Enzootic Pneumonia in Pigs: Propagation of a Causative Mycoplasma in Cell Cultures and in Artificial Medium

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SUMMARY

Three strains of a new species of mycoplasma were recovered from pneumonic pig lungs, known free of Mycoplasma hyorhinis, by prolonged incubation in pig testicle cell cultures. The three strains produced a characteristic cytopathic effect in the cell cultures. A highly enriched meat-infusion-broth medium was evolved and permitted regular propagation of these organisms. Pneumonia could consistently be produced by intratracheal inoculation of pigs with the mycoplasma propagated in the enriched broth medium or in cell cultures. The mycoplasma were recovered from the lungs of experimentally infected pigs by inoculation into the broth medium. Comparative studies of the pneumonia producing mycoplasma and of M. hyorhinis were carried out in cell cultures, broth media, and in pigs infected experimentally by different routes. The morphological characteristics of the mycoplasma, grown in the different media, are described and illustrated.

RÉSUMÉ

Après des périodes d'incubation prolongées nous avons isolé en cultures cellulaires de testicule de porc, trois souches d'une nouvelle espèce de mycoplasma. Ces souches furent isolées à partir de pneumonies porcines démontrées exemptes de contamination par Mycoplasma hyorhinis, et elles produisent en cultures cellulaires un effet cytopathogène caractéristique. La culture de ces trois souches a été possible en milieu de culture de cellules et en un bouillon complexe à base d'infusion de viande lequel nous a permis d'obtenir d'assez fortes concentrations d'organismes. La pneumonie a été produite de façon régulière chez des porcs sains par inoculation intratchéale de suspensions des trois souches cultivées en cultures cellulaires ou en bouillon à infusion. Les souches propagées en milieu de culture de cellules furent moins infectieuses pour les porcs peut-être à cause de la moins forte concentration des organismes. Le réisolement des mycoplasmas à partir des lésions pulmonaires produites a réussi en utilisant le bouillon à infusion. Des études portant sur les cultures cellulaires, les milieux de culture liquides, et les porcs inoculés par différentes voies ont été faites entre les mycoplasma de la pneumonie et M. hyorhinis. Les caractéristiques de la morphologie et de la croissance de ces mycoplasmas en divers milieux de culture sont décrites et illustrées.

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INTRODUCTION

Mycoplasma have been widely isolated from enzootic pneumonia (EP) of swine (Virus Pneumonia of Pigs) although their primary etiologic significance in this disease has not been clearly demonstrated in every instance.

The work published prior to 1962 has been reviewed (13, 14). Bontscheff (3) obtained a cytopathic effect in calf and pig kidney cell cultures inoculated with an organism isolated in embryonated chicken eggs and produced pneumonia in pigs inoculated with eighth cell culture passage material. L'Ecuyer and Switzer (14) cultured two isolates from pneumonic lungs in Hela and in pig kidney cells. Cytopathic effects were not observed but early passage cultures were infective for pigs. Subsequently Maré and Switzer (16, 17) recovered a mycoplasma in cell-free broth medium and used it to produce pneumonia experimentally. This mycoplasma could be recovered from the lesions, it formed colonies on special solid medium, it did not multiply in media used routinely for the other swine species, and the name *Mycoplasma hyopneumoniae* was suggested.

Betts and Whittlestone (2) using the

Cambridge "J" strain, detected a cytopathic effect in plasma clot cultures of lung and nasal mucosa from an infected pig. They could produce pneumonia with early passage cell culture material but not with twentieth passage materials. Goodwin and Whittlestone (8) inoculated a similar plasma clot isolate of this strain in pig lung monolayers and produced a cytopathic effect. They could detect pleomorphic organisms in stained preparations of these cell cultures. Seventh passage cell culture fluids, representing a 10^{-16} dilution of the original material, were used to produce pneumonia in four pigs. Subsequently, Goodwin *et al*, in a series of publications (9, 10, 11, 12) reported propagation of this pleomorphic organism in boiled pig lung cell cultures and in cell-free medium. Growth was promoted by enrichment with infusion broth and yeast extract. Pleomorphic organisms were seen consistently in these cultures and in touch preparations of experimentally produced pneumonias. Pneumonia was produced with relative consistency using the various liquid media and was serially transmissible in pigs. The organism was propagated on solid medium, the name *Mycoplasma suipneumoniae* was proposed although it could not be distinguished from *M. hyopneumoniae* by growth inhibition tests.

