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# Further observations on the problem of isolating *Mycoplasma suipneumoniae* from field cases of enzootic pneumonia in pigs

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SUMMARY

In previous work in this laboratory, *Mycoplasma suipneumoniae* was recovered in liquid medium from 13 % of individual cases and 18 % of outbreaks of enzootic pneumonia in pigs. In the work now described, however, these recovery rates, when judged by the same criteria, were 45 and 75 %, respectively. As there was evidence to suggest that this second series of pneumonic cases was less suitable for cultural examination than the first series, some of the other factors that might have improved the recovery rate were investigated.

Some improvement was probably achieved by inoculating the liquid medium with three or four different dilutions of pneumonic tissue, each dilution always being in duplicate, and by incubating the inoculated tubes for over 3 weeks before discarding them.

A second advantage could have derived from the fact that all batches of liquid medium were tested for their ability to support the growth of *M. suipneumoniae* before being used to culture field material.

The effect of varying one constituent at a time was observed in controlled experiments: different batches of pig sera had a marked, variable effect on the growth of both *M. suipneumoniae* and *Mycoplasma hyorhinis*; medium made with purchased Hartley's broth was found to be superior to medium incorporating broth made in this laboratory, more so for the growth of *M. suipneumoniae* than *M. hyorhinis*; the incorporation of yeast extract made in this laboratory gave a marginal advantage for the growth of *M. hyorhinis*; and both mycoplasmas grew equally well in medium containing or lacking thallium acetate.

Some batches of medium were, by chance, markedly selective for the growth of *M. suipneumoniae* compared with *M. hyorhinis*. As the full reasons for this were not known, attempts were made to develop selective media in a more direct way. One such medium contained 5 % pig serum and 15 % horse serum, and a second was of similar composition, except that the pig serum used inhibited preferentially the growth of *M. hyorhinis* compared with *M. suipneumoniae*. Both media markedly favoured the growth of *M. suipneumoniae* when tested separately with cultures of *M. suipneumoniae* and *M. hyorhinis*. The second medium yielded *M. suipneumoniae* when inoculated with a $10^{-1}$ dilution of a culture of *M. suipneumoniae* and a $10^{-2}$ dilution of a culture of *M. hyorhinis*, whereas a standard batch of liquid medium, similarly inoculated with *M. suipneumoniae* did not yield this

mycoplasma until the *M. hyorhinis* culture included in the inoculum was diluted to $10^{-6}$.

Both selective media, when tested on a small number of field cases, gave improved isolations of *M. suipneumoniae* compared with the routine batches of liquid medium used initially.

Considerable difficulty was experienced in producing a sufficiently high level of antibodies to *M. hyorhinis* in pig sera and to *M. suipneumoniae* in rabbit sera. This exacerbated the problem of isolating and identifying *M. suipneumoniae* from field cases of enzootic pneumonia by this cultural method.

## INTRODUCTION

Goodwin, Pomeroy & Whittlestone (1965, 1967) established that enzootic pneumonia of pigs could be reproduced with a mycoplasma, which they named *Mycoplasma suipneumoniae*. Although other infective agents, especially *Mycoplasma hyorhinis* and secondary bacteria, are commonly present concurrently in the lesions from field cases, this does not invalidate the essential connexion between *M. suipneumoniae* and enzootic pneumonia: the most direct way of diagnosing the disease, therefore, is to isolate and identify *M. suipneumoniae*. Hitherto, however, this has proved difficult.

The two main media used routinely in this laboratory have been solid medium and liquid medium. With the present solid medium, *M. suipneumoniae* can rarely be recovered on primary isolation, even from experimental cases of the disease where *M. hyorhinis* and secondary bacteria are apparently absent (Goodwin, Pomeroy & Whittlestone, 1968). Using liquid medium, *M. suipneumoniae* was recovered from 91% of such experimental cases but, in a series of 12 field outbreaks, a positive cultural diagnosis was obtained in only three of 24 cases of pneumonia (12·5%) from two outbreaks (Goodwin *et al.* 1968). If the cultural diagnosis of enzootic pneumonia is to become a routine matter, therefore, it is essential to obtain a higher isolation rate of *M. suipneumoniae* from field material.

