

Infection of pigs by Aujeszky's disease virus via the breath of intranasally inoculated pigs

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SUMMARY

Aujeszky's disease is a worldwide problem in the pig industry. In this experiment, four pigs chosen to act as shedder pigs were intranasally infected with Aujeszky's disease virus. Next, on three consecutive days, eight recipient pigs were exposed to the breath of a pair of shedder pigs via a mask-to-mask module. Except for the virtual absence of CNS signs, shedder pigs expressed clinical signs that were similar to pigs infected naturally or experimentally. Only mild respiratory signs occurred in recipient pigs, but all were infected by aerosols of Aujeszky's disease virus as evidenced by seroconversion. The pig is a much more sensitive indicator of airborne virions than our aerosol collection methods. We conclude that the mild respiratory disease acquired by the aerogenous route in recipient pigs is an easily managed model for studying the transmission of airborne respiratory infections and the immune responses to this type of infection.

DURING the past 25 years, the pig industry has changed dramatically, such that the stocking density of large numbers of pigs in closed environments facilitates airborne transmission of respiratory pathogens (Christensen and Mousing 1992). Without evidence for the other methods of spread of Aujeszky's disease, it has been postulated that the virus is able to infect 'receptor' herds over considerable distances because it is suspended in aerosols from 'source' herds (Gloster et al 1984, Christensen et al 1990, 1993, Scheidt et al 1991). Like pathogens in other species, Aujeszky's disease virus in pigs may be excreted in the breath or the spray that is created as pigs cough and sneeze or by resuspension and entrainment of the virus from surfaces on which it has settled (Donaldson et al 1983, Cox 1987).

Aujeszky's disease virus that was given experimentally as an intranasal dose or as an aerosol was shed by the infected pigs and carried, via ventilation ducts or across rooms, to sentinel pigs that, subsequently, developed antibodies against the virus (Donaldson et al 1983, Gillespie et al 1996). Baskerville et al (1971) found that although exposure to experimental aerosols that contained a strain of Aujeszky's disease virus with an affinity for the respiratory system caused a milder clinical syndrome than did intranasal inoculation, both methods of infection resulted in coughing and sneezing. Later, Baskerville (1972) concluded that the onset, severity and persistence of clinical signs, including neurological signs, was dosage dependent.

By studying the response of pigs to airborne Aujeszky's disease virus, we believe that we can better elucidate the process by which organisms spread among pigs and pig

farms. Only then can changes in environments and other interventions that might reduce the shedding and susceptibility of airborne pathogens be implemented. Hence we designed this experiment with the objective of answering three questions. Firstly, could we give an intranasal dose of Aujeszky's disease virus that would initiate respiratory disease without causing severe neurological signs or the death of relatively young pigs? Secondly, using a closed breathing system, would virus shed as a 'primary' aerosol in the breath of intranasally inoculated pigs infect other pigs? Thirdly, was our methodology, which used an all-glass impinger, as effective as the respiratory system of the pig for identifying the presence of virus in a 'primary' aerosol?

MATERIALS AND METHODS

Origin and management of the pigs

Weaned pigs (5 to 6 weeks of age and about 15 kg body weight) were collected from the nursery on a farm validated free of Aujeszky's disease. However, on the basis of regular herd health visits and diagnostic tests on samples from pigs, many common respiratory and enteric pathogens were identified in the herd, i.e. typical of a commercial swine herd in terms of health status (Kluge et al 1992).

All pigs were reared and procedures were performed in isolation buildings in the research animal housing complex at Purdue School of Veterinary Medicine, in accordance with protocols approved by the University Animal Care and Use Committee. The pigs were housed in isolation rooms in pens with solid concrete floors. Each room was maintained at 25°C and had a volume of about 28 m³ with a ventilation rate of 12 air changes per hour. Daily, after faeces were removed, the floors were cleaned with minimal amounts of water at low pressure to minimise formation of aerosols.

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Water from nipple drinkers and a dry, commercial weaner meal containing 16% protein were provided ad libitum.

Pigs were randomly assigned to one of two categories i.e., shedder pigs that were to be infected with virus and recipient pigs that were exposed to the breath of shedder pigs. All shedder pigs were housed in one room, but recipient pigs were housed as pairs in four other rooms. Caretakers were assigned to manage shedder or recipient pigs. To avoid cross-contamination between different categories of pigs, investigators washed their hands and arms in antiseptic soap and changed boots, overalls and gloves between handling pigs from different rooms.