The relationship of the above discussed agents to the agents isolated from this disease first in Sweden remains to be determined. Organisms have been recovered by a number of European workers from cases of EP and isolations are most easily accomplished through yolk-sac inoculation of embryonated chicken eggs. Bakos and Dinter (1) could not produce typical disease in pigs inoculated with these isolates and they showed (4) that they were antigenically similar to the serositis producing species, *M. hyorhinis*. Workers in Rumania (21, 22) have recovered a pararickettsia from the lungs of pigs with pneumonia by yolk-sac inoculation of embryonated chicken eggs. The organism caused pneumonia when inoculated into pigs, it could be identified in stained preparations by light microscopy and it was lethal to mice following intranasal inoculation. Several serologic techniques indicated that the agent shared antigens with ornithosis virus. MacKay *et al* (18) produced enzootic pneumonia-like lesions in pigs experimentally inoculated with protoplasts of *Hemophilus parainfluenzae*.

MATERIALS AND METHODS

Source and Preparation of Inocula

Pneumonic swine lungs from field cases or collected at slaughter were examined for freedom from *M. hyorhinis*. Lungs found to be free of *M. hyorhinis* were tested for infectivity by intratracheal (IT) inoculation into susceptible minimal-disease piglets. Pneumonic lung suspensions used for inoculation were ground and diluted 1:10 to 1:20 in Dulbecco's (5) phosphate buffer (DPB). The suspensions were left standing for 30 minutes and the supernatant fluid portion was used. The pigs were examined at regular intervals for at least four weeks before euthanasia and necropsy.

Source of Pigs

The pigs used in these trials were from a minimal-disease swine herd maintained under strict quarantine and shown by repeated examinations to be free of respiratory diseases. This herd was established from caesarean-derived, colostrum-deprived pigs reared in isolation. The pigs used were from subsequent generations and were farrowed and reared naturally.

Inoculation of Pigs

All pigs were between three and eight weeks of age when inoculated. Intratracheal administration followed intravenous anesthesia with thiopental sodium. The anesthetized pigs were held in an upright position, the mouth was held open and the larynx exposed with a Guedel laryngoscope[1]. An open end tracheal catheter was then inserted between the vocal cords and into the trachea for a distance of two to three inches. Four to six ml of the test suspension was then inoculated through the tracheal catheter and the liquid was followed by an adequate volume of air to ensure complete emptying of the catheter tube. Pigs inoculated intraperitoneally (IP) were hung by the hind legs and inoculated through the abdominal wall between the midline and the last teat.

Assessment of Pneumonia

Pigs were killed with an overdose of pentobarbitol sodium by the intracardiac

[1]Ingram and Bell, Surgical Instruments, Montreal, Quebec.

route and were exsanguinated. At necropsy the lungs were examined closely for the presence of areas of plum-colored consolidation. Consolidated areas generally occurred in the anteroventral portions of the lungs and if the disease was advanced they were depressed in relation to adjacent unaffected areas. Selected areas were collected and fixed in ten per cent formol saline for histological study.

Bacteriological Methods

Isolation and propagation of *M. hyorhinis* were attempted in a modification of the beef-heart-infusion broth (BHI) described by Ross and Switzer (20) which contained 20.0 per cent minimal-disease pig serum and 0.01 per cent yeast extract. Growth was recognized by clouding and sediment formation in liquid medium, by microscopic examination of Giemsa stained sediments of the liquid medium and by the production of typical colonies on agar.

Liquid media. The basic components of the various media employed were Madin's modification (15) of Hank's balanced salt solution and PPLO Broth[2].

The following supplements were tested for growth stimulating properties when added to one or both of the above medium bases: minimal-disease pig serum either fresh or heated at 56°C for 30 minutes, yeast extract[2], Eagle's enrichments[3], lactalbumin hydrolysate[2], chick embryo extract[4], gastric mucin[5], hemoglobin[2], DPN[5], Tween 80[6], cholesterol[5].

The final composition of the liquid medium evolved during this study and hereafter referred to as "Broth A" is listed in Table I.

Liquid medium was clarified by passage under negative pressure through a Whatman No. 1 filter paper. The medium was then pre-filtered (Selas 015)[7] and sterilized (Selas 03) under positive pressure. All media were incubated for 48 hours at 37°C to check for sterility and were stored at 4°C until used. Transfers were made using a 1:5, or 1:10 dilution of inoculum from the previous passage.

Aeration was attempted in boiling flasks using a mixture of air and five per cent CO_2 according to the method of Newing (19). A second method involved the use of one liter Erlenmeyer flasks equipped with air filters and containing 500 ml of broth. The flasks were mounted on a wrist shaker over a water bath, set at 36°C, so that the broth in the flask was submerged. They were shaken so as not to cause excess frothing of the medium.

The effect of initial pH and of buffering on the broth medium was determined by preparing duplicate flasks at pH 7.8, 7.2 and 6.8. One flask at each pH was buffered with Sorensen's phosphate buffer 0.01 molar. The inoculated flasks were incubated on a shaker for four days.

TABLE I Composition of the Broth Medium (Broth A) Used for the Propagation of the Pneumonia Producing Porcine Mycoplasma.

