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Evaluationof *Mycoplasma muris* and *Mycoplasma pulmonis* infections in Genital and Respiratory Tracts

FatemehAbedini[1]; Mohammad Reza Zinatizadeh[2]; Abbas Ashtari[3]; Mostafa Jafarpoor [2]; Mohammad Ebrahimi[4]; Gholam Reza Shokri[1]; Roozbeh Fallahi[1]

1. Research and development Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Educationand Extension Organization, Hesarak, Karaj, Alborz, Iran.
2. Faculty of Biological Science, Islamic Azad University, Tonekabon Branch, Mazanderan, Tonekabon, Iran.
3. Mycoplasma reference laboratory, Razi vaccine and serum research institute, Agricultural Research, Educationand Extension Organization, Hesarak, Karaj, Alborz, Iran.
4. Quality Assurance, Razi Vaccine and Serum Research Institute Agricultural Research, Educationand Extension Organization, Hesarak, Karaj, Alborz, Iran.

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ABSTRACT

Mycoplasma pulmonis and *muris* infections have been associated with several diseases in conventionally housed laboratory rat and mice colonies. In naturally infected of *mycoplasma pulmonis* in mice and rat colonies, the respiratory organ appears to be the favored site of colonization; so, it was not surprising to see that infection spread from the site of urogenital tract to the respiratory tract. In this research, PPLO broth culture and polymerase chain reaction (PCR) assay was used to detect *Mycoplasma pulmonis* and *muris* contamination in mice strain NIH. A new species of *Mycoplasma muris* (MYMORazi) have been identified in the nasopharyngeal, lung and vaginal samples of mice strain NIH in Razi Vaccine and serum research institute. Despite using two pair's specific primers targeting 16SrRNA gene of *Mycoplasma pulmonis*, detection was failed in both organs. We propose that the *Mycoplasma muris* strain MYMO Razi can cause infection in both organs of mouse strain NIH without presence of *Mycoplasma pulmonis*.

1. Introduction

The *Mollicutes* are smaller and simplest free-living and self-replicating bacteria. They are phylogenetically similar to gram positive bacteria and are evolved from Clostridia by genome reduction. *Mollicutes* have been isolated more than 100 species from vertebrates, insects and plants. Among *Mycoplasma, M. genitalium* has the smallest genome in this group (5.77 $\times 10^5$bp), and strain LB12 has the largest genome (2.2 $\times 10^6$ bp) (Razin and Freundt, 1984; Tully and Whitcomb, 1989). The *Mollicute* cells are motile, chemotactic and exhibiting a rich repertoire of cell movements. They are characterized by a complete lack of cell walls, internal or external flagella. They are being enveloped by only a cholesterol-containing unit membrane, and having a well-defined internal cytoskeleton (Kirchhoff *et al.*, 1992).

Mycoplasma infection may cause various problems in tissue cultures and laboratory rodent colonies. Five *Mycoplasma* species have been isolated from laboratory rodents including: *M.*

*Corresponding author: Dr. Fatemeh Abedini
Tel: +98-9123112958
E-mail address: ftmhabedini@yahoo.com

pulmonis, *M. arthritidis*, *M. muris*, *M. collis* and *M. neurolyticum* (Davidson *et al.*, 1994). These species have been associated with manifestations of several disease but the infections often remain unapparent. *Mycoplasma* infections are known to affect the immune system and physiological mechanisms, thereby influencing the experimental results obtained with these infected laboratory animals (Cassell *et al.*, 1981; van Kuppeveld *et al.*, 1993).

M.pulmonisis a common pathogen in many conventionally maintained colonies. It can cause respiratory mycoplasmosis and persistent infections in laboratory murine, and in later stages of infection it characterized by chattering in mice, snuffles in rats, weight loss, polypnea, inactivity and head tilt (Cassell *et al.*, 1981).Contamination of cell cultures, transplantable tumors, serum, cells and other biological materials with *M. pulmonis* and other *Mycoplasma* species is very common and problematic for the maintenance and use of these materials (Collins and Parker, 1972; Nicklas *et al.*, 1993). In addition to its role in respiratory infection, recently more research indicated the possibility of urogenital transmission and production of disease in all parts of the female genital tract (Cassell *et al.*, 1981; Jacoby and Lindsey, 1998). It has been estimated that infection with *M. pulmonis* can cause genital infections with decreased birth rate approximately 50 to 100% in laboratory rats and mice (Cassell *et al.*, 1981; Kamatani *et al.*, 1983; Steiner and Brown, 1993).

