Effects of intranasal inoculation with *Bordetella bronchiseptica*, porcine reproductive and respiratory syndrome virus, or a combination of both organisms on subsequent infection with *Pasteurella multocida* in pigs

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Objective—To determine effects of intranasal inoculation with porcine reproductive and respiratory syndrome virus (PRRSV) or *Bordetella bronchiseptica* on challenge with nontoxigenic *Pasteurella multocida* in pigs.

Animals—Seventy 3-week-old pigs.

Procedure—In experiment 1, pigs were not inoculated (n= 10) or were inoculated with PRRSV (10), *P multocida* (10), or PRRSV followed by challenge with *P multocida* (10). In experiment 2, pigs were not inoculated (n = 10) or were inoculated with *B bronchiseptica* (10) or PRRSV and *B bronchiseptica* (10); all pigs were challenged with *P multocida*. Five pigs from each group were necropsied 14 and 21 days after initial inoculations.

Results—*Pasteurella multocida* was not isolated from tissue specimens of pigs challenged with *P multocida* alone or after inoculation with PRRSV. However, in pigs challenged after inoculation with *B bronchiseptica, P multocida* was isolated from specimens of the nasal cavity and tonsil of the soft palate. Number of bacteria isolated increased in pigs challenged after coinoculation with PRRSV and *B bronchiseptica*, and all 3 agents were isolated from pneumonic lesions in these pigs.

Conclusions and Clinical Relevance—Infection of pigs with *B bronchiseptica* but not PRRSV prior to challenge with *P multocida* resulted in colonization of the upper respiratory tract and tonsil of the soft palate with *P multocida*. Coinfection with PRRSV and *B bronchiseptica* predisposed pigs to infection of the upper respiratory tract and lung with *P multocida*. Porcine reproductive and respiratory syndrome virus and *B bronchiseptica* may interact to adversely affect respiratory tract defense mechanisms, leaving pigs especially vulnerable to infection with secondary agents such as *P multocida*. (*Am J Vet Res* 2001; 62:521–525)

Respiratory disease in pigs is arguably the most important health concern for swine producers today. Isolation of multiple agents from pigs with pneumonia and upper respiratory tract disease is commonplace, and it is thought that the interaction among pathogens and environmental or management conditions plays a key role in etiopathogenesis. Numerous infectious organisms are associated with respiratory disease in pigs, including viral agents such as **porcine reproductive and respiratory syndrome virus (PRRSV)**, swine influenza virus, and porcine respiratory coronavirus and bacterial agents such as *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis, Pasteurella multocida*, *Bordetella bronchiseptica,* and *Streptococcus suis*. The presence of multiple infectious agents complicates diagnostic processes and disease control, because treatments or management changes may control only one aspect of a multifactorial disease.

Porcine reproductive and respiratory syndrome virus is a widely disseminated pathogen of swine associated with reproductive and respiratory disease. In addition, PRRSV exacerbates the adverse effects of other pathogens.[1-10] This may be related to the ability of PRRSV to replicate in alveolar macrophages, the cellular component of innate and acquired immune responses in the respiratory tract. *Bordetella bronchiseptica* also is commonly found in swine and is considered a cause of bronchopneumonia and atrophic rhinitis. It colonizes the nasal cavity, causing mucosal inflammatory lesions with loss of cilia and atrophy of the turbinates. Infection with *B bronchiseptica* predisposes pigs to disease with secondary pathogens such as toxigenic *P multocida* and *S suis*.[11-13] *Pasteurella multocida* is a common secondary infective agent in pigs with respiratory tract diseases. Toxigenic strains of *P multocida* cause progressive atrophic rhinitis in pigs, often

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secondary to a primary infection with *B bronchiseptica*. *Pasteurella multocida* is one of the most common secondary invaders in pigs with pneumonia. Although serotypes A and D and toxigenic and nontoxigenic strains have been isolated from pneumonic lesions, the most common *P multocida* isolates from such lesions belong to nontoxigenic serotype A strains. Because PRRSV, *B bronchiseptica*, and *P multocida* are common pathogens of pigs, the purpose of the study reported here was to determine whether these agents interact in causing respiratory tract disease in pigs.