Product	Concentration
PPLO-Broth	2.1% W/V
Lactalbumin hydrolysate	0.5% W/V
Gastric mucin	0.5% W/V
Yeast extract	0.01% W/V
Thallium acetate[1]	0.025% W/V
Penicillin[1]	2000 IU/ml
Pig serum[2]	20%

[1]Inhibitors were employed only for initial isolation and were omitted for regular transfer of strains.
[2]Heat inactivated at 56° C for 30 minutes.

Measurement of Growth

Growth was estimated by sedimentation of the suspended organisms at 5,400 G for 30 minutes in an angle-head centrifuge. The sediment was washed and recentrifuged once, it was then resuspended in an appropriate amount of saline and centrifuged at 2,200 G for one hour in a horizontal centrifuge using either Hopkins vaccine or Kolmer graduated tubes.

Staining Procedures

Coverslips immersed in the culture medium during incubation or smears of sediments obtained by centrifugation were stained for one hour in 1:50 Giemsa after fixation in methyl alcohol for five minutes. All preparations were examined by bright field microscopy using oil immersion.

Cell Cultures

The swine testicle cell cultures were

[2]Difco Laboratories, Detroit, Michigan.
[3]Microbiological Associates, Bethesda, Maryland.
[4]Baltimore Biological Laboratories, Baltimore, Maryland.
[5]Nutritional Biochemical Corp., Cleveland, Ohio.
[6]Atlas Powder Co.
[7]Selas Filter Corporation, Philadelphia, Pennsylvania.

prepared as described by L'Ecuyer and Switzer (14) with ten per cent minimal-disease pig serum substituted for calf serum. In prolonged incubation trials maintenance medium was changed as the pH of the supernatant and the condition of the cell sheet warranted. Cell culture coverslips were stained with May-Grunwald Giemsa stain according to the method of Eaton *et al* (7).

Two series of primary pig testicle cell cultures were inoculated with a 1:50 dilution of pneumonic lung suspensions from experimentally infected pigs. One series was passaged at five day intervals and the other at 14 day intervals.

Chicken Embryo Inoculation

The method of Eaton *et al* (6) was used to inoculate chicken-embryos aged 13 days in the amniotic sac. The inoculum was an 0.65 micron filtrate[8] of lung suspension from infected pigs. Lung, trachea and spleen from surviving embryos was pooled and re-inoculated into fresh embryos by the same route. A suspension of tissues harvested from fourth passage embryos was inoculated into two pigs and two pigs received a suspension from control embryos.

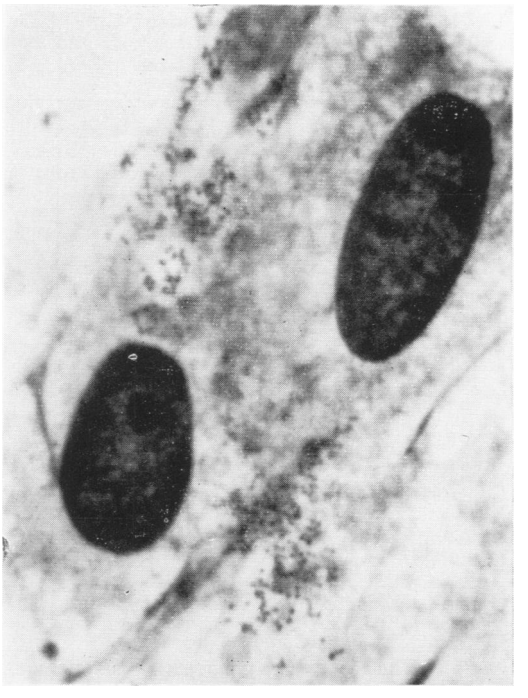

Fig. 1. Primary pig testicle cell culture infected with a pneumonic lung suspension and showing numerous mycoplasma bodies apparently located on the cell membrane. May-Grunwald and Giemsa stain. X 1750.

RESULTS

Lung specimens (EP strains) 28, 29, 31, and 33 were apparently free, on the basis of routine broth culture, of *M. hyorhinis* and produced typical lung consolidation when inoculated IT into pigs.

Recovery of Mycoplasma in Cell Cultures

Four mycoplasma isolates were recovered, one each from the four infective lungs (28, 29, 31, 33). On the first passage isolate 31 produced typical mycoplasma cytopathic effect (CPE), Switzer and L'Ecuyer (23), at three days post-inoculation (PI) in both series. This effect was maintained and appeared in two to three days PI in all subsequent passages. Stained coverslips from these passages contained numerous coccoid bodies apparently on the surface and in the cytoplasm of the cells. This cell culture isolate produced typical, granular, colonies with raised centers on BHI agar and was classified as a strain of *M. hyorhinis*.

[8]Millipore Filter Corporation.

