Iranian Journal of Veterinary Science and Technology

Received: 2025-Nov-29
Accepted after revision: 2026- Feb-02
Published online: 2026- Feb-02

RESEARCH ARTICLE

DOI:10.22067/ijvst.2026.96662.1621

Clinical, pathological, and molecular investigation and isolation of *Mycoplasma pulmonis* in a laboratory animal production and breeding colonies with murine respiratory mycoplasmosis

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ABSTRACT

This study investigated the prevalence of *Mycoplasma pulmonis* (*M. pulmonis*) contamination and disease within a 6 month period in a laboratory animal production center housing rats, mice, hamsters, guinea pigs, and rabbits. A total of 66 samples from different breeding colonies were subjected to culture, molecular diagnosis (PCR), and pathological examination. Sampling of the involved organs was performed in animals showed clinical signs. Clinical symptoms including tilt head and turning towards the affected ear, ruffled coat, chromodacryorrhea, and cobblestone lung, were observed in four adult rats. PCR revealed *M. pulmonis* DNA in 33 samples (50%), indicating widespread contamination. However, positive cultures and isolation were confirmed in only 12 samples (18% of total samples), all originating from rat colonies. Pathological changes such as atelectasis and bronchiectasis were observed in the samples of rats. The facility utilized a conventional breeding system with a shared air conditioner and also lacked biosecurity barriers, facilitating easy transmission of infection from infected rat to other animals. Due to the lack of sensitivity of those animals to this microorganism, no apparent issues exhibited in them, suggesting the absence of stressful and predisposing conditions for opportunistic infections such as *M. pulmonis*. Although microbial monitoring should be done regularly and periodically for various infectious agents.

Keywords

Mycoplasma pulmonis, Laboratory animals, Diagnosis, Isolation, Histopathology

Number of Figures: 7
Number of Tables: 1
Number of References:: 23
Number of Pages: 9

Abbreviations

SPF: specific pathogen-free
CAR: cilia-associated respiratory
FELASA: Federation of European Laboratory Animal Science Associations

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Introduction

M. pulmonis is recognized as a potential rodent pathogen, even within advanced laboratory animal breeding facilities [1]. Mycoplasma represents the smallest self-replicating and polymorphic microorganism, typically ranging from 200-500 nm in diameter. Their gram-negative nature, lack of cell wall, combined with their diminutive size and flexibility, enables them to easily traverse filter membranes, posing a contamination risk [1-5]. In rats and mice, *M. pulmonis* colonizes the respiratory tract, leading to respiratory mycoplasmosis. This condition is frequently undiagnosed due to its subclinical presentation. Clinical manifestations typically emerge only when the disease has progressed significantly. Factors such as elevated ammonia levels, various stressors, and the presence of other pathogenic agents can influence the clinical progression and severity of the disease [1, 2, 6]. The prevalence of respiratory mycoplasmosis in conventional rat colonies has been reported up to 100%, while in colonies equipped with biosecurity measures, the prevalence ranges from 20% to 60%. In general, the prevalence of mycoplasma infections in laboratory rat colonies is higher than in other laboratory animals such as mice, hamsters, guinea pigs, and rabbits. Nevertheless, mycoplasmas have been identified in these other species, and they may serve as significant potential sources of infection, primarily in a subclinical form. In specific pathogen-free (SPF) colonies, the presence of mycoplasma infections is expected to be absent [3, 7, 8]. Hill (1972) noted that transmission risk is considerably higher from rats exhibiting overt disease compared to those with subclinical infections. Typical clinical manifestations include sneezing, nasal discharge, shortness of breath, and head tilt, particularly when middle or inner ear infections are present. Other common symptoms include weight loss, hunchback, wrinkled skin, and the presence of chromodacryorrhea in the eyes and nostrils, which are red secretions from harderin gland. Nasal secretions rarely become mucous and purulent, but sometimes purulent exudate may be seen in the nasal passages, ears, and trachea. Mycoplasmas usually affect the respiratory tract [1, 2, 4, 6, 9, 10]. In infected rats, the lungs may appear normal in the early stages. However, in the later and advanced stages of the infection, particularly in older rats, the lungs can develop a cobblestone texture with gray or yellow-purple discoloration. Pneumonia and chronic inflammatory infiltrates resulting from bronchiectasis abscesses may also be present [11]. Microscopic histological alterations often do not correlate with clinical symptoms or external observations. Common diagnostic methods include serology, ELISA, indirect immunofluorescence, culture, histopathology, and molecular methods like PCR. However, serological assays may be imprecise due to cross-reacting or interfering antibodies, potentially leading to false positive across different species. ELISA detects mycoplasma infections through antibody detection in serum, but cannot identify subclinical infections and may also produce cross-reactions with other species. PCR, using specific primers, can detect various mycoplasma genera and species with as little as 0.5-1 picogram of DNA. Employing the RT-PCR method to amplify ribosomal RNA sequences enhances sensitivity by up to 1000 times, effectively identifying M. pulmonis in throat swab samples from rats. While culture can confirm *M. pulmonis* presence, a negative culture result might indicate a subclinical infection. Isolating mycoplasma from rats exhibiting mild or moderate disease poses significant challenges [1, 7]. The species identified in laboratory mice and rats include *M. pulmonis*, *M. arthritidis, M. neurolyticum, M. collis,* and *M. muris*, with *M. pulmonis* being the most significant [3, 4, 7, 8, 12].

