SPF 仔豚への Pasteurella multocida および Bordetella bronchiseptica 鼻腔内接種試験

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Changes in the Nasal Mucosa of Specific-Pathogen-Free Neonatal Pigs Infected with *Pasteurella multocida* or *Bordetella bronchiseptica*

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ABSTRACT. Specific-pathogen-free neonatal pigs were intranasally inoculated with serotype D strains of *Pasteurella multocida* or with strain L3 of *Bordetella bronchiseptica*, and changes in the nasal mucosae were examined with a scanning electron microscope (SEM) or a light microscope. *P. multocida* caused slight chronic catarrhal inflammatory changes in the infected nasal mucosa, but most of the epithelial cells appeared intact. A small number of lymphocytes and neutrophils infiltrated the lamina propria and submucosa, and a small amount of a mucopurulent substance accumulated on the mucosa. *P. multocida* was not readily observed on the nasal mucosa by SEM, though a low number of the organisms were constantly recovered from the nasal cavities of the infected pigs throughout the experimental period. In contrast, marked inflammatory changes were observed after the infection by *B. bronchiseptica*. Degeneration and desquamation of the nasal epithelial cells were noted throughout the infected nasal mucosa. Cilia were lost from most of the ciliated epithelial cells. Marked hyperplasia of the epithelial cells was also observed in many parts of the epithelium. *B. bronchiseptica* was frequently noted on the remaining cilia or on the microvillous cells by SEM, and numerous organisms were constantly recovered from the nasal cavities of the infected pigs during the period. Clinical atrophic rhinitis was induced only in the pigs given *B. bronchiseptica*.—KEY WORDS: *Bordetella bronchiseptica*, dermonecrotic toxin, *Pasteurella multocida*, swine atrophic rhinitis, swine nasal mucosa.

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Swine atrophic rhinitis (AR) is characterized by deformity and reduction both in volume and in size of the nasal turbinates and snouts [2, 5, 10, 12, 14, 24, 31]. *Bordetella bronchiseptica* at phase I [18] causes marked loss of cilia accompanied by the characteristic morphologic changes of the nasal mucosa when it is intranasally inoculated into gnotobiotic or specific-pathogen-free (SPF) neonatal pigs [2, 5, 10, 12, 14, 24, 31, 34]. A heat-labile dermonecrotic toxin (DNT) extracted from the phase I organisms of *B. bronchiseptica* has been suggested as a virulence factor for the production of the turbinate atrophy in neonatal pigs [9] or in young mice [27].

The toxigenic strain of *Pasteurella multocida* has also been implicated as a causative agent of swine AR or as a component responsible for the production of the turbinate atrophy [4, 7, 11, 20, 21, 25, 26]. Our previous data [23], however, failed to confirm the results reported by Pedersen and Elling [21] and by Pedersen and Barfod [20] that the inoculation of *P. multocida* alone resulted in severe nasal turbinate atrophy in neonatal pigs, and that severe lesions observed in naturally occurring AR had not been reproduced experimentally with *B. bronchiseptica* alone. Hence, our observation is consistent with the hypothesis that *B. bronchiseptica* alone is responsible for pathogenesis of swine AR.

Adherence of pathogenic microorganisms

to mucosal surfaces has been considered as an important initial step in infections of the respiratory tract [13, 14, 28], gastrointestinal tract [30], and urogenital system [33]. The adhering capacity may influence the degree of colonization and thus it may be closely related to pathogenicity of the organism [6, 28]. Since *B. bronchiseptica* is known to multiply on the surface of the swine nasal mucosa [34], the adherence to the mucous membranes must play an important role in its pathogenicity. However, there is little information available on the process by which either *B. bronchiseptica* or *P. multocida* colonizes on normal swine nasal mucosa [8, 14, 26].

The purpose of the present study was to describe ultramicroscopic lesions induced in the nasal mucosa of neonatal pigs by *P. multocida* or *B. bronchiseptica* in relation to colonization of the organisms on the infected nasal mucosa.