The first cell culture passage of lungs 29 and 33 produced typical mycoplasma CPE on day ten PI. The CPE was evident in six to ten days PI during the second passage and occurred thereafter in two to three days PI. Coverslips prepared from the first passage contained a few coccoid mycoplasma-like bodies three days PI in strain 29 and six days PI in strain 33. These bodies were very numerous at the time of appearance of CPE on day ten PI, Figure 1. Morphologically these bodies were indistinguishable from *M. hyorhinis* strain 31. Repeated attempts to propagate these organisms in BHI broth or agar using three blind passages at each attempt, gave negative results. On the initial attempt lung 28 failed to produce detectable CPE through five passages at 14 day intervals. On a subsequent attempt this strain showed a few mycoplasma-like bodies at the end of the second 14 day passage and showed typical CPE on day three of the third passage. Stained preparations from this third passage in pig testicle cells contained numerous mycoplasma-like bodies resembling those seen with isolates 29 and 33.

TABLE II Results of the Inoculation of Pigs Using Pig Testicle Cell Cultures Infected with Suspensions of Pneumonic Lung.

Isolate	Cell Culture Transfer	Cytopathic Effect	Route of Inoculation[1]	Pneumonia
29	3	+	IT	2/2[2]
29	3	+	IP	0/1
29[3]	NA[4]	NA	IT	2/2
28	3	−	IT	0/2
28	3	−	IP	0/1
31	3	+	IT	0/2
31	3	+	IP	0/1[5]
33	4	+	IT	0/2
33	4	+	IP	0/1
28	8	+	IT	2/2
28	8	+	IP	0/1
29	10	+	IT	1/2
33	10	+	IT	2/2
Control[6]	NA	−	IT	0/2

[1]IP — intraperitoneally; IT — intratracheally.
[2]Number of pigs with pneumonia over number inoculated.
[3]Inoculum was lung suspension from the pigs inoculated IT and shown in first line.
[4]NA — not applicable.
[5]This pig had polyserositis but no pneumonia at necropsy.
[6]Uninoculated pig testicle cell culture.

The cell cultures inoculated with strains 28, 29 and 33, and subjected to five day passage intervals failed to show bodies in stained preparations or to develop CPE through three blind passages.

The results of the inoculation of pigs with these cell cultures are summarized in Table II. Pneumonia was produced in seven of ten pigs inoculated IT with cell cultures of isolates 28, 29 and 33, which had undergone CPE and contained mycoplasma bodies. Such cell cultures produced no lesions when inoculated IP. *M. hyorhinis* strain 31 did not produce lesions in pigs when inoculated IT but it produced polyserositis following IP inoculation. Cell cultures which did not develop CPE following inoculation with lung 28 and control cell cultures failed to induce pneumonia in pigs.

Broth Culture

The mycoplasma-like organisms present in pig testicle cell cultures exhibiting CPE could be propagated in cell culture medium (CCM) free of cells. A color change was produced within 48 hours of incubation and was due to glucose fermentation and also to reduction of the phenol-red indicator. Initially both CCM and BHI broth were used. After six passages in CCM, growth could be demonstrated by the color change and by the presence of coccoid organisms on submerged coverslips from isolates 29 and 33. No growth was evident from lungs 28 or 31. Only *M. hyorhinis* strain 31 grew in BHI broth as evidenced by opacity in the medium and a typical sediment. All control cultures remained negative. After six transfers in the liquid medium the isolates were inoculated onto BHI agar and CCM containing one per cent agar. Strain 31 grew on both types of agar medium whereas isolates 29 and 33 did not produce any recognizable colonies.

TABLE III Results of Inoculation of Pigs with Mycoplasma Propagated in Cell Culture Medium. (CCM)

Inoculum Strain No.	Inoculum Passage No.	Route of Inoculation[1]	Pneumonia
29	5	IT	2/2[2]
29	13	IT	1/2
29	13	IP	0/1
29	15	IT + IP	1/2
33	5	IT	2/2
33	14	IT	0/1
33	14	IP	0/1
33	15	IT + IP	1/2
28	15	IT + IP	0/2
Control Medium		IT	0/1

[1]IT — intratracheal; IP — intraperitoneal.
[2]Number positive over number inoculated.

The concentration of viable organisms of isolates 29 and 33 in CCM appeared to be very low and dropped off very rapidly with increasing acidity. This made it essential to transfer cultures at 48 hour intervals.

Subsequent studies on isolates 28, 29 and 33 in CCM indicated that glucose was not essential to propagation. The addition of lactalbumin hydrolysate, yeast extract, swine gastric mucin and the use of pig serum heated at 56°C for 30 minutes, markedly stimulated growth and allowed sustained propagation. It was also found that the balanced salt solution could effectively be replaced by PPLO Broth[9] with a resulting increase in growth. The following products when added to broth medium were found to have no stimulatory effect on growth or in some cases to be slightly inhibitory; Eagle's enrichments, chick embryo extract, hemoglobin, diphosphopyridine nucleotide, Tween 80, and cholesterol.

