Interaction of *Haemophilus parasuis* with Nasal and Tracheal Mucosa Following Intranasal Inoculation of Cesarean Derived Colostrum Deprived (CDCD) Swine

J.L. Vahle, J.S. Haynes, and J.J Andrews

ABSTRACT

Twenty-three cesarean derived, colostrum deprived pigs were obtained at 5 wk of age and inoculated intranasally with either 1.4 × 10^8 colony forming units of *Haemophilus parasuis* or sterile phosphate buffered saline. Pigs were euthanized at 4, 8, 12, 18, 26, or 36 h post-inoculation and tissues from the oropharynx and respiratory tract were obtained for qualitative bacterial culture, immunohistochemistry for *H. parasuis* antigens, and light and transmission electron microscopy. *Haemophilus parasuis* was consistently isolated from the nasal cavity (17/17, 100%) and trachea (13/17, 76%) and rarely isolated from the lung (3/17, 18%) and blood stream (1/17, 6%) of infected pigs. Antigens of *H. parasuis* were sporadically detected on the nasal mucosa (6/17, 35%) and trachea (8/17, 47%). Light microscopic lesions included submucosal and intraepithelial infiltrates of neutrophils and infrequent, patchy loss of cilia. Ultrastructural changes in nasal mucosal epithelial cells included cell protrusion, loss of cilia, and dilation of the cytocavitary network. Bacteria were infrequently identified and were either within an amorphous material at the apical surface of the cilia or were between individual cilia. These results suggest *H. parasuis* associates with the nasal mucosa and can induce a suppurative rhinitis with nasal mucosal epithelial cell degeneration. This process may represent an initial event in the pathogenesis of *H. parasuis* infection of swine.

RÉSUMÉ

Vingt-trois porcs obtenus par césarienne et privés de colostrum furent élevés jusqu'à l'âge de cinq semaines puis inoculés par voie intranasale avec soit 1,4 × 10^8 unités formant des colonies d'*Haemophilus parasuis* ou avec de la saline tamponnée. Les animaux furent euthanasiés à 4, 8, 12, 26 ou 36 h suivant l'inoculation et des échantillons de tissus provenant de l'oropharynx et du tractus respiratoire furent prélevés pour analyse bactériologique qualitative, pour la détection d'antigènes d'*H. parasuis* par immunohistochimie, de même que pour examen par microsopies photonique et électronique. *Haemophilus parasuis* fut constamment isolé de la cavité nasale (17/17) et de la trachée (13/17), mais rarement du poumon (3/17) et de la circulation sanguine (1/17) chez les animaux infectés. Les antigènes d'*H. parasuis* ont été détectés de façon sporadique sur la muqueuse nasale (6/17) et dans la trachée (8/17). Les lésions observées en microscopie photonique incluaient une infiltration sous-mucosale et intra-épithéliale de neutrophiles, de même qu'une perte peu fréquente et inégale de cils.

Les changements ultrastructuraux observés au niveau des cellules épithéliales de la muqueuse nasale incluaient la protrusion de cellules, la perte de cils et une dilatation du réseau cytocavitaire. Des bactéries étaient très peu fréquemment identifiées et étaient soit à l'intérieur d'un matériel amorphe à la surface apicale des cils, soit entre des cils individuels. Ces résultats suggèrent qu'il y a association entre *H. parasuis* et la muqueuse nasale et que le micro-organisme peut induire une rhinite suppurative avec une dégénérescence des cellules épithéliales de la muqueuse nasale. Ce processus pourrait représenter une des étapes initiales dans la pathogénie de l'infection à *H. parasuis* chez le porc.

(Traduit par docteur Serge Messier)

INTRODUCTION

Haemophilus parasuis is a common cause of fibrinous polyserositis and polyarthritis in young swine (1). In addition *H. parasuis* can cause septicemia and myositis in naive swine and is often cultured from the lungs of pigs with pneumonia and from the nasal cavity of conventional swine (2,3,4,5,6). In recent years there has been increased interest in disease caused by *H. parasuis*, in part due to the severity of disease when *H. parasuis* is introduced into naive populations (7,8). Expanded use of high-health animal husbandry technologies in the swine industry has apparently resulted in greater numbers of swine susceptible to disease caused by *H. parasuis*.

