JOURNAL OF BACTERIOLOGY, Oct. 1970, p. 295-299
Copyright © 1970 American Society for Microbiology
Vol. 104, No. 1
Printed in U.S.A.

# Characterization of Canine Mycoplasmas by Polyacrylamide Gel Electrophoresis and Immunodiffusion

D. ARMSTRONG AND B. YU

*Department of Medicine and the Microbiology Laboratory, Memorial Hospital for Cancer and Allied Diseases, New York, New York 10021*

Received for Publication 16 July 1970

Canine mycoplasmas which had been characterized by biological and serological methods were further studied by using polyacrylamide gel electrophoresis (PGE) and double diffusion in agar gel. The three dog mycoplasmas previously characterized, *Mycoplasma canis*, *M. maculosum*, and *M. spumans* showed distinctive patterns by PGE. Five additional representative isolates from dogs had been characterized serologically and biologically into three new groups, A, C, and D. An additional mycoplasma (group B) was indistinguishable from *M. canis* by growth inhibition and PGE but was more broadly reactive with field isolates serologically. The group A organisms were distinctive in pattern and similar to those studied by Razin and Rottem, tentatively designated *M. edwardii.* The group C organisms were represented by two isolates which were similar by fluorescent-antibody studies but different by growth inhibition tests. These two isolates were also different from each other by PGE. The group D serotypes were also distinctive by PGE from all other dog mycoplasmas tested. It was found, during these studies, that two different mycoplasmas showed different PGE patterns at different intervals during incubation. Immunodiffusion studies showed a relationship among all the canine mycoplasmas, and bands of nonidentity between the two group C mycoplasmas were demonstrated.

In an earlier paper (1) previously uncharacterized canine mycoplasmas were described and compared with the known serotypes *Mycoplasma spumans* (PG-13), *Mycoplasma canis* (PG-14), and *Mycoplasma maculosum* (PG-15). From the new isolates four groups (A through D) were separated on the basis of either biological or serological differences, and some isolates within these groups showed certain distinctive characteristics. Group B appeared serologically and biologically related to *M. canis*. Group D isolates differed in that one (MH4603) grew better aerobically and metabolized arginine, whereas another (MH5408) did not; however, these isolates were serologically identical. The group C organisms showed serological cross reactions by immunofluorescence, but differed by growth inhibition and biologically in that MH4609 grew on yeast-free agar and more strongly hemolyzed (alpha) mammalian erythrocytes than MH2919. There then appear to be at least three (A, C, and D) if not four (group C, types MH2919 and MH4609) new serotypes of mycoplasmas isolated from dogs which have not appeared to be related to the other well described mycoplasmas (1). Polyacrylamide gel electrophoresis has been used and found reliable (6, 8) in differentiating serotypes of mycoplasmas including canine mycoplasmas. Polyacrylamide gel electrophoresis and immunodiffusion (Ouchterlony) tests were employed in further characterizing and comparing all of these canine mycoplasmas. Further evidence for difference between the two group C mycoplasmas and a possible pitfall in the use of the polyacrylamide gel technique were found.

## MATERIALS AND METHODS

General cultural and serological techniques were previously described (1). Polyacrylamide gel electrophoresis of *Mycoplasma* cell proteins was performed by using the techniques of Razin and Rottem (6) and Takayama et al. (7) with some modifications.

**Preparation of cell extract.** *Mycoplasma* cultures were grown in 300 ml of broth for 48 to 72 hr at 37 C. Cells were harvested by centrifugation at 15,400 × *g* for 15 min in a Sorvall angle centrifuge, washed twice in 0.9% sodium chloride, and suspended in 1 ml of the same saline. One volume of cells containing 50 to 100 μg of protein per ml (4) was extracted by adding

two volumes of phenol-acetic acid-water (2:1:0.5, v/v/v). Insoluble material was removed by centrifugation at 23,000 × *g* for 15 min, 0.1 ml of the supernatant fluid was mixed with 0.05 ml of 40% (w/v) sucrose solution in 35% acetic acid, and 0.12 ml of the mixture was used for electrophoresis.

