

## Interaction between *Bordetella bronchiseptica* and Toxigenic *Pasteurella multocida* on the Nasal Mucosa of SPF Piglets

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**ABSTRACT.** The interaction between *Bordetella bronchiseptica* and type D toxigenic *Pasteurella multocida* was studied in five groups of 4 specific-pathogen-free (SPF) piglets each. At 28 days of age, piglets of groups 3 and 4 were inoculated into both nostrils with  $10^8$  colony-forming-units (CFU) of a non-dermonecrotic toxin (DNT)-producing, phase I strain of *B. bronchiseptica*. Piglets of groups 1 and 3 were treated intranasally with a sonic extract of the non-toxic strain of *B. bronchiseptica* and those of groups 2 and 4 with *B. bronchiseptica* DNT into the left nostril. Sonic extract and DNT treatment was started at 33 days of age and lasted for 5 days. Piglets of group 5 served as controls. At the age of 37 days, piglets of all groups except group 5 were inoculated into both nostrils with  $5 \times 10^7$  CFU of toxigenic *P. multocida*. At slaughter at 50 days of age, *P. multocida* was recovered from the left nasal cavity of 3 piglets of group 2 and all piglets of group 4. In piglets inoculated with *B. bronchiseptica* DNT the mucosal epithelial cells of the left nasal cavity showed loss of cilia, regressive lesions such as vacuolation, karyopycnosis and necrosis, hypertrophy of the epithelium, infiltration of the epithelium and submucosa by inflammatory cells, could also be seen. The results suggest that action of the *B. bronchiseptica* DNT on the nasal mucosa is a precondition of the growth of *P. multocida* in the nasal cavity. It appears that colonization by *P. multocida* presupposes presence of a mucosal injury inflicted by *B. bronchiseptica* DNT in itself, without involvement of the other virulence factors.—**KEY WORDS:** atrophic rhinitis, *Bordetella bronchiseptica*, dermonecrotic toxin (DNT), interaction, *Pasteurella multocida*.

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The combination of toxigenic *Bordetella bronchiseptica* [13, 15] and toxigenic *Pasteurella multocida* [17, 21] in causing atrophic rhinitis (AR) in swine is well known. The importance of *P. multocida* became clear when its toxigenic strains were discovered [9] and found to induce progressive AR following experimental infection [16, 20]. Gnotobiotic piglets experimentally infected with toxigenic *B. bronchiseptica* sometimes developed lesions with a tendency to heal [19]. Combined infection of the experimental piglets nasal cavity with both toxigenic bacteria led to severe atrophy of the nasal turbinates [20]. In the absence of *B. bronchiseptica*, *P. multocida* cannot grow on the healthy nasal mucosa [4, 17]. After experimental infection of gnotobiotic piglets only a small number of *P. multocida* colonized on the nasal mucosa and produced only mild atrophy [20], confirming that the presence of *B. bronchiseptica* is important for the growth of *P. multocida* in the nasal cavity. In the absence of *B. bronchiseptica*, colonization of the porcine nasal mucosa by toxigenic *P. multocida* could be enhanced by intranasal inoculation of 1% acetic acid before infection [7]. Chanter *et al.* [2] suggested that, besides the *B. bronchiseptica* toxin, other

products of *B. bronchiseptica* and the interaction between the two species may be important in colonization of the nasal mucosa by *P. multocida*. However, the role of the virulence factors of *B. bronchiseptica* (adhesins, DNT, adenylate cyclase) in this process was not studied in detail previously.

The aims of the experiments described here were to determine whether the simultaneous presence of *B. bronchiseptica* is needed for colonization of the porcine nasal mucosa by toxigenic *P. multocida*, and to characterize the potential interaction, if any, between the two bacteria.

### MATERIALS AND METHODS

**Bacterial strains:** *B. bronchiseptica* strain 219 was isolated from pig affected with clinical AR [5]. *B. bronchiseptica* strain N-95 was isolated from the nasal cavity of a pig free from the clinical signs and gross pathological lesions of AR at slaughter. Both strains showed phase I colony morphology and were hemolytic on Bordet-Gengou (BG) agar (Difco, U.S.A.) containing 10% bovine blood [6]. They produced adenylate cyclase and hemagglutinin for horse, calf and sheep (titres of strain 219: 1:16, 1:16

and 1:32; titres of strain N-95: 1:16, 1:32 and 1:8, respectively). Both strains possessed all K antigenic determinants except factor 13. Strain 219 also produced mouse lethal factor and dermonecrotic toxin in guinea pigs (necrosis 19 mm in diameter), whereas strain N-95 did not [6].