There is relatively little known about the pathogenesis of *H. parasuis* disease. Phenotypic features do not necessarily correlate with virulence and specific virulence factors have not been identified (1,9,10). We recently studied the sequence of events that follow intranasal inoculation of cesarean derived, colostrum deprived (CDCD) pigs with *H. parasuis* (11). In that study, *H. parasuis* infection produced fibrinopurulent polyserositis and polyarthritis. In

Department of Veterinary Pathology (Vahle, Haynes), Veterinary Diagnostic Laboratory (Andrews), College of Veterinary Medicine, Iowa State University, Ames, Iowa.

Correspondance to J.L. Vahle. Reprint requests to J.S. Haynes.

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addition, *H. parasuis* was consistently isolated from the nasal cavity, but was not recovered from other mucosal sites (11). A study that examined isolates obtained from slaughter swine determined that *H. parasuis* was a common inhabitant of the nasal cavity, but was not recovered from the tonsil (12). These studies indicate that the nasal mucosa may be the primary site of mucosal colonization for *H. parasuis*.

Determining the interactions of a bacterium with mucosal surfaces is important in understanding the pathogenesis of bacterial infections and identifying virulence factors. This study was designed to investigate the interaction of *H. parasuis* with respiratory mucosal surfaces in CDCD swine as determined by qualitative bacterial culture, immunohistochemistry, and light and transmission electron microscopy.

MATERIALS AND METHODS

BACTERIAL INOCULUM

An isolate of *H. parasuis* recovered from the pericardium of a pig with severe polyserositis, polyarthritis, and meningitis was used in this study. The isolate was passed twice and stored at −70°C. To prepare the challenge inoculum, the isolate was thawed at room temperature, grown overnight in supplemented M96 broth, and inoculated onto supplemented pleuropneumonia-like organism (PPLO) agar (10). Inoculated plates were incubated at 37°C for 18–24 h. Bacteria were harvested in 5.0 mL cold, sterile, phosphate buffered saline solution (PBSS), pH 7.2. The bacterial suspension was centrifuged, the supernatant discarded, and the cells resuspended in 4.0 mL of cold sterile PBSS. The standard plate count of this suspension was 1.4×10^8 colony forming units (CFU) per mL. In a previous experiment, inocula prepared in this manner were virulent in a CDCD pig model (11).

EXPERIMENTAL ANIMALS

Twenty-three cesarean derived colostrum deprived pigs of uniform weight were obtained from a commercial source at 5 wk of age. The experimental pigs had been housed under strict isolation procedures since birth. Nasal swabs obtained upon arrival were negative for *H. parasuis*, *Streptococcus suis*, *Bordetella bronchiseptica*, *Pasteurella multocida*, and *Actinobacillus pleuropneumoniae*. Pigs were housed in an isolation facility, fed an 18% protein corn-soybean meal ration, and provided water ad libitum. All animal related procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University.

EXPERIMENTAL INFECTIONS

Control and experimental pigs were housed in separate isolation rooms. Seventeen experimental pigs were inoculated with 0.5 mL of challenge inoculum slowly dripped into each nostril to give a total inoculum dose of 1.4×10^8 CFU. Six control pigs were sham inoculated with sterile PBSS. At 4, 8, 12, 18, and 26 h post-inoculation (HPI) 3 experimental pigs and 1 control pig were euthanized and necropsied. At 36 HPI only 2 experimental pigs and 1 control pig were euthanized and necropsied. At necropsy, samples were obtained for bacterial culture and light and electron microscopic examination. Specific anatomic locations were used to maintain consistency in sampling the nasal cavity. Nasal cavity samples were obtained from the following locations: the nasal vestibule and rostral 20 percent of the nasal turbinate, a region immediately caudal to the midpoint of the nasal turbinate, and the caudal 20% of the nasal turbinate.

