Characterization of Mycoplasma Strains Isolated from House Musk Shrews (*Suncus murinus*) and Their Infectivity in Mice and Rats

Kazuo GOTO, Toshio ITOH, Susumu EBUKURO, Naoko KAGIYAMA,
and Ryo HARASAWA*

Central Institute for Experimental Animals, 1,430 Nogawa, Miyamae-ku, Kawasaki-shi,
Kanagawa 216, and *Animal Center for Biomedical Research, Faculty of
Medicine, The University of Tokyo, 7-3-1 Hongo,
Bunkyo-ku, Tokyo 113, Japan

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Five mycoplasma strains isolated from house musk shrews (*Suncus murinus*) in the Central Institute for Experimental Animals were characterized and compared with three murine mycoplasma strains, *Mycoplasma pulmonis* m 53, *M. arthritidis* PG6, and *M. neurolyticum* Type A, and with reference strain G3-5 previously isolated from a house musk shrew. These isolates fermented glucose, but did not hydrolyze urea and arginine, passed through membrane filters of 450 nm pore size, were sensitive to digitonin, and formed minute (115 to 231 μ m in diameter) colonies on agar medium. All the five unclassified house musk shrew mycoplasma strains and strain G3-5 used as a reference constituted a homogeneous group based on (i) their antigenic properties (determined using the metabolism inhibition test), (ii) their polypeptide profiles (determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the Western blotting assay), and (iii) their genomic properties (determined using DNA cleavage pattern analysis), but were quite distinct from the established murine mycoplasmas on the basis of these findings. In a survey of 56 house musk shrews aged 2 to 45 weeks in our colonies, mycoplasmas were isolated from the oral cavities of all animals examined. No gross or microscopic lesions were observed in the five animals from which the mycoplasma strains were isolated. In experimental infection, the mycoplasma was not infective for mice and rats. The results suggest that this group of mycoplasmas is a common inhabitant of house musk shrews. —

KEY WORDS : characterization, mycoplasma, *Suncus murinus*

Mycoplasmas are widely distributed in nature and have been isolated from many laboratory animal species including nonhuman primates, dogs, mice and rats [3,8,14,20]. Moreover, they frequently produce chronic respiratory disease, arthritis and genital tract infections in these animal species. The house musk shrew (*Suncus murinus*) is a species of insectivore, and has recently attracted attention as a new experimental animal [9,10,12,18], but little information is available on the infectious agents in this animal species. Isolation of mycoplasma from house musk shrew was first reported by Ito *et al.* in 1988 [11]. However, the organisms from the house musk shrew have not been characterized further, and their rela-

tionship with other mycoplasma from rodents has not been established. For this reason, it is necessary to define their characteristics and prevalence of infection in the house musk shrew.

The purposes of the present studies were to examine the properties of the mycoplasmas isolated from house musk shrews, and to investigate the actual state of mycoplasma infection in a house musk shrew colony and their pathogenicity in mice and rats.

Materials and Methods

Animals. The SUN strain of house musk shrews maintained in four rooms of the Central

Institute for Experimental Animals (CIEA) as a closed breeding colony was used for these studies. The history of the SUN strain of house musk shrew was previously reported by Mastuzaki *et al.* [17].

Mycoplasmas. Five mycoplasma strains from house musk shrews were examined. Each strain was isolated from the trachea (G677-2T, G666-16R, G666-16A), the lung (G677-1L) or the prepuce (G677-1P) of three animals aged 12–16 weeks maintained in different breeding rooms of the facilities. The unidentified isolates were cloned five times by filtration through filters with pore size of 450 nm to produce pure cultures. Among the reference strains employed as controls in various tests for characterization of the isolates, strain G3-5 isolated from a house musk shrew, *Mycoplasma pulmonis* m53 and *M. neurolyticum* Type A were kindly provided by Dr. K. Koshimizu, Department of Veterinary Medicine, Kago-shima University, Japan. *M. arthritis* PG6 was generously provided by Dr. M. Nakagawa, Department of Veterinary Science, National Institute of Health, Japan.