*P. multocida* strain 9591 was isolated from the nasal cavity of a piglet affected with AR. The strain belonged to type D and produced dermonecrotic toxin (necrosis 24 mm in diameter) [5].

**Purification and testing of dermonecrotic toxin (DNT):** Preparation of DNT from toxigenic *B. bronchiseptica* strain 219 and determination of its activity were carried out by the methods described previously [5]. The preparation was checked for purity by two-dimensional double gel diffusion [5] and by PAGE. To rule out any nonspecific activity, the concentrated DNT was dialysed before use. DNT gave a single precipitation line by gel diffusion and a double band by PAGE using Coomassie blue staining. Its biological activity was ca. 150 ng in the guinea pig skin test and its LD<sub>50</sub> to mice was ca. 500 ng.

**Preparation of non-toxic extract:** The bacterial extract was prepared from a non-toxic suspension of *B. bronchiseptica* strain N-95 containing 10<sup>12</sup> CFU/ml by sonication at 50 Hz for 10 min, then filtered through a 0.22 µm Millipore filter.

**Inocula for piglets:** *B. bronchiseptica* inoculum was prepared from *B. bronchiseptica* strain N-95. The isolate was cultured on BG agar containing 10% bovine blood at 37°C for 24 hr. The inoculum was prepared with phosphate buffer of pH 7.2 and adjusted to a concentration of 2×10<sup>8</sup> colony-forming-units (CFU)/ml by spectrophotometry (SPECOL-10, Karl Zeiss Jena, Germany). *P. multocida* inoculum was prepared by the method of Éliás *et al.* [4]. The inoculum was adjusted to 10<sup>8</sup> CFU/ml.

**Experimental infection in SPF piglets:** Four-week-old SPF piglets were assigned to 5 groups of 4 piglets each and kept isolated in climatic chambers up to 50 days of age when they were killed.

The piglets were obtained from hysterectomy-derived sows free from *B. bronchiseptica*, *P. multocida*, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, and from antibodies to these microorganisms.

From the age of 33 days on, piglets of group 1 were inoculated into the left nostril with a non-toxic bacterial extract containing 2 µg protein. The

inoculum was given 5 times daily, on 5 consecutive days. Piglets of group 2 received, into the left nostril, 2 µg (0.5 ml) *B. bronchiseptica* DNT 5 times daily, on 5 consecutive days, starting when the piglets were 33 days old.

At the age of 28 days, piglets of group 3 were inoculated with *B. bronchiseptica* N-95 into both nostrils. The inoculum contained 10<sup>8</sup> CFU/0.5 ml per nostril. From the age of 33 days, the piglets received, into the left nostril, *B. bronchiseptica* N-95 bacterial extract containing 2 µg protein 5 times daily, on 5 consecutive days.

When 28 day-old, piglets of group 4 were inoculated with *B. bronchiseptica* N-95 into both nostrils. The inoculum contained 10<sup>8</sup> CFU/0.5 ml per nostril. From the age of 33 days, the piglets received, into the left nostril, 2 µg *B. bronchiseptica* DNT 5 times daily, on 5 consecutive days.

At the age of 37 days, piglets of all groups except group 5 were inoculated, into both nostrils, with toxigenic, type D *P. multocida* (5 × 10<sup>7</sup> CFU/0.5 ml per nostril).

Piglets of group 5 served as untreated controls.

**Bacteriological examination:** Nasal swab samples for bacteriological examination were taken from the piglets at 5-day intervals. At slaughter, swabs from the surface of the pharyngeal tonsils were also cultured for bacteria. *B. bronchiseptica* was isolated from nasal swabs after plating on BG agar plates containing 10% bovine blood. Plates were incubated at 37°C for 24 hr. *P. multocida* was isolated on YPC agar (cystine agar medium containing yeast—proteose peptone, Difco) containing 10% sterile bovine blood after incubation at 37°C for 18 hr [14].