MATERIALS AND METHODS

Bacteria and culture media: *Pasteurella multocida,* capsular serotype D [3], strain SP-72 [16] and 47459 (provided by Dr. de Jong, Central Veterinary Institute, Rotterdam, The Netherlands) and *Bordetella bronchiseptica* strain L3 of pig origins [9] were used. These strains produced dermonecrotic toxin (DNT) [16] and were preserved as lyophilized cultures. The cystine agar medium containing yeast-extract-Proteose peptone (YPC) [19] and Bordet-Gengou's (BG) agar medium [1] supplemented with 20% horse blood were used for the propagation of *P. multocida* and *B. bronchiseptica,* respectively, throughout this investigation.

Neonatal pigs: Fourteen seven-day-old, specific-pathogen-free (SPF) pigs derived from 3 sows were used. These pigs were removed immediately after birth from their dams and housed in a separate room in each infection group (Table 1). They were fed only sterile milk (Nippon Formula Feed Manufacturing Corp., Tokyo, Japan) without antibiotics during the experimental period. Prior to the experiment, serum and nasal swab samples of each pig were collected, and these samples were checked serologically and bacteriologically for the absence of *P. multocida* and *B. bronchiseptica* infections according to the methods and the criteria described previously [22, 29, 32].

Preparation of inocula: *Pasteurella multocida* organisms grown on the YPC agar plates at 37°C for 6 h were harvested and suspended in 0.02 *M* phosphate-buffered solution, pH 7.0, containing 0.85% NaCl (PBS). Viable cells of the suspention were adjusted to approximately 5×10^7 colony-forming units (CFU)/0.5 m*l* by spectrophotometry [23]. Phase I of *B. bronchiseptica* grown on the BG agar plates at 37°C for 18 hr was also collected and viable cells of the suspension were adjusted to approximately 5×10^4 CFU/0.5 m*l* by spectrophotometry [23]. Inoculation was done within 30 min after each inoculum was prepared.

Experimental infection: The experimental design is summarized in Table 1. Inoculation of *P. multocida* or *B. bronchiseptica* into neonatal pigs was performed *via* the nasal route with 0.5 m*l* of each inoculum. These pigs were randomly distributed into 3 groups: A; inoculation of *P. multocida* (8 pigs from 3 sows), B; inoculation of *B. bronchiseptica* (4 pigs from 3 sows), and C; controls (2 pigs from 2 sows). All the pigs were bled and sacrificed postinoculation days (PID) 49.

Clinical observations: Clinical signs including sneezing and coughing were recorded daily. Gross lesions of atrophic rhinitis (AR) characterized by shortening and deviation of the snouts in these pigs was graded at necropsy on a scale from 0 to 3 (0 =normal, 1=mild, 2=moderate, and 3=

Table 1. Experimental design

Infection group	Inoculation[a]			No. of neonatal pigs tested	PID[b] necropsy
	Viable cell numbers (CFU)/ neonatal pig				
	P. multocida		*B. bronchiseptica*		
	SP-72	47459	L3		
A	5×10^7	—	—	4	49
	—	5×10^7	—	4	49
B	—	—	5×10^4	4	49
C	—	—	—	2	(56)[c]

a) Seven days of age neonatal pigs were intranasally inoculated.
b) Postinoculation days.
c) Days of age at necropsy.

severe) [23]. The pigs with the scores of 2 and 3 were considered as positive clinical AR.

Bacterial isolation: Isolation of *B. bronchiseptica* and *P. multocida* was attempted PID 7, 21, or 49 from the nasal cavity of each neonatal pigs as described previously [23]. Numbers of the recovered organisms (CFU/g of sample) were averaged in each infection group, and the results were expressed on a scale from − to +++ (− =no recovery, + =$<10^2$ CFU, ++ =$<10^6$ CFU, and +++ =$\geq10^6$ CFU), according to the criteria described in a previous paper [23]. Identification of the recovered organisms was done by the criteria described previously [22, 29]. The DNT-producing ability of recovered *P. multocida* or *B. bronchiseptica* was investigated in guinea pigs [16].

Macroscopic observation of the nasal cross section: The nasal cross sections cut at a middle level between the cannine and the first permolar tooth were obtained from neonatal pigs within one hr after euthanasia. Production of the nasal turbinate atrophy and distortion of the nasal septum were macroscopically evaluated and scored from 0 to 4 by using these cross sections, according to the criteria described by *Maeda et al.* [10]. The scores above 2 were considered as "positive" atrophy.

