Effects of Ammonia Inhalation and Acetic Acid Pretreatment on Colonization Kinetics of Toxigenic Pasteurella multocida within Upper Respiratory Tracts of Swine

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Pigs reared in intensive production systems are continuously exposed to ammonia released by the microbial degradation of their excrement. Exposure to this gas has been shown to increase the severity of the disease progressive atrophic rhinitis by facilitating colonization of the pig's upper respiratory tract by Pasteurella multocida. The etiological mechanism responsible for this synergy was investigated by studying the colonization kinetics of P. multocida enhanced by ammonia and comparing them with those evoked by an established disease model. Three-week-old Large White piglets were weaned and allocated to five experimental groups (groups A to E). Pigs in groups A and B were exposed continuously to ammonia at 20 ppm for the first 2 weeks of the study. Pigs in group C were pretreated with 0.5 ml of 1% acetic acid per nostril on days −2 and −1 of the study. On day 0 all the pigs in groups A, C, and D were inoculated with 1.4 × 10⁸ toxigenic P. multocida organisms given by the intranasal route. The kinetics of P. multocida colonization were established by testing samples obtained at weekly intervals throughout the study. The study was terminated on day 37, and the extent of turbinate atrophy was determined by using a morphometric index. The results of the study showed that exposure to aerial ammonia for a limited period had a marked effect on the colonization of toxigenic P. multocida in the nasal cavities of pigs, which resulted in the almost total exclusion of commensal flora. In contrast, ammonia had only a limited effect on P. multocida colonization at the tonsil. The exacerbation of P. multocida colonization by ammonia was restricted to the period of ammonia exposure, and the number of P. multocida organisms colonizing the upper respiratory tract declined rapidly upon the cessation of exposure to ammonia. During the exposure period, the ammonia levels in mucus recovered from the nasal cavity and tonsil were found to be 7- and 3.5-fold higher, respectively, than the levels in samples taken from unexposed controls. Acetic acid pretreatment also induced marked colonization of the nasal cavity which, in contrast to that induced by ammonia, persisted throughout the time course of the study. Furthermore, acetic acid pretreatment induced marked but transient colonization of the tonsil. These findings suggest that the synergistic effect of ammonia acts through an etiological mechanism different from that evoked by acetic acid pretreatment. A strong correlation was found between the numbers of P. multocida organisms isolated from the nasal cavity and the severity of clinical lesions, as determined by using a morphometric index. The data presented in the paper highlight the potential importance of ammonia as an exacerbating factor in respiratory disease of intensively reared livestock.

Livestock reared in intensive housing systems are continuously exposed to high concentrations of aerial pollutants in the form of organic dust, noxious gases, microorganisms, and bacterial endotoxins (26). The effects of these pollutants on the health, welfare, and productivity of livestock are as yet largely unknown (12, 20, 31). Epidemiological surveys have shown an association between the severity of atrophic rhinitis, an infection of the upper respiratory tract of swine, and the level of aerial pollutants within the buildings in which the pigs were reared (2, 26). Our previous experimental studies (15) have begun to explore the mechanisms responsible for this association.

Atrophic rhinitis is a contagious disease of the upper respiratory tract of swine. The disease is characterized clinically by atrophy and degeneration of the bony and cartilaginous structures of the upper airways, in particular, the nasal turbinates and septum. In severe cases the pig's snout becomes visibly shortened or twisted (10). The disease is associated with reduced daily weight gain, decreased feed conversion efficiency, and an increase in the time taken to reach finishing weight (14, 18, 24). The etiology of this disease is complex, with toxigenic strains of Bordetella bronchiseptica and Pasteurella multocida types A and D implicated as primary etiological agents in experimental studies (8, 16, 22, 28). The more severe form of the disease is attributed specifically to colonization of the pig's nasal cavity by toxigenic strains of P. multocida and is known as progressive atrophic rhinitis (22, 28, 32). Purified toxin from these strains of P. multocida causes turbinate atrophy when the toxin is administered to pigs by either aerosol inhalation or parenteral injection (4). Nevertheless, it has proven to be difficult to reproduce the clinical disease experimentally in pigs without either depriving them of passive immunity by withholding colostrum (21) or pretreating their nasal cavity with a mild irritant such as 1% acetic acid prior to bacterial challenge (23).