BACTERIOLOGY

Specimens collected for bacterial culture included blood, lung, tonsil, and swabs from the rostral, middle, and caudal nasal cavity, and trachea. Tubes of supplemented M96 broth and blood agar were inoculated (10). Blood agar plates were cross-streaked with *Staphylococcus aureus*. Broth cultures were incubated overnight at 37°C and then inoculated on blood agar plates with a *S. aureus* nurse streak. All media were examined for growth after 24 and 48 h incubation at 37°C. *H. parasuis* was identified by colony morphology and satellitosis from the *S. aureus* nurse. A representative isolate from each experimental pig was submitted to the Iowa State University Veterinary Diagnostic Laboratory and confirmed as *H. parasuis* based on colony morphology, satellitosis from the *S. aureus* nurse, gram-stain characteristics, and a negative urease reaction.

PATHOLOGY

Tissues from the rostral, middle, and caudal nasal cavity, tonsil, trachea, and lung were fixed in 10% neutral buffered formalin for 24 h, processed routinely, embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin. For electron microscopy, 3–6 specimens from each tissue were cut to 1 mm thickness in at least one dimension and placed in McDowell and Trump's 4F:1G fixative (4% formaldehyde, 1% glutaraldehyde) in 0.1 M Sorenson's sodium phosphate buffer (13). Tissues were rinsed in distilled water, post-fixed in 1% osmium tetroxide, rinsed in distilled water, dehydrated in acetones, and embedded in epoxy resins. Semi-thin (1 micron) sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections of selected areas were stained with lead citrate and uranyl acetate and examined on a Hitachi electron microscope.

IMMUNOHISTOCHEMISTRY

Five micron sections from formalin-fixed, paraffin-embedded blocks were placed on poly L-lysine coated microscope slides, deparaffinized in xylenes, and rehydrated. Endogenous peroxidase activity was quenched by reacting the slides with a 3% hydrogen peroxide solution for 20 min. Following washing in Tris-buffered saline solution slides were overlaid with 10% normal goat serum for 20 min to prevent background staining. A rabbit antisera to the *H. parasuis* isolate used for inoculation was applied as the primary antibody at a 1:1000 dilution. Slides were overlaid with primary antibody and incubated in a humidified chamber overnight at 4°C. After washing in Tris-buffered saline, a 1:200 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, California, USA) adsorbed with 2% normal swine serum was applied to the slides for 30 min. Slides were washed with Tris-buffered saline and a 1:200 dilution of peroxidase conjugated streptavidin (Zymed Laboratory Inc, San Francisco, California, USA) was applied for

TABLE I. Summary of bacteriologic and immunohistochemical findings in pigs inoculated with *H. parasuis*

	Rostral Nasal		Mid Nasal		Caudal Nasal		Tonsil		Trachea		Lung		Blood
HPI	BACT[a]	IHC[b]	BACT	IHC	BACT	IHC	BACT	IHC	BACT	IHC	BACT	IHC	BACT
4	3/3	0/3	3/3	1/3	1/3	2/3	0/3	0/0	2/3	2/3	0/3	0/0	0/3
8	3/3	0/3	2/3	2/3	0/3	1/3	0/3	0/0	2/3	2/3	0/3	0/0	0/3
12	1/3	0/3	3/3	1/3	2/3	0/3	0/3	0/0	2/3	1/3	0/3	0/0	0/3
18	1/3	0/3	3/3	1/3	1/3	1/3	0/3	0/0	3/3	1/3	0/3	0/0	0/3
26	1/3	0/3	3/3	0/3	2/3	0/3	0/3	0/0	3/3	2/3	2/3	0/3	0/3
36	0/2	0/2	2/2	0/2	2/2	0/2	0/2	0/0	1/2	0/3	1/2	0/0	1/2

[a] Bacterial Culture: Number culture positive for *H. parasuis*/number sampled
[b] Immunohistochemistry: Number *H. parasuis* antigen positive/number sampled

30 min. Streptavidin was detected with a 0.4% 3-amino-9-ethylcarbazole (AEC) solution. Slides were counterstained with hematoxylin. Negative controls included using normal rabbit serum and Tris buffered saline in place of the anti-*H. parasuis* antisera.