In later work with liquid medium in this laboratory, the percentage of successful recoveries of *M. suipneumoniae* from natural cases of the disease has been markedly increased and the purpose of this paper is to describe some of the factors that probably contributed to this more favourable situation.

## MATERIALS AND METHODS

### *Pneumonic-lung samples*

These were obtained from outbreaks of enzootic pneumonia which arose in herds that had previously shown no sign of the disease, except for the two from Herd EK, which was a chronically infected herd. The disease in all the herds satisfied the clinical and epidemiological characteristics of enzootic pneumonia (Goodwin & Whittlestone, 1967) and in nearly all the cases listed in Table 1, organisms with the morphology of *M. suipneumoniae* were seen in touch preparations examined by the method of Whittlestone (1967).

All the lung samples had been stored either at −30° C. or −60° C. before being examined culturally.

*Cultural examinations*

The basic liquid medium used was as previously described (Goodwin, Hodgson, Whittlestone & Woodhams, 1969). Any variations on this are described in the text. The solid medium was made from the above liquid medium as described earlier (Goodwin *et al.* 1967).

About 1 g. of pneumonic tissue was ground with sterile sand and liquid medium in a Griffith's tube; $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions of lung in liquid medium were prepared, always in duplicate, for incubation. If growth occurred, further passages (usually one or two) were made in liquid medium before passaging into tubes for a metabolic-inhibition test.

In many instances, 0·02 ml. of each primary dilution in liquid medium and of later passages in this medium was seeded onto solid medium.

*Serological techniques*

The growth-inhibition and metabolic-inhibition tests were carried out with the same rabbit antisera (R 1, R 2) and in the same manner as before (Goodwin *et al.* 1968), the metabolic-inhibition tests being performed in small glass tubes (Goodwin *et al.* 1969).

## RESULTS

Table 1 shows the results of culturing 45 cases of pneumonia from 16 outbreaks. The herds are listed in the order in which they were examined, as are the individual cases of pneumonia within each herd. All the serological results apply to liquid medium, for in only eight cases was a mycoplasma cultured directly on solid medium, and in none of these instances were the colonies inhibited by either the *M. hyorhinis* or *M. suipneumoniae* antiserum.

Where both *M. suipneumoniae* and *M. hyorhinis* are bracketed together, an atypical result was obtained: at first, the higher concentrations of each rabbit serum seemed to be inhibiting growth of the homologous antigen but shortly thereafter growth occurred even in these tubes. We concluded from this that both mycoplasmas were probably present in the primary inoculum, and in such a combination initially that each eventually grew through its heterologous serum.

It can be seen that *M. suipneumoniae* was recovered from 19 of the 45 cases (42 %). If the less clear-cut results are included, where the isolation of *M. suipneumoniae* appeared to be only partly inhibited by *M. hyorhinis*, the positive cases of enzootic pneumonia by cultural examination are increased to 24 (53 %). On an outbreak basis a positive diagnosis was obtained in 14 out of 16 instances. In the earlier study, however, only two cases of pneumonia were usually examined from each outbreak (Goodwin *et al.* 1968) and, in order to compare the present work more strictly, not more than the first two cases of pneumonia from each herd in Table 1 should be considered. When this is done, however, the positive diagnoses on an individual case basis become at least 13 out of 29 (45 %) and on an outbreak

Table 1. *Attempts to isolate* M. suipneumoniae *from field cases of enzootic pneumonia*