In a previous paper (15) we identified a synergistic role of ammonia in facilitating colonization of the pigs' upper respi-
ratory tract by *P. multocida*, thereby contributing to the severity of the clinical disease atrophic rhinitis. However, the basis of this synergy is as yet unclear. Ammonia could facilitate colonization by influencing one or more of the following mechanisms: (i) the mucosal defenses of the pig’s upper respiratory tract, (ii) the normal commensal flora, or (iii) the growth or (iv) the virulence of *P. multocida*. The aim of the current study was to explore further these alternative possibilities by comparing the colonization kinetics of *P. multocida* within the upper respiratory tracts of pigs exposed to ammonia at 20 ppm with those induced by acetic acid pretreatment (23).

MATERIALS AND METHODS

Animals. Thirty minimal-disease piglets were derived from three Large White sows obtained from the Institute of Animal Health, Compton Laboratory, Newbury, England. The piglets were weaned at 3 weeks of age and were randomly assigned to five experimental groups (groups A to E). The number of pigs in each group was as follows: group A, n = 6; group B, n = 6; group C, n = 7; group D, n = 7; group E, n = 4). Each group was housed in a separate Rochester exposure chamber.

Exposure chambers. The exposure chambers were 1.4-m³ stainless steel Rochester exposure chambers built to the design of Timbrell et al. (33). Each chamber was operated at negative atmospheric pressure (−70 kPa), giving 60 air changes per hour. Air was drawn into the chamber via a HEPA filter and, after traversing the chamber, was vented via a HEPA filter to prevent the release of biological material. With the chamber the air was maintained at a temperature of 25 ± 1.0°C and at a relative humidity of 50% ± 5%. Ammonia from a cylinder containing compressed gas (BOC Special Gases, London, United Kingdom) was introduced into the air inlet pipe of the chambers when required. The concentration of ammonia within the chamber breathing zone was maintained at 20 ppm (±2 ppm) by using a regulator and a flow tube (BOC Special Gases) and was measured twice daily with gas diffusion tubes (Ammonia 5A/20501; Drager, Lubeck, Germany).

Experimental protocol. In the exposure chambers the groups of pigs were treated as follows. Pigs in groups A and B were exposed to air containing gaseous ammonia at a concentration of 20 ppm from day −3 until day 10 of the experiment. Five days after ammonia exposure commenced (day 0) all the pigs in group A were given a bilateral intranasal inoculum containing 1.4 × 10⁶ CFU of *P. multocida*. The pigs in group C were given 1 ml of 1% acetic acid into both nostrils on days −2 and −1 of the study, followed on day 0 by a bilateral intranasal inoculum containing 1.4 × 10⁶ CFU of *P. multocida*. The pigs in group D were given a bilateral intranasal inoculation of 1.4 × 10⁶ CFU of *P. multocida* on day 0. Pigs in the control group (group E) received no chemical or bacterial challenge.

At weekly intervals throughout the study, commencing on day −5, nasal lavage and tonsillar biopsy specimens were taken from all of the pigs. To facilitate these procedures the pigs were anesthetized with sodium thiopentone, which was administered into the brachial vein at a dose of 20 mg/kg. The study was concluded on day 10, when the pigs were sacrificed and postmortem examination for macroscopic signs of disease were noted. The snout was removed by making a transverse cut at the level of the second premolar (17) with a band saw and were fixed by immersion in 10% neutral buffered formalin for 1 week.

Results. A toxigenic isolate of *P. multocida* (strain LFB3) from *B. bronchiseptica* and *B. bronchiseptica* isolated on their respective media and confirmed as *P. multocida*. The recovered fluid was cultured at 37°C overnight on HBA and presumptive *P. multocida* organisms on the NAB plates and presumptive *B. bronchiseptica* organisms on the CH plates were identified by colony morphology and counted, and the identities of representative colonies were confirmed with API 20E and API 20 NE test strips (API-BioMerieux, Basingstoke, Hampshire, United Kingdom), respectively.