Each pig was clinically examined daily whilst in the experiment. Coughing, sneezing and the quantity and nature of nasal discharge were assigned a grade between 0 and 2. The severity of dyspnoea was assigned a grade between 0 and 3. With increasing severity, higher grades were assigned. For shedder pigs, the rectal temperatures were monitored between days 3 to 8 post infection (PI), whereas recipient pigs were monitored on the days of exposure to the breath of shedder pigs. A pig was regarded as febrile if its rectal temperature was $\geq 40^{\circ}\text{C}$. Each day the pigs were handled in the sequence, recipient then shedder pigs.

Experimental design

Twelve pigs were randomly assigned to one of three equally sized groups and to one of two pairs within each group. One group (group 1) was then randomly assigned to be the shedder pigs and on the day the pigs were collected from the farm (day zero of the experiment), each of the pigs in group 1 was intranasally inoculated with a suspension of Aujeszky's disease virus. Each pig was restrained vertically with its head uppermost whilst the viral suspension was instilled.

Each pair of pigs in recipient groups 2 and 3 was matched with a pair of shedder pigs. On days when attempts were made to infect recipient pigs, a shedder pig and a recipient pig were placed in hammocks, samples of nasal mucus were collected and rectal temperatures were measured. The recipient pig was then exposed to the breath of a shedder pig via

a closed mask-to-mask module (Fig 1). After 15 minutes, the first pig was replaced by the second of a pair of shedder pigs and the recipient pig was exposed to the breath of this pig for a further 15 minutes. The procedure was repeated on three consecutive days.

After each recipient pig had been exposed to the breath of a pair of shedder pigs, it was returned to the isolation room. Between each pair of recipient pigs, the hammock was exchanged for a laundered one. For each recipient pig, the mask and connecting tube were sterilised by immersion in 95 per cent ethyl alcohol and drying in a stream of heated air.

Intranasal inoculum

An inoculum was prepared from stock cultures of Aujeszky's disease virus in CRFK cells suspended in phosphate buffered saline (PBSS, pH 7.4). The pigs were inoculated with 0.5 ml of suspension in each nostril.

Exposure of recipient pigs to an aerosol in the breath of shedder pigs

With the snouts of the two pigs sealed in face masks, the pump generated a vacuum and a unidirectional flow of air such that the exhaled breath from a shedder pig went to the mask and, therefore, the nostrils of a recipient pig. As exposure of the recipient pigs to the breath of shedder pigs was staggered, pigs from group 2 were exposed on days 3 to 5 PI for shedder pigs, whereas pigs from group 3 were exposed on days 6 to 8 PI.

Aerosol sampling

In an effort to capture Aujeszky's disease virus from the breath of the pigs, every time a recipient pig was exposed to the breath of a shedder pig, the air that went through the mask-to-mask module was also passed through two all-glass impingers (AGI-30, Gillespie et al 1996). Each AGI-30 contained 20 ml of PBSS and was held in an ice/water mixture. For each recipient, paired samples of breath were pooled.