The two methods of aeration employed

[9]Difco Laboratories, Detroit, Michigan.

TABLE IV Results of Inoculation of Pigs with Mycoplasma Propagated in Broth Media.

Medium[1]	Inoculum Isolate No.	Inoculum Pass No.	Route of Inoculation[2]	Pneumonia	Reisolation
Broth A	29	30	IT	2/2[3]	2/2[3]
Broth A	29	80	IT	2/3	ND[4]
Broth A	28	26	IT	2/3	2/2
Broth A	28	3	IT	3/3	ND
ND	28	ND	CT	1/2	1/2
Broth A	28	37	IT + IP	2/2	2/2
BHI	31	14	IT	0/2	....
BHI	31	14	IP	0/1[5]	....
BHI	31	5	IT + IP	0/2[5]	1/2[6]
BHI	Control medium		IT	0/1	....
Broth A	33	26	IT	3/3	2/3
Broth A	Control medium		IT + IP	0/2	....

[1]Broth A — see Table I; BHI — beef heart infusion broth.
[2]IT — intratracheal; IP — intraperitoneal; CT — contact infection.
[3]Number positive over number inoculated or cultured.
[4]ND — not done.
[5]The lungs of these pigs were normal but they had lesions of polyserositis at necropsy.
[6]M. hyorhinis was recovered from the joint of one pig.

had a stimulatory effect on growth and increased the yield of packed cells. The method of Newing (19) resulted in more than a two-fold increase in yield of packed cells but the degree of aeration possible was limited by the amount of foam produced by the gas bubbling through the medium. The use of shaker cultures resulted in more than a twenty-fold increase in yield of packed cells in comparison to stationary cultures.

The optimum pH for growth based on yield of packed cells was pH 7.2 without buffer. The final pH of this medium was 6.6 after four days of incubation. The buffer, particularly at the higher pH appeared to have an inhibiting effect on the organisms.

Exerimental infections Table III, summarizes the results of the inoculation of pigs with mycoplasma strains propagated in CCM. Pneumonia was produced only irregularly with mycoplasma strains 28, 29 and 33, propagated in this medium. Only seven of 13 pigs inoculated IT developed pneumonia and none of the pigs inoculated IP developed pneumonia or lesions of any of the other organs. One pig which received control medium IT was negative.

Table IV, summarizes the results of the inoculation of pigs with mycoplasma isolates 28, 29 and 33 propagated in Broth A and of *M. hyorhinis* strain 31 propagated BHI broth. A clear-cut distinction, based on the results of pig inoculation, can be made between mycoplasma isolates 28, 29 and 33 on the one hand and strain 31 on the other. Pneumonia was produced with isolates 28, 29 or 33 in 12 out of 12 pigs inoculated IT. The highest passage used to inoculate pigs was the eightieth passage. None of four pigs inoculated IT with strain 31 developed pneumonia. In addition, none of the pigs inoculated IT or IP with strains 28, 29 or 33 developed lesions other than the typical lung consolidation. The three pigs inoculated IP with *M. hyorhinis* strain 31 had chronic lesions of polyserositis at necropsy four weeks PI and the organism was recovered from the arthritic joint of one pig. The pigs who received uninoculated BHl broth or Broth A had no lesions at necropsy. Histopathologically the lung lesions produced by infection with mycoplasma isolates were similar to those obtained following the inoculation of suspensions of pneumonic lung.

Mycoplasma indistinguishable from those used to inoculate the pigs were recovered in most cases where it was attempted by inoculation of pneumonic lung suspensions directly into Broth A. Three to four transfers were necessary before growth was recognizable, however, typical organisms were usually seen in stained smears of the second passage.

Chicken Embryo Inoculation

Strains 28, 29 and 33 were inoculated into chicken embryos. No definite pattern of mortality could be detected during four

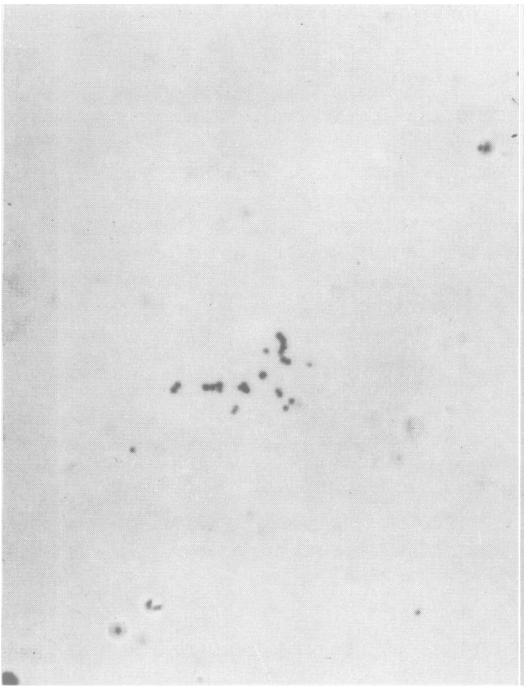

Fig. 2. Microbiology of coccoid pneumonia producing mycoplasma on a submerged coverslip from an early passage in CCM. Giemsa stain. X 1750.

