

## Dilution Technique for Isolation of *Haemophilus* from Swine Lungs Collected at Slaughter

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A total of 307 lungs obtained from a slaughterhouse were cultured by a dilution technique for the isolation of *Haemophilus* spp. The technique consisted of performing serial (10-fold) dilutions of the tissue samples to a dilution of  $10^{-5}$ . Two selective media were used. L broth consisted of a basal brain heart infusion broth containing 5% horse serum, 5% yeast extract, and 100  $\mu\text{g}$  of NAD and 0.5  $\mu\text{g}$  of lincomycin per ml. L-B broth was identical to L broth, except 1.5  $\mu\text{g}$  of bacitracin per ml was included. The broths were incubated overnight and then plated onto blood agar. A total of 83 (27%) isolates were obtained, and both media proved to be necessary, as a proportion of isolates grew in one of the media employed but not in the other. Of the isolates, 66.3% were urease positive and most of these (98%) were classified as "minor group" strains. Urease-negative strains (27.7%) were classified as *Haemophilus parasuis*.

The isolation of *Haemophilus* spp. in swine lung samples is readily achieved from swine with acute pleuropneumonic disease (7). In such lungs, *Haemophilus pleuropneumoniae* is present in large numbers, and direct plating onto blood agar with a *Staphylococcus epidermidis* nurse colony streak yields high isolation rates (7). However, direct plating from chronic pneumonic tissue samples usually yields negative *Haemophilus* spp. isolations. According to Little and Harding (6), this phenomenon is due to an inhibitory effect caused by the concurrent presence of *Pasteurella multocida*. These authors have also described the use of a dilution technique for the isolation of *Haemophilus* spp. from field cases of chronic pneumonia.

The purpose of the present experiment was to evaluate a modified dilution technique for the isolation of *Haemophilus* spp. from swine lungs at slaughter. The technique was compared with a standard direct plating method.

### MATERIALS AND METHODS

A total of 307 lungs were obtained at two local slaughterhouses. The lungs were collected at the slaughter line without regard to the presence or absence of lesions.

**Media.** The following media were used: (i) serum broth and agar consisted of brain heart infusion broth or agar (Difco Laboratories, Detroit, Mich.) containing 5% horse serum (GIBCO Laboratories, Grand Island, N.Y.), 5% fresh yeast extract (GIBCO), and 100  $\mu\text{g}$  of NAD (Eastman Kodak Co., Rochester, N.Y.) per ml; (ii) L broth consisted of serum broth with 5  $\mu\text{g}$  of lincomycin (The Upjohn Co., Kalamazoo, Mich.) per ml; (iii) L-B broth was the same as L broth,

but with 1.5  $\mu\text{g}$  of bacitracin (Sigma Chemical Co., St. Louis, Mo.) per ml added; (iv) sheep blood agar.

**Bacteriological evaluation.** After scorching the lung surfaces with a hot spatula, a sample of ca. 1  $\text{cm}^3$  was collected and put in a tube containing 10 ml of L broth. This tube was labeled ( $10^{-1}$  dilution) and then shaken vigorously on a Vortex-Genie mixer. A 1-ml amount was then transferred to a second (9-ml) tube of L-broth and shaken. Four such dilutions were made in L broth. A fifth dilution ( $10^{-5}$ ) was then made in L-B broth.

The whole set of dilution broths was then incubated overnight at 37°C. The highest dilution of L broth showing turbidity and the L-B broth (regardless of turbidity) were then plated onto sheep blood agar with an *S. epidermidis* nurse strain and incubated overnight at 37°C.

A lung sample obtained from an adjacent area was plated directly onto sheep blood agar, a slant of *S. epidermidis* as a nurse strain was streaked at right angles, and the plate was incubated overnight at 37°C.

Colonies showing satellitism around the nurse streak were tested for urease activity, subcultured into serum broth, and frozen at  $-70^\circ\text{C}$  for further biochemical characterization. Five strains were lost upon passage and could not be characterized.

**CO<sub>2</sub>.** During the first half of the experiment, blood agar plates were inoculated in duplicate from the dilution broths; one set was incubated in an ordinary incubator under air, and the other was incubated under a partial CO<sub>2</sub> atmosphere in a CO<sub>2</sub> incubator. No difference in the isolation rates was noted when CO<sub>2</sub> was used, and this practice was discontinued.

**Characterization of isolates.** A total of 40 urease-positive and 5 urease-negative strains were further characterized by the methods proposed by Kilian et al. (2, 4) and Biberstein et al. (1). Dextrose, sucrose, lactose, xylose, and mannitol fermentations were assessed by inoculating 0.5 ml of phenol red sugar broth medium with 0.05 ml of the bacterial growth washed

TABLE 1. Isolation of *haemophilus* spp. in L and L-B broths ( $n = 307$ )

Isolates from (broth):	No. of isolates	% of lungs
L	46	15.0
L-B	62	20.2
L, but not L-B	21	6.8
L-B, but not L	37	12.1
Both L and L-B	83	27.0

from a 6-h serum agar plate. Urease and  $\delta$ -aminolevulinic acid media were seeded with a similar inoculum after they were washed and centrifuged three times in sterile phosphate-buffered saline. After overnight incubation, carbohydrate fermentation was assessed by the appropriate color change in phenol red indicator, as urease-positive by alkalization of bromthymol blue indicator, and as producing porphyrins from  $\delta$ -aminolevulinic acid (a requirement of X factor) by conversion of Kovac reagent.