The agents that cause primary infections of the genitourinary tract in laboratory rats and mice are *Leptospira interrogans serovarballum*, *Mycoplasma muris* and *Mycoplasma pulmonis*. McGarrity and colleagues for the first time has been isolated *M. muris* from vaginas of 42 female mice strains RIII and C57BL/6 in a single colony. Their study indicated that the *Mycoplasma* isolates recovered on SP-4 medium are serologically distinct from 82 *Mycoplasma* species and unclassified serotypes (McGarrity *et al.*, 1976).

Recently, several methods are used to detect *M. pulmonis* and *muris* infections in rodents and contaminated biological materials. Diagnosis of *M. pulmonis* infection can be done using serological methods for the presence of anti-*Mycoplasma* antibodies. However, *in vitro* isolation is time-consuming, and serological assays often give incorrect results due to lack of sensitivity, cross-reactivity between different species of rodent Mycoplasmas and a low level of antibody in immunodeficient animals. Detection of *Mycoplasma pulmonis* and *muris* at an early stage or the animal with immunodeficient by polymerase chain reaction is important because it is very specific and sensitive(McGarrity *et al.*, 1976; Cassell *et al.*, 1981; Alexander, 1985; Davis *et al.*, 1987; Davidson *et al.*, 1994).

2. Materials and Methods

2.1. Animal and Tissues preparations

Animal usage was approved by the Animal Care and Use Committee of Razi Vaccine and Serum Research Institute, karaj, Iran. Female NIH mice between 4 to 6 weeks old were taken from a department of animal breeding in Razi Vaccine and Serum Research Institute under conventional premises. Vaginal, nasopharyngeal and lung tissue samples were obtained from 29 female mice strain NIH and tissues were immediately frozen in liquid nitrogen for later molecular works.

2.2. Culture of Mycoplasma pulmonis and muris

All samples were obtained from vaginal, nasopharyngeal and lung of 29 mice strain NIH. In order to process specimens for culture, samples were first homogenized with 1 ml sterile PBS using a mortar and pestle. The homogenized tissues were centrifuged and the supernatant were used for culture in transfer media at 37°C incubator overnight. All samples were filtered into the fresh PPLO broth and were kept in incubator at 37°C in 5% Co2 for 4-5 weeks (BBL, Becton Dickinson and company, Cockeyville, Sparks, MD, USA). Samples were observed daily for signs of growth and contamination and uncultured PPLO broth were used as a negative control. Samples with signs of growth mycoplasma inoculated on to PPLO agar medium.

2.3. DNA extraction

DNA extraction was performed by phenol/chloroform method according to the procedures of Sambrook and colleagues (Sambrook and Russell, 2001). All samples were treated with lysis buffer and proteinase K to an equal volume of samples, and were kept at 56ºC for 4 hrs. Saturated phenol (300µl) were added to all samples, mixed well and centrifuged at 13000 rpm for 15 min, then the upper aqueous phase was transferred to a new microtubes. Equal volumes of phenol/chloroform (1/1) were added to the samples and centrifuged. The aqueous layers were transferred to a new microtube. Equal volumes of chloroform was added to all samples and centrifuged. The aqueous layers were transferred to a new microtube. Sodium acetate 3M (1:10 volume) and absolute ethanol double volumes were added to the samples, and were kept on 20ºC for 20 min and were centrifuged. Remove supernatant completely. Wash the DNA pellet by adding 1 ml of 75% ethanol. DNA pellets were dried and dissolved in DEPC-treated water and were kept at -20°C.

2.4. Design Positive Control for M. pulmonis

Positive control for *Mycoplasma pulmonis* was prepared based on our previous work (Abedini *et al.*, 2014). Briefly, 103 bp from conserved sequence of 16S rRNA (353-458) of *M. pulmonis* (accession number AF125582) was synthesized and cloned into pUC57 vector. *Escherichia coli* DH5 cells were made competent by a standard CaCl2 transformation protocol (Sambrook and Russell, 2001). Plasmid extraction was done by using GF-1 plasmid DNA extraction kit (Vivantis, Malaysia) based on the manufacturer's protocol. DNA was stored at -20°C until use.

2.5. PCR amplification with specific primers

In this research, published primers were used for the specific detection of genus, species of *the M. pulmonis* and *M.* muris. Primers for a genus of *Mycoplasma* were used based on *M. arginini* strain EF-Hungary targets to 16S ribosomal RNA gene, accession number (HM179556.1) as follows: forward primer M1F: 5'-GCT GCG GTG AAT ACG TTC T-3' (1321- 1339), rivers primer M3R: 5'-TCC CCA CGT TCT CGT AGG G-3' (1462-1481), PCR product 163 bp (Kojima *et al.*, 1997). Primer sequences, with the corresponding nucleotides of the *M. pulmonis* 16S rRNA (accession number AF125582) were used as follows: forward primer, 5’ CAG CGTGCA GGA TGA AGG T 3’ (363–381); reverse primer, 5’ CCG TCATAC TTA GGG CAT TTC C 3’ (448–427), PCR product 83bp.