Culture media and growth conditions. Media for cultivation of mycoplasmas have been described previously by Cassell *et al.* [2]. Briefly, the broth medium contained 70 ml of PPLO broth (Difco Laboratories, U.S.A.) supplemented with 10 ml of 25% (wt/vol) yeast extract, 20 ml of heat-inactivated horse serum (GIBCO Laboratories, U.S.A.), 1 ml of 100,000 units of penicillin G, 1 ml of 2.5% thallium acetate, 0.5 g of glucose and 0.002% phenol red and pH was adjusted at 7.8. The agar medium consisted of the same ingredients except that 1.3% Bacto-agar (Difco) was added, and the glucose and the phenol red were omitted. Specimens were collected with sterile cotton swabs from each site of the animals, inoculated to the broth and agar media and incubated at 37°C in a humid chamber. The broth media were checked for the organisms in the mid-to late-log phase (pH 7.0 to 7.1).

Preparation of rabbit antisera. For serological tests, hyperimmune rabbit antisera to each mycoplasma were prepared. Each antigen was produced from about 1,000 ml of 24-hr broth culture. The sediment was washed by centrifugation at 8,000xg for 20 min with three changes of PBS. The final sediment was resuspended in 20 ml of PBS and stored at

–20°C. Rabbits were injected intravenously with 2 ml of the antigen solution six times weekly for 5 consecutive weeks. Sera were dispensed in 2 ml aliquots and stored at –20°C until use.

Characterization of the isolates.

(i) Morphological studies. Mycoplasma colonies grown on the agar medium were examined after 2 days of incubation. The colonies were transferred to slides, stained with Gram's stain and examined by light microscopy.

(ii) Reversion studies. Each of the five mycoplasma strains examined was serially subcultured five times in broth media without antimicrobial agents, and each broth subculture was inoculated onto antimicrobial agent-free agar medium and observed for reversion to bacteria [13].

(iii) Filtration studies. Cultures (after 20 hr of incubation) were tested for filtrability with membrane filters (Sartorius, Germany) of different pore sizes 450 and 200 nm in diameter. Filtrability was determined by color change of the medium after filtration [13].

(iv) Sterol requirement. Growth responses to cholesterol were determined by using a technique that was modified slightly from the recommended indirect method described by Hill *et al.* [7]. Briefly, the isolates were tested for digitonin susceptibility by using disks containing 0.025 ml of a 1.5% ethanolic solution of digitonin (Wako Pure Chemicals, Osaka, Japan). The width of the zone of growth inhibition was measured in millimeters.

(v) Tests for biochemical and biological properties. Glucose fermentation, arginine and urea hydrolysis were assessed by the methods of McGarrity *et al.* [15] with some modifications. All procedures were performed with broth or agar cultures that were grown aerobically for 1 to 3 days. Adsorption of erythrocytes was tested with guinea pig, sheep and house musk shrew cells using the method of Manchee *et al.* [16].

(vi) Metabolism inhibition (MI) test. The test was based on the procedure of Purcell *et al.* [19] with some modifications. This test was performed using glucose as the substrate and was carried out as follows. In tubes containing 0.5 ml each of serial two-fold dilutions of the antiserum, 0.5 ml of broth containing approximately 10^2 to 10^3 color-changing units (ccu)/ml

of the organism was added and then the tubes were incubated at 37°C. Each antiserum was heated at 60°C for 20 min before use and the tests were performed in duplicate.

(vii) Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of whole-cell proteins. The centrifuged mycoplasma cells were washed with PBS, resuspended in PBS, and diluted 1:1 with a solution containing 2% SDS, 5% 2-mercaptoethanol and 20% glycerol, and then boiled for 5 min. Electrophoresis was performed on 10% polyacrylamide gel. Gels were stained with Coomassie brilliant blue R-250 [5].

