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Isolation and identification of *Mycoplasma gallisepticum* in chickensbn from industrial farms in Kerman province

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Abstract

Mycoplasma gallisepticum is the most important and infectious *Mycoplasmosis.* It is caused lots of economic losses for poultry's industry of Iran. The target of this study is comparison of culture and nested PCR techniques to detect *Mycoplasma gallisepticum* infection of chicken's from industrial farms in Kerman province of Iran. 88 isolates received from industrial farms of Kerman province of Iran were measured by culture and nested PCR techniques. Two primers were used to identify Mycoplasma, but two pair's primer for gallisepticum species. *Mycoplasma galisepticum* isolation and PCR results on samples from infected chickens received from Kerman province of Iran showed that PCR test was more sensetive than culture and this protocol can use as a remarkable way to diagnose *Mycoplasma gallisepticum* infection in birds. On the other hand, nested PCR is analytical test that its sensitivity showes two pairs of nested primer (the external primers and internal primers) can amplify two rigions of GapA gene and be more sensetive. Many routine tests DNA amplification methods have been developed for diagnosis of *Mycoplasma gallisepticum* and used in many in many laboratiries. PCR method based on the GapA can amplify DNA from *Mycoplasma gallisepticum*. The PCR method can that target surface protein gene. Nested gapA PCR is more sensetive test from PCR of GapA gene. This technique is a simple and fast method to detect and isolate infected birds, so it is a way to decrease economic losses in poultry industry. Four genes were recommended to *Mycoplasma gallisepticum* PCR that GapA gene is one of them that by nested PCR can detect *Mycoplasma gallisepticum*

Key word*: Mycoplasma gallisepticum,* nested PCR, chicken.

Introduction

Mycoplasma is the simplest and smallest bacterial cell. This organism can infect and grow in plant, animal, human and insect host. The Mycoplasmosis of poultry is one of the most diseases which makes problem in our country. *Mycoplasma gallisepticum* is responsible for disease in chickens. This contagious disease probably occurs throughout the world (Kleven, S.H., 2003). Precise isolation and identification of *Mycoplasma gallisepticum* among poultry (fowl) flocks (population) improves prevention of pathogen's spreading. *Mycoplasma gallisepticum* has serotypes, but there are some heterogeneous among strains and

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they have different infectious and tissues priorities (Pourbakhsh , S.A,2005). *Mycoplasma gallisepticum* is a slow-growing organism in culture test, although the culture test can help for direct diagnosing of these bacteria. The detection of *Mycoplasma gallisepticum* requires one or more weeks for growth and diagnosis in chicken isolates (Branton, S.L, 2009). Garcia compared molecular method for *Mycoplasma gallisepticum* detection. He recommended four gene for diagnosis including LP1, GapA, mgc2 and 16srRNA. Lysnyansky used mgc1–PCR-RFLP for this purpose (Johansson, K. E, 1993). Ferqosan worked on *Mycoplasma gallisepticum* received from America and France. He analyzed mgc2, GapA and PvpA genes for GTS (gene targeting sequencing technique). Result showed that this test was more sensetive than RAPD technique (Kojima et al., 1997).

Serologic tests were used for *Mycoplasma gallisepticum* testing, but it needs at least of 1 week after infection for antibodies production. This test can be resulted by agglutination and hemagglutination inhibition. hemagglutination inhibition test requires 3 week (Stipkovits , L,1994).
Therefore, Polymerase Chain Reaction (PCR) is a rapid test, since the process can be completed in 1 day or less and sensitive method to detect organism's nucleic acid for diagnosis (Afshrifar. A.R,2005) In the most cases, the 16S rRNA gene is used for *Mycoplasma gallisepticum* PCR base, but the other PCR assay are based on other genes of this bacterial cell (Pourbakhsh , S.A,2005).
In this study, we performed nested PCR test based on GapA gene of *Mycoplasma gallisepticum*. This technique can amplify GapA gene in tracheal swabs isolates received from industrial farms in Kerman province of Iran.

MATERIALS AND METHODS

Isolation of Mycoplasma

All of isolates were tracheal swabs in PPLO broth culture. After culture tube filtration, this media were incubated in PPLO broth at 37 C ^{o} for colour change because of contamination. These isolates were inoculated in PPLO media agar plate at 37 C ^{o} for 1 week examining the presence of Mycoplasma colonies (Branton, S.L, 2009).