Primer sequences, with the corresponding nucleotides of the *M. muris* 16S rRNA (accession number M23939.2), are as follows: forward primer, 5'-TTA AAG TTC CGT TTG GAA CG-3 (193-202)'; reverse primer, 5'-ATC ATT TCC TAT TCC TAC CA-3' (467-448), PCR product 275 bp (Kuppeveld *et al.*, 1993)(Table 1).

The PCR amplification was done by using master mix (Biosystems Company, UK, 2x PCRBIO Taq Mix Red) according to the manual instructions in a total volume of 25µl per sample (Table 2).

Table 1. Primers used for fragment amplification of the 16S rRNA gene of *M. genus*, *M. pulmonis* and *M. muris* by PCR. Primers for M1F and M3R were derived and modified from 16S ribosomal RNA gene of *M. arginini, M. orale,* and other *Mycoplasma* spp.

Genus/Species	Primers Sequence	Fragment size	References
M. genus			
Primer-F1	5´ - GCTGCGGTGAATACGTTCT- 3´	163 bp	(Kojima *et al.*, 1997)
Primer-M3R	5´ –TCCCCACGTTCTCGTAGGG-3´		
M. Pulmonis			
Primer-F1	5´-CAGCGTGCAGGATGAAGGT-3´	83 bp	(Loganbill *et al.*, 2005)
Primer-R1	5´-CCGTCATACTTAGGGCATTTCC-3´		
Primer-F2	5'-AGCGTTTGCTTCACTTTGAA-3'	266 bp	(Kuppeveld *et al.*, 1993)
Primer-R2	5'-GGGCATTTCCTCCCTAAGCT-3'		
M. muris			
Primer-F	5'-TTAAAGTTCCGTTTGGAACG-3'	275 bp	(Kuppeveld *et al.*, 1993)
Primer-R	5'-ATCATTTCCTATTCCTACCA-3'		

Table 2. PCR amplification with specific primers for *M. genus*, *M. pulmonis* and *M. muris.*

PCR Program		*M.genus*	*M. pulmonis*	*M. muris*
Initial Denaturation		95°C (7.5 min)	95°C (10 min)	95°C (10 min)
45 cycles	Denaturation	95°C (30 sec)	95°C (30 sec)	95°C (30 sec)
	Annealing	56°C (30 sec)	60°C (1 min)	54°C (1 min)
	Extensions	72°C (1 min)	72°C (30 sec)	72°C (30 sec)
Final Extensions		72°C (5 min)	72°C (7 min)	72°C (7 min)

3. Results

Culture and characterization of *M. pulmonis* and *M. muris* were performed by PPLO broth, PPLO agar and PCR. In this study, a total of 29 tissue samples from vaginal, nasopharyngeal and lung tissues female mice was cultured in PPLO broth and agar to isolate the *M. Muris* and *M. pulmonis*. The results of PPLO agar showed 55% positive in nasopharyngeal, and lung samples and 68% positive in vaginal samples (Table 3).

The DNA extracted was used for PCR amplification of *M. arginini* 163 bp fragment of 16S rRNA gene as positive control, results showed that there were 48% positive samples from nasopharyngeal and lung and 65% positive in vaginal samples (Figure 1).

Table 2. The results of the PPLO agar culture and PCR assay in different samples

Tests	Vaginal		nasopharyngeal & lung	
	Positive	Negative	Positive	Negative
PPLO Agar	20 (68%)	9 (31%)	16 (55%)	14 (48%)
PCR *M. genuse*	19 (65%)	7 (58%)	15 (51%)	14 (48%)
PCR *M. pulmonis*	0	29 (100%)	0	29% (100%)
PCR *M. muris*	19 (65%)	7 (24%)	13 (44%)	16 (55%)