Materials and Methods

Animals—At 2 weeks of age, 70 pigs were transferred from a herd that was certified free of brucellosis and pseudorabies to an animal isolation facility at the National Animal Disease Center. No medications or vaccinations were administered. The source herd was monitored quarterly by use of serologic tests and periodically by inspection of tissues at slaughter for evidence of swine dysentery, swine influenza, mange, lice, and infection with rotavirus, PRRSV, transmissible gastroenteritis, *Mycoplasma* spp, *Salmonella spp*, and *B bronchiseptica*. Swab specimens were obtained from the tonsil of the soft palate and nasal cavity from all pigs before the study, and neither *B bronchiseptica* nor *P multocida* were isolated. Porcine reproductive and respiratory syndrome virus was not isolated from serum obtained from any pig prior to the study.

Preparation of inocula—Porcine reproductive and respiratory syndrome virus strain NADC-21[a] was isolated from the serum of a premature pig born on a farm on which an acute outbreak of abortions and a concomitant increase in sow mortality were reported. The virus was isolated on MARC-145 cells, passaged 2 times, and frozen at –70 C.

Bordetella bronchiseptica strain KM22,[b] a virulent phase-I swine isolate, was cultured from a herd with atrophic rhinitis. To prepare the inoculum, *B bronchiseptica* was cultured on Bordet-Gengou agar supplemented with 10% ovine blood at 37 C for 40 hours and suspended in PBS solution.

Pasteurella multocida strain P-3480, a nontoxigenic serotype A:3 isolate, was cultured from the lung of a pig with pneumonia. To prepare inocula, *P multocida* was cultured on dextrose starch agar for 18 hours at 37 C and suspended in cold tryptose broth.

Experiment 1—Forty pigs were randomly assigned to 1 of 4 groups (10 pigs/group). After 1 week of acclimation, on day 0 of the experiment, pigs in groups 1 and 2 were inoculated intranasally with 4 ml (2 ml/nostril) of cell culture medium containing PRRSV (10^4 **median cell culture infectious dose [CCID$_{50}$]**/ml). On day 7, pigs in groups 2 and 3 were inoculated intranasally with 2 ml (1 ml/nostril) of tryptose broth containing *P multocida* ($10^{8.4}$ **colony forming units [CFU]**/ml). Pigs in group 4 served as noninoculated controls. Clinical signs of disease and rectal temperature were recorded daily, and body weight was recorded twice a week. Five pigs from each group were euthanatized and necropsied on days 14 and 21.

Experiment 2—Pigs were randomly assigned to 1 of 3 groups (10 pigs/group). After 1 week of acclimation, on day 0 of the experiment, pigs in groups 1 and 2 were inoculated intranasally with 1 ml (0.5 ml/nostril) of PBS solution containing *B bronchiseptica* ($10^{5.9}$ CFU/ml). Pigs in group 2 were also inoculated intranasally at the same time with 4 ml (2 ml/nostril) of cell culture medium containing PRRSV (10^4 CCID$_{50}$/ml). On day 7, pigs in all 3 groups were inoculated intranasally with 2 ml (1 ml/nostril) of tryptose broth containing *P multocida* ($10^{8.8}$ CFU/ml). Clinical signs and rectal temperature were recorded daily, and body weight was recorded twice a week. Five pigs from each group were euthanatized and necropsied on days 14 and day 21.

Necropsy—An estimate of gross lung involvement was assigned on the basis of percentage of each lung lobe affected and percentage of total lung volume each lobe represented. Percentage of total lung volume of each lobe was estimated as 5% for the left cranial, 6% for the left middle, 29% for the left caudal, 11% for the right cranial, 10% for the right middle, 34% for the right caudal, and 5% for the intermediate lung lobes.[14]

Snouts from pigs in experiment 2 were transversely sectioned at the level of the first premolar tooth, and each of the 4 scrolls of the ventral nasal turbinate was assigned a score from 0 to 4 according to the following scale: 0 = normal; 1 = less than half of turbinate atrophied; 2 = at least half of turbinate atrophied; 3 = turbinate straight and only a small portion remained; and 4 = total turbinate atrophied. A score of 0 to 2 was assigned to the nasal septum according to the following scale: 0 = normal; 1 = slight deviation; and 2 = severe deviation. The total score for lesions of the turbinate and nasal septum was derived by addition of the four turbinate scores and the septal score; total score ranged from 0 to 18.