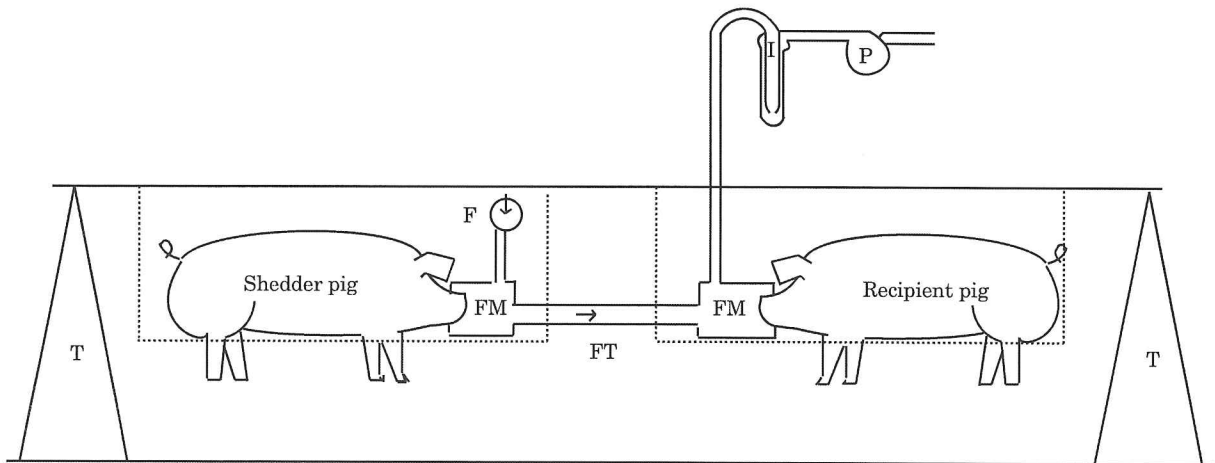


FIG 1: Arrangement of equipment used to expose a recipient pig to the breath of a shedder pig that had been intranasally inoculated with Aujeszky's disease virus. F, HEPA filter cartridge; FM, acrylic face mask; FT, stainless steel flexible tube (earthed). I, all-glass impinger; P, vacuum pump; T, trestle; H, hammock (dotted line is the outline; the hammock is suspended from two steel tubes). Arrows indicate the air flow.

Collection of nasal swabs and procedures for quantification of Aujeszky's disease virus

Procedures for the collection of nasal mucus on weighed swabs and for the quantification of virus in the intranasal inoculum, nasal mucus and tissues have been reported (Gillespie et al 1996). However, samples of aerosols that were collected in the AGI-30 were examined differently. Briefly, CRFK cells were cultured in 75 ml flasks until confluent and then trypsinised. Harvested cells were suspended in 10 ml KSM 0004 (laboratory medium, CLK). Next, 2.5 ml of the CRFK cell suspension was added to the fluid from the AGI-30 and the mixture rolled at room temperature for 1 hour. After centrifuging the suspension at 250 × g for 10 min at 5°C, the pellet of cells was re-suspended in 20 ml of KSM 0004, set in a 75 ml flask, incubated at 37°C and observed daily for cytopathic effects for up to 7 days.

Serological tests

Blood was collected by venepuncture of the anterior vena cava whilst the pig was restrained in dorsal recumbency in the room in which it was housed. Serum was then separated from coagulated blood and tested for antibodies against Aujeszky's disease virus using the serum neutralisation test.

Sampling regimen

The schedule for procedures is shown in Fig 2. For shedder pigs, nasal mucus was collected on days 3 to 8 PI and for recipient pigs, on days 2, 5, 8 and 11 after their first exposure to the breath of shedder pigs (post exposure, PE). These were days 5, 8, 11 and 14 of the experiment for pigs from group 2 and days 8, 11, 14 and 17 for pigs from group 3. The shedder pigs were monitored until day 16 PI and then euthanased. Serum from recipient pigs was collected 14 and 17 days PE. On day 25 of the experiment, recipient pigs were also euthanased.

Post-mortem evaluation of recipient pigs

All pigs were electrically stunned and bled out. The procedures in this section were similar to those reported elsewhere (Gillespie et al 1996). Briefly, macroscopic abnormalities in the major body systems were noted and

samples of tonsil, lung, olfactory lobe of the brain, and trigeminal ganglia were used for isolation of Aujeszky's disease virus.

RESULTS

Clinical signs

Between days 3 and 8 PI, clinical signs in the shedder pigs included dyspnoea, coughing and sneezing, depression, inappetence and anorexia (Fig 3). The pig that had the most severe clinical signs also exhibited forced expirations in the hammock and, based on material on nasal swabs, one pig developed a muco-purulent to caseous rhinitis. Except for one pig on day 4 and one pig on day 8 of the experiment, all shedder pigs were febrile over this period (Fig 4). On the seventh day PI, one of the shedder pigs developed mild ataxia and shook when handled for sampling, but these signs resolved by the following day.

Among recipient pigs, clinical signs were minimal. On the third day of exposure, three of four recipient pigs in group 2 had forced expirations whilst they were restrained in the hammock. On days 12 and 13 of the experiment, one pig from group 2 coughed and at least two pigs, one of which had a muco-purulent ocular discharge, sneezed. None of the recipient pigs developed a consistent febrile response and their appetites were unaffected.

Aujeszky's disease virus in intranasal inoculum, breath of pigs and nasal mucus

The titre of the Aujeszky's disease virus was 4.6 log₁₀TCID₅₀ ml⁻¹ and the average intranasal dose per pig was, therefore, the same or 3.42 log₁₀TCID₅₀ kg⁻¹ bodyweight. Aujeszky's disease virus was not detected in any of the samples of breath. The virus was found in nasal mucus from all the shedder pigs, as early as 3 days PI in some (Fig 4), but it was not isolated from any of the recipient pigs.

