Pulmonary Clearance of *Bacillus subtilis* Spores in Pigs

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ABSTRACT

The pulmonary clearance rate of *Bacillus subtilis* was determined in ten pigs (23-39 kg) exposed simultaneously for 15 minutes to an aerosol generated by an ultrasonic nebulizer. Two pigs were killed at each interval of zero, two, four, eight and 12 hours and the concentrations of *B. subtilis* in lungs (all lobes), dorsal and ventral nasal turbinates, trachea, pharyngeal and bronchial lymph nodes were determined. The mean percent (± standard error) pulmonary clearance of *B. subtilis* was 54.2 ± 11.7, 53.0 ± 11.8, 77.4 ± 5.2 and 88.1 ± 3.7 at two, four, eight and 12 hours, respectively. The numbers of *B. subtilis* retained in the posterior (caudal and accessory) lobes at zero time were significantly greater than those in the anterior (cranial and middle) lobes ($P<0.05$). However, by 12 hours postinoculation the numbers of organisms retained in the two regions did not differ significantly ($P>0.05$). The mean percentage of *B. subtilis* retained by the turbinates, trachea, pharyngeal and bronchial lymph nodes varied between pigs at each time interval, but was usually less than that retained by the lungs.

It was concluded that deposition of *B. subtilis* spores took place in all parts of the respiratory tract when pigs were exposed to aerosols and that the spores were progressively cleared by the normal lung.

RÉSUMÉ

Cette expérience consistait à déterminer le taux de clairance pulmonaire de *Bacillus subtilis*, chez dix porcs dont le poids variait de 23 à 29 kg et qu'on avait soumis simultanément, durant 15 minutes, à des aérosols produits par un nébuliseur ultrasonique. On sacrifia ensuite deux porcs à la fois, aux intervalles suivants: immédiatement après la nébulisation, ainsi que deux, quatre, huit et 12 heures plus tard. On détermina ensuite la concentration de *B. subtilis* dans tous les lobes pulmonaires, les cornets nasaux dorsaux et ventraux, la trachée et les ganglions lymphatiques pharyngiens et bronchiques. Le pourcentage moyen de clairance pulmonaire s'établit comme suit: 54,2 ± 11,7; 53 ± 11,8; 77,4 ± 5,2 et 88,1 ± 3,7 au bout de respectivement deux, quatre, huit et 12 heures. Le nombre de *B. subtilis* retenus dans les lobes pulmonaires caudaux et accessoire, immédiatement après la nébulisation, se révéla sensiblement plus élevé que dans les lobes craniaux et moyen ($P<0,05$). Au bout de 12 heures, le nombre de bacilles retenus dans ces deux régions pulmonaires n'accusa cependant pas de différence appréciable ($P>0,05$). Le pourcentage moyen de *B. subtilis* retenus par les cornets, la trachée et les ganglions pharyngiens et bronchiques varia d'un porc à l'autre et à chacun des intervalles précités; il s'avéra toutefois ordinairement inférieur à celui des poumons.

L'expérience permet de conclure que le dépôt de spores de *B. subtilis* se produisit dans toutes les parties des voies respiratoires, lorsque les porcs furent soumis à des aérosols, et que les spores subirent une élimination progressive par les poumons sains.

INTRODUCTION

Although many bacteria are being increasingly incriminated in serious respiratory infections in pigs kept under intensive management (1, 2), there have been few studies undertaken of pulmonary bacterial clearance in the pig to aid understanding of the pathogenesis of these infections (3, 4). The purpose of the present study was to define some parameters of the pulmonary clearance of *Bacillus subtilis*, a nonpathogen, in young pigs to serve as a basis for possible future comparisons with bacterial respiratory pathogens.

MATERIALS AND METHODS

PIGS

Ten pigs were obtained from a local SPF herd and weighed (23-39 kg) at the start of the experiment. The pigs were kept in an isolation unit [21°C average environmental temperature, and relative humidity 62%] and fed antibiotic free feed and water *ad libitum*.

