Evaluation of mosquitoes, *Aedes vexans*, as biological vectors of porcine reproductive and respiratory syndrome virus

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Abstract

The objective of this study was to determine whether mosquitoes, *Aedes vexans* (Meigen), could serve as biological vectors of porcine reproductive and respiratory syndrome virus (PRRSV). Specifically, the study assessed the duration of viability and the site of PRRSV within mosquitoes, and evaluated whether PRRSV could be transmitted to a susceptible pig by mosquitoes following a 7- to 14-day incubation period after feeding on an infected pig. For the first experiment, a total of 100 mosquitoes were allowed to feed on a pig, experimentally infected with PRRSV (day 7 post-inoculation) and were then maintained alive under laboratory conditions. A set of 10 mosquitoes were collected at 0 hour (h), 6 h, 12 h, 24 h, 48 h, 72 h, 5 days (d), 7 d, 10 d, and 14 d post-feeding (pf). Samples of exterior surface washes, salivary glands, thorax carcasses, and gut homogenates were collected from each set of mosquitoes, and tested for PRRSV. Infectious PRRSV was detected by polymerase chain reaction and swine bioassay only from the gut homogenates of mosquitoes collected at 0 h and 6 h pf. For the second experiment, a total of 30 mosquitoes were allowed to feed on a pig, experimentally infected with PRRSV and the mosquitoes were then maintained under laboratory conditions. On each of day 7, 10, and 14 pf, a set of 10 mosquitoes were allowed to feed on a susceptible pig. Transmission of PRRSV to susceptible pigs did not occur, and PRRSV was not detected from the mosquitoes. These findings indicate that mosquitoes are not likely to serve as biological vectors of PRRSV.

Résumé

Cette étude avait comme objectif de déterminer si les moustiques Aedes vexans *(Meigen) pouvaient servir de vecteur biologique du virus du syndrome respiratoire et reproducteur porcin (PRRSV). Plus spécifiquement, la durée de la viabilité et de la localisation du PRRSV à l'intérieur des moustiques ont été évaluées, ainsi que la possibilité que le PRRSV soit transmis à un porc susceptible par des moustiques suivant une période d'incubation de 7 à 14 jours après s'être nourris à partir d'un animal infecté. Au cours de la première expérience un total de 100 moustiques se sont nourris sur un porc infecté expérimentalement avec le PRRSV (jour 7 post-inoculation) et ont été maintenus vivants en conditions de laboratoire. Des échantillons de 10 moustiques ont été prélevés après 0, 6, 12, 24, 48 et 72 h, ainsi que 5, 7, 10 et 14 j suivant le repas de sang (pf). À partir de chacun des groupes de moustiques, on a vérifié la présence de PRRSV à partir des échantillons suivants : lavage de la surface externe, glandes salivaires, carcasses de thorax et intestins homogénéisés. Du PRRSV infectieux a été détecté par réaction d'amplification en chaîne par la polymérase et par bio-essai chez le porc seulement à partir des intestins homogénéisés de moustiques prélevés aux temps 0 et 6 h pf. Au cours de la deuxième expérience, un total de 30 moustiques se sont nourris sur un porc infecté expérimentalement avec le PRRSV et les moustiques ont par la suite été maintenus vivants dans des conditions de laboratoire. Aux jours 7, 10 et 14 pf, un groupe de 10 moustiques ont été mis en contact avec un porc susceptible afin de se nourrir. La transmission du PRRSV aux porcs susceptibles ne s'est pas produite et le PRRSV n'a pas été détecté à partir des moustiques. Ces résultats indiquent que les moustiques ont peu de capacité à agir comme vecteur biologique du PRRSV.*

(Traduit par Docteur Serge Messier)

Introduction

Successful control and eradication of porcine reproductive and respiratory syndrome virus (PRRSV) is of great importance to the global swine industry today (1). To reduce the risk of PRRSV entry, swine producers utilize stringent measures to enhance the biosecurity of their farms; however, infection of PRRSV in swine herds still frequently occurs. Current known routes of transmission of PRRSV are infected pigs, semen, needles, and fomites (2–5). The term "area spread" or "local spread" has been used to describe transmission of PRRSV throughout swine-dense areas without an identification of the source of the virus (6). Airborne spread is sometimes used to speculate a cause of area spread outbreaks of PRRSV; however, it has been documented that airborne transmission of PRRSV over long distances (greater than 1 m) was an infrequent event under field conditions (7,8). These recent studies have raised the question

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regarding the role of insects in transmission of PRRSV throughout commercial swine producing areas. Furthermore, biosecurity protocols, such as placing of bird screen on the sidewall opening of buildings, do not prevent the entry and spread of insects to farms.