| Herd | Pig | Growth in liquid medium | Serological identification |
|---|---|---|---|
| XI | 983 | + | *M. hyorhinis* |
| | 984 | + | *M. suipneumoniae* |
| | 982 | + | *M. suipneumoniae* |
| | 985 | + | *M. hyorhinis* |
| EO | 989 | + | NR |
| | 991 | + | *M. suipneumoniae* + *M. hyorhinis* |
| | 990 | + | *M. suipneumoniae* |
| | 992 | + | *M. suipneumoniae* |
| ME | 998 | + | *M. suipneumoniae* + *M. hyorhinis* |
| | 999 | + | *M. suipneumoniae* |
| UN | 1003 | + | *M. suipneumoniae* |
| | 1001 | + | *M. suipneumoniae* |
| | 1002 | + | NR |
| ZD (1) | 1022 | + | *M. hyorhinis* |
| | 1023 | + | *M. suipneumoniae* |
| | 1026 | + | *M. hyorhinis* |
| ZD (2) | 1030 | + | *M. hyorhinis* |
| | 1031 | + | *M. hyorhinis* |
| TF | 1049 | + | *M. suipneumoniae* |
| | 1048 | + | *M. hyorhinis* |
| | 1050 | + | *M. suipneumoniae* |
| LC | 1051 | + | DR |
| | 1052 | + | DR |
| | 1053 | + | *M. suipneumoniae* |
| ME | 1056 | + | *M. suipneumoniae* |
| | 1057 | + | *M. suipneumoniae* + *M. hyorhinis* |
| | 1072 | + | *M. suipneumoniae* + *M. hyorhinis* |
| | 1070 | + | DR |
| | 1069 | + | *M. suipneumoniae* |
| | 1071 | + | DR |
| DN | 1076 | + | *M. suipneumoniae* |
| TZ | 1067 | − | |
| | 1068 | + | *M. suipneumoniae* |
| DT | 1058 | + | *M. suipneumoniae* |
| | 1060 | − | |
| | 1059 | − | |
| ZC | 1061 | + | *M. suipneumoniae* |
| CI | 1080 | + | *M. suipneumoniae* |
| EK | 1084 | + | *M. suipneumoniae* |
| | 1085 | + | *M. hyorhinis* |
| TL | 1063 | + | DR |
| | 1064 | + | *M. hyorhinis* |
| | 1065 | + | *M. suipneumoniae* + *M. hyorhinis* |
| | 1065A | − | |
| | 1065B | − | |

NR = no result; DR = doubtful result.

basis 12 out of 16 (75%). As the corresponding rates of isolation in our previous study were 13% for individual cases and 18% for outbreaks, this is a striking improvement, and some of the possible reasons for it are considered below.

*Persons making the isolations*

The laboratory manipulations made in this series of attempted isolations were performed by different persons from those similarly concerned in the previous study (Goodwin *et al.* 1968). In such an event, it is always possible to obtain different results, sometimes for reasons that are not very obvious. In the present work, however, two people performed the primary inoculations and the subsequent passages, one dealing with 19 cases of pneumonia and the other with 26. Both persons isolated *M. suipneumoniae* from 42% of their cases, and this remarkable degree of conformity suggests that factors other than personal skill were probably involved in the more frequent isolation of *M. suipneumoniae* in the work now being reported.

*Selection of pneumonic samples*

In the previous work (Goodwin *et al.* 1968), the pneumonic samples chosen for cultural examination were preselected, in that they were nearly all cases in which many organisms with the morphology of *M. suipneumoniae* were seen in the touch preparations.

Table 2. *Relationship between the mycoplasma recovered and mycoplasmas seen in touch preparations*

| Mycoplasmas of *M. suipneumoniae* – type | Total cases | Recovery of mycoplasmas | | | |
|---|---|---|---|---|---|
| | | *M. suipneumoniae* alone | *M. hyorhinis* alone | *M. suipneumoniae* + *M. hyorrhinis* | Negative or doubtful |
| Moderate numbers or above | 20 | 12 | 2 | 3 | 3 |
| Less than moderate numbers | 25 | 7 | 9 | 2 | 7 |

In the present work, since the outbreaks were studied soon after they arose, there was less opportunity for selecting pneumonic cases with numerous organisms of the *M. suipneumoniae*-type in touch preparations. The effect of this is shown in Table 2. Many fewer isolations of *M. suipneumoniae*, and many more isolations of *M. hyorhinis*, were made from those cases of pneumonia where organisms of the *M. suipneumoniae*-type were sparse in the touch preparations. The isolation rate of *M. suipneumoniae* from the 20 cases that were more comparable with those examined by Goodwin *et al.* (1968) was 60%. The more frequent recovery of *M. suipneumoniae* in the present work, therefore, was achieved despite the generally less favourable pneumonic samples.

