

Identification of *Mycoplasma Muris* Isolated from Vaginal Samples of NIH Mice

Mohammad Reza Zinatizadeh¹, Fatemeh Abedini^{*2}, Mostafa Jafarpour³, Zahra Masoumalinejad⁴

1. Department of Genetics, Faculty of Science, Tonekabon Branch, Islamic Azad University, Mazandaran, Iran

2. Department of Animal Breeding, Razi Vaccine and Serum Research Institute, Karaj, Iran

3. Department of Microbiology, Faculty of Science, Tonekabon Branch, Islamic Azad University, Mazandaran, Iran

4. Department of Microbiology, Sirjan Branch, Islamic Azad University, Sirjan, Iran

KEYWORDS

Mycoplasma muris
PCR
New strain
16S rRNA sequences
NIH Mice

Article Info

Received 2019/02/01;

Accepted 2019/03/12;

Published Online 2019



ABSTRACT

Mycoplasma muris (M.M) is a small pathogenic bacterium that lives in the female mouse genital tract. *Mycoplasma muris* may have harmful effects on the reproductive health of female. This research was performed to optimize the detection of *M. muris* in NIH mice in the Department of Animal Breeding, Razi Vaccine and Research Institute, Iran. In this cross-sectional study, 29 vaginal samples of NIH mice were selected through simple random sampling. For detection of the mycoplasma, the vaginal tissue removal of samples was done. First, samples were crushed using mortar and pestle with PBS 1ml, then were cultured in the PPLO broth and incubated at 37°C for 24h, they were passed through 0.45 µm pore-size filters and inoculated into specific PPLO broth and agar media for 3-4 weeks. In the next section, the PCR test was used with primers of 16S rRNA gene of *M. muris*. From 29 tested samples, 17.24% samples were positive for *M. muris* by PCR method, while 35.93% cultures showed positive. The phylogenetic analysis indicated a new strain of *M. muris*. The results of culture and PCR methods displayed the contamination in NIH mice. Therefore, more researches are needed regarding the presence of mycoplasma for treatment and clinical signs.

Corresponding Information: Agricultural Research, Education and Extension Organization (AREEO), Research & Development Department, Razi Vaccine and Serum Research Institute, E-mail: fmhabedinii@gmail.com

Copyright © 2019. This is an open-access article distributed under the terms of the Creative Commons Attribution-noncommercial 4.0 International License which permits copy and redistribute the material just in noncommercial usages, provided the original work is properly cited.

Introduction

Mycoplasmas were first isolated from rodents almost 80 years ago, and exist generally in all creatures (1, 2) and are recognized via various diseases, including pneumonia, arthritis, meningitis and chronic urogenital tract diseases (3). They are the members of the class Mollicutes with more than 120 species; 23-40% of the G+C content, the range of a genome size is about 600-1350 kb, they require cholesterol for growth and cause the diseases in humans and animals (4). *Mycoplasmas* are substantial and chronic bacterial pathogens for humans and animals that often colonize in the respiratory and genitourinary tracts (5).

A mycoplasma infection typically induces persistence of subclinical infections in mice

colonies. Recent surveys have demonstrated that approximately 60% of all the conventional laboratory rat and mice colonies are infected with one or more mycoplasmas (6). The microorganisms lack a cell wall and are highly polymorphic, which makes them resistant to antimicrobials that are effective on the cell structure, such as penicillin. These bacteria are probably derived from Gram-positive bacteria and their phylogeny is similar to *Clostridium*, however, the mycoplasma bacterium is Gram-negative (7).

The diagnosis of *Mycoplasma* species based on modern techniques requires a relatively large amount of time to be identified (8). The 16S rRNA gene sequencing plays an important role to identify

isolates of bacteria particularly rare bacteria, slow-growing bacteria, and the discovery of novel bacteria (9).

M. muris is a small pathogenic bacterium that lives in the genital tracts of mice. There is a specific relationship between *M. muris* and *U. urealyticum* clusters, which was first reported in 1983, and the mycoplasma infection is extremely rare among the mice (10). At present, there is no sufficient information regarding *M. muris* pathological state (11). The aim of this research study was to set up and optimize specific PCR method for detection of *Mycoplasma muris* in NIH mice.

Materials and Methods

Animals

All NIH mice were kept under standard conditions in Department of Animal Breeding Razi Vaccine and Research Institute. The protocol was approved by the animal care committee of Razi Vaccine and Serum Research Institute (MED.0309.QGL) that was performed on 29 vaginal samples of NIH mice from the Animal Breeding Center Razi Institute.