The strains were identified by the criteria of Bergey's Manual [18, 22]. Phase type, capsular antigens, haemagglutinin of *B. bronchiseptica* were determined as described by Éliás and Kruger [6] and adenylate cyclase activity was tested by the method of Kruger *et al.* [11]. The capsular antigens of *P. multocida* strains were determined by indirect-haemagglutination and by the acriflavine and hyaluronidase tests [1].

*B. bronchiseptica* DNT activity was determined by the skin test in guinea pigs and by a mouse test. Albino guinea pigs weighing 500 g were inoculated intracutaneously with 0.2 ml of the DNT. The minimal dose of DNT was the highest dilution of the DNT which caused cutaneous necrosis 10 mm in diameter in 72 hr. To determine the LD<sub>50</sub> of the DNT, four SPF mice weighing 20 g were inoculated

Table 1. The effect of various pretreatments of pigs with *Bordetella bronchiseptica* extract or cells on the subsequent colonization of the nasal mucosa by *Pasteurella multocida*

Group (n=4)	Pretreatment			Challenge	Recovery				
	<i>B. bronchiseptica</i>			<i>P. multocida</i>	<i>B. bronchiseptica</i>		<i>P. multocida</i>		
	Live culture <sup>a)</sup> (non-toxicogenic)	Non-toxic extract <sup>b)</sup>	DNT <sup>c)</sup>	Live culture <sup>d)</sup> (toxigenic)	Nasal cavity		Tonsils		Tonsils
					Right	Left	Right	Left	
1	—	+	—	+	0/4 <sup>e)</sup>	0/4	0/4	0/4	0/4
2	—	—	+	+	0/4	0/4	0/4	0/4	3/4
3	+	+	—	+	4/4	4/4	0/4	0/4	0/4
4	+	—	+	+	4/4	4/4	0/4	0/4	4/4
5	—	—	—	—	0/4	0/4	0/4	0/4	0/4

a) Inoculated into both nostrils at 28 days of age.

b) Treated into the left nostril on 5 consecutive days, from 33 to 37 days of age.

c) Treated as above (b).

d) Inoculated into both nostrils at 37 days of age.

e) Infected piglets/tested.

See text for further details.

intraperitoneally with 0.2 ml of the serial dilution of each DNT. The test was read 72 hr after inoculation [5].

*Pathological and histological examination:* The head of the piglets was split longitudinally and examined for lesions of the nasal turbinates, nasal septum and ethmoid bone.

Tissue specimens secured for histopathological examination, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were treated and examined by the method described previously [5].

## RESULTS

*Bacteriological findings:* The nasal swabs of group 1 and group 5 piglets were free from *B. bronchiseptica* and *P. multocida* throughout.

The left nasal cavity of 3 and tonsil of 4 group 2 piglets yielded *P. multocida* at 40 and 45 day-old and at slaughter at the age of 50 days.

*B. bronchiseptica* strain N-95 was consistently cultured from both the right and the left nasal cavities of group 3 piglets beyond the age of 30 days. *P. multocida*, however, was not isolated from these piglets.

*B. bronchiseptica* strain N-95 was consistently isolated from both the right and the left nasal cavities of group 4 piglets on every 5th day after 30 days of age. In the period between 40 days of age and slaughter, the left nasal cavity and tonsil of 4 piglets yielded *P. multocida* (Table 1).

*B. bronchiseptica* and *P. multocida* strains isolated from the experimental piglets had the same properties as those contained in the inocula.

*Histopathological findings:* Piglets of all groups were free from gross lesions in the nasal cavity at slaughter. Neither light microscopic nor electron microscopic lesions were demonstrable in either of the nasal turbinates of piglets of groups 1, 3 and 5 and in the right nasal cavities of group 2 and 4 piglets. In piglets intranasally treated with *B. bronchiseptica* DNT the mucosal epithelial cells of the left turbinate showed loss of cilia and of microvilli (Fig. 1). Regressive lesions such as vacuolation, karyopycnosis and necrosis, hypertrophy of the epithelium, widening of the intracellular spaces, and infiltration of the epithelium and submucosa by inflammatory cells such as lymphocytes, plasma cells and granulocytes could also be seen (Fig. 2).