Toxin testing of *B. bronchiseptica* and *P. multocida*. Representative colonies of *P. multocida* and *B. bronchiseptica* isolated on their respective media and confirmed as the API 20 E and API 20 NE strips, respectively, were subjected to the cytotoxic toxin test by an agar overlay technique on embryonic bovine lung cells (5).

Snout sections. After fixation in 10% formalin, the pig snouts were sectioned transversely at 5-mm intervals with a band saw. Snout sections cut at the level of the second premolar teeth were viewed under a dissecting microscope (MSB; Wild, Heerbrugg, Switzerland) linked to a computerized image analysis system (VIDIS III; Analytical Measuring Systems, Oxford, United Kingdom). A morphometric index of atrophy was calculated on the basis of the area of the nasal cavity to that of the ventral turbinate (11).

Ammonia concentration in mucus coating the upper respiratory tract. Mucus samples were collected on day 3 of the experiment by using ear, nose, and throat swabs (Medical Wire Company, Corsham, Eng, mod). Samples were obtained from the ventral meatuses of the left and right nasal cavities and from the tonsils of all pigs. Immediately prior to the swabs were washed in distilled water, dried in an oven at 110°C, and preweighed to a precision of 0.1 mg. Sampling was commenced at the outside of the exposure chamber by positioning the pig’s snout in an air lock fitted with a rubber diaphragm which sealed around the pig’s head. This system avoided contamination of the swab by the ammonia in the chamber air. Care was taken to ensure that sampling was conducted with a minimum of delay, previous experience having shown that the levels of ammonia in mucus decline rapidly once exposure ceases (unpublished data). After sampling, each swab was immediately immersed in 1 ml of distilled water in the swab tube (preweighed), and the tube and the swab were weighed. The concentration of ammonia in the water was determined with a combination pH electrode (CE7L; Russell pH, Aucktembury, Scotland).

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Statistics. Statistical analysis of the data was performed by using a general linear model of variance (GLM), one-way analysis of variance (AV), and regression analysis (RA).

Results. Clinical signs. Throughout the experiments all animals retained a healthy appetite and exhibited normal behavior. Clinical signs of disease were restricted to sporadic sneezing by some of the pigs inoculated with *P. multocida*. Macrophscopic findings. Intranasal inoculation of *P. multocida* in the absence of either acetic acid pretreatment or ammonia exposure (group D) resulted in a mean ± standard deviation (SD) morphometric index of 43.28 ± 2.28 and failed to induce any macroscopically detectable changes in the architecture of the nasal cavity compared with that of the noninfected control group (group E). In contrast, intranasal inoculation of *P. multocida* into pigs either pretreated with acetic acid (group C) or exposed to ammonia (group A) resulted in a significant level of turbinate damage, as assessed by the morphometric index (P < 0.05 [GLM]). The severity of damage was greatest in the group pretreated with acetic acid (group C) with a mean ± SD morphometric index of 96.57% ± 2.90% compared to a mean ± SD morphometric index of 52.00% ± 5.00% for the group exposed to ammonia (group A). There was no statistical difference between the control group (group E) or the group exposed to ammonia alone (group B), with morphometric indices of 42.50% ± 4.76% and 42.14% ± 3.14%, respectively.

Exposure to ammonia in the absence of *P. multocida* inoculation had no detectable effect on the architecture of the nasal cavity.

Bacteriological findings. Figure 1 presents the numbers of *P. multocida* organisms recovered from the upper respiratory tract of all pigs.
tracts of the pigs infected by using three different regimens (groups A, C, and D). In the absence of any chemical challenge, intranasal inoculation of \textit{P. multocida} induced only transient colonization (2 weeks) of both the tonsil and the nasal mucosa to approximately $10^2$ CFU g$^{-1}$ (or CFU ml$^{-1}$), which then declined to undetectable levels (below 10 CFU g$^{-1}$ or CFU ml$^{-1}$) (Fig. 1A).

In contrast, acetic acid pretreatment (Fig. 1B) resulted in colonization of the nasal mucosa to approximately $10^5$ CFU g$^{-1}$ or CFU ml$^{-1}$, which persisted throughout the 6-week study. Colonization of the tonsil reached a peak at about 2 weeks following challenge, before declining to undetectable levels by week 5 of the study (Fig. 1B).

