Mechanical transmission of porcine reproductive and respiratory syndrome virus by mosquitoes, *Aedes vexans* (Meigen)

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Abstract

The objective of this study was to determine whether porcine reproductive and respiratory syndrome virus (PRRSV) could be transmitted to naïve pigs by mosquitoes following feeding on infected pigs. During each of 4 replicates, mosquito-to-pig contact took place on days 5, 6, and 7 after PRRSV infection of the donor pig. A total of 300 mosquitoes [*Aedes vexans* (Meigen)] were allowed to feed on each viremic donor pig, housed in an isolation room. After 30 to 60 s, feeding was interrupted, and the mosquitoes were manually transferred in small plastic vials and allowed to feed to repletion on a naïve recipient pig housed in another isolation room. Prior to contact with the recipient pig, the mosquitoes were transferred to clean vials. Swabs were collected from the exterior surface of all vials, pooled, and tested for PRRSV. Separate personnel handled the donor pig, the recipient pig, and the vial-transfer procedure. Transmission of PRRSV from the donor to the recipient pig occurred in 2 out of 4 replicates. The PRRSV isolated from the infected recipient pigs was nucleic-acid-sequenced and found to be 100% homologous with the virus used to infect the donor pigs. Homogenates of mosquito tissues collected in all replicates were positive by either polymerase chain reaction or swine bioassay. All control pigs remained PRRSV negative, and PRRSV was not detected on the surface of the vials. This study indicates that mosquitoes (*A. vexans*) can serve as mechanical vectors of PRRSV.

Résumé

*Afin de déterminer si le virus du syndrome respiratoire et reproducteur porcin (PRRSV) pouvait être transmis par des moustiques de porcs infectés à des porcs immunologiquement naïfs, des contacts moustique-porc ont été permis aux jours 5, 6 et 7 suivant l'infection d'un porc donneur lors de 4 réplications de l'expérience. Un total de 300 moustiques (*Aedes vexans*) purent se nourrir sur le porc donneur virémique gardé dans une pièce en isolement. Le repas des moustiques fut interrompu après 30 à 60 secondes et les moustiques transférés manuellement dans de petits pots de plastique. Les moustiques purent continués leur repas jusqu'à satiété en se nourrissant sur un porc receveur immunologiquement naïf gardé en isolement dans une autre pièce. Avant le contact avec le porc receveur les moustiques furent transférés dans des contenants propres. La surface extérieure de tous les contenants fut écouvillonnée et les écouvillons mis ensemble et testés pour la présence de PRRSV. La manipulation du porc donneur, du porc receveur et de la procédure de transfert des contenants furent effectuées par du personnel différent. La transmission du PRRSV du porc donneur au porc receveur s'est produite en 2 occasions lors des 4 tentatives. La séquence de l'acide nucléique du PRRSV isolé des porcs receveurs infectés était homologue à 100 % à celle du virus utilisé pour infecter le porc donneur. Des homogénats de tissus de moustiques prélevés lors des différentes réplications se sont avérés positifs pour la présence du PRSV par réaction d'amplification en chaîne par la polymérase ou par bio-essai chez le porc. Tous les porcs témoins sont demeurés négatifs à la présence de PRRSV et aucun virus ne fut détecté à partir de l'extérieur des contenants utilisés. Les résultats obtenus démontrent que les moustiques peuvent servir de vecteur mécanique au PRRSV.*

(Traduit par Dr Serge Messier)

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically significant pathogen in the global swine industry (1). Introduction of PRRSV into naïve herds can occur through infected pigs, semen, contaminated needles, fomites (coveralls and boots), and hands of personnel (2–5). In order to reduce the risk of the entry of PRRSV into naïve swine populations, swine producers use stringent measures to enhance the biosecurity of their farms; however, infection of naïve herds frequently occurs through unidentified

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routes. Potential routes that have not been explored include insects. Insects have long been known to serve as mechanical or biologic vectors for certain swine pathogens (6–9). Currently used methods of biosecurity, such as shower in/out, personnel downtime (no swine contact for 48 to 72 h before entering the farm), quarantine and testing of incoming stock, and bird-proofing of facilities, do not regulate the entry of insects into swine herds. Since PRRSV infection results in prolonged viremia in infected pigs (10), and since bloodborne transmission of PRRSV by contaminated needles has been proven (4), it was hypothesized that insects, particularly hematophagous species such as mosquitoes, may be vectors of PRRSV. Additionally, mosquitoes are commonly observed in swine farms during the summer and are ubiquitous in tropical pig-raising regions.

