

## Flat Gel Polyacrylamide Electrophoresis of Porcine Mycoplasmas

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Received for publication 29 May 1974

The flat gel acrylamide electrophoresis technique was standardized and applied to the comparison of four species of porcine mycoplasmas. Clear differences were observed between these species, and differences were seen among the strains of *Mycoplasma hyosynoviae*. The clarity of the patterns and the number of bands developed was influenced by the amount of protein in the extract and the age of the culture. The technique allows the comparison of several protein extracts in parallel without the problems associated with the rearrangement of separate gel columns.

Polyacrylamide gel electrophoresis of mycoplasma proteins is a generally accepted technique for the detection of interspecies differences (13). It has been used to assist the identification of new species (5, 6) and has been applied to subspecies differentiation (7, 10, 14, 16). The method commonly used for mycoplasma studies is based on the tubular gel system first described by Davis (4) and Takayama et al. (19). The flat gel system devised by Ackroyd (1), despite some advantages, has been used for mycoplasma studies only by Zola et al. (20), so we re-investigated this technique and applied it to the study of porcine mycoplasmas.

### MATERIALS AND METHODS

**Mycoplasma.** Several species were studied: *Mycoplasma hyosynoviae* strains S16 (17), M60 (8), and P45 (15); *Mycoplasma hyorhinis* strains MH31 (11) and S218 (9); *Mycoplasma suis pneumoniae* NCTC 10110; and *Acholeplasma granularum* NCTC 10128. In addition, *M. hyorhinis* strains 483, 499, and 508, isolated at these laboratories, were used.

**Culture media and preparation of antigens.** *M. hyorhinis* and *A. granularum* were grown in PPLO broth (Difco) enriched with 10% horse serum, 10% boiled defibrinated horse blood extract, 10% of a 25% bakers' yeast extract, 0.002% deoxyribonucleic acid (DNA), 200 IU of penicillin per ml, 1/4,000 thallos acetate, 0.002% phenol red, and 0.1% glucose.

*M. hyosynoviae* was cultured in this medium containing 0.1% arginine in place of glucose, and *M. suis pneumoniae* was grown in phosphate-buffered saline (PBS), pH 7.2, supplemented with 20% horse serum, 0.1% lactalbumin hydrolysate with yeast extract, penicillin, thallos acetate, phenol red, and glucose as above. *M. hyorhinis*, *M. hyosynoviae*, and *A. granularum* strains were cultured in 2-liter vol-

umes of broth inoculated with 50-ml amounts of actively growing broth cultures. *M. suis pneumoniae* was grown in 1-liter volumes of medium inoculated with 100-ml cultures. *A. granularum* cultures were harvested after 24 h of incubation at 37 C, *M. hyorhinis* and *M. hyosynoviae* after 2 days of incubation, and *M. suis pneumoniae* cultures after 4 days of incubation. Antigen was sedimented by centrifugation at  $2,500 \times g$  for 1 h at 4 C, washed three successive times in 40-ml amounts of PBS, and finally resuspended in PBS. Volumes (5 ml) of the antigen suspension were stored at -70 C for polyacrylamide gel electrophoresis, and the protein content of 0.5 ml of each antigen was estimated by the method of Lowry et al. (12). Where sequential harvests were required, appropriate samples were removed at intervals up to 11 days and harvested for antigen as above. The sediment from 2-liter volumes of uninoculated media, incubated for 4 or 6 days at 37 C, was also examined.

**Solubilization of protein.** Antigen was thawed and after centrifugation ( $2,500 \times g$ , 30 min, 4 C) 0.1 or 0.2 ml of a solution of phenol-acetic acid-water (2 g:1 ml:0.5 ml) was added per mg of protein (18). The suspension was thoroughly mixed with a pipette and incubated at room temperature for 60 min before the addition of 0.02 ml of a 1% solution of Rhodamine-G in phenol-acetic acid-water. This solubilization procedure sometimes left a trace of insoluble residue. Sediments from the uninoculated broths were also solubilized. The protein concentrations of extracts applied to any one gel were always the same.

**Polyacrylamide gel electrophoresis.** Gel containing 7.5% acrylamide, 35% acetic acid, and 5 M urea (19) was polymerized in a rectangular glass cell (100 by 120 by 1.5 mm) at 50 C for 30 min. The electrophoretic apparatus used was a modification of that reported by Ackroyd (1). Silicone rubber spacers (8 by 4 mm) were pushed down onto the gel surface 8 mm apart and covered with 10% acetic acid, and 0.04-ml volumes of the protein extracts were introduced between the spacers. Samples were electrophoresed at

10 mA for 15 min; the spacers were then removed and electrophoresis was continued at 10 mA for about 7 h, until the leading edge of the indicator dye reached the bottom edge of the gel.

The gel was removed from the glass cell and stained for 30 min in an aqueous solution of 0.05% Coomassie blue and 10% trichloroacetic acid (3). Gels were destained over a period of 1 to 2 days in several changes of an aqueous solution of 2% acetic acid, 20% methanol, and 0.1% glycerol.

### RESULTS

A modification of the method of Zola et al. (20), which reduced the gel thickness to 1.5 mm and increased the electrophoresis time to 6 to 7 h at a constant current of 10 mA, produced a horizontal protein migration front, provided the wick was exactly the width of the gel. Antigens could be stored at  $-70^{\circ}\text{C}$  prior to solubilization without any alteration in the number and intensity of the bands produced. Solubilization of most strains with 0.1 ml or 0.2 ml of phenol-acetic acid-water per 1 mg of protein gave the same number of detectable bands, but with *A. granularum* more bands were detected when the antigen was solubilized with 0.1 ml of phenol-acetic acid-water per 1 mg of protein. Uninoculated media yielded small amounts of sediment, extracts of which produced a few very faint bands, none of which appeared to correspond with bands from mycoplasma extracts.

With most of the strains investigated there was a general weakening of the protein patterns with increased age of culture (Fig. 1), and this sometimes resulted in a diminution in the number of detectable bands. However, this attenuation was not found with the patterns produced by *M. suis pneumoniae* extracts. Extracts of *M. hyosynoviae* (S16) antigen harvested after incubation for 1 day formed a strong band (arrowed) near the top of the gel (Fig. 2). This band faded after an additional day of incubation, whereas most of the other bands became more intense before finally fading. This phenomenon was not observed with the two other strains of *M. hyosynoviae* (M60 and P45), but it was reproducible by using another culture of *M. hyosynoviae* (S16).

The patterns of the four different species revealed marked differences although there were a few shared bands (Fig. 3, columns C-F). When strains of particular species were compared, the five strains of *M. hyorhinis* gave identical patterns, but several differences were observed among the three strains of *M. hyosynoviae*, although these shared the majority of bands (Fig. 3, columns A-C) and these characteristics were reproducible using different cultures of the organisms.

### DISCUSSION

There is some dispute about the influence of age upon the number and quality of protein bands obtained in gel electrophoresis. Many

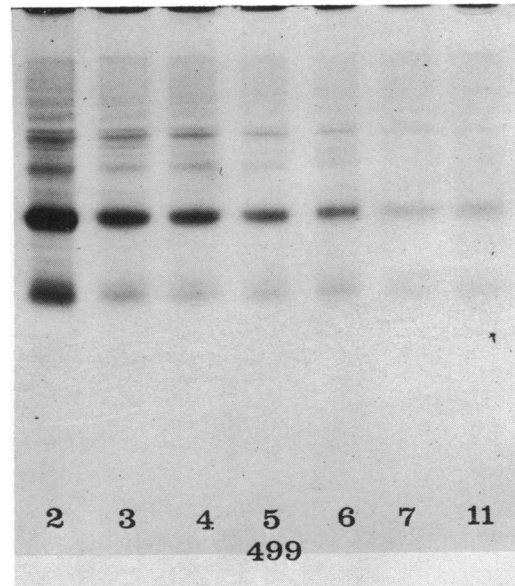


FIG. 1. Electrophoretic patterns of extracts of *M. hyorhinis* (499) antigen from broth culture harvested after 2, 3, 4, 5, 6, 7, and 11 days of incubation (0.1 ml of phenol-acetic acid-water per 1 mg of protein).

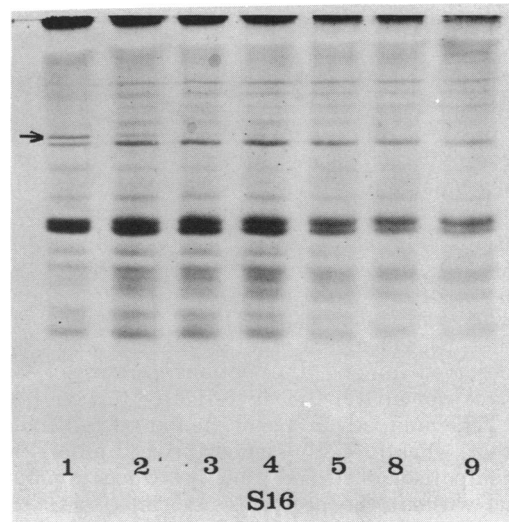


FIG. 2. Electrophoretic patterns of extracts of *M. hyosynoviae* (S16) antigen from broth culture harvested after 1, 2, 3, 4, 5, 8, and 9 days of incubation (0.2 ml of phenol-acetic acid-water per 1 mg of protein).

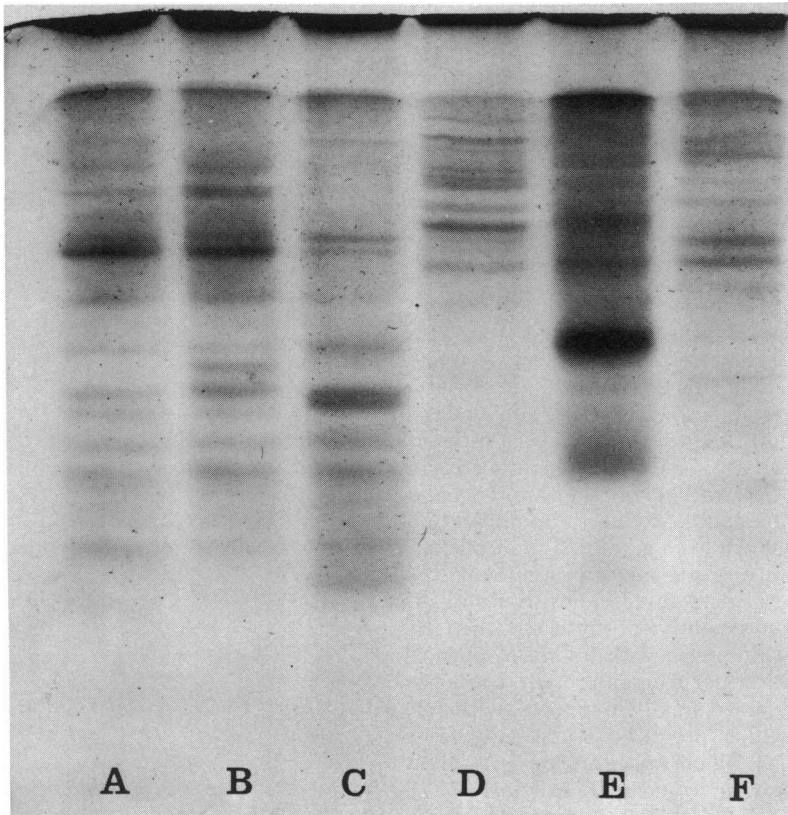


FIG. 3. Electrophoretic patterns of extracts of (A) *M. hyosynoviae* (M60), (B) *M. hyosynoviae* (P45), (C) *M. hyosynoviae* (S16), (D) *M. suisneumoniae* (10110), (E) *M. hyorhinis* (508), and (F) *A. granularum* (10128) (0.2 ml of phenol-acetic acid-water per 1 mg of protein).

workers have reported that the age of culture does not influence the number of bands obtained (7, 13, 20), but these observations are contradicted by Armstrong and Yu (2), and Rosendal (16) with whom we agree. With the strains investigated here not only could aging result in apparent loss of bands, but early growth of one strain, *M. hyosynoviae* (S16), revealed a distinct but transient extra band. The significance of this band, its subsequent disappearance, and the differences which we observed among the various strains of *M. hyosynoviae* were not investigated further.

The main advantage of the flat gel method is that, when properly standardized, it allows the comparison of several protein extracts in parallel without the problems associated with the rearrangement of separate gel columns.

#### ACKNOWLEDGMENTS

We would like to thank those who kindly supplied cultures for study, E. A. Jones for photography, A. E. Pink for protein estimations, and Joy Bushby for the typing of this manuscript.

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