Serological findings

Sera collected from recipient pigs at 14 days PE were all positive for antibodies against Aujeszky's disease virus (Fig 5).

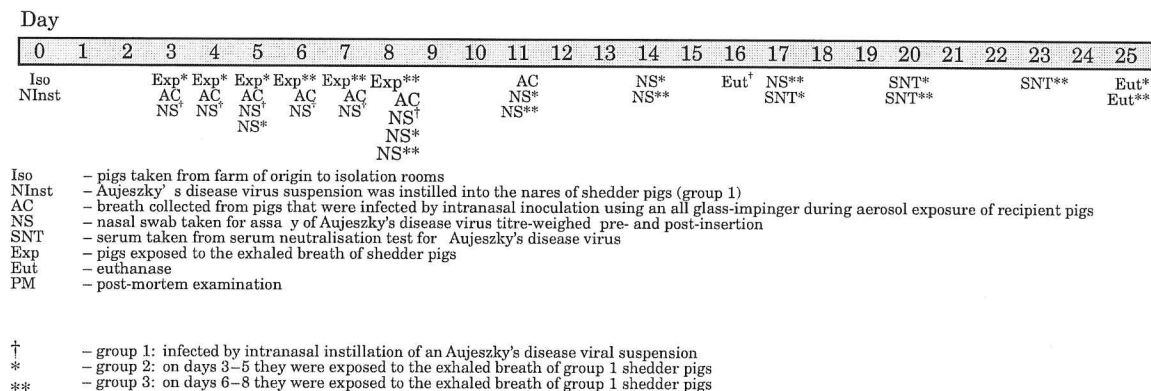


FIG 2: Timeline for sampling pigs that were intranasally infected with Aujeszky's disease or were exposed to breath of these pigs.

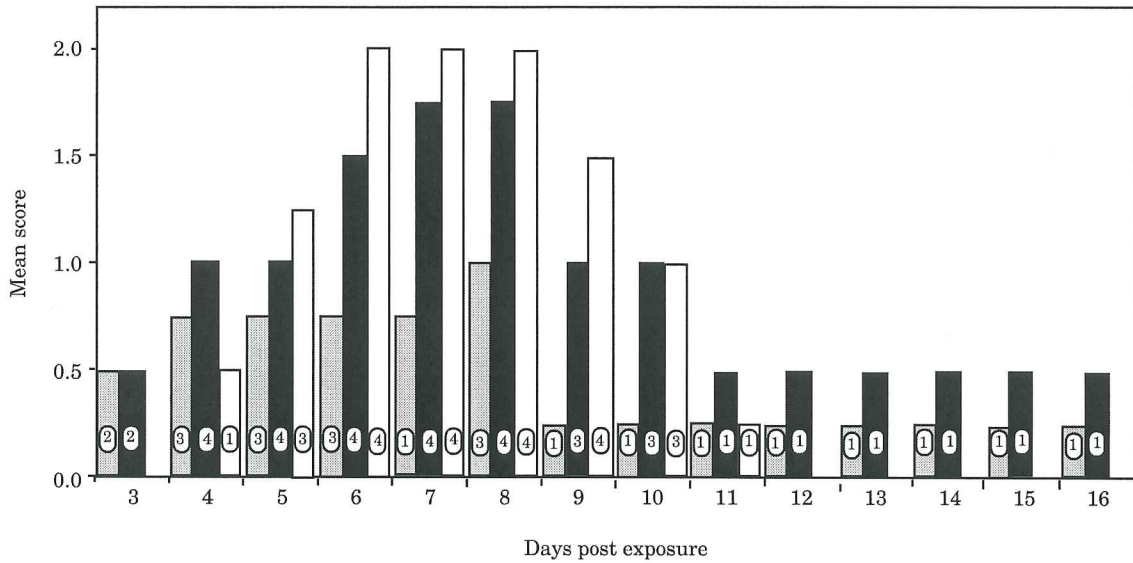


FIG 3: The mean scores for clinical signs among shedder pigs in which higher grades were assigned with increasing severity. The number in the box represents the number of pigs with a score greater than 0. Score for coughing and sneezing (□) or nasal discharge (■) between 0 and 2. Score for dyspnoea (▤) between 0 and 3.