(viii) Western blotting analysis. Proteins were transferred from the gels to nitrocellulose paper (PALL BIODYNE) by the method of Towbin *et al.* [21]. Non-specific binding sites were blocked by overnight incubation at 4°C in PBS containing 3% BSA. The rabbit hyperimmune serum prepared against the mycoplasma strain G666-16R was diluted 1:100 in PBS. These antibody preparations were then reacted with the nitrocellulose filter at 37°C for 1 hr. After being washed three times with PBS, the filter was incubated with horseradish peroxidase-linked protein A diluted 1:1,000 in PBS. After three further washes in PBS, the filter was stained with 0.025% (wt/vol) diaminobenzidine tetrahydrochloride (Wako Pure Chemicals, Osaka, Japan) in 50 mM Tris-hydrochloride (pH 7.2) containing 0.01% (vol/vol) hydrogen peroxide.

(ix) Restriction endonuclease analysis. The cell pellets were gently lysed in a solution containing 100 mM EDTA, 0.5% SDS, and 50 mM Tris-hydrochloride (pH 8.0). The cell lysates were treated successively with proteinase K and then extracted with phenol, phenol-chloroform (1:1), and chloroform-isomyl alcohol (24 : 1). Individual DNA preparations were precipitated with two volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.4). Each DNA pellet was dissolved in Tris-EDTA buffer [6]. DNA cleavage by restriction endonuclease was carried out using *Hind* III purchased from Takara Shuzo Co., Ltd. About 5 µg of mycoplasma DNAs was digested with 10 to 15 units of the enzyme. The reaction mixture was incubated at 37°C for 2 hr and subjected to 1.0% agarose gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide [4].

(x) Survey of mycoplasma in house musk shrews. A total of 56 swab samples of the oral cavity were obtained from 56 house musk shrews, 2 to 45 weeks old, bred and maintained in the CIEA. The isolates were identified by the growth inhibition (GI) test using antiserum against the G666-16 strain. The test was performed by the method of Black [1]. The gross and histopathological lesions were examined in five of the 56 house musk shrews.

(xi) Experimental mycoplasmal inoculation in rats and mice. Six SPF female Jcl:Wistar rats aged 4 weeks and six female SPF Jcl:ICR mice aged 4 weeks were inoculated orally and intranasally. The inoculum contained 3.2×10^4 colony-forming units (cfu) of a mycoplasma strain (G677-2T). The rats and mice were examined 3 and 7 days after inoculation for the cultivation of mycoplasma using the swabs from their oral cavity.

Results

Characterization of the isolates.

(i) Morphological studies. Colonies of the five strains grown on agar medium had a fried-egg appearance and measured about 115 to 231 µm in diameter. The cells were pleomorphic and gram negative. Most cells were coccoid or cocobacillary.

(ii) Reversion studies. None of these strains tested showed any evidence of reversion to a bacterial form when they were serially subcultured in antimicrobial agent-free agar media.

(iii) Filtration studies. These strains passed through membrane filters of a pore size of 450 nm but failed to pass through filters of a pore size of 200 nm.

(iv) Sterol requirement. The growth of each of the strains examined was inhibited by 375 µg of digitonin when the disk test procedure was used. The inhibition zones surrounded disks with digitonin ranged from 2.4 to 2.5 mm. Colony formation was not inhibited by control disks without digitonin.

(v) Biochemical and biological properties. The biochemical and biological characteristics of these strains were as follows: they fermented glucose, did not hydrolyze arginine and urea, and did not show hemadsorption ability for guinea pig, sheep and house musk shrew erythrocytes.

Table 1. Serological relationships among the established mycoplasma species and unclassified house musk shrew strains by the MI test

Strain	Antiserum against					Type A
	G666-16R	G677-1L	G3-5	m53	PG6	
<i>Mycoplasma</i> sp.						
G666-16R	80	40	80	—	—	—
G677-1L	80	80	80	—	—	—
G3-5	20	20	160	—	—	—
<i>M. pulmonis</i>						
m53	— a)	—	—	1280	—	—
<i>M. arthritidis</i>						
PG6	—	—	—	—	5120	—
<i>M. neurolyticum</i>						
Type A	—	—	—	—	—	80