## DISCUSSION

The poor ability of *P. multocida* to colonize on the porcine nasal mucosa [12] inspired investigations into the factors promoting colonization in given conditions. Studies along this line demonstrated the favorable influence of mucosal infection by *Haemophilus parasuis* [8] and of painting with 1% acetic acid [7] in experimental conditions. Pedersen and Barfod [17] studied the influence of non-infectious factors in natural *P. multocida* infections in which isolation experiments failed to detect the

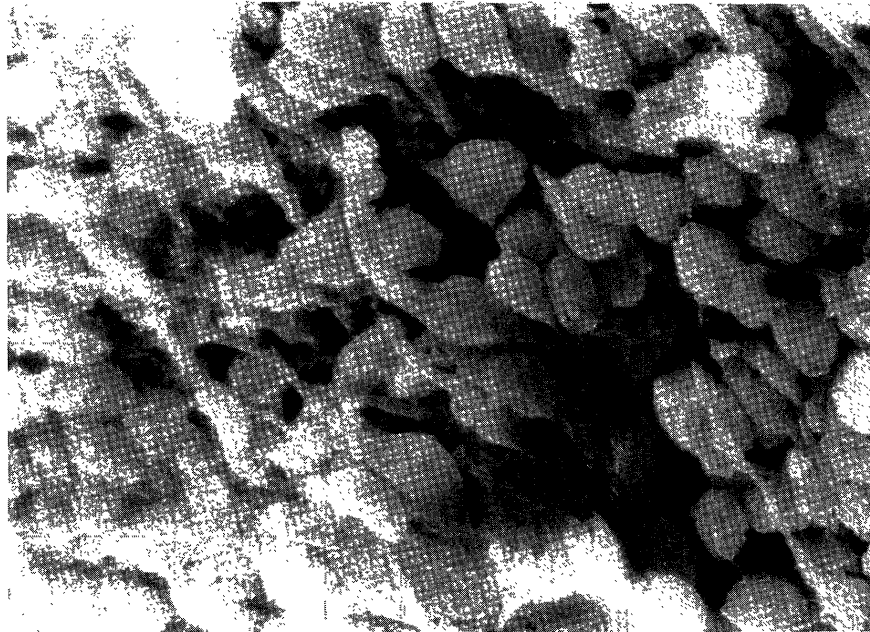


Fig. 1. Scanning electron micrograph of the ventral nasal turbinate from a 50-day-old SPF piglet intranasally treated with 10 µg of *Bordetella bronchiseptica* DNT five times. The mucosal epithelial cells of the turbinate show loss of cilia and microvilli. × 7,500.

