Isolation and detection of *Mycoplasma gallisepticum* by polymerase chain reaction and restriction fragment length polymorphism

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Summary

Mycoplasmas were isolated from tracheal and air sac samples of the suspected flocks to have mycoplasmosis and cultured on Frey's medium. Forward and reverse primers were selected on the basis of known sequences of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) 16S rRNA genes. These primers successfully amplified a 780 bp fragment of the target DNA in MG. Restriction fragment length polymorphism (RFLP) by three restriction enzymes (REs), *HpaI*, *HpaII*, and *MboI* was performed for each PCR product. According to the RFLP results, 55 samples were detected as MG; no MS was detected. The PCR results were confirmed by sequencing of a selected amplicons. Results showed that PCR and RFLP are rapid and useful for diagnosis of both cultured as well as field samples of suspected flocks to have infection with MG. In addition, PCR could be performed with at least 100 CFU of MG per each PCR reaction.

Key words: Mycoplasma, PCR, RFLP, Poultry

Introduction

Mycoplasma gallisepticum (MG) is a pathogen that affects commercial poultry, causing significant economic burdens. This bacteria is responsible for chronic respiratory disease in chickens and infectious sinusitis in turkeys (Ley and Yoder, 1997).

Avian mycoplasmosis in poultry may be occurred as a result of vertical or horizontal transmission. Horizontal transmission usually is happened by direct contact between infected and susceptible birds. However, indirect transmission via humans or fomites may also play an important role because of the possible persistence of MG in the environment (Christensen *et al.*, 1994). Due to the horizontal and vertical transmissions, special control programs have been adopted which are mainly depend on serological and/or bacteriological tests. However, inter-species cross-reactions and nonspecific reactions (Bradley *et al.*, 1988; Kleven, 1997) often hamper serological procedures.

In addition, the isolation procedure may not always be successful alone due to the overgrowth of nonpathogenic mycoplasmas in culture (Zhao and Yamamoto, 1993). To solve these problems, species-specific recombinant DNA probes for the diagnosis of MG have been developed (Khan *et al.*, 1987; Santha *et al.*, 1987; Hyman *et al.*, 1989). Despite excellent specificity of these probes, under standard hybridization conditions, their sensitivity is 1–2 ng of the target DNA, which is equivalent to approximately 10^6 organisms (Zhao and Yamamoto, 1993). Cell numbers of this magnitude probably occur only in acute infections. Therefore, MG DNA probes may not be able to detect the organisms from specimens taken during the late stages of infection.

In order to develop sensitive diagnostic and laboratory diagnostic methods, we used the polymerase chain reaction (PCR)

(Nascimento *et al*., 1993; Maroies *et al.*, 2000; Maroies *et al.*, 2002) in combination with restriction fragment length polymorphism (RFLP) for detection and identification of MG. Herein, we report on an *in vitro* DNA amplification assay using primers designed on the basis of the 16S rRNA genes of MG (Weisburg *et al.*, 1989; Van Kuppeveld *et al.*, 1992; Kempf *et al.*, 1993; Van Kuppeveld *et al.*, 1993; Garcia *et al*., 1995; Silveria *et al*., 1996) and the RFLP analysis of PCR products by restriction endonucleases, *HpaI*, *HpaII*, and *MboI* with unique restriction sites.

Materials and Methods

Mycoplasma cultures and DNA isolation

One-hundred and twenty-three tracheal and air sac samples from different chicken flocks suspected to have mycoplasmosis were collected and cultured on Frey's medium (Frey *et al.,* 1968). Only 100 samples produced mycoplasmas colonies on agar Frey's medium.

Five-ml of each culture from each purified sample was centrifuged in a microcentrifuge (Genofuge, Techne, Germany) at 14000 rpm for five minutes. Cell pellets were washed twice with 100 µl of 150 mM phosphate buffered saline (PBS) solution (pH = 7.2) and re-suspended in 25 µl PBS.

The cell pellets was then heated directly at 95°C for 10 minutes in a water bath and incubated in -20˚C for five minutes. Finally, the cell suspension was centrifuged at 14000 rpm for five minutes. The supernatant containing mycoplasma DNA was collected and stored at -20˚C until used.