For pigs inoculated with \textit{P. multocida} and exposed to gaseous ammonia (Fig. 1C), colonization within the nasal cavity was about the same level as that for pigs in which \textit{P. multocida} colonization was induced by acetic acid treatment, but the numbers declined after 2.5 weeks, becoming undetectable by week 5 of the study. This treatment induced a very low level of colonization of the tonsil by \textit{P. multocida} during weeks 1 and 2 of the study only (Fig. 1C).

A direct correlation was found between the level of pathogenic challenge (i.e., the mean area under the graphs in Fig. 1) and the severity of clinical lesions, as measured by the morphometric index ($R^2 = 0.950$, $P < 0.05$ [RA]). Statistical analysis showed that acetic acid pretreatment had a significant effect on the numbers of \textit{P. multocida} organisms isolated from the nasal cavity at all time points and from the tonsil until day 31 of the study ($P < 0.05$ [GLM]). The numbers of \textit{P. multocida} organisms isolated from the nasal cavities of pigs exposed to ammonia were significantly elevated on days 2 to 23 ($P < 0.05$ [GLM]); however, exposure to ammonia had no significant effect on the numbers of \textit{P. multocida} organisms isolated from the tonsils.

The total number of bacteria isolated on HBA from the tonsils and nasal cavities of pigs in all groups remained constant throughout the experiment: $6.25 \pm 0.67$ and $5.32 \pm 0.74$ log$_{10}$ CFU ml$^{-1}$ (means $\pm$ SDs), respectively; analysis revealed no statistical difference in the number of bacteria isolated over the time course of the study or between experimental groups. At time points when high numbers of \textit{P. multocida} were isolated, this organism constituted almost exclusively the total number of bacteria isolated.

All the tested isolates of \textit{P. multocida} from the infected pigs were found to be toxigenic. No \textit{P. multocida} organisms were isolated from the noninfected groups, but small numbers of \textit{B. bronchiseptica} organisms were isolated from pigs in all groups at different time points during the experiment ($50$ to $100$ CFU ml$^{-1}$ from the nasal lavage and $25$ to $80$ CFU g$^{-1}$ from the tonsillar biopsy specimens). These isolates proved not to be toxigenic and had no significant effect on the numbers of \textit{P. multocida} isolated from the upper respiratory tract.

\textbf{Ammonia levels in mucus.} The mucus from pigs exposed to ammonia (groups A and B) contained significantly more ammonia ($P < 0.05$ [AV]) than the mucus from those not exposed to ammonia (groups C and E) (Table 1). The concentration of ammonia was approximately 3.5-fold higher in the nasal mucus and approximately 7-fold higher in the tonsillar mucus.

\textbf{pH of mucus.} Exposure to ammonia did not affect the pH of the mucus from either the tonsil or the nasal cavity, as indicated in Table 1.

\section*{DISCUSSION}

Pigs reared in intensive production systems are continuously exposed to gaseous ammonia generated by microbial degrada-
tion of their excrement. Ammonia levels within commercial pig building are typically in the region of 10 to 25 ppm (19, 26); however, in some instances levels as high as 90 ppm have been recorded (30). Ammonia is a corrosive, highly toxic irritant gas. It is extremely soluble in water and consequently is rapidly concentrated in the aqueous layer covering the mucous membranes of the eyes, nose, and throat (9). Our previous study showed that exposure to ammonia at concentrations within the range encountered in commercial piggeries contributes to the severity of the clinical condition progressive atrophic rhinitis by facilitating colonization of the pig’s upper respiratory tract with the pathogenic bacterium *P. multocida* (15).