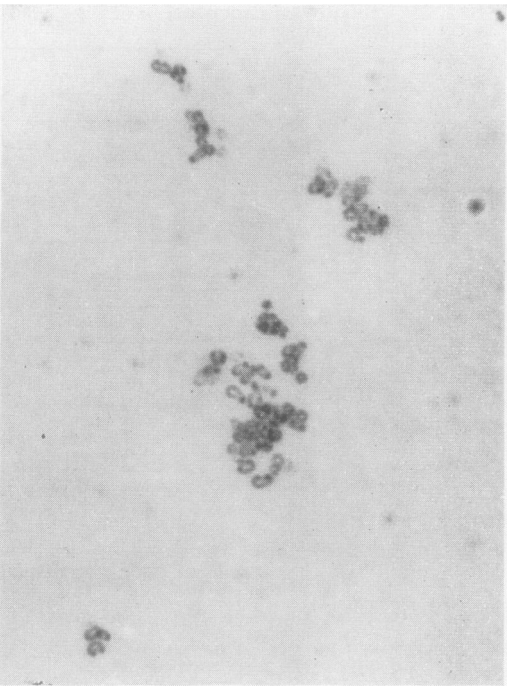

Fig. 3. Several microcolonies of ring-form organisms on a submerged coverslip of a later passage of a strain of pneumonia producing mycoplasma in CCM. Giemsa stain. X 1750.

blind passages and the only change seen was irregular stunting of some of the embryos. None of the pigs inoculated with chick embryo material had lung or other lesions at necropsy.

Growth and Morphological Characteristics

Growth of isolates 28, 29 and 33 in CCM was recognized macroscopically on the basis of the color change produced; no cloudiness or sediment was formed during the multiplication of these organisms. On initial propagation in this medium microcolonies of coccoid organisms measuring approximately 0.5 microns were seen on Giemsa stained coverslips, Figure 2. Within two to three passages the microcolonies became more abundant and were made up of pleomorphic, round to oval, ring-form organisms which frequently showed mono- or bi-polar dense areas. The smallest bodies measured 0.85 to 1.05 microns in diameter, but some were double that size, Figure 3. Smears prepared from centrifuged cultures contained similar ring-form organisms, Figure 4.

Propagation of these isolates in Broth A resulted in a fine cloudiness in the medium but no sediment. The cloudiness was uniform throughout the depth of the tube and could best be seen by swirling the tubes using direct transillumination. Stained smears prepared from recently isolated strains or from cultures passaged at regular 48 hour intervals contained mostly oval, ring-form organisms measuring approximately 0.85 to 1.0 micron by 0.05 micron and showing polar condensations. Cultures subjected to prolonged incubation or shaker cultures incubated for 72 to 96 hours contained extremely pleomorphic organisms varying from bodies similar to those described to extremely large bodies measuring up to 5.5 microns in diameter and showing deeply stained internal structures or condensations, Figures 5 and 6.

M. hyorhinis strain 31, Figure 7, was propagated in BHI broth or agar from the outset. This species was most commonly seen as coccoid bodies measuring 0.5 to 0.9 micron in diameter but on some occasions it produced ring-form organisms.

Agar Culture

Attempts at colonization of isolates 28, 29 and 33 on the different media used, with

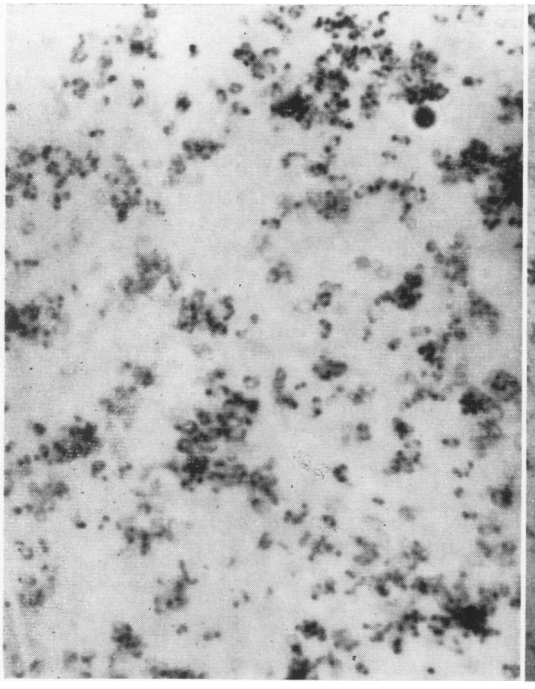

Fig. 4. Smeared centrifuge sediment of a pneumonia strain propagated in Broth A. Note the presence of ring-forms, some showing mono- or bi-polar condensations. Giemsa stain. X 1750.

