).

The product of PCR by specific primers of mgc2 F/2R, only reacts with *Mycoplasma gallisepticum* and doesn't react with any one of the other 22 different species of *Mycoplasma gallisepticum* (Lysnyansky *et al*. 2005; Biro *et al*. 2006).

Nor does it react with the 2 species of acholeplasma isolated from poultry or with the 9 species of bacteria which are present in the respiration system of poultry (Lysnyansky *et al*. 2005; Biro *et al*. 2006). According to these studies the sensitivity of mgc2-PCR 15 (fg) in pure extracted DNA in every reaction of strains of MG like R strain and OR2 is reliable. According to Lysnyansky *et al*. (2005), the stability of mgc2 gene in passages 154, 74, 13 in the R strain, pa-

ages 255, 16 in the F strain, passage 50 in the vaccine strain ts-11 and passage 29 in the vaccine strain 6/85, is invariable and 100% accurate (Lysnyansky *et al*. 2005). The sensitivity of this method of determination has also been proved for 40 CCU strain ts-11 in every reaction (Gharaibeh and Roussan, 2008). In this study, from 372 serum samples which were taken from 26 farms, 17 farms and 137 serum samples in RSA test became positive. *Mycoplasma gallisepticum* in 14 farms out of 17 farms proved positive in RSA test and was isolated by culture method. From 14 farms, 10 farms were chosen and 109 sterilized swaps were obtained from different parts of respiratory system. A total of 46 samples in mgc2 PCR test for determination of *Mycoplasma gallisepticum* with specific primers mgc2-2F were used.

PCR product of 300 basic pair's bands on agarose gel became positive, concurring with previous studies. According to previous studies and the results of this study in Iran, it seems that the use of PCR of 16S rRNA and especially mgc2 genes, can be a useful method for the detection of *Mycoplasma gallisepticum* in clinical samples.

CONCLUSION

It can be concluded that mgc2-PCR test can be used in laboratories for rapid and exact determation of *Mycoplasma gallisepticum* in clinical samples. More studies on different strains of *Mycoplasma* in different regions seem necessary for evaluating the efficiency of mgc2-PCR.

ACKNOWLEDGEMENT

Great thanks to Dr. Ashtari at reference *Mycoplasma* laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran for his technical assistance.

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