Isolation of virus and bacteria—Necropsy specimens were collected from the tonsil of the soft palate and the right cranial lung lobe from each pig in experiment 1 and from the nasal turbinate, tonsil of the soft palate, and right cranial lung lobe from each pig in experiment 2. Specimens were weighed and ground individually in PBS solution, using a tissue grinder. Number of CFU of *P multocida* or *B bronchiseptica* per gram of tissue was determined by plating serial 10-fold dilutions of homogenates on duplicate selective blood agar plates containing 5 µg of clindamycin/ml for *P multocida* or 20 µg of penicillin/ml, 10 µg of amphotericin B/ml, 10 µg of streptomycin/ml, and 10 µg of spectinomycin/ml for *B bronchiseptica*.

Serum and pulmonary lavage fluid samples for virus isolation were obtained from each pig at necropsy. Pulmonary lavage was performed by injecting 40 ml of PBS solution, 10 ml at a time, into the lumen of the main bronchus of the right lung at the bifurcation of the trachea. The right lung was massaged gently and the PBS solution collected by aspiration. Serum samples and lavage fluid were stored at –80 C. Isolation of PRRSV was performed by inoculation of 100 µl of serum or lavage fluid onto a monolayer of MARC-145 cells in a 24-well plate. Cells were examined for cytopathic effects daily for 1 week.

Verification of *Pasteurella multocida* strain—To determine whether *P multocida* organisms isolated at necropsy were derived from the inoculating strain (P-3480), **restriction endonuclease analysis (REA)** of *P multocida* DNA was performed, using *Hha*I as described.[15]

Statistical analyses—Mean CFU of *B bronchiseptica* isolated per gram of nasal turbinate tissue was compared between groups by use of a Student *t*-test. In experiment 2, mean weight gain and mean turbinate scores of pigs euthanatized on day 21 were compared among groups by use of Student *t*-tests. Level of significance was set at $P < 0.05$.

Results

Experiment 1—Pigs in the control group remained clinically normal throughout the experiment. Pigs inoculated with *P multocida* alone did not develop clinical signs of disease or a febrile response **(Fig 1)**. These pigs gained weight at rates similar to

pigs in the control group (**Fig 2**). Clinical signs of disease, including lethargy, anorexia, increase in respiratory rate, sneezing, febrile response, and reduced rate of weight gain, were of similar severity between pigs inoculated with PRRSV alone or prior to challenge with *P multocida*.

Gross lesions were not observed in noninoculated pigs or pigs inoculated with *P multocida* alone. All pigs inoculated with PRRSV alone or prior to challenge with *P multocida* had generalized lymphadenopathy and gross lesions consistent with interstitial pneumonia. Mean percentage of lung involvement was similar for pigs in both groups (PRRSV alone, 24.3 and 18.2% [days 14 and 21, respectively]; PRRSV and *P multocida*, 28.3 and 19.7% [days 14 and 21, respectively]).

Pasteurella multocida was not isolated from tissue specimens of tonsil or lung obtained from any pig in experiment 1. Porcine reproductive and respiratory syndrome virus was not isolated from serum or pulmonary lavage fluid of pigs in the control group or pigs inoculated with *P multocida* alone; however, PRRSV was isolated from serum or pulmonary lavage fluid of all pigs inoculated with PRRSV alone or prior to challenge with *P multocida*.

Experiment 2—Clinical signs of disease did not develop in the 10 pigs inoculated with *P multocida* alone; mean rectal temperature remained < 40 C throughout the experiment (**Fig 3**). At necropsy, gross lesions were not observed in any of these pigs, and there was no evidence of turbinate atrophy. The challenge strain of *P multocida* was not isolated from any pig. However, a noncapsulated strain of *P multocida* that yielded REA results different from those of the challenge strain was isolated from the tonsil of 4 of 10 pigs. Porcine reproductive and respiratory syndrome virus and *B bronchiseptica* were not isolated from any of the pigs in this group.