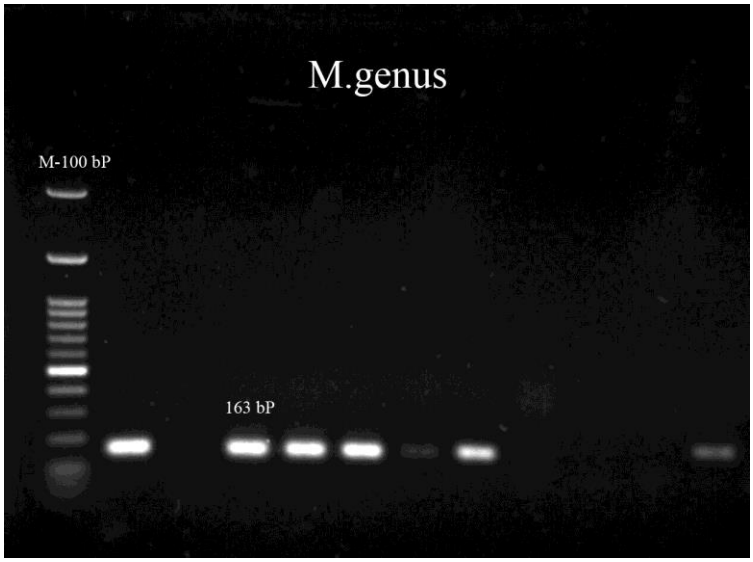

Figure 1. PCR amplification of *M.genus*.

The *M. genus* was prepared by the Mycoplasma reference laboratory of Razi Vaccine and Serum Research Institute, Karaj, Iran. The results of PCR amplification of 83 bp and 266 bp fragment of 16S rRNA gene of *M. pulmonis* showed that all samples from vaginal, nasopharyngeal and lung have been negative. While, PCR amplification of 275 bp fragment targets 16S rRNA gene of *M. muris* was 65% positive from the vaginal samples and of 55% positive from the nasopharyngeal and lung samples (Figure 2 ,3) (Table 3).

The 275 bp of PCR products from *M. muris* was sequenced by an automated DNA sequence analyzer (Bioneer, Korea), and the gene sequence was submitted to GenBank (GenBank accession No: KX792083.1). The phylogenetic tree was obtained by using alignment with MUSCLE, clustering method (UPGMA), Root method (Pseudo) and Distance method (PctIdKimura). Figure 4 shows that the *M. muris* strain MYMO Razi accession number KX792083.1 isolated from the NIH mice from Razi vaccine and serum research institute are grouped with the *M. muris* NR_044664.2 and *M. muris* M23939.2 together in one clade. The *M. pulmonis* strains, NR_041744.1 and NR_113692.1 cluster together. Moreover, the evolution route of 3 strains of *M. muris* suggested that three strains of *M. muris* most probably have separate origin from *M. pulmonis* (Figure 4).

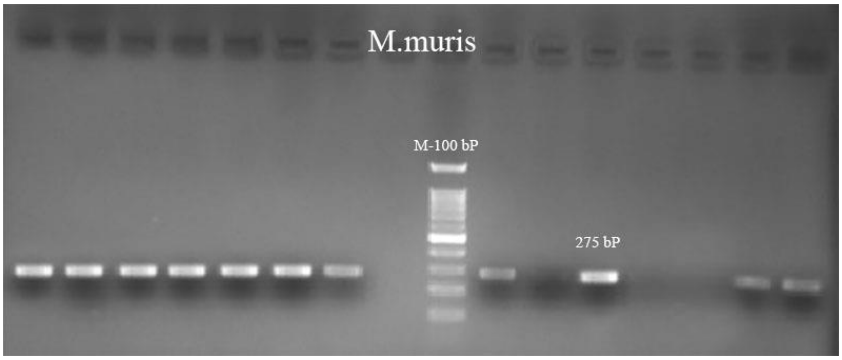

Figure 2. PCR amplification of *M.muris* from vaginal samples.

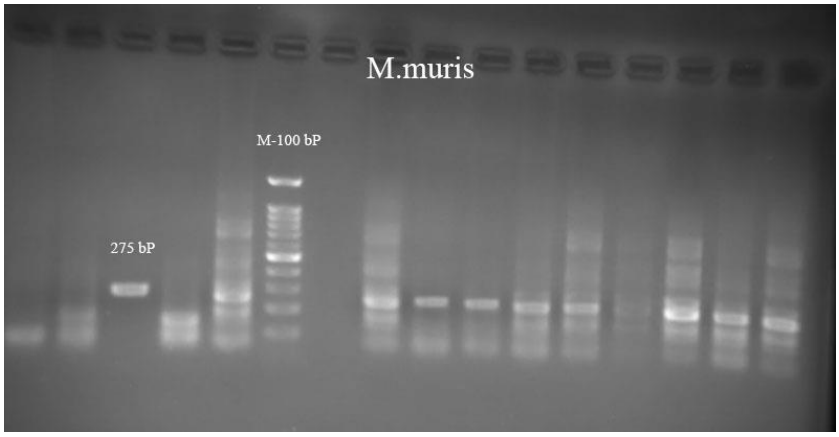

Figure 3. PCR amplification of *M.muris* from nasopharyngeal and lung samples.