Scanning electron microscopic (SEM) observation: The nasal mucosae removed from the cross sections mentined above were infused with 20 ml/sample of 0.5% glutaraldehyde (Eastman Kodak Corp.) solution. These pre-fixed specimens were cut into small pieces. Portions of the nasal tissues were fixed in 1% OsO_4 solution for 2 hr, dehydrated in a graded series of alcohol and isoamyl acetate, and dried by the CO_2 critical point method. The dried nasal tissue sections were cracked, processed for platinum shadow coasting and examined using SEM (Model S-45, Hitachi Ltd., Tokyo, Japan) [28].

Light microscopic observation: The nasal mucosa removed from the transverse sections of the snout mentioned above were examined. Tissues were fixed in 10% neutral formalin solution, dehydrated in alcohol, and embedded in paraffin. They were cut into thin sections approximately 6 μ thick, and the sections were stained with hematoxylin and eosin (H & E).

RESULTS

Clinical findings: None of the neonatal pigs in group A that were given *P. multocida*, strains SP-72 or 47459 (Table 1) showed

Table 2. Gross lesions of neonatal pigs inoculated intranasally with *P. multocida* or *B. bronchiseptica*

Infection group	No. of neonatal pigs tested	Occurrence of the clinical AR		Production of the nasal turbinate atrophy	
		Range of the score[a] (Mean of the score)	No. of positive neonatal pigs	Range of the score[b] (Mean of the score)	No. of positive neonatal pigs
A	8	0 (0.0)	0	0 (0.0)	0
B	4	2–3 (2.5)	4	2–4 (3.0)	4
C	2	0 (0.0)	0	0 (0.0)	0

a) The socre was graded from 0 to 3 (0=normal, 1=mild, 2=moderate, and 3=severe). The pigs with the scores 2 and 3 were considered as positive clinical AR.
b) According to the criteria described by Maeda *et al.* [10]. The scores above 2 were considered as positive nasal turbinate atrophy.

Table 3. Recovery of the inoculated organisms from the nasal cavities of infected neonatal pigs

Infection group	No. of neonatal pigs tested	No. of the neonatal pigs with positive recovery of the organisms (Mean CFU numbers of the recovered organisms[a])							
		P. multocida				*B. bronchiseptica*			
		0[b]	PID 7	PID 21	PID 49	0[b]	PID 7	PID 21	PID 49
A	8	0 (−)	8 (+)	8 (+)	8 (+)	0 (−)	0 (−)	0 (−)	0 (−)
B	4	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	4 (++)	4 (+++)	4 (+++)
C	2	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)

a) Divided into 4 categoria (from–to +++).
b) Before inoculation.

clinical signs, and their snouts appeared normal during the experimental period (Table 2). In contrast, sneezing and coughing were occasionally noted in all the pigs in group B that were given *B. bronchiseptica.* These pigs showed moderate to severe clinical AR signs at necropsy. All the controls in group C remained clinically normal during the period.

Recovery of the organisms: The inoculated organisms were recovered from the nasal cavities of all the infected neonatal pigs (Table 3). From all the pigs in group A, the toxigenic *P. multocida* was constantly recovered in small numbers during the experimental period. Throughout the period, *B. bronchiseptica,* phase I, was constantly recovered in abundant numbers from all the pigs in group B. Bacterial isolations from control animals (group C) were consistently negative for both species of the bacteria.

Macroscopic findings: No gross lesion was found in the nasal cross sections of all the pigs in group A (Table 2). In contrast, moderate to severe nasal turbinate atrophy was induced in all the pigs in group B. The nasal turbinates of the controls were normal.