The current study has highlighted a number of points relevant to the etiology of progressive atrophic rhinitis and the synergistic role of inhaled ammonia. The study revealed that irrespective of the numbers of *P. multocida* organisms isolated from the upper respiratory tract of a pig, the total bacterial count remained relatively constant, even when *P. multocida* comprised 100% of the organisms recovered. This demonstrates that *P. multocida* colonized the nasal cavity principally by displacing the normal flora. It follows that both acetic acid pretreatment and ammonia exposure altered the conditions within the nasal cavity to enable *P. multocida* to compete successfully with the normal flora for the available nutrients. The synergistic effect of inhaled ammonia on the colonization of a pig’s nasal cavity with *P. multocida* was such that by the end of the 2-week exposure period *P. multocida* had almost totally displaced the normal commensal flora. Despite this, once ammonia exposure ceased, *P. multocida* numbers declined to undetectable levels within 3 weeks, and the commensal flora was reestablished at preexposure levels. These findings indicate that the changes to the environment of a pig’s nasal cavity which facilitate *P. multocida* colonization are dependent on the continued presence of ammonia for the maintenance of the changed environment.

The effect of ammonia was less apparent at the tonsil, with only low numbers of *P. multocida* being isolated from this site. This is in contrast to the findings of our previous study (15), in which high numbers of this organism were isolated following a prolonged exposure period (5 weeks). Taken together, these findings suggest that exposure to ammonia facilitates a gradual colonization at the tonsil, in contrast to a more rapid effect within the nasal cavity. Supportive evidence for this hypothesis comes from the observation that the average number of *P. multocida* organisms isolated from the tonsil following inoculation increased during the exposure period, while over the same period the average number in the unexposed control group declined.

A possible explanation for the differences in the colonization kinetics of *P. multocida* on the nasal mucosa and tonsil could relate to the concentration of ammonia in the mucus at these sites. The high solubility of ammonia and the internal architecture of the nasal cavity results in a high proportion of inhaled ammonia being absorbed into the mucus coating the nasal cavity (25). Consequently, the concentrations of ammonia within mucus recovered from the nasal cavities of pigs exposed to ammonia at 20 ppm was approximately 7 times higher than that in the mucus taken from control animals maintained in pure air, while at the tonsil ammonia concentrations were approximately 3.5 times that at the tonsils of controls. This distribution is undoubtedly largely due to the anatomical differences between these two sites. Further supportive evidence linking the concentration of ammonia in mucus with the level of colonization by *P. multocida* comes from our previous study (15). That study revealed a progressive increase in the number of *P. multocida* organisms isolated from the tonsil after exposure to ammonia at levels of up to 35 ppm, while within the nasal cavity the numbers peaked after exposure to ammonia at 10 ppm (15).

Ammonia inhalation could facilitate colonization by *P. multocida* via one or more mechanisms affecting the host, the pathogen, or the competing commensal flora. Prolonged exposure to ammonia has been shown to evoke a range of histopathological changes to the mucus lining of the upper respiratory tract (9, 13, 15). Exposure to high concentrations of ammonia has also been shown to compromise the mucociliary clearance rate and to affect mucin secretion (7, 13). The extent to which these changes contribute to colonization with *P. multocida* is unclear. Two potential etiological mechanisms investigated as part of this study were the effect of ammonia exposure on the normal commensal flora and the pH of the mucus within the upper respiratory tract. In the case of the former we were unable to demonstrate statistically significant alterations in the composition of the commensal flora as a consequence of ammonia exposure. Furthermore, the absence of a statistically significant change in the number of bacteria isolated from pigs in the ammonia control group, i.e., pigs exposed to ammonia but not infected with *P. multocida*, suggests that exposure to ammonia at 20 ppm had little effect on either the growth or the rate of clearance of the commensal flora. Measurement of the pH of mucus recovered from the nasal cavity and tonsil revealed no significant change in pH as a consequence of exposure to ammonia; however, there was a small but significant difference in the pH of mucus at the two sites (*P < 0.05 [AV]*) (Table 1). It is unlikely that this difference will significantly influence the growth of *P. multocida*. Taken together the findings described above lend support to the hypothesis that ammonia brings about its synergistic effect by directly enhancing the growth of *P. multocida*, thereby enabling it to compete successfully with the commensal flora, possibly by providing a preferential nitrogen source for protein and nucleic acid synthesis.

