한국가축위생학회지 제38권 제2호 (2015)
Korean J Vet Serv, 2015, 38(2), 101-106
ISSN 1225-6552, eISSN 2287-7630
http://dx.doi.org/10.7853/kjvs.2015.38.2.101

Korean Journal of
Veterinary Service
Available online at http://kjves.org

< Original Article >

A 16S rDNA polymerase chain reaction assay to detect *Mycoplasma pulmonis* in rats model

Sunhwa Hong[1#], Hyun-A Lee[1#], Yeon-Shik Choi[2], Yungho Chung[3], Okjin Kim[1]*

[1]*Center for Animal Resource Development, Wonkwang University, Iksan 570-749, Korea*
[2]*Department of Bio-Medical Analysis, Bio Campus of Korea Polytechnics, Nonsan 320-905, Korea*
[3]*Department of Companion Animal and Animal Resources Science, Joongbu University, Geumsan-gun 312-702, Korea*

(Received 17 May 2015; revised 16 June 2015; accepted 23 June 2015)

Abstract

Murine mycoplasmosis, caused by *Mycoplasma* (*M.*) *pulmonis*, is a prominent disease in rodent animals. The aim of this study was to develop a sensitive and specific PCR assay to detect *M. pulmonis* in animals and to assess the suitability of this assay for the detection of mycoplasmal infection in rats experimentally infected with *M. pulmonis*. A new PCR assay using the *M. pulmonis*-specific primer pairs MPul-F and MPul-R was developed. The primers and probe for the assay were designed from regions in the 16S rRNA gene that are unique to *M. pulmonis*. The novel PCR assay was very specific and sensitive for *M. pulmonis*, detecting the equivalent of 5 pg of target template DNA. It detected only *M. pulmonis* and no other *Mycoplasma* species or other bacterial species. The newly developed PCR assay also effectively detected *M. pulmonis* infection in rats. These results suggest that this PCR assay using *M. pulmonis*-specific primer pairs of MPul-F and MPul-R will be useful and effective for monitoring *M. pulmonis* infection in animals.

Key words : Mycoplasma, *Mycoplasma pulmonis*, 16S rRNA, Pneumonia, Rat

INTRODUCTION

Murine mycoplasmosis, caused by *M. pulmonis*, is a prominent disease in laboratory rats and mice. Because of its chronicity and slow cumulative mortality, *M. pulmonis* has profound impact on many parameters in rodent studies (Lindsey et al, 1971; Cassell et al, 1986). One of the primary diseases caused by *M. pulmonis* is murine respiratory mycoplasmosis or chronic respiratory disease, which involves the nasal passages, middle ears, trachea, and lungs, and causes rhinitis, otitis media, tracheitis, and pneumonia (Lindsey et al, 1971; Cassell et al, 1981a). Apart from respiratory disease, this organism can also cause genital infections, resulting in reduced birth rates (Cassell et al, 1981a; Cassell et al, 1981b; Cassell, 1982). Despite their pathogenic potential, mycoplasmal infections can remain imperceptible and be very dangerous because mycoplasmas possess immunomodulatory activities that can influence the outcome of experiments (Cassell et al, 1981b; Cassell et al, 1986).

Identification of *Mycoplasma* species as the causative agent of disease is often hindered by the lack of rapid diagnostic tests, along with similarities in the clinical features of the diseases caused by them. Conventional methods of *Mycoplasma* species diagnosis are based on microbiological and serological tests such as complement fixation test, enzyme-linked immunosorbent assay (ELISA), and immunoblotting; these methods can be time-consuming, insensitive, and nonspecific (Muthomi and Rurangirwa, 1983; Ball and Finlay, 1998; Nicholas et al, 1996). It is necessary to clarify the status of *Mycoplasma* contamination in animal colonies because of its prevalence in commercial and animal facilities

*Corresponding author: Okjin Kim, Tel. +82-63-850-6668, Fax. +82-63-850-7308, E-mail. kimoj@wku.ac.kr
#Both authors contributed equally to this work.