Mycoplasma cultures can be labor-intensive, time-consuming, and may lack sensitivity for certain species. Several factors influence the successful isolation of mycoplasmas, including the selection of appropriate tissues, proper sampling techniques, appropriate transportation under standard conditions, the presence of growth-inhibiting factors in the sampled tissue, and the microorganism's nutritional requirements [12]. It is advisable to collect samples from multiple tissues of the same animal. While isolation of *M. pulmonis* from rats with clinical signs is not difficult, concurrent primary or secondary bacterial and viral infections can aggravate the lesions and complicate diagnosis [7]. *M. pulmonis* is also considered as a zoonosis agent in person who deal with contaminated rats. Piasecki et al. (2017) reported a 76% infection rate in breeding technicians working with rats infected with *M. pulmonis*, detected via PCR on nasopharyngeal samples. Strain sensitivities also exist with laboratory mice. For example, the C57BL/6 is more resistant than the BALB/c and DBA/2 [8, 11, 13].

Abbreviations-Cont'd

PPLO: Pleuropneumonia-like organisms
H & E: hematoxylin and eosin
BLAST: Basic Local Alignment Search Tool
PC: Positive Control
NC: Negative Control
PS: Positive sample
fnPCR: Fluorogenic Nuclease PCR

Results

Clinical observations

Animals were monitored for clinical signs of the infection over a period of six months. Among all the tested laboratory animal colonies including Wistar and Sprague Dawley rats, non-inbred NIH and NMRI mice, BALB/c, C57BL/6 and DBA/2 inbred mice, golden Syrian hamsters, Pirbright albino and colored guinea pigs and Dutch albino rabbits, only the rat colony exhibited the related clinical signs (Table 1). Four adult male and female rats from both Wistar and Sprague Dawley strains were observed with clinical signs including head tilt and rotational movement towards the affected ear (in the direction of bending the head) when the animal was lifted by the tail (Figure 1)

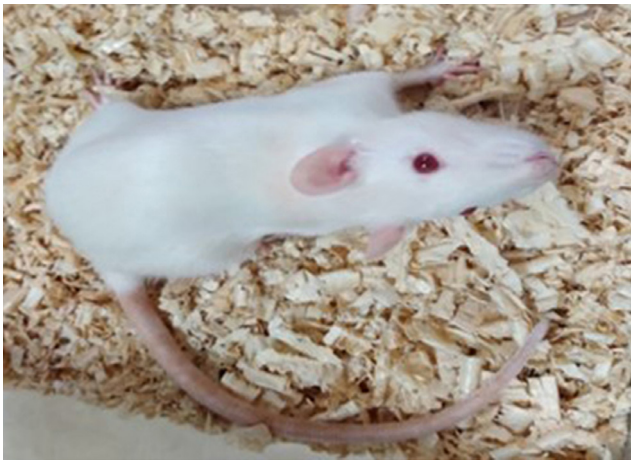

Figure 1.
Head tilt in rat with respiratory mycoplasmosis towards the right ear.

