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Survival of Toxigenic *Pasteurella multocida* in Aerosols and Aqueous Liquids

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The survival of toxigenic *Pasteurella multocida* in air and liquids was studied to identify possible risk factors in the etiology of atrophic rhinitis. In aerosols, at low relative humidity (28%), the viability of toxigenic *P. multocida* 5 min after aerosolization was at least 22% of its initial value. Viability at low relative humidity declined to 8% after 45 min. Viability at high relative humidity (79%) was 69% after 5 min and declined to 2% after 45 min. Survival of toxigenic *P. multocida* in liquids depended on storage and constituents in the liquid. Toxigenic *P. multocida* became nonculturable 1 to 14 days after inoculation in water and artificial seawater, depending on the storage temperature. Toxigenic *P. multocida* stored at 37°C could be detected for up to 6 days in pig slurry and more than 36 days in Bacto Tryptose broth and nasal lavages. However, in Bacto Tryptose broth and nasal lavages stored at 4°C, *P. multocida* was detected for up to 14 days whereas at 15 and 37°C it was detected for more than 49 days. These results suggest that aerosols and fomites can play a role in the transmission of atrophic rhinitis.

Severe porcine atrophic rhinitis is characterized by reduction in the size of the nasal turbinates accompanied by brachygnathia superior or twisting of the snout (5, 13, 24, 25, 28, 32). Atrophic rhinitis is a multifactorial disease involving herd management, environment, heredity, and infectious agents, including *Bordetella bronchiseptica* and *Pasteurella multocida*. The ability of *P. multocida* to cause atrophic rhinitis is associated with production of a dermonecrotic toxin (11, 18). Cell extracts of toxigenic *P. multocida* used to inoculate pigs intranasally (18) or intraperitoneally (30) or intraperitoneal inoculation of purified toxin (6) reproduced atrophic rhinitis. However, gnotobiotic pigs infected with toxigenic *P. multocida* or *B. bronchiseptica* alone developed only mild turbinate damage, while combined inoculation with both pathogens resulted in severe atrophic rhinitis. The important difference in these experiments was that much greater numbers of toxigenic *P. multocida* organisms were present in the nasal cavities of pigs (26, 29, 31). The requirement for *B. bronchiseptica* to predispose pigs to infection with toxigenic *P. multocida* can be replaced by previous treatment of the nares with a mild chemical irritant (27). However, the natural routes of inoculation and transmission between pigs are unknown. If transmission is other than by direct contact, then it must be by the airborne route and/or by contact with fomites. Toxigenic *P. multocida* has been detected in the air of piggeries (3), but the ability of these bacteria to survive in aerosols or aqueous liquids is not known.

In this report, we describe the survival of *P. multocida* after aerosolization over a range of relative humidities and in various aqueous liquids. The ability of *P. multocida* to survive in these media can indicate the likelihood of aerosols or specific fomites as transmission routes in the spread of atrophic rhinitis.

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MATERIALS AND METHODS

Bacterial strains. Toxigenic *P. multocida* LFB3 and nontoxigenic *P. multocida* P37 were isolated from pigs with or without disease, respectively. *Bacillus subtilis* subsp. *niger*, in the endospore form, was used to estimate physical losses in the aerosolization study (2, 9).

Collection of nasal lavage. Nasal washings were collected from piglets 2 weeks after weaning by introducing approximately 10 ml of distilled water into one nostril and collecting the mucus-water mixture from the other nostril. The washings were then pooled and sterilized by autoclaving and diluted by half in distilled water.

Preparation of *P. multocida* for aerosolization. LFB3 and P37 were grown for 18 h on 5% horse blood agar (HBA) at 37°C. Bacteria were then suspended in distilled water or diluted nasal washings at approximately 10^{10} CFU/ml. A *B. subtilis* subsp. *niger* stock culture, stored at 4°C, was heated to 60°C for 30 min to stimulate sporulation. Endospores were added to *P. multocida* suspensions at a sample-to-tracer ratio of 100:1.

Recovery and enumeration of *P. multocida* organisms from aerosols. The number of viable organisms in each sample was determined on 5% HBA and on selective medium containing 5% HBA with neomycin, actidione, and bacitracin by using a modification of the method of Miles and Misra, and the weighted mean count was calculated (7).

