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A newly developed consensus polymerase chain reaction to detect *Mycoplasma* species using 16S ribosomal RNA gene

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Abstract

Mycoplasmas are highly fastidious bacteria, difficult to culture and slow growing. Infections with *Mycoplasma* species can cause a variety of problems in living organisms and *in vitro* cell cultures. In this study, we investigated the usefulness of a genus-specific consensus PCR analysis method to detect *Mycoplasma* species. The developed consensus primer pairs MycoF and MycoR were designed specifically to amplify the 16S ribosomal RNA gene (rRNA) of *Mycoplasma* species by the optimized PCR system. The developed consensus PCR system effectively amplified 215 bp of *Mycoplasma* genus-specific region of 16S rRNA. In conclusion, we recommend this consensus PCR for monitoring *Mycoplasma* species in animals, human and cell culture system.

Key words : *Mycoplasma*, PCR, Consensus, 16S rRNA, Genus-specific

INTRODUCTION

Mycoplasmas are among the smallest free-living microorganisms capable of autoreplication. Mycoplasmas are highly fastidious bacteria, difficult to culture and slow growing (McAulifffe et al, 2003). The class Mollicutes consists of wall-less prokaryotes, which are small in size and have unusually small genomes. More than 100 species have been isolated from vertebrates, plants, and insects. The largest group is formed by the genus *Mycoplasma*, of which more than 90 species have been described (Hong et al, 2011). Many species are important veterinary pathogens causing respiratory infection, mastitis, conjunctivitis, arthritis, and occasionally abortion (McAulifffe et al, 2003). Also, in the field of laboratory animal medicine, it has been reported that mycoplasma infections are very common and considered highly contagious (Hong et al, 2011). Major mycoplasmas of animals include *Mycoplasma* (*M.*) *hyopneumoniae*, *M. pulmonis*, *M. collies*, *M. neurolyticum*, *M. arthritiditis*, *M. hyorhinis*, *M. mycoides* (Razin and Hayflick, 2010). In humans, mycoplasmas have been recognized either as pathogenic organisms or as commensals. The best known pathogen is *Mycoplasma pneumoniae*, which causes a primary atypical pneumonia (Ieven et al, 1996). *M. hominis* and *Ureaplasma urealyticum* may cause a variety of genitourinary diseases (Taylor-Robinson et al, 1981) when they invade beyond the genital tract.

Mycoplasmas, which are invisible to light microscopy, are frequent contaminants of *in vitro* animal cell cultures. These cryptic contaminations can be recognized via several specific procedures, including the recognition of color changes in a dye applied to the culture media, ELISA for the detection of specific proteins, or PCR methods (Hopert et al, 1993; Uphoff and Drexler, 2011;

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Volokhov et al, 2011). As mycoplasma infections in cultured cells tend to induce both biochemical and genetic changes, experimental results can often be misinterpreted. Therefore, contamination surveys should be periodically conducted in laboratories working with susceptible cultures.

Identification of mycoplasmas as the causative agents of disease is often hindered by the lack of rapid diagnostic tests together with similarities in the clinical diseases that they cause. Conventional methods of mycoplasma diagnosis are based on culture and serological tests, such as the complement fixation test (Muthomi and Rurangirwa, 1983), ELISA (Ball and Finlay, 1998), and immunoblotting (Nicholas et al, 1996), and can be time-consuming, insensitive, and nonspecific. It is necessary to clarify the current status of mycoplasma contamination in animal colonies, because they are prevalent in commercial and animal facilities (Lindsey et al, 1971). PCR provides a powerful technique of identifying mycoplasmas and studying homology between their nucleic acids. However, those kinds of PCR assays require multiple assays because of a lot of *Mycoplasma* species (McAulifffe et al, 2003).

In this study, we investigated the usefulness of a genus-specific PCR analysis method targeting a region of 16S ribosomal RNA (rRNA) gene to detect *Mycoplasma* species.