PREPARATION OF CULTURES FOR AEROSOLIZATION

A *B. subtilis* spore culture (con-

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centration about 1×10^{10} organisms/mL) was obtained from the Defence Research Board, Suffield, Alberta, and stored at 4°C until required. The culture was diluted in sterile physiological saline (SPS) (0.85% NaCl) before aerosolization. Viable counts of the organism aerosolized were determined before and after aerosolization on tryptose agar (5). The plates were incubated aerobically overnight at 37°C. Viable counts were expressed in colony forming units (CFU) and recorded as arithmetic means.

AEROSOL EXPOSURE

Ten pigs were exposed simultaneously for 15 minutes to *B. subtilis* aerosols (median particle diameter 4.2 μm)[1] generated by an ultrasonic nebulizer[2] (Fig. 1) (6). The bacterial suspension (50 ml) was placed in the nebulizer chamber and the nebulizer was operated at maximum capacity [33.9 KPa (10 p.s.i.), average aerosol output 3 mL of culture per minute]. The exposure chamber consisted of a metal frame supported polyethylene enclosed chamber measuring 107 cm wide × 122 cm long × 107 cm high (7). The nebulizer output was directed into the exposure chamber through an opening at the top of the chamber. At the end of aerosolization, all pigs were removed from the exposure chamber; two pigs (zero time) were killed immediately and the remaining eight were transferred back to the isolation unit.

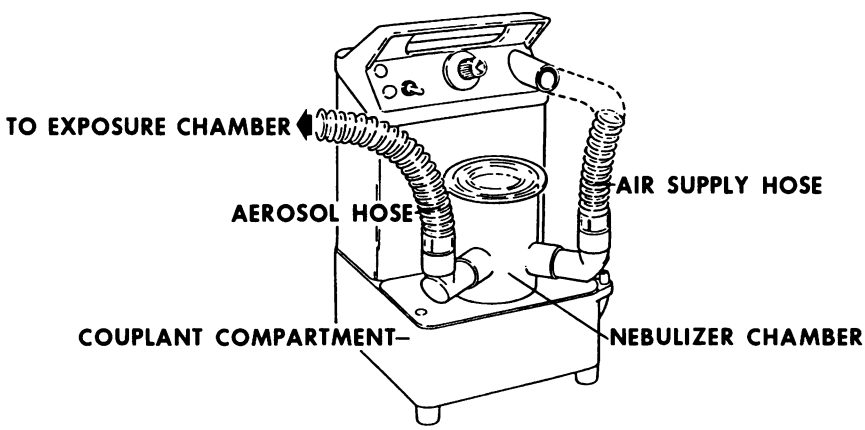

Fig. 1. Model 65 ultrasonic nebulizer.

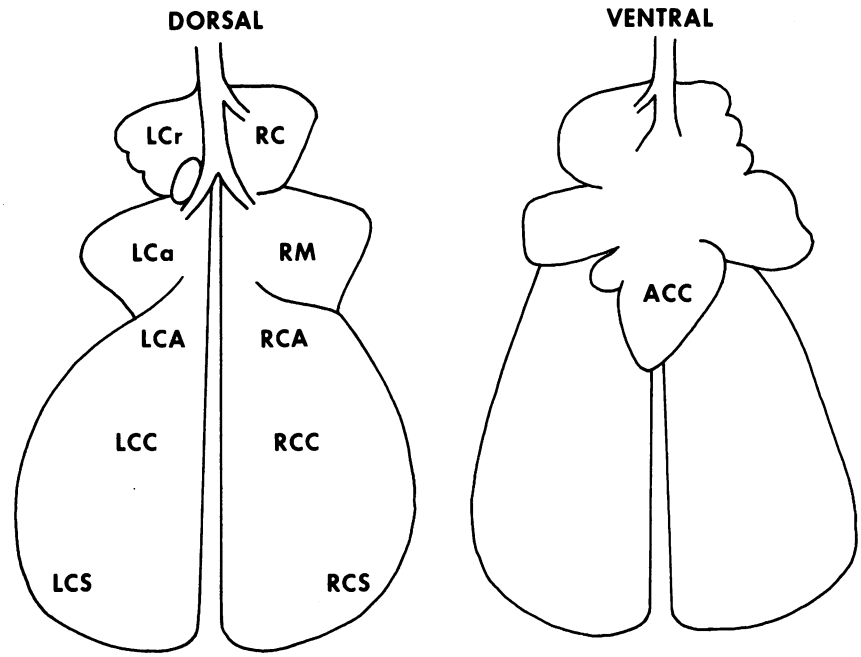

Fig. 2. Areas of pig lungs sampled at intervals following *Bacillus subtilis* aerosol exposure (zero to 12 hours).