Growth conditions

In this cross-sectional study, 29 Vaginal Samples of NIH mice were selected through simple random sampling. For detection of the mycoplasma, the vaginal tissue removal of samples was done and crushed using mortar and pestle with PBS 1ml. Samples were placed in the PPLO broth solution (3ml) at 37°C for 24h. 15% of hours serum, thallium acetate (1:4000), penicillin (1000IU/ml), 0.0125% β-Nicotinamide adenine dinucleotide (NAD) and 0.0125% cysteine hydrochloride were added into PPLO broth and PPLO agar medium (BBL, Becton Dickinson and company, Cockeysville, Sparks, MD, USA) as necessary requirements for *M. muri*. After one day, all samples were filtered with CHROMAFIL® CA-45/25S, then were transferred into the new PPLO broth (1ml) and kept in an incubator at 37°C under 5% CO₂ for 3-4 weeks. 0.5ml of samples were transferred to PPLO agar media and checked for typical mycoplasma colonies.

DNA extraction

DNA extraction was accomplished using phenol-chloroform method (12). Briefly, 1 ml of each sample were centrifuged at 13000 rpm for 15 min. The supernatant fluid was discarded, PBS (150 µl) and Lysozyme (100 µl) were added, then the tubes were kept on the hot plate at 37°C for 30-40 min. All of the samples with Lysis buffer (Tris-HCl 50 mM pH=8, SDS 1%, NaCl 100mM, EDTA 50 mM, proteinase K 20 µl to 100µl) were shifted to the tubes and placed in a 56 °C bath for 4h. Then 200 µl saturated phenol was added and centrifugation was done (13000 rpm or 15700 g) for 15 min while supernatant was transferred to a new microtube and mixed, phenol /chloroform (1:1) was added with an equal volume. After centrifugation at 13000 rpm for 15 min, the aqueous phase was transferred and added to equal volume of pure chloroform. Samples were centrifuged (13000 rpm) for 15 min. Upper phase was mixed with 1/10 volume of acetate sodium solution 3M and were precipitated with 2 times volume of cool ethanol (it was kept at -20 °C for 20 min and centrifuged for 15 min at 13000 rpm). The next,

discarded ETOH and drying tubes then added 50 µl distilled water to them.

Amplification with specific primers (PCR)

In this study, published primers were used for the specific diagnosis of genus and species of *M. muris*. The primer of Mycoplasma genus is as follows: M1F: 5'-GCTGCGGTGAATACGTTCT-3', M3R: 5'-TCCCCACGTTCTCGTAGGG-3' (13). Specific amplification primers of *M. muris* were used, MMF: 5'-TTAAAGTTCCGTTTGGAAACG-3' and MMR: 5'-ATCATTTTCCTATTCCTACCA-3' that described by Van Kuppeveld et al (6). The PCR master mix (Biosystems Company, 2x PCRBIO Taq Mix Red) was prepared in a total volume of 25µl per sample, containing (12.5µl of 2X PCR Master Mix, 1µl+1µl primer 10µM, 3µl DNA template, and 7.5µl water). The PCR reaction was conducted in a master cycler (Eppendorf, Germany) as follows: for detection of genus: 7.5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C, with a final extension cycle of 5 min at 72°C. For

detection of *mycoplasma muris*: 10 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 1 min at 54°C and 30 sec at 72°C, with a final extension cycle of 7 min at 72°C.

PCR and Phylogenic Analysis

A 10µl aliquot of each PCR products was mixed with 2µl loading buffer (6X) and separated using electrophoresis (in 1.7% agarose gel 1xTris–acetic acid–EDTA (TAE) buffer) with 0.5 µl /ml SYBR Safe (100 volts for 1 hour) and UV-transillumination. Positive samples of the PCR products were purified from agarose gel (High pure PCR product purification Kit, Roche) and sequenced by Bioneer Company, Korea. All sequences were analyzed by multiple sequence alignments and phylogenetic analysis using Clustal Omega

(<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

RESULTS

Isolation of mycoplasma

Vaginal samples obtained from 29 NIH mice and were grown on PPLO broth medium at 37°C under aerobic conditions. First, colonies of the organisms were detected, then the incubation was conducted in the aerobic condition of broth cultures at 37°C for 3-4 weeks. Each isolate was purified by standard filtration. Some samples showed the growth on PPLO agar plates after 7 days of incubation and colonies were observed.

Bacteriology

Bacterial growth was observed in 11 samples out of 29 (35.93%), in the PPLO broth and PPLO agar The *Mycoplasma* genus PCR (MGPCR) and *Mycoplasma* species PCR (MMPCR) produced 163 bp amplicons (12 samples out of the 29, 41.37%) and 236 bp amplicons (5 samples out of 29,17.24%) which are displayed in figure 1 and figure 2 respectively. The full results are presented in Figure 3 and Table 1.



