Evaluation of the aerosol transmission of a mixed infection of *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus

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To evaluate the transmission of *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus (PRRSV) by aerosol as either a single or mixed infection, 28 pigs were inoculated intratracheally with *M hyopneumoniae* on day 0 and infected intranasally with PRRSV on day 35; they were housed together in a barn. To assess the aerosol transmission of *M hyopneumoniae* as a single infection, one trailer (A) containing 10 five-week-old sentinel pigs was placed along the south side of the barn (1 m from the fans) on day 28. To assess the mixed infection, two trailers (B and C), each containing 10 five-week-old sentinel pigs, were placed along each side of the barn on day 42. The sentinel pigs in the three trailers were exposed to the exhaust from the fans for seven days. No *M hyopneumoniae* infection was detected in the sentinel pigs in trailer A, but it was detected in the sentinel pigs in trailers B and C. No PRRSV was detected in any of the sentinel pigs.

RESPIRATORY diseases in pigs are a major health concern in most pork-producing regions of the world. Recently, a new respiratory syndrome, the porcine respiratory disease complex (PRDC), has emerged as a serious problem in the late finishing stage of production, and it has been suggested that pneumonia in pigs with PRDC is due to a combination of both bacterial and viral agents. Two of the most important agents associated with PRDC are *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus (PRRSV) (Thacker and others 1999).

*M hyopneumoniae* has been reported to be transmitted by different routes, the most likely of which is through direct contact (Done 1996). However, indirect transmission and spread by aerosols have also been proposed (Goodwin 1984). It has been suggested that *M hyopneumoniae* may be transmitted by aerosols when the population density and infection levels are high (Done 1996). On the basis of a study of the risk factors associated with unexplained outbreaks in *M hyopneumoniae*-free herds, Goodwin (1985) proposed that the agent could be transmitted by moist, cold air over long distances. The agent has been detected by PCR in air samples under experimental and field conditions (Stark and others 1998); under field conditions, the agent was detected only on farms with acute clinical problems. These observations have provided evidence that *M hyopneumoniae* can be transmitted in the air, but the possibility has not been proved experimentally under field conditions.

It is not clear whether PRRSV can be transmitted by aerosols. Under experimental conditions it has been shown that the virus can spread over distances of 1 m (Tormorell and others 1997), but attempts to transmit it by aerosols under field conditions have been unsuccessful (Otaké and others 2002, Trincado and others 2003). In a recent study, Dee and others (2003) made a further attempt by using a straight-tube, negative-pressure model to determine whether PRRSV could move from one point to another solely in the air. Viable virus was transported over 150 m and infected three of six sentinel pigs exposed to the exhaust from the model, suggesting that the virus might occasionally be spread by aerosols under field conditions.

There have been no studies of the potential transmission of mixed infections of *M hyopneumoniae* and PRRSV. It has been shown that *M hyopneumoniae* may potentiate PRRSV (Thacker and others 1999), but it is not known whether this could increase the probability of aerosol transmission of the virus. The objectives of this study were to evaluate the aerosol transmission of *M hyopneumoniae* either as a single infection or as a mixed infection with PRRSV.

**Source of animals and facilities**

A total of 63 two-month-old pigs were obtained from a source known to be free of PRRSV and *M hyopneumoniae*. The pigs were housed at the research farm of the University of Minnesota Swine Disease Eradication Center, in a mechanically ventilated finishing building containing 11 pens. The pens were 10 x 2·5 m in size, had partially slatted floors, and were separated from each other by a combination of solid walls and vertical rod (open) gating. The ventilation system consisted of 20 inlets (each 0·3 x 0·6 m) and seven exhaust fans, each 0·83 m in diameter and capable of extracting 6100 cubic feet per minute (cfm) of air; four of them were on the north side and three were on the south side. The animals were placed, 10 per pen, in pens 1, 3, 5, 7, 9 and 11, leaving an empty pen between them and allowing 1 m² of space per pig (Fig 1). During the study, the animals were cared for under the guidelines of the University of Minnesota Institutional Animal Care and Use Committee.

**Infection model and monitoring protocol**

Twenty-eight of the pigs were inoculated intratracheally with *M hyopneumoniae* strain 232 (10³ colour-changing units [CCU]/ml) on day 0, and infected with PRRSV strain MN 30-100 (10²·5 TCID50) intranasally on day 35. The infected animals were in pens 3, 5, 7 and 9. In order to investigate the shedding and activity of the agents among the animals housed in the infected barn, the remaining animals were not inoculated, and 12 were used as direct-contact controls and 20 as indirect-contact controls. Three of the direct-contact pigs were placed in each of the four infected pens, and the indirect-contact controls were placed in pens 1 and 11 (Fig 1). Three two-month-old pigs negative for *M hyopneumoniae* and PRRSV were housed in another facility on the same site, to serve as negative controls.
M *hyopneumoniae* single infection trial