Recently we documented that mosquitoes (*Aedes* spp.) can harbor homologous, infectious PRRSV following feeding upon viremic pigs raised under commercial farm conditions (11). During this investigation, approximately 550 mosquitoes were collected from a farm experiencing an acute outbreak of PRRS. Mosquitoes were pooled (30 mosquitoes/pool), and PRRSV nucleic acid was detected by polymerase chain reaction (PCR) in 1 out of 22 mosquito pools. The open reading frame (ORF) 5 region of the nucleic acid was sequenced and found to be 100% homologous with the same region in the PRRSV isolated from pigs during the acute outbreak. The supernatant from the PCR-positive sample was tested by swine bioassay and was also found to contain infectious PRRSV. Following inoculation, the bioassay pig produced PRRSV antibodies, as detected by enzyme-linked immunosorbent assay (ELISA), and the serum was positive for PRRSV by PCR and virus isolation (VI). Nucleic acid sequencing of the ORF 5 region of the PRRSV isolate's nucleic acid indicated 100% homology with the PRRSV previously recovered from the commercial swine and the mosquito homogenate.

Although the results from that investigation indicated that homologous infectious PRRSV could be transferred from viremic pigs to mosquitoes, they did not prove that mosquitoes can transmit PRRSV from infected to naïve pigs. Therefore, we conducted a study to evaluate whether PRRSV could be transmitted to naïve pigs by mosquitoes following feeding on infected pigs under experimental conditions.

Materials and methods

Infection model

Twelve 6-wk-old pigs were purchased from a farm known to be PRRSV negative on the basis of 7 years of diagnostic data and the absence of clinical signs of PRRS. The study design consisted of 4 replicates, each replicate involving 3 pigs. Each pig was housed in a separate room at the isolation facility of the University of Minnesota College of Veterinary Medicine. This facility consisted of a series of rooms that were ventilated separately and contained individual slurry pits, which prevented cross-contamination of pathogens between rooms. Upon arrival at the facility, all animals were blood-tested to ensure a PRRSV-naïve status. During the study period, all pigs were cared for under the guidelines of University of Minnesota Institutional Animal Care policies. To initiate each replicate, 1 pig was intranasally inoculated with 5 mL of cell culture fluid containing a total dose of 10^4 $TCID_{50}$ of a field isolate (MN-30100) of PRRSV (12). The inoculated pig was designated as the donor pig. The remaining 2 pigs were not inoculated and served as the recipient pig and the protocol-control pig, respectively.

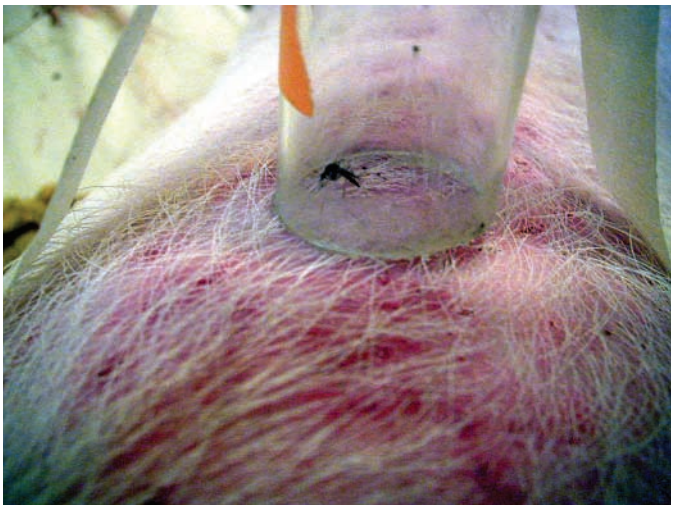

Figure 1. Method of mosquito containment during the manual vector-transmission protocol: a plastic transfer vial containing an individual mosquito is fastened with masking tape over the dorsal surface of the pig; the mosquito has access to the pig's skin through a nylon screen fixed to the bottom of the vial.