MATERIALS AND METHODS

Microorganisms and growth conditions

M. hyopneumoniae, M. hyorhinis, M. pneumonia, M. hominis, M. pulmonis, M. arthritiditis, M. bovis, M. fluccurare and *M. mycoides* were obtained from the American Type Culture Collection (Rockville, MD, USA). The mycoplasmas were grown in modified Friis medium (Friis, 1973), containing 20% porcine serum (Gibco-BRL, Korea), 5% fresh yeast extract (Gibco-BRL), methicillin (0.15 mg/ml; Sigma-Aldrich Canada, Oakville, Ontario, Canada), bacitracin (0.15 mg/ml; Sigma-Aldrich), and thallium acetate (0.08 mg/ml; Sigma-Aldrich). The cells were harvested by centrifu-

gation at 12,000×g for 30 min at 4°C, washed three times, and suspended in 0.1 M phosphate-buffered saline (PBS, pH 7.4).

Nucleic acid extraction and designation of the consensus primers

DNAs were extracted from the cultures of *M. hyopneumoniae, M. hyorhinis, M. pneumonia, M. hominis, M. pulmonis, M. arthritiditis, M. bovis, M. fluccurare* and *M. mycoides*. Mycoplasma DNAs were extracted from the cultured *Mycoplasma* species using an AccuPrep Genomic DNA extraction kit (Bioneer Corporation, Korea) according to the manufacturer's instructions. The DNA was eluted in Tris-EDTA buffer (pH 8.0), and an aliquot was used for the PCR amplification.

Consensus PCR primer pairs were designed from the mycoplasma 16S rRNA sequence (NC 019395.1) for the detection of versatile species in the *Mycoplasma* genus (Fig. 1). Forward primer MycoF was 5'-CTC TTT GTA CCG GCC ATT GT-3' (20 mer, nucleotide 699283-699302). Reverse primer MycoR was 5'-GAA TGA CAG ATG GTG CAT GG-3' (20 mer, nucleotide 699478-699497).

PCR

The template DNA (50 ng) and 20 pmol of each primer were added to a PCR mixture tube (*AccuPower* PCR PreMix; Bioneer Co., LTD., Korea) containing 2.5 U of Taq DNA polymerase, 250 μM each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM MgCl$_2$, and the gel loading dye. The volume was adjusted with distilled water to 20 μl. The reaction mixture was subjected to denaturation at 94°C for
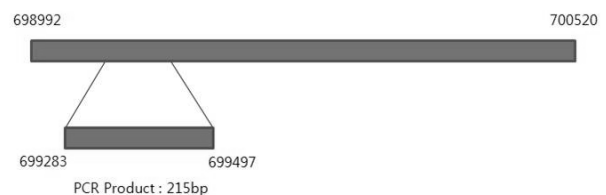

Fig. 1. The location of consensus PCR primer pairs MycoF and MycoR designed from the mycoplasma 16S ribosomal DNA sequence.

5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension step of 72°C for 3 min, and samples were kept at 4°C until analysis. Reactions were conducted using My Genie 32 Thermal Block PCR (Bioneer). After amplification, a 5 μl aliquot of each PCR was separated by electrophoresis on 2% agarose gels followed by ethidium bromide staining and UV transillumination.

Assay specificity and sensitivity

DNAs were extracted from the cultures of *M. hyopnenmoniae*, *M. hyorhinis*, *Staphylococcus aureus*, *Escherichia coli*, *Helicobacter pylori*, *Pasteurella multocida*, *Salmonella pullorum*, *Salmonella gallinarum and Actinobacillus pleuropneumoniae*. Amplification of the 16S rRNA gene was performed with the same PCR procedure. In order to determine the sensitivity of the PCR assay, serial 10-fold dilutions of the purified genomic DNA of *M. hyopnenmoniae* were tested.

Clinical samples

To evaluate the PCR system under field conditions, nasal were collected from conventional animal facility. Sampling was performed from the nares with a dry, unmoistened swab. The tip of the collection swab was inserted into the nares and rolled five times in each nostril. Collected specimens were transported and stored at room temperature. Specimens were processed for PCR analysis within 24 h of being collected. Each collection swab was put into 2 ml of 0.1 M PBS buffer

and vortexed and discarded, and the PBS was submitted to extract genomic DNAs for PCR assay.

