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Characterization of Mycoplasma Strains from Cats

B. C. COLE, L. GOLIGHTLY, AND J. R. WARD

Department of Internal Medicine, Division of Arthritis, University of Utah Medical Center, Salt Lake City, Utah 84112

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Mycoplasma strains (B1, B2, CS, and S1A) were isolated from the saliva of normal cats. These were compared with a strain (CO) isolated from the eye of a cat with severe conjunctivitis. On the basis of morphology, biochemical reactions, and antigenic composition, two distinct species were recognizable. Strains CO, B1, and B2 were antigenically unrelated to the other species tested; strains CS and S1A possessed antigenic components in common with *Mycoplasma arthritidis*, *M. salivarium*, *M. hominis*, type 1, and *M. orale*, types 1 and 2. It was tentatively suggested that the two cat species be called *M. felis* and *M. gateae*, respectively.

The isolation of potentially pathogenic mycoplasma strains from laboratory and domestic animals has been reported by many workers. Although *Mycoplasma arthritidis* is a well-known pathogen for rats, it may be harbored by apparently healthy animals (13). Similarly, *M. neurolyticum*, *M. pulmonis*, and *M. hyorhinis*, all known to be pathogenic species, may occur in normal animals (12, 21, 22, 25). Recent evidence suggests that *M. hominis*, type 1, a common human commensal, is pathogenic under certain circumstances (19).

Switzer, in 1954, isolated a mycoplasma from the pneumonic lung of a 6-week-old kitten, but could not induce disease experimentally (24). This study describes the isolation and characterization of mycoplasma from cats, and discusses their role in cat conjunctivitis.

MATERIALS AND METHODS

Strains. The original cat strain, designated CO, was isolated by J. K. Williams, Department of Pharmacology, University of Utah, from the eye of a cat with severe conjunctivitis. Strains B1, B2, CS, and S1A were isolated from cat salivas by the authors. *M. arthritidis*, strain 14124, and *M. hominis*, type 1, strain 14027, were obtained from the American Type Culture Collection (Rockville, Md.). *M. orale*, types 1 and 2, *M. salivarium*, strain 156, and *M. hyorhinis*, strain DGL, were kindly supplied by M. F. Barile (Bureau of Biologic Standards, National Institutes of Health, Bethesda, Md.). *M. arthritidis*, strain PN, was isolated by the authors from a spontaneous rat abscess.

Biochemical tests. Unless otherwise stated, the methods used were as described previously (4). PPLO Agar or Broth (Difco), supplemented with 0.25% (w/v) yeast extract (BBL), 0.2 μg of deoxyribonucleic acid per ml (calf thymus, Mann Research Laboratories, New York, N.Y.), and 10% (v/v) horse serum were used as the basal media throughout the study.

Fermentation of the following carbohydrates was tested: glucose, maltose, mannose, starch, fructose, and dextrin.

Hemolytic activity towards sheep, guinea pig, chicken, and duck erythrocytes was tested by growing the organisms on base agar supplemented with 4% (v/v) of the various bloods. The plates were incubated at 37 C for 48 hr and were then stored at 4 C for another 24 hr before recording the results.

Peroxide production was tested by a modification of the method of Kraus et al. (15). Base serum agar was supplemented with 4% (v/v) sheep blood and 0.01% (w/v) benzidine [added from a 1% (w/v) sterile aqueous solution]. The plates were inoculated and examined 5 to 7 days later for the development of a brown coloration to the growth. Earlier studies (B. C. Cole, Ph.D. Thesis, University of Birmingham, England, 1964) indicated the value of this test in the differentiation of mycoplasma.

The reduction of methylene blue was performed by use of concentrated suspensions of the strains. Cultures were centrifuged at 27,000 × *g* for 15 min, concentrated 100-fold in serum broth, and stored at −20 C in duplicate. Viable counts (18) were carried out on one tube of each of the strains. Final suspensions were adjusted to contain 10^9 and 10^8 colony-forming units (CFU) per ml. The suspensions, in 0.1-ml amounts, were mixed with 0.1 ml of sterile 0.01% (w/v) aqueous methylene blue in precipitin tubes. The tests were done in duplicate; one set of tubes was incubated anaerobically, and one set was covered with sterile mineral oil and incubated aerobically. Decolorization was recorded after 24 and 48 hr of incubation at 37 C.

Antibiotic sensitivites were performed by seeding agar plates from broth cultures of the strains to be tested, and overlaying these with Multi-discs (Consolidated Laboratories, Chicago Heights, Ill.). Antibiotics and the amounts used were: erythromycin, 2 μg; tetracycline, 5 μg; neomycin, 5 μg; novobiocin,

5 μg; lincomycin, 2 μg; chloramphenicol, 5 μg; dihydrostreptomycin, 2 μg; and penicillin G, 2 units.

Antigenic composition. Antigens for immunization and subsequent testing were cloned three times and grown in 750-ml amounts of rabbit infusion broth (27), supplemented with 10% (v/v) sterile rabbit serum. The cultures were incubated in 2-liter low-form flasks on a reciprocating shaker. The cells were harvested by centrifugation, washed three times in saline, and finally resuspended in 5 ml of saline containing 0.2% (v/v) Formalin. Total nitrogen estimations of the suspensions were made by use of a Coleman Nitrogen Analyzer, model 29. Antisera were produced in rabbits by foot-pad injection (H. E. Morton and R. J. Roberts, Bacteriol. Proc., p. 139, 1966) and subcutaneous injection (20) for each strain. By use of these procedures, strains CO and B1 would not induce antibody formation as detected by agar gel double diffusion, although low titers of 1:160 were detected by complement fixation. An improved immunological response to these strains was obtained by reinjecting the same animals, but reversing the two procedures.

All antigens were subjected to 1 min of cavitation by use of a Branson Sonifier, model S75, prior to the serological determinations. Agar gel double diffusion tests were set up to determine individual antigenic components. The sterile media used for PPLO growth were centrifuged and all sera were tested against the resuspended deposits for possible false reactions. Precipitin bands were recorded after 4 to 5 days of incubation at room temperature.

Growth inhibition tests were carried out by the method of Clyde (3). Complement-fixation tests were carried out by the method of Kolmer et al. (14). Antigens of two different strains were adjusted to the same optical density by use of a Beckman DB Spectrophotometer at 530 mμ. The dilution of antigen giving maximal complement fixation against the homologous sera was determined. As the antigen titer was identical in these two cases, all other antigens were adjusted to the same optical density. Both antigens and antisera were heated at 56 C for 30 min prior to use in the complement-fixation tests. Titers were recorded as the reciprocal of the dilution of antiserum which gave 100% fixation of complement.

RESULTS

Isolation and growth characteristics. The original cat strain, CO, was isolated in pure culture on Columbia Agar Base (BBL), supplemented with 5% (v/v) horse blood, from the eye of a cat with severe conjunctivitis. Minute α-hemolytic colonies developed after 24 hr of incubation in a candle jar. Microscopic examination by use of Dienes stain (6) revealed the typical morphology of mycoplasma growth. The conjunctivitis responded to tetracycline therapy (J. K. Williams, *personal communication*).

All other isolations were carried out on PPLO Agar supplemented with 10% (v/v) horse serum and 1,000 units of penicillin G per ml. Swabs from the eyes and mouth of six normal cats were inoculated in duplicate onto the base agar, and were incubated aerobically and anaerobically at 37 C. All eye cultures were negative for mycoplasma. Three out of six of the oral cultures contained mycoplasma after both aerobic and anaerobic incubation. The isolates were designated B1, B2, and S1A, respectively. Two kittens to be used in pathogenicity experiments were similarly cultured. One contained mycoplasma, designated CS, in its saliva.

On initial isolation, all strains grew well, producing visible colonies after 24 hr of either aerobic or anaerobic incubation. No growth occurred on serum-free agar. Strains CO, B1, and B2 did not survive refrigeration at 4 C for more than 1 week. The colonies of CO, B1, and B2 were somewhat rough in appearance on initial isolation, but possessed well-defined central regions of growth into the agar (Fig. 1). In contrast, the colonies of S1A and CS did not possess well-defined regions of agar growth (Fig. 2). For comparative purposes, the colonies of *M. hominis*, type 1, are recorded in Fig. 3. Colony impression preparations were fixed with Bouin's fixative and were stained for 15 to 30 min in dilute carbol fuchsin. Colonies of strains CS and S1A were characterized by the presence of numerous large bodies in various stages of differentiation into minute elementary bodies (Fig. 2a). Such large bodies were almost completely absent from strains CO, B1, and B2 (Fig. 1a). Large bodies were infrequent in stained preparations of *M. hominis*, type 1.

Pathogenicity. Drops of a broth culture of strain CO, each containing approximately 10^7 CFU, were implanted in the eyes of two kittens every 48 hr. The mycoplasma could be reisolated from the eyes up to 48 hr after implantation, but not later. After 2 weeks of such treatment, one of the kittens developed a conjunctivitis with a pronounced mucous discharge. Inoculation ceased at this point. During the course of the infection, which lasted 2 weeks, mycoplasma could be isolated from the eyes of the infected kitten, but not from the normal kitten. The reisolated strain was designated CO-R. When the apparent infection had subsided, mycoplasma could no longer be isolated.

The reisolated mycoplasma was tested biochemically and serologically and was found to be identical with the strain implanted. The saliva of both animals was cultured for mycoplasma at the completion of the experiment. Both contained mycoplasma which produced colonies resembling strain CO, and one produced, in addition, colonies resembling strain CS, which had been previously isolated at the beginning of the experiment.

One week after the infection had subsided, both animals were bled, and the sera were tested by complement fixation against antigens of strains CO and CS. No titers were detected.

Concentrated suspensions (100-fold) of strains S1A and CS were injected subcutaneously and intravenously into groups of six rats. Neither abscesses nor arthritis developed.

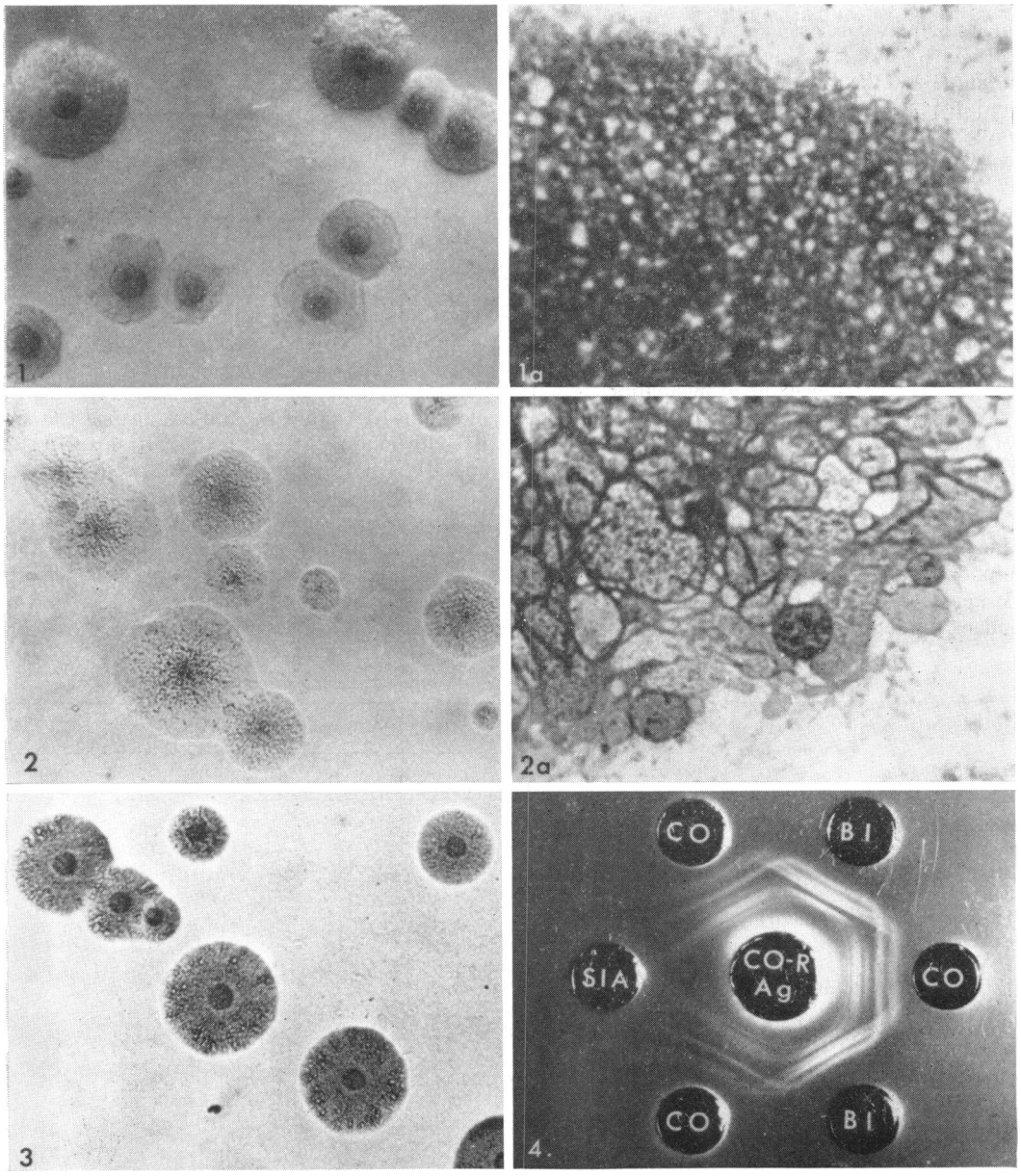

FIG. 1. *Colonial morphology of cat strain CO after 2 days of aerobic growth.* × *30.*

FIG. 2. *Colonial morphology of cat strain S1A after 2 days of aerobic growth.* × *30.*

FIG. 1*a* and 2*a*. *Impression preparations stained with carbol fuchsin, showing peripheral regions of colonies.* × *1,125.*

FIG. 3. *Colonial morphology of Mycoplasma hominis, type 1, after 3 days of aerobic growth.* × *30.*

FIG. 4. *Gel diffusion plate showing relationship between cat strains CO, CO-R, B1, and S1A. Antigen of CO-R is in the center well; antisera are in the outer wells.*

Biochemical properties. The results are summarized in Table 1. None of the strains attacked carbohydrates, and peroxide production was not detected. On egg yolk-agar, strains CO, B1, and B2 produced a "film and spots" (7) reaction and a clearing of the emulsion around the growth; no lipase activity was detected on horse serum-agar. Strains S1A and CS produced ammonia from arginine, but the others did not. Tetrazolium blue and triphenyltetrazolium chloride were weakly reduced by all strains anaerobically, but not aerobically.

Strains CO, B1, and B2 reduced methylene blue anaerobically in 24 hr when 5×10^8 CFU per ml of reaction mixture was used. Partial reduction occurred after 24 hr of aerobic incubation when the reaction mixture was covered with oil. With 5×10^7 CFU per ml, and 48 hr of anaerobic incubation, only CO and B2 reduced the dye. S1A and CS were negative in all of the methylene blue tests, even after 48 hr of incubation.

Strains S1A and CS produced weak β-hemolysis of sheep and guinea pig blood after 24 hr of refrigeration of the 2-day aerobic cultures. Chicken and duck cells were not lysed. The other strains showed greater hemolytic activity, producing marked α-hemolysis of sheep and chicken bloods and strong α-hemolysis of duck cells. Strains CO, B1, and B2 produced β-hemolysis of guinea pig cells, even without refrigeration.

All strains were resistant to erythromycin and dihydrostreptomycin, and were inhibited by tetracycline, chloramphenicol, and lincomycin. Strains S1A and CS were resistant to neomycin and novobiocin, to which the others were sensitive.

Serology. Complement fixation. The results are recorded in Table 2. On the basis of complement fixation, antigens of strains S1A and CS reacted identically with S1A antiserum. Similarly, antigens of B1, B2, and CO produced titers comparable with the homologous systems by use of B1 and CO antisera. The other species, *M. arthritidis*, *M. salivarium*, and *M. hominis*, type 1, produced the highest titers against their own sera, although there was some evidence of cross-reaction between them and also with strains S1A and CS. For example, *M. hominis*, type 1, serum cross-reacted significantly with S1A and CS antigens.

Growth inhibition. The results (Table 3) confirmed those obtained by complement fixation. Serum prepared against S1A inhibited the growth of strains S1A and CS. Antisera against CO and B1 both inhibited the growth of strains CO, B1, and B2. The other species tested were inhibited only by the homologous antisera.

Agar gel double diffusion. The number of precipitin bands observed between the various antigens and sera are recorded in Table 4. By absorption of sera with antigens and vice versa, and by alternating one serum or antigen with those con-

TABLE 1. *Biochemical reactions of mycoplasma strains isolated from cats*

Biochemical tests	Mycoplasma strains[a]				
	CO	B1	B2	S1A	CS
Ammonia from arginine	−	−	−	+	+
Egg yolk reaction	+	+	+	−	−
Methylene blue reduction[b]	+	+	+	−	−
Carbohydrate fermentation	−	−	−	−	−
Tetrazolium blue reduction					
Aerobic	−	−	−	−	−
Anaerobic	+	+	+	+	+
Triphenyltetrazolium chloride reduction					
Aerobic	−	−	−	−	−
Anaerobic	s	s	s	s	s
Neomycin	+	+	+	−	−
Novobiocin	+	+	+	−	−
Hemolysis[c]					
Sheep	α+	α+	α+	βs	βs
Guinea pig	β+	β+	β+	βs	βs
Chicken	α+	α+	α+	−	−
Duck	α++	α++	α++	−	−

[a] Reactions graded as: ++, strongly positive; +, positive; s, slight; −, no reaction. For neomycin and novobiocin, + denotes inhibition of growth.

[b] Methylene blue reduction, recorded after 48 hr of anaerobic incubation, 5×10^8 CFU/ml of reaction mixture.

[c] Hemolysis recorded after 24 hr of refrigeration at 4 C.

TABLE 2. *Comparison of mycoplasma strains as measured by complement fixation through use of specific antisera*

Antigen	Reciprocal of complement-fixing antibody titer[a] against					
	Cat strains			Others		
	CO	B1	S1A	*M. arthritidis*	*M. salivarium*	*M. hominis type 1*
CO	1,280	320	20	20	40	
B1	1,280	320	<20	20	40	40
B2	640	320	40	40	40	
S1A	20	20	320	80	40	160
CS	40	20	320	80	40	160
M. arthritidis 14124	20	<20	40	1,280	40	160
M. salivarium 156	80	40	80	80	640	80
M. hominis, type 1, 14027	40	20	40	80	40	640

[a] Titer read as the highest dilution of serum showing complete fixation of complement.

TABLE 3. *Comparison of mycoplasma strains by inhibition of growth with specific antisera*

Strain	Antisera[a]					
	CO	B1	S1A	*M. arthritidis*	*M. salivarium*	*M. hominis, type 1*
CO	+	+	−	−	−	−
B1	+	+	−	−	−	−
B2	+	+	−	−	−	−
S1A	−	−	++	−	−	−
CS	−	−	++	−	−	−
M. arthritidis, 14124	−	−	−	+	−	−
M. salivarium, 156	−	−	−	−	++	−
M. hominis, type 1, 14027	−	−	−	−	−	++

[a] Symbols: +, denotes zone of growth inhibition 2 mm or less; ++, denotes zone of growth inhibition greater than 2 mm.

TABLE 4. *Number of precipitin bands observed between mycoplasma strains in agar gel double diffusion studies*

Antigens	Antisera against cat strains			Antisera against other species		
	CO	B1	S1A	*M. arthritidis*	*M. salivarium*	*M. hominis, type 1*
CO	5	5	0	0	0	0
CO-R	7	6	0	0	0	0
B1	5	5	0	0	0	0
B2	5	5	0	0	0	0
S1A	0	0	4	2	1	3
CS	0	0	6	2	1	3
M. arthritidis, 14124	0	0	1	4	1	2
M. salivarium, 156	0	0	1	0	5	2
M. hominis, type 1, 14027	0	0	3	1	2	4
M. arthritidis, PN	0	0	1	3	1	2
M. orale, type 1	0	0	2	1	1	2
M. orale, type 2	0	0	2	1	1	2
M. pulmonis, T1	0	0	0	0	0	0
M. hyorhinis, DGL	0	0	0	0	0	0

taining common components, it was possible to analyze the antigenic structure of each organism. The results of these analyses are recorded in Table 5. A complete analysis of strains other than those of cat origin was not undertaken, and only the antigens which were shared with the latter are recorded. It is clear, however, from Table 5 that the other species all contained, in addition, their own specific components.

Strains CO, B1, B2, and CO-R were closely related to each other and distinct from all of the other strains tested (Fig. 4). The seven antigenic components identified were designated a through g.

Strains S1A and CS were closely related, producing at least four precipitin bands, respectively, when tested against S1A serum. The latter also produced: one precipitin band with antigens of *M. arthritidis* and *M. salivarium*; two bands with *M. orale*, types 1 and 2; and three bands with *M. hominis*, type 1 (Fig. 5). By alternating S1A serum with sera of the other species around *M.*

TABLE 5. *Antigenic components of mycoplasma strains as identified by gel diffusion*

Strains	Components
Cat strains	
B1	a, b, c, d, e, g
B2	a, b, c, d, g
CO	a, b, c, d, e, f, g
CO-R	a, b, c, d, e, f, g
S1A	h, i, j, k, l
CS	h, i, j, k, l
Other species	
M. arthritidis, 14124	h, i, (j)[a]
M. salivarium, 156	h, (i)
M. hominis, type 1, 14027	h, i, j
M. orale, type 1	h, i, (j)
M. orale, type 2	h, i, (j)

[a] Antigens in parentheses demonstrable by absorption only.

arthritidis antigen, a continuous band was produced (Fig. 7). A similar continuous band was also produced by use of *M. hominis*, type 1, antigen. This component was designated h. The two other components which S1A and CS shared with *M. hominis*, type 1, were designated i and j, respectively. Absorption of S1A serum with *M. hominis*, type 1, antigen removed components h, i, and j, but S1A serum still produced two bands with its own antigen and that of CS (Fig. 5 and 6). These components were designated k and l. Absorption of S1A serum with *M. arthritidis* also removed components h, i, and j, indicating that small amounts of components i and j also occurred in *M. arthritidis*. Absorption with *M. salivarium* antigen removed only components h and i. Absorption with *M. orale*, types 1 and 2, removed all three components.

Similar reactions occurred in the opposite direction by use of antigens of CS and S1A with sera against *M. arthritidis*, *M. salivarium*, and *M. hominis*, type 1 (Table 4 and Fig. 8). In this instance, two precipitin bands were produced with *M. arthritidis* serum.

None of the sera tested reacted with antigens of *M. pulmonis* or *M. hyorhinis*.

DISCUSSION

Switzer (24) reported the isolation of mycoplasma from the pneumonic lung of a 6-week-old kitten. The disease could not be produced experimentally, however, and the isolate was not characterized.

The cat strains isolated in the present study could be placed in two distinct groups on the basis of physiology, morphology, and antigenic composition. Group 1, comprising strains CO, B1, and B2, was characterized by the reduction of methylene blue, lipase activity towards egg yolk emulsion, sensitivity to neomycin and novobiocin, marked hemolysis of various animal erythrocytes, and lack of ammonia production from arginine. Colonies produced well-defined regions of agar growth.

Group 2, strains S1A and CS, did not reduce methylene blue, possessed no lipase activity towards egg yolk emulsion, were resistant to neomycin and novobiocin, produced only weak hemolysis of sheep and guinea pig erythrocytes, but produced ammonia from arginine. The colonies were vacuolated without well-defined central regions. Groups 1 and 2 were serologically unrelated, as tested by complement fixation, gel diffusion, and growth inhibition. The presence of common antigens between *M. arthritidis*, *M. salivarium*, *M. hominis*, type 1, and *M. orale* is well established (2, 5, 16, 17, 20, 26, 27). By use of agar gel double diffusion, it was shown that three of these antigens were also common to the Group 2 cat strains. However, on the basis of complement fixation, growth inhibition, and the presence of two antigens specific to the Group 2 strains, it was concluded that the latter formed a distinct group. This was also confirmed by the characteristic colonial morphology. On the basis of previous reports, the cat strains could also be differentiated from *M. gallinarum*, *M. gallisepticum*, *M. canis*, *M. maculosum*, *M. mycoides*, *M. fermentans*, *M. laidlawii*, *M. neurolyticum*, and *M. pneumoniae* (1, 8–10, 23). The physiological characteristics of most of these species have been confirmed by the present authors (*unpublished observations*).

The original isolation and apparent induction of conjunctivitis with strain CO suggests mycoplasma as one of the possible etiological agents of cat conjunctivitis. The common occurrence of identical mycoplasma strains in the saliva of healthy cats suggests this as the potential source of the infection. This is supported by the finding that strain CO survived in the saliva of both kittens, even after they could no longer be isolated from the eyes. Further studies are required, however, to confirm the pathogenicity of these strains. It is interesting to note that mycoplasma have also been isolated from cases of human conjunctivitis (11).

As a result of these studies, it is tentatively suggested that Groups 1 and 2 be called *M. felis* and *M. gateae*, respectively.

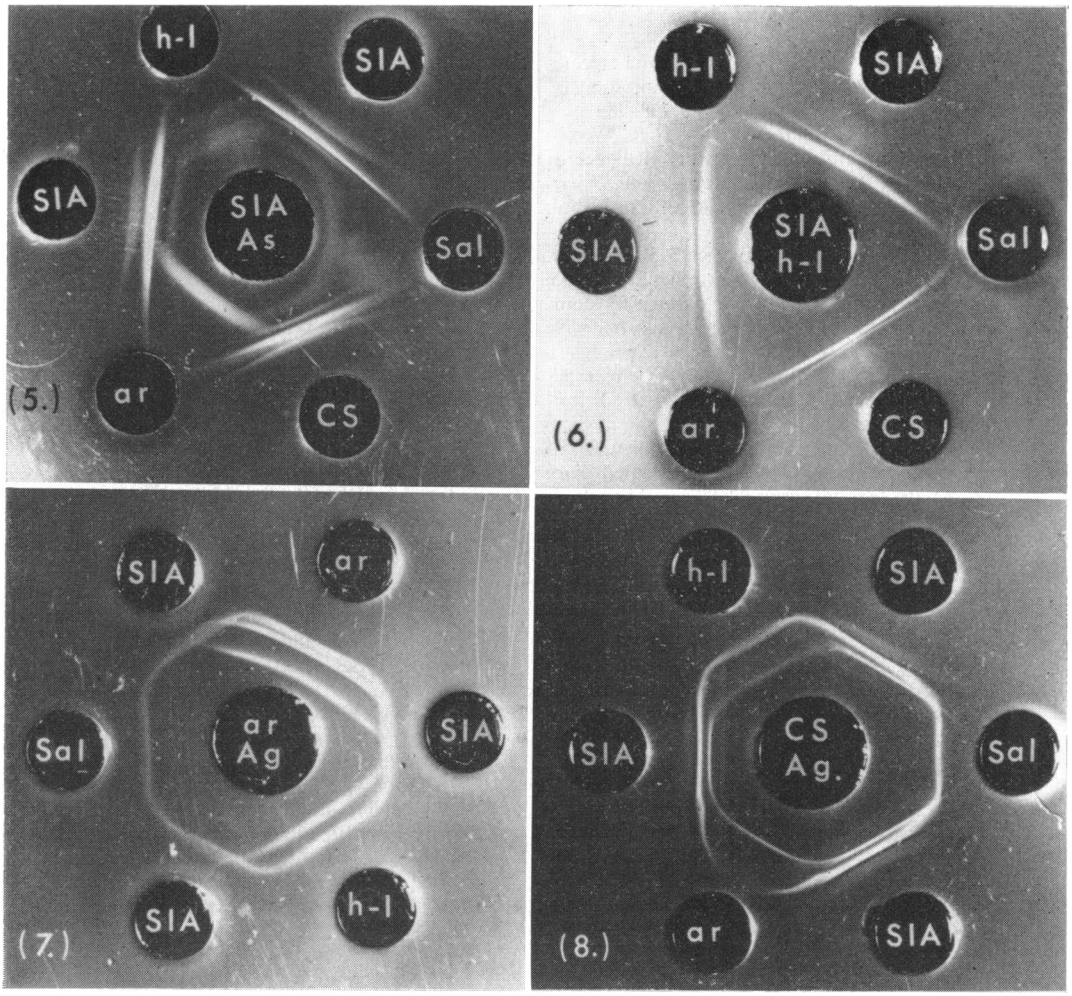

FIG. 5. *Gel diffusion plate illustrating antigens shared between cat strains S1A and CS, and Mycoplasma arthritidis (ar), M. hominis, type 1 (h-1), and M. salivarium (Sal). S1A antiserum is in the center well; antigens are in the outer wells*

FIG. 6. *Arrangement as for Fig. 5, except that S1A antiserum in the center well has been absorbed with Mycoplasma hominis, type 1 (h-1) antigen.*

FIG. 7. *Gel diffusion plate illustrating a common antigen to Mycoplasma arthritidis (ar), M. hominis, type 1 (h-1), M. salivarium (Sal), and cat strain S1A. M. arthritidis antigen is in the center well; antisera are in the outer wells.*

FIG. 8. *Gel diffusion plate illustrating common components between strains CS, S1A, Mycoplasma arthritidis (ar), M. hominis, type 1 (h-1), and M. salivarium (Sal). Antigen of strain CS is in the center well; antisera are in the outer wells.*

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