*Number of cultures put up and duration of primary incubation*

In this work, several dilutions of lung were incubated in duplicate, compared with the two single dilutions (1/200 and 1/2000) used by Goodwin *et al.* (1968). Also, the primary dilutions were incubated for a longer period than previously. Because growth was often obtained in only one of a pair of duplicate dilutions and sometimes at only one particular dilution, and because growth was sometimes not apparent until 14–21 days after primary inoculation, it is likely that these later procedures contributed to the improved isolation rate.

*Variability of liquid medium*

Titrations of standard cultures of *M. suipneumoniae* and *M. hyorhinis* in different batches of liquid medium showed a considerable batch variability in the ability to support the growth of these organisms, particularly *M. hyorhinis*

Table 3. *Variation in growth of* M. suipneumoniae *and* M. hyorhinis *in different batches of liquid medium*

| Batch of liquid medium | Highest dilution of culture producing growth | |
|---|---|---|
| | *M. suipneumoniae* | *M. hyorhinis* |
| 1 | $10^{-8}$ | No growth |
| 2 | $10^{-7}$ | $10^{-3}$ |
| 3 | $10^{-6}$ | $10^{-7}$ |
| 4 | $10^{-6}$ | $10^{-4}$ |
| 5 | $10^{-6}$ | $10^{-2}$ |
| 6 | $10^{-8}$ | $10^{-3}$ |
| 7 | $10^{-9}$ | $10^{-8}$ |
| 8 | $10^{-5}$ | $10^{-1}$ |
| 9 | No growth | No growth |

(Table 3). An attempt to correlate the general variability of the liquid medium with the more frequent isolations of *M. suipneumoniae* in the present work was unsuccessful; but it was known that all the batches of liquid medium used would support the growth of *M. suipneumoniae* well, whereas complete information of this type is not available for the earlier work (Goodwin *et al.* 1968). It is possible, therefore, that the chances of isolating *M. suipneumoniae* from field material can be considerably increased by using batches of liquid medium that have been pretested for the more favourable growth of this mycoplasma compared with *M. hyorhinis.*

*Attempts to improve the liquid medium in favour of* M. suipneumoniae

The general plan in this part of the work was to make up batches of liquid medium in which only one constituent was varied, and to test such batches together under identical conditions for their ability to support the growth of *M. suipneumoniae* and *M. hyorhinis.* In the first stage, the object was to improve the growth of *M. suipneumoniae*; thereafter, experiments were undertaken to inhibit selectively the growth of *M. hyorhinis.*

*Factors influencing the growth of* M. suipneumoniae

*Serum.* Table 4 shows the results obtained with five samples of a medium in which a different batch of pig serum had been used in each sample. It can be seen that, in general, *M. suipneumoniae* grew much better than *M. hyorhinis*, and that both these organisms grew variably in the five batches of medium. In a parallel experiment, in which four batches of pig serum, different from those listed in Table 4, were compared using *M. hyorhinis* alone, rather better growth of *M. hyorhinis* was obtained but there was still an obvious variability.

Table 4. *Variation in growth of* M. suipneumoniae *and* M. hyorhinis *in a medium made with different batches of pig serum*

| | Highest dilution of culture at which growth occurred | |
|---|---|---|
| Batch of serum | *M. suipneumoniae* | *M. hyorhinis* |
| 1 | $10^{-8}$, $10^{-7}$ | No growth, no growth |
| 2 | $10^{-5}$ ($10^{-7}$), $10^{-5}$ ($10^{-7}$) | ($10^{-1}$), No growth |
| 3 | $10^{-8}$, $10^{-8}$ | ($10^{-1}$), No growth |
| 4 | $10^{-7}$, $10^{-7}$ | No growth, no growth |
| 5 | $10^{-6}$, $10^{-7}$ | ($10^{-8}$), No growth |

*Note.* All titrations were made in duplicate. The figures in parentheses indicate the maximum titre of partial growth.