RESULTS

BACTERIOLOGIC FINDINGS

H. parasuis was consistently isolated from the nasal cavity and trachea (Table I) and only sporadically isolated from the lung and blood. *H. parasuis* was not recovered from the tonsil. From sites within the nasal cavity, *H. parasuis* was recovered from the mid-portion of the turbinate of all infected pigs. Cultures from the rostral portion of the turbinate were positive for *H. parasuis* at 4 and 8 HPI while positive *H. parasuis* cultures from the caudal nasal cavity were present at 12, 26, and 36 HPI. *H. parasuis* was isolated from the lung at 26 and 36 HPI and from the blood of 1 pig at 36 HPI.

IMMUNOHISTOCHEMICAL FINDINGS

Antigens of *H. parasuis* were sporadically detected in sections of middle and caudal turbinate and trachea and were most frequently detected at 4 and 8 HPI (Table I). *Haemophilus parasuis* antigens were not detected in sections of rostral turbinate, tonsil, or lung. Immunoreactivity occurred as patchy focal areas of intense staining at the apical surface of mucosal epithelial cells with minimal background staining. Immunoreactivity was not present within the mucosa or nasal submucosal glands.

MACROSCOPIC FINDINGS

There were no gross lesions detected in pigs examined at 4, 8, 12, and 26 HPI. The 2 experimental pigs examined at 36 HPI had mild to moderate amounts of fibrinous exudate loosely adherent to the pleural surface of the lung.

LIGHT MICROSCOPIC FINDINGS

Microscopic lesions were consistently present at all time points in sections from the middle and caudal nasal cavity of experimental pigs. At 4 HPI the nasal submucosa was diffusely infiltrated by large numbers of neutrophils (Figure 1). Neutrophils were present as multifocal intraepithelial aggregates or aggregates accompanied by cell debris within submucosal glands. Lesions present at 8 and 12 HPI were less severe and consisted of moderate numbers of neutrophils within the submucosa and increased numbers of intraepithelial neutrophils. At 26 and 36 HPI the submucosal infiltrate consisted of decreased numbers of neutrophils compared to early time points and scattered aggregates of lymphocytes. Focal loss of cilia and irregularity of the mucosal epithelium overlying areas of inflammatory cell infiltrate were observed in at least 1 pig at each time point, but were most consistent at 18 and 26 HPI (Figure 2). Microscopic lesions of the rostral nasal mucosa were limited to submucosal infiltrates of low numbers of neutrophils in all pigs examined 4 HPI.

Microscopic lesions of the trachea were observed at 12, 18, 26, and 36 HPI. At 12 HPI there were widely scattered, focal, submucosal infiltrates of neutrophils. This lesion was more prominent at 18 HPI and there were also rare intraepithelial aggregates of neutrophils. By 26 and 36 HPI there were multifocal aggregates of lymphocytes and macrophages, but lesser numbers of neutrophils. Significant lesions were not observed in the tonsil and lung of infected pigs or in tissues from control pigs.