Microbial survival in air. The survival of *P. multocida* was examined in air at four relative humidities and in distilled water and nasal lavage fluid. Suspensions of *P. multocida* were aerosolized by using a Henderson apparatus (12) with a Collison triple-jet nebulizer (20). The aerosol was then stored in a 75-liter rotating drum (14) for 45 min. Aerosol samples were collected at 1 s and 3, 7, 12, 17, 24.5, 32, and 47 min after aerosolization by using Porton AGI30 impingers containing phosphate-buffered saline. Phosphate-buffered saline was also used to make serial dilutions to enumerate bacteria. Physical losses within the Henderson apparatus were calculated from the reduction in numbers of *B. subtilis* subsp. *niger* organisms (2).

Each treatment (four humidities and two suspension flu-

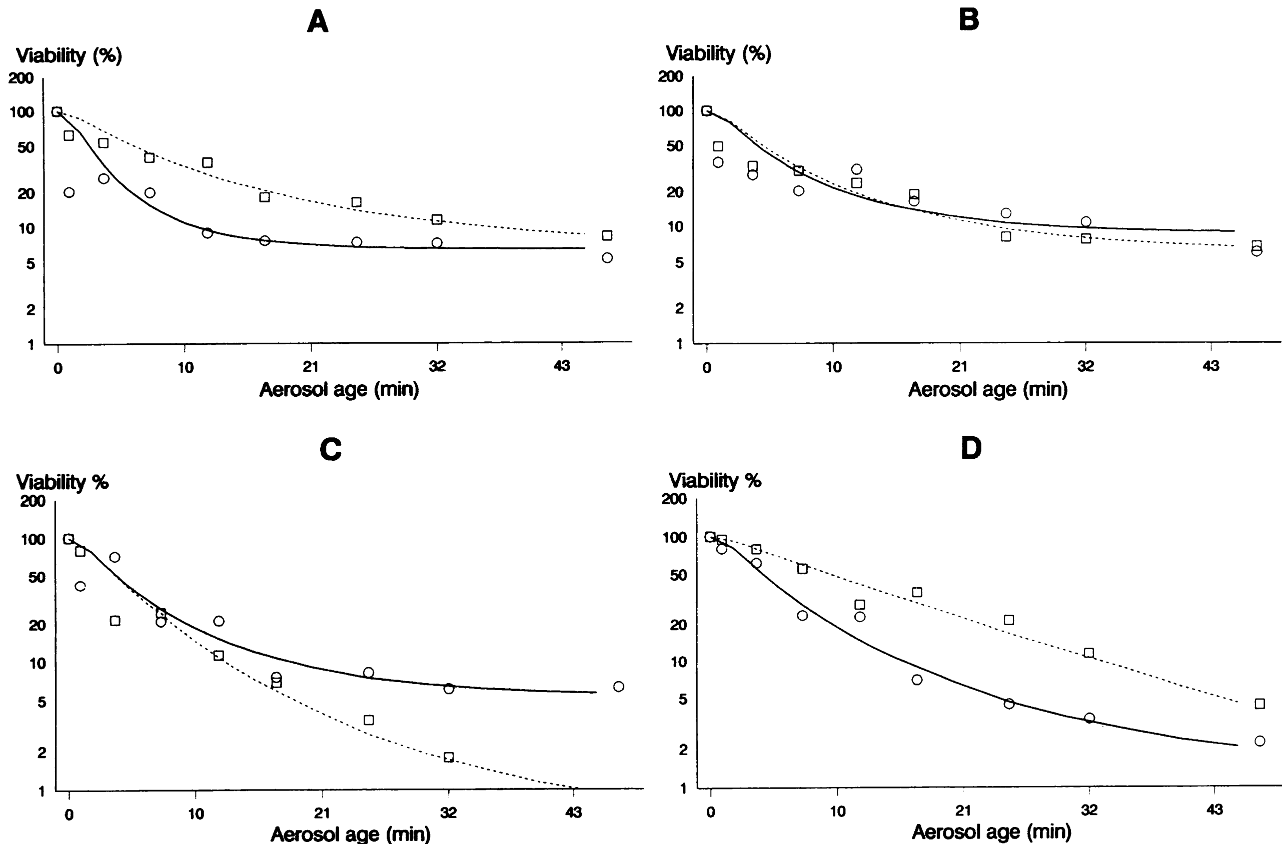

FIG. 1. Mean viability of *P. multocida* in air at four relative humidities when suspended in either water (○) or nasal washings (□). The lines represent best-fit equations calculated from a theory of microbial A, 28% relative humidity; B, 40% relative humidity; C, 59% relative humidity; D, 79% relative humidity.

ids) was replicated three times. Nominally, the relative humidities were 25, 40, 60, and 80%, with actual means of 28, 40, 59, and 79%, respectively. The mean temperature of the experiments was 22.6°C. The data were analyzed by using a theory of microbial inactivation kinetics (9).

Survival in liquids. The survival of *P. multocida* in liquids was examined at 4, 15, and 37°C in each of five aqueous liquids: distilled water, instant ocean (concentration, 33.3 g/liter; Aquarium Systems) Bacto Tryptose broth (Difco), sterile pig slurry (composition: feces, urine, water, and a minimal trace of straw bedding), and sterile nasal washings. *P. multocida* was grown on 5% HBA for 18 h at 37°C. The cells were suspended in phosphate-buffered saline at approximately 10^9 CFU/ml. This suspension was then used to inoculate 50 ml of each liquid at 10^7 CFU/ml. The different cultures were then sampled at regular intervals. The number of viable bacteria was estimated by serial dilution of the sample in phosphate-buffered saline as described above.

In a preliminary investigation of the survival of *P. multocida* in nasal lavages allowed to dry, 50 ml of nasal lavage fluid was inoculated with *P. multocida* at 10^7 CFU/ml, placed in a 250-ml beaker, and incubated at 37°C. The lavage fluids were then sampled at 0, 2, 3.5, 7.5, and 24 h, and the numbers of viable bacteria were estimated as described above.