*Hartley's broth.* Four comparisons were made between two different batches of purchased Hartley's broth (Oxoid) and the broth made in this laboratory. The medium made with the purchased broth supported the growth of both *M. suipneumoniae* and *M. hyorhinis* better than the medium made with our own broth, the main difference being the much higher speed of growth in the former medium. This improvement in growth, however, was much more marked with *M. suipneumoniae* than with *M. hyorhinis.*

*Yeast extract.* Two comparisons were made between a purchased batch of yeast extract (DIFCO) and the yeast extract made in this laboratory. In both cases, our own yeast extract seemed slightly better for the growth of *M. hyorhinis*, but there was no growth difference with *M. suipneumoniae.*

*Presence of thallium acetate.* A comparison was made between medium containing the usual concentration of thallium acetate and the same medium without this inhibitor. No significant difference in the growth rate of either *M. suipneumoniae* or *M. hyorhinis* was observed with the two types of medium.

*Attempts to inhibit the growth of* M. hyorhinis

The first approach under this heading was to compare some variants of the standard liquid medium for their ability to support the growth of *M. suipneumoniae* more readily than the growth of *M. hyorhinis* (Table 5). Because there was sometimes a limit to the number of titrations that could be performed in parallel, these were not always taken to the limit of growth. It can be seen that during this phase of the work the standard medium containing 20 % pig serum supported the

growth of *M. hyorhinis* quite well (Expts. 1, 5), but the reduction in the percentage of pig serum in favour of horse serum depressed the growth of *M. hyorhinis*, in that this mycoplasma did not produce a complete change in pH in medium D beyond the $10^{-1}$, $10^{-1}$ and $10^{-3}$ tubes, respectively, in Expts. 2, 4 and 5. Insufficient results are available for medium E to say what the effect of the complete substitution of pig serum was, but Expt. 5 indicates that the growth of *M. suipneumoniae* was partly depressed in that medium, so that the maximum difference in growth between *M. hyorhinis* and *M. suipneumoniae* which we were seeking had now been lost.

Table 5. *Comparison between five media containing varying proportions of pig and horse serum*

| | | Media | | | | | |
|---|---|---|---|---|---|---|---|
| Serum | Culture titrated | A | B | C | D | E | Exp. no. |
| Pig | — | 20 % | 15 % | 10 % | 5 % | None | |
| Horse | — | None | 5 % | 10 % | 15 % | 20 % | |
| | *M. hyorhinis* | $10^{-7}$ | ND | ND | ND | $10^{-6}$ | 1 |
| | *M. hyorhinis* | *$10^{-3}$* | *$10^{-3}$* | *$10^{-3}$* | $10^{-1}$ | ND | 2 |
| | *M. suipneumoniae* | *$10^{-3}$* | *$10^{-3}$* | *$10^{-3}$* | *$10^{-3}$* | ND | |
| | *M. hyorhinis* | *$10^{-3}$* | $10^{-1}$ | $10^{-1}$ | ND | ND | 3 (passage |
| | *M. suipneumoniae* | *$10^{-3}$* | *$10^{-3}$* | *$10^{-3}$* | ND | ND | of Exp. 2) |
| | *M. hyorhinis* | ND | ND | ND | $10^{-1}$ | ND | 4 (passage |
| | *M. suipneumoniae* | ND | ND | ND | $10^{-7}$ | ND | of Exp. 2) |
| | *M. hyorhinis* | * | ND | ND | $10^{-3}$ | $10^{-3}$ | 5 |
| | *M. suipneumoniae* | * | ND | ND | *$10^{-8}$* | $10^{-5}$ | |

* These cultures were also titrated in parallel in a routine batch of liquid medium containing 20 % pig serum: *M. hyorhinis* gave a titre of $10^{-6}$; *M. suipneumoniae* gave $10^{-7}$.

ND = not done.

*Note.* Italicized titres mean that the titration was not taken beyond this tube. Where lines are bracketed together, all the titrations were made in parallel on the same day.

Table 6. *Mycoplasmas recovered with three selective media compared with original routine media*

| | | Organism recovered using each medium below | | |
|---|---|---|---|---|
| Pig | Result from Table 1 | E | D | SLM |
| 1022 | *M. hyorhinis* | *M. suipneumoniae* | *M. hyorhinis* | *M. suipneumoniae* |
| 1048 | *M. hyorhinis* | *M. hyorhinis* | *M. hyorhinis* | *M. hyorhinis* |

SLM = Standard liquid medium with depressant effect on the growth of *M. hyorhinis*.