DETERMINATION OF THE AEROSOL CONCENTRATION IN THE EXPOSURE CHAMBER

An Andersen sampler (8) was used for a period of one minute after ten minutes of nebulization in an attempt to quantitate the particle size and the concentration of *B. subtilis* in aerosols within the chamber. Glass petri dishes (100 × 15 mm) containing 27.0 mL of tryptose agar were used to culture the organism.

NECROPSY PROCEDURE

Two pigs were killed at times zero, two, four, eight and 12 hours after aerosolization by intravenous injection of pentobarbitone sodium followed by exsanguination. Standard necropsy procedures were followed and the lungs were removed aseptically and placed on aluminum trays.

QUANTITATION OF *B. SUBTILIS* IN THE PORCINE RESPIRATORY TRACT

Duplicate samples of tissue (over 1 g each) were removed from all areas of the lung (Fig. 2) (9) and from the bronchial and pharyngeal lymph nodes. One complete set of samples was processed immediately and the second one

[1]Determined by the Physics Division, Saskatchewan Research Council, on a Sierra Cascade Impactor, Carmel Valley, California.
[2]Model 65, DeVilbiss Company, Somerset, Pennsylvania.

was stored at -70°C while awaiting processing. A gram sample was weighed from each portion of lung and homogenized in a glass grinder (7 mL capacity) with 2 mL of SPS. One milliliter samples of blended suspensions were diluted from 10^{-1} to 10^{-3} and duplicate 0.1 mL samples were spread by means of a glass spreader on 100 × 15 cm plastic Petri dishes containing tryptose agar. Samples of trachea (middle third) and dorsal and ventral nasal turbinates approximately 2.5 cm in length were washed repeatedly (five times) with 2 mL of SPS which were then serially diluted, plated and incubated as before. The plates were examined after overnight aerobic incubation and plates with 30 to 300 CFU were counted.

HISTOPATHOLOGICAL EXAMINATIONS

Representative samples of lung were fixed in 10% buffered formalin, sectioned and stained with hematoxylin eosin stain and occasionally with Brown and Bren Gram method.

DETERMINATION OF PULMONARY BACTERIAL CLEARANCE

Mean (± standard error) bacterial counts at each time interval were calculated by averaging the CFU of the one gram samples taken from all regions of the lungs from both pigs. Pulmonary retention was calculated as the number of viable organisms remaining at a given time divided by the number deposited at time zero and expressed as percentage. The percentage pulmonary clearance was obtained by subtracting the percent retention from 100.

STATISTICAL ANALYSIS

The number of *B. subtilis* retained in the anterior (cranial and middle) and posterior (caudal and accessory) lobes at each time interval were compared by the Student's t-test.

RESULTS

During aerosolization, the humidity inside the chamber appeared to increase gradually to the saturation point as evidenced by the condensation on the inner side of the polyethylene sheet. However, the pigs did not appear to experience discomfort during the aerosolization period.

The amount of *B. subtilis* culture aerosolized in 15 minutes was 40 mL and the average concentration was 1.2×10^9 organisms per mL. The number of CFU seen on plates following sampling by the Andersen sampler were too numerous to count.

The deposition (zero time) of *B. subtilis* in the porcine lung averaged $(1.52 \pm 0.1) \times 10^3$ organism (± standard error) per gram of lung for the two pigs (Table I). The CFU means for duplicate lung samples, the average counts per pig and percentage retention and clearance at subsequent time intervals are also shown in Table I. The mean percentage retention of *B. subtilis* at zero time in (a) the cranial plus middle, (b) caudal and (c) accessory lobes with respect to the mean density at zero time was 82.6, 110.5 and 115.0, respectively. The numbers of *B. subtilis* retained in the posterior (caudal and accessory) lobes at zero and eight hours postinfection were significantly greater than those in the anterior (cranial and middle) lobes ($P<0.05$), but at two, four and 12 hours postinoculation the numbers of organisms retained in the two regions did not differ significantly ($P>0.05$). An initial rapid clearance of the organism (54.2 ± 11.7 at two hours) was followed by a two hour lag period in clearance (53.0 ± 11.8% at four hours). Subsequently, however, the organism was cleared progressively, but not