(Lindsey et al, 1971). Polymerase chain reaction (PCR) is a powerful technique for identifying mycoplasmas and for studying homology between their nucleic acids (McAulifffe et al, 2003).

The aim of this study was to develop a sensitive and specific PCR assay to detect *M. pulmonis* in laboratory rodent animals and to assess the suitability of this assay for the detection of *mycoplasma*l infection in rats experimentally infected with *M. pulmonis*.

MATERIALS AND METHODS

Microorganisms and growth conditions

M. pulmonis (ATCC19612), *M. hyopneumoniae* (ATCC25934), *M. hyorhinis* (ATCC27717), *M. hominis* (ATCC23114), and *M. arthritiditis* (ATCC19611) were obtained from the American Type Culture Collection (Rockville, Md.). *Mycoplasma*s were grown in modified Friis medium (Friis, 1973), containing 20% porcine serum (Gibco-BRL, USA), 5% fresh yeast extract (Gibco-BRL), 0.15 mg · mL^{-1} methicillin (Sigma-Aldrich, USA), 0.15 mg · mL^{-1} bacitracin (Sigma-Aldrich), and 0.08 mg · mL^{-1} thallium acetate (Sigma-Aldrich). Cells were harvested by centrifugation at 12,000 x g for 30 min at 4°C, washed 3 times, and suspended in 0.1 M phosphate-buffered saline (PBS; pH 7.4).

Animals and infection

We acquired 5-week-old male specific pathogen-free Sprague Dawley rats from Samtako (Osan, Korea). Rats were acclimatized and kept in an isolated specific pathogen-free (SPF) barrier room with regulated temperature (23°C±1°C), humidity (50%±5%), and light/dark cycle (12/12 h). The animals were fed sterilized pellet diet by 2 M rad radiation (Purina, Korea) and sterilized water *ad libitum*. After an adaptation period of 1 week, the animals were divided into 2 groups (infected and control) and maintained in an opaque polypropylene cage in an isolated ventilated cage system. Five rats (rats 1 through 5) were experimentally infected with 10^6 colony-forming units (CFU) of *M. pulmonis* (in 100 μL of culture medium) by intranasal inoculation. Five non-infected control rats (rats 6 through 10), housed separately from the infected rats, were used as controls. After 1 week, these rats were sacrificed by cervical dislocation. Fresh pulmonary tissues were collected and subjected to the *M. pulmonis*-specific PCR assay developed in this study.

All studies were performed in accordance with the Guide for Animal Experimentation by Wonkwang University and approved by the Institutional Animal Care and Use Committee of Wonkwang University (Approval No. WKU13-35). All efforts were made to minimize the pain or discomfort to the animals used.

Nucleic acid extraction and designation of the *M. pulmonis*-specific PCR primers

DNA was extracted from the *M. pulmonis* cultures. In addition, the pulmonary tissues were homogenized and resuspended in PBS and subjected to DNA extraction as described previously (Cho et al, 2011). Genomic DNA was isolated using an *AccuPrep* Genomic DNA extraction kit (Bioneer Corporation, Daejeon, Korea) according to the manufacturer's instructions. DNA was eluted in Tris-EDTA buffer (pH 8.0), and an aliquot was used for PCR amplification. All DNA samples were stored at −20°C until the PCR assays were performed.

Species-specific PCR primer pairs were designed from *Mycoplasma* 16S rRNA sequence for the detection of *M. pulmonis* (Fig. 1). The forward primer MPul-F was 5'-CAG TAC TTG AGT TAG AAA ATG GA-3' (23-mer, nucleotide 256498-256520). The reverse primer MPul-R was 5'-ATC TGA AAG TTT TGA AGA GTT TTG-3' (24-mer, nucleotide 257383-257405).