Clinical signs of disease in pigs inoculated with *B bronchiseptica* prior to challenge with *P multocida* consisted mainly of sneezing and mild coughing; mean rectal temperature was ≥ 40 C for 2 days (Fig 3). Mean weight gain for this group was significantly ($P = 0.02$) less than for the group inoculated with *P multocida* alone (**Fig 4**). At necropsy, lesions in pigs inoculated with *B bronchiseptica* prior to challenge with *P multocida* consisted of mucopurulent nasal discharge and mild atrophy of the turbinates (mean turbinate score, 3.0 and 2.8 [days 14 and 21, respectively]), but gross lung lesions were not observed. *Bordetella bronchiseptica* was isolated from the nasal turbinates of 5 of 5 pigs on day 14 (mean number of organisms, $10^{5.9}$ CFU/g of tissue) and 4 of 5 pigs on day 21 ($10^{6.2}$ CFU/g of tissue) and from the lungs of 1 of 5 pigs on day 14 and 0 of 5 pigs on day 21. The challenge strain of *P multocida* was recovered from nasal turbinate or tonsil specimens of 3 of 5 pigs on day 14 and 4 of 5 pigs on day 21 but not from the lung of any pig in this group. A noncapsulated strain of *P multocida* that yielded REA results different from those of the challenge strain was isolated from the tonsil of 1 of 10 pigs at necropsy. Porcine reproductive and respiratory syndrome virus was not isolated from any of the pigs in this group.

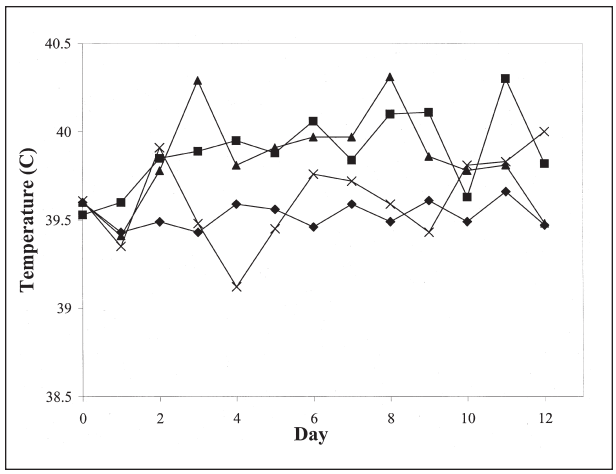

Figure 1—Mean rectal temperature of 3-week-old pigs inoculated intranasally with porcine reproductive and respiratory syndrome virus (PRRSV) on day 0 (n = 10; ■), PRRSV on day 0 and *Pasteurella multocida* on day 7 (10; ▲), or *P multocida* alone on day 7 (10;◆). Ten pigs served as controls; these pigs were not inoculated (X).

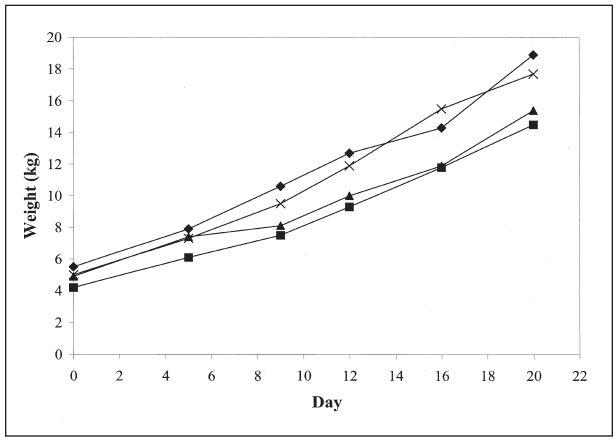

Figure 2—Mean body weight of 3-week-old pigs inoculated intranasally with PRRSV on day 0, PRRSV on day 0 and *P multocida* on day 7, or *P multocida* alone on day 7. Ten pigs served as controls; these pigs were not inoculated. *See* Figure 1 for key.

Clinical signs of disease, including a febrile response and reduced rate of weight gain, was most severe in pigs inoculated with PRRSV and *B bronchiseptica* prior to challenge with *P multocida*. Pigs developed lethargy, anorexia, increased respiratory rate, frequent sneezing and coughing, conjunctivitis, mucopurulent nasal discharge, epistaxis, and vomiting. Mean rectal temperature for these 10 pigs was ≥ 40 C for 11 days (Fig 3), and mean weight gain was significantly less than mean weight gain for the groups challenged with *P multocida* alone or after inoculation with *B bronchiseptica* ($P = 0.003$ and $P < 0.001$, respectively; Fig 4). At necropsy, lesions consisted of copious mucopurulent and, at times, hemorrhagic nasal discharge and mild to moderate turbinate atrophy (mean turbinate score, 2.6 and 6.0 [days 14 and 21, respectively]). Mean turbinate score on day 21 for pigs inoculated with all 3 agents was significantly ($P = 0.037$) greater than for pigs inoculated with *B bronchiseptica* and *P multocida*. All pigs had gross lesions of pneumo-