The nasal mucosa of neonatal pigs given P. multocida or controls: The nasal mucosa of the controls showed almost normal structure when observed by SEM, and light microscope. The infected nasal mucosa in group A showed essentially normal structure when observed by SEM (Fig. 1A and 1B) and light microscope (Fig. 1C). Slight infiltrations of lymphocytes and neutrophils

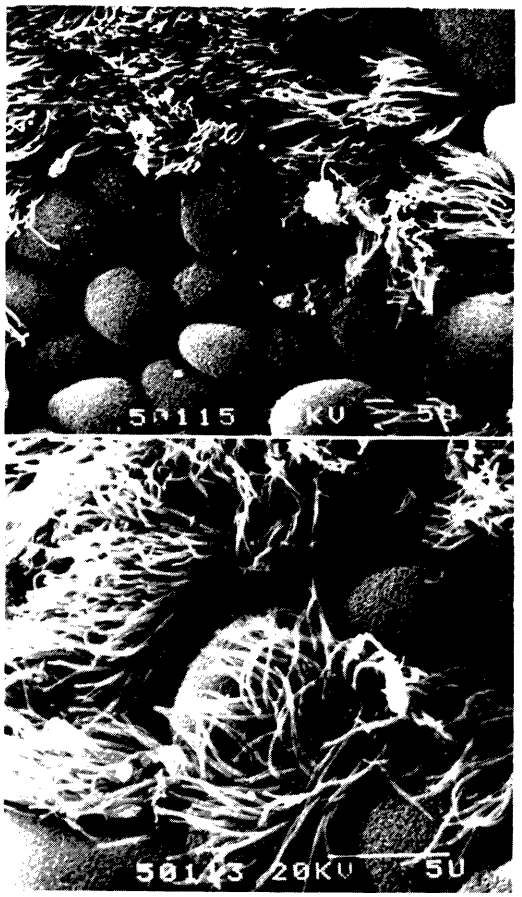

Fig. 1. Scanning electron (A and B) and histologic (C) micrographs of the nasal mucosa of neonatal pigs intranasally inoculated with *P. multocida*. A and B: The infected nasal mucosa of all pigs showed essentially the normal structure. Most of the epithelial cells appeared intact. C: The infected nasal mucosa showed essentially normal structure. Slight infiltrations of lymphocytes and neutrophils were observed in the lamina propria and submucosa. H & E stain; ×720.

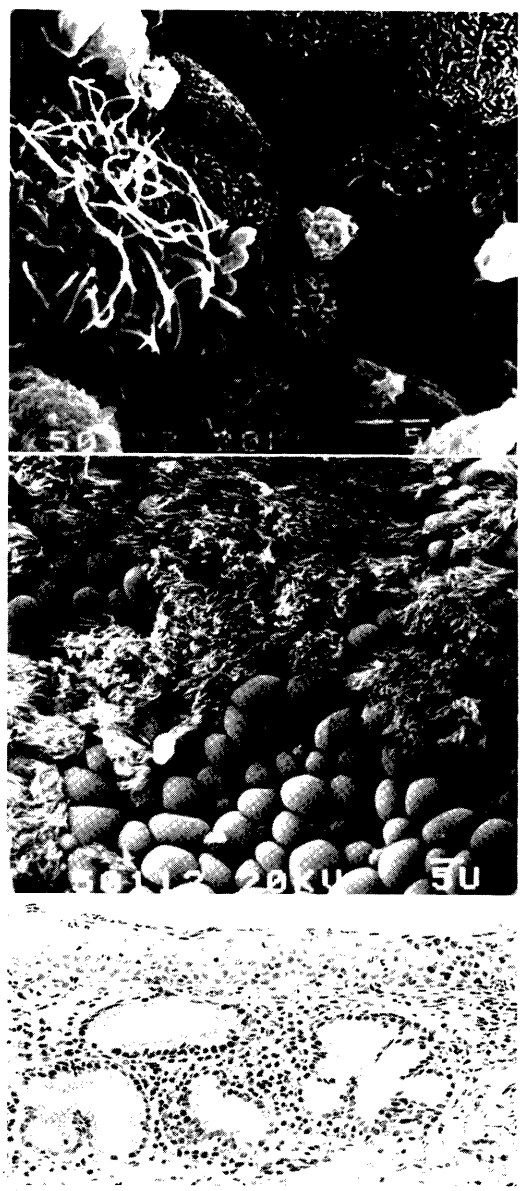

Fig. 2. Scanning electron (A and B) and histologic (C) micrographs of the nasal mucosa of 8 weeks of age neonatal pigs intranasally inoculated with *B. bronchiseptica*. A: Marked degeneration and marked desquamation of the nasal epithelial cells were shown in most parts of the infected nasal mucosa of all pigs. B: *B. bronchiseptica* (arrow) was frequently noted near the remaining cilia. Cilia were lost from most of the epithelial cells. C: Marked hyperplasia of the epithelial cells were observed in many parts of the epithelium. Marked infiltrations of lymphocytes and neutrophils were observed in the lamina propria and submucosa. H & E stain; ×720.

were observed in the lamina propria and submucosa, and a small amount of a mucopurulent substance was found on the mucosa. Most of the epithelial cells appeared intact. Microvillous cells were slightly increased in numbers compared with those of the controls. A small number of *P. multocida* was infrequently observed on the nasal mucosa by SEM.

