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Attempts to recover *Mycoplasma suipneumoniae* from experimental and natural cases of enzootic pneumonia in pigs

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Goodwin & Whittlestone (1964, 1966) induced enzootic pneumonia in pigs with a mycoplasma-like agent grown in liquid medium. Using essentially this medium, Maré & Switzer (1965) also reproduced enzootic pneumonia with liquid-medium cultures, and from these cultures they isolated a mycoplasma on solid medium, which they called *Mycoplasma hyopneumoniae*. However, at least one mycoplasma other than the causal agent of enzootic pneumonia commonly occurs in the respiratory tract of pigs affected with enzootic pneumonia and it cannot be assumed, therefore, that a mycoplasma isolated on solid medium is the same as a pneumonia-inducing agent grown in parallel in liquid medium. Goodwin, Pomeroy & Whittlestone (1965) proved that the mycoplasma isolated from their pneumonia-inducing fluids was indeed the causal agent of enzootic pneumonia by passaging it serially on solid medium until a dilution of at least 10^{-15} of the primary seed inoculum was reached; these final colonies induced enzootic pneumonia. This mycoplasma was named *Mycoplasma suipneumoniae*. Subsequently, *M. suipneumoniae* was shown to be serologically different from a wide range of other mycoplasmas (Goodwin, Pomeroy & Whittlestone, 1967), and hence it is probably a new species.

The relationship and nomenclature of these porcine mycoplasmas is complicated by the fact that the culture distributed as *M. hyopneumoniae* did not derive from the colonies on solid medium that were named *M. hyopneumoniae* by Maré & Switzer (1965), but from their pneumonia-inducing fluids (W. P. Switzer, 1968, personal communication). The cross-neutralization between *M. suipneumoniae* and *M. hyopneumoniae* in the growth-inhibition and metabolic-inhibition tests, therefore, shown by Goodwin *et al.* (1967), indicates a relationship between *M. suipneumoniae* and a mycoplasma derived from pneumonia-inducing fluids in the United States, and not between *M. suipneumoniae* and the colonies originally published as *M. hyopneumoniae*. Because of this difficulty, and because the name *M. suipneumoniae* refers to a mycoplasma of known pathogenicity, which has been compared with other mycoplasmas serologically, we are continuing to use this name. A precise link then exists with our previous work, and this should prevent further confusion.

Enzootic pneumonia of pigs appears from clinical evidence to be a widespread disease, both in this country and abroad. Hitherto, however, because there has been no precise method of diagnosis, the limits and distribution of the disease

have not been defined. Clearly, the isolation of the causal agent offers a considerable advantage in this respect, especially when there is no published information on the diagnostic value of specific antibodies in pig sera. This paper presents a preliminary assessment of the extent to which the isolation of *M. suipneumoniae* might be routinely possible.

Table 1. *Attempts to isolate* Mycoplasma suipneumoniae *from experimentally induced cases of enzootic pneumonia*

Pig	Type*	Strain of experimental infection	Isolation of mycoplasmas: on solid medium	Isolation of mycoplasmas: in liquid medium	Serological identification
2856	HPCD	J	–	+	
2857	HPCD	J	–	+	
2860	HPCD	J	–	+	
2878	HPCD	J	–	+	
2958	HPCD	J	–	+	
2959	HPCD	J	–	+	
2895	HPCD	J	–	+	
2896	HPCD	J	–	+	
2805	HPCD	J	–	+	
3012	HPCD	J	ND	+	
3013	HPCD	J	ND	+	*M. suipneumoniae*
3014	HPCD	J	ND	+	
2912	MH	CZ	–	+	
2915	HPCD	CZ	–	+	
2922	MH	CZ	–	+	
2923	MH	CZ	–	+	
2897	HPCD	CZ	–	+	
2910	HPCD	CZ	–	+	
2911	HPCD	CZ	–	+	
2913	MH	CZ	–	+	
2932	HPCD	CZ	–	–	Not applicable
2898	HPCD	CZ	–	–	

* See Materials and Methods (p. 599) for definitions.
ND = not done.

MATERIALS AND METHODS

Pneumonic-lung samples

The two strains (J and CZ) of *M. suipneumoniae* that were in the lung samples used to induce the cases of enzootic pneumonia shown in Table 1 originated from two adult sows in separate herds; both these cases of enzootic pneumonia were chosen because, when first studied, the lung lesions did not yield any readily cultivable mycoplasmas. For further information on the history of the J strain, see Goodwin & Whittlestone (1963). The CZ strain has been cultured repeatedly in the laboratory (in tissue cultures, in liquid media and on solid media) and re-isolated from transmission experiments in pigs, without the appearance of any mycoplasma other than *M. suipneumoniae*; it is specifically neutralized in the metabolic-inhibition and growth-inhibition tests by serum prepared against the J strain of *M. suipneumoniae*.