DNA extraction

DNA extraction was performed on 88 collected isolates from industrial farms.
First, 0.5 ml of cultures was transferred into microtubes and was centrifuged for at 13000 rpm 5 min. supernatants were discarded but precipitate were collected. 100 ml lysis buffer was added to per microtubes and mixed. This suspension incubated at 56 C ^{o} for 4 hours. Microtubes were centrifuged at 13000 rpm for 15 min. Following centrifuge, two phases was observed in microtubes. Upper phase was included total DNA. This phase was transferred into new micritube. Equal volume of Phenol/chloroform were added to microtubes, voterxed and centrifuged at 13000 rpm for 15 min. upper phase was tranfered into new microtube, added chloroform and centrifuged for 5 min. 0.1 time of upper phase was added Sodium acetate 3 M. after mix, cold ethanol was added 2 time of volume in microtube. Microtubes were incubated at -20 C ^{o} for 20 min and centrifuged at 13000 rpm for 15 min. washing step was performed on precipitate. Total DNA of isolates were stored in -70 C ^{o} after drying in 56 C ^{o}- (Evans, J. D, 2005).

Nested PCR

This test was amplified16s rRNA gene to identify *Mycoplasma* and GapA gene for species *gallisepticum* diagnosis (Thornton, D. L2009). Primers for 16s rRNA is: (Kojima et al., 1997).
M1F:5’GCTGCGGTGAATACGTTCT3’
M3R:5’TCCCCACGTTCTCGTAGGG3’

Reaction had predenature step at 94 C ^{o}, 33 cycles including denature step at 94 C ^{o} for 1 min, annealing at 55 C ^{o} in 1 min, extension at 70 C ^{o} in 1 min (Bardbury, J.M.,2001).

Table 1: sequences 4 primers in nested PCR for amplification of GapA

GapA 5FA	5'TCARCGTTTCTAAGATTCCTTTTG 3'
GapA 6RA	5'GCATCAAAACCAGTAAATTCTTGG 3'
GapA 9FB	5'TTCTAGCGCTTTARCCCTAAACCC 3'
GapA 4RB	5'CTTGTGGAACAGCAACGTATTCGC 3'

Nested amplification reaction was conducted separately in a final volume of 25 µL (10x PCR buffer, 2 mM $MgCl_2$, 10 µm deoxynucleoside triphosphate (dNTP), 14 nMconcentration of each primers (external and internal), 5 U of enzyme and 7 ng of genomic DNA as a template). Taq DNA Polymerase was used with the following amplification program: The first amplification of the GapA PCR was 94 C ^{o} for 3 min followed with 35 cycles of 94 C ^{o} for 30 S, 58 C ^{o} for 1 min and 72 C ^{o} for 30s. In the second reaction, isolates were heated for 5 min at 94°C to denature two strands DNA prior to amplification of DNA fragment for 35 cycles. Each cycle included 30 seconds of denaturation phase at 94 C °, 30 s at 56 C ° (the annealing temperature of the primers) and a 1min extension phase, that the best result was achieved at 72°C, then one 5 min at 72°C. The PCR products were run on 1% agarose gel stained with Syber safe.

Result

Culture of Mycoplasma

In PPLO culture, *Mycoplasa* survive for 15 days. Difficulties have been encountered in isolating and propagating this germ. Turbidity was indicative of bacterial contamination, so contaminated cultures were filtered and passaged. Some of broth cultures were subcultured by inoculation of fresh both media**.** In other cultures were observed any color change. In PPLO media agar plates were observed *Mycoplasma* colonies in 61 plates of 88 or % 69.

Nested PCR

Total percentages estimated in other search for single and nested PCR methods were 100% by GapA and 16srRNA genes.

For this purpose, 16srRNA PCR was performed to confirm that all isolates belonged to mycoplasma. The aim of this stage is the formation of 307 bp fragment in 88 isolates. 100 percent of these isolates showed the expected band (Fig 1).