Fig 1. *Mycoplasma* genus PCR: PCR electrophoresis analysis in 1.7% gel agarose. M: Marker (100bp DNA ladder). Lane C+: Positive control (163bp band, *Mycoplasma* genus, NCTC 10123). Lane C-: Negative control (uncultured PPLO broth) and Lane 1-8: the isolated *Mycoplasma*.

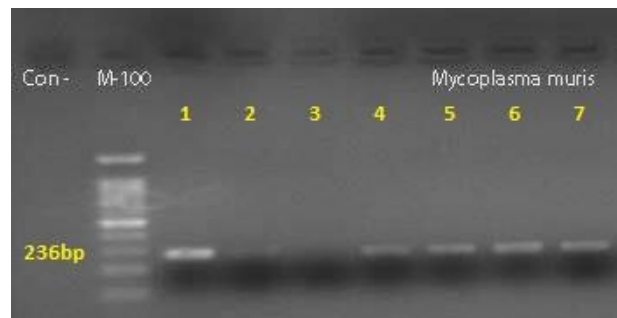


Fig 2. *M. muris* PCR: PCR electrophoresis analysis in 1.7% gel agarose. M: Marker (100bp DNA ladder). Lane C-: Negative control (uncultured PPLO broth) and Lanes 1, 4-7: Positive results (236bp). Lanes 2, 3: Negative results.

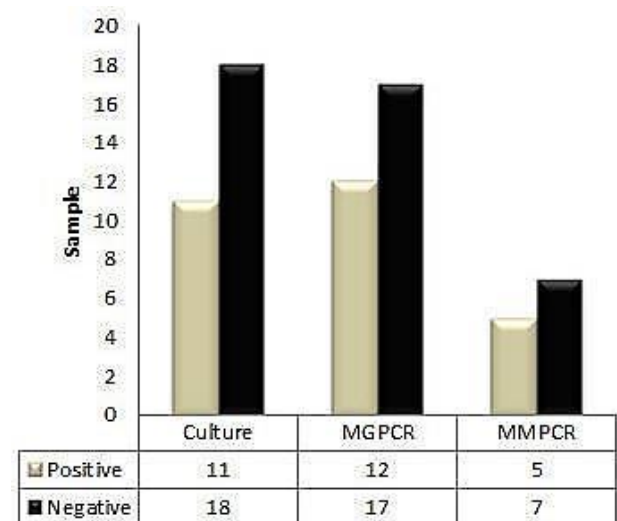


Fig 3. Sensitivity of the methods used for the diagnosis of *M. muris* in mice

Table 1. The results of the culture and PCR methods in the vaginal samples

Tests	Result	Total
Culture	Positive	(35.93%)
	Negative	(62.06%)
MGPCR	Positive	(41.37%)
	Negative	(58.62%)
MMPCR	Positive	(17.24%)
	Negative	(24.13%)

Calculation (Coefficient, Sensitivity, Specificity)

In table 2, the results have indicated that the sensitivity of PCR method is more than 80% to detect the *Mycoplasma* and the specificity of PCR assay is 94.44% (14).

Table 2. Distribution of samples for culture and the results of Mycoplasma-PCR methods (coefficient, sensitivity, specificity)

No. Sample	Culture	MGPCR
10	+	+
16	-	-
2	-	+
1	+	-
29Total		

$$\text{Coefficient} = \frac{(MGPCR\ positive, Culculture\ positive) + (MGPCR\ Negative, Culculture\ Negative)}{Total} \times 100$$

$$\frac{26}{29} \times 100 = 89.65\%$$

$$\text{Sensitivity} = \frac{(MGPCR\ positive, Culculture\ positive) - (MGPCR\ Negative, Culculture\ positive)}{(MGPCR\ positive, Culculture\ positive) + (MG\ PCR\ Negative, Culculture\ positive)} \times 100$$

$$\frac{9}{11} \times 100 = 81.81\%$$

$$\text{Specificity} = \frac{(MGPCR\ Negative, Culculture\ Negative) + (MGPCR\ Negative, Culculture\ positive)}{(MGPCR\ Negative, Culculture\ Negative) + (MGPCR\ positive, Culculture\ Negative)} \times 100$$

$$\frac{17}{18} \times 100 = 94.44\%$$

Phylogenetic Analysis

Phylogenetic analysis was done by the obtained data from the sequences of the *M. muris* isolates and were published in GenBank (Fig.4). Our sequence data have been submitted to GenBank and assigned the accession number of KX792083 for a new strain of *M. muris* (MYMORazi) from Iran. The accession numbers of the nucleotide sequence for other *M. muris* strains (16s rRNAs) in GenBank, that were applied in this study are as

follows: NR-044664.2 (strain RIII-4), AY753215.1, and D89507.1. In the phylogenetic analysis, MYMORazi, AY753215.1, and D89507.1 were joined together in a monophyletic clade that was most closely related to the *Mycoplasma muris*. As expected, as expected, AY753215.1 and D89507.1 are located in the MYMORazi cluster.