To assess the possibility of the transmission of *M hyopneumoniae* by aerosol as a single infection, a trailer (A) containing 10 five-week-old sentinel pigs was placed on day 28 along the south side of the infected barn, 1 m from the exhaust fans. The trailer was 9 m long, 2.5 m wide and 2 m high, and its walls contained 76 air inlets, each 0.08 m x 0.23 m. To prevent other forms of transmission, the personnel in charge of the sentinel pigs were disposable boots, gloves and coveralls whenever they were inside the trailer, and a footbath containing 100 per cent sodium hypochlorite was placed at its entrance. The air inlets into the trailers were covered with insect screens to prevent transmission by flies and mosquitoes. The sentinel pigs were exposed to the barn air exhaust for seven days, and were then moved to a building approximately 30 m from the infected barn where they were kept for 21 days; the personnel in charge of this building were required to wear disposable boots, coveralls and gloves. After this period the pigs were sent to the diagnostic laboratory for postmortem examination and collection of samples.

*M hyopneumoniae* and PRRSV mixed infection trial

Forty-two days after the *M hyopneumoniae* challenge and seven days after the PRRSV challenge, two trailers (B and C), like A, each containing 10 five-week-old sentinel pigs, were placed along the north and south sides of the infected barn. Trailer B was placed on the south side (1 m from the fans), and trailer C was placed 6 m from the fans on the north side of the barn. In order to maximise the contact of the sentinel pigs with the barn air exhaust for seven days, and to prevent other forms of transmission, each trailer was 9 m long, 2.5 m wide and 2 m high, and its walls contained 76 air inlets, each 0.08 m x 0.23 m. To prevent other forms of transmission, the personnel in charge of the sentinel pigs were disposable boots, gloves and coveralls whenever they were inside the trailer, and a footbath containing 100 per cent sodium hypochlorite was placed at its entrance. The air inlets into the trailers were covered with insect screens to prevent transmission by flies and mosquitoes. The sentinel pigs were exposed to the barn air exhaust for seven days, and were then moved to a building approximately 30 m from the infected barn where they were kept for 21 days; the personnel in charge of this building were required to wear disposable boots, coveralls and gloves. After this period the pigs were sent to the diagnostic laboratory for postmortem examination and collection of samples.

Animal sampling and diagnostic analysis

In the infected barn, blood and nasal swabs were taken from 12 of the infected pigs, the 12 direct-contact pigs and 10 of the indirect-contact pigs; these animals were randomly selected, ear tagged, and tested repeatedly throughout the experiment, on days 0, 28, 35, 42 and 49. The same schedule was followed with the negative control pigs. For the aerosol transmission trials, the *M hyopneumoniae* and PRRSV status of the sentinel pigs was monitored on the day they were placed in the trailers and 28 days later. The blood samples were tested for antibodies to *M hyopneumoniae* by using an ELISA (Dako Laboratories) (Sorensen and others 1993), for PRRSV nucleic acid by PCR (TaqMan) and for PRRSV antibodies by ELISA (IDEXX Laboratories). The nasal swabs were tested for *M hyopneumoniae* by using the nested PCR technique described by Calsamiglia and others (1999).

The sentinel pigs from trailer A were euthanased and their lungs were examined for the presence of lesions suggestive of *M hyopneumoniae* infection. Bronchial swabs were collected and tested for PRRSV by nested PCR. In the mixed infection trial, tissue samples from the lungs, tonsils and lymph nodes from at least two sites were collected from each of the sentinel pigs in trailers B and C. The lungs were evaluated macroscopically for the presence of lesions suggestive of PRRSV and *M hyopneumoniae* infection, and bronchial swabs were collected. The homogenised tissues were tested for PRRSV by PCR. Bronchial swabs were tested for *M hyopneumoniae* by nested PCR.

Air sampling

Air samples were collected daily during the exposure period from inside and outside the infected barn by using an all-glass impinger (Thorne and others 1992, Terzieba and others 1996) at a sampling rate of 12.5 l/minute and a pressure of 600 mmHg (Otake and others 2002). The impinger contained 30 ml minimal essential medium, and the air samples were collected for 10 minutes (Torremorell and others 1997). Independent samples were taken for each agent, and tested for *M hyopneumoniae* and PRRSV by PCR.