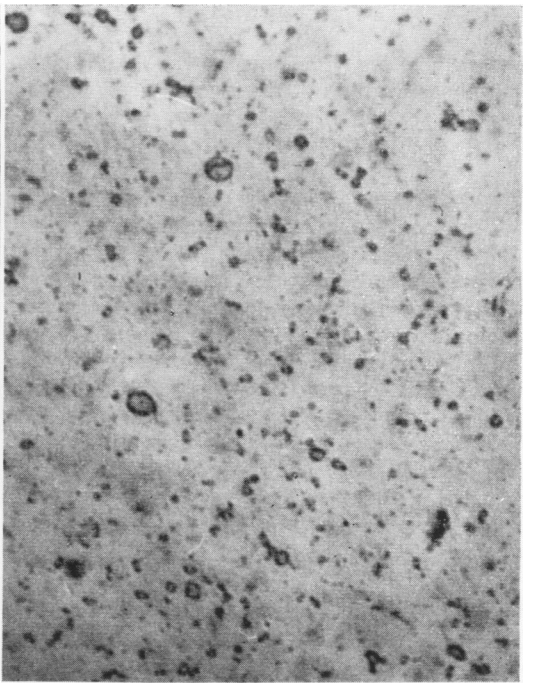

Fig. 5. Smeared Broth A sediment of a pneumonia strain prepared after 96 hours of incubation on a shaker. Note pleomorphism and large bodies, some with internal condensations. Giemsa stain. Phase contrast, X 1300.

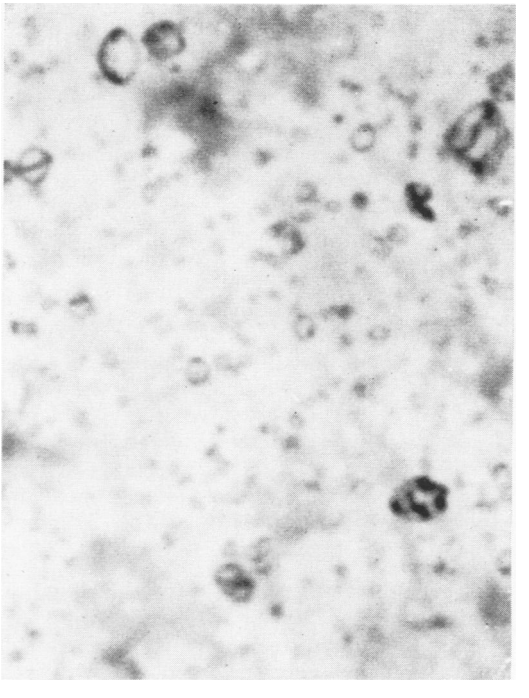

Fig. 6. Similar to Figure 5. Giemsa stain. X 2000.

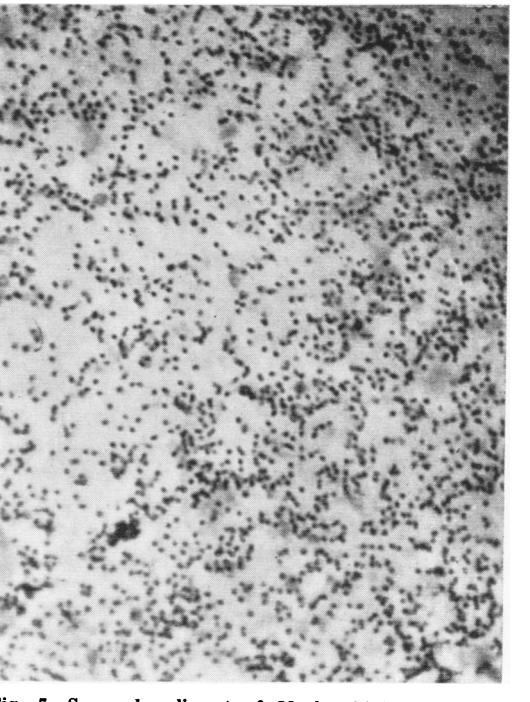

Fig. 7. Smeared sediment of M. hyorhinis strain 31. Note the dense coccoid bodies. Giemsa stain. Phase contrast, X 1300.

1 to 1.5% agar[10] added, yielded essentially negative results. On a few occasions colony-like structures developed on the agar plates but these could not be transferred subsequently to fresh agar plates.