**Preparation of gel.** Stock solution A was composed of 6 g of acrylamide, 0.16 g of *N,N'*-methylenebisacrylamide, 12 g of urea, 28 ml of glacial acetic acid, and distilled water to a volume of 60 ml. Stock solution B was prepared fresh daily with 0.30 g of ammonium persulfate, 12 g of urea, and distilled water to a volume of 20 ml. The working solution was a mixture of stock solution A (7.5 ml), stock solution B (2.5 ml), and *N,N,N',N'*-tetramethylethylene diamine (0.05 ml) for a final concentration of 7.5% (w/v) acrylamide, 35% (v/v) acetic acid, and 5 M urea.

**Glass gel tubes.** Glass gel tubes were 100 mm long with an outside diameter of 6 mm and an inside diameter of 4 mm.

**Electrophoresis.** Electrophoresis was performed in a Canalco Disc Electrophoresis model 6. The tubes were filled with 0.8 ml of the working solution, overlaid with 0.3 ml of 75% acetic acid, and incubated at 45 C for 30 min to polymerize the acrylamide. After rinsing with 75% acetic acid, the *Mycoplasma* cell extract sucrose-acetic acid mixture (0.12 ml) was layered on top of the gel column and overlaid with 0.3 ml of 75% acetic acid; the remainder of the column was filled with 10% acetic acid. The upper and lower reservoirs of the electrophoresis tank were also filled with 10% acetic acid. Electrophoresis was carried out for 1.75 hr at 2 to 4 ma per tube at room temperature. The use of the narrower 4 mm (inside diameter) gel tubes permitted the use of a smaller amount of sample and reduced electrophoresis time. The polyacrylamide gel columns were stained with 1% Amido Black in 7% acetic acid for 40 min, rinsed in water, and washed overnight in two changes of 7% acetic acid.

**Immunodiffusion tests.** Immunodiffusion (Ouchterlony) tests were performed in 1% agarose in borate buffer (13.4 g of boric acid, 26.8 g of $Na_2B_4O_7 \cdot 10H_2O$, and water to 1 liter) by using lucite matrices made according to template 2 by Macy et al. (5) except that the matrix was laid directly over the agar without the use of plastic tapes. Wells were filled with 30 μliters of each reagent and plates were read after 72 hr at room temperature. The plates were first read by indirect light and then stained with Triple stain (2).

**Antigens and antisera.** Antigens and antisera were prepared as previously described (1).

## RESULTS

**Polyacrylamide gel electrophoresis.** The results of the polyacrylamide gel electrophoresis support the growth inhibition and serological results previously described (1). The patterns were consistent for each serotype on multiple isolates, e.g., nine of PG-13, six of PG-14, and two of PG-15. PG-13, -14, and -15 showed distinctive patterns by electrophoresis and resembled those patterns already published (6, 8). Of the group A mycoplasmas, four separate isolates were tested as follows: Razin and Rottem (6), PG-24 of Edwards, MH5270 (A), MH6471 (A'). The latter two mycoplasmas isolated in this laboratory came from the lungs of dogs with pneumonia. All of these appeared similar to each other and to the patterns published (6, 8). The group B mycoplasmas, MH4942 (B) and MH6010 (B'), showed one-way crosses by growth inhibition with PG-14 (antisera to these two mycoplasmas inhibited PG-14, whereas the reverse was not true), and were very similar by electrophoresis (Fig. 1). Two additional isolates inhibited by group B antisera (but not PG-14) also showed similar patterns. Of the remaining, group C isolates MH4609 (C) and MH2919 (C') were distinctive, and group D MH5408 and MH4603 were similar, as would be anticipated by their reactions in growth inhibition (Fig. 2). Additional group C isolates (a total of three C and three C') from this and other laboratories (1) were also tested, and the PGE patterns remained consistent and distinctive, correlating with the growth inhibition serotype.

