Proposal for Classifying Human Strain Navel and Related Simian Mycoplasmas as *Mycoplasma primum* sp. n.

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Mycoplasmas isolated from simian (*Cercopithecus aethiops*) tissues were shown to have biological, biochemical, and serological properties and electrophoretic cell protein patterns similar to strain Navel isolated from man 15 years ago. The simian and human Navel strains comprised a single serogroup, distinct from the established *Mycoplasma* and *Acholeplasma* species of the class *Mollicutes*. It is proposed that strains with the properties described be named *Mycoplasma primum*.

The first mycoplasmas isolated from simians (*Cercopithecus*) were identified as *Mycoplasma hominis* (27) and *M. salivarium* (26). The first systematic investigation of the oral and urogenital mycoplasma flora of monkeys (*Cercopithecus aethiops*) was reported by DelGiudice et al. (7). Of 70 mycoplasmas isolated, 66 (94%) were related to *Mycoplasma* species previously isolated only from man (10), including *M. orale* 2, *M. orale* 3, *M. salivarium*, *M. fermentans*, and strain Navel. The most common simian strain isolated from urogenital tissues was related to strain Navel and was present in 13 of the 18 (72%) positive specimens. Five simian Navel-related strains were isolated also from oropharyngeal tissues. The presence of Navel-related strains in *C. aethiops* was confirmed later by other workers (15) who isolated 10 strains from oral and urogenital tissues. These findings suggest that *M. primum* is a common inhabitant of the oral and urogenital tract of *C. aethiops*. By contrast, this mycoplasma has been isolated only once from man.

In 1955, Ruiter and Wentholt (24) isolated strain Navel from an infected human umbilicus. The organism was culturally and morphologically distinct from mycoplasmas isolated from the vagina of the same patient. On the basis of glucose fermentation, Edward (24) reported that strain Navel was also distinct from the G-strains previously isolated from human urogenital tissues by Ruiter and Wentholt (22, 23). Subse-

quently, the G-strains were named *M. fermentans* (9), whereas strain Navel was not classified. Lemcke (12, 13) examined strain Navel and reported that it was serologically distinct from 16 human and animal mycoplasmas. In this report, further characterizations of the human and simian Navel-related group of mycoplasmas are presented. The biological, biochemical, and serological properties of these mycoplasmas indicate that they are a distinct group and merit species designation. We are proposing, therefore, that mycoplasmas with the properties described in this report be named *M. primum*.

MATERIALS AND METHODS

Mycoplasmas. Two cloned lines of the *M. primum* strain Navel were used. The Lister line was lyophilized in 1964 and described in earlier studies (12, 13). The second line, ATCC 15497, was obtained from the American Type Culture Collection.

Some of the 18 simian strains isolated earlier (7) were used including vaginal strains M35, P084, and B328 and prostatic-urethral strains I26, I30, and HRC292. Strain HRC292 was selected as the simian prototype strain.

The other species and unclassified strains of mycoplasmas used are listed in Table 1.

Media. Several broth and agar media were used (3, 7, 10, 12, 30). Some were similar and were composed of a basal medium containing 20% horse serum and yeast extract (7, 10). In others, the horse serum was replaced with either inactivated, pooled, human serum (12), human blood (3), or Serum Fraction (Difco, reference 30). Cultures were incubated aerobically or in a

TABLE 1. *Mycoplasmatales* used as antigens and for preparation of antisera in serological tests with *Mycoplasma primatum* strains Navel and HRC292 by fluorescent antibody, growth inhibition, and complement fixation test