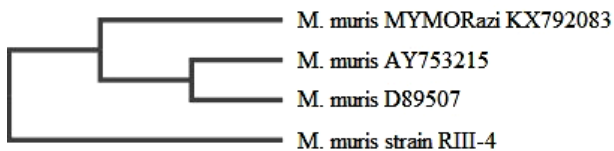


Fig 4. Phylogenetic analysis of *Mycoplasma muris* 16S rRNA. Phylogenetic relationships of MYMORazi with other related strains. The bar indicates 5% sequence divergence.

Discussion

In this study, *M. muris* was detected from vaginal samples of NIH mice using culture and PCR methods in Razi Vaccine and Serum Research Institute, Alborz, Iran. In previous studies, SP-4 medium and strict anaerobic conditions were used for *M. muris* diagnosis while in the present study, PPLO medium under aerobic condition was applied (11). The presence of bacterial pollution, PCR assay can be used on mycoplasma growth in the culture (24h of Mycoplasma enrichment for growth in the culture medium). (15).

PCR is routinely used as a molecular detection method in many laboratories, because of its high sensitivity (6). The 16S rRNA gene has been shown as a noteworthy marker for species identification using different molecular biological techniques such as denaturing gradient gel electrophoresis (DGGE), restriction analysis, pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) and gene sequencing (16-20). Numerous bacterial 16S rRNA sequences have been consigned in databases in GenBank or (EMBL) for nucleotide sequences (21).

Based on the phylogeny analysis of the 16S rRNA gene, *M. muris* is the common and ancestral cluster of the pneumonia group (10), in fact, *M. muris* 16S rRNA gene sequence clusters with the other previously identified mycoplasmas on a branch related to the pneumoniae group of Mycoplasma genus (10). The pneumoniae group comprises three distinct clusters represented by *M. pneumoniae*, *M. muris*, and *Ureaplasma urealyticum*.

Three agents cause main infections of the genitourinary tract in the mice. *Leptospira interrogans* serovar ballum (LISB) leads to renal

infection and has been reported in laboratory mice. *Mycoplasma muris* has been isolated from the vaginas of mice and has occurred in the female genital tract infections with *M. pulmonis*.

Amplification and sequencing of the *M. muris* 16S rRNA gene were obtained according to Van Kuppeveld' study. (6). Our data demonstrated that 17.24% of samples were positive by PCR method with the Mycoplasma species primer. Sequencing of the 16S rRNA has shown that the sequence analysis of the gene is a valuable tool for tracing *M. muris* isolated from the NIH mice.

In this study, one of the samples was positive for PCR method but showed negative in culture. Therefore, this study indicates that contamination level is close to the results of PCR test compared to culture method. The sequences were compared in GenBank using BLASTN program and aligned by Clustal Omega software. Our results displayed that MYMORazi strain (accession number KX792083) was phylogenetically related to the members of the muris species with the 98% similarity sequence. The phylogenic relationships can be determined between *M. muris* strains as a new strain. The phylogenic confirms a new strain for epidemiological aims and the phylogenic studies are essential to understanding the relationships between strains.

Conclusion

Studies have demonstrated that *M. muris* is an emerging sexually transmitted infection. Additional more researches are needed regarding their pathogenicity and resistance for clinical detection and treatment. Clinical detection and pathogenicity for *M. muris* infection should be considered.

Acknowledgments

We thank all the staff of the Mycoplasma Reference Laboratory, Animal Breeding Center, Razi Vaccine, and Serum Research Institute, Karaj, Iran. This study was supported by a grant of Research and Development Deputy of Razi Institute. The authors declare no conflict of interest.