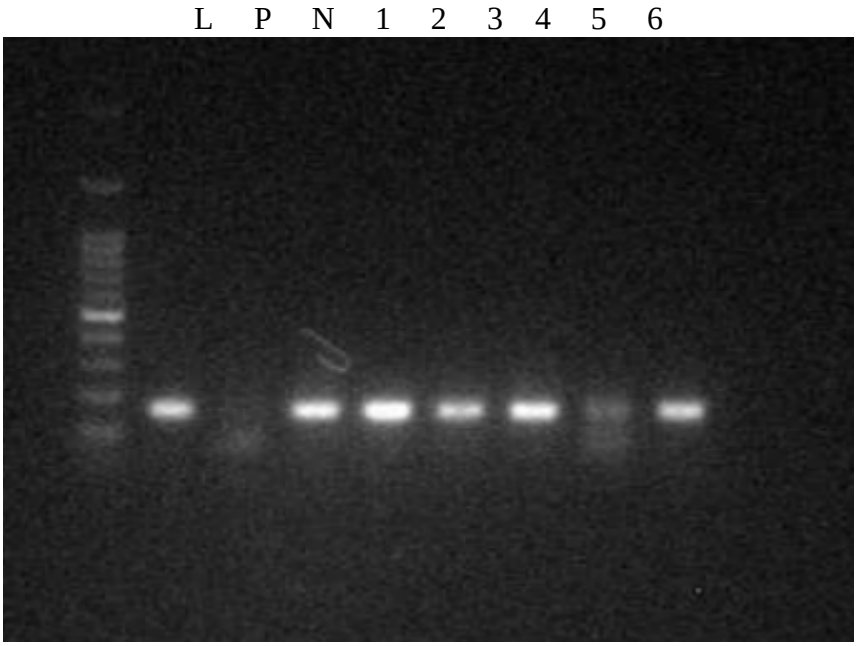

Fig 1: PCR product using specific primers to detect Mycoplasma genus.
L: ladder. P: positive control. N: negative control. Lane 1-6: positive sample

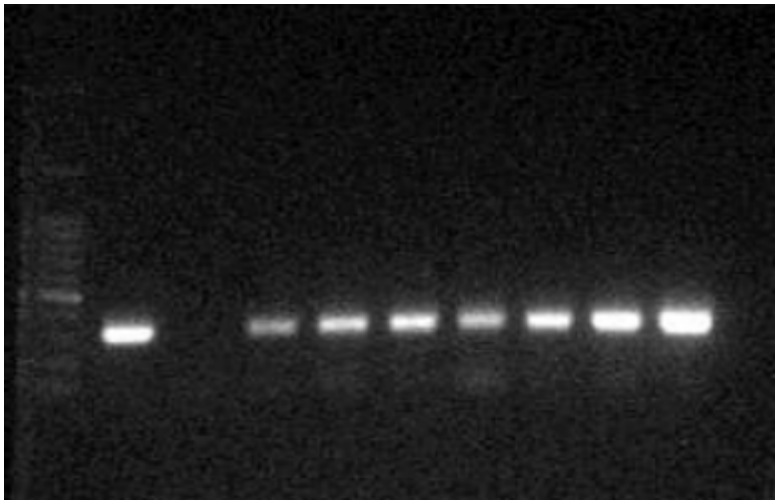

Fig2): PCR product using specific primers to detect Mycoplasma species.
L: ladder. P: positive control. N: negative control. Lane 1 -6: positive sample

Discussion

Mycoplasmas are distinguished from other prokaryotes by their specific morphology and lack of cell wall, so this strain of organism shows a great pleomorphism.Comparative genomic analyses of 16SrRNA

sequencing classified this bacteria in Mollicutes class (Bardbury, J.M.,2001, Thornton, D. L., Branton, S.L,2009).

Many PCR methods have been developed for diagnosis of *Mycoplasma gallisepticum*.This technique is used as part of routine tests for detection of Mycoplasma *gallisepticum*. PCR as a tool to detection of *Mycoplasma gallisepticum* causes a comparison of several generic and licensed PCR methods. Genes like mgc2, LP, GapA and l6S rRNA can use in many laboratories for diagnosis of *Mycoplasma gallisepticum* (Fernandez et al., 1993). PCR methods based on GapA gene only amplified *Mycoplasma gallisepticum* DNA, while the 16S rRNA PCR can use to identify this *Mycoplasma* and others (Fernandez et al., 1993). In this study, we used GAPA gene for isolation of Mycoplasma *gallisepticum* from chickens of Kerman province of Iran. This PCR test showed that can use as a tool for *Mycoplasma gallisepticum* diagnosis and isolation.

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