The field strains listed in Table 2 all came from sudden outbreaks of enzootic pneumonia which arose in 12 separate herds that had been in a control scheme for herds believed to be free from this disease (Goodwin & Whittlestone, 1967). These outbreaks had the clinical and epidemiological characteristics of enzootic pneumonia (Goodwin & Whittlestone, 1967), and the pneumonic cases used satisfied the pathological and touch-preparation criteria that are referenced later. Pigs 2750, 2645, 2644, 2901, 2902, 2903 and 603 were specially killed, in order to harvest lung specimens aseptically; all the other pigs in Table 2 were killed at slaughterhouses, and the lesions were sampled after the lungs had been removed in the routine way on the slaughter line.

Lung samples that were not cultured when fresh were stored at −30° C. or −60° C.

Table 2. *Attempts to isolate* Mycoplasma suipneumoniae *from field cases of enzootic pneumonia*

Herd of origin	Pig	Isolation of mycoplasmas: on solid medium	Isolation of mycoplasmas: in liquid medium	Serological identification
EO	9952	−	+	*M. hyorhinis*
	9954	−	+	
HU	9949	−	+	*M. suipneumoniae*
	9948	−	+	
IT	9920	−	+	*M. hyorhinis*
	9921	+	+	
US	9906	−	+	*M. hyorhinis*
	9909	−	+	*M. suipneumoniae*
TZ	9902	−	−	NA
	9901	−	+	*M. hyorhinis*
CL	9769	−	−	NA
	9770	−	+	*M. hyorhinis*
TE	2750	−	+	*M. hyorhinis*
	2752	−	−	NA
XI	2645	−	−	NA
	2644	−	−	
TP	2901	−	+	*M. hyorhinis*
	2902	−	+	
	2903	−	+	
CO	9960	+	+	*M. hyorhinis*
	9962	−	−	NA
PF	2982	−	+	*M. hyorhinis*
	2983	−	−	NA
MG	603	−	ND*	*M. hyorhinis**

* This isolation was made by a different method (see Materials and Methods p. 599).
ND = not done; NA = not applicable.

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Disease-transmission experiments in pigs

Two types of pig were used: hysterectomy-produced, colostrum-deprived (HPCD) animals, which had been reared in strict isolation, and naturally born pigs (MH) from a herd established only from HPCD pigs; the lungs of routinely slaughtered pigs from this herd are regularly checked for the absence of enzootic pneumonia. Apart from the first passage from herd MG (Table 3), which was made in ordinary isolation accommodation, all the disease-transmission experiments

Table 3. *Disease-transmission experiments with six field strains of enzootic pneumonia*

Herd of origin	Material providing inoculum	Experimental inoculations: Pigs	Presence of enzootic pneumonia	Recovery of mycoplasmas: on solid medium	Recovery of mycoplasmas: in liquid medium	Serological identification
EO	9952, 9954	2994	+	−	+	*M. suipneumoniae*
		2995	+	−	+	
	Controls for EO and IT	2992	−	.	.	.
		2993	−	.	.	.
IT	9920, 9921	2996	+	−	+	*M. hyorhinis*
TZ	9901, 9902	2873	+	ND	−	NA
		2874	+	ND	−	
	Control	2875	−	.	.	.
	2873, 2874	2925	+	−	+	*M. suipneumoniae*
		2926	+	ND	ND	
	Control	2927	−	.	.	.
XI	2644, 2645	2658, 2661, 2663	+	−	−	NA
	Control	2660	−	.	.	.
TP	2900, 2901, 2902, 2903	2975	+	+	+	*M. hyorhinis*
MG	603	2029*	+	−	ND	.
		2039*	+	−		
		2044*	+	ND		
	Controls	162*, 163*, 164*	−	.	.	.
	2029, 2039	2264	+	−	ND	.
	2264	2321	+	−	ND	.
		2324	+	−	ND	*M. suipneumoniae*
	Control	2322	−	.	.	.

ND = not done; NA = not applicable.
* These six pigs were type MH: all the others in this Table were HPCD.
† This isolation was made by a different method (see Materials and Methods).
Note. No attempts were made to recover mycoplasmas from the control pigs.

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shown in the tables were made 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.

Apart from pigs 2029, 2039 and 2044 in Table 3, which received 20 ml. each of a 10^{-2} lung suspension intra-tracheally, all the experimentally infected pigs in Tables 1 and 3 were inoculated intranasally with 4–30 ml. of a 1/25 to 1/4 suspension of ground pneumonic lung in broth.