Recently, we have documented mechanical transmission of PRRSV by houseflies (*Musca domestica*) under experimental conditions, and the survival of infectious PRRSV within the intestinal tract of houseflies for up to 12 h following feeding on an infected pig (9,10). Identification of homologous infectious PRRSV from mosquitoes (*Aedes vexans*) collected at a commercial swine farm experiencing acute PRRSV outbreak, and mechanical transmission of PRRSV by the mosquitoes under experimental conditions has been documented (11). However, the duration of viability and the site of PRRSV in mosquitoes are not currently understood. Furthermore, it is unknown whether PRRSV is capable of replicating within mosquitoes, and whether mosquitoes could potentially serve as biological vectors of PRRSV.

Mosquitoes (Meigen) serve as biological vectors of arboviruses, primarily of the families *Bunyaviridae*, *Flaviviridae*, *Reoviridae*, *Rhabdoviridae*, and *Togaviridae* (12). Biological transmission of arboviruses by mosquitoes requires 4 steps: 1) Ingestion of a blood meal containing the virus and infection/replication in epithelial cells lining the midgut of the mosquitoes, 2) escape of the virus from the midgut epithelium into the hemocele, 3) infection/replication in the salivary gland of the mosquitoes, and 4) secretion of the virus in saliva and transmission to susceptible animals by feeding on them (13). Numerous studies have been conducted to determine the fate of arboviruses within mosquitoes. Dissection-detection, titration, or both methods; histopathological techniques, including immunofluorescent antibody or immunoperoxidase assays; and electron microscopy have been used to determine the site of arboviruses in mosquitoes (14–17). An important principle of all biological vectors of arboviruses is demonstration of an extrinsic incubation period. The extrinsic incubation period is defined as "the period between the initial feeding and the time at which the mosquitoes are capable of inducing infections" (18). Although the extrinsic incubation period can vary depending on the virus genetics, the initial dose of the virus, species of mosquito, and environmental temperature, it has been documented that a sufficient concentration of the virus for transmission to a susceptible vertebrate host will be generally developed following a period of 7 to 14 d (19). During this critical period, recovery of the virus from midgut; salivary gland; and other tissues, such as muscle, fat bodies, or legs, is the proof of replication and dissemination of the virus throughout the body of mosquito, thereby verifying the capacity of that mosquito species to serve as a biological vector of the virus (20,21).

The goal of the present study was to further evaluate the role of mosquitoes as vectors of PRRSV. As a follow-up to our previous study, which demonstrated mechanical transmission of PRRSV by mosquitoes (*A. vexans*) (11), we attempted to evaluate the potential for mosquitoes to serve as biological vectors of PRRSV. The specific objectives of this study were to determine the duration of PRRSV viability in mosquitoes following feeding on an infected pig, document the site of PRRSV within the mosquitoes, and to determine whether PRRSV could be transmitted to a susceptible pig by mosquitoes following 7- to 14-day incubation period after feeding on an infected pig.

Materials and methods

Experiment I: Assessment of the duration of viability and site of PRRSV within mosquitoes

Infection model — Two 6-week-old pigs were purchased from a farm known to be PRRSV-negative based on 7 y of diagnostic data and the absence of clinical signs. Each pig was housed in a separate room at the isolation facility at 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, preventing cross-contamination of pathogens between rooms. Upon arrival at the facility, both animals were tested by polymerase chain reaction (PCR) (22), virus isolation (VI) (23), and enzyme linked immunosorbent assay (ELISA) (24) to verify their PRRSV-negative status. One pig was intranasally inoculated with 5 mL of a PRRSV field isolate (MN-30100) at a concentration of 10^4 $TCID_{50}$/mL (25). This PRRSV isolate had been used in our previous study that demonstrated mechanical transmission of PRRSV by mosquitoes (11). The remaining pig was not inoculated and served as a negative control. Both animals were cared for following the standards of the University of Minnesota Institutional Animal Care and Use Committee during the entire period of the study.