All four affected rats displayed these symptoms; one animal additionally showed hunchback, a crumpled body cover and chromodacryorrhea around the eyes. The red secretions contain porphyrin and are related to harderin gland. These secretions appeared bright fluorescent red under the UV light (Figure 2) and from this method it can be determined whether it is porphyrin or blood. Despite the presence of symptoms, fertility and reproductive in performance remained unaffected across all examined laboratory animal colonies and the breeding output continued at normal levels.

Necropsy observations

In some of the infected rats with clinical signs, lung cobblestone was observed (Figure 3).

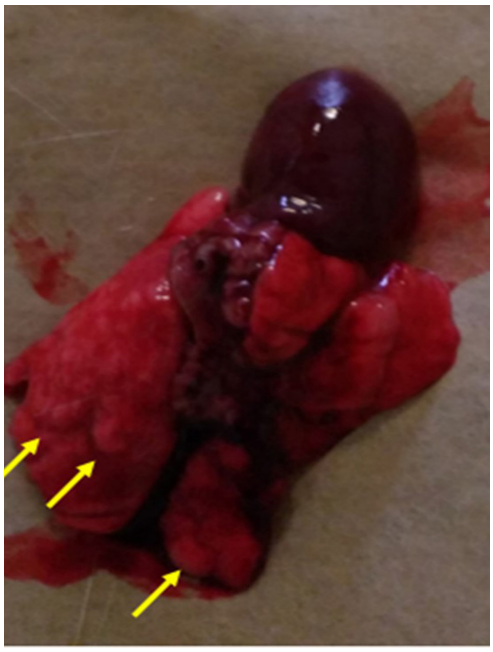

Figure 3.
Cobblestone lung in a rat with respiratory mycoplasmosis.

Cultural observations

Out of a total of 66 swab samples collected from different colonies, only 12 samples (18%) showed color change to orange and yellow in PPLO liquid medium, without any observed turbidity (Table 1). Colonies grown on PPLO agar medium appeared in the

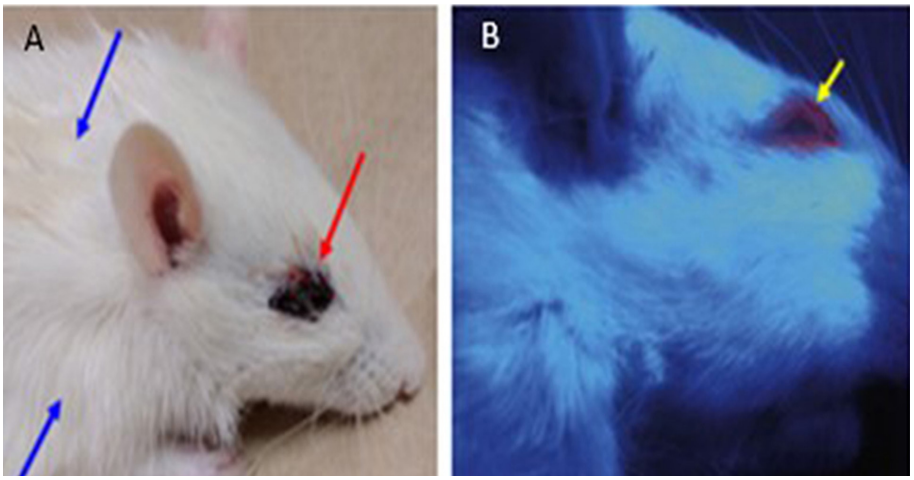

Figure 2.
A) Chromodacryorrhea around the eyes (red arrows) and ruffled coat (blue arrows), B) Observation of harderian gland discharges under the UV lamp appearing bright fluorescent red.

Table 1.
Type and number of animals and the results obtained.

Type of animal	Strain of animal	Number of male	Number of female	Number and percentage of animals with symptoms	Number and percentage of histo-pathological changes	Number and percentage of PCR-positive animals	Number of animals with positive culture results
Rat	Wistar	3	3	3 (50 %)	3 (50 %)	6 (100 %)	6 (100 %)
	Sprague Dawley	3	3	4 (66.7 %)	4 (66.7 %)	6 (100 %)	6 (100 %)
Mice	NIH	3	3	0	0	4 (66.7 %)	0
	NMRI	3	3	0	0	3 (50 %)	0
	BALB/c	3	3	0	0	0	0
	C57BL/6	3	3	0	0	0	0
	DBA/2	3	3	0	0	0	0
Hamster	Syrian	3	3	0	0	2 (33.3 %)	0
Guinea pig	Albino Pirbright	3	3	0	0	5 (83.3 %)	0
	Color Pirbright	3	3	0	0	4 (66.7 %)	0
Rabbit	Albino Dutch	3	3	0	0	3 (50 %)	0
	Total	**33**	**33**	**7 (10.6 %)**	**7 (10.6 %)**	**33 (50 %)**	**12 (18.1 %)**
		66					

form of fried-eggs (Figure 4).