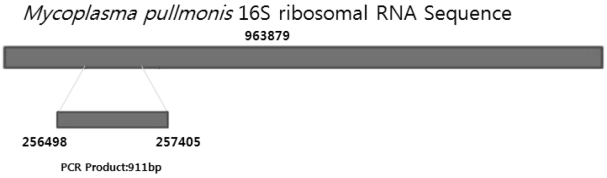

Fig. 1. Location of species-specific PCR primer pairs MPul-F and MPul-R designed from the *Mycoplasma pulmonis* 16S ribosomal RNA sequence.

Assay specificity

DNA was extracted from the cultures of *M. hyopneumoniae*, *M. hyorhinis*, *M. pulmonis*, *M. hominis*, and *M. arthritiditis*. Amplification of the *M. pulmonis*-specific gene was performed with the primer pairs of MPul-F and MPul-R designed from the *Mycoplasma* 16S rRNA sequence in this study. The template DNA (50 ng) and 20 pmol each primer were added to a PCR mixture tube (*AccuPower* PCR PreMix; Bioneer Corp., Korea) containing 2.5 U of Taq DNA polymerase, 250 μM each deoxynucleoside triphosphate (dNTP), 10 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$, and the gel loading dye. The volume was adjusted to 20 μL with distilled water. The reaction mixture was subjected to denaturation at 94°C for 5 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension step of 72°C for 3 min; the samples were kept at 4°C until analysis. Reactions were conducted using My Genie 32 Thermal Block PCR (Bioneer, Korea). After amplification, a 5-μL aliquot of each PCR reaction mixture was separated by electrophoresis on 2% agarose gels, followed by ethidium bromide (EtBr) staining and ultraviolet (UV) transillumination.

Assay sensitivity

DNA was extracted from *M. pulmonis* and *M. hominis* cultures. To determine the sensitivity of the newly developed PCR assay, serial dilutions of purified chromosomal DNA of *M. pulmonis* strains were tested. Amplification of the *M. pulmonis*-specific gene was performed with the primer pairs of MPul-F and MPul-R designed from the *Mycoplasma* 16S rRNA sequence in this study. The reaction condition of PCR assay After amplification, a 5-μL aliquot of each PCR reaction mixture was separated by electrophoresis on 1.5% agarose gels, followed by EtBr staining and UV transillumination.

Applicability to animal samples

To evaluate the PCR system under field conditions, pulmonary tissues were collected from the rats experimentally infected with *M. pulmonis*. The pulmonary tissues were homogenized, resuspended in PBS, and subjected to DNA extraction, as described previously (Cho et al, 2011). The PCR assay was performed with the *M. pulmonis*-specific primer pairs of MPul-F and MPul-R designed in this study. After amplification, a 5-μL aliquot of each PCR reaction mixture was separated by electrophoresis on 1.5% agarose gels, followed by ethidium bromide (EtBr) staining and UV transillumination.

RESULTS

Assay specificity

A PCR assay using the *M. pulmonis*-specific primer pairs MPul-F and MPul-R was developed and evaluated for its specificity and sensitivity. Because of PCR amplification, 911-bp amplicons were detected from the extracted DNA of *M. pulmonis* (Fig. 2). The targeted 911-bp of the 16S rRNA gene of *M. pulmonis* were specifically amplified by the optimized PCR system with the *M. pulmonis*-specific primer pairs MPul-F and MPul-R designed in this study. The specificity of the newly developed *M. pulmonis*-specific primer pairs was confirmed using other bacterial DNA with a high level of homology in their sequences (Fig. 2). No positive signals were observed in the template DNA samples of *M. hyopneumoniae*, *M. hyorhinis*, *M. hominis*, and *M. arthritiditis* (Fig. 2). However, the DNA of *M. pulmonis*