Source of mosquitoes — Mosquitoes were trapped at the field of the University of Minnesota St. Paul campus, using CO_2-baited CDC light traps (26). The mosquitoes were collected 3 d prior to the initiation of the study, placed into humidified incubation cages, and maintained alive using sucrose solution under laboratory conditions (27°C). Approximately 300 randomly sampled mosquitoes were identified for their genus and species, and the majority (95.6%) of the population was *A. vexans*. A total of 100 mosquitoes were randomly sampled and tested by PCR and VI to ensure the absence of PRRSV in the mosquito population prior to the initiation of the experiment.

Mosquito contact protocol — Mosquito-to-pig contact took place 7 d after the pig was experimentally infected with PRRSV. This time period was selected based on published data indicating consistent development of PRRSV-viremia in nursery age pigs on days 5, 6, and 7 post-inoculation (pi) (4,5,9–11). To allow mosquitoes to feed on the infected pig, a manual vector transmission protocol was employed. This method had been previously used to study transmission of bovine leukosis virus from infected to susceptible cattle by stable flies (*Stomoxys calcitrans*) (27), and mechanical transmission of PRRSV by houseflies (*Musca domestica*) and mosquitoes (*A. vexans*) (9,11). The pig was consciously restrained in a sling (Panepinto sling; Asset Inventory Project, University of Minnesota, St. Paul, Minnesota, USA), and transparent plastic screw-cap vials (3 cm diameter, 5 cm height) each containing an individual mosquito were adhered with masking tape (Highland 2600 tape; 3M, St. Paul, Minnesota, USA) on the dorsal surface of the pig. These vials contained a nylon screen (1 mm diameter hole, 64 holes per cm^2) on the bottom, allowing the mosquito access to the pig's skin.

On day 7 pi, a total of 100 mosquitoes were allowed to feed to repletion on the infected pig. For each individual mosquito, the insertion of the proboscis and the initiation of abdominal swelling were observed. Following completion of feeding (3 to 5 min), all blood-fed mosquitoes were placed into humidified incubation cages

(27°C) and maintained alive with sucrose solution at the entomology laboratory of the University of Minnesota.

Sampling methods — The experimentally infected pig was blood-tested on day 7 pi to confirm the presence of PRRSV-viremia. A set of 10 blood-fed mosquitoes were randomly collected at each of the following times: 0 h, 6 h, 12 h, 24 h, 48 h, 72 h, 5 d, 7 d, 10 d, and 14 d post-feeding (pf). A set collected at 0 h pf was placed on dry ice immediately following feeding on the infected pig. For assessment of PRRSV on the exterior surface of the mosquitoes, 10 mosquitoes of each subset were pooled into a sterile plastic tube (1.5 mL micro-centrifuge tube; Dot Scientific, Burton, Michigan, USA) containing 1 mL of minimum essential medium (MEM). Pooled insects were washed with MEM by vortexing (Vortex-Genie Mixer; Scientific Industries, Bohemia, New York, USA) at 8000 × *g* for 10 s. The washing fluid (MEM) was transferred into a separate sterile tube, labeled according to each sampling time, and tested for PRRSV. These samples were designated as exterior surface washes. Following collection of the exterior surface wash, the mosquitoes were disinfected with 70% ethanol, rinsed twice with MEM for 5 s, and dissected. The salivary glands were removed from the individual mosquitoes, pooled into a sterile tube containing 1 mL of MEM, and labeled according to each sampling time. These samples were designated as salivary glands. Following the collection of salivary glands, other thoracic tissues including heart, muscles, fat bodies, wings, and legs were then pooled into a sterile tube containing 1 mL of MEM, labeled according to each sampling time. These samples were designated as thorax carcasses. Finally, digestive organs, including midgut, hindgut, and ventral diverticulum (crop), were removed from each individual mosquito, pooled into a sterile tube containing 1 mL of MEM, and labeled according to each sampling time. These samples were designated as gut homogenates. All samples of the salivary glands, thorax carcasses, and gut homogenates were compressed against tube wall, centrifuged at 4200 × *g* for 5 min, and supernatant tested for PRRSV.