As medium D appeared to favour the growth of *M. suipneumoniae* compared with *M. hyorhinis* to a marked degree it was used, along with medium E, to re-examine two of the field cases of enzootic pneumonia (1022, 1048) in Table 1, from which only *M. hyorhinis* had been isolated previously. In addition, a batch of standard liquid medium (SLM) was included which, by chance, had the ability to depress the growth of *M. hyorhinis* (Table 6). It can be seen that media E and SLM allowed the isolation of *M. suipneumoniae* from case 1022, but *M. hyorhinis* was

still the only mycoplasma isolated from case 1048. As this was only a partial improvement, an attempt was made to prepare an even more selective medium, based on medium D, as described below.

*Incorporation of serum containing antibodies to* M. hyorhinis

The first difficulty in this connexion was to find pigs with sera that did not already inhibit the growth of mycoplasmas. It had previously been observed that many pig sera contained non-specific inhibitory substances (Goodwin *et al.* 1969) and we preferred to produce antisera against *M. hyorhinis* in animals that were initially free from such inhibitors, to allow the possibility of obtaining the maximum difference between the inhibition of *M. hyorhinis* and *M. suipneumoniae*. Such pigs, however, were difficult to find. A total of 23 colostrum-deprived, hysterectomy-produced pigs, and eight pigs born naturally in a herd established entirely from hysterectomy-produced pigs, were bled; the sera from all of them after inactivation (56° C. for 30 min.) had some inhibitory effect on the growth of *M. hyorhinis*, and only six of the sera (all from hysterectomy-produced pigs) had no inhibitory effect on the growth of *M. suipneumoniae*. Nevertheless, because it had previously been observed that the non-specific inhibitory effect fluctuated with time, nine of the hysterectomy-produced pigs were selected for three experiments in which they were inoculated with *M. hyorhinis*.

There were two pigs in the first experiment, four in the second and three in the third; two of the pigs in the second experiment and all three in the third experiment had preinoculation sera which did not inhibit the growth of *M. suipneumoniae*. Cultures of *M. hyorhinis* that had grown in liquid medium at high dilutions were given by various routes: intranasally (as living cultures), subcutaneously (with and without Freund's complete adjuvant), intraperitoneally and intramuscularly (with and without Freund's complete adjuvant); all the pigs received *M. hyorhinis* antigen by at least two different routes and two of the pigs were inoculated by four different routes. When the antigen was given by injection it had previously been centrifuged, washed, and resuspended in a more concentrated form in phosphate-buffered saline.

The outcome of these three separate attempts to produce a pig antiserum to *M. hyorhinis* was disappointing. All the sera were tested after inactivation and the best sample inhibited the growth of *M. hyorhinis* to a serum dilution of only 1/64, but it also partially inhibited the growth of *M. suipneumoniae* to a dilution of 1/8; furthermore, when this pig was killed 13 days later to harvest a large quantity of serum, the above titres had fallen to 1/4 (partial inhibition to 1/12), and partial inhibition to 1/4, respectively. The second point of note is that four of the five pigs that had serum with no inhibitory effect on *M. suipneumoniae* before their course of inoculations developed such an inhibitory effect afterwards (up to a serum dilution of 1/24); the single pig that did not react in this way, did not produce any inhibitory effect to *M. hyorhinis* either.

The serum sample which inhibited the growth of *M. hyorhinis* when diluted 1/64 was now incorporated into the earlier selective medium, type D. The horse-serum component in medium D avoided the problem of finding a pig serum that

did not contain inhibitory substances to *M. suipneumoniae* when diluting out such inhibitory substances in the antiserum against *M. hyorhinis*. The new medium (F) contained 6% (1/17) of the latter antiserum and 14% horse serum; it was thus only marginally different in percentage composition from the original medium D.

Titrations of *M. suipneumoniae* and *M. hyorhinis* cultures were made in medium F alone, and in both medium F and the batch of standard liquid medium (SLM) shown in Table 6, in parallel (Table 7). Medium F seemed promising, in that *M. hyorhinis* did not produce a full pH change beyond the $10^{-1}$ tube in all three experiments, whereas *M. suipneumoniae* grew well in the same medium; secondly, when medium F was compared with a standard liquid medium in parallel, the latter medium supported the growth of *M. hyorhinis* almost as well as *M. suipneumoniae*.