a) No inhibition at serum dilution of 1 : 20

Fig. 1. SDS-polyacrylamide gel electrophoresis of cell protein from rodents and house musk shrew mycoplasma strains. *M. pulmonis* m 53, lane 1 ; *M. arthritidis* PG 6, lane 2 ; *M. neurolyticum* type A, lane 3 ; unclassified house musk shrew mycoplasmas G 3-5, lane 4 ; G 666-16 A, lane 5 ; G 666-16 R, lane 6 ; G 677-1 L, lane 7 ; G 677-2 T, lane 8 ; G 677-1 P, lane 9. Molecular mass markers used : rabbit muscle phosphorylase b (97.4 Kdal), bovine serum albumin (66.2 Kdal), ovalbumin (42.7 Kdal), and carbonic anhydrase (31.0 Kdal) .

(vi) MI test. As shown in Table 1, the three house musk shrew mycoplasma strains did not react with the antisera against *M. pulmonis* m53, *M. arthritidis* PG6 and *M. neurolyticum* Type A. Conversely, the antisera prepared against the three house musk shrew mycoplasma strains (strains G3-5, G666-16R and G677-1L) reacted with three house musk shrew mycoplasmas but not with the three murine mycoplasma species.

(vii) SDS-polyacrylamide gel electrophoresis of whole-cell proteins. As shown in Fig.1, the five strains and strain G3-5 gave similar SDS-polyacrylamide gel electrophoretic protein patterns, and the patterns of house musk shrew mycoplasmas were distinct from those of the previously described three murine mycoplasma species.

(viii) Whole-cell polypeptides of mycoplasmas analyzed by immunoblotting with antiserum

Fig. 2. Immunoblot analysis of whole-cell polypeptides from the same species as shown in Fig. 1, reacting with antibodies against strain G 677-1 L.

Fig. 3. Cleavage patterns of the chromosomal DNA of mycoplasmas with *Hind*III. Lane 10 indicates molecular mass references of the phage λ DNA cleaved with *Hind*III. Lanes 1 through 9 are the same as in Fig. 1.

against G677-1L strain are shown in Fig.2. Immunoanalyses of electrophoretic blots in-

dicated similar protein profiles for our isolates and strain G3-5. In addition, the strain G677-1 L antiserum demonstrated only a few cross-reactions with murine mycoplasmas.

(ix) Cleavage patterns of chromosomal DNAs from the strains after digestion with *Hind* III are shown in Fig.3. All house musk shrew mycoplasmas exhibited remarkably similar reaction patterns. On the other hand, the DNA cleavage patterns produced were distinct from those of the murine mycoplasma species.

(x) Epidemiological study. Mycoplasmas were isolated from all oral cavities of the 56 house musk shrews, although no gross lesions were observed in respiratory tracts, joints, or reproductive organs of the house musk shrews in the colony. Histopathologically, no abnormalities were seen in the lungs, tracheas, and spleens of five house musk shrews.

(xi) Experimental infection in mice and rats. Mice and rats inoculated intranasally with the mycoplasma isolated from the house musk shrew were sacrificed, and oral swabs were taken at 3 or 7 days postinoculation. Neither clinical signs nor gross lesions were observed in the mice and rats, and no mycoplasmas were reisolated from any of the animals.

Discussion

House musk shrew mycoplasma strains were first isolated in 1988 from various sites of

experimental and wild house musk shrews by Ito *et al.* [11]; two strains of mycoplasma isolated from house musk shrews have been shown to be serologically related to each other but distinct from the nine established mycoplasma species using the GI test [11]. Our findings confirm the previous study [11] and support the notion that our isolates are also serologically related to each other and to a mycoplasma strain isolated previously from a house musk shrew but distinct from the murine mycoplasma species. However, there were some differences in MI titers among the house musk shrew mycoplasma strains when antisera prepared against the house musk shrew strains G666-16 R, G677-1L and G3-5 were used.