Source of mosquitoes

Mosquitoes were trapped (indoors and outdoors) at a PRRSV-positive commercial swine farm in Minnesota, using a CO_2-baited CDC light trap (13). The PRRSV strain on the farm had been previously determined to be 100% homologous with the strain used to infect the donor pigs in this study. Mosquitoes were collected 3 d prior to the initiation of each replicate. They were placed into humidified incubation cages at 27°C and kept alive with sucrose solution. Then, 50 randomly sampled mosquitoes (approximately 1% of the population collected) were classified by genus and species and tested to ensure the absence of PRRSV.

Mosquito-transmission protocols

Mosquito-to-pig contact took place on days 5, 6, and 7 after inoculation of the donor pig. This period was selected on the basis of both published data and our experience indicating that the peak of PRRSV viremia in nursery-age pigs occurred at this time (4,5,10). To attempt transmission of PRRSV by mosquitoes from the donor to the recipient pig, a manual vector-transmission protocol was applied (14) (Figure 1). This method had previously been used to study transmission of bovine leukosis virus from infected to susceptible cattle by stable flies (*Stomoxys calcitrans*). The pigs were consciously restrained in a Panepinto sling (Asset Inventory project, University of Minnesota, St. Paul, Minnesota, USA), and transparent plastic screw-cap vials (3 cm in diameter, 5 cm in height), each containing an individual mosquito, were fastened with masking tape (Highland 2600 tape; 3M Company, St. Paul, Minnesota, USA)

Table I. Diagnostic data for the pigs and the mosquito homogenates in a study of transmission of porcine reproductive and respiratory syndrome virus (PRRSV)

	Donor pigs			Recipient pigs			Mosquito homogenates		
Replicate no.	PCR	VI	ELISA	PCR	VI	ELISA	PCR	VI	ELISA
1	+	+	+	−	−	−	Suspect	−	+[a]
2	+	+	+	−	−	−	Suspect	−	+[a]
3	+	+	+	+[a]	+	+	−	−	+[a]
4	+	+	+	+[a]	+	+	+[a]	−	NT

PCR = polymerase chain reaction; VI = virus isolation; ELISA = enzyme-linked immunosorbent assay; + = positive results recorded during 21-d study period; − = negative results recorded during 21-d study period; NT = not tested

[a] samples in which the virus had 100% homology in the open reading frame 5 region of its nucleic acid with the PRRSV isolate used to infect the donor pigs

over the dorsal surface of the pig. The vials had on the bottom a nylon screen with 64 holes per cm^2, each hole being 1 mm in diameter, which allowed the mosquito access to the pig's skin.

During each replicate, 300 mosquitoes, 100 per day, were allowed to feed for 30 to 60 s on the donor pig. Non-feeding mosquitoes were discarded and not included in the daily count. Following the observation of insertion of the mosquito proboscis and initiation of abdominal swelling, feeding was interrupted by removing the vial from the back of the pig. The vials were transferred to a neutral site and the mosquitoes transferred to new, alcohol-disinfected vials prior to delivery to the recipient pig's room. The neutral transfer area was separate from the donor and recipient isolation rooms, and the vial transfer procedure took approximately 30 s per vial. The person carrying out the transfer did not contact the donor pig or the recipient pig at any time. Immediately following transfer of the vials to the recipient pig's room, the mosquitoes were allowed to feed to repletion on the recipient pig. Following the cessation of feeding, vials containing blood-fed mosquitoes were immediately placed on dry ice and submitted for PRRSV testing.

Extensive effort was made to rule out the possibility of non-mosquito transmission of PRRSV. To ensure that the vials that contacted the recipient pig were not contaminated with PRRSV from the donor pig, swabs (Dacron fiber-tipped plastic applicator swabs; Fisher Scientific Company, Hanover Park, Illinois, USA) were collected from the exterior surface of the vials containing the mosquitoes that contacted the recipient pig and tested for PRRSV. Additionally, on days 5, 6, and 7 after infection of the donor pig in each replicate, a protocol-control procedure was conducted to determine if contamination could occur by means of the transfer process. The identical procedure of transferring vials from the donor to the recipient pig was carried out; however, the vials were empty, and the negative-control pig, housed in a separate room, was used. This procedure was conducted prior to any contact with the donor pig or the recipient pig.