Diagnostic analysis — All samples (n = 40) were tested twice for PRRSV nucleic acid by reverse transcriptase polymerase chain reaction (RT-PCR) (TaqMan RT-PCR assay; Perkin-Elmer Applied Biosystem, Foster City, California, USA) (22). In order for samples to be considered positive, PRRSV nucleic acid had to be detected during both tests. Samples with only 1 positive reaction were considered suspect. All samples were also tested for viable PRRSV by virus isolation (VI) on both MARC-145 cells and porcine alveolar macrophages (PAM) (23). If the virus was isolated in MARC-145, PAM, or both, the sample was considered VI-positive. Additionally, all samples that were PCR-positive and VI-negative were tested for the presence of infectious PRRSV by swine bioassay (28). The purpose of the swine bioassay was to verify the presence of infectious PRRSV in the samples with evidence of PRRSV nucleic acid but no evidence of viable virus according to VI. For this procedure, 4-week-old pigs were obtained from a PRRSV-negative farm, and housed in separate rooms of the isolation facility at the University of Minnesota College of Veterinary Medicine. Individual pigs were inoculated intramuscularly with 1 mL of each sample that was PCR-positive and VI-negative. The inoculated pigs were then blood-tested on days 0, 7, 14, and 21 pi by PCR, VI, and ELISA (IDEXX Laboratories, Westbrook, Maine, USA) (24). All animals were cared for following the standards of the University of Minnesota Institutional Animal Care and Use Committee during the entire period of this procedure.

Experiment II: Attempts to transmit PRRSV to a susceptible pig by mosquitoes that had a 7- to 14-day incubation period after feeding to repletion on an infected pig

Infection model — This procedure was based on the method previously described (9–11,27). A total of 3 6-week-old pigs were obtained from the same source as described in experiment I. Each pig was housed in a separate room at the University of Minnesota College of Veterinary Medicine isolation facility. Upon arrival, all animals were tested by PCR, VI, and ELISA to insure a PRRSV-negative status. One pig was intranasally inoculated with the same PRRSV isolate and concentration used in the experiment I (a donor pig). The remaining pigs that were not inoculated served as a recipient pig and a negative control pig, respectively. All animals were cared for following the standards of the University of Minnesota Institutional Animal Care and Use Committee during the entire period of the study. On a daily basis, the designated employee at the facility always cared for the protocol control pig first, the recipient pig second, and the donor pig last.

Source of mosquitoes — The source of mosquitoes described in experiment I was also used in this experiment.

Mosquito transmission protocols — Mosquito-to-pig contact took place 7 d after the donor pig was inoculated. To facilitate transmission of PRRSV from the donor to the recipient pig via mosquitoes, the manual vector transmission protocol was applied, as previously described (9–11,27). A total of 30 mosquitoes were allowed to feed to repletion on the donor pig. For each individual mosquito, the insertion of the proboscis and the initiation of abdominal swelling were observed. Following the completion of feeding (3 to 5 min), all blood-fed mosquitoes were placed into a humidified incubation cage (27°C) and maintained alive with sucrose solution at the entomology laboratory at the University of Minnesota. On days 7, 10, and 14 pf, a set of 10 mosquitoes were randomly selected at each sampling time, and allowed to feed to repletion on the recipient pig by using the manual vector transmission protocol, as previously described. Following the feeding, vials containing an individual mosquito were immediately placed on dry ice and the mosquitoes were tested for PRRSV.

Sampling and diagnostic analysis — The donor pig was bled on the exposure day (day 7 pi) to document PRRSV-viremia. The recipient pig was bled weekly and PRRSV infection status of the recipient pig was monitored for 28 d post-exposure (pe). Day 7 pf of mosquitoes was equal to day 0 pe of the recipient pigs. The control pig was bled at the beginning and the end of the experiment (days 0 and 28 pe). Samples of exterior surface washes, salivary glands, thorax carcasses, and gut homogenates were collected from each mosquito, as previously described.