Primer selection

Based on previous report (Garcia *et al.*, 1995), two 17-mer oligonucleotide primer sets [M16S PCR 5′ (MYC-1) and M16S PCR 3′ (MYC-2)] common in all avian mycoplasmas were selected. These primers were designed from 16S rRNA gene sequences of MG (Gen Bank No. M 22441). The 5′ primer (forward primer or MYC-1) was chosen from a highly conserved region (U_2). The selected sequence (MYC-1 primer) was common between MG and *M. synoviae* (MS) 16S rRNA gene. The 3′ primer (backward primer or MYC-2) had been selected from the variable region (V_4) (Neefs *et al.*, 1990). PCR primers were prepared by Cinna Gen Inc, Tehran, Iran.

Optimization of PCR for avian mycoplasmas

After optimization of the PCR reaction in terms of titration with varying magnesium chloride concentrations and selection of an appropriate amplification program (Silveria *et al*., 1996), PCR was carried out as follows: 2 µl of DNA extract was added to 48 µl of the reaction mixture consisting of 5 µl of 10x PCR buffer [500 mM KCl, 200 mM tris HCl (pH = 8.4)]. One µl of 10 mM dNTPs, 1 µl Tag DNA polymerase, 1.5 µl of 50 mM $MgCl_2$ (Cinna Gen Inc. Tehran, Iran), 1 µl of each primer (0.5 µg/ml), and 37.5 µl double distilled water (DDW) in a total volume of 50 µl of the reaction mixture. Alternatively, for testing of the non-cultivated clinical samples, 3 µl of DNA extract and 36.5 µl DDW were used in the PCR reaction mixture as mentioned above.

The PCR mixture was initially denatured at 94°C for five minutes, before performing 35 cycles consisting of 94 ؛C/1′, 56 ؛C/1′, 72؛C/1′ followed by a final 10 minutes extension at 72°C using a Genius model thermal cycler (Techne, Germany).

Detection of PCR products

Five µl of each PCR product was electrophoresed on 1% agarose gel containing ethidium bromide (0.5 µg/ml) at a constant 100 volts for 30 minutes and exposed to ultraviolet light using gel documentation set (Uvitec, Germany) to detect the amplified products. The size of the PCR products was determined by comparison with an appropriate gene marker (Ladder 100, MBI Fermentas, Germany).

RFLP analysis

Following amplification, PCR products were digested with three restriction endonucleases (REs)—*HpaI*, *HpaII*, and *MboI*— (MBI Fermentas, Germany) according to the manufacturer's instructions.

The REs were used to generate unique

RFLP patterns for the related species. Ten µl of PCR product from each sample was digested with 0.5 µl (10 units) of each RE and 2 µl of the recommended buffer and 7.5 µl DDW at 37°C for two hours. Restriction patterns were observed using 2% agarose gel electrophoresis containing ethidium bromide (0.5 µg/ml) with TBE buffer at a constant voltage of 100 volts for 30 minutes and visualized under ultraviolet light. The data saved by gel documentation set.

PCR specificity

DNA from other species of mycoplasmas, such as *M. agalactiae* (Ma) as a member of genus Mycoplasma, *E. coli* (Ec) and *Salmonella enteritidis* (Se) were tested by similar PCR reaction; no band was observed (Fig. 1).

Fig. 1: PCR Specificity. C+ and C- are positive and negative controls of PCR reaction. Lane 1–3 are PCR products and M is DNA marker (ladder 100) which each band is for 100 bp. Ec, Se and Ma lanes are PCR products corresponding to PCR reaction products with DNA obtained from *E. coli* (Ec), *Salmonella enteritidis* (Se), *Mycoplasma agalactiae* (Ma); no bands are visible

PCR sensitivity

To determine the detection limit of this PCR test, an overnight culture of MG was titrated to determine the colony forming units (CFU) per ml (Rodwell and Whitcomb, 1983). Twelve 10-fold serial dilutions were made in Frey's medium for each culture and immediately 20 µl of each dilution was cultured on Frey's medium agar plates and kept at 37°C. Colonies were counted one week later. One-ml aliquots from each dilution were collected for DNA extraction. Mycoplasma cells were lysed in 20 µl PBS (as mentioned above), 2 µl of which was used for PCR reaction as mentioned before (Fig. 2).