Sampling and diagnostic analysis

During each replicate, the donor pig was bled on each exposure day (days 5, 6, and 7 postinfection) to document PRRSV viremia. Blood was collected from the recipient pig on days 7, 14, and 21 postexposure to monitor its PRRSV-infectious status. Day 5 after infection of the donor pig was equal to day 0 after exposure of the recipient pig. The protocol-control pig was bled at the beginning and the end of each replicate. All blood-fed mosquitoes collected on each exposure day were pooled in sterile tubes (BD Falcon; Becton Dickinson and Company, Franklin Lakes, New Jersey, USA) containing 3 mL of minimum essential medium (MEM). The pooled mosquitoes were compressed against the tube wall with sterile swabs and centrifuged at 6500 rpm for 5 min, then the supernatant was tested for PRRSV. Swabs from the exterior surface of the vials that contacted the recipient pig were immediately placed into sterile tubes containing 3 mL of MEM, pooled according to exposure day, and tested for PRRSV.

All serum samples from the pigs, mosquito homogenates, and swabs from the vials were tested twice for PRRSV nucleic acid by reverse transcriptase PCR (RT-PCR), with the TaqMan RT-PCR assay (Perkin-Elmer Applied Biosystems, Foster City, California, USA) (15). For samples to be considered positive, PRRSV nucleic acid had to be detected in both tests. Samples with only 1 positive reaction were considered suspect. All samples were tested for viable PRRSV by VI with the use of MARC-145 cells and porcine alveolar macrophages (16). Samples from the recipient pigs and the mosquito homogenates that were positive for PRRSV by PCR or VI were nucleic-acid-sequenced to compare the ORF 5 region with that of the virus used to infect the donor pig (17). Serum was tested for PRRSV antibodies by the IDEXX ELISA (IDEXX Laboratories, Westbrook, Maine, USA) (18). Mosquito homogenates and swabs collected from the vials that were not positive by either PCR or VI were pooled by replicate and tested for the presence of infectious PRRSV by swine bioassay (7).

Results

All the animals were determined to be seronegative for PRRSV by ELISA prior to the initiation of each replicate, and PRRSV was not detected by PCR, VI, or swine bioassay in randomly sampled mosquitoes from the swine farm. The majority (96%) of the mosquitoes collected from the farm were classified as *A. vexans* (Meigen). Diagnostic data for the donor pigs, the recipient pigs, and the mosquito homogenates are summarized in Table I. PRRSV viremia was detected in the donor pig on each exposure day (days 5, 6, and 7 postinfection) during all 4 replicates by PCR and VI. Transmission of PRRSV from the donor to the recipient pig was demonstrated in replicates 3 and 4: the recipient pigs in replicates 3 and 4 were positive by PCR and VI on day 7 postexposure and by ELISA

on day 14. PRRSV isolated from the recipient pig in replicates 3 and 4 was 100% homologous (by sequencing of the ORF 5 region of the nucleic acid) with the virus used to infect the donor pig.

The mosquito homogenate from replicate 4 was PCR positive, and nucleic-acid-sequencing of the ORF 5 region of the virus from this sample indicated 100% homology with the virus used to infect the donor pig. Homogenates from replicates 1 and 2 were PCR suspect but positive by swine bioassay. The homogenate from replicate 3 was PCR negative; however, it too was positive by swine bioassay. The ORF 5 region of the nucleic acid of the virus isolated by swine bioassay in replicates 1, 2, and 3 was 100% homologous with that of the virus used to infect the donor pig. All the homogenates were negative by VI. All swabs collected from the exterior surface of the transfer vials were negative by PCR, VI, and swine bioassay. The protocol-control pigs remained negative by PCR, VI, and ELISA during the entire study.

Discussion

This is the first report documenting transmission of PRRSV by mosquitoes under experimental conditions. Our data clearly demonstrate that PRRSV can be mechanically transmitted to naïve pig by mosquitoes (*A. vexans*) with a manual vector-transmission protocol. These results support our previous recovery of PRRSV from mosquitoes that were allowed to feed on PRRSV-infected commercial swine (11) and indicate that mosquitoes (*A. vexans*) could contribute to horizontal transmission of PRRSV among pigs within infected commercial farms. Although it is not known whether *A. vexans* and other mosquitoes can serve as a source of PRRSV transmission between farms, their capacity for traveling extended distances (2.5 to 10 km) and for multiple blood-feedings in their life (19), along with their ability to harbor and transmit PRRSV, may support this theory. PRRSV nucleic acid was detected by PCR in the mosquito homogenate from replicate 4, and the homogenates from replicates 1 and 2 were PCR suspect. The homogenate from replicate 3 was negative by PCR and VI, yet positive by swine bioassay. Although the PCR assay used in this study (TaqMan RT-PCR) is highly sensitive, its inability to detect PRRSV in mosquito homogenates must be considered and has been hypothesized to be due to inhibition by the presence of residual heme pigments or other impurities found in insects (20). We attempted to overcome this problem through the use of multiple diagnostic tests, including swine bioassay, to enhance the accuracy of our results.