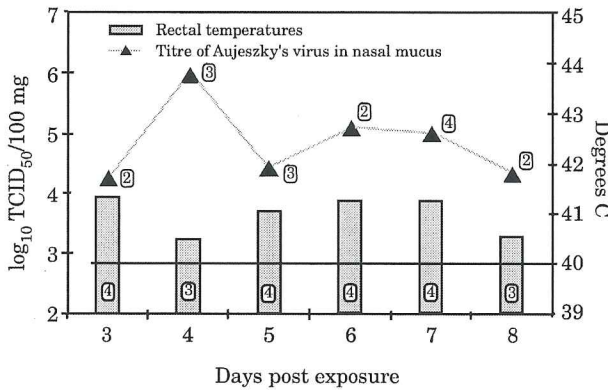


FIG 4: The mean titre of Aujeszky's disease virus in the nasal mucus (left axis ▲) and the mean rectal temperature (right axis □) of shedder pigs. The numbers in boxes represent the number of pigs on a particular day from which the mean of the measured parameter was derived.

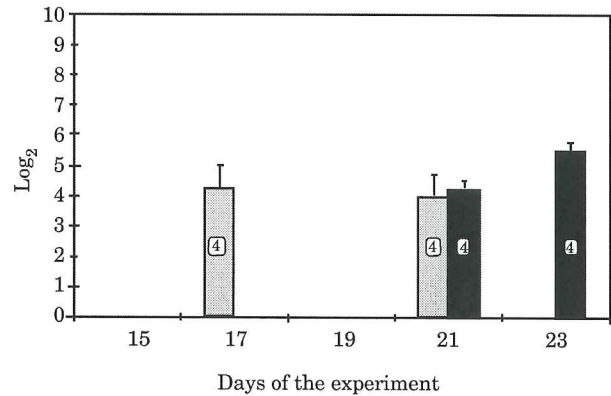


FIG 5: The mean serum neutralising antibody titre (log₂) for each group of recipient pigs. Bars represent standard error of the mean. The number in each box represents the number of pigs with serum antibody against Aujeszky's disease virus. □ Group 2; recipients; 2 pairs (n = 4), ■ Group 3; recipients; 2 pairs (n = 4).

Post-mortem findings

In one recipient pig, small focal areas of the lungs appeared collapsed and, in another, fibrinous pericarditis and pleuritis had developed. Organs and tissues were unremarkable in all the other pigs. Aujeszky's disease virus was isolated from the lung, tonsil and olfactory bulb of all shedder pigs. Among recipient pigs, virus was only found in three tonsils.

DISCUSSION

Our first objective was achieved when all pigs exposed to a single intranasal dose of Aujeszky's disease virus became infected and survived. Like Donaldson et al (1983) and Bourgueil et al (1992), we detected Aujeszky's disease virus in nasal secretions from shedder pigs. In contrast to the

febrile response in the days immediately PI, the numbers of pigs shedding virus and titres of virus in nasal mucus were less consistent. Maes et al (1983) had estimated that 12.5 log₁₀ TCID₅₀ of virus was necessary for a 50 per cent chance of isolating the virus from nasal mucus on the swab. As we used procedures comparable with those of Maes et al (1983), it is possible that there were infectious virions in all samples of nasal mucus collected from shedder pigs, but our methods were too insensitive for detecting them in the quantity shed.

The respiratory signs among shedder pigs and their duration compared with those described by Donaldson et al (1983), Bourgueil et al (1992) and Maes et al (1983). Using a virulent strain of Aujeszky's disease virus (P-2208, Kanitz 1972), which killed pigs of all ages in the herd from which it was isolated, Maes et al (1983) typically found that

experimentally infected pigs were febrile, anorexic and depressed. During the second week PI, three of eight, 6-week-old pigs that were given a low dose ($2.7 \log_{10} \text{TCID}_{50}$) of Aujeszky's disease virus, developed severe neurological signs, and two died. Although the virus was not detected in nasal mucus until 9 days PI, it persisted in four pigs to 21 days PI. By comparison, 2 days after receiving a high dose ($4.7 \log_{10} \text{TCID}_{50}$) experimentally infected, 10-week-old pigs were febrile, anorexic and depressed, but severe neurological signs were absent and all pigs survived.