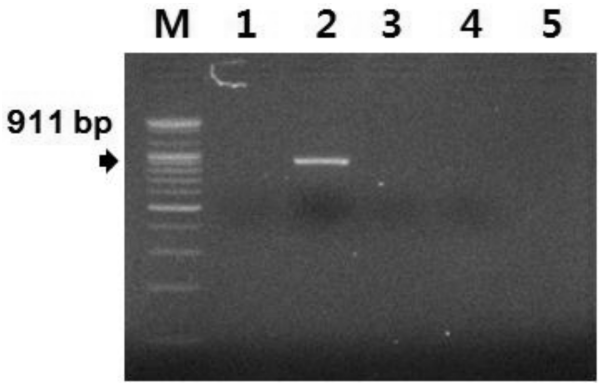

Fig. 2. Specificity of the developed species-specific PCR targeted with the *Mycoplasma pulmonis* 16S ribosomal RNA sequence. Lane M: 100-bp marker, N: negative control, 1: *M. hyopneumoniae*, 2: *M. pulmonis*, 3: *M. hyorhinis*, 4: *M. hominis*, 5: *M. arthritiditis*.

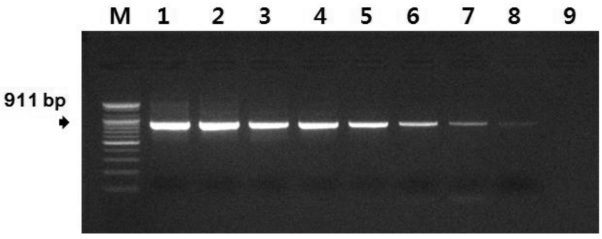

Fig. 3. Sensitivity of the developed species-specific PCR targeted with the *Mycoplasma pulmonis* 16S ribosomal RNA sequence. Lane M: 100-bp marker, 1: 10^4 pg of *M. pulmonis* DNA, 2: 5×10^3 pg of *M. pulmonis* DNA, 3: 10^3 pg of *M. pulmonis* DNA, 4: 5×10^2 pg of *M. pulmonis* DNA, 5: 10^2 pg of *M. pulmonis* DNA, 6: 5×10 pg of *M. pulmonis* DNA, 7: 10 pg of *M. pulmonis* DNA, 8: 5 pg of *M. pulmonis* DNA, 9: 1 pg of *M. pulmonis* DNA.

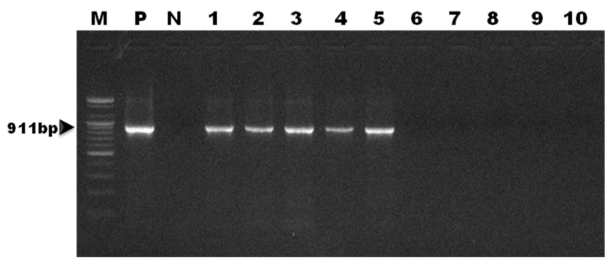

Fig. 4. Amplified products of sample DNAs obtained by the developed species-specific PCR for *Mycoplasma pulmonis* were identified using 1.5% agarose gel electrophoresis. Lane M: 100-bp marker, P: positive control, N: negative control, 1～5: DNAs extracted from the pulmonary tissues of *M. pulmonis*-infected rats, 6～10: DNAs extracted from the pulmonary tissues of non-infected control rats.

yielded a strong positive signal (Fig. 2).

Assay sensitivity

To evaluate the sensitivity of the newly developed *M. pulmonis*-specific PCR assay, serial dilutions of the DNA extracted from the cultured *M. pulmonis* were tested. Results revealed the presence of at the least 5 pg of genomic DNA in the 5-μL aliquots of PCR product by EtBr staining (Fig. 3).

Applicability to animal samples

A newly developed PCR assay using primers designed from the *Mycoplasma* 16S rRNA sequence was employed to detect *M. pulmonis* infection in rats. The targeted 911-bp gene was specifically amplified by the PCR assay with the *M. pulmonis*-specific primer pairs MPul-F and MPul-R. The target nucleic acid fragments were specifically amplified in the pulmonary tissue samples of *M. pulmonis*-infected rats by the newly developed PCR analysis (Fig. 4). No positive signals were detected in the tissue samples of non-infected control rats (Fig. 4).