RESULTS

Amplification of the consensus region of the 16S rRNA was performed with mycoplasma consensus primers. MycoF and MycoR was performed for primary amplification of target DNA. In this study, 215 bp amplicons were amplified using the extracted DNAs from *M. hyopneumoniae*, *M. hyorhinis*, *M. pneumonia*, *M. hominis*, *M. pulmonis*, *M. arthritiditis*, *M. bovis*, *M. fluccurare* and *M. mycoides* (Fig. 2). The targeted 215 bp of 16S rRNA gene of *Mycoplasma* species were specifically amplified by the optimized PCR system with the developed consensus primers, MycoF and MycoR.

The specificity of the developed consensus primers, MycoF and MycoR was confirmed by the study using other bacterial DNAs with high homology in their sequences. No positive signals were observed in the template DNA samples of *Staphylococcus aureus*, *Escherichia coli*, *Helicobacter pylori*, *Pasteurella multocida*, *Salmonella pullorum*, *Salmonella gallinarum* and *Actinobacillus pleuropneumoniae* (Fig. 3). However, the DNAs of *M. hyopnenmoniae* and *M. hyorhinis* were resulted in strong positive signal (Fig. 3).

Serial 10-fold dilutions of DNA from *M. hyopnenmoniae* were tested with our PCR assay. With our assay, we could detect 10 pg of genomic DNA in 5 μl aliquots of PCR product by ethidium bromide staining (Fig. 4). Nasal swabs were collected from conventional
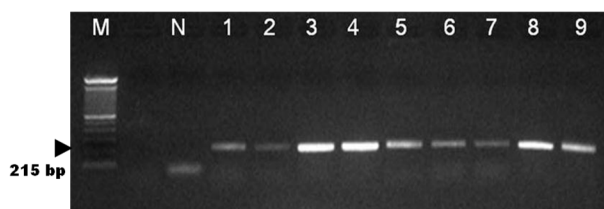

Fig. 2. Amplification of 16S rRNA gene from *Mycoplasma* species. Their PCR products were electrophoresed on a 2 % agarose gel. Lane: M: 1 kb marker, N: negative control, 1: *M. hyopneumoniae*, 2: *M. hyorhinis*, 3: *M. pneumonia*, 4: *M. hominis*, 5: *M. pulmonis*, 6: *M. arthritiditis*, 7: *M. bovis*, 8: *M. fluccurare*, 9: *M. mycoides*.
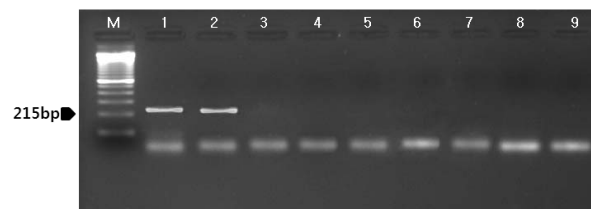

Fig. 3. Specificity of the developed consensus PCR targeted with 16S rRNA mycoplasma gene. Lane: M: 1 kb marker, 1: *M. hyopneumoniae*, 2: *M. hyorhinis*, 3: *Staphylococcus aureus*, 4: *Escherichia coli*, 5: *Helicobacter pylori*, 6: *Pasteurella multocida*, 7: *Salmonella pullorum*, 8: *Salmonella gallinarum*, 9: *Actinobacillus pleuropneumoniae*.
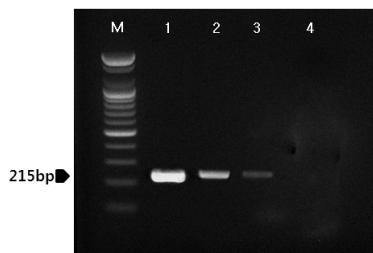
Fig. 4. Sensitivity of genus-specific PCR targeted 16 SrRNA Mycoplasma gene. Lanes: M: 1 kb marker, 1: 10^3 pg DNA of *M. hyopnenmoniae*, 2: 10^2 pg DNA of *M. hyopnenmoniae*, 3: 10 pg DNA of *M. hyopnenmoniae*, 4: 1 pg DNA of *M. hyopnenmoniae*.

animal facility and submitted to PCR analysis (Fig. 5).