In our study, pigs at about 6 weeks old received a greater intranasal dose than those in the study of Maes et al (1983). However, neurological signs of short duration developed in only one of four shedder pigs in our study. As our pigs were infected on the day of arrival, a stressful time compared to that for the pigs acclimatised in rooms about 1 week prior to infection (Maes et al 1983), this finding was all the more interesting.

In contrast to overt disease in shedder pigs, clinical signs in recipient pigs were minimal. Although we anticipated that Aujeszky's disease virus would be in the breath of shedder pigs, we were uncertain of the timing. Therefore, recipient pigs were exposed to the breath from two different shedder pigs and groups 2 and 3 were exposed on different days PI. As by 14 days PE all recipient pigs had seroconverted to Aujeszky's disease virus, our second objective was also achieved. At least one pig must have shed virus during each exposure period. Furthermore, because of the uniformity of seroconversion among recipient pigs, it is probable that there was relatively uniform exposure to Aujeszky's disease virus. In future studies with our model, we would confidently use the same intranasal dose of virus to infect shedder pigs and then expose recipient pigs to 'primary' aerosols of Aujeszky's disease virus.

Enough viable virions were shed in the breath to infect recipient pigs, but Aujeszky's disease virus was not detected in breath from any of the shedder pigs. In other studies, the viability of various titres of Aujeszky's disease virus was tested *in vitro* by placing stock cultures of virus in PBSS in an AGI-30 and operating the collector for 15 minutes. After adjusting for the initial titres of virus, agitation in the AGI-30 inactivated about 96.5 per cent of the virions (unreported data). As we attempted to increase the likelihood of detecting virions by adding CRFK cells to a minimum of 30 ml of fluid taken from the AGI-30, we estimated that $0.033 \text{TCID}_{50} \text{ml}^{-1}$ of Aujeszky's disease virus would have been detected. Using a conservative estimate that 99.5 per cent of Aujeszky's disease virus is inactivated in the collection liquids in the AGI-30, the minimum threshold for detection of airborne virions with our methods would be 6.6TCID_{50} .

When compared to the first and second stages of a three-stage liquid impinger, Donaldson et al (1977) had found that some lipid-coated viruses in fluid in the third stage were susceptible to inactivation after 20 minutes of agitation. Furthermore, they found that by adding 1.0 per cent (w/v) peptone to the virus mixture in an impinger, the virions were partially protected. Similarly, when we added 0.125% (w/v) mucin and 0.025% (v/v) calf serum to stock suspensions of Aujeszky's disease virus in PBSS, the virions were partially protected in an operating AGI-30 (unreported data). Although we operated a different type of impinger for shorter periods

(15 vs 20 minutes) and the herpesvirus that we used was different from those used by Donaldson et al (1977), both methods demonstrated partial protection of lipid-coated viruses in operating impingers. These findings are consistent with the suggestion of Donaldson et al (1983) that the nature of the medium in which virus is associated is an important factor in the stability of Aujeszky's disease virus in aerosols. Although we were unsuccessful at measuring any quantities of Aujeszky's disease virus in 'primary' aerosols, we did, however, demonstrate that the pig is a much more sensitive indicator of virions in aerosols than our aerosol collection methods and our third question was, therefore, answered.

In projects in which other types of collectors were successfully used to gather virus-laden aerosols within rooms, coughing and sneezing among large pigs may have elevated the concentration of virions (Donaldson et al 1983, Bourgueil et al 1992). Alternatively, dust-associated, 'secondary' aerosols may have increased the chances of detection of Aujeszky's disease virus. Despite the limitations of our methods for detecting Aujeszky's disease virus (Gillespie et al 1996), we inferred that the mild clinical response in recipient pigs was a result of relatively low doses of Aujeszky's disease virus in 'primary' aerosols. Although we found that the shedding of infectious 'primary' aerosols probably occurred over 6 days among pairs of shedder pigs, the necessity for repeated exposures of recipient pigs remains to be determined.