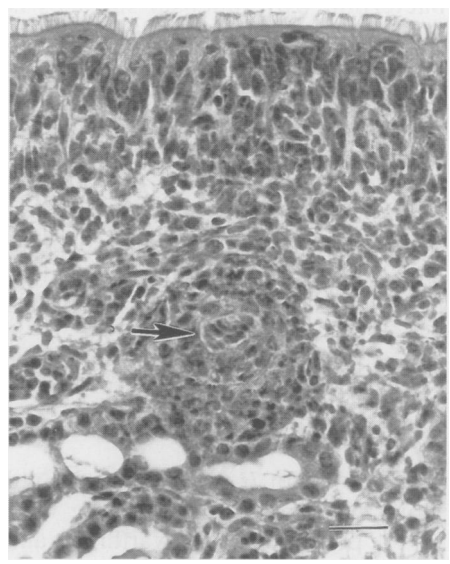

Figure 1. Nasal turbinate; CDCD pig 4 h post-*H. parasuis* infection. Note submucosal and intraepithelial infiltrates of neutrophils and aggregates of neutrophils and cellular debris within the lumen of a nasal submucosal gland (arrow). H&E. Bar = 20 microns.

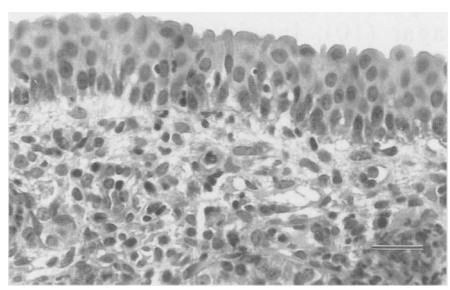

Figure 2. Nasal turbinate; CDCD pig 18 h post-*H. parasuis* infection. Note mild submucosal and intraepithelial infiltrates of lymphocytes. There are decreased number of cilia present. H&E. Bar = 20 microns.

ULTRASTRUCTURAL FINDINGS

Ultrastructural changes were limited to the nasal and tracheal mucosa.

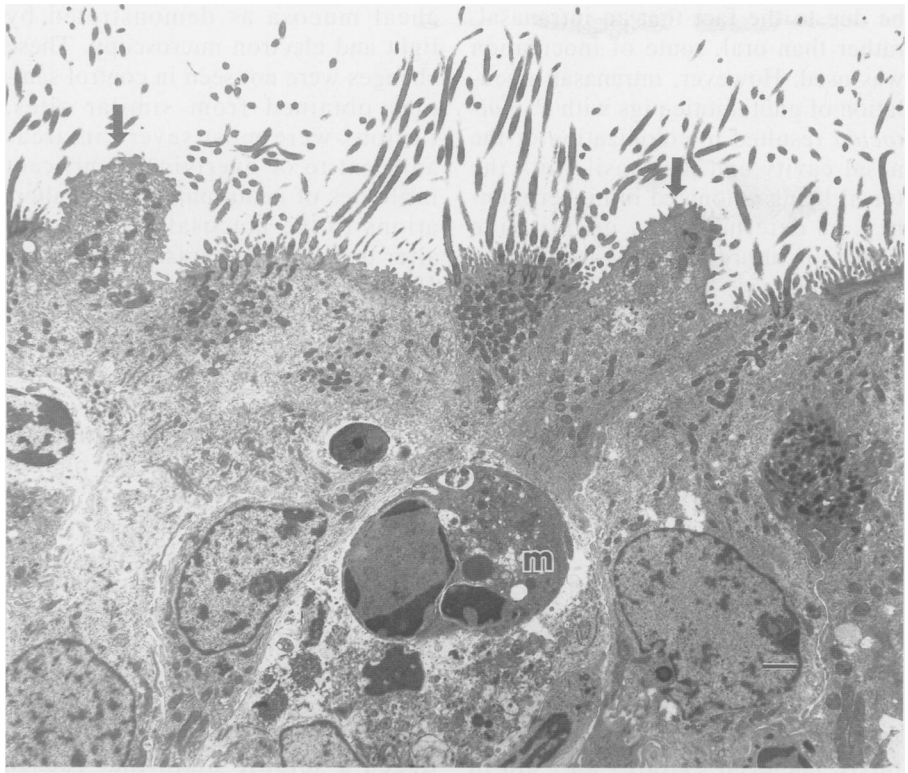

Figure 3. Nasal mucosa; CDCD pig 4 h post-inoculation. There is multifocal cell protrusion (arrows). There are decreased numbers of cilia. A cell containing phagolysosomes within the mucosa is identified as a macrophage (m). Bar = 1 micron.