Results

From 100 mycoplasma samples which assayed by PCR in this research, only 43 produced a 780 bp fragment. In other words, the 16S rRNA gene of MG was successfully amplified with the MYC-1 and MYC-2 primers. Additionally, some bacterial species, including *E. coli*, *S. enteritidis* and

Fig. 2: PCR Sensitivity. PCR products corresponding to serial dilutions of an MG isolate broth culture with a primary titer of 1.2×10^{8} CFU/ml. Lanes 1–7: the PCR product dilutions of 10^{-7} through 10^{-1}. C+ and C- are positive and negative controls of PCR reaction. Lane M: 100 bp molecular weight ladder. Diagnostic band is 780 bp

M. agalactiae were also tested with no PCR product produced (Fig.1. Lanes Ec, Se and Ma). The DNAs from mycoplasma isolates were successfully amplified under the condition described (Fig. 1. Lanes 1-3).

The sensitivity of the PCR method for detecting of MG species using serial dilution of templates showed (Fig. 2) that this method is able to detect the MG to 10^{-6} dilution, which was equivalent to about 100 CFU/PCR reaction mixture (Fig. 2, Lane 2).

PCR analysis of non-cultivated samples collected from chickens suspected to have mycoplasmosis showed that 12 out of 15 samples, produced a specific 780 bp band.

RFLP

To survey the possibility of using RFLP for differentiation of mycoplasma isolates, the 780 bp products obtained from amplification of the mycoplasmas DNA was digested with three REs. According to the literatures (Fan *et al.*, 1995; Garcia *et al.*, 1995), the demonstrated RFLP pattern for MG is unique that can differentiate MG from MS. They disclosed that RFLP pattern for MS PCR product is two fragments of 680 bp and 100 bp in length if cut by *HpaI*, while *HpaII* and *MboI* cut this product into 600-bp and 180-bp fragments. Fig. 3 shows the RFLP for the collected samples. As can be seen, digestion of MG PCR product with *HpaI* produces 500-bp and 280-bp fragments.

HpaII digestion of the PCR product showed two restriction fragments of 600-bp and 180-bp while *MboI* was not able to cut that product (Fig. 3, Lane 1–3 and 4–6).

In conclusion, 43 of 100 samples and 12 tracheal samples showed MG pattern in RFLP analysis.

Nucleotide sequencing

To confirm that the assay was targeting MG, a selection of amplicons was sequenced. The sequencing results were subsequently compared to the

Fig. 3: RFLP analysis of MG PCR product. Lanes 1 and 4 are digested with *HpaI*. Lanes 2 and 5 are digested with *HpaII* and lanes 3 and 6 are digested with *MboI*. Lane M is the DNA marker

corresponding sequence submitted to the Gene Bank. It indicated that there was 99% homology between the nucleotide sequences of Iranian MG isolate and two MG strains (R and 5969). The sequencing result is submitted to GenBank under access number of AY705443. In this way, the full sequencing of MG or MS PCR products was not necessary.

Discussion

We have used a simple diagnostic PCR-RFLP assay for detection and differentiation of two avian mycoplasmas. The method has a number of significant advantages. One of the advantages is using the boiling method in DNA isolation procedure which in comparison to the commercial DNA preparation kit and lysis method, was found to be more efficient and provided an easy, rapid and inexpensive way for the disruption of the cells.

Another advantage comes from the use of pure culture of the two mycoplasmas without the need for immunodiagnostic methods. Pure cultures were simply amplified using a primer pairs, specific for a segment of 16S rRNA in a PCR program as mentioned above. In this study, PCR products of 16S rRNA gene from 44 samples (32 culture samples and 12 tracheal washing samples) avian mycoplasmas were checked based on the unique RFLP patterns produced by three REs. In addition, it was found that for detection of avian mycoplasmas using PCR-RFLP method, there is no need for incubation of tracheal washings collected from suspected chickens, which simplified the template preparation step—especially in large scale control and eradication programs.

Furthermore, PCR assay using serial dilutions of the templates showed that the sensitivity of the test for pure culture of MG is around 100 CFU/PCR reaction.

Finally, limited sequencing analysis approved the reliability of the method. In conclusion, the described procedure provides a reliable sensitive assay for

isolation and detection of *M. gallisepticum*.

In clinical samples, besides the pathogenic mycoplasmas, saprophytic avian mycoplasmas such as *M. gallinarum* and *M. gallinaceum* commonly exist (Keleven, 1985). Not all mycoplasma colonies observed on agar medium are either MG or MS, hence, only 55 samples showed 780-bp band.

Also it was found that an annealing temperatures higher than 56°C or lower than 54°C reduces the sensitivity of the assay.

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