The nasal mucosae of neonatal pigs given B. bronchiseptica: Marked degeneration and marked desquamation of the epithelial cells were observed in the nasal mucosa of all the pigs in group B by SEM (Fig. 2A and 2B) and light microscope (Fig. 2C). Cilia were lost from most of the epithelial cells. Marked hyperplasia of the epithelial cells were observed in many parts of the epithelium. Microvillous cells were increased in numbers compared with those of the controls. Marked infiltrations of lymphocytes and neutrophils were noted in the lamina propria and submucosa and deposition of a mucopurulent substance on the nasal mucosa were extensive. Numerous organisms were observed on the remaining cilia (Fig. 2B) and on a mucopurulent substance.

DISCUSSION

The aim of the present investigation was to investigate the aetiological roles of the two species of bacteria in the development of swine AR. To approach this goal, we characterized the ultramicroscopic and histologic lesions induced in the nasal mucosa of SPF neonatal pigs that were infected with *P. multocida* or *B. bronchiseptica* alone in relation to colonization of these organisms on the mucosa. The infected nasal mucosa of the neonatal pigs given *B. bronchiseptica* had extensive ultrastructural and histologic changes, whereas those of the pigs given *P. multocida* showed few changes, though the numbers of the pigs employed in the present study were limited. *Bordetella bronchiseptica* was found in large numbers on the infected nasal mucosa, but *P. multocida* organism was scarce on the mucosa by SEM. There has been a close correlation between the colonization of *B. bronchiseptica* on the nasal mucosa and the appearance of the mucosal damage. The ultramicroscopic and/or histologic lesions induced in the nasal mucosa of neonatal pigs by *B. bronchiseptica* were similar to those reported by Yokomizo and Shimizu [34] and by Oyamada *et al.* [23].

Bordetella bronchiseptica caused clinical signs of swine AR and produced both gross and histologic lesions in the nasal turbinates of the infected neonatal pigs [2, 5, 10. 12, 14, 23, 24, 31, 34]. Histologically, the mucosal epithelial cells lost cilia, and lymphocytes and neutrophils infiltrated the submucosal areas, resulting in the chronic catarrhal inflammatory changes [5, 10, 14, 23]. These observations were consistent with the present ultramicroscopic and light microscopic observations. The results of the previous [23] and the present study with bacterial recovery also clearly demonstrated that *B. bronchiseptica* colonized in a large number on the infected nasal mucosa after the inoculation. *Bordetella bronchiseptica* attached in an abundant number to the epithelial cells or on a mucopurulent like substance even at PID 49, at a late stage of the infection, both by SEM and TEM. The firm adherence of *B. bronchiseptica* to the nasal epithelial cells may be the factor for colonization of the organisms on the nasal mucosa as previously demonstrated by Yokomizo and Shimizu [34]. Thus, our present findings suggest that the ability for the adherence of *B. bronchiseptica* to the swine nasal epithelial cells may be one of the important factors responsible for the virulence in the upper respiratory tract infection caused by *B. bronchiseptica*. In addition, the colonization of *B. bronchiseptica* in site also seems to be essential for production of

the gross and histologic turbinate lesions and persistence of the disease caused by *B. bronchiseptica.*

The nasal mucosa of SPF neonatal pigs infected with *P. multocida* appeared essentially normal in morphology. The colonization of the organisms was infrequently observed by SEM. The present findings agreed well both with the previous findings on the bacterial recovery tests [8, 14, 34] and with the light microscopic observations [14, 23]. The present findings, however, may not completely eliminate the possibility that *P. multocida* can adhere to the nasal epithelial cells during the course of *P. multocida* infection, since limited numbers of the organisms were constantly recovered from the nasal cavities of the inoculated pigs (Table 3). The lower affinity of *P. multocida* to the nasal epithelium may account for the markedly lower virulence of the organisms compared with that of *B. bronchiseptica* [14, 23]. The hypothesis is consistent with the previous observations that the application of acetic acid or inoculation of *B. bronchiseptica* were prerequisite in order to colonize toxigenic *P. multocida* on the nasal mucosa of SPF or gnotobiotic neonatal pigs [4, 7, 11, 16, 20, 21, 25, 29].