Species	Strain	Species	Strain
<i>Mycoplasma hominis</i> ^a	PG-21, ^{a, b} Botte, ^b SC4 ^b	<i>M. spumans</i>	PG-13 ^{a, b}
<i>M. fermentans</i> ^a	PG-18, ^{a, b} G-2 ^b	<i>M. canis</i> ^a	PG-14 ^{a, b}
<i>M. salivarium</i> ^a	PG-20, ^{a, b} B3 ^b	<i>M. maculosum</i>	PG-15 ^{a, b}
<i>M. pneumoniae</i>	FH ^{a, b}	<i>M. edwardii</i>	PG-24 ^{a, b}
<i>M. orale</i> 1 ^a	CH19299, ^{a, b} 837 ^b	<i>Mycoplasma</i> sp.	689 ^a
<i>M. orale</i> 2 ^a	CH20247 ^a	<i>M. felis</i>	27, ^a CO ^b
<i>M. orale</i> 3 ^a	DC333 ^{a, b}	<i>M. gatae</i>	Mart, ^a CS ^b
<i>M. lipophilum</i>	MaBy ^{a, b}	<i>M. feliminutum</i>	Ben ^a
<i>Mycoplasma</i> sp. ^d	Simian 291 ^{a, b}	<i>Mycoplasma</i> sp.	KDA ^a
<i>M. mycoides</i> var <i>mycoides</i>	Gladysdale ^{b, c}	<i>M. gallisepticum</i>	PG-31, ^a H1344, ^b X95 ^b
<i>M. bovigenitalium</i>	PG-11 ^{a, b}	<i>M. gallinarum</i>	PG-16 ^{a, b}
<i>M. bovirhinis</i>	PG-43 ^{a, b}	<i>M. iners</i>	PG-30 ^{a, b}
<i>Mycoplasma</i> sp.	DBS188 (calf) ^b	<i>M. anatis</i>	1340 ^{a, b}
<i>M. mycoides</i> var <i>capri</i>	PG-3, ^b pp. goat ^{b, c}	<i>M. meleagridis</i>	17529 ^{a, b}
<i>M. agalactiae</i>	Agalactia ^{b, c}	<i>Mycoplasma</i> sp. (group D)	A36 ^b
<i>M. arginini</i>	G-230, ^a C-506 ^b	<i>M. neurolyticum</i>	Type A; ^{a, b} KSA ^b
<i>Mycoplasma</i> sp.	DBS189 (goat) ^b	<i>M. pulmonis</i>	ASH, ^a Negron, ^b M1 ^b
<i>Mycoplasma</i> sp.	BBL-G145 ^a	<i>M. arthritis</i>	PG-6, ^a PG-27 ^{a, b}
<i>Mycoplasma</i>	HRC581 ^a	<i>Acholeplasma laidlawii</i> ^d	PG-8, ^{a, b} PG-9 ^{a, b}
<i>M. hyorhinis</i>	GDL, ^b BTS-7 ^a	<i>A. granularum</i>	BTS-39 ^{a, b}
		<i>A. axanthum</i>	S-743 ^b

^a Tested by fluorescent antibody.^b Tested by growth inhibition.^c Tested by complement fixation.^d *Mycoplasmatales* isolated from primates.

5% carbon dioxide in nitrogen (3) atmosphere at 36 ± 1 C.

Test for sterol dependence was performed as described previously (20).

Filterability. Filterability of strain HRC292 was determined by a method reported previously (30). In brief, actively growing broth cultures were diluted 10-fold in a phosphate-buffered saline solution, pH 7.4, with 0.2% gelatin added. Filterability was determined by use of a Swinney hypodermic adaptor and membrane filters (average porosity sizes of 100, 220, 300, and 450 nm. Millipore Corp., Bedford, Mass.). Colony-forming units (CFU) were determined on the original suspension and on the filtrates from each filter used.

Biochemical tests. The procedures used to determine dextrose fermentation, arginine and urea hydrolysis, phosphatase activity, tetrazolium reduction, and serum digestion were reported elsewhere (1, 2).

Action on erythrocytes. Hemolytic activity for guinea pig and sheep erythrocytes was determined by adding a 3% suspension of washed erythrocytes in 1.5% agar as an overlay to colonies grown on 20% horse serum-agar medium (25). The ability of guinea pig and sheep erythrocytes to hemadsorb to mycoplasma colonies was determined as described elsewhere (17; R. A. DelGiudice and R. Pavia, *Bacteriol. Proc.*, p.71, 1964).

Polyacrylamide gel disc electrophoresis. The method of Razin (19) was used for polyacrylamide gel disc electrophoresis. In brief, 48-hr broth cultures were sedimented, the cell proteins were solubilized in phenol-acetic acid-water (2:1:0.5, w/v/v), and the extracts were examined in polyacrylamide gels containing 5 M urea and 35% acetic acid.

Serologic tests. The plate immunofluorescence (FA, reference 8), growth inhibition (GI, reference 4), and complement fixation (CF, reference 12) tests used were performed essentially as described elsewhere, with some modifications. For the GI test, the serum content of medium was reduced to 5% or replaced with 1 to 2% of Serum Fraction (Difco), and antisera were tested against dilutions of the antigen. In the CF test, the Microtiter system containing 1-unit volume of 0.025 ml was used. The final concentration of sheep erythrocytes in the hemolytic system, after addition of hemolysin, was 1.0%. Anticomplementary sera were adsorbed with one-tenth complement for 24 hr at 4 C.

Antisera to mycoplasmas were prepared in rabbits (12, 13) or in goats. Preimmunization sera controls were included in all serological tests.

RESULTS

Mycoplasma primatum cultural characteristics.

All the strains grew well in the various broth and agar media when incubated in either air or CO₂-N₂. However, titers (colony-forming units per milliliter) were higher on the human blood-agar medium (3) than on horse serum-agar (10) and when the cultures were incubated in CO₂-N₂ instead of air. Broth cultures produced turbid growth with titers of 10⁸ CFU per ml or greater. Film and spots were not produced.