Results

Experiment I: Assessment of the duration of viability and location of PRRSV within mosquitoes

The inoculated pig demonstrated PRRSV-viremia at the time that mosquito-pig contact took place (day 7 pi), confirmed by PCR and VI.

Table I. Diagnostic data from mosquito samples in experiment I

Sampling time	Mosquito sample	PCR	VI	Bioassay
0 hour pf	Wash	−	−	NT
	Gut	+	−	+
	Salivary gland	−	−	NT
	Thorax	Suspect	−	−
6 hours pf	Wash	−	−	NT
	Gut	+	−	+
	Salivary gland	−	−	NT
	Thorax	−	−	NT
12 hours to	Wash	−	−	NT
14 days pf[a]	Gut	−	−	NT
	Salivary gland	−	−	NT
	Thorax	−	−	NT

pf — post-feeding; Wash — exterior surface washes of mosquitoes; Gut — gut homogenates of mosquitoes; Salivary gland — salivary gland homogenates of mosquitoes; Thorax — homogenates of thorax carcass of mosquitoes except for salivary gland; PCR — polymerase chain reaction; PCR (+) — a sample that is PCR-positive in 2/2 tests; PCR-suspect — a sample that is PCR-positive in 1/2 tests; Bioassay (+) — a PCR(+)/VI(−) sample that induced PRRSV-viremia and seroconversion in a susceptible pig during 21-day monitoring period following intramuscular inoculation; NT — not tested

[a] Specific sampling points included in this period are 12 h, 24 h, 48 h, 72 h, 5 d, 7 d, 10 d, and 14 d

Diagnostic results from mosquitoes in experiment I are summarized in Table I. The PRRSV nucleic acid was detected by PCR from the gut homogenates collected at 0 h and 6 h pf. The thorax carcasses collected at 0 h pf was PCR-suspect. The gut homogenates collected at 0 and 6 h pf were confirmed to contain infectious PRRSV by swine bioassay, while the thorax carcasses collected at 0 h pf did not. All other samples were negative by all tests.

Experiment II: Attempts to transmit PRRSV to a susceptible pig by mosquitoes that had a 7- to 14-day incubation period following feeding to repletion on an infected pig

The donor pig demonstrated PRRSV-viremia at the time that mosquito-pig-contact took place (day 7 pi), confirmed by PCR and VI. The recipient pig remained PRRSV-negative during the 28-day monitoring period following the mosquito exposure. Porcine respiratory and reproductive syndrome virus was not detected by PCR and VI in any mosquito samples collected on days 7, 10, and 14 pf.

Discussion

The data from the study strongly suggest that mosquitoes (*A. vexans*) cannot serve as biological vectors of PRRSV. We attempted to assess viral replication and dissemination through analysis of numerous anatomical sites of mosquitoes using multiple diagnostic tests. Furthermore, we conducted a transmission experiment that included an incubation period of 7 to 14 d. The first experiment in the study clearly demonstrated that infectious PRRSV could survive in the intestinal tract of mosquitoes for up to 6 h following feeding on an infected pig. However, PRRSV was not detected in the salivary glands at any sampling points, and from thorax carcasses no later than 6 h following feeding on an infected pig. These findings suggest that PRRSV was not disseminated in the body of the mosquito during the 14-day incubation period (20,21). Moreover, inability of the mosquitoes that had fed on the PRRSV-viremic pig to transmit the virus to a susceptible pig following the 7- to 14-day incubation period in the 2nd experiment, indicates that PRRSV is not capable of replication within mosquitoes to establish a sufficient concentration of the virus to infect a naïve animal (19). This finding also supports the previously published evidence of the failure of the equine arteritis virus, which belongs to the same family as PRRSV (family *Arteriviridae*, genus *Arterivirus*), to replicate within intrathoracically inoculated mosquitoes (29). Therefore, we concluded that mosquitoes are not likely to serve as biological vectors of PRRSV.

