Title: Evaluation of the Role of Mosquitoes as Biological Vectors of PRRSV - NPB #02-091

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Abstract: The objectives of the study were 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 an experimentally PRRSV-infected pig (day 7 post-inoculation) and 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 (PCR) 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 an experimentally PRRSV-infected pig and the mosquitoes 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 (*Aedes vexans*) are not likely to serve as biological vectors of PRRSV.
Introduction: Vectors of porcine reproductive and respiratory syndrome virus (PRRSV) include infected pigs, semen, contaminated needles, and fomites. To reduce the risk of PRRSV-introduction, swine producers practice strict biosecurity measures, i.e. quarantine and testing of replacement stock, 48-72 hours downtime for visitors, with shower-in/out policies. Despite these efforts, infection of naïve herds frequently occurs through unidentified routes. A potential route of PRRSV that has not been explored is a mosquito. Mosquitoes can serve as mechanical or biological vectors of numerous animal pathogens can travel 2.5-10 km, and current biosecurity measures cannot regulate their entry into swine herds. We have recently detected PRRSV in mosquitoes that fed upon infected pigs raised under commercial farm conditions. The PRRSV RNA from the mