J. Hyg., Camb. (1967), **65**, 85
Printed in Great Britain
With 1 *plate*

Characterization of *Mycoplasma suipneumoniae*: a mycoplasma causing enzootic pneumonia of pigs

By R. F. W. GOODWIN, ANNETTE P. POMEROY
and P. WHITTLESTONE
School of Veterinary Medicine, University of Cambridge

(*Received* 3 *November* 1966)

Enzootic pneumonia of pigs is a common disease in Britain; it has also been reported from many other countries and it will probably become even more apparent as the trend to keep pigs in larger numbers in close indoor confinement continues. The essence of the problem in the field derives from the fact that the pneumonic lesions may persist throughout the main growing life of the pig, during which time the affected animal usually remains infective to other pigs. Although rarely fatal, the condition is often associated with a reduction in growth rate and the cumulative economic loss is so great, both nationally and internationally, that enzootic pneumonia has been described as the most important disease of pigs today.

From one strain of enzootic pneumonia (the J strain) studied in this laboratory, Goodwin & Whittlestone (1963) grew an agent in tissue culture which induced the typical disease. Later, this agent was grown in an acellular liquid medium, from which it likewise induced enzootic pneumonia experimentally (Goodwin & Whittlestone, 1964, 1966). By this time it was widely assumed that the causal agent of enzootic pneumonia was a *Mycoplasma*, but the problem of growing the agent on solid medium and reproducing the disease with the resulting colonies remained. Although mycoplasma colonies could occasionally be isolated on solid medium from our liquid-medium cultures at this stage, we could not passage these colonies on solid medium. Eventually, however, by using improved cultural methods, enzootic pneumonia was induced with mycoplasma colonies that, after several passages on solid medium, were at least a 10^{-15} dilution of the first seed inocula (Goodwin, Pomeroy & Whittlestone, 1965), the mycoplasma being named *Mycoplasma suipneumoniae*. In the present paper, the pathogenic role of this mycoplasma forms one part of a fuller description of the organism.

MATERIALS AND METHODS

Strains of mycoplasma

M. suipneumoniae was derived from the J strain of enzootic pneumonia, which originated from a field outbreak of the disease. This strain has been shown to be consistently infective during serial transmissions in pigs over a period of several years and it regularly contained pleomorphic organisms. One of these transmitted pneumonias yielded an organism in tissue cultures (Goodwin & Whittlestone,

1963), from which the disease was induced in pig 2206 (Goodwin & Whittlestone, 1965). Pneumonic material from pig 2206 was inoculated into plasma-clot cultures prepared from the normal lung of a hysterectomy-produced colostrum-deprived pig, and the fluids from these cultures, harvested after 3 days, were used to initiate the passages on solid medium.

Mycoplasma strain 603 was isolated from a field case of enzootic pneumonia but has not itself induced the disease experimentally (Goodwin & Whittlestone, unpublished).

The sources of the various other mycoplasmas used in this work are shown in the appropriate parts of the text.

Cultural media

Our original solid medium, which had been used for producing the pig inocula, contained lung broth. Subsequently, the lung broth was replaced by Hartley's broth (made with ox heart). In limited comparative experiments, the Hartley's-broth medium produced better growth of *M. suipneumoniae*; furthermore, because this gave a clearer medium, it was used for nearly all the growth-inhibition tests. To make about 100 ml. of medium, 1 g. of Oxoid Ionagar no. 2 was added to 40 ml. of balanced salt solution (Hanks's with 0·001 % phenol red) at 56° C. and autoclaved at 1·2 atm. (gauge pressure) for 15 min. The following sterilized constituents were then warmed to 56° C. and mixed together before adding to the Hanks's solution plus agar at 56° C.: either lung broth or Hartley's broth (30 ml.), serum from enzootic-pneumonia-free pigs (20 ml.) and 5 % lactalbumin hydrolysate (10 ml.). The final medium also contained penicillin (200 units/ml.), thallium acetate (1/8000) and yeast extract (0·5 %). The yeast extract was prepared as described by Herderscheê (1963). The lung broth was made by mincing lung from hysterectomy-produced colostrum-deprived pigs and leaving this overnight at 4° C. in twice the weight of water; this suspension was then filtered through double gauze, simmered for 15 min., filtered again through gauze, and centrifuged (900 *g* for 20 min.), the supernatant fluid being autoclaved thereafter (0·5 atm., gauge pressure, for 30 min.) and tested for sterility. With *M. hominis* type I, *M. orale* type II and *M. pulmonis*, which did not grow readily on this medium, the medium used was that of Taylor-Robinson, Somerson, Turner & Chanock (1963).

The main liquid medium used was the same as the solid medium containing Hartley's broth, but without the agar. For the pig infectivity work, however, the liquid medium contained lung broth instead of Hartley's broth. The use of an earlier liquid medium (Goodwin & Whittlestone, 1964, 1966) is referred to in the text.

Samples for routine bacteriology were plated on horse-blood agar, chocolate agar and Edward's medium (with 1/8000 thallium acetate); incubation was aerobic at 37° C.

Routine incubation of the mycoplasma cultures was as follows: the liquid-medium tubes were rolled at 37° C.; the solid-medium plates were incubated in a moist atmosphere containing CO_2 (5–10 % in air).

Antisera

The sera used in the immuno-fluorescent work and for precipitation in agar gel (rabbit sera R 3 and R 4, both against *M. suipneumoniae*, and R 14, against mycoplasma 603; pig sera P 2268 and P 2269, both against *M. suipneumoniae*) were made before *M. suipneumoniae* could be grown on solid medium. The mycoplasmas were therefore grown either in pig-lung-monolayer cultures (Goodwin & Whittlestone, 1963) or cell-free fluids (Goodwin & Whittlestone, 1964). The sera used in the growth-inhibition and metabolic-inhibition tests (rabbit sera R 2 and R 7, both against *M. suipneumoniae*, R 43 against *M. hyopneumoniae* of Maré & Switzer (1965) and R 1 against mycoplasma 603) were made using mycoplasmas grown in the liquid medium containing Hartley's broth: the antigens for rabbits R 7, R 43 and R 1 were grown in liquid medium after at least four serial single-colony passages on solid medium. In all cases the antigen was centrifuged, washed several times in phosphate-buffered saline (PBS) and resuspended in PBS.

The rabbits, after bleeding, received one subcutaneous injection of antigen with Freund's complete adjuvant, followed by intravenous injections, except that the first inoculation of rabbit R 2 was intradermal, and rabbits R 7 and R 43 received an intramuscular injection (with incomplete Freund's adjuvant) at the same time as their final intravenous inoculations. The pigs received live cultures of *M. suipneumoniae* in tissue-culture fluids intranasally, followed by subcutaneous injections of antigen with Freund's complete adjuvant. For precipitation in agar gel, the rabbit sera were absorbed with normal pig serum; for immunofluorescence they were absorbed with pig lung-liver powder (Nairn, 1962). The globulins in the pig sera were precipitated with sodium sulphate (Marrack, Hoch & Johns, 1951) and reconstituted in one fifth of the original volume.

The antiserum to rabbit gamma-globulin was prepared in a goat by injecting alum-precipitated globulin intramuscularly; the globulins were concentrated as above.

The sera supplied by other laboratories are identified in the text. All sera were stored at about $-20°$ C.

Serological techniques

Growth inhibition

The method of Clyde (1964) was used, except that the plates were inoculated either directly with colonies from solid medium or with a suspension of such colonies and, secondly, the disks were soaked in 0·01–0·025 ml. of antiserum, allowed to dry (Stanbridge & Hayflick, in preparation), and stored at $-20°$ C. until required.

Metabolic inhibition

The method of Taylor-Robinson, Purcell, Wong & Chanock (1966) was used.

Precipitation in agar gel

The double-diffusion method of Ouchterlony (1964) was used.

Immuno-fluorescence

Control and infected cover-slips were rinsed in PBS and fixed in dry acetone for 5 min.; they were then stained immediately or stored at −20° C., or lower temperatures, until required. Acetone-fixed smears of concentrated antigen were also used.

Conjugation with fluorescein isothiocyanate (Gurr) was according to Marshall, Eveland & Smith (1958), the conjugate being purified by filtration on a G 25 coarse-grade Sephadex column. The fluorescent filtrate was concentrated by dialysis against Carbowax (Kohn, 1959) and then absorbed with reconstituted pig lung-liver powder by shaking at room temperature for 1 hr. (100 mg./ml. of serum); the powder was then removed by centrifugation (30,000*g*). Normal goat serum was conjugated to lissamine rhodamine B and added to the anti-rabbit-globulin conjugate (ratio 1:5) as a counterstain. Only pig serum P 2268 was conjugated for use in the direct method.

Staining (37° C. for 30 min.) was as described by Nairn (1962); in the indirect method the initial incubation with rabbit serum was at 37° C. for 1 hr. Preparations were examined with Leitz Ortholux equipment; the UV light source was HBO 200 W, the screening filter was UG 1 (1·5 mm.), and a dark-field condenser was used. Assessment of the number of organisms, and their colour and brightness compared with the background, was made without the observer knowing the identity of the specimens.

Pig inoculations

All the pigs were hysterectomy-produced, colostrum-deprived animals. They were reared and infected under conditions of strict isolation in a specially designed building: each cubicle is approached through an ante-room in which protective clothing is put on, and before each experiment all the equipment in the cubicles is first steamed and then fumigated with formalin within the cubicle itself. The pigs that were infected with cultures and the pigs used in the first pig-passages were 3–5 weeks old; the pigs used in the second pig-passage were 24 weeks old; and the pigs used to produce sera P 2268 and P 2269 were 7 weeks old. All the attempts to produce enzootic pneumonia were by intranasal inoculation; the dose of lung tissue was 6–8 ml. of a 10^{-1} suspension in broth.

Enzootic pneumonia was diagnosed by the macroscopic appearance of the lesions, the histological picture, and the presence of pleomorphic organisms in touch preparations (Whittlestone, 1967).

RESULTS

General characteristics of Mycoplasma suipneumoniae

On the solid media incorporating lung-broth or Hartley's broth, minute colonies (*ca.* 20–100 μ diam.) were first seen at 3–5 days; these enlarged to a maximum diameter of 400 μ at 7–10 days, when they appeared as convex mycoplasma-type colonies, devoid of a central nipple (Plate 1, fig. 1). There was considerable variability in colony size. No growth into the medium was observed. Colonies

could not be passaged on Edward's medium; nor could they be grown on our standard medium anaerobically. In liquid medium, growth was associated with the production of acid and a faint opalescence was occasionally seen in the best cultures. In such cultures the mycoplasma appeared to be non-motile. On some occasions, and particularly when liquid medium was seeded with colonies from solid medium, the organisms did not adhere to the cover-slips, so that the growth assessments were made initially from the pH change, coupled with the examination of stained, dried drops of the liquid cultures. The mycoplasma appeared to grow as well when the Hanks's solution contained no glucose.

Whatever the medium (solid media, liquid media, tissue cultures, or the living pig), the individual elements of the mycoplasma were very pleomorphic, but in each of these culture systems the organism tended to grow in a manner characteristic for the system concerned. Thus, in touch preparations from lung, ring and bipolar forms (0·5–0·8 μ diam.) predominated and the organism occurred singly or in groups. In tissue cultures (Goodwin & Whittlestone, 1963) the organism was usually in diffuse groups, mainly as cocci (*ca.* 0·5 μ diam. and commonly in short chains) or rings (up to 3 μ diam.) containing a single coccus-like structure. In liquid media (Goodwin & Whittlestone, 1966), the main forms were cocci strung on fine branching filaments (0·1 μ diam.), or globular structures, usually in colonies; under the best conditions, as in the medium containing Hartley's broth, growth was more confluent and the globular structures were both larger (up to 16 μ) and more plentiful (Plate 1, fig. 2).

The mycoplasma grew well in liquid media and on solid media in the presence of thallium acetate (1/8000) and penicillin (200 units/ml.), whereas it was inhibited on solid medium by tetracycline (10 μg. disk), in a zone just over 3 cm. wide.

Serological tests

Growth inhibition

Rabbit sera R 2 and R 7, prepared against *M. suipneumoniae*, both gave good results in this test against the homologous culture, as also did the serum prepared by us against *M. hyopneumoniae* (Maré & Switzer); the zones of inhibition varied from 5 to 11 mm., measured from the edge of the disk to the beginning of colony growth. The sera prepared against *M. suipneumoniae* inhibited *M. hyopneumoniae*, and the serum prepared against the latter organism inhibited *M. suipneumoniae* to about the same extent.

The mycoplasmas listed in Table 1 were all inhibited by their respective homologous antisera but not by antiserum R 2 or R 7 prepared against *M. suipneumoniae*; also, in each case, the respective homologous antiserum to the mycoplasma named failed to inhibit a cloned culture of *M. suipneumoniae*. The sources of the cultures and antisera are shown.

The mycoplasmas listed in Table 2 were not inhibited by antiserum prepared against *M. suipneumoniae*. Although cultures of *M. suipneumoniae* were not inhibited by antisera prepared against these other mycoplasmas, no inhibition was obtained when these same antisera were used against their homologous cultures. The sources of the cultures are shown.

The mycoplasmas listed in Table 3 were not inhibited by antiserum prepared against *M. suipneumoniae*. No homologous antisera were available. The sources of the cultures are shown.

Table 1. *Mycoplasmas differing from* Mycoplasma suipneumoniae: *homologous system working*

Name of organism	Source and identity of culture	Source and identity of antiserum
M. hyorhinis F*	Dinter, Danielsson & Bakos (1965)	Dinter, Danielsson & Bakos (1965)
M. granularum B 2	Dinter, Danielsson & Bakos (1965)	Dinter, Danielsson & Bakos (1965)
B 3	Dinter, Danielsson & Bakos (1965)	Dinter, Danielsson & Bakos (1965)
B 4	Dinter, Danielsson & Bakos (1965)	Dinter, Danielsson & Bakos (1965)
B 6	Dinter, Danielsson & Bakos (1965)	Dinter, Danielsson & Bakos (1965)
M. pneumoniae	National Institutes of Health (Eaton FH)	Hayflick (Eaton FH)
M. salivarium	Taylor-Robinson (PG 20)	Hayflick (H 145)
M. orale I	Taylor-Robinson (CH 19299)	National Institutes of Health (CH 19299)
M. orale II*	Taylor-Robinson (CH 20247)	Taylor-Robinson (CH 20247)
M. fermentans	Taylor-Robinson (PG 18)	Edward & Leach (PG 18)
M. hominis I*	Taylor-Robinson (PG 21)	National Institutes of Health (PG 21)
M. pulmonis	Edward & Leach (PG 34)	Hayflick (Negroni)
*M. arthritidis**	Edward & Leach (Campo, PG 27)	Edward & Leach (PG 6)
M. mycoides var. *mycoides*	Edward & Leach (PG 1)	Edward & Leach (PG 1)
M. laidlawii A	Edward & Leach (PG 8)	Hayflick (41)
M. laidlawii B	Edward & Leach (PG 9)	Hayflick (41)
*M. agalactiae**	Edward & Leach (PG 2)	Edward & Leach (PG 2)
M. bovirhinis	Edward & Leach (Bovine group 4, PG 43)	Edward & Leach (PG 43)
Bovine group 7	Edward & Leach (PG 50)	Edward & Leach (PG 50)
Bovine group 8	Edward & Leach (PG 51)	Edward & Leach (PG 51)
M. canis	Edward & Leach (PG 14)	Edward & Leach (PG 14)
M. gallisepticum	Edward & Leach (X 95, PG 31)	Chu (A 5969)
M. gallinarum	Edward & Leach (PG 16)	Chu
Iowa 695	Roberts (2)	Chu (Iowa 695)

* Weak result; that is, not a complete absence of colonies in the zone of inhibition.

Table 2. *Mycoplasmas differing from* Mycoplasma suipneumoniae: *homologous system not working*

Name of organism	Source and identity of culture
M. hyorhinis S 7	Dinter, Danielsson & Bakos (1965)
GDL	Edward & Leach (PG 44)
M. bovigenitalium	Edward & Leach (B 2, PG 11)
M. neurolyticum	Edward & Leach (Sabin A, PG 39)
M. spumans	Edward & Leach (PG 13)
M. 'agalactiae var. *bovis'*	Edward & Leach (Bovine group 5, PG 45)
Bovine group 6	Edward & Leach (PG 49)
M. mycoides var. *capri*	Edward & Leach (PG 3)

Table 3. *Mycoplasmas differing from* Mycoplasma suipneumoniae: *no homologous serum available*

Name of organism	Source and identity of culture
M. iners	Edward & Leach (PG 30)
M. maculosum	Edward & Leach (PG 15)
M. meleagridis	Roberts
M. anatis	Roberts (4)
WR 1	Roberts (3)
186	Roberts (6)
658	Roberts (7)
Avian 8	Roberts

Table 4. *Summary of reactions in agar gel and with immuno-fluorescence*

	Reaction in agar gel					Titre with immuno-fluorescence		
	Pig antisera		Rabbit antisera			Pig antiserum	Rabbit antisera	
Antigen	P 2268	P 2269	R 3	R 4	R 14	P 2268	R 3	R 14
M. suipneumoniae	+	+	+	+	−	1/160	1/320	−
Mycoplasma 603	−	−	−	−	+	NT	−	1/640

NT = not tested.

NOTE. Serum R 14 was prepared against mycoplasma 603; all the other sera were prepared against *M. suipneumoniae*.

Relationship of mycoplasma 603 *with other porcine mycoplasmas.* Mycoplasma 603 and *M. hyorhinis* S 7 were not inhibited by their respective homologous sera in the growth-inhibition test; mycoplasma 603 was also not inhibited by sera prepared against the following porcine strains: *M. suipneumoniae, M. granularum, M. hyorhinis* S 7, *M. hyorhinis* F, B 2, B 3, B 4 and B 6. The last seven strains were from Dinter, Danielsson & Bakos (1965). Nor did rabbit serum R 1 prepared against mycoplasma 603 inhibit any of these eight mycoplasmas, except for *M. hyorhinis* F. Thus, by this test, there seems to be some relationship between mycoplasma 603 and *M. hyorhinis* F.

Metabolic inhibition

M. suipneumoniae and *M. hyopneumoniae* were indistinguishable in the metabolic-inhibition test: both organisms were inhibited by rabbit serum R 2 (against *M. suipneumoniae*) and rabbit serum R 43 (against *M. hyopneumoniae*) to titres of 1/640–1/1280. Both the pre-inoculation rabbit sera did not inhibit the metabolism of either mycoplasma at the lowest dilution (1/40).

Mycoplasma 603 and *M. hyorhinis* (Edward & Leach) were indistinguishable by the same test, in that both organisms were inhibited by rabbit serum R 1 against mycoplasma 603 to a titre of 1/2560. No inhibition was obtained with the pre-inoculation serum at a dilution greater than 1/40.

Precipitation in agar gel

The main findings in the double-diffusion tests are summarized in Table 4.

Antisera R 3 and R 4 against *M. suipneumoniae* gave at least three precipitation lines against the homologous mycoplasma grown either in tissue cultures or in cell-free fluids. No lines were obtained with cells from control cultures centrifuged at 30,000 *g* for 60 min., or with mycoplasma 603.

Concentrated globulin fractions of the pig sera gave rise to two precipitation lines on reaction with *M. suipneumoniae* and these seemed to be continuous with one of the lines arising from the reactions between *M. suipneumoniae* and rabbit antiserum R 3. These concentrated fractions gave no lines with either centrifuged cells from control cultures or with mycoplasma 603.

Serum R 14, prepared against mycoplasma 603, formed a single line against mycoplasma 603; this line crossed those formed between *M. suipneumoniae* and its homologous antisera, showing that the two antigens were unrelated. No lines were obtained with centrifuged cells from control cultures.

Immuno-fluorescence

In general, it was easier to see the mycoplasmas in the fluorescent preparations than in Giesma-stained cover-slips. In the direct method, *M. suipneumoniae* grown in both living tissue cultures and cell-free fluids could be stained to a pig-serum titre of 1/160; no such staining was seen with control coverslips. In the indirect method, *M. suipneumoniae* grown in the same two systems could be stained to a rabbit-serum titre of 1/320. Negative results were obtained in parallel tests with pre-inoculation rabbit sera, with control cover-slips, with the conjugate alone, and with mycoplasma 603.

Mycoplasma 603 was stained to a rabbit-serum titre of 1/640. In parallel tests with pre-inoculation serum, conjugate alone, and *M. suipneumoniae*, no such staining occurred.

All these results are summarized in Table 4.

Production of enzootic pneumonia in pigs

Two main attempts were made to induce enzootic pneumonia with colonies of *M. suipneumoniae*. The general plan was to passage the agent on solid medium and inoculate pigs with several doses of the final mycoplasma colonies washed from this medium. In case such colonies were less effective at inducing pneumonia than the same agent grown in liquid medium, the inoculum prepared from the solid medium was also inoculated into liquid medium and, after passage in this medium, further pig inocula were prepared which were given to other pigs in each of the two experiments. The details concerning the preparation of all these inocula have been given elsewhere (Goodwin *et al.* 1965).

In the first experiment, the mycoplasma was maintained on solid medium for 42 days and the dilution of the inoculum for the first solid-medium passage in the final material given to the pigs was at least 10^{-15}. None of the three pigs that received the mycoplasma directly from solid medium developed pneumonia. Two

of the three pigs that received the mycoplasma after subsequent passage in liquid medium, however, developed typical enzootic pneumonia. Two control pigs, which had been inoculated with the same batch of liquid medium as that which had been used to prepare the infected inocula, were killed at about the same time: they showed no sign of pneumonia. No significant bacteria were isolated from the lungs of any of the pigs in this experiment. Thus, the disease was produced only after passaging the mycoplasma from solid medium further in liquid medium, despite the fact that it had thereby been passaged outside the pig for a longer period.

In the second experiment, fewer passages were made on solid medium before gaining the required dilution of the primary solid-medium inoculum (at least 10^{-15}); because of this, the mycoplasma was maintained on the solid medium for only 35 days. A further difference in this experiment was that one of the pig inocula prepared directly from solid medium was made with very young colonies, washed off after only 3 days of incubation. Two pigs received the mycoplasma directly from solid medium and both developed typical lesions of enzootic pneumonia. One of these cases of pneumonia was passaged to two further pigs, and thence (in a second passage in pigs) to pig 2805, which also developed typical lesions (Plate 1, fig. 3). Two pigs received the mycoplasma after it had been passaged in liquid medium from solid medium; both developed enzootic pneumonia, but the lesions were more extensive than in the pigs inoculated directly from solid medium. These two cases of pneumonia were combined to make an inoculum for two further pigs, both of which developed enzootic pneumonia. Litter-mate controls were kept in all the pig passages; they received inocula prepared from the lung tissue of the appropriate previous control animals. The only control pig with lung lesions was the final one (pig 2807, which was the control for pig 2805), but these lesions were not typical of enzootic pneumonia histologically, and no pleomorphic organisms were found in the touch preparations. No significant bacteria were found in the lungs of any of the pigs in this experiment. Thus, although lesions were produced in the second main experiment by the inocula prepared directly from solid medium, once again the mycoplasma seemed more effective at inducing pneumonia after subsequent passage in liquid medium.

Recovery of Mycoplasma suipneumoniae

The pneumonia in pig 2805 was the second and final pig passage of a pneumonia induced directly with colonies from solid medium, and *M. suipneumoniae* was recovered from the lung lesions in this animal. The recovered agent was inhibited in the metabolic-inhibition test to a rabbit-serum titre of 1/320. *M. suipneumoniae* was not recovered from the control (pig 2807).

DISCUSSION

The general characteristics of the agent studied in this paper suggest that it is indeed a mycoplasma: the colonial form, the size of the elementary particles, the marked pleomorphism (with the development of filamentous forms), the requirement of a complex medium, and growth in the presence of penicillin and thallium

acetate, but not tetracycline, are all in keeping with this view. The difficulty of growing this particular mycoplasma, and the relatively slow growth of the colonies, indicate that with further work the cultural media might be improved.

The most important property of *M. suipneumoniae* is that it induces enzootic pneumonia in pigs. The mycoplasma was passaged on solid medium to a point where the final colonies which gave rise to the pig inocula were at least a 10^{-15} dilution of the first seeding onto solid medium; the induced pneumonia was then passaged serially in pigs—when it retained all the characteristics of enzootic pneumonia—and from the pneumonia in the second pig passage, a mycoplasma was recovered that was inhibited by serum prepared against *M. suipneumoniae*. It is not known, however, whether *M. suipneumoniae* is the sole cause of the disease known as enzootic pneumonia in the field. To investigate this question, we are now screening a variety of pneumonic samples obtained from outbreaks of enzootic pneumonia for strains of mycoplasma that differ serologically from *M. suipneumoniae*: it will then be necessary to see whether such strains, in pure culture, will induce a disease similar to enzootic pneumonia.

As judged by the growth-inhibition and metabolic-inhibition tests, *M. suipneumoniae* is indistinguishable from *M. hyopneumoniae* of Maré & Switzer (1965).

The comparisons with the other mycoplasmas suggest that *M. suipneumoniae* is probably a new species. We would have liked all these comparisons to have been individually complete, with all the homologous sera working well against their specific mycoplasmas. Some of the sera received from other laboratories, however, gave either a weak result or no result against their homologous culture. The simplest explanation for this is that these sera, which had often been prepared for use in other serological tests, were unsuitable for the growth-inhibition test. However, in the eight cases where the homologous serum did not inhibit growth, and in the eight cases where no homologous serum was available, none of the mycoplasmas supplied was inhibited by our sera against *M. suipneumoniae*. In all these tests, no qualitative difference was observed between the reactions obtained with the rabbit antiserum prepared against the cloned culture of *M. suipneumoniae* and the rabbit antiserum prepared against the uncloned culture. It should be pointed out that the mycoplasmas compared with *M. suipneumoniae* in the present study have not yet been compared among themselves in the literature to an extent that establishes that they are all separate species.

M. suipneumoniae was distinguishable from mycoplasma 603 in agar gel and by immuno-fluorescence, but these tests are inconvenient at the moment for routine diagnosis. It is simpler to use the growth-inhibition test for testing mycoplasma isolates from porcine respiratory disease but a problem may arise when other mycoplasmas are present concurrently; for these organisms commonly outgrow *M. suipneumoniae* in culture. The problem would be greatly simplified if a selective medium could be developed for *M. suipneumoniae*. This particular difficulty is not fully appreciated in several laboratories, where the isolation of mycoplasmas from pneumonic tissue in the pig, regardless of the pathogenicity of these organisms for pigs or their relationship to known pathogenic strains, is now being taken to indicate the presence of enzootic pneumonia.

The results obtained with the hyperimmune pig sera, using the immuno-fluorescent and double-diffusion techniques, are encouraging. We are now extending this work to include other tests, such as metabolic inhibition, working in the first instance with sera prepared against *M. suipneumoniae* in hysterectomy-produced colostrum-deprived pigs. The longer-term objective is to use sera from the field in an attempt to evaluate the role of *M. suipneumoniae* in the respiratory-disease complex of pigs.

SUMMARY

A micro-organism, previously known as the J agent, was grown on solid medium: its various characteristics suggested that it was a mycoplasma and it was provisionally named *Mycoplasma suipneumoniae.*

In the growth-inhibition and metabolic-inhibition tests, *M. suipneumoniae* was indistinguishable from *M. hyopneumoniae* (Maré & Switzer, 1965).

By the growth-inhibition test, *M. suipneumoniae* seemed unrelated to all of a wide range (42 strains) of other mycoplasmas examined. These results suggest that, if the similarity between *M. suipneumoniae* and *M. hyopneumoniae* is substantiated in further work, these latter two strains are probably a new species.

M. suipneumoniae was also identified by the metabolic-inhibition test, by precipitation in agar gel, and by immuno-fluorescence. Using the last two methods, *M. suipneumoniae* was distinguished from a second porcine mycoplasma (strain 603).

The most important property of *M. suipneumoniae* is its ability to induce enzootic pneumonia experimentally in pigs.

The immuno-fluorescent and double-diffusion studies were made during work with Mrs Carol Barton. Miss Janet Leech and Mrs Rosemary Trimmer provided the main technical assistance. We are grateful to Dr R. M. Chanock, Dr H. P. Chu, Prof. Z. Dinter, Dr D. G. ff. Edward, Dr L. Hayflick, Dr R. H. Leach, Dr R. H. Purcell, Mr D. H. Roberts and Dr D. Taylor-Robinson for kindly supplying us with cultures and antisera, and we are particularly indebted to three people for advice on the techniques used: Dr Eva Orlans (the double-diffusion technique), Dr D. Taylor-Robinson (the growth-inhibition and metabolic-inhibition tests) and Prof. B. P. Marmion (immuno-fluorescence). Most of the expenses of this work, including the salary of one of the authors (A.P.P.), were met by a grant from the Agricultural Research Council.

REFERENCES

Clyde, W. A. (1964). Mycoplasma species identification based upon growth inhibition by specific antisera. *J. Immun.* **92**, 958.

Dinter, Z., Danielsson, D. & Bakos, K. (1965). Differentiation of porcine mycoplasma strains. *J. gen. Microbiol.* **41**, 77.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1965). Production of enzootic pneumonia in pigs with a mycoplasma. *Vet. Rec.* **77**, 1247.

Goodwin, R. F. W. & Whittlestone, P. (1963). Production of enzootic pneumonia in pigs with an agent grown in tissue culture from the natural disease. *Br. J. exp. Path.* **44**, 291.

GOODWIN, R. F. W. & WHITTLESTONE, P. (1964). Production of enzootic pneumonia in pigs with a micro-organism grown in media free from living cells. *Vet. Rec.* **76**, 611.

GOODWIN, R. F. W. & WHITTLESTONE, P. (1965). Experiments on the possible relationship between inclusion-body rhinitis, enzootic pneumonia and type-XI pneumonia of pigs. *Res. vet. Sci.* **6**, 100.

GOODWIN, R. F. W. & WHITTLESTONE, P. (1966). Enzootic pneumonia of pigs: growth and behaviour of the causal mycoplasma in liquid media. *Br. J. exp. Path.* **47**, 518.

HERDERSCHEÊ, D. (1963). An improved medium for the cultivation of the Eaton agent. *Antonie van Leeuwenhoek* **29**, 154.

KOHN, J. (1959). A simple method for the concentration of fluids containing protein. *Nature, Lond.* **183**, 1055.

MARÉ, C. J. & SWITZER, W. P. (1965). New species: *Mycoplasma hy pneumoniae*—a causative agent of virus pig pneumonia. *Vet. Med.* **60**, 841.

MARRACK, J. R., HOCH, H. & JOHNS, R. G. S. (1951). The valency of antibodies. *Br. J. exp. Path.* **32**, 212.

MARSHALL, J. D., EVELAND, W. C. & SMITH, C. W. (1958). Superiority of fluorescein isothiocyanate (Riggs) for fluorescent-antibody technique with a modification of its application. *Proc. Soc. exp. Biol. Med.* **98**, 898.

NAIRN, R. C. (1962). Immunological tracing: general considerations. In *Fluorescent Protein Tracing*. Edinburgh and London: E. and S. Livingstone Ltd.

OUCHTERLONY, Ö. (1964). Gel-diffusion techniques. In *Immunological Methods*. Oxford: Blackwell Scientific publications.

TAYLOR-ROBINSON, D., SOMERSON, N. L., TURNER, H. C. & CHANOCK, R. M. (1963). Serological relationships among human mycoplasmas as shown by complement-fixation and gel diffusion. *J. Bact.* **85**, 1261.

TAYLOR-ROBINSON, D., PURCELL, R. H., WONG, D. C. & CHANOCK, R. M. (1966). A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. *J. Hyg., Camb.* **64**, 91.

WHITTLESTONE, P. (1967). Mycoplasmas in enzootic pneumonia of pigs. *Ann. N.Y. Acad. Sci.* (In the Press.)

EXPLANATION OF PLATE

Fig. 1. Eight-day-old growth of colonies of *Mycoplasma suipneumoniae*; these colonies were used to inoculate pigs that developed enzootic pneumonia. × 50.

Fig. 2. Culture of *Mycoplasma suipneumoniae* in liquid medium containing Hartley's broth, showing chains of cocci and globular structures of varying sizes up to 16 μ. May–Grünwald–Giesma, × 2000.

Fig. 3. Histology of pneumonia in pig 2805: final pig passage of a pneumonia induced with cultures of *Mycoplasma suipneumoniae*. Haematoxylin and eosin, × 160.

ADDENDUM

A cloned type-culture of *Mycoplasma suipneumoniae* has been deposited with Dr B. E. Andrews, Mycoplasma Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London N.W. 9.

Fig. 1

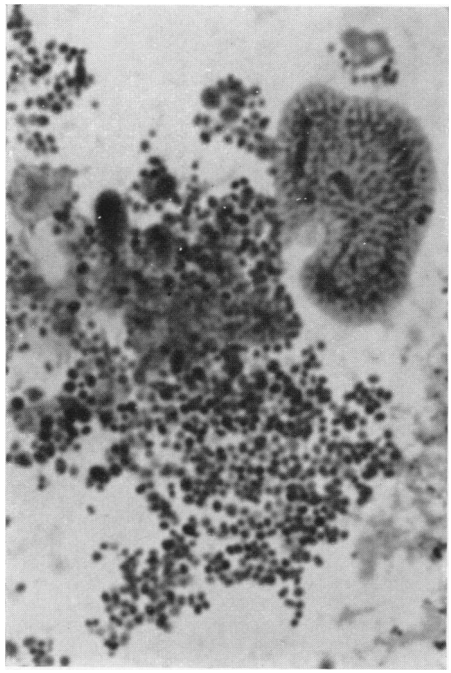

Fig. 2

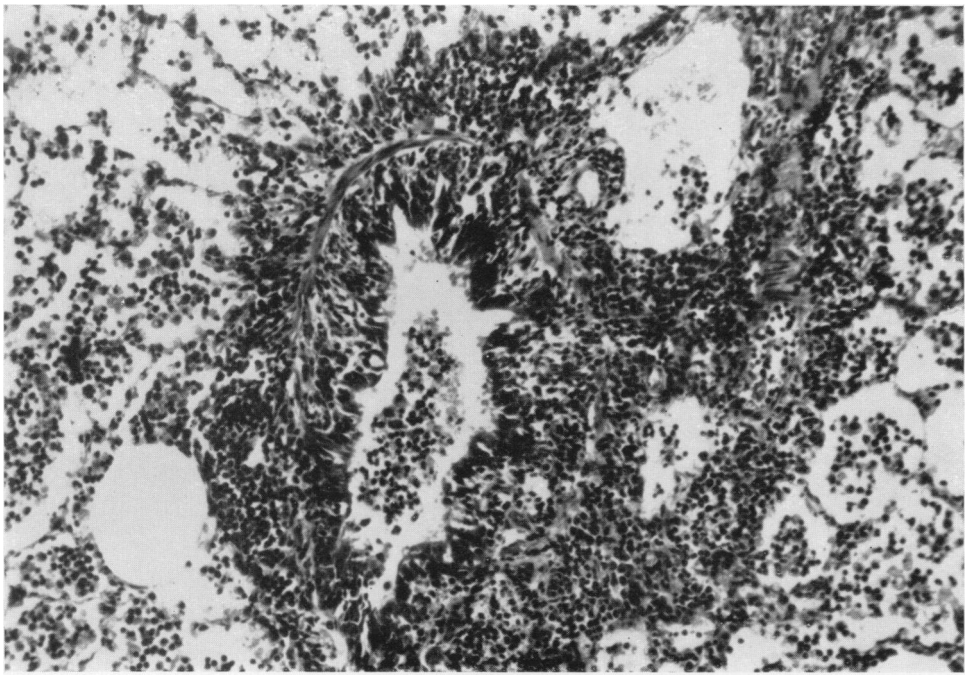

Fig. 3