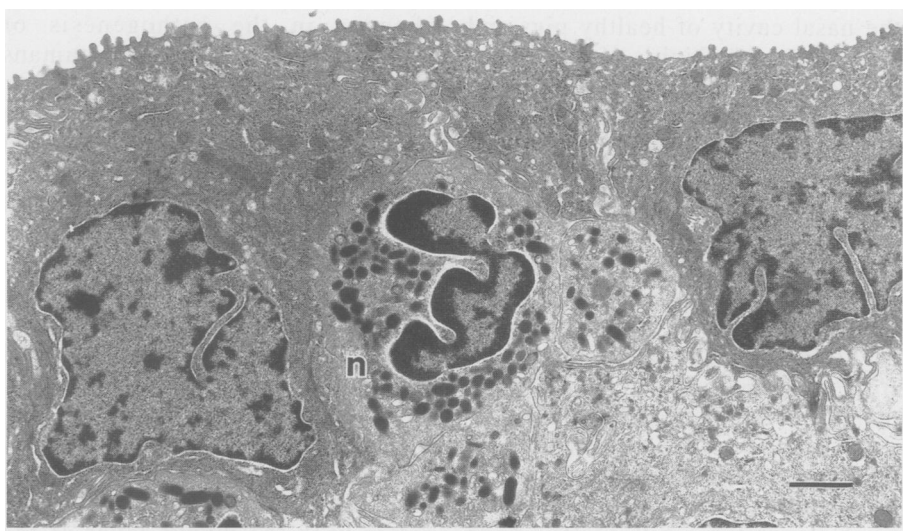

Figure 4. Nasal mucosa; CDCD pig 36 h post-inoculation. An intraepithelial neutrophil (n) is present. There is diffuse loss of cilia and basal bodies, microvilli are irregular and reduced in size, and there is dilation of the cytocavitary network within the apical cytoplasm. Bar = 1 micron.

Lesions were detected in the nasal mucosa of all infected pigs. At 4 and 8 HPI there were scattered areas in which the apical surface of the mucosa was irregular due to cells that protruded above the surface of adjacent epithelial cells (Figure 3). These cells lacked cilia and basal bodies and had apical cytoplasm with decreased electron density. In these areas there was an apparent decrease in the numbers of cilia compared to tissues from control pigs. At all other time points there was more extensive loss of cilia. Affected cells lacked basal bodies and had irregular microvilli. There was mild dilation of the cytocavitary network which was most prominent in the apical portion of the cytoplasm. Multivesicular bodies were prominent in many of these cells. Although there were focal areas of increased intercellular space, tight junctions remained intact. The most severely affected cells were often overlying aggregates of intraepithelial neutrophils (Figure 4). In addition, there were cells within the epithelium, interpreted as macrophages, that contained multiple phagolysosomes.

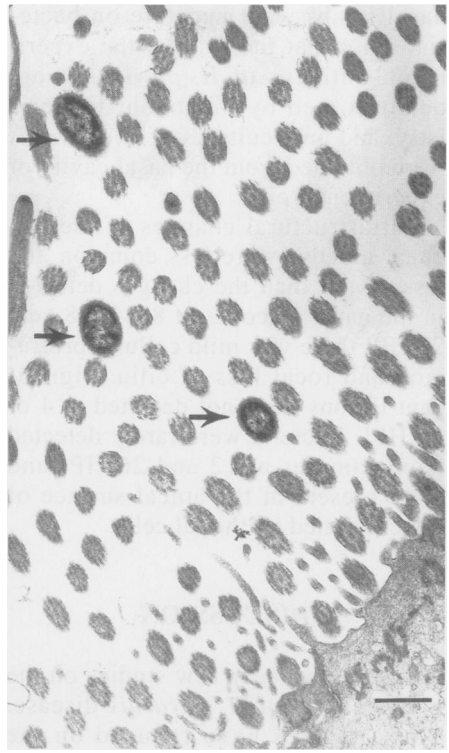

Figure 5. Nasal mucosa; CDCD pig 26 h post-inoculation. Rare bacteria (arrows) are present between cilia. Bacteria are not closely apposed to cilia. Bar = 0.5 micron.