Histopathology

Histopathological examination of lung tissue samples form symptomatic rats revealed hyperemia of the lung lobes. Thickening of vascular and alveolar walls was observed due to the infiltration and accumulation of lymphocytes and macrophages, which indicated infectious interstitial pneumonia (Figure 5A). The parenchyma of the lung tissue in some areas showed atelectasis, and bronchiectasis and bronchiolectasis were present in a number of bronchi. Epithelial thickening with the accumulation of nuclei (hyperplasia) and narrowing of the duct caused by a layer of cuboidal to columnar epithelium (obstructive bronchiolitis) were also observed (Figure 5B).

Identification of M. pulmonis

During PCR analyses, 33 out of 66 swab samples showed the respective DNA band of 500 bp and hence were regarded positive (Figure 6). According to these results 50% of the samples were contaminated with *M.*

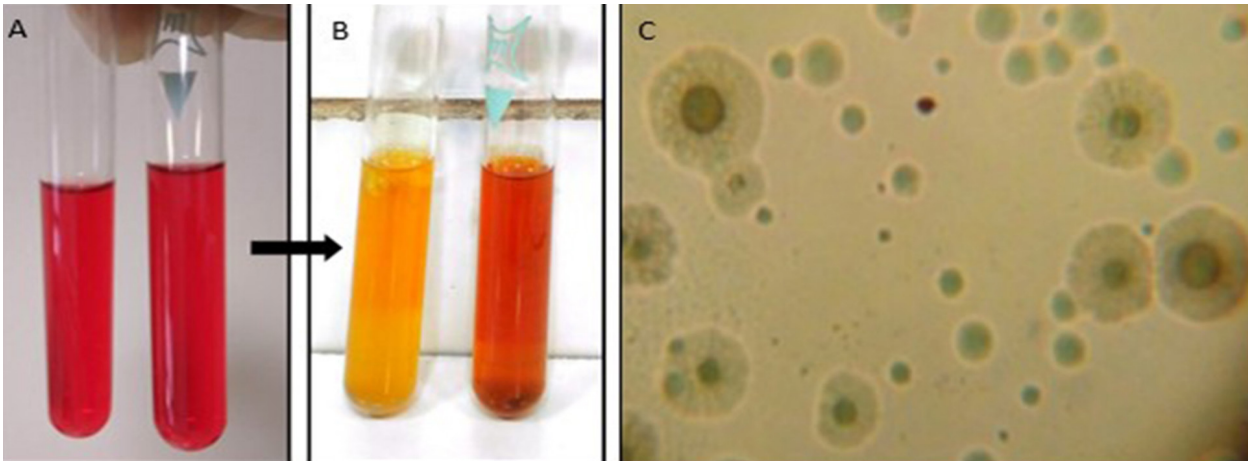

Figure 4.
PPLO broth medium A) uncultured, B) cultured with color change orange and yellow, C) typical Mycoplasma colonies on PPLO agar medium appearing as fried-egg.