During the design of the study, a perceived risk involved in the manual vector-transmission protocol was the possibility of PRRSV infection of the recipient pigs by contaminated personnel, fomites, or transfer vials. We attempted to minimize this risk by using designated personnel, a protocol-control procedure, and the testing of transfer vials for residual PRRSV. During each replicate, separate personnel were designated to handle the donor pig, the recipient pig, or the vial-transfer procedure. All swabs collected from the exterior surface of the vials that contacted the recipient pigs were PRRSV negative by PCR, VI, and swine bioassay. The fact that the protocol-control pigs remained negative throughout the study suggests that the transfer protocol was not a mechanical means of contamination.

Although we demonstrated transmission in 2 out of 4 replicates, this study was not designed to measure the frequency of the observed events, and the quantity of PRRSV within an individual mosquito was not calculated. Therefore, no conclusions can be drawn regarding the probability of transmission of PRRSV by an individual mosquito, the minimum number of mosquitoes required to infect a naïve pig, or the dose of PRRSV-infected blood that an individual mosquito must consume for transmission to occur. The reason transmission did not occur in replicates 1 and 2, despite detection of the virus in the mosquito homogenates by swine bioassay, is unknown. Possible explanations are that a sufficient quantity of the virus was not present within the mosquitoes that contacted the recipient pigs. The methods used to detect PRRSV in this study were not quantitative; therefore, again, no conclusion can be made regarding the amount of virus that the mosquitoes must carry to infect naïve pigs.

The source of the mosquitoes used for this study was a commercial swine farm. We preferred to use wild mosquitoes collected from a commercial swine setting, rather than laboratory-colonized mosquitoes, to better represent species of mosquitoes found under field conditions. In fact, *A. vexans* is the most commonly seen mosquito in the midwestern United States (Moon, unpublished data). However, the use of wild mosquitoes had certain limitations, including the inability to standardize the age of the mosquitoes. The source farm was PRRSV positive. We selected it because of convenient access, the fact that we had been observing tremendous numbers of mosquitoes both inside and outside the facility, and the fact that the facility was mechanically ventilated. Additionally, the virus isolated from the farm had been found to be 100% homologous (in the ORF 5 region of its nucleic acid) with the virus used for this study. Our selection of the source farm might be considered a limitation, since the virus isolated from the infected recipient pigs could have originated from the farm. However, clinical signs, such as coughing, fever, and depression, were not observed in any pigs housed on the farm, and viremia was not detected by PCR or VI in any pigs tested during the collection of mosquitoes. Furthermore, prior to initiation of each replicate, the collected mosquitoes were housed in the laboratory for 3 d, and representative samples (50 insects per replicate) were found to be negative by PCR, VI, and swine bioassay. Therefore, we feel that it is very unlikely that the mosquitoes contained PRRSV when collected.

An important consideration when designing this study was the welfare of the pigs while they were in contact with the mosquitoes. During this period, the pigs were consciously restrained for approximately 2 h in Panepinto slings, which were specifically designed for surgical procedures. The pigs were upright, and the leg openings in the slings were lined with soft cloth to avoid irritation. A small fan propelled fresh air onto the pig's face, and the animals were frequently removed for exercise throughout the mosquito-contact period. They were given treats and fruit juice, and they appeared to be very comfortable in the slings, several falling asleep during the procedure. Finally, despite the numerous mosquito bites on the dorsal surface of the pigs, evidence of pruritus or secondary staphylococcal infection was not observed.

In conclusion, our results indicate that PRRSV can be mechanically transmitted from infected to naïve pigs by mosquitoes (*A. vexans*).

This study did not address the question whether mosquitoes could also serve as biologic vectors of PRRSV. This virus is a member of the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (21). Although it is unlikely that *Arterivirus* members can replicate within insects (22), further studies are needed to determine whether PRRSV has a potential to replicate within *A. vexans* or any other mosquitoes and to evaluate the role of mosquitoes in the transmission of PRRSV throughout commercial swine-producing areas.

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