The electrophoretic patterns of cell proteins showed that our five isolates and strain G3-5 have many polypeptides in common. Moreover, the immunoblotting analysis indicated that the polypeptides of similar size specific for house musk shrew mycoplasma strains share common antigenic determinants. Although the protein patterns among the house musk shrew mycoplasmas were specific, there were a few polypeptides of house musk shrew strains which migrated along with the protein bands of murine mycoplasma strains. Thus, the findings indicate that the five isolates and strain G3-5 have many common, antigenically similar proteins and that they also share a few antigenic determinants with murine mycoplasma polypeptides.

The DNA cleavage patterns revealed close genomic relationships among the house musk shrew mycoplasma strains. Moreover, these strains were genomically distinct and were unrelated to the three murine mycoplasma species.

The combined data from the morphological, biological and serological analyses, the SDS-polyacrylamide gel electrophoresis patterns of the cellular proteins and the DNA cleavage patterns indicated that the five house musk shrew mycoplasma strains and strain G3-5 comprised a homogeneous cluster of related organisms that were quite distinct from the previously described murine mycoplasma species.

The epidemiological study showed that although mycoplasma was isolated from the oral cavity of all 56 house musk shrews, they had no gross lesions in the respiratory tracts,

joints or reproductive organs. Previous report [11] also showed that house musk shrews from which mycoplasma was isolated were apparently healthy. The isolation rate of the mycoplasma from the oral cavity was also high (100%) in comparison with 83% in the previous report [11]. Moreover, the strains neither produced evidence of disease nor colonized in experimentally inoculated mice and rats.

On the basis of these findings and other biological and serological properties of the organisms, we consider that mycoplasma strains with these characteristics are a peculiar organism present as indigenous microflora in the oral cavity of house musk shrews.

References

- [1] Black, F. T. (1973). Modification of growth inhibition test and its application to human T-mycoplasmas. *Appl. Microbiol.*, **25**, 528-533.
- [2] Cassell, G. H., Lindsey, J. R., Davis, J. K., Davidson, M. K., Brown, M. B., and Mayo, J. G. (1981). Detection of natural *Mycoplasma pulmonis* infection in rats and mice by an enzyme linked immunosorbent assay (ELISA). *Lab. Anim. Sci.*, **31**, 676-682.
- [3] Davis, J. K. and Cassell, G. H. (1982). Murine respiratory mycoplasma in LEW and F344 rats; Strain difference in lesion severity. *Vet. Pathol.*, **19**, 280-293.
- [4] Harasawa, R., Koshimizu, K., Pan, I.-J., and Barile, M. F. (1985). Genomic and phenotypic analyses of avian ureaplasma strains. *Jpn. J. Vet. Sci.*, **47**, 901-909.
- [5] Harasawa, R., Imada, Y., Ito, M., Koshimizu, K., Cassell, G. H., and Barile, M. F. (1990). *Ureaplasma felinum* sp. nov. and *Ureaplasma cati* sp. nov. isolated from the oral cavities of cats. *Int. J. Syst. Bacteriol.*, **40**, 45-51.
- [6] Harasawa, R., Stephens, E. B., Koshimizu, K., Pan, I.-J., and Barile, M. F. (1990). DNA relatedness among established *Ureaplasma* species and unidentified feline and canine serogroups. *Int. J. Syst. Bacteriol.*, **40**, 52-55.
- [7] Hill, A. C. (1983). *Mycoplasma collis*, a new species isolated from rats and mice. *Int. J. Syst. Bacteriol.*, **33**, 847-851.
- [8] Hill, A., Turton, J. A., and Bleby, J. (1978). Bacterial and mycoplasma flora of the common marmoset (*Callithrix jacchus*). *Vet. Rec.*, **103**, 824-827.
- [9] Ishikawa, A. and Namikawa, T. (1991). Postnatal growth pattern of F₁ hybrids of a cross between two strains of large and small musk shrews, *Suncus murinus*. *Exp. Anim.*, **40**, 223-230.
- [10] Ishikawa, A., Hirunagi, K., Oda, S., Namikawa, T., and Tomita T. (1992). Kinky coat, a new autosomal recessive mutation in the musk shrew, *Suncus murinus*. *Exp. Anim.*, **41**, 203-214.
- [11] Ito, M., Yamamoto, K., Kotani, H., and Koshimizu, K. (1988). Isolation of mycoplasmas from house