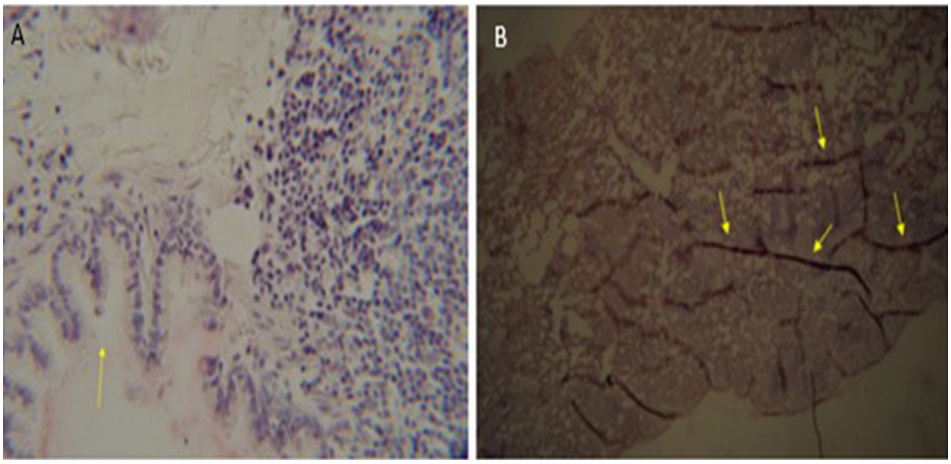

Figure 5.
A) Bronchiectasis and bronchiolectasis, thickening of the epithelium with the accumulation of nuclei (hyperplasia) and narrowing of the ducts by a layer of cuboidal to columnar epithelium (obstructive bronchiolitis) in the lung tissue of the affected rat (hematoxylin-eosin staining, magnification X 200). B) Areas of the alveolar wall thickened with the infiltration and accumulation of lymphocytes and macrophages, in the lung tissue of the infected rat, which indicates infectious pneumonia (magnification X 32).

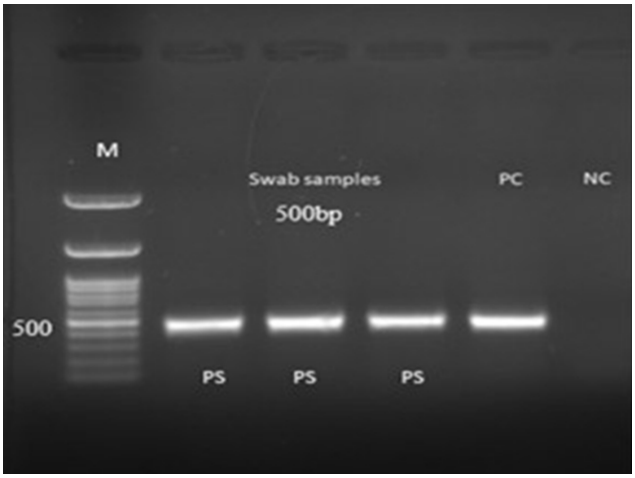

Figure 6.
PCR product performed on swab samples for *M. pulmonis* 16S/23S rRNA intergenic spacer region. [M: The Ladder DNA, 100 bp (GeneRulerTM 100 bp DNA Ladder-Fermentas) as a marker, PC: Positive Control, NC: Negative Control, PS: Positive sample].

pulmonis. According to obtained results, 50% of Wistar rats and 66.6% of the Sprague Dawley rats exhibited respiratory symptoms, while none of the other laboratory animal species showed clinical sign. Animals that showed histopathological symptoms, also showed histopathological changes (10.6%). Comparison of microbiological culture and PCR analysis a significant difference were recorded, and PCR analysis appeared more sensitive compare to microbiological culture. By culture only 18% of samples appeared positive, while PCR detected 50% positive samples. The phylogenetic tree of *M. pulmonis* is shown in Figure 7.