The virtual absence of clinical disease in recipient pigs may have been a function of a regional deposition of airborne virus in the respiratory tract of the pigs (Cox 1987). Baskerville (1971) and Donaldson et al (1983) considered that aerosols containing pathogens in large particles ($\geq 6 \mu\text{m}$) that emanated from coughs and sneezes and that were deposited in the upper airways of contact or sentinel pigs were an important source of the virus within groups and among pigs separated by short distances. However, none of our shedder pigs sneezed in the hammocks and, therefore, it is not surprising that the virions were not deposited and replicating in detectable quantities in the nasal mucosa of all recipient pigs. We believe the most likely source of virions for recipient pigs was small particles from the lungs of shedder pigs that were inhaled directly into the lungs (Cox 1987, Fairchild and Stampfer 1987, Gillespie et al 1996). During the natural spread of airborne diseases, large particles, e.g., from coughing, are much more susceptible to settling close to their source, whereas smaller particles are more likely to spread directly among pens, rooms, buildings and farms (Donaldson et al 1983, Cox 1987, Scheidt et al 1991). Therefore, we concur with Cox (1987) and believe that small particles from the lungs ($\leq 6 \mu\text{m}$) are also pertinent in spreading respiratory diseases.

Studies of epizootic diseases generally are retrospective and, therefore, the interval between entry of Aujeszky's disease virus and expression of clinical signs can only be inferred. An estimated 4 to 8 day interval between entry of virus into a herd and onset of Aujeszky's disease was utilised when aerosol transmission was implicated as the method of spread within an area around 'source' premises (Gloster et al 1984, Scheidt et al 1991). Gloster et al (1984), however, postulated that pigs in the 'source' herd received only a low airborne dose and, therefore, the interval between entry of virus and manifestation of disease was longer.

In Aujeszky's disease surveillance programmes in the USA, investigators have identified one or more seropositive breeding pigs in herds qualified free of the disease (Anelli et al 1991, Scherba and Jin 1994). Although attempts to isolate virus were unsuccessful, viral DNA was detected in trigeminal ganglia or tonsils in pigs from nine herds; however, the pigs in these herds did not succumb to clinical disease (Scherba and Jin 1994). In contrast, virus was isolated from pigs from five of 19 herds in which there was no evidence of Aujeszky's disease (Anelli et al 1991). Furthermore, in three of the five herds, within 3 to 24 months after removal of the last reactor, pigs developed antibodies against Aujeszky's disease virus, and in another herd pigs developed clinical signs of Aujeszky's disease (Anelli et al 1991).

Although Scherba and Jin (1994) and Anelli et al (1991) never identified the origin of Aujeszky's disease virus, re-entry or recrudescence of Aujeszky's disease virus just prior to the outbreak were possibilities. In fact, inclement weather conditions in wintertime were implicated in the recrudescence and shedding of airborne virus in a herd that was the likely 'source' of virus for the epizootic that Scheidt et al (1991) described. As further evidence for this concept, in the absence of clinical signs, to recrudescence virus was isolated from peripheral blood cells 4 to 5 weeks after young pigs were infected with a low dose of Aujeszky's disease virus (Balasch et al 1998). On the basis of the findings of Anelli et al (1991), Scherba and Jin (1994), and Balasch et al (1998), we believe that entry of airborne virus into the breeding herd does not always result in the immediate manifestation of clinical Aujeszky's disease.

Apparently, low concentrations of virus in 'primary' aerosols were shed by our pigs. Therefore, on the basis of this finding and epidemiological evidence from outbreaks of Aujeszky's diseases, we propose that exposure to a 'primary' aerosol is relevant for prospective studies that examine immune responses to the virus and the shedding of Aujeszky's disease virus over prolonged periods. Ultimately, clinical paradigms related to the methods of entry of virus into a herd, latency, recrudescence, and outbreaks of respiratory disease can be examined.

This is the second model in which we have transmitted Aujeszky's disease virus in aerosols and caused subclinical or mild clinical signs in naïve pigs (Gillespie et al 1996). The clinical picture resembles that in herds in which Aujeszky's disease is only diagnosed during routine serological surveillance. In the current model, recipient pigs were infected with the breath of shedder pigs, whereas in the other, shedder pigs were infected directly by nebulised aerosols (Gillespie et al 1996). Perhaps a better comparison with 'primary' airborne transmission of respiratory infections could be achieved if the two models were integrated so that shedder pigs, infected with experimentally nebulised aerosols, were the source of pathogens for recipient pigs. Investigations of cell-mediated responses in the lungs, i.e., 'the first line of defence', against viruses that have a propensity for the respiratory system could then be investigated to contribute to our knowledge of how to enhance resistance to these infections.

ACKNOWLEDGEMENTS

The encouragement and advice of S. Albrechts is gratefully acknowledged.

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