In two instances with two different *Mycoplasma* species, PG-14 and the group D MH4603 *Myco-*

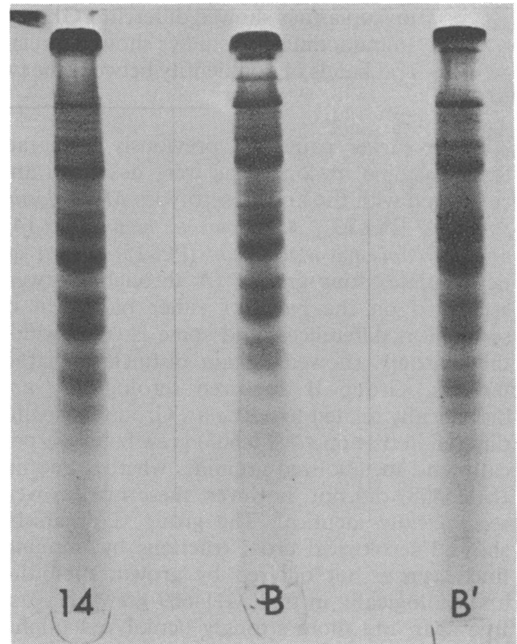


FIG. 1. *Polyacrylamide gel electrophoresis pattern of M. canis (PG-14) and of two similar isolates, B-MH4942 and B'-MH6010, which were more broadly serologically reactive.*

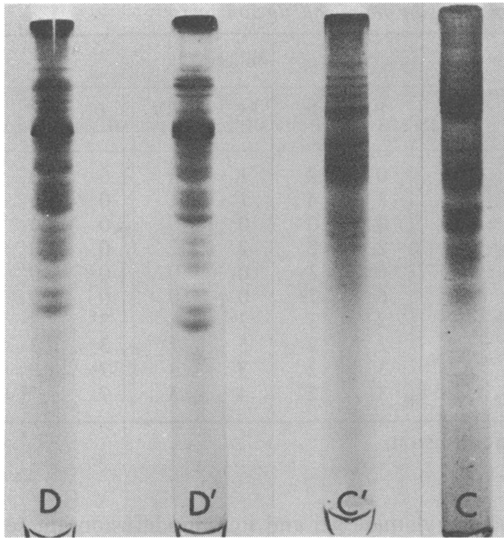


FIG. 2. *Polyacrylamide gel electrophoresis pattern of the two group D isolates which were identical serologically and by PGE. Group C (MH2919) and group C′ (MH4609) differed by growth inhibition and also by PGE.*

*plasma*, each showed different patterns on prolonged incubation (Fig. 3). Electrophoresis of PG-14 after 48 hr of incubation resulted in the usual pattern resembling group B and other PG-14 serotypic isolates. The same strain of PG-14 after 120 hr of incubation resulted in a different pattern on electrophoresis (Fig. 3). In addition, the group D *Mycoplasma* on prolonged incubation (72 hr versus 120 hr) also resulted in a different pattern on electrophoresis (Fig. 3). This organism did not ferment glucose, whereas the group B organism did.

**Immunodiffusion tests.** The results of immunodiffusion tests are recorded in Table 1. Each specific serotype (by growth inhibition) usually showed two to eight more bands with the homologous antiserum with the exceptions noted below.

The groups C and C′ organisms showed differences in immunodiffusion and by polyacrylamide gel electrophoresis. Each of the group C and C′ serotypes developed three to four more lines with its own antiserum, as did the other distinct serotypes. In tests in which reagents were reacted in adjacent wells, there were one or two lines of crossing between C (MH2919) and C′ (MH4609), suggesting nonidentical antigens (Fig. 4).

The group D organisms which were identical by polyacrylamide gel electrophoresis also showed marked similarity by immunodiffusion. Crossing lines of nonidentity were never seen.

The relationship of group B organisms to PG-14 was also supported by immunodiffusion tests.

## DISCUSSION

Characterization of canine mycoplasmas by biological factors, growth inhibition, complement fixation, and fluorescent-antibody techniques was previously reported (1), and four canine mycoplasmas were studied by polyacrylamide gel electrophoresis (6, 8). Further studies by us, using polyacrylamide gel electrophoresis and immunodiffusion techniques, confirm and add depth to the existence of four distinct canine mycoplasma species: *M. spumans*, *M. canis*, *M. maculosum*, and *M. edwardii*. Our group A isolates appeared identical by polyacrylamide gel electrophoresis to the prototype, PG-24, and to published photographs of other isolates (6, 8). The distinctiveness of this *Mycoplasma* species was also supported by immunodiffusion tests.