Bacto Tryptose broths were inoculated with toxigenic *P. multocida* at 10^7 and 10^2 CFU/ml and incubated at 37°C for 3 days. Supernatants from these cultures were added to fresh Bacto Tryptose broths inoculated with toxigenic *P. multocida* at 10^2 and 10^7 CFU/ml. The broths were then sampled at 0.8 h and 1, 2, 3, 6, and 8 days, and the number of viable bacteria was estimated as described above.

RESULTS

Survival in air. An initial sharp decrease in viable bacteria was observed in the first 10 min after aerosolization, followed by a much slower rate of decline as the aerosol aged. Typically, the viability was 80% at an age of 1 min, decreasing to about 5% at 45 min. This pattern was similar for all four humidities and both suspension fluids (Fig. 1).

Statistical analysis (paired *t* test) of the data showed that at 28, 40, and 59% humidity there was no significant difference in the survival of *P. multocida* suspended in water or nasal washing aerosols. However, once the aerosol age reached approximately 17 min at 79% humidity, survival of toxigenic *P. multocida* in aerosols of nasal washing was significantly greater than in aerosols of water.

The viability of toxigenic *P. multocida* was affected slightly by the relative humidity of the air. As humidity decreased, the initial rate of decline of viable bacteria increased for aerosols suspended in water. However, as the aerosol aged, the differences in viability at the various relative humidities became smaller such that at 45 min they were statistically insignificant (analysis of variance) and predicted viabilities ranged between 2 and 9%. This implies that a substantial minority of the original population of airborne cells was able to survive for comparatively long

TABLE 1. Estimated parameters of the viability of toxigenic *P. multocida* aerosolized in water[a]

Mean relative humidity (%)	Mean ± SEM of parameter:		Residual mean square	Predicted % viability at aerosol age (min) of:		
	a	*b*		1	5	45
28	−9.7 ± 5.43	2.8 ± 0.38	0.39	65.8	21.8	6.4
40	−5.9 ± 4.80	2.5 ± 0.60	0.30	79.1	38.5	8.9
59	−5.1 ± 1.80	2.9 ± 0.37	0.17	78.3	36.1	5.7
79	−2.9 ± 0.55	4.4 ± 0.43	0.05	81.1	39.3	2.1

[a] The fitted equation was $V = e^{b(e^{at} - 1)}$, where a is the rate constant of the inactivation process, b is a parameter that is proportional to the mean concentration of an essential chemical moiety, V is the microbial viability (in percent; assumed to be 100% at an aerosol age of 0 h), and t is aersol age in hours.

periods. A similar pattern of change in viability as the aerosol age increased was observed with nontoxigenic *P. multocida* (data not shown).