Bacteria were only rarely detected by transmission electron microscopy in sections of nasal mucosa. At 4 HPI low numbers of bacteria were present at the luminal surface of ciliated epithelial cells, often associated with an amorphous, variably electron dense material. At 26 and 36 HPI rare bacteria were identified between cilia (Figure 5). Bacterial cells were not in direct contact with cilia and no fimbria-like structures were observed. When bacteria were observed, they were associated with normal epithelial cells adjacent to areas of epithelial cell degeneration. Although the bacterial cells were not specifically labeled, they were presumed to represent *H. parasuis* as bacterial cells were not seen in samples from control pigs,

nasal swabs were negative on bacterial culture at the start of the experiment, antigens of *H. parasuis* were demonstrated by immunohistochemistry, and pure cultures of *H. parasuis* were obtained from the nasal cavity of experimental pigs.

Ultrastructural changes of the tracheal mucosa were less common and less severe than the changes detected in the nasal mucosa. At 8, 12, 18, and 26 HPI there was mild cellular protrusion and focal loss of cilia. Significant lesions were not detected at 4 or 36 HPI. Bacteria were rarely detected from samples at 12 and 26 HPI and were present at the apical surface of intact ciliated epithelial cells.

DISCUSSION

There have been few studies on the pathogenesis of *H. parasuis* disease. These studies have focused on the clinical signs and systemic lesions of *H. parasuis* and have not examined mucosal colonization (5,14,15,16,17). In a recent study utilizing a CDCD pig model of *H. parasuis* infection, *H. parasuis* was recovered from the nasal cavity, but not from other mucosal sites (11). That study did not examine multiple sites within the nasal cavity, obtain samples at early post-inoculation times, or utilize immunohistochemistry and transmission electron microscopy to study the interaction of *H. parasuis* with mucosal surfaces. The present study demonstrated that *H. parasuis* is consistently recovered from the middle and caudal nasal cavity and *H. parasuis* can be demonstrated on the nasal mucosa by immunohistochemistry and transmission electron microscopy. In addition, *H. parasuis* was sporadically associated with the tracheal mucosa. Bacterial cultures and immunohistochemistry failed to demonstrate *H. parasuis* within the tonsil. In most cases, immunohistochemistry was less sensitive than bacterial culture. In only 1 pig at 4 HPI and 1 pig at 8 HPI, antigens of *H. parasuis* were detected by immunohistochemistry at a site which was culture negative. Other investigators have recovered *H. parasuis* from nasal secretions and identified *H. parasuis* antigens within the tonsil (18). Failure of the current study to demonstrate tonsilar colonization may be due to the fact that an intranasal, rather than oral, route of inoculation was used. However, intranasal inoculation of gnotobiotic pigs with *P. multocida* resulted in colonization of the nasal cavity and the tonsil, with the tonsil being colonized in higher numbers as determined by quantitative bacterial culture (19). The tonsil is an important site of colonization for *S. suis* and *P. multocida* in swine (19,20,21). In contrast, *H. parasuis* may preferentially colonize the nasal mucosa. This is consistent with culture results from slaughter swine in which *H. parasuis* was frequently isolated from the nasal cavity but was recovered from the surface or cut surface of the tonsil in only one case (12). The present study did not use quantitative bacterial cultures to determine the magnitude of colonization. Further studies using oral and intranasal routes of inoculation, different *H. parasuis* strains, and quantitative bacterial cultures may aid in determining the relative importance of the tonsil as a site of colonization of *H. parasuis*.