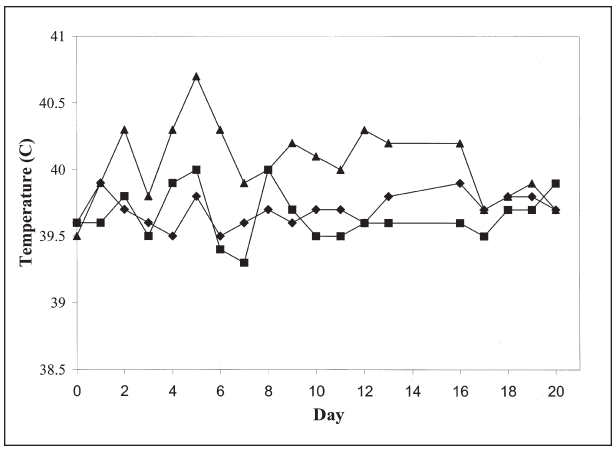

Figure 3—Mean rectal temperature of 3-week-old pigs inoculated intranasally with *Bordetella bronchiseptica* on day 0 and *P multocida* on day 7 (n = 10;■), PRRSV and *B bronchiseptica* on day 0 and *P multocida* on day 7 (10; ▲), or *P multocida* alone on day 7 (10;◆).

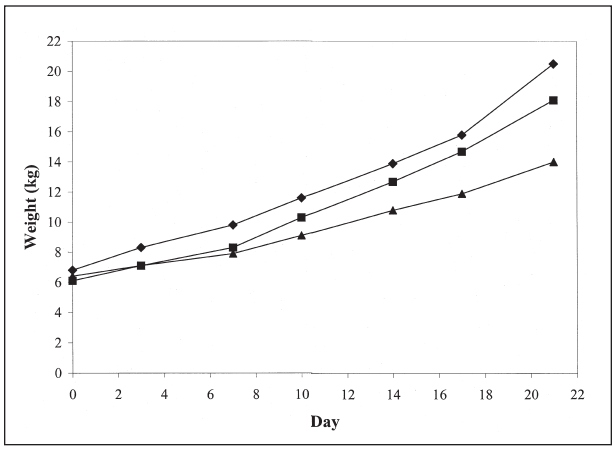

Figure 4—Mean body weight of 3-week-old pigs inoculated intranasally with *bronchiseptica* on day 0 and *P multocida* on day 7, PRRSV and *B bronchiseptica* on day 0 and *P multocida* on day 7, or *P multocida* alone on day 7. *See* Figure 3 for key.

nia (mean percentage of lung affected, 34.5 and 40.5% [days 14 and 21, respectively]) and generalized lymphadenopathy. *Bordetella bronchiseptica* was isolated from the nasal turbinates of all pigs (mean number of organisms, $10^{7.3}$ and $10^{8.0}$ CFU/g tissue [days 14 and 21, respectively]) and from the lung of 1 of 5 pigs on day 14 and 5 of 5 pigs on day 21. Number of *B bronchiseptica* recovered per gram of nasal turbinate tissue was significantly (P = 0.008 and 0.013 [days 14 and 21, respectively]) greater for pigs inoculated with all 3 agents, compared with pigs inoculated with *B bronchiseptica* and *P multocida*. The challenge strain of *P multocida* was recovered from nasal turbinate or tonsil specimens of 4 of 5 pigs on day 14 and 5 of 5 pigs on day 21 and from the lungs of 0 of 5 pigs on day 14 and 3 of 5 pigs on day 21. A noncapsulated strain of *P multocida* that yielded REA results different from those of the challenge strain was isolated from the tonsil of 4 of 10 pigs. Porcine reproductive and respiratory syndrome virus was isolated from serum or pulmonary lavage fluid from all pigs in this group.