Data from the three replicates were pooled and fitted to an equation derived from a first-order probability theory by using a method suitable for a generalized nonlinear model (9). An initial viability of 100% was assumed. Estimates of the parameters and their standard errors and the predicted viabilities for particular aerosol ages are shown in Tables 1 and 2. The overall fits to the data were satisfactory, as shown by the residual mean squares, but were comparatively poor for ages of less than 3 min.

P. multocida may have been sublethally injured by aerosolization, since we observed that petite colonies accounted for about one-third of the number of bacteria growing on HBA. Incubation for another 24 h at 37°C allowed the small colonies to grow to normal size. Subculture of petite colonies also resulted in normal-sized colonies. Sublethal injury was also demonstrated with selective media. Smaller numbers of bacteria grew on 5% HBA containing neomycin, actidione, and bacitracin.

On several occasions at high relative humidity, viabilities of greater than 150% were obtained with the first few samples, although the pre- and postspray samples showed no difference in the sample-to-tracer ratio. This may have been due to loss of viability of the tracer.

Survival in liquids. The survival of LFB3 in different liquids is shown in Table 3. *P. multocida* LFB3 declined in instant ocean and distilled water at a faster rate at higher temperatures. For example, when stored at 4°C it could be recovered for up to 14 days but at 37°C bacteria could not be cultured after 24 h. Conversely, in sterile pig slurry at the higher temperatures LFB3 survived slightly longer; for example, at 4°C it could not be cultured after 3 days but at 37°C viable LFB3 was detected for up to 6 days.

TABLE 2. Estimated parameters of the viability of toxigenic *P. multocida* aerosolized in nasal washings[a]

Mean relative humidity (%)	Mean ± SEM of parameter:		Residual mean square	Predicted % viability at aerosol age (min) of:		
	a	*b*		1	5	45
28	−3.1 ± 0.97	2.7 ± 0.42	0.05	87.1	54.1	8.6
40	−4.4 ± 1.76	2.8 ± 0.44	0.14	81.7	42.3	6.7
59	−2.7 ± 0.76	5.4 ± 0.80	0.13	78.8	34.3	1.1
79	−0.2 ± 0.20	9.4 ± 15.49	0.03	92.6	68.9	4.5

[a] See the footnote to Table 1 for a description of the terms listed.

TABLE 3. Survival of toxigenic *P. multocida* in various liquids

Liquid and temp (°C)	Log_{10} CFU/ml after storage time (days) of:								
	0	1	2	3	6	8	14	28	36
Distilled water									
4	7.6	6.2	5.7	4.9	3.1	1.8	0		
15	7.0	5.7	4.1	3.4	2.4	0			
37	7.1	0							
Bacto Tryptose broth									
4	7.8	7.7	7.5	7.1	6.1	5.6	5.1	0	
15	6.8	7.5	8.6	7.3	6.9	6.7	6.5	7.4	7.4
37	7.8	6.1	6.8	7.1	7.4	7.2	6.9	6.1	5.5
Instant ocean									
4	7.8	6.7	6.5	6.0	5.0	4.2	1.2	0	
15	6.9	3.9	0						
37	7.7	0.3	0						
Slurry									
4	7.8	7.5	6.4	4.8	0				
15	7.0	6.6	4.7	4.3	0				
37	7.5	6.9	7.5	6.4	4.3	0			
Nasal washings									
4	7.9	7.9	7.8	7.9	7.2	6.8	5.2	1.8	0
15	7.0	7.2	7.2	7.1	6.4	7.0	7.3	7.4	7.5
37	7.7	6.9	4.9	6.1	5.7	5.5	4.4	4.6	5.1