Environmental monitoring

It has been suggested that environmental conditions play an important role in the transmission of *M hyopneumoniae*...
**RESULTS**

*Infected barn*

The diagnostic data from the infected barn are shown in Tables 1 and 2. All the animals tested were negative to *PRRSV* and *M. hyopneumoniae* by serology and PCR on day 0, but on day 28 five of 12 infected pigs were positive by nested PCR on a nasal swab; on day 35 six of 11 were positive and on day 42 nine of 10 were positive. *M. hyopneumoniae* was detected by ELISA and nested PCR in both the direct- and indirect-contact controls housed inside the barn, but in the indirect-contact animals it was not detected until day 42, by nested PCR. On day 42, seven days after they had been infected with *PRRSV*, all the infected pigs tested were positive by PCR of serum. *PRRSV* was detected in both the direct- and indirect-contact controls, on day 42 by PCR and on day 49 by ELISA.

*M. hyopneumoniae single infection trial*

No *M. hyopneumoniae* was detected in any of the sentinel pigs from trailer A (Table 3) after their seven days’ exposure and 21 days in another building. *M. hyopneumoniae* was detected in air samples collected from inside the barn, but not in samples collected from the exhaust fan (Table 4).

**DISCUSSION**

Under the conditions of this study *M. hyopneumoniae* was transmitted via aerosols to the sentinel pigs in trailers B and C. *M. hyopneumoniae* was detected by nested PCR in nasal and bronchial swabs; however, all the sentinel pigs were negative by ELISA. Antibodies to *M. hyopneumoniae* may take five to nine weeks to appear after a natural infection (Sorensen and others 1993, Sitjar and others 1996), and the period of three weeks for which the pigs were left after they had been exposed may not have been adequate for antibodies to develop.

On the other hand, *M. hyopneumoniae* was not transmitted to the sentinel pigs in trailer A in the single infection trial, although it was recovered from air samples collected inside the infected barn. One explanation for this observation could be that between days 28 and 35 the microbial load present in the infected population was insufficient for transmission to occur; the microbial load is related to the proportion of infected animals and the acute phase of the disease (Kobish and others 1993). The diagnostic data from the infected barn showed that there was a smaller proportion of PCR-positive pigs during this period than later. There was no evidence of infection in the indirect-contact pigs until day 42. It can therefore be assumed that at least 33 per cent of the population was not shedding the mycoplasma during this first trial period. A second explanation could be associated with the higher relative humidity in the infected barn during the single infection trial (86-12 per cent) than in the infected barn during the mixed infection trial (67-23 per cent). *M. hyop-
M hyopneumoniae has been identified in air samples taken under conditions of low relative humidity (Stark and others 1999, Cardona and others 2003). In a humid environment, particles tend to aggregate into larger particles with a higher rate of sedimentation ( Cox and Wathes 1995). There were also differences in temperature between the trials. However, even though the concentration of airborne particles increases at low temperature ( Stark 1999), it is unlikely that this was a factor in the different results between the trials, because the lowest mean temperature was registered in the single infection trial, in which M hyopneumoniae was not transmitted. A third explanation could be that the co-infection with PRRSV stimulated higher levels of shedding of M hyopneumoniae. However, this is not clear, because the failure to transmit the mycoplasma on its own appeared to be related more to the smaller number of clinically affected animals shedding it at the time. Although M hyopneumoniae was successfully transmitted by aerosols, further studies are needed to evaluate the factors that may favour the process, such as microbial loads, environmental conditions and possibly mixed infections.

In the mixed infection assessment, PRRSV was not transmitted to the sentinel pigs by aerosol even though it was given at the time of an acute M hyopneumoniae infection and despite the fact that the direct- and indirect-contact animals inside the barn were infected. The potentiation of PRRSV pneumonia by M hyopneumoniae has been described by Thacker and others (1999), but in this study it was not possible to determine whether PRRSV shedding increased as a result of the mixed infection. In order to increase the probability that PRRSV would be transmitted by aerosol, a rigid PVC pipe that had successfully moved the virus 150 m in a previous study ( Dee and others 2003) was used. The fact that it was not successful in the present study may have been due to differences in the titre of virus. In the previous study, the aerosol was generated mechanically and may have had a higher titre of virus than that shed naturally by infected animals. These results are similar to the results of Otake and others (2002) and Trincado and others (2003), who also failed to detect PRRSV in air samples and sentinel pigs under controlled field conditions. Since the present study was successful in transmitting M hyopneumoniae but not PRRSV, the data suggest that the transmission of PRRSV by aerosol under field conditions may be difficult to prove. Further studies to quantify the agent in aerosols produced by experimentally and naturally infected pigs, and to assess the minimal infected population required to transmit the virus by aerosol, will be needed.

The results of this study show that M hyopneumoniae can be transmitted by aerosols under controlled field conditions, by identifying it in air samples and in sentinel pigs. However, its transmission seems to be dependent on the microbial load and possibly on the environmental conditions. Under the conditions of this study, a mixed infection of M hyopneumoniae and PRRSV did not result in the aerosol transmission of PRRSV, suggesting that the airborne transmission of PRRSV may be a rare event under field conditions.

**References**


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