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# Proposal for Classifying Strain PG-24 and Related Canine Mycoplasmas as *Mycoplasma edwardii* sp. n.

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Mycoplasmas recovered recently from dogs were found unrelated to three classified canine *Mycoplasma* serotypes but were similar in biological and serological properties to a *Mycoplasma* strain (C21, PG-24) isolated 18 years earlier. It is proposed that strains with the characteristics described be designated *Mycoplasma edwardii* sp. n.

The first systematic investigation of the mycoplasmal flora of dogs was performed by Edward and Fitzgerald in 1951 (8). On the basis of differences in colonial appearance and serological properties, a majority of mycoplasmas recovered from the canine throat and genital tract could be separated into three types. These serotypes were later designated *Mycoplasma spumans*, *M. canis*, and *M. maculosum* in the classification system proposed by Edward and Freundt (9). However, one throat isolate (strain C21) appeared to be serologically distinct from other mycoplasmas recovered from dogs. Although this culture was not given a species designation at the time, it was distributed to other investigators and placed in various national culture collections under the label of the "PG-24 strain".

Seventeen years later, Razin and Rottem (12) reported the recovery of a *Mycoplasma* from the lung of a dog dying of pneumonia that appeared, by acrylamide-gel electrophoretic pattern of cell proteins, to resemble the PG-24 (C21) strain. Repeated isolation of mycoplasmas with the serological and biological properties of the PG-24 strain from other canine hosts was also reported recently (1; M. Barile et al., *in preparation*). A characterization of various PG-24 related strains recovered in the latter two studies, and particularly an extensive serological comparison of these strains against other *Mycoplasma* serotypes, reaffirmed their distinct properties. We are therefore proposing that mycoplasmas with the serological relationships and biological characteristics described below be named *Mycoplasma edwardii*.

## MATERIALS AND METHODS

**Mycoplasmas.** Two PG-24 strains (C21 and ATCC 23462) were obtained from D. G. ff. Edward and the American Type Culture Collection, respectively. Strain C21 was stored in a dried state since 1953. Two dog lung isolates, MH 5270 (1) and dog G (12), were also examined. For comparative purposes prototype strains of *M. spumans* (PG-13), *M. canis* (PG-14), and *M. maculosum* (PG-15) were also included in this study. These strains were obtained from Ruth Wittler, Walter Reed Army Institute of Research, Washington, D.C.

**Media.** Mycoplasmas were grown on several broth and agar formulations, including the 20% horse serum medium of Hayflick (10) and modified Edward medium (7). A serum fraction medium prepared from BBL Mycoplasma Broth base supplemented with 1% PPLO Serum Fraction (Difco), 10% fresh yeast extract, and 500 units/ml of penicillin was also employed. A serum-free medium was prepared by omitting the PPLO Serum Fraction in the latter preparation. Mycoplasmas were grown under both aerobic and anaerobic [5% $CO_2$ in nitrogen (4)] conditions at 37 C.

**Test for sterol dependence.** The sterol requirements for the growth of *M. edwardii* was determined by the procedure previously reported (14). The organisms were grown at 37 C for 4 days in 100-ml quantities of BBL Mycoplasma Broth medium without PPLO Serum Fraction. The medium was supplemented with 0.5% bovine serum albumin (fraction V), 0.5% glu-

cose, and various amounts of cholesterol dissolved in Tween 80.

**Filtration studies.** An actively growing 18-hr broth culture of *M. edwardii* strain PG-24 was diluted 10-fold in phosphate-buffered saline at *p*H 7.4 with 0.2% gelatin added. Filterability of the diluted mycoplasma suspension was determined with the use of Swinney hypodermic adapter and membrane filters (Millipore Corp., Bedford, Mass.) types VC (100 nm), GS (220 nm), PH (300 nm), and HA (450 nm). Colony-forming units (CFU) were determined on the original suspension and on the filtrates from each filter used.

**Biochemical tests.** Carbohydrate fermentation tests were carried out in serum fraction broth supplemented with 0.5% of the tested carbohydrate (13), but arginine utilization was determined in Hayflick broth (2).

**Serological tests.** Plate (6) and indirect (13) fluorescent-antibody (FA) procedures as well as growth inhibition tests (5) were performed with the C21 and PG-24 strains of *M. edwardii*. Reciprocal tests were carried out with both antigens and rabbit antisera to the *Mycoplasma* serotypes and unclassified strains listed in Table 1.

**Polyacrylamide gel electrophoresis.** Mycoplasmas grown in serum fraction broth were sedimented, the cell proteins were solubilized in phenol-acetic acid-water (2:1:0.5, w/v/v), and the extract was run in polyacrylamide gels containing 5 M urea and 35% acetic acid (11).