DISCUSSION

The most serious cause of confusion in studies on EP of swine is the fact that more than half of the affected pigs carry *M. hyorhinis* in their lungs along with the etiologic agent(s) of the disease. Before the present study was undertaken, pneumonic lungs from eight pigs selected at random were examined for absence of *M. hyorhinis*. Four of these were eliminated from the study on the basis of typical growth in BHI broth and the production of colonies on BHI agar. The other four strains produced pneumonia following experimental IT inoculation of pigs. However, when these four lungs were inoculated into pig testicle cell cultures, one was found also to be contaminated with *M. hyorhinis*. The cell cultures had apparently acted as an enrichment medium and allowed *M. hyorhinis* to overgrow the more fastidious pneumonia producing organism. Our experience indicates that unless some method can be developed to specifically inhibit the multiplication of *M. hyorhinis*, it will not be possible to recover the pneumonia producing mycoplasma, in pure culture, from pneumonic lungs which carry both species. Much of the apparent confusion in published reports on EP may be due to studies carried out with *M. hyorhinis* or with infectious lungs contaminated with this species.

A strain of *M. hyorhinis* was included throughout the present study so that direct comparisons could be made in parallel with pneumonia producing mycoplasma as regards their morphology and growth requirements or characteristics. It appears impossible to distinguish these two species when they are propagated in cell cultures or in CCM. However, in meat-infusion medium they could be easily distinguished since *M. hyorhinis* grew abundantly in BHI broth and produced typical colonies on BHI agar whereas it grew only poorly in Broth A, perhaps due to its requirement for hemoglobulin. In contrast, mycoplasma isolates 28, 29 and 33 apparently did not multiply in BHI broth but grew fairly abundantly in Broth A.

[10]Noble agar — Difco Laboratories.

The use of long term passage intervals in cell cultures proved to be the key to the success of this work. L'Ecuyer and Switzer (14) made transfers at seven day intervals and obtained multiplication of an infectious agent in cell cultures without detectable effects on the cells. In this study five day intervals were equally useless whereas 14 day intervals were effective on the three strains studied. Goodwin and Whittlestone (8) used plasma clot cultures prepared from pneumonic lungs to infect cell monolayers and produce CPE. It would appear that prolonged incubation either allows the mycoplasma to adapt to growth in an artificial environment or simply allows sufficient numbers to accumulate and attack the cell substrate. Considerable variation was seen in the ability of strains to adapt to cell cultures, for example isolate #28 produced CPE only after the third transfer on the second attempt.

It was evident that, as noted by Goodwin and Whittlestone (11), broth media based on cell culture medium would not be very satisfactory for routine propagation and isolation of EP mycoplasma. Although propagation was rapid in such media, the viability as well as the infectivity for pigs were very low. Broth A was evolved by a process of gradual addition and elimination starting from cell culture medium. The criteria applied were yield of packed cells from a given volume, incubated for a fixed period. It was found that two to three day passage intervals, using stationary cultures, were ideal for routine maintenance of cultures and that for increased yields of antigen, three to four days of incubation using shaker cultures were preferable. Broth A as presently formulated appears to provide an excellent medium for direct isolation from infected pig lungs and for regular propagation of strains in the laboratory.

Pneumonia could consistently be produced in pigs inoculated with mycoplasma propagated in cell cultures or in Broth A. The higher concentrations of viable organisms obtained with these media appear to explain their greater infectivity by comparison to CCM.

The negative results obtained following amniotic sac inoculation of chicken embryos with infectious lung suspensions, are in line with previously reported negative results following yolk sac, allantoic sac or amniotic sac inoculation (14) or following

yolk sac and chorioallantoic membrane inoculation (17). These results contrast with numerous reports particularly on the use of the yolk sac as the route of choice for primary isolation.

The pneumonia producing isolates have tentatively been classified among the Mycoplasmataceae for the following reasons: (1) morphology and size; (2) extreme pleomorphism indicating absence of a rigid cell wall or internal structure; (3) very faint Gram negative staining but fairly good staining with the metachromatic dyes (Giemsa); (4) requirement for serum in growth medium; and (5) ability to propagate in the presence of penicillin and thallous acetate and morphologic stability when these are withdrawn. The other properties such as colony formation and growth into the agar, and growth inhibition by antiserum have not as yet been satisfied.

That a species of mycoplasma exemplified by isolates 28, 29 and 33 is the etiologic agent of EP would appear to be proven by: (1) the isolation of this organism from typical field cases of EP; (2) the experimental production of pneumonia in pigs with extremely high dilutions of these organisms obtained after long culture intervals; (3) the subsequent infectivity of the produced pneumonias for other pigs either by contact or by inoculated long suspensions; and (4) the recovery in broth medium of typical organisms from the lungs of experimentally infected pigs. These results support the conclusions of Maré and Switzer (16, 17) and of Goodwin *et al* (9, 10, 11).

Studies on EP carried out at two laboratories have resulted in essentially the same conclusions concerning the etiology of the disease and the proposing of two names for this new species of porcine mycoplasma. Goodwin *et al* (12) have shown by growth inhibition studies that their isolate *M. suipneumoniae* was antigenically related to *M. hyopneumoniae*. It would appear that the species designation *M. hyopneumoniae* has precedence in publication.

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