Fig. 5. Direct PCR from *M. hyopnenmoniae* infected clinical samples. Lanes: M: 1 kb marker, 1: sample-1, 2: sample-2, 3: sample-3, 4: sample-4, 5: sample-5, 6: sample-6.

DISCUSSION

Mycoplasma species can induce a variety of problems in animals and human and in *in vitro* cell cultures. Many species that can exist as commensal organisms are associated with certain diseases, but mycoplasma infections are often subtle or subclinical in nature. These non-apparent infections tend to be quite insidious, as they may affect a variety of biochemical and genetic aspects of the infected cells, thereby resulting in unreliable experimental results and the possible transmission of diseases (Armstrong et al, 2010; Uphoff and Drexler, 2011; Degeling et al, 2012). Therefore, it is necessary to establish a routine diagnostic protocol for mycoplasma infection in order to ensure reliable research results, as well as the safety of commercial biological products. However, the detection of *Mycoplasma* species in cell cultures remains a problem, despite the substantial improvements that have been made in recent years in biochemical, immunological, and molecular biological methods (Jung et al, 2003).

The diagnosis of *Mycoplasma* species is usually done by cultivation of the organism or by immunofluorescence tests performed on frozen thin lung sections with polyclonal antibodies (Kobisch and Friis, 1996; Maes et al, 1996). However, due to the fastidious nature of *Mycoplasma* species, its culture and serological identification may take up to 1 month. Serological detection methods are further hampered by
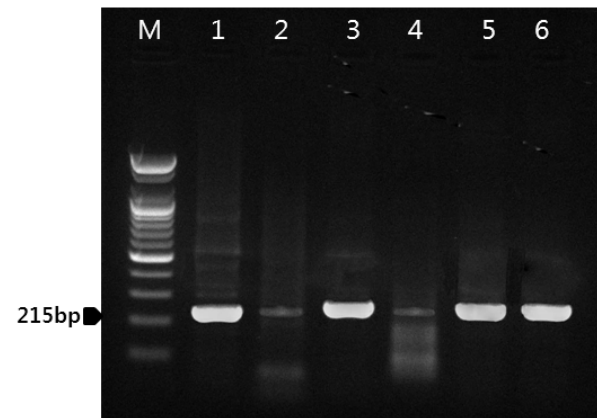
cross-reactions which have been reported between *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* (Armstrong et al, 1987; Freeman et al, 1984). With the advances made in molecular biology during the last few years, more is known about *Mycoplasma* species genes. Hence, other methods can be used as diagnostic tools for this organism. Recently, PCR methods have been used to detect *Mycoplasma* species (Hong et al, 2011). In order to circumvent those limitations, many nucleic acid technology-predicated procedures have been developed. PCR-based methods for the detection of certain DNA regions of the mycoplasma genome have proven both rapid and specific (Hu et al, 1995; Harasawa and Kanamoto, 1999; Kong et al, 2001; Loens et al, 2003; Khanna et al, 2005).

Classical diagnosis methods do not encompass a wide range of mycoplasmas, and have some limitations, due primarily to the fact that they require experience and skill, as well as a series of time-consuming and laborious steps (Chen, 1977). Despite its many limitations, then, the time-consuming practice of microbiological cultivation has traditionally constituted the method of choice, in cases in which the other methods do not yield conclusive results. However, certain mycoplasmas, such as non-culturable *M. hyorhinis* strains, have proven difficult to cultivate, and may elude such diagnostic tests. In order to circumvent the problems posed by the aforementioned classical methods, other methods have been developed, some employing nucleic acid

technology. The results presented in this study indicate that 16S rRNA gene from 9 *Mycoplasma* species could be detected by the developed consensus PCR, using highly specific primers, MycoF and MycoR.

We suggested that this consensus PCR will be useful and effective for monitoring *Mycoplasma* species in various kinds of animals and human and also cell cultures and could be used for detection of a new *Mycoplasma* species.

ACKNOWLEDGMENTS

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