TABLE I. Pulmonary Clearance and Retention of *B. subtilis* in Pigs Following Aerosolization

Post exposure time (hrs)	Pig No.	Average counts per duplicate pig sample	Average counts per gram per pig (± standard error)	Average counts per gram (2 pigs)	Percentage retention per duplicate pig sample	Average % retention for 2 pigs (% cleared ± standard error)
		1.88×10^3				
0	881 (1)	1.92×10^3	$(1.90 \pm 0.1)10^3$		NA	NA
		7.30×10^2				
	886	1.63×10^3	$(1.13 \pm 0.4)10^3$	$(1.52 \pm 0.1)10^3$	NA	NA
		1.15×10^3			75.6	
2	881 (2)	8.10×10^2	$(1.00 \pm 0.1)10^3$		53.3	45.8 ± 11.7
		4.58×10^2			30.1	
	886K	3.69×10^2	$(4.10 \pm 0.4)10^2$	$(7.00 \pm 0.6)10^2$	24.3	(54.2 ± 11.7)
		9.36×10^2			61.6	
4	881K	1.10×10^3	$(1.00 \pm 0.1)10^3$		72.4	47.0 ± 11.8
		4.60×10^2			30.3	
	884 (3)	3.60×10^2	$(4.29 \pm 0.4)10^2$	$(7.20 \pm 0.4)10^2$	23.7	(53.0 ± 11.8)
		1.65×10^2			10.9	
8	884K	2.57×10^2	$(2.10 \pm 0.2)10^2$		16.9	22.6 ± 5.2
		4.98×10^2			32.8	
	879(1)	4.50×10^2	$(4.80 \pm 0.4)10^2$	$(3.50 \pm 0.3)10^2$	29.6	(77.4 ± 5.2)
		1.38×10^2			9.0	
12	879 (2)	6.18×10	$(9.86 \pm 1.2)10$		4.0	11.9 ± 3.7)
		2.01×10^2			13.2	
	884 (1)	3.25×10^2	$(2.63 \pm 0.25)10^2$	$(1.81 \pm 0.19)10^2$	21.4	(88.1 ± 3.7)

NA = Not applicable

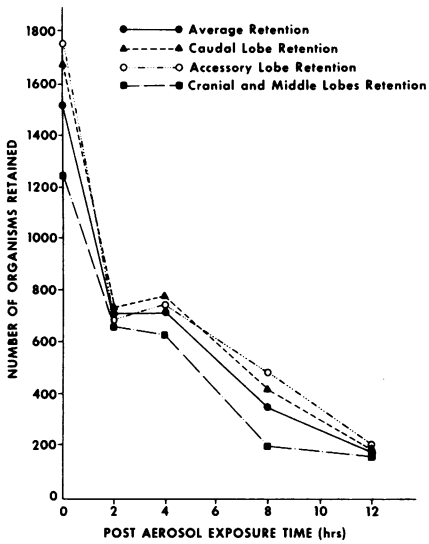

Fig. 3. Pulmonary retention of *Bacillus subtilis* by the cranial and middle, caudal and accessory lobes of pigs' lungs from zero to 12 hours.

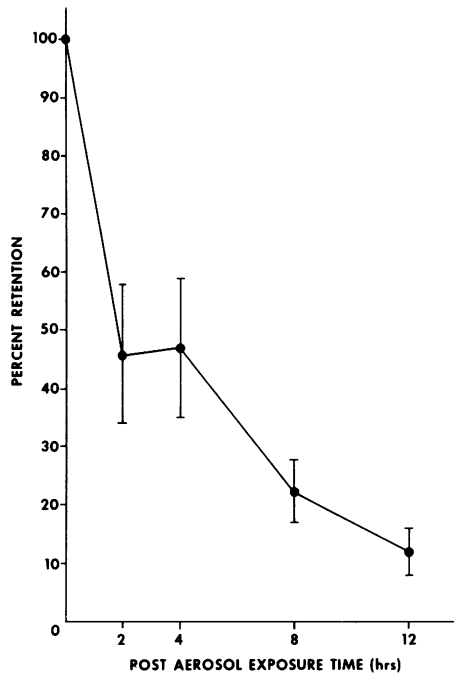

Fig. 4. The clearance of *Bacillus subtilis* by pig lungs from zero to 12 hours (± standard error).

completely, from all areas of the lung at 12 hours postaerosolization (Table I, Figs. 3 and 4).