Table 7. *Titrations in selective medium (F) and in a routine batch of medium (SLM)*

| Experiment | Medium | Highest dilution of culture at which growth occurred | |
|---|---|---|---|
| | | *M. suipneumoniae* | *M. hyorhinis* |
| 1 | F | *$10^{-8}$* | $10^{-1}$ ($10^{-4}$) |
| 2 | F | *$10^{-6}$* | $10^{-1}$ ($10^{-3}$) |
| 3 | F | $10^{-7}$ ($10^{-8}$) | $10^{-1}$ ($10^{-2}$) } Titrated in |
| | SLM | *$10^{-8}$* | $10^{-7}$ ($10^{-8}$) } parallel |

*Note.* Italicized titres mean that the titration was not taken beyond this tube; titres in parentheses indicate the maximum titre of partial growth.

SLM = Standard liquid medium with depressant effect on the growth of *M. hyorhinis*.

Medium F was next tested with mixed cultures of *M. hyorhinis* and *M. suipneumoniae*, but before doing this a trial experiment of this type was performed in an ordinary routine batch of liquid medium. A culture of *M. suipneumoniae* and a culture of *M. hyorhinis* were titrated in this medium, and both grew at the highest dilution tried ($10^{-6}$). Tubes of the same medium were put up in parallel with these titrations, each tube containing a $10^{-1}$ dilution of the *M. suipneumoniae* culture but with varying dilutions of the *M. hyorhinis* culture (from $10^{-1}$ to $10^{-6}$). In all three branches of this experiment there were control tubes and every dilution was made in duplicate. The object was to see how much the *M. hyorhinis* culture had to be diluted in a mixture before it ceased to outgrow *M. suipneumoniae*. The tubes inoculated with the *M. hyorhinis* culture at the $10^{-1}$ to $10^{-5}$ dilutions yielded *M. hyorhinis* from the mixture, and only the two tubes inoculated with the $10^{-6}$ dilution of *M. hyorhinis* yielded *M. suipneumoniae*.

The above experiment was now repeated with medium F. The culture of *M. suipneumoniae* when titrated alone again grew at the $10^{-6}$ dilution (the highest dilution tried), but the culture of *M. hyorhinis* when titrated alone grew well in this medium only at the $10^{-1}$ dilution. There was some growth of *M. hyorhinis* at $10^{-2}$ and $10^{-3}$, but none thereafter. With the mixed cultures (*M. suipneumoniae* at $10^{-1}$ throughout, and *M. hyorhinis* varying from $10^{-1}$ to $10^{-6}$), growth occurred in both the tubes inoculated with the $10^{-1}$ dilution of *M. hyorhinis*, but

no result could be obtained on either of these cultures with the metabolic-inhibition test. It was concluded from this that both mycoplasmas were probably present, each growing through the heterologous rabbit antiserum. *M. suipneumoniae*, however, was recovered from all 10 tubes inoculated with *M. hyorhinis* in dilutions from $10^{-2}$ to $10^{-6}$. It seemed, therefore, that medium F allowed the isolation of *M. suipneumoniae* from mixed cultures when it was inoculated with ten times more of a culture of *M. suipneumoniae* than of a culture of *M. hyorhinis*, whereas with the routine batch of liquid medium tried, the dose of the *M. suipneumoniae* culture had to be 100,000 times that of the *M. hyorhinis* culture before *M. suipneumoniae* was recovered.

Table 8. *Mycoplasmas recovered with two selective media compared with original routine media*

| Pig | Result from Table 1 | Organism recovered using each selective medium below: F | SLM |
|---|---|---|---|
| 1026 | *M. hyorhinis* | *M. hyorhinis* | *M. suipneumoniae* |
| 1030 | *M. hyorhinis* | *M. suipneumoniae* | *M. hyorhinis* (possibly with *M. suipneumoniae*) |
| 1031 | *M. hyorhinis* | *M. suipneumoniae* | *M. hyorhinis* and *M. suipneumoniae* |

SLM = Standard liquid medium with depressant effect on the growth of *M. hyorhinis*.