All the lung samples from the experimentally infected pigs were harvested aseptically, after removal of the sternum.

The initial laboratory diagnosis of all the cases of enzootic pneumonia mentioned in this paper was based on the nature of the gross lesions, the histological picture and the examination of touch preparations for organisms with the morphology of *M. suipneumoniae*, as described elsewhere (Goodwin & Whittlestone, 1966).

Cultural examinations

When studying the outbreaks of enzootic pneumonia listed in Table 2, apart from herd MG, samples of pneumonic lesions from several pigs in each outbreak were usually available. The general procedure was to select first a case where the stained touch preparations made from the lesions showed many mycoplasmas morphologically similar to *M. suipneumoniae*: if this case did not yield a mycoplasma on culture, or if *Mycoplasma hyorhinis* was cultured, the next best case (as judged by touch preparations) was then examined. To this extent, therefore, the lesions cultured from the field were not random samples.

Media

Except for pig 603 (Table 2) and pig 2324 (Table 3), the liquid and solid media used contained Hartley's broth, and were prepared and incubated as previously described (Goodwin *et al.* 1967). Mycoplasma 603 was isolated first in pig-lung monolayer cultures, from which it was passaged via Edward's medium to pig-kidney monolayer cultures and then to solid medium containing Hartley's broth; after five single-colony passages on this solid medium, it was grown in the Hartley's-broth liquid medium. Mycoplasma 2324 was initially isolated in plasma-clot cultures prepared from the freshly harvested pneumonic lesions; it was then passaged to pig-lung monolayer cultures and subsequently to boiled-tissue-culture medium (Goodwin & Whittlestone, 1966), from which it was passaged to liquid medium for identification.

The solid medium used for the cultural examinations relating to herd MG in Tables 2 and 3 was serum-brucella-agar medium described elsewhere (Goodwin & Whittlestone, 1962).

Inoculations and assessments

Fresh lung, or rapidly thawed lung from the deep freeze, was ground in a Griffith's tube with liquid medium, to make a 1/10 dilution. Further dilutions were then made in liquid medium, usually to give final lung dilutions of 1/200–

1/2000 and, before incubating these, samples from them were seeded on to solid medium.

Growth in liquid medium was assessed by the production of acid but, before carrying out a metabolic-inhibition test, the acid-producing agent was passaged two or three times.

In several instances, mycoplasma-type colonies were isolated directly on solid medium but these isolations are not noted in the text and tables, unless the colonies could be passaged on solid medium.

Serological techniques

The growth-inhibition test of Huijsmans-Evers & Ruys (1956), as modified by Clyde (1964) and Stanbridge & Hayflick (1967), was used: the disks were soaked in 0·025 ml. of antiserum, allowed to dry, and stored at −20° C. until required. The metabolic-inhibition tests (Taylor-Robinson, Purcell, Wong & Chanock, 1966), using either microtitre plastic plates or small glass tubes, were made with rabbit antiserum R 1 against *M. hyorhinis*, or R 2 against *M. suipneumoniae* (Goodwin *et al.* 1967); medium and organism controls were included with each test.

RESULTS

Experimental cases of enzootic pneumonia

Twenty-two cases of experimentally induced enzootic pneumonia were cultured for mycoplasmas; these cases were unselected and all the examinations made during a certain period of time are included. The results are shown in Table 1.

In no instance was a primary isolation made directly on solid medium. In 20 of the 22 cases, however, a mycoplasma was cultured in liquid medium, and in all of these cases the mycoplasma was identified as *M. suipneumoniae*.

Field cases of enzootic pneumonia

After finding that *M. suipneumoniae* could be cultured from a high percentage of experimentally induced cases of enzootic pneumonia, 11 field outbreaks of the disease were studied in the same way. These results are shown in Table 2, together with a twelfth outbreak (strain MG); this outbreak, which is included because it provided relevant results in disease-transmission experiments (see Table 3) and because it yielded our working strain (603) of *M. hyorhinis*, had been studied earlier by a different method, in 1961.

It can be seen that in only two of the 12 outbreaks was *M. suipneumoniae* isolated. In seven of the total number of 24 pigs examined, no mycoplasma was recovered, and with herd XI both pneumonic cases came into this category; this outbreak will be referred to again in the next section (disease-transmission experiments). In nine of the 12 outbreaks, the only mycoplasma identified was *M. hyorhinis* and the question arose, therefore, whether these nine outbreaks were truly cases of enzootic pneumonia, even though they simulated the disease in other respects. To throw some light on this question, material from six of the outbreaks that had not yielded *M. suipneumoniae* was used in disease-transmission experiments.