Discussion

Results of a previous study indicate that infection with PRRSV predisposes pigs to pulmonary infection with *B bronchiseptica*.[16] In that study, *B bronchiseptica* was not isolated from the lungs of pigs infected with bacteria alone but was isolated from the lungs of pigs infected with both agents. The clinical course of disease, including respiratory signs, febrile response, and decreased weight gain, was more pronounced in pigs inoculated with both agents than in pigs infected with either agent alone. Results of the study reported here confirm and extend these previous results. *Bordetella bronchiseptica* was isolated from the lungs of only 1 of 10 pigs inoculated with *B bronchiseptica* and *P multocida*, but it was isolated from 6 of 10 pigs inoculated with PRRSV, *B bronchiseptica*, and *P multocida*. Prior infection with PRRSV did not predispose pigs to infection with *P multocida*, and disease was no more severe in pigs inoculated with PRRSV and *P multocida* than in pigs inoculated with PRRSV alone. These results agree with results of Carvalho et al, who also reported that there was no interaction between PRRSV and *P multocida*.[17] In the present study, prior inoculation with *B bronchiseptica* resulted in colonization of nasal and tonsilar tissue with *P multocida*. Prior inoculation with both *B bronchiseptica* and PRRSV not only resulted in colonization of the nasal cavity and tonsil with *P multocida* but also the lungs. Although *P multocida* was not isolated from pigs in experiment 2 prior to the initial inoculation, pigs were apparently subclinically infected, because a strain of *P multocida* different from that inoculated was isolated from some pigs in all groups at necropsy. It is not known whether subclinical infection with other strains of *P multocida* affect the ability of strain P-3480 to colonize tissue or cause disease; however, this does not alter the fact that prior inoculation of pigs with *B bronchiseptica* and PRRSV exacerbated disease caused by infection with *P multocida* strain P-3480.

Anecdotal evidence from field outbreaks of respiratory tract disease suggest that reemergence of many pathogens is associated with swine herds becoming seropositive for PRRSV.[1-6,8,9] This observation has led to the belief that PRRSV acts synergistically with other pathogens. The virus replicates in alveolar macrophages and may affect macrophage function. Impaired ability of macrophages to clear infectious agents or to aid in the induction of inflammatory and immune responses could increase severity of lung disease. However, evidence to prove a link between PRRSV and other pathogens has been mixed. Results of some studies indicate that disease severity increases in pigs coinfected with PRRSV and *S suis*, porcine respiratory coronavirus, or swine influenza virus.[7,10] Also, it has been reported that infection with *M hyopneumoniae* results in more severe PRRSV lesions rather than more severe mycoplasma lesions.[18] Other studies evaluating dual infections with PRRSV and *S suis*, *H parasuis*, *Salmonella cholerasuis*, *P multocida*, *A pleuropneumoniae*, *M hyopneumoniae*, or transmissible gastroenteritis virus have failed to demonstrate a synergistic interaction between agents.[10,17-23]

In the study reported here, inoculation with

P multocida alone did not result in colonization of the respiratory tract. Although PRRSV causes some mechanical damage to the upper respiratory tract, prior inoculation with PRRSV also did not result in increased colonization of respiratory tract tissue with *P multocida*. One reason for the ability of PRRSV to predispose to pulmonary infection with *B bronchiseptica* but not *P multocida* is that *B bronchiseptica*, unlike *P multocida*, is capable of colonizing the upper respiratory tract without aid from other infectious agents. Prior infection with *B bronchiseptica* enabled *P multocida* to colonize the upper respiratory tract but did not enable *P multocida* to gain access to the lung. However, prior infection with PRRSV and *B bronchiseptica* allowed *P multocida* to gain access to the lung. Possible mechanisms by which *B bronchiseptica* predisposed pigs to infection with *P multocida* include use of adhesins produced by *B bronchiseptica*, toxin-induced turbinate atrophy, and damage to ciliated epithelium or other innate defense mechanisms. Thus, field cases of respiratory disease may be more complex than just 1 agent predisposing pigs to infection with another agent. Instead, it may be that combinations of primary pathogens attack multiple defenses causing severe debilitation of the respiratory tract and resulting in the complex infections seen in the field. As *B bronchiseptica* and PRRSV are found in a high percentage of swine herds, simultaneous infection with PRRSV and *B bronchiseptica* may severely inhibit resistance to secondary respiratory tract pathogens such as *P multocida*.

[a]Gift of Dr. Kelly Lager, USDA, Agricultural Research Service, National Animal Disease Center, Ames, Iowa.

[b]Gift of Dr. Tibor Magyar, Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary.

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