The survival of LFB3 in Bacto Tryptose broth was also greatest at the higher temperatures tested. At 4°C it could not be cultured after 14 days, but at 37°C it remained viable for more than 49 days. In one experiment at 15°C, the suspension was viable for more than 155 days, at which stage there were still 2×10^5 CFU/ml. Similarly, in sterile nasal washings, the survival of LFB3 was greatest at the higher temperature. At 4°C it could not be cultured after 28 days, but at 37°C it remained viable for more than 49 days. Survival of LFB3 in unsterilized nasal washings showed the same pattern as in sterile nasal washings, although after 21 days it became difficult to detect colonies of *P. multocida* because of growth of the background flora. The presence of nontoxigenic type D or A *P. multocida* naturally present in nasal washings did not appear to affect the survival pattern of toxigenic *P. multocida* (data not shown). In a preliminary investigation of drying of nasal lavages, toxigenic *P. multocida* declined rapidly and was not recovered 24 h after inoculation and the start of drying.

Addition of culture supernatant from either a culture that had undergone logarithmic growth or a culture that had been inoculated at 10^7 CFU/ml and had slowly declined to 10^2 CFU/ml after 3 months had no effect on the survival pattern. Conversely, addition of fresh nutrients to a culture that had declined to 10^2 CFU/ml resulted in fresh growth.

DISCUSSION

P. multocida has traditionally been considered a fragile organism because it does not survive particularly well during routine laboratory practice unless subcultured regularly. However, in our experiments *P. multocida* survived for relatively long periods in both liquids and aerosol.

The survival of airborne microorganisms is governed by many physical and biochemical factors, including conditions

of growth, methods of aerosol generation and collection, and climate, gaseous atmosphere, and radiation (see reference 33 for a review). Stresses arising during aerosol sampling can make assessment of the viable catch inaccurate because of lethal effects of sampling per se. Half-lives of microbial aerosols range from seconds to hours, depending on the species and the determinants of viability. By this comparison, therefore, toxigenic *P. multocida* is a robust organism similar to other gram-negative bacteria (9).

Physical forces involved in collection of the sample may play an important part in determining the numbers of bacteria recovered. No one aerosol sampler mimics the physical and biochemical environment of the respiratory tract in all its essential features. In particular, aerosols are not warmed to 37°C or subjected to vapor phase rehydration over a period of several seconds, while a gentle landing on nutritious mucous surfaces is not provided in conventional samples, e.g., the Porton AGI30 used here. Taken together, these and other limitations of aerosol samplers can alter the apparent viability of airborne microbes and therefore assessment of the risk of airborne transmission.

Viabilities of greater than 100% have also been observed by Anderson (1) working with *Escherichia coli*, who attributed this anomaly to bacterial clumping or batch variation of *B. subtilis*. Similar results obtained in our study may also have been due to endospores settling from the aerosol during storage in the drum and adhering to the surface of the spray tube or inactivation. The latter explanation seems unlikely, because endospores have been shown to be very resistant to the normal effects of aerosolization (23). The ideal tracer for studies has yet to be discovered, although a number of alternatives have been examined (2, 9).

The fit of the data to the equation based on first-order inactivation kinetics was satisfactory, except for the observations at 1 s, which were consistently lower than the predicted values. The reason for this is unclear. Cox's (9) theory of the first-order inactivation kinetics contains two parameters, a and b. Parameter a represents the rate constant of the inactivation process, which was affected by humidity. Parameter b represents the mean concentration of a chemical moiety on which microbial survival depends. Aerosolization results in a series of hydrates of this moiety which are irreversibly denatured by a first-order process. Constancy of the estimates of b provides support for Cox's theory of microbial inactivation. The values of b at the highest relative humidity had large standard errors, perhaps because of failure of the endospore to act as an efficient tracer (vide infra).