Hemolysis was assessed both directly on sheep and horse blood agars and by a Camp test with a *Staphylococcus aureus* strain producing beta-hemolysin. Morphology was assessed by standard Gram staining.

## RESULTS

Although L-B broth yielded a greater number of isolates, many were recovered only from L broth (Table 1). Direct plating did not yield any positive isolations. Of the cultures, 55 (66.3%) were urease positive, 23 (27.7%) were urease negative, and 5 were not tested for urease.

The results from the characterization of the selected strains are shown in Table 2. Practically all (98%) urease-positive strains were classified as *Haemophilus* sp. taxon "minor group," and only one (2%) isolate proved to be *H. pleuropneumoniae*. The five selected urease-negative strains were classified as *H. parasuis*.

**Pathological findings.** The 83 isolates were obtained from 82 lungs; one lung yielded 2 isolates. Of these 82 lungs, 11 (13.4%) had no macroscopic lesions and yielded 8 minor group strains, 2 *H. parasuis*, and 1 untyped strain. Two lungs (2.4%) showed signs of pleuritis but not of pneumonia, 22 (26.8%) showed signs of both pleuritis and pneumonia, and the rest had macroscopic lesions of pneumonia only.

## DISCUSSION

There are few reports on the isolation of *Haemophilus* spp. from lungs with chronic lesions. Little (5), using a selective medium, reported a prevalence of 14.2% *Haemophilus parainfluenzae* (now classified as taxon minor group) and 16.7% *H. parasuis* from 120 lungs. He did not, however, isolate any *Haemophilus* spp. from lungs with no macroscopic pneumonia. The present trial demonstrated the need of

looking for *Haemophilus* spp. even in apparently healthy lungs. This will be of special relevance in epidemiological studies of the disease.

The high rate of isolation in this trial demonstrated the importance of performing a careful bacteriological study when working with chronic lesions. The fastidious nature of *Haemophilus* spp. makes the use of some selective substances in the media a necessity, as overgrowth by commensal organisms usually masks the presence of *Haemophilus* spp. The selective antibiotics used in the present study were chosen because of their low activities against *Haemophilus* spp. (5; R. A. Schultz, M.S. thesis, Iowa State University, Ames, 1981). These antibiotics proved to be useful in the majority of lungs studied, but some plates still had an overgrowth of contaminants that completely prevented isolation of *Haemophilus* spp. It is possible, therefore, that the media described above might still be improved with the addition of other selective substances; this is a point which merits further investigation.

It is also interesting that 62 isolates were recovered from the  $10^{-5}$  dilution, suggesting that the apparent failure of ordinary media and techniques in the isolation of *Haemophilus* spp. is not due to their presence in low numbers, but rather to overgrowth by less fastidious organisms. In all samples from which *Haemophilus* spp. were isolated from L-B broth only, there was extensive overgrowth in L broth. The superior performance of L-B broth could have been due to a tail-end effect of the dilution scheme, but it also seems likely that bacitracin has an important role in decreasing the overgrowth.

The high prevalence of both *H. parasuis* and minor group strains in lungs with chronic lesions suggests that these strains might be involved in the etiology of chronic swine pneumonia, probably interacting with other organisms, notably *P. multocida* and *Mycoplasma hyopneumoniae*, a view that has already been suggested (6). The detailed pathology and bacteriology of the minor

TABLE 2. Characterization of selected isolates

Test or substrate	Reaction of		
	<i>Haemophilus</i> sp. taxon minor group ( $n = 39$ )	<i>H. parasuis</i> ( $n = 5$ )	<i>H. pleuropneumoniae</i> ( $n = 1$ )
Glucose	+	+	+
Sucrose	+	+	+
Lactose	+	+	-
Xylose	-	+/-	+
Mannitol	-	+/-	-
Urease	+	-	+
Porphyrin	+	+	+
Camp	-	-	+
Hemolysis	-	-	+

group strains is being studied presently by our group and will be discussed in a later communication. There is a need for research on the role of *H. parasuis* in diseases other than polyserositis, and specifically in chronic swine pneumonia.

Another important problem is the similarity of the minor group strains to *H. pleuropneumoniae*. *H. pleuropneumoniae* is of great importance to the swine industry and is usually identified by its requirement for V factor, presence of hemolysis, and positive urease and Camp tests. Hemolysis is an unreliable characteristic of the *Haemophilus* spp., and it is not uncommon to find non-hemolytic *H. pleuropneumoniae* strains (3). Because of this, undue importance may be given to the urease test, which distinguishes *H. pleuropneumoniae* from *H. parasuis*. The minor group strains are also urease positive and require V factor but are Camp negative. Therefore, it is important that complete characterization be performed to ascertain the identity of the isolates obtained from swine lungs.

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