This information is important because it can help swine practitioners to understand the potential role of mosquitoes in transmission of PRRSV. It has been documented that mosquitoes can travel for the distances of 2.5 to 10 km and can feed on blood multiple times in their life (30). Furthermore, mosquitoes frequently inhabit the interior of transport vehicles and livestock trailers, increasing the chance of contact with pigs that are potentially infected with PRRSV and allowing them to travel greater distances during shorter periods of time. All this information, along with previously published evidence of the ability of mosquitoes to mechanically transmit PRRSV from infected to naïve pigs under experimental conditions (11), suggests that, while mosquitoes may play a role in area spread of PRRSV during warm weather, they serve as strictly mechanical vectors, not biological vectors. Their inability to serve as biological vectors may limit the significance of the mosquitoes in the transmission of PRRSV; however, further studies, such as on-farm investigations and large scaled epidemiological studies, are needed to make a final conclusion

regarding the significance of mosquitoes throughout commercial swine producing areas.

The sample of thorax carcasses from mosquitoes collected at 0 h pf was PCR-suspect. Considering the fact that the virus was not detected from thoracic tissues collected any longer than 0 h pf, we speculate that PRRSV may have been present in upper digestive tract, perhaps strictly localized to the esophagus. Another interpretation of the PCR-suspect reading from the thorax carcass sample could be either a false positive reaction, or indicative of a very small amount of PRRSV nucleic acid that was present in the sample. Finally, despite careful attention to avoid cross-contamination between samples during dissection of the individual mosquitoes, the possibility exists that the thorax tissues may have been contaminated with the virus originating from the gut tissues of the same individual mosquito.

Regarding the exterior surface washes of mosquitoes, PRRSV was not detected from the samples collected during any sampling time. The method used to collect the exterior surface washes of mosquitoes in the study has been documented to be able to recover PRRSV nucleic acid from the exterior surface of houseflies (*M. domestica*) that had contact with an infected pig (10). A possible explanation for this finding is that the proboscis of mosquitoes are structurally much smaller than the mouthparts of houseflies, so that a sufficient quantity of the virus was not present on the proboscis or elsewhere on the exterior surface of mosquitoes. It is also logical to assume that the virus that resided on the exterior surface of mosquitoes may have been destroyed by environmental factors, such as ultraviolet light or drying (31).

Mosquitoes used in the study were identified for their genus and species, and the majority (95.6%) of the insect population was *A. vexans*. It has been reported that *A. vexans* is generally distributed over the whole of North America including Canada, the United States, and Mexico (32). However, this species is especially abundant in the Midwest United States and makes up the majority of wild mosquito populations observed in Minnesota (R. Moon; personal communication 2002). Therefore, we believe that the insect population used in this study was representative of the wild mosquito population in the Midwest United States.

As with all scientific studies, the present study also involved some recognized limitations. Since we processed all of the samples as pools of 10 mosquitoes at each collection time and did not quantitatively assess PRRSV in an individual mosquito, no conclusion could be made regarding the concentration of the virus present in the intestinal tract of mosquitoes collected at 0 and 6 h pf. It has been previously documented that PRRSV could be detected in insect samples at concentrations as low as 10^1 $TCID_{50}$/mL by PCR, 10^2 $TCID_{50}$/mL by swine bioassay, and 10^3 $TCID_{50}$/mL by VI (33). Therefore, we may speculate that the concentrations of the virus detected from the gut homogenates of mosquitoes collected at 0 h and 6 h pf may have been in a range of 10^1 to 10^2 $TCID_{50}$/mL. Additionally, it is possible that the variation in the amount of blood that each individual mosquito ingested may have affected the outcome of the study. However, the use of transparent plastic vials allowed us to observe the entire feeding process of each individual mosquito in order to minimize this variability. Finally, VI results from all mosquito samples were negative in this study. Similar findings also have been observed in previous studies (9–11), a possible explanation for this finding is, again, the relative lower sensitivity of VI as compared to PCR (33).

In conclusion, PRRSV can survive within the intestinal tract of mosquitoes for up to 6 h following feeding on an infected pig; however, infectious PRRSV is restricted to the intestinal tract and does not replicate or disseminate systemically within mosquitoes during a 14-day incubation period. These findings support the previous study demonstrating the inability of Arteriviruses (equine arteritis virus) to replicate in mosquitoes (29). This study, along with our previous study (11), suggest that while mechanical transmission of PRRSV by mosquitoes is possible, mosquitoes (*A. vexans*) are not likely to serve as biological vectors of PRRSV.

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