Suspension fluid appeared to have no effect on the eventual survival of *P. multocida* in aerosols, except at high humidities. Relative humidity appeared to affect the initial decline in viability because the decline of *P. multocida* was similar to that observed with *E. coli* (8). However, a higher proportion of the population of *P. multocida* was found to be more stable at low relative humidity than was *E. coli*. As a general rule, gram-negative bacteria aerosolized in air are less stable at low relative humidity than at high relative humidity, because of the toxic effect of oxygen on airborne bacteria.

In general, the composition of the suspension fluid affects the survival of airborne microbes (9). Upon aerosolization, the concentration of solutes increases as water is lost through dehydration, so that some solutes may eventually become toxic (4). Additionally, substances such as chloride ions can displace water or take up water sites in some proteins (21, 22). The ability of a compound to displace bonded water appears to correspond to its toxicity for airborne cells. Desiccation of bacteria during freeze-drying—a process somewhat akin to aerosolization of dry powders—results in leaky, repairable membranes, and prolonged storage produces additional inactivation of the membrane of dehydrated bacteria (19). The lethal effects have been attributed to amino-carbonyl reactions between the cell membrane proteins and reducing sugars (10). Removal of protective water molecules enhances such reactions, while addition of compounds such as nonreducing sugars can protect by reversibly competing for binding sites for reducing sugars. However, disulfide bonds which can also form between cell membrane proteins during storage are essentially irreversible, adversely affecting the structure of the cell membrane. Denaturation of the surface protein and lipoprotein of *E. coli* occurs to its greatest extent at high relative humidities (9). *P. multocida* appears to follow the same pattern of change in viability, so it is likely to be due to the same denaturation process. Consequently, there may be a protective compound in nasal washings which enables *P. multocida* to survive longer at high relative humidity, even though nasal washings, like other secretions, must contain isotonic salts.

The presence of petite colonies of *P. multocida* suggests that *P. multocida* was sublethally injured by aerosolization. Injured bacteria may have an absolute peptide requirement for repair and growth, such as that observed for *Streptococcus lactis* and *S. faecalis*. Stressed bacteria may have different biochemical requirements for growth or be more susceptible to selective agents (9). Consequently, selective agents may inhibit growth of aerosolized bacteria because of cell membrane or lipoprotein-lipopolysaccharide damage. Another aspect to consider is the time needed for repair of particular mechanisms, which may require several minutes to upwards of 6 h for completion (19). Osmotic pressure of the collection fluid must also be considered to prevent osmotic lysis of bacteria which are already stressed by the airborne state.

Survival of toxigenic *P. multocida* in liquids appeared to depend on nutrient limitation and temperature. Experience in this laboratory has shown that cultures of *P. multocida* grown in commercially available liquid or solid medium do not survive longer than 7 to 10 days at 4°C. However, toxigenic *P. multocida* survived for several months in Bacto Tryptose broth at 15 and 37°C. Addition of culture supernatant from either a culture that had or had not undergone logarithmic growth had no effect on the survival pattern. This discounts the possibility that the presence of dermonecrotic toxin or a metabolic byproduct was toxic to *P. multocida* growth.

The ability of *P. multocida* to survive in all liquids also depended on the storage temperature. The only consistent pattern observed from the data was that water and instant ocean were lethal as the temperature increased, while suspension fluids containing nutrients were protective. The reason for this is unclear, although temperature may affect the ability of *P. multocida* to utilize certain constituents in the growth medium.

This study has shown that toxigenic *P. multocida* can survive the stresses of aerosolization extremely well. Consequently, inadequate ventilation, combined with intensive stocking of pig units, may result in spread of infection by the aerial route in pig houses. Toxigenic *P. multocida* also survives well in liquids, thus implicating other routes of transmission, e.g., fomites contaminated with nasal mucus. However, subsequent desiccation of fomites contaminated

with nasal mucus greatly reduces the risk of spread of infection. Contagion may not be the only route of transmission. For example, herds free from enzootic pneumonia commonly become reinfected without introduction of infected pigs (15), whereas no herd in the Pig Health Control Association checked for the absence of atrophic rhinitis has yet developed the disease (17). This indicates the relative likelihood of indirect transmission of *Mycoplasma hypopneumoniae* and toxigenic *P. multocida*. Indirect transmission of atrophic rhinitis has, however, been suggested in one herd (16).

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