That the group B mycoplasmas were related to *M. canis* was confirmed by these studies. It is interesting that the B (MH4942) isolate which was most reactive by growth inhibition (1)

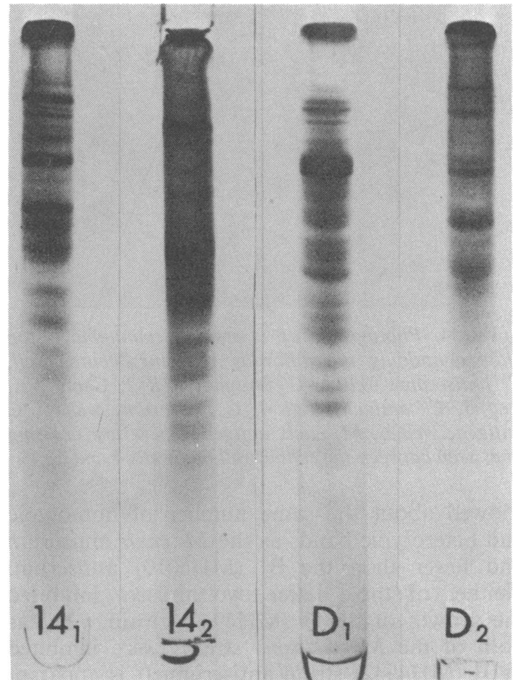


FIG. 3. *Polyacrylamide gel electrophoresis pattern of M. canis after 48 hr of incubation (PG-$14_1$) and after 120 hr of incubation (PG-$14_2$). The group $D_1$ Mycoplasma was incubated for 72 hr, whereas the group $D_2$ identical organism was incubated 120 hr. Differences on prolonged incubation are evident.*

TABLE 1. *Comparison of canine mycoplasmas by immunodiffusion tests*[a]

| Antiserum | *M. spumans* PG-13 | *M. canis* PG-14 | *M. maculosum* PG-15 | Antigen | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | A, MH5270 | B, MH4942 | B', MH6010 | C, MH2919 | C', MH4609 | D, MH5408 | D', MH4603 |
| *M. spumans* PG-13 | 6[b] | 1 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 |
| *M. canis* PG-14 | 0 | 6[b] | 2 | 0 | 3 | 4 | 1 | 1 | 0 | 0 |
| *M. maculosum* PG-15 | 0 | 0 | 4[b] | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| A, MH5270 | 0 | 3 | 0 | 5[b] | 2 | 2 | 2 | 2 | 0 | 0 |
| B, MH4942 | 4 | 4 | 0 | 0 | 6[b] | 3 | 0 | 0 | 0 | 0 |
| B', MH6010 | 0 | 5 | 0 | 2 | 6 | 7[b] | 0 | 0 | 0 | 0 |
| C, MH2919 | 3 | 2 | 3 | 2 | 2 | 3 | 7[b] | 4 | 3 | 3 |
| C', MH4609 | 3 | 4 | 4 | 2 | 2 | 2 | 5 | 7[b] | 3 | 3 |
| D, MH5408 | 5 | 5 | 5 | 2 | 3 | 2 | 3 | 3 | 7[b] | 8 |
| D', MH4603 | 2 | 1 | 2 | 2 | 3 | 2 | 4 | 3 | 7 | 7[b] |

[a] Number of precipitin lines shown by double immunodiffusion.
[b] Homologous system.

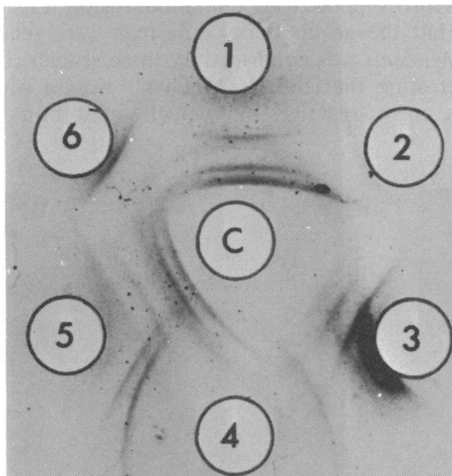


FIG. 4. *Photograph of a stained immunodiffusion plate containing the following reagents: center well, C' antiserum; well 1, C' antigen; well 2, C antigen; well 3, C' antigen; well 4, C antiserum; well 5, C antigen; well 6, M. canis antigen. There are crossing lines seen between the center well and wells 4 and 5.*

showed about the same number of homotypic and heterotypic bands as the *M. canis* antiserum and fewer than the B' (MH6010) antiserum. Neither of these latter two antisera inhibited the growth of the B (MH4942) strain, whereas both of the *Mycoplasma* strains were inhibited by B (MH4942) strain antiserum. It is apparent that the ability to inhibit growth of the *Mycoplasma* strain cannot be predicted from the number of precipitin bands.