Medium F was now tested with three field cases that had previously yielded only *M. hyorhinis*; at the same time, it was compared with the batch of liquid medium (SLM) shown in Table 6. The results summarized in Table 8 show that medium F allowed the isolation of *M. suipneumoniae* from both pigs 1030 and 1031; it was not greatly superior to medium SLM, however, as this gave a better result with pig 1026 and was almost successful with pig 1030.

### *Production of antisera in rabbits*

This paper has emphasized the difficulties of isolating *M. suipneumoniae* from field cases of enzootic pneumonia. With these cultural techniques, however, there seems to be yet another difficulty, and that is the need to have good diagnostic antisera against *M. suipneumoniae* and *M. hyorhinis*. We have not been able to produce antisera to *M. suipneumoniae* very readily, despite several attempts. In all, 16 rabbits have recently been injected with concentrated antigen subcutaneously (plus Freund's complete adjuvant) followed by intravenous injections of antigen alone. In half the rabbits this course of injections was followed by intramuscular injections and intravenous injections; furthermore, in case the antigenicity of the culture of *M. suipneumoniae* had been grossly affected by the repeated centrifugation and resuspension, fresh antigen was prepared and after only one centrifugation the deposit was resuspended in one-hundredth of its original volume. The rabbits that received this unwashed antigen all developed anaphylactic shock and one of them died, but the highest serum titre produced was only 1/160.

## DISCUSSION

Because *M. suipneumoniae* cannot yet be cultivated directly on solid medium from cases of enzootic pneumonia, it is necessary to make the primary isolations in liquid medium. It would seem that the isolation rate of *M. suipneumoniae* can be improved by preselecting the pneumonic tissue: active, moist cases of pneumonia with *M. suipneumoniae*-type organisms in touch preparations are likely to give better results, but the chances of success can also be improved by inoculating the liquid medium with several different dilutions of pneumonic tissue, at least two tubes being put up at each dilution. Whether the isolation rate could be improved still further by culturing from very fresh tissue which has not been frozen remains to be investigated.

The greatest problem, however, is still the blocking action of *M. hyorhinis*, when this mycoplasma is concurrently present in the lung. Some batches of liquid medium made in this laboratory appear to be much more selective than others for *M. suipneumoniae*, but this chance variation cannot be relied upon, nor can it be readily controlled. Known variations in the inhibitory capacity of normal pig serum seem to account for a large part of this over-all variability, but non-serum factors are probably also involved. Awareness of this variability could be important in those laboratories which are still having difficulty in growing *M. suipneumoniae*, even in pure culture. Most of our media will at least support the growth of *M. suipneumoniae* well, but where this organism is only being grown fitfully and in low titre, there is probably less chance of cultivating *M. suipneumoniae* in the presence of *M. hyorhinis*.

The fact that our normal liquid medium yielded *M. hyorhinis* when inoculated with 10,000 times the dose of *M. suipneumoniae* culture as *M. hyorhinis* culture indicates how formidable the task of isolating *M. suipneumoniae* from all field cases may be. The high degree of success that we have now been able to achieve, however, may mean that *M. suipneumoniae* usually vastly outnumbers *M. hyorhinis* in lesions of enzootic pneumonia, and this would explain why organisms with the morphology of *M. suipneumoniae* are usually more obvious in touch preparations.

A more selective medium than our medium F might be made if a pig serum with a much greater inhibitory effect on *M. hyorhinis* could be produced. Although we have not been very successful in this regard, other workers might find a way of producing such sera. Arising from this part of the work, it is interesting that four out of five pigs produced inhibitory substances to *M. suipneumoniae* after being injected with *M. hyorhinis*; this may indicate shared antigens or merely a non-specific stimulatory effect.

We have mentioned our difficulty in producing good antisera against *M. suipneumoniae* in rabbits because we have heard that other workers are experiencing the same problem. *M. suipneumoniae* may yet prove to be a not very antigenic mycoplasma in this context.

Although a notable improvement in isolation rate has been achieved, we have not yet reached the stage where we can regularly culture *M. suipneumoniae* from

nearly all field cases of enzootic pneumonia and, while there are many obvious ways in which this approach may be developed further, there is no way of knowing how long it might take. In the meantime, therefore, a different method of diagnosis has been studied and this will be reported separately (Goodwin & Hodgson, 1970).

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