H. parasuis is often isolated from the nasal cavity of healthy pigs and from pigs with rhinitis (4,22). In the present study *H. parasuis* was only transiently recovered from the rostral portion of the nasal cavity. This portion of the nasal cavity is covered by a stratified squamous epithelium with a gradual transition to the ciliated psuedostratified columnar epithelium of the middle to caudal nasal turbinates (23). This type of epithelium may be more resistant to colonization. *Haemophilus parasuis* was consistently isolated from the middle portion of the nasal cavity at all time points and induced light microscopic lesions of an acute suppurative rhinitis. Colonization of *P. multocida* in gnotobiotic pigs was not associated with lesions of the respiratory mucosa; however, in that study bacterial numbers in the inoculum were much lower than the high dose of 10^8 CFU used in this experiment. The severity and extent of the mucosal lesion in this study may be due to the high number of bacteria in the inoculum.

In addition to producing a suppurative rhinitis, *H. parasuis* infection produced focal loss of cilia and acute cell swelling within the nasal and tracheal mucosa as demonstrated by light and electron microscopy. These changes were not seen in control samples obtained from similar sites. Lesions were most severe in areas adjacent to or overlying significant infiltrates of neutrophils. These alterations to the mucosal surface may alter defense mechanisms and allow *H. parasuis* to invade the mucosa and gain access to the blood stream. Bacteria were not detected in association with the areas of cellular degeneration, however macrophages within the epithelium contained multiple phagolysosomes indicating phagocytic activity. The failure to detect bacteria associated with the areas of cilia loss and cellular degeneration may have been due to the small sample size examined or bacteria on the surface may have been lost during processing. An alternate explanation is that *H. parasuis* may have associated with the mucous layer and produced a soluble toxin that caused the cellular changes. *Haemophilus influenzae* lipopolysaccharide and an *H. influenzae* derived low molecular weight substance may produce similar changes in the pathogenesis of *H. influenzae* infections in humans (24,25).

Haemophilus influenzae has been shown to colonize a mucous layer and nonciliated cells, does not colonize intact ciliated epithelial cells, and induces ciliostasis, loss of cilia, and epithelial cell degeneration in human nasopharyngeal tissue (26,27,28). *Haemophilus influenza* has also been shown to break down epithelial cell tight junctions, invade between cells, and pass into the submucosa (28). Based on the evidence presented in this study we hypothesize that *H. parasuis*, like *H. influenzae*, associates with the mucus layer of the nasal cavity and damages the mucosal epithelium and induces inflammation by release of one or more toxic compounds. This cellular damage compromises the mucosal barrier and may lead to invasion and systemic spread of the infection.

This study did not determine a specific cell type or structure colonized by *H. parasuis*. Bacterial cells presumed to be *H. parasuis* were seen only rarely by transmission electron microscopy. When present, bacterial

cells were not closely apposed to cilia or other cell structures. This is in contrast to *Mycoplasma hyopneumonia* and *B. bronchiseptica* which colonize ciliated epithelial cells in high numbers and are often intimately associated with the cilia (29,30,31,32).

Mechanisms of colonization of *H. parasuis* remain poorly defined and attachment structures were not seen during transmission electron microscopic examination of tissue sections in this study. Fimbriae of *H. parasuis* have been observed on bacteria inoculated on chicken chorioallantoic membranes, but not on cells from culture media (33). Fimbriae can be important attachment structures in bacterial pathogenesis and have been demonstrated in *S. suis* (34) and *P. multocida* (35) from swine. Capsular material may be important in attachment to mucosal surfaces (36). Initial reports suggested that virulent strains of *H. parasuis* did not possess a capsule (9). In a study using a guinea pig model of *H. parasuis* infection, encapsulation of a virulent *H. parasuis* strain was demonstrated by capsular staining (10). The isolate used in this study possessed a capsule as determined by Maneval staining (37). (J. Vahle, Data not shown) Further efforts to determine the presence of fimbria or capsular material may aid in identifying virulence factors of *H. parasuis*.

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