Table 3. The results of the PPLO agar culture and PCR assay in different samples.

Tests	Vaginal		nasopharyngeal & lung	
	Positive	Negative	Positive	Negative
PPLO Agar	20 (68%)	9 (31%)	16 (55%)	14 (48%)
PCR *M. genuse*	19 (65%)	7 (58%)	15 (51%)	14 (%48)
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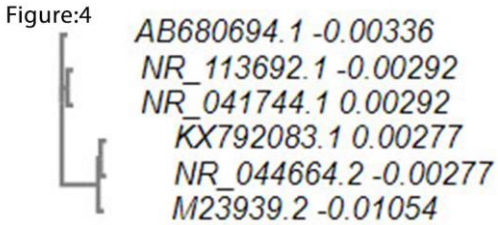

Figure 4. Phylogeny tree (real) of *M. muris* 16SrRNA gene. Maximum identity matrix was shown between KX792083.1 from Iran and NR_044664.2. from USA**.**

4. Discussion

Himmelreich and colleagues were compared the sequenced genomes of the human *Mycoplasma pneumonia* (816 kb) and human *Mycoplasma genitalium* (580 kb), the two closely related bacteria, with emphasis on coding capacity and genome organization (Himmelreich *et al.*, 1997). The genomes of *M. genitalium* and *M. pneumoniae* were subdivided into six segments (Fraser *et al.*, 1995; Peterson *et al.*, 1995). The sequenced genomes revealed all the 470 open reading frames (ORFs) of the genome of *M. genitalium* were the same in the larger genome of *M. pneumoniae*. In human *Mycoplasma genitalium* is naturally found in the genitourinary tract and *Mycoplasma pneumoniae* in the upper and lower airways (Hu *et al.*, 1977; Tully *et al.*, 1981).

Although there is an exception and researchers have been reported that *M. pneumoniae* has been isolated from urogenital specimens (Goulet *et al.*, 1995) and *M. genitalium* were isolated from the respiratory tract of patients (Baseman *et al.*, 1988). This shows that both bacteria can exist in the same organs (Himmelreich *et al.*, 1997). Researchers indicated that *M. pulmonis* is the most common pathogenic bacteria which usually produces respiratory infections in laboratory rats and mouse colonies and it would infect the genital tract of female mice as well (Collins and Parker, 1972; Furr and Taylor-Robinson, 1984; Nicklas *et al.*, 1993; Baker *et al.*, 1998).

Loganbill and colleagues showed that by using fnPCR assay, they were able to detect the *M. pulmonis* DNA in nasopharyngeal wash fluid from seronegative mice. Alignment of nucleotide sequences for design of primers (275 bp) targeting 16S rRNA gene of *M. pulmonis* (GenBank accession M23941), 17 other *Mycoplasma* species, and more than 13 bacterial species from genera was done by ClustalW and Pretty software programs (Genetics Computer Group, Madison, Wis). The nucleotide sequences revealed several regions in the 16S rRNA gene that were unique to *M. pulmonis*. Their finding indicated that the fnPCR assay could be used to identify seronegative mice infected with *M. pulmonis* (Loganbill *et al.*, 2005).

In conventionally rat colonies, the prevalence of respiratory Mycoplasmosis are approximately 100% (Lindsey and Morse, 1986; Brown and Reyes, 1991), while, the incidence of genital Mycoplasmosis are reported more than 40%(Cassell *et al.*, 1981; Cassell *et al.*, 1981; Lindsey and Morse, 1986; Cox *et al.*, 1988). Brown and colleagues reported that the time of *M. pulmonis* infection plays an important role in determination of pregnancy outcome and dissemination of infection from the urogenital organ to the respiratory organ (Brown and Reyes, 1991).

In our research despite this unique region that was used to detect *M. pulmonis* by PCR assay, we could not detect *M. pulmonis* in respiratory and genital tracts of 29 mice strain NIH, instead for the first time we have identified *M. muris* in both organs. More research is needed to know more information about the genome of *M. muris* and *M. pulmonis*. In conclusion, the PCR assay provides a very specific and sensitive assay for the detection of *M. muris* in laboratory mice.

Conclusion

In addition to the role of *M. muris* in genital infection, our data indicated the possibility of respiratory transmission without present of *M. pulmonis* in mice strain NIH. By using specific primers and PCR, *Mycoplasma muris* can be easily detected in both organs. We suggested that more research is needed to obtain the complete genome information of *M. muris* and compare it with *M. pulmonis*.

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Refereces

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