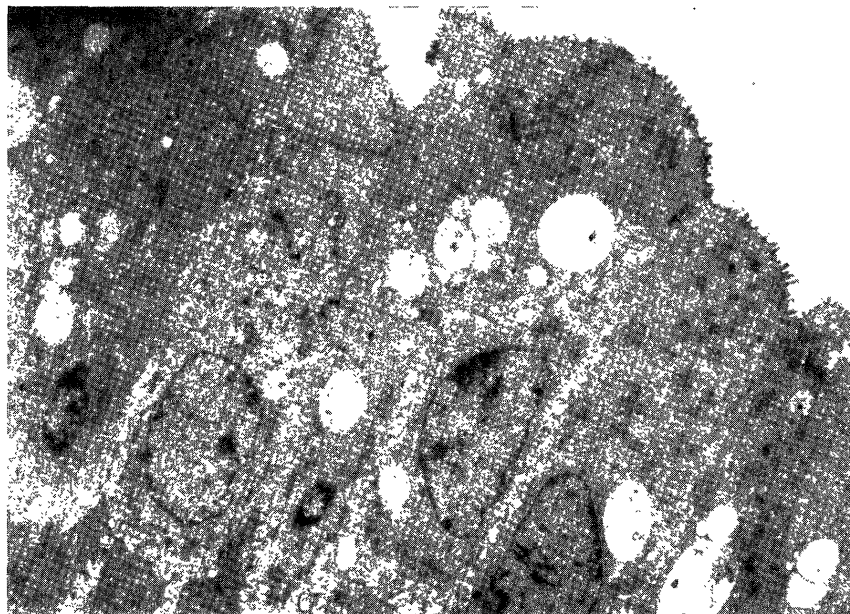


Fig. 2. Transmission electron micrograph of the ventral nasal turbinate from a 50-day-old SPF piglet intranasally treated with 10 µg of *Bordetella bronchiseptica* DNT five times. The nasal mucosal epithelial cells show regressive lesions such as vacuolation, karyopycnosis and necrosis. Widenings of the intracellular spaces are seen. × 5,000.

simultaneous presence of *B. bronchiseptica*. Chanter and Rutter [3] reported that the extent of a *P. multocida* infection induced in acid-painted nasal mucosa could be reduced by simultaneous administration of *P. multocida* antitoxin. This supports the

conclusion that the DNT produced by *P. multocida* promoted colonization of the nasal mucosa by the DNT-producing strain.

In addition, authors generally agree that the presence of toxin-producing *B. bronchiseptica*

promotes the establishment and growth of *P. multocida* on the nasal mucosa [20].

In view of this, we investigated the influence of *B. bronchiseptica* DNT in itself, and the joint influence of non-toxic factors, on colonization of the nasal mucosa by *P. multocida*. Four experimental groups were formed to study the effect of a non-toxic *B. bronchiseptica* extract (group 1), a non-DNT-producing live *B. bronchiseptica* strain (group 3) and of *B. bronchiseptica* DNT alone (group 2) and in combination (group 4).

Treatment with the non-toxic *B. bronchiseptica* extract (group 1) failed to promote the growth of *P. multocida* and infection with a non-DNT-producing *B. bronchiseptica* strain (group 3) had no influence either on colonization by *P. multocida*, unlike the pertinent observations of Chanter *et al.* [2]. The inconsistent results may have been due to the fact that not only the initial age of the experimental piglets, but also the period of study, the method of bacterial isolation and the properties of the non-DNT-producing *B. bronchiseptica* strains used for experimental infection differed between the experiments performed by Chanter *et al.* [2] and ourselves.

We isolated *P. multocida* colonies from the nasal mucosa of the piglets intranasally treated with *B. bronchiseptica* DNT. It follows that the mucosal injury inflicted by DNT was sufficient in itself, without a simultaneous presence of *B. bronchiseptica* organisms, to promote colonization by *P. multocida*. It also follows that other virulence factors, such as adenylate cyclase and adhesins, played no role whatever in the process studied.

The positive response of the piglets infected with non-DNT-producing *B. bronchiseptica* and treated additionally with *B. bronchiseptica* DNT by the intranasal route (group 4) can be explained as follows: *P. multocida* may have "realized" the fact of being "cheated" by us if its colonization had in fact depended on interaction between the two bacteria. In that case no colonization would have taken place. Since, however, the *P. multocida* organisms did grow to the same extent as in the nasal mucosa of the piglets of group 2, the results obtained in group 4 unequivocally supported the independent action of DNT.

The bacteriological and pathological results obtained for the nasal cavities of group 2 and group 4 piglets give answers to further two important questions. Firstly, it has become obvious that *P. multocida* can colonize and grow only on a nasal

mucosa the epithelial cells of which show regressive changes detectable at least by microscopy. This statement is supported by the finding that *P. multocida* grew only in the piglets left nasal cavity showing pathological changes, and could not be recovered throughout the experiment from the right nasal cavity having an intact mucous membrane. Secondly, it has been revealed that in piglets older than 33 days a 5-day treatment of the nasal mucosa with *B. bronchiseptica* DNT and a toxigenic *P. multocida* infection existing for 13 days are not sufficient to produce gross lesions of the nasal bones.

The present experimental observations pose the question whether the action of *B. bronchiseptica* DNT is a specific prerequisite of the growth of *P. multocida*, or just represents an effect that may as well be exerted by other factors promoting colonization. The clarification of this problem would be of great practical importance, despite the fact that *B. bronchiseptica* infection is omnipresent in conventional pig herds all over the world [10, 20].

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