The two group D strains which differed somewhat biologically had proved serologically identical (1). These findings were confirmed by the polyacrylamide gel and immunodiffusion studies (Fig. 2 and Table 1). These would appear to be minor strain variants of the same species. In contrast, the group C isolates, C (MH2919) and C' (MH4609), showed minor biological differences but were also different by growth inhibition. They did, however, cross by immunofluorescent studies (1). The polyacrylamide gel patterns of the two prototype strains (Fig. 2) were different. In addition, two other isolates each that typed with either MH2919 or MH4609 antiserum by growth inhibition also resembled the prototype strain by polyacrylamide gel pattern. The difference in these two *Mycoplasma* isolates was supported by immunodiffusion tests in which each organism showed two to three more precipitin bands with its homologous antiserum than with each other or any other of the mycoplasmas tested (Table 1). Finally, there were definite crossing lines of nonidentity when the isolates were tested (Fig. 4), demonstrating that the two mycoplasmas contain distinct separate antigens. With the data available (e.g., that isolates C (MH2919) and C' (MH4609) differ by growth inhibition, polyacrylamide gel electrophoresis, and immunodiffusion), it seems appropriate to regard them as two separate mycoplasmas. Only further studies in different laboratories on new isolates will clarify this issue.

The changes which can occur in polyacrylamide gel patterns on prolonged incubation and growth of the same mycoplasma species is apparent in Fig. 3. This occurs both with glucose fermenters, *M. canis*, and nonfermenters, the group D mycoplasma. This was also observed with other *M. canis* and group D isolates. After extraction, the 48-hr incubated cultures were held at 4 C (which did not change their patterns) until the prolonged

incubation cultures were extracted and both were electrophoresed and stained together, excluding variables during these procedures. Increase in the quantity of protein present results only in increased intensity of bands using the same *Mycoplasma*. Although there appears to be more material staining in PG-$14_2$ and less in $D_2$, the bands are very different, suggesting a qualitative difference in proteins present. Perhaps the change is due to enzymatic breakdown of *Mycoplasma* proteins, but this was not delineated in our studies. This phenomenon does point out the necessity for rigorous control of methods using polyacrylamide gel electrophoresis to characterize mycoplasmas within individual laboratories and a possible pitfall in comparing patterns from different laboratories in which the methods may differ.

## LITERATURE CITED

1. Armstrong, D., J. G. Tully, B. Yu, V. Morton, M. H. Friedman, and L. Steger. 1970. Previously uncharacterized mycoplasma isolates from an investigation of canine pneumonia. Infect. Immun. **1**:1–7.
2. Crowle, A. J. 1961. Immunodiffusion. Academic Press Inc., New York.
3. Edward, D. G. ff., and W. A. Fitzgerald. 1951. The isolation of organisms of the pleuropneumonia group from dogs. J. Gen. Microbiol. **5**:566–575.
4. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:262–275.
5. Macy, N. E., M. B. O'Sullivan, and G. J. Gleich. 1968. Comparison of sensitivities of immunodiffusion methods. P.S.E.B.M. **128**:1098–1102.
6. Razin, S., and S. Rottem. 1967. Identification of mycoplasma and other microorganisms by polyacrylamide gel electrophoresis of cell proteins. J. Bacteriol. **94**:1807–1810.
7. Takayama, K., D. H. MacLennan, A. Tzagoloff, and C. D. Stoner. 1964. Studies on the electron transfer system LXZII 67: polyacrylamide gel electrophoresis of the mitochondrial electron transfer complexes. Arch. Biochem. Biophys. **114**: 223–230.
8. Tully, J. G., M. F. Barile, R. A. Del Guidice, T. R. Carski, D. Armstrong, and S. Razin. 1970. Proposal for classifying strain PG-24 and related canine mycoplasmas as *Mycoplasma edwardii* sp. n. J. Bacteriol. **101**:346–349.