Table II shows the number of *B. subtilis* in the various tissues sampled at specified times. The number of organisms deposited on the mucosal membrane of turbinates and trachea (zero time) varied in the two pigs but was less than the mean CFU/g retained in the lung. Few organisms were detected in the bronchial and pharyngeal lymph nodes and none in the tonsils at zero time. At subsequent intervals, the organism was often detected in the turbinates and trachea, but to a lesser extent in the pharyngeal and bronchial lymph nodes and only occasionally in the tonsils.

Grossly and histologically all the lungs appeared normal.

DISCUSSION

The pulmonary clearance of *B. subtilis* in porcine lungs was determined following aerosolization of the organism. A simple exposure system with a continuous flow of aerosol was devised that allowed natural breathing and permitted sampling of the aerosol. The DeVilbiss ultrasonic nebulizer is commonly used in human respiratory therapy (6, 10). It was, therefore, envisaged that the size of infective droplets generated by the nebulizer would, when inhaled by the pig, penetrate throughout the lung. A possible disadvantage of this equipment for generating aerosols containing microorganisms might be loss of viability in the aerosols due to disruption of the organisms by sonic energy (11). In the present experiments, the Andersen sampler proved unsuitable for quantitating viable bacteria. It was then recognized that other sampling devices such as glass impingers might be more suitable when high concentrations of bacteria were present in aerosols (12, 13).

Exposure of pigs to *B. subtilis* aerosols resulted in the desired high concentration of organisms deposited in the lung. As with these pigs, other studies have shown that calves exposed to aerosols of *B. subtilis* spores had higher counts in the lungs than on the nasal mucosa and trachea (14). Hence, it appears that in the study of porcine pulmonary infections the aerosol route is a more appropriate route of inoculation than the intratracheal or intranasal routes which have previously been used.

In these studies, the numbers of *B. subtilis* present in the lung at zero time probably reflect retention rather than deposition (15), since the data conclusively show that a few organisms had reached the bronchial lymph nodes, possibly *via* the pulmonary lymphatics (16, 17). Although the present experiments demonstrated that *B. subtilis* is cleared by the normal porcine lung in a pattern similar to that reported for other bacterial species in calves and mice (18, 19), it is realized (Figs. 3 and 4) that the slight increase of organisms between two and four hours may not be real but could be due to pig-to-pig variation with only two pigs sampled per time period. Addi-

TABLE II. Mean Colony Forming Units of *B. subtilis* in Various Tissues of Pigs Following Aerosol Exposure

	At hours postexposure and pig number									
	0 hours		2		4		8		12	
	#881(1)	886	881(2)	886K	881K	884(3)	884K	879(1)	879(2)	884(1)
Turbinates[a]	250	58	10	35	-[b]	10	50	+[c]	36	10
Tonsil[d]	—	—	48	—	+	10	—	—	—	—
Trachea[c]	140	10	190	—	10	5	200	50	65	10
Pharyngeal[d] lymph nodes	25	20	—	10	20	460	—	15	—	—
Bronchial[d] lymph nodes	—	1	—	—	10	—	—	—	30	—

[a]Mean CFU in approximately 2.5 cm of tissue
[b]Negative for *B. subtilis*
[c]Positive for *B. subtilis* but not counted due to contamination
[d]Mean CFU per gram of tissue

tional experimental animals at each point would likely result in a conventional clearance curve. However, the mean pulmonary clearance rate of *B. subtilis* in the pig was slightly slower than that of the nonpathogenic *Serratia marcescens* in calves (18). The host species and age and the bacterial species and strain are important variables in apparent pulmonary bactericidal capacity (4, 20, 21).

A simple aerosol exposure procedure was developed for the study of the pulmonary clearance of *B. subtilis* in pigs. The system allows for effective simultaneous exposure of pigs to aerosols within a short period of time. It is envisaged that this system of aerosol exposure will offer a useful alternative experimental system for the study of porcine respiratory infections.

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