Disease-transmission experiments

The six herd outbreaks of enzootic pneumonia investigated in this way comprised five of the 11 recent outbreaks, plus outbreak MG from 1961. The results are shown in Table 3.

In the first passage of the disease from herd TZ, the areas of induced pneumonia were extremely small in both pigs. A second passage was made, therefore, and as the lesions in pig 2925 in this passage were much more extensive than in pig 2926, the former lesions were chosen for cultural examination.

The pig inoculum for the second passage from herd MG was clarified through a Seitz no. 5 pad and then taken through a Millipore filter (APD 0·8 μ).

Table 3 shows that the lesions from herd XI, from which no mycoplasmas had been cultured, transmitted as enzootic pneumonia, as did the lesions from all the other five outbreaks, which had been complicated originally by *M. hyorhinis*. Two of the latter five transmissions (IT and TP) continued to yield *M. hyorhinis* from the first-passage pneumonias, but the remaining three transmissions yielded *M. suipneumoniae* at the first (EO), second (TZ) and third (MG) passages, respectively.

DISCUSSION

In 10 of the 12 outbreaks listed in Table 2, a diagnosis of enzootic pneumonia could not be reached by isolating and identifying *M. suipneumoniae*. Six of these 10 outbreaks, however, were selected at random and lung lesions from each of them readily induced a disease indistinguishable from enzootic pneumonia in animal transmission experiments. It seems very probable, therefore, that the remaining four herd outbreaks, which had the same features as the previous six, were also due to enzootic pneumonia. If this assumption is correct, the failure to make a diagnosis in 10 out of 12 outbreaks was due in one instance to the inability to isolate any mycoplasmas, and in nine instances to the simultaneous presence of *M. hyorhinis*. In our experience, therefore, *M. hyorhinis* is commonly present in field cases of enzootic pneumonia and hence the isolation of an unidentified mycoplasma is of no value in the diagnosis of this disease, particularly when the presence of *M. hyorhinis* appears to prevent the isolation of *M. suipneumoniae*. Conversely, when *M. hyorhinis* appeared to be absent, *M. suipneumoniae* was isolated in 24 out of 31 experimental cases, and three out of 10 field cases, making a total of 27 out of 41 cases, or 66 %. In the 22 experimental transmissions using the J or CZ strains (Table 1), the isolation rate of *M. suipneumoniae* was 91 %.

M. suipneumoniae was not isolated directly on solid medium in any of the 26 experimental and field cases where it was isolated in liquid medium, and there could be several reasons for this. Perhaps either the present solid medium or the way it is used is unsuitable for such direct isolation. On the other hand, there could be inhibiting factors in the lung which could still present a problem, even with an improved medium or better cultural methods. If a way could be found of isolating *M. suipneumoniae* directly on solid medium, without the intervention of liquid medium, it should be possible to distinguish it, even if it were mixed with *M. hyorhinis* or other mycoplasmas.

It is interesting that in three of the five transmission experiments in pigs where the presence of *M. hyorhinis* was an initial complication, this mycoplasma disappeared from the lung lesions on passage. It may be that in the early stages of the disease at least, the pig—when inoculated intranasally—is able to eliminate *M. hyorhinis* or confine this mycoplasma to the upper respiratory tract. In this sense, the live pig can be used as a selective medium, but in our limited experience the failure rate is high (at least 40 % on first passage); the method is also expensive and, clearly, a simple and much more uniformly reliable selective laboratory medium would be very helpful at this stage.

SUMMARY

Two strains of enzootic pneumonia, which are regularly free from mycoplasmas other than *Mycoplasma suipneumoniae*, were transmitted experimentally to a total of 22 pigs. *M. suipneumoniae* was recovered in liquid medium from the lung lesions in 20 of these animals (91 %).

Twelve field outbreaks of enzootic pneumonia were likewise examined for *M. suipneumoniae*: from each outbreak, cases were selected where many mycoplasmas with the morphology of *M. suipneumoniae* were seen in touch preparations made from the lung lesions. *M. suipneumoniae* was recovered from only two of these outbreaks, but *M. hyorhinis* was obtained from nine of them.

To establish whether the outbreaks yielding only *M. hyorhinis* were indeed enzootic pneumonia, material from five of them was used in disease-transmission experiments: enzootic pneumonia was induced in every instance, and *M. suipneumoniae* was recovered from three of the five transmitted strains.

It is concluded that *M. hyorhinis* is commonly present in the lung lesions of enzootic pneumonia and that this mycoplasma may often prevent the isolation of *M. suipneumoniae*. The problem of recovering *M. suipneumoniae* in this situation is made more difficult by the fact that in no instance where *M. suipneumoniae* was isolated directly in liquid medium was it cultured in parallel on solid medium.

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