DISCUSSION

M. pulmonis can have a significant detrimental impact on research that utilizes infected mice and rats by causing morbidity and mortality, and through interference with respiratory function and altered respiratory carcinogenesis and immunity (Baker, 1998; Institute of Laboratory Animal Resources, 1991). Contamination of transplantable tumors, cell cultures, and other biological materials with *M. pulmonis* and other *Mycoplasma* spp. is quite common and problematic for the maintenance and use of these materials (Collins and Parker, 1972; Nicklas et al, 1993).

Diagnosis of contamination by *Mycoplasma* species is usually accomplished by microbiological studies or immunofluorescence tests performed on frozen, thin pulmonary tissue sections by using polyclonal antibodies (Kobisch and Friis, 1996; Maes et al, 1996). However, because of the fastidious nature of the *Mycoplasma* spp., its microbiological and serological identification may take up to 1 month. Serological detection is further hampered by cross-reactions, which have been reported among *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* (Freeman et al, 1984; Armstrong et al, 1987).

With the advances made in molecular biology during the last few years, more is known about *Mycoplasma* spp. genes, and other diagnostic tools have been developed for this organism. Recently, PCR methods have been used to detect *Mycoplasma* spp. (Hong et al, 2011). PCR-based methods for the detection of certain regions in the *Mycoplasma* genome have proven to be both rapid and specific (Hu et al, 1995; Harasawa and Kanamoto, 1999; Kong et al, 2001; Loens et al, 2003; Khanna et al, 2005).

In this study, a new PCR assay using the *M. pulmonis*-specific primer pairs MPul-F and MPul-R was

developed. The primers and probe for the assay were designed from regions in the 16S rRNA gene that are unique to *M. pulmonis*. The novel PCR assay was very specific and sensitive for the detection of *M. pulmonis*. The assay was able to detect the equivalent of 5 pg of target template DNA, indicating that the assay was very sensitive. The *M. pulmonis* PCR assay was shown to be highly specific as only *M. pulmonis* and no other *Mycoplasma* spp. or other bacterial species was detected. In addition, the newly developed PCR assay effectively detected *M. pulmonis* infection in rats.

Thus, results from this study suggest that this newly developed PCR assay using the *M. pulmonis*-specific primer pairs MPul-F and MPul-R will be useful and effective for monitoring *M. pulmonis* infection in animals.

ACKNOWLEDGEMENTS

This study was supported by the research fund of Wonkwang University in 2015. We wish to appreciate Gi-Wook Oh, research assistants of Center for Animal Resources Development, Wonkwang University, for carrying out the technical support.

REFERENCES

Armstrong CH, Freeman MJ, Sands-Freeman L. 1987. Crossreactions between *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*: practical implications for the serodiagnosis of *mycoplasma*l pneumonia of swine. Isr J Med Sci 23: 654-656.

Baker DG. 1998. Natural pathogens of laboratory mice, rats, and rabbits and their effects on research. Clin Microbiol Rev 11: 231-266.

Ball HJ, Finlay D. 1998. Diagnostic application of monoclonal antibody (MAb)-based sandwich ELISAs. Methods Mol Biol 104: 127-132.

Cassell GH. 1982. The Derrick Edward Award Lecture: The pathogenic potential of mycoplasmas: *Mycoplasma pulmonis* as a model. Rev Infect Dis 4(Suppl): 18-34.

Cassell GH, Davis JK, Simecka JW, Lindsey JR, Cox NR, Ross S, Fallon M. 1986. *Mycoplasma*l infections: disease pathogenesis, implications for biomedical research and control. pp. 87-130. In: Bhatt PN, Jacoby RO, Morse III HC, New AE (ed.). Viral and *mycoplasma*l infections of laboratory rodents effects on biomedical research. Academic Press, Orlando.

Cassell GH, Lindsey JR, Davis JK. 1981a. Respiratory and genital mycoplasmosis of laboratory rodents: implications for biomedical research. Isr J Med Sci 17: 538-554.