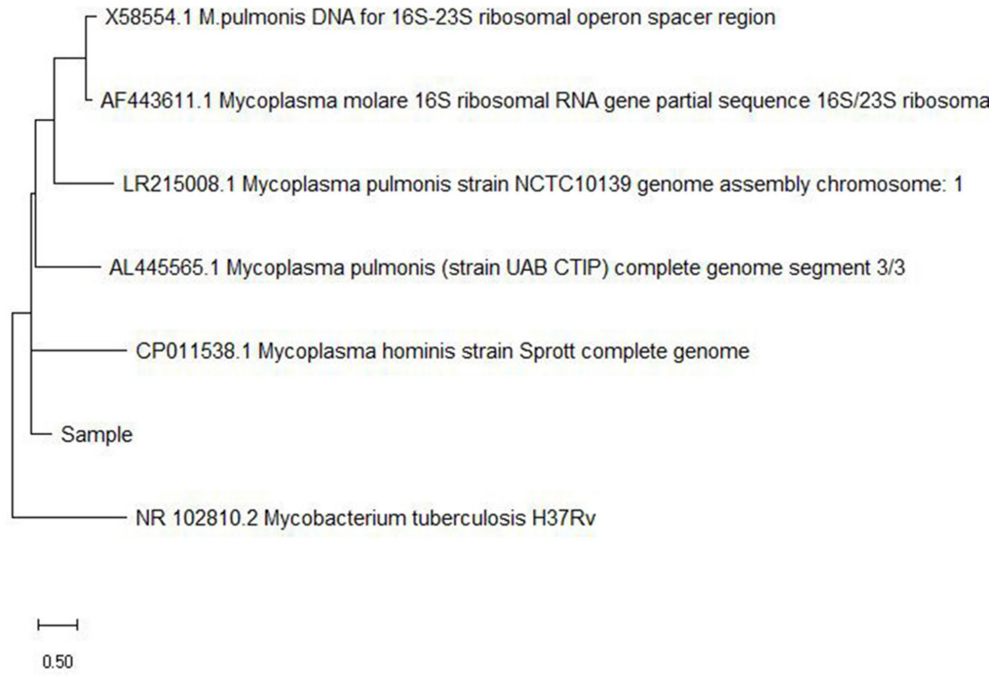

Figure 7.
Evolutionary analysis in the phylogeny tree of the samples from the current study, was constructed using the maximum likelihood method, utilizing the Mega version X (10) software. The phylogenetic tree was constructed using the nucleotide sequence of the 16S ribosomal RNA sequence gene of the *M. pulmonis*. The sample and the other nucleotide sequences Mycoplasma were grouped together in a Clustal (cluster), indicating their genetic similarity. *Mycobacterium tuberculosis* was the out group.

Discussion

Mycoplasma was initially isolated from rodents in the late 1940s, with *M. pulmonis* recognized as the predominant species in these hosts. Infection with this microorganism is of considerable importance because it can interfere with research results [7]. Rossini et al. (1983) reported the isolation of mycoplasmas from laboratory mice exhibiting symptoms of pneumonia and arthritis [7]. Furthermore, Timenesky et al. (1992) successfully isolated both *M. pulmonis* and *M. arthritidis* from rats and mice in conventional colonies [7]. Additionally, Barreto et al. (2002) identified *M. pulmonis* in mice presenting respiratory symptoms [7]. This study was investigated the precence of murine respiratory mycoplasmosis in a laboratory animal production and breeding colonies. In the study, clinical signs were observed only in four adult Wistar and Sprague Dawley rats. These signs included head tilt and a rotational movement towards the affected ear. Additionally, one rat exhibited a ruffled coat and chromodacryorrhea. In certain infected rats displaying clinical symptoms also exhibited characteristic cobblestone appearance of the lung. Overall, the incidence of mycoplasma infections in laboratory rat colonies is greater than that in other laboratory animals, such as mice, hamsters, guinea pigs, and rabbits. While mycoplasmas can be isolated from these other species, they may serve as significant potential sources of subclinical infection [3, 7, 8]. In the present study, by using culture methods, *M. pulmonis* was isolated from 12 samples from rat colonies, representing 18% of the total samples analyzed. The lung tissue samples from rats exhibiting clinical symptoms displayed pathological alterations such as hyperemia, vascular and alveolar wall thickening, atelectasis, bronchiectasis and bronchiolectasis. In contrast to culture results, PCR detected *M. pulmonis* in 33 out of 66 samples (50%) collected from colonies of rats, mice, hamsters, guinea pigs, and rabbits. According to Reyes et al. (2000), *M. pulmonis* is naturally present in the respiratory and reproductive system of rats, although susceptibility varies among strains; for example, LEW inbred rats are more sensitive to *M. pulmonis* than F344. Also, the Sprague Dawley is more sensitive to genital mycoplasmosis than the Wistar [14]. Hence, the PCR technique employed in this study might have detected the *M. pulmonis* that are natural residents of the respiratory system of these animals. In contrast, by culture technique these were not identified which might be due to the small number of these bacteria or lower sensitivity of the method used compared to PCR analysis. Shafaati et al. (2016) detected the presence of *M. pulmonis* in the respiratory system of laboratory rats through PCR targeting the SrRNA gene and successfully isolated the organism. While, many other techniques have also been employed for the identification of *M. pulmonis* in laboratory animals [2]. Goto et al. (2012) employed MALDI-TOF MS spectrophotometric technique for rapid identification of *M. pulmonis* [12]. Loganbill et al. (2005) used fnPCR (Fluorogenic Nuclease PCR) method to detect *M. pulmonis* in laboratory rats [9]. Conversely, Disastra (2019) by using PCR method, reported no detection of *M. pulmonis* infection in samples prepared from the lungs and trachea of mice, rats, guinea pigs and laboratory hamsters at Mahidol National Center of Thailand, indicating that probably animals of this facility were free of this mycoplasma [15]. *M. caviae* and *M. pulmonis* cause latent infection in the nasopharynx, brain and genital tract of guinea pigs [11]. Guinea pigs are often asymptomatic and carriers, but poor hygienic conditions and overcrowding it can elevate ammonia levels, irritates the respiratory tract and increases the risk of mycoplasma infection. In advanced cases, *M. caviae* and *M. pulmonis* cause acute lymphadenitis, metritis and arthritis in guinea pigs [16]. *M. pulmonis* has also been isolated from the rhinopharynx of rabbits with upper respiratory tract symptoms, likely due to proximity to infected rat colonies [16]. Abd-Algawad et al. (2021) reported that infection of rabbits with *M. pulmonis* increases their vulnerability to bacterial infections such as *Pasteurella multocida* and *Staphylococcus* aureus [17]. In rabbits, respiratory diseases are the second most important after digestive diseases. Respiratory bacterial infections in rabbits cause rhinitis, sinusitis, otitis media, conjunctivitis, pneumonia, lung abscess and septicemia. Although mycoplasma infections are usually subclinical; for example, *M. pulmonis* infection in rabbits is very rare, but in some cases it can lead to respiratory disorders [17]. The breeding system examined in the present study used conventional housing with shared ventilation and no biosecurity barriers between different areas, these conditions likely facilitated the transmission of infection from infected rats to other laboratory animals. The insensitivity of these animals to the microorganism did not result in any adverse effects, suggesting a lack of stressful or predisposing conditions for opportunistic infections. However, regular and periodic microbial monitoring is essential for various infectious agents.