There is a substantial amount of evidence supporting that *B. bronchiseptica* is a major cause of the disease [2, 5, 10, 12, 14, 23, 24, 31], although many bacteria and/or factors responsible for the occurrence of swine AR have been suggested. Some studies positively suggest that a DNT of *B. bronchiseptica* may act as an active component to produce the nasal turbinate atrophy in neonatal pigs [9]. The toxigenic strains of *P. mutocida* have also been considered as causative agents for swine AR [4, 7, 11, 20, 21, 25, 26], and their DNT have been claimed for their virulence. However, our previous data failed to support the claim [23]. When the nasal fragments removed from the cross sections of SPF neonatal pigs were cultured in a medium containing *P. multocida,* the organisms did not colonize on the cultured fragments, and surface of the infected fragments appeared normal in morphology by SEM (unpublished data). However, morphologic changes in the nasal mucosa characteristic to the AR cases, as shown in the present study, were observed in the nasal fragments cultured in a medium containing purified DNT of *P. multocida.* The results suggest that *P. multocida* DNT is capable of producing the mucosal damage on the swine nasal mucosa. It is likely that the mucosal damage is induced *in vivo,* if the production of *P. multocida* DNT is sufficient in amount to cause the damage on the infected nasal mucosa of neonatal pigs given *P. multocda* alone. *Pasteurella multocida* DNT and *B. bronchiseptica* DNT were not produced from the actively growing cells *in vitro,* but a low amount of the DNT was released from the cells by autolysis [15]. Therefore, proliferation of the organisms is essential for the production of the DNT in a sufficient quantity by the two species of the bacteria. If *P. multocida* DNT is responsible for production of the nasal turbinate atrophy in neonatal pigs as reported by some researchers [4, 7, 11, 20, 21, 25, 26], colonization of *P. multocida* on the nasal mucosa may be essential for the production of its DNT *in vivo.* The lower frequency of colonization and the small amount of mucosal damage on the infected nasal mucosa of neonatal pigs and on the infected nasal fragments (unpublished data) by *P. multocida* alone suggest that the organisms may not colonize to such an extent as to produce its DNT on the normal nasal mucosa, as previously demonstrated under natural and experimental conditions [8, 14, 23]. In conclusion, the present ultramicroscopic findings support previous hypothesis [23, 29] that *B. bronchiseptica* induces swine AR *in vivo,* whereas *P. multocida* alone does not.

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要　約

SPF 仔豚への *Pasteurella multocida* および *Bordetella bronchiseptica* 鼻腔内接種試験：中井豊次・久米勝己[1)]・吉川博康[2)]・小山田敏文[2)]・吉川堯[2)]（北里研究所付属家畜衛生研究所，[1)]北里研究所，[2)]北里大学獣医畜産学部病理学教室）——壊死毒産生血清型 D・*P. multocida*（Pm）接種豚の鼻粘膜は軽度な炎症反応を呈するが，上皮細胞はよく保持され，ほぼ正常な組織像を示した。Pm は試験期間中鼻腔内から少量ずつ回収されたが，鼻粘膜への Pm の付着は走査型電顕では認められなかった。一方，*B. bronchiseptica*（Bb）接種豚の鼻粘膜は顕著な慢性鼻炎像を呈した。上皮細胞は変性剝離し，残存する変性した線毛上皮細胞は線毛をほとんど欠いていた。Bb の付着は残存上皮細胞や微絨毛付近に多数認められ，Bb は試験期間中鼻腔から多量に回収された。鼻甲介の萎縮および変形を特徴とする，いわゆる萎縮性鼻炎は Bb 接種豚でのみ認められた。