- musk shrews (*Suncus murinus*). *Exp. Anim.*, **37**, 339-340.
- [12] Kaji, T., Saito, H., Ueno, S., and Matsuki, N. (1990). Comparison of various motion stimuli on motion sickness and acquisition of adaptation in *Suncus murinus*. *Exp. Anim.*, **39**, 75-79.
- [13] Koshimizu, K., Harasawa, R., Pan, I.-J., Kotani, H., Ogata, M., Stephens, E. B., and Barile, M. F. (1987). *Ureaplasma gallorale* sp. nov. from the oropharynx of chickens. *Int. J. Syst. Bacteriol.*, **37**, 333-338.
- [14] Koshimizu, K. and Ogata, M. (1979). Characterization and differentiation of mycoplasmas of canine origin. *Jpn. J. Vet. Sci.*, **36**, 391-406.
- [15] McGarrity, G. J., Rose, D. L., Kwiatkowski, V., Dion, A. S., Phillips, D. M., and Tully, J. G. (1983). *Mycoplasma muris*, a new species from laboratory mice. *Int. J. Syst. Bacteriol.*, **33**, 350-355.
- [16] Manchee, R. J. and Taylor-Robinson, D. (1968). Haemadsorption and haemagglutination by mycoplasmas. *J. Gen. Microbiol.*, **50**, 465-478.
- [17] Matsuzaki, T., Tanaka, T., Saito, R., Yamanaka, M., Saito, M., and Nomura, T. (1992). Establishment of an outbred strain (Jic : SUN) in the house musk shrew, *Suncus murinus*. *Exp. Anim.*, **41**, 167-172.
- [18] Ohno, T., Oda, S., Ishikawa, A., and Namikawa, T. (1992). Red-eyed dilution (rd), a novel coat-color mutant gene in the musk shrew (*Suncus murinus*, insectivora). *Exp. Anim.*, **41**, 173-179.
- [19] Purcell, R. H., Taylor-Robinson, D., Wong, D., and Chanock, R. M. (1966). Color test for the measurement of antibody to T-strain mycoplasmas. *J. Bact.*, **92**, 6-12.
- [20] Saito, M., Nakayama, K., and Nakagawa, M. (1976). Localization of *Mycoplasma pulmonis* in mice. *Exp. Anim.*, **25**, 265-272.
- [21] Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; Procedure and some application. *Proc. Natl. Acad. Sci. USA.*, **76**, 4350-4354.

スunksから分離されたマイコプラズマの性状および マウス、ラットへの感染性

後 藤 一 雄・伊 藤 豊 志 雄・江 袋 進・鍵 山 直 子・原 澤 亮*

財団法人実験動物中央研究所

*東京大学医学部附属動物実験施設

財団法人実験動物中央研究所において生産、維持されている実験用スunksから分離されたマイコプラズマ5株の性状を、これまでに報告されているスunks由来のマイコプラズマ株(G 3-5)および齧歯類由来のマイコプラズマ(*M. pulmonis* m 53, *M. arthritidis* PG 6, *M. neurolyticum* Type A)と比較した。今回分離された5株はいずれもブドウ糖を分解し、アルギニンおよび尿素非分解、孔径450-nmの濾過膜通過性、ジギトニン感受性であり、寒天培地上のコロニーの直径は115~231 μ mであった。また、(i)代謝阻止試験、(ii) SDS-PAGE, ウェスタンブロッティングによるタ

ンパク分析、および(iii)制限酵素によるDNA分析の結果、これら5株はG 3-5株と同様の結果を示したが、齧歯類由来のマイコプラズマとは明確な差異が観察された。さらに、我々の施設で生産、維持されている、2週齢から45週齢のスunks 56匹のマイコプラズマ汚染調査では全例の口腔からマイコプラズマが分離された。しかし、これらのうち5匹の病理学的検索では呼吸器等に病変は見出されなかった。また、マウス、ラットに対する感染性も認められなかったことから、本株はスunksに固有なマイコプラズマであることが示唆された。