Conclusion

This study identified *M. pulmonis* in almost 50% of the animals that showed clinical sign of respiratory mycoplasmosis. Mycoplasma infection is a recognized concern, and although preventive measures exist, it remains imperative for breeding centers to safeguard the health of their laboratory animals. Regrettably, in numerous countries, animal health certificates issued by various centers are often not authentic. Beyond the

symptoms and complications associated with mycoplasma infection, it adversely affects the outcomes of research involving these animals. Consequently, it is essential to investigate the presence of mycoplasma in the target animal population. Subclinical infections are common, and significant number of laboratory animals may carry mycoplasma infections without exhibiting clinical symptoms. The absence of SPF conditions and the use of shared ventilation likely facilitated the spread of infection within the facility examined. Therefore, the implementation of continuous and permanent health monitoring programs is crucial for quality control of these animals.

Materials & Methods

Ethical approval

The present study was conducted in accordance with the guidelines set by the Animal Ethics Committee of Razi Vaccine and Serum Research Institute, and all experiments were carried out in accordance with relevant guidelines and regulations.

Animals

During a period of 6 months, from October 2023 to March 2024, different breeds of laboratory animals from a Laboratory animal breeding facility in Iran were examined for possible contamination and disease caused by *M. pulmonis*. Laboratory animals included Wistar and Sprague Dawley rats, NIH and NMRI outbred mice, BALB/c, C57BL/6 and DBA/2 inbred mice, golden Syrian hamsters, albino and colored Pirbright guinea pigs, and albino Dutch rabbits. Each breed was housed in a separate room.

Animal Housing and management

The breeding system was conventional and the airflow was provided by a shared air conditioner. No biosecurity barriers were in place between rooms. All animals appeared clinically healthy, were free from external and internal parasite, and were provided standard compressed food (pellets) and tap water ad libitum. Laboratory mice, rats, hamsters and guinea pigs were kept in shoebox polycarbonate cages, while rabbits were kept in aluminum cages with mesh floors and standard dimensions. For polycarbonate cages, sterile aspen tree wood shavings were used as bedding. Changing the cage and wood shavings was done twice a week. The temperature of the breeding rooms was 22-24°C, the humidity was 45-55%, the air ventilation was 8-10 three minutes/hour and the light/dark period was 12:12 hours cycle with light intensity was below 325 lux.