Dependence on sterol for growth. All strains tested required cholesterol or serum for growth. Growth did not occur in cholesterol-free medium

even when supplemented with Tween 80 and albumin. The addition of cholesterol stimulated growth (Table 2).

Morphological characteristics. Typical mycoplasma colonies were produced on agar. Gram stains of broth cultures revealed fragile, gram-negative, pleomorphic, spherical, coccobacillary, and filamentous forms. Clumping of cells was noted also. Phase-contrast microscopy of unstained broth cultures showed a similar cellular morphology. Thin-section electron micrographs

of sedimented broth culture pellets showed the classic mycoplasma cell bounded by a triple-layered membrane and devoid of a cell wall (Fig. 1)

Filterability characteristics. The suspension of strain HRC292 used for filtration contained 9.8×10^8 CFU per ml and the filtrates from the 450-, 300-, 220-, and 100-nm filters yielded 3.9×10^7 , 3.0×10^7 , 9.4×10^5 , and 0 CFU per ml, respectively. Strain Navel showed a similar pattern of filterability.

Biochemical characteristics. The properties of strains Navel and HRC292 are summarized in Table 3. Appropriate positive and negative control cultures were used. Strains Navel and HRC292 produce positive reactions for arginine hydrolysis and phosphatase activity and negative reactions for dextrose fermentation, urease, hemolysis and hemadsorption activity, and tetrazolium reduction.

Electrophoretic pattern of cell proteins. The cell protein electrophoretic patterns of strains Navel (ATCC 15497 and Lister) and HRC292 were examined independently in two laboratories (National Institutes of Health, Bethesda, Md., and Lister Institute). In both laboratories, the patterns for the two lines of strain Navel were found to be identical but distinct from the patterns of other mycoplasmas (19, 28, 29). In the American Laboratory, strain HRC292 was found

TABLE 2. Effect of cholesterol on the growth of *Mycoplasma primatum* strains Navel and HRC292 in a serum-free medium

Cholesterol in medium ($\mu\text{g/ml}$)	Cell protein ^a	
	Strain Navel	Strain HRC292
0 ^b	0.01	0.01
0 ^c	0.01	0.01
1	0.25	0.43
5	1.37	1.94
10	1.04	1.76
20	3.33	2.28

^a Expressed in milligrams per 100 ml of medium and determined in 6-day-old cultures.

^b Serum-free medium alone.

^c Serum-free medium with 0.5% albumin and 0.01% Tween 80.

FIG. 1. Thin-section electron micrograph of liquid culture pellet of strain HRC292. Prepared by P. McGrath, Bethesda, Md.

TABLE 3. *Properties of Mycoplasma primum*

Properties	Strain Navel	Strain HRC292
Dextrose fermentation	—	—
Arginine hydrolysis	+	+
Urea hydrolysis	—	—
Hemolysis (sheep and guinea pig red blood cells)	—	—
Hemadsorption (sheep and guinea pig red blood cells)	—	—
Phosphatase activity	+	+
Tetrazolium reduction (aerobic)	—	—
Serum digestion	—	—
Film and spots	—	—
Serum or cholesterol requirement	+	+
Pathogenicity for man and animals	Unknown.	
Habitat and source	Strain Navel was isolated from an infected human umbilicus; other strains were isolated from the respiratory and genitourinary tracts of <i>Cercopithecus aethiops</i> .	
Broth cultures	Produces marked turbidity.	
Agar cultures	Produces classical mycoplasma colonies, at times with "fried-egg appearance."	
Preferred atmosphere	5% CO ₂ in N ₂ atmosphere.	
Cell protein electrophoretic patterns	Strains Navel and HRC292 are similar to each other but distinct from the patterns of the other established <i>Acholeplasma</i> and <i>Mycoplasma</i> species (Table 1).	
Antigenicity	Strains Navel and HRC292 are similar to each other but distinct from the other established <i>Mycoplasma</i> and <i>Acholeplasma</i> species (Table 1) by FA, CF, and GI procedures. ^a	
Type culture	HRC292 (isolated from prostatic urethral tissues of <i>C. aethiops</i>).	
Other properties of strain Navel	Produces negative reactions for tellurite reduction, methylene blue reduction, gelatin hydrolysis, and casein digestion (1). The deoxyribonucleic acid base composition (moles per cent guanine plus cytosine) by the T _m procedure is 28.6 (31).	

^a Abbreviations: FA, fluorescent antibody; CF, complement fixation; GI, growth inhibition.

to give patterns identical to those obtained with the Navel lines (Fig. 2). Although the pattern given by HRC292 in the Lister Laboratory had a general similarity to that of the Navel strain, there were certain differences by which the two could be distinguished (Fig. 3).

Serological characteristics. Serological relatedness of human strain Navel to 18 simian strains was established earlier (7) by FA procedures with antisera against strain Navel (ATCC 15497). The serological relatedness of strain Navel to some of these simian strains was examined further by FA, CF, and GI procedures. In cross-serological reactions, the titers obtained ranged from 1:20 to 1:1,280 by FA (Table 4), 1:80 to 1:10,240 by CF (Table 5), and 3 to 11 mm by GI (Table 6). These findings indicate that the human and simian strains form a single serological group.

The serological relationship of *M. primum* to established species was examined by either CF, GI, or FA tests.

Tests were used to compare strains Navel and HRC292 with at least one strain of the mollicutes listed in Table 1. None of these strains, representing 41 established *Mycoplasma* and *Acholeplasma* species or serogroups, exhibited a serological relationship with either strain of *M. primum*.

DISCUSSION

These studies show that the human and simian Navel-related strains comprise a single serogroup and that the group is serologically distinct from the recognized *Mycoplasma* and *Acholeplasma* species tested. A comparison of the cultural and biochemical properties and an electrophoretic analysis of the membrane proteins are compatible with the serological results. We are therefore proposing the name *M. primum* for this group and designating strain HRC292 (ATCC 25948) as the type culture.

M. primum is classified in the class Molli-

FIG. 2. Electrophoretic patterns of membrane proteins of *Mycoplasma primatum*. Results from the National Institutes of Health.

TABLE 4. Plate immunofluorescence (FA) tests with strains of *Mycoplasma primatum*^a

Antisera	Reciprocal of FA titer for antigen of				
	Strain Navel		Simian strains		
	ATCC 15495	Lister	HRC 292	B328	PO84
Strain Navel (ATCC 15497)	1,280	640	80	20	20
Strain HRC292	160	80	320	80	80

^a Appropriate negative controls were included in these studies. See Table 1.

cutes order *Mycoplasmatales* (9) because of its cellular and colonial morphology, filterability, resistance to penicillin, lack of bacterial reversion, and its cultural characteristics. Classification in the family *Mycoplasmataceae* is based on its growth requirement for cholesterol (20).
The variation in results obtained by the two laboratories in comparing strain Navel and HRC292 by polyacrylamide gel disc electrophoresis is of interest because of the lack of information about the reproducibility of this technique in different laboratories. Variations between Navel and HRC292 were noted also in serological tests.
The intraspecies variations among strains of *M. primatum* are no more than those observed

FIG. 3. Electrophoretic patterns of membrane proteins of *Mycoplasma primatum*. Results from the Lister Institute of Preventive Medicine.

TABLE 5. Complement fixation (CF) tests with strains of *Mycoplasma primatum*^a

Antisera	Reciprocal of CF titer for antigens of		
	Navel (Lister)	Navel (ATCC 15495)	HRC292
Strain Navel (Lister)	10,240	10,240	1,280/2,560
Strain Navel (ATCC 15497)	320/640	320	80
Strain HRC292	160	80/160	320/640

^a Appropriate negative controls were included in these studies. See Table 1.

with strains of *M. hominis* (11, 21) and *M. pulmonis* (14).
Seven of the 9 “human” *Mycoplasma* species have now been isolated from nonhuman primates, and they include *M. hominis* (5, 6, 16, 18, 26, 27), *M. salivarium* (5–7, 15, 16, 26, 27), *M.*

TABLE 6. Growth inhibition tests with strains of *Mycoplasma primatum*^a

Antisera	Zones of growth inhibition for antigens of (mm)							
	Strain Navel		Simian strains					
	ATCC 15495	Lister	HRC292	B328	PO84	I26	I30	M35
Strain Naval (Lister)	10	10	5	3	5	7	7	7
Strain Navel (ATCC 15497) .	9	9	5	5	5	5	4	7
Strain HRC292	5	6	4	3	3	5	7	5

^a Appropriate negative controls were included in these studies. See Table 1.

fermentans (7), *M. orale* 1 (6), *M. orale* 2 (5–7, 16, 18), *M. orale* 3 (6, 7), and *M. primatum* (5, 7). The notable exceptions are *M. pneumoniae* and *M. lipophilum*. Three other mycoplasmas have been isolated from nonhuman primates, i.e., *Acholeplasma laidlawii* (15, 16), *M. canis* (18) and strain HRC291 (7). The nonhuman primates harboring human *Mycoplasma* species include *Cercopithecus* (7, 15, 26, 27), *Macaca* (16), chimpanzee (5, 6, 18), gorilla (6), and orangutan (6).

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