References

1. Nelson JB. Infectious catarrh of mice: ii. the detection and isolation of coccobacilliform bodies. *The Journal of experimental medicine*. 1937; 65(6): 843-849.
2. Razin S, Yogeve D, Naot Y. Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular Biology Reviews*. 1998; 62(4): 1094-1156.
3. Waites KB, Katz B, Schelonka RL. Mycoplasmas and ureaplasmas as neonatal pathogens. *Clinical microbiology reviews*. 2005;18(4): 757-789.
4. Razin S. Molecular biology and genetics of mycoplasmas (Mollicutes). *Microbiological reviews*. 1985; 49(4): 419-455.
5. Simmons WL, Dybvig K. Mycoplasma biofilms ex vivo and in vivo. *FEMS microbiology letters*. 2009; 295(1): 77-81.
6. Van Kuppeveld FJ, Van der Logt JT, Angulo AF, Van Zoest MJ, Quint WG, Niesters HG, Galama JM, Melchers WJ. Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. *Applied and environmental microbiology*. 1992; 58(8): 2606-2615.
7. Taylor-Robinson D, Jensen JS. *Mycoplasma genitalium*: from Chrysalis to multicolored butterfly. *Clinical Microbiology Reviews*. 2011; 24(3): 498-514.
8. Yamamoto M, Asahara M, Tamura T, Matsumura M, Hayashimoto N, Makimura K. Rapid identification of *Mycoplasma pulmonis* isolated from laboratory mice and rats using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Journal of Veterinary Medical Science*. 2012; 74(8): 1083-1086.
9. Woo PC, Lau SK, Teng JL, Tse H, Yuen KY. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infection*. 2008; 14(10): 908-934.
10. Weisburg WG, Tully JG, Rose DL, Petzel JP, Oyaizu H, Yang D, Mandelco L, Sechrest J, Lawrence TG, Van Etten J. A phylogenetic analysis of the mycoplasmas: basis for their classification. *Journal of bacteriology*. 1989; 171(12): 6455-6467.
11. McGarrity GJ, Rose DL, Kwiatkowski V, Dion AS, Phillips DM, Tully JG. *Mycoplasma muris*, a new species from laboratory mice. *International Journal of Systematic and Evolutionary Microbiology*. 1983; 33(2): 350-355.
12. Pourbakhsh SA, Shokri GR, Banani M, Elhamnia F, Ashtari AB. Detection of *Mycoplasma synoviae* infection in broiler breeder farms of Tehran province using PCR and culture methods. *Archives of Razi Institute*. 2010; 65(2): 75-81.
13. Kojima A, Takahashi T, Kijima M, Ogikubo Y, Nishimura M, Nishimura S, Harasawa R, Tamura Y. Detection of *Mycoplasma* in Avian Live Virus Vaccines by Polymerase Chain Reaction. *Biologicals*. 1997; 25(4): 365-371.
14. Florkowski CM. Sensitivity, specificity, receiver-operating characteristic (ROC) curves and likelihood ratios: communicating the performance of diagnostic tests. *Clin Biochem Rev*. 2008; 29(1): 83-87.
15. Nicholas R, Ayling R, McAuliffe L. *Mycoplasma diseases of ruminants*. USA. CABI; 2008; 1-14.
16. McAuliffe L, Ellis RJ, Lawes JR, Ayling RD, Nicholas RA. 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma* species. *Journal of Medical Microbiology*. 2005; 54(8): 731-739.
17. Stakenborg T, Vicca J, Butaye P, Maes D, De Baere T, Verhelst R, Peeters J, de Kruif A, Haesebrouck F, Vaneechoutte M. Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of *Mycoplasma* species. *BMC infectious diseases*. 2005; 5(1): 1-10.
18. Marois C, Dufour-Gesbert F, Kempf I. Comparison of pulsed-field gel electrophoresis with random amplified polymorphic DNA for typing of *Mycoplasma synoviae*. *Veterinary microbiology*. 2001; 79(1): 1-9.

19. Gray LD, Ketring KL, Tang YW. Clinical use of 16S rRNA gene sequencing to identify *Mycoplasma felis* and *M. gateae* associated with feline ulcerative keratitis. *Journal of clinical microbiology*. 2005; 43(7): 3431-3434.

20. Clarridge JE. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious

diseases. *Clinical microbiology reviews*. 2004; 17(4): 840-862.

21. Pettersson B, Tully JG, Bölske G, Johansson KE. Updated phylogenetic description of the *Mycoplasma hominis* cluster (Weisburg et al. 1989) based on 16S rDNA sequences. *International journal of systematic and evolutionary microbiology*. 2000; 50(1): 291-301.

How to cite this article:

Zinatizadeh M R, Abedini F, Jafarpour M, Masoumalinejad Z. Identification of *Mycoplasma Muris* Isolated from Vaginal Samples of NIH Mice . *Mod Med Lab J*. 2019; 2 (1) :100-106