Cassell GH, Wilborn WH, Silvers SH, Minion FC. 1981b. Adherence and colonization of *Mycoplasma pulmonis* to genital epithelium and spermatozoa in rats. Isr J Med Sci 17: 593-598.

Cho SJ, Lee HA, Hong S, Kim O. 2011. Uterine adenocarcinoma with feline leukemia virus infection. Lab Anim Res 27: 347-351.

Collins MJ Jr, Parker JC. 1972. Murine virus contaminants of leukemia viruses and transplantable tumors. J Natl Cancer Inst 49: 1139-1143.

Freeman MJ, Armstrong CH, Freeman-Sands LL, Lopez-Osuna M. 1984. Serological cross-reactivity of porcine reference antisera to *Mycoplasma hyopneumoniae*, *M. flocculare*, *M. hyorhinis* and *M. hyosynoviae* indicated by the enzyme-linked immunosorbent assay, complement fixation and indirect hemagglutination tests. Can J Comp Med 48: 202-207.

Friis NF. 1973. The pathogenicity of *Mycoplasma flocculare*. Acta Vet Scand 14: 344-346.

Harasawa R, Kanamoto Y. 1999. Differentiation of two biovars of *Ureaplasma urealyticum* based on the 16S-23S rRNA intergenic spacer region. J Clin Microbiol 37: 4135-4138.

Hong S, Lee HA, Park SH, Kim O. 2011. Sensitive and specific detection of *Mycoplasma* species by consensus polymerase chain reaction and dot blot hybridization. Lab Anim Res 27: 141-145.

Hu M, Buck C, Jacobs D, Paulino G, Khouri H. 1995. Application of PCR for detection and identification of *mycoplasma* contamination in virus stocks. In Vitro Cell Dev Biol Anim 31: 710-715.

Institute of Laboratory Animal Resources. 1991. Infectious diseases of mice and rats. National Academy Press, Washington D.C.

Khanna M, Fan J, Pehler-Harrington K, Waters C, Douglass P, Stallock J, Kehl S, Henrickson KJ. 2005. The pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia* (Chlamydophila) *pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and its real-time counterpart. J Clin Microbiol 43: 565-571.

Kobisch M, Friis NF. Swine mycoplasmoses. 1996. Rev Sci Tech 15: 1569-1606.

Kong F, James G, Gordon S, Zelynski A, Gilbert GL. 2001. Species-specific PCR for identification of common contaminant mollicutes in cell culture. Appl Environ Microbiol 67: 3195-3200.

Lindsey JR, Baker HJ, Overcash RG, Cassell GH, Hunt CE. 1971. Murine chronic respiratory disease. Significance as a research complication and experimental production with *Mycoplasma pulmonis*. Am J Pathol 64: 675-708.

Loens K, Ursi D, Goossens H, Ieven M. 2003. Molecular diagnosis of *Mycoplasma pneumoniae* respiratory tract infections. J Clin Microbiol 41: 4915-4923.

Maes D, Verdonck M, Deluyker H, de Kruif A. 1996. Enzootic pneumonia in pigs. Vet Q. 18: 104-109.

McAulifffe L, Ellis RJ, Ayling RD, Nicholas RA. 2003. Differentiation of *Mycoplasma* species by 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis fingerprinting. J Clin Microbiol 41: 4844-4847.

Muthomi EK, Rurangirwa FR. 1983. Passive haemagglutination and complement fixation as diagnostic tests for contagious caprine pleuropneumonia caused by the F-38 strain of *mycoplasma*. Res Vet Sci 35: 1-4.

Nicholas RA, Santini FG, Clark KM, Palmer NM, De Santis P, Bashiruddin JB. 1996. A comparison of serological tests and gross lung pathology for detecting contagious bovine pleuropneumonia in two groups of Italian cattle. Vet Rec 139: 89-93.

Nicklas W, Kraft V, Meyer B. 1993. Contamination of transplantable tumors, cell lines, and monoclonal antibodies with rodent viruses. Lab Anim Sci 43: 296-300.