Sampling collection

Sampling was done according to the Federation of European Laboratory Animal Science Associations (FELASA) guidelines [18-21]. Assuming the lowest probability of *M. pulmonis* presence in the mentioned colonies, for maximum sampling, 10% prevalence of infection at the animal colonies level was considered. On this basis and with the highest level of confidence (99.9%), 66 adult animals (Table 1) were randomly selected from both sexes for sampling. Swab samples from the nasopharynx of animals (two samples from each animal) were taken according to the laboratory animal ethical principles. Samples from animals with clinical signs were taken according to the animal ethical principle. After euthanasia (intraperitoneal injection of combination of ketamine and xylazine at the rate of 2-3 times the anesthesia dose) samples from the bifurcation of the trachea was taken [22].

Culturing of Samples

The collected samples were inoculated into PPLO (Pleuropneumonia-like organisms) broth medium (BBL, Sparks, MD, USA). The sample swabs were then taken out of PPLO liquid media and 1 ml of the contaminated media was passed through a 0.45µm syringe filters (Sartorious, USA) and transferred to fresh PPLO broth culture and incubated at 37°C for almost a week. If the color of the medium changed to orange or yellow (due to acidity) on three consecutive weeks with the renewal of the liquid culture medium, the results of mycoplasma culture are considered positive. Positive samples were further cultured on PPLO agar medium and observed daily up to 21 days for typical *M. pulmonis* colonies. Samples from symptomatic animals were cultured on PPLO broth, blood agar, MacConkey agar, sabouraud dextrose agar and anaerobic cultured in blood agar and placed in a 37°C.

Histopathological Examinations

In order to investigate the histopathology of lung tissue, samples from infected animals were prepared and placed in 1% neutral formaldehyde buffer solution. Tissues were routinely processed and embedded in paraffin. Paraffin blocks were cut in sections of 5-6µm and stained with hematoxylin and eosin (H & E) for observation with a light microscope. The used animal carcasses were destroyed using an infectious waste disposal device (hydroclave) [22].

DNA Extraction and PCR analysis

The swab samples were subjected to DNA extraction using the high pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers' instructions. DNA concentration and purity were determined by absorbance using a Nano® spectrophotometer (Maestrogen, Las Vegas, USA).

The assay was performed on an ABI Veriti 96 Thermal Cycler (Applied Biosystems, Foster, CA, USA) using a primer amplifying a specific region, (16S/23S rRNA intergenic spacer region) as described earlier [23]. The reaction mixture (50 µl) contained 33 µl of nuclease-free water, 5 µl of 10X Ampliqon Ammonium Buffer, 1µl of 25mM $MgCl_2$, 2µl 10 mM of dNTP mix, 2µl of 10µM each primer, 1µl of 2.5 units/µl Ampliqon AccuPOLTM DNA Polymerase (AMPLIQON, Odense M, Denmark), and 4 µl of extracted viral DNA. The PCR conditions included; 94°C for 2 min, 35 cycles of (94°C for 30 sec, 54°C for 45 sec, and 72°C for 1 min) and 72°C for 5 min. The PCR amplified products were run and analyzed on 1% agarose gel electrophoresis.

The obtained PCR products were sequenced in both directions by a commercial DNA service company (Macrogen, Seoul, South Korea). The obtained sequences were analyzed and compared with *M. pulmonis* reference sequences in a database using Basic Local Alignment Search Tool (BLAST) at NCBI.

Authors' Contributions

All authors contributed to the study conception and design. R. F. acquisition of data: R. F. analysis and interpretation of data: R. F. drafting of the manuscript: R. F. critical revision of the manuscript for important intellectual content: M. M. review and editing: R. F. administrative, technical, and material support: M.M.

Acknowledgements

All authors would like to thank the staff of the Laboratory Animal Production and Breeding, Pathology, and Microbiology Departments of Razi Vaccine and Serum Research Institute for their cooperation.

Conflict of interest

The authors declare that there is no conflict of interest.

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How to cite this article
Fallahi R, Moharrami M, Bashashati M, Bozorgi S. Clinical, pathological, and molecular investigation and isolation of *Mycoplasma pulmonis* in a laboratory animal production and breeding colonies with murine respiratory mycoplasmosis. Iran J Vet Sci Technol.2026; 18(2): 59-67.
DOI: https://doi.org/10.22067/ijvst.2026.96662.1621
URL: https://ijvst.um.ac.ir/article_47848.html