Pretreatment of pigs with acetic acid induced high levels of colonization of the pig’s nasal cavity with *P. multocida*, and this high level of colonization persisted throughout the 5 weeks of the study. During this period the flora of the nasal cavity became almost purely *P. multocida*. The persistence of this colonization was somewhat unexpected given the transient nature of the acetic acid pretreatment and is in contrast to the colonization evoked by the ammonia exposure regimen. Other

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**TABLE 1. Mean concentration of ammonia in nasal cavity and tonsil of pigs on day 3 and mean pH of mucus in nasal cavity and tonsil of pigs on day 4 of the experiment**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD pH</th>
<th>Mean ± SD ammonia concn (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nasal</td>
<td>Tonsillar</td>
</tr>
<tr>
<td>A and B</td>
<td>7.2 ± 0.288</td>
<td>6.9 ± 0.235</td>
</tr>
<tr>
<td>C and E</td>
<td>7.2 ± 0.310</td>
<td>6.9 ± 0.296</td>
</tr>
</tbody>
</table>

*The numbers of pigs in each group were as follows: group A, n = 6; group B, n = 6; group C, n = 7; group D, n = 7; group E, n = 4.*
investigators (4) have reported only transient colonization of 2 to 3 weeks’ duration following acetic acid pretreatment of gnotobiotic pigs. An explanation for these differences is uncertain. One possibility is that the colonization kinetics in gnotobiotic pigs given an artificial flora are different from those in conventionally reared pigs, which were used in this study. It is also possible that the repeated sampling procedures conducted throughout our study may have influenced *P. multocida* colonization; however, this latter explanation seems unlikely considering the low level of transient colonization seen in the infected control group. A notable exception to the transient colonization pattern previously reported for the acetic acid pretreatment model occurred when pigs were concurrently infected with toxigenic strains of *B. bronchiseptica* (4). In the current study toxigenic isolates of this organism were not isolated, despite extensive screening; consequently, it seems unlikely that this explanation could account for the persistence of the *P. multocida* colonization found in the current study.

The importance of the tonsil both as a reservoir of infection and as a site for osteolytic toxin absorption has been discussed by others (1). However, the findings of this study and our previous study (15) suggest that colonization of the nasal mucosa is more important than that of the tonsil in the development of clinical lesions associated with atrophic rhinitis. This is shown by regression analysis of the morphometric index against the numbers of *P. multocida* organisms isolated from the two sites.

The mechanism by which acetic acid facilitates colonization of the pig's upper respiratory tract is unclear. A limited histological survey failed to reveal significant pathological changes following treatment (23). It has been proposed that the acetic acid regimen induces only subtle biochemical changes unaccompanied by overt tissue damage (4). However, cytological examination of lavage fluid collected from pigs following acetic acid treatment prior to pathogenic challenge reveals an approximate threefold increase in the numbers of mammalian cells recovered compared to the numbers recovered from PBS-challenged controls (unpublished data). This increase was largely due to a marked rise in the proportion of polymorphonuclear cells, which increased from 5 to 60% of the cells isolated following acetic acid pretreatment. This demonstrates that acetic acid pretreatment provokes an inflammatory cell infiltration indicative of overt damage to the epithelium of the upper respiratory tract.

Given the differences in the colonization kinetics induced by ammonia exposure and acetic acid pretreatment, it seems doubtful that they invoke a common etiological mechanism. Further studies are needed to clarify this point, in particular, the effect of these treatments on mucciliary clearance and the population and activity of leukocytes within the nasal cavity. The possibility that ammonia evokes its effect by a mechanism completely unrelated to acetic acid treatment, such as by the direct enhancement of the growth or viability of *P. multocida*, needs to be evaluated.

In conclusion, exposure to ammonia at levels within the range commonly encountered in commercial pig buildings facilitates colonization of the pig’s nasal cavity by toxigenic *P. multocida*. Inhaled ammonia is concentrated in the mucus lining of the nasal cavity, creating an environment in which *P. multocida* can compete successfully with the normal commensal flora for the available nutrients. Colonization with *P. multocida* displaces the normal flora; however, once ammonia exposure ceases, the flora is reestablished, simultaneously displacing *P. multocida*. Acetic acid pretreatment evokes an inflammatory cell effusion indicative of overt tissue damage. The severity of the clinical lesions correlates closely with the numbers of *P. multocida* organisms colonizing the pig's nasal cavity, suggesting that local absorption of toxin is more important in causing turbinate atrophy than absorption at remote sites such as the tonsil.

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