## RESULTS

**M. edwardii cultural characteristics.** The PG-24, MH 5270, and dog G strains appeared to grow well, aerobically or anaerobically, in either liquid or solid media containing 20% horse serum or 1% PPLO Serum Fraction. Classical *Mycoplasma* colonies were observed with all three strains, particularly with PG-24 (Fig. 1). Some early subcultures of the MH 5270 and dog G strains exhibited only the central portion of the usual diphasic colony and, on occasion, the colony centers contained only vacuoles or globules. This colonial form was lost on continued subcultivation. Broth cultures of the three strains showed a rather uniform light turbidity after 24 hr. Gram stains of the mycoplasmas revealed gram-negative bacillary-like pleomorphic elements and some filamentous forms. The growth of *M. edwardii* was unaffected by the presence or absence of penicillin (500 units/ml) in the medium.

**Dependence on sterol for growth.** The growth response of *M. edwardii* (PG-24) to cholesterol can be seen in Table 2. No growth was obtained in the serum-free medium even when supplemented with Tween 80 and albumin. Similar results were obtained with the C21 strain.

TABLE 1. *Mycoplasmas employed as antigens and for preparation of antisera in serological tests with Mycoplasma edwardii*

| Species | Strain |
|---|---|
| Primate | |
| *Mycoplasma hominis* | PG-21, Botte |
| *M. fermentans* | PG-18 |
| *M. salivarium* | PG-20 |
| *M. pneumoniae* | FH |
| *M. orale* 1 | CH 19299 |
| *M. orale* 2 | CH 20247 |
| *M. orale* 3 | DC 333 |
| *M. lipophilum* | MaBy |
| *M.* sp. | Navel |
| Caprine and ovine | |
| *M. arginini* | BBL-88, G 230 |
| *M.* sp. | B3 |
| *M.* sp. | BBL-G-145 |
| *M.* sp. | KS-1 (ATCC 15718) |
| *M.* sp. | Goat 189 |
| Canine | |
| *M. spumans* | PG-13 |
| *M. canis* | PG-14 |
| *M. maculosum* | PG-15 |
| Murine | |
| *M. neurolyticum* | Type A |
| *M. pulmonis* | PG-34, Negroni |
| *M. arthritidis* | PG-6, PG-27 |
| Avian | |
| *Mycoplasma gallisepticum* | S 6, PG-31 |
| *M. gallinarum* | PG-16 |
| *M.* sp. (C) | 859 |
| *M.* sp. (D) | 887 |
| *M.* sp. (F) | 1197 |
| *M. iners* (G) | 640, PG-30 |
| *M. meleagridis* (H) | 886, 529 |
| *M.* sp. (I) | Iowa 695 |
| *M. anatis* | 1340 |
| Bovine | |
| *M. bovigenitalium* | PG-11 |
| *M. bovirhinis* | PG-43 |
| *M.* sp. | Calf 188 |
| *M.* sp. | Conn. |
| Swine | |
| *M. hyorhinis* | BTS-7, GDL |
| Feline | |
| *M. felis* | Cat 27, CO |
| *M. gateae* | Mart, CS |
| *M.* sp. | Bennett |
| Miscellaneous | |
| *M. laidlawii* | PG-8 |
| *M. laidlawii* | PG-9 |
| *M. granularum* | BTS-39 |
| *M.* sp. | S-743 |

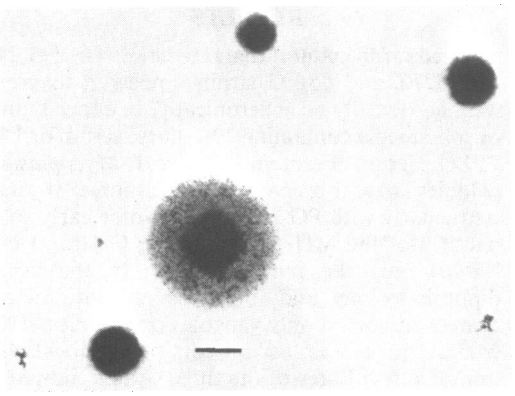

FIG. 1. *Mycoplasma edwardii (PG-24). Five-day culture on horse serum agar stained with Dienes stain. Marker, 100 nm.*

TABLE 2. *Effect of cholesterol on the growth of M. edwardii in a serum-free medium*

| Cholesterol in medium (μg/ml) | Cell protein[a] |
|---|---|
| 0[b] | 0.01 |
| 0[c] | 0.01 |
| 1.0 | 0.10 |
| 5.0 | 1.10 |
| 10.0 | 1.65 |
| 20.0 | 2.15 |

[a] Expressed in milligrams per 100 ml of medium.
[b] Serum-free medium alone.
[c] Serum-free medium with 0.5% albumin and 0.01% Tween 80.

**M. edwardii filterability characteristics.** The mycoplasma suspension employed contained $2.0 \times 10^9$ CFU per ml and the filtrates yielded $1.9 \times 10^7$; $2.3 \times 10^7$; $7.4 \times 10^4$ and 0 CFU/ml for 450-, 300-, 220-, and 100-nm filters, respectively.

**M. edwardii biochemical characteristics.** A summary of the results of the glucose fermentation and arginine utilization tests by *M. edwardii* strains and other canine mycoplasmas is presented in Table 3. In addition to the fermentation of glucose, *M. edwardii* strains also fermented maltose, glycogen, starch, and dextrin. No acid was produced from mannose, sucrose, or galactose. *M. canis* PG-14 also gave the same fermentation pattern as *M. edwardii* strains. The nonfermenting *M. spumans* and *M. maculosum* cultures were the only strains capable of degrading arginine (3).

**Serology of canine mycoplasmas.** The results of reciprocal indirect FA tests with various canine *Mycoplasma* antigens and antisera are shown in Table 4. The distinct serological characteristics of *M. edwardii* from other canine mycoplasmas were also confirmed with reciprocal tests in the direct FA plate procedure. Colonies of PG-24, MH 5270 and dog G stained only with fluorescein-conjugated antisera prepared against the PG-24 strain. The other canine mycoplasmas stained only with their respective homologous antiserum. Reciprocal growth inhibition tests with the canine mycoplasmas gave results similar to the FA procedures. In addition, *M. edwardii* strains were compared against antisera to other *Mycoplasma* serotypes (Table 1), by both the indirect and plate FA test, without any evidence of a serological relationship to established species or unclassified strains of *Mycoplasma*. Similar results were obtained with growth inhibition tests when anti-

TABLE 3. *Glucose fermentation and arginine utilization by Mycoplasma edwardii and other canine mycoplasmas*

| Serotype | Strain | Glucose fermentation | Arginine utilization |
|---|---|---|---|
| *Mycoplasma spumans* | PG-13 | − | + |
| *M. canis* | PG-14 | + | − |
| *M. maculosum* | PG-15 | − | + |
| *M. edwardii* | PG-24 | + | − |
| | MH 5270 | + | − |
| | Dog G | + | − |

TABLE 4. *Indirect fluorescent antibody (FA) tests with canine mycoplasmas*

| Antigens | Reciprocal of FA titer for antisera to | | | | |
|---|---|---|---|---|---|
| | *M. spumans* PG-13 | *M. canis* PG-14 | *M. maculosum* PG-15 | *M. edwardii* | |
| | | | | PG-24 | MH 5270 |
| *Mycoplasma spumans* PG-13 | *512* | 16 | <16 | <16 | 16 |
| *M. canis* PG-14 | <16 | *512* | 16 | 16 | 16 |
| *M. maculosum* PG-15 | 32 | 16 | *512* | 16 | 32 |
| *M. edwardii* PG-24 | 16 | 16 | 16 | *1,024* | 512 |
| *M. edwardii* MH 5270 | <16 | <16 | <16 | 512 | *1,024* |
| *M. edwardii* dog G | 32 | 16 | 32 | 512 | 512 |

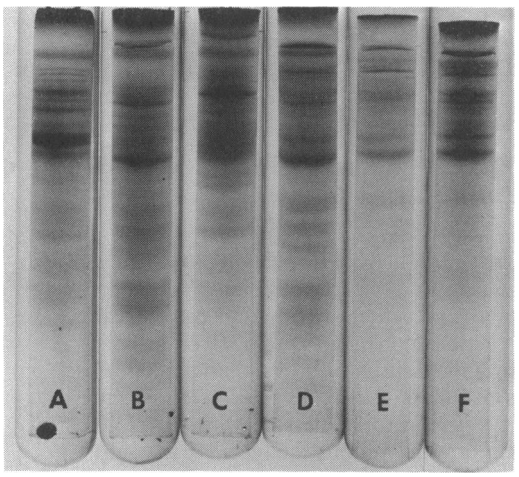


FIG. 2. *Electrophoretic patterns of Mycoplasma cell proteins. (A) M. spumans (PG-13), (B) M. canis (PG-14), (C) M. maculosum (PG-15), (D) M. edwardii (PG-24), (E) M. edwardii (MH 5270), and (F) M edwardii (dog G).*

serum to PG-24 was compared to the mycoplasmas listed in Table 1 or when antisera to these mycoplasmas were compared to *M. edwardii* C21.

**Electrophoretic patterns of cell proteins.** A comparison of the cell protein patterns of the canine mycoplasmas is shown in Fig. 2. The three *M. edwardii* strains have similar patterns and this pattern is clearly distinct from each of the other three canine *Mycoplasma* species.

## DISCUSSION

A comparison of various canine mycoplasmas has shown that the PG-24 strain recovered 18 years ago is biologically similar and serologically related to several mycoplasmas recently isolated from dogs. This group of related mycoplasmas was also found to be serologically distinct from all other established canine and animal *Mycoplasma* serotypes and unclassified strains examined. *M. canis* strains originally isolated from dogs were reported to be non-fermenters (8). However, the PG-14 strain and other recent isolates of *M. canis* (1) are clearly capable of fermenting glucose.

On the basis of the properties outlined here, we propose that mycoplasmas exhibiting biological and serological characteristics similar to PG-24 be designated as *M. edwardii*, with the representative or type strain being the PG-24 (ATCC 23462) culture initially recovered by D. G. ff. Edward. *M. edwardii* has been recovered from the throat (1, 8; M. Barile et al., *in preparation*), lung (1, 12), and genital tract (M. Barile et al., *in preparation*) of healthy dogs and dogs with respiratory disease. The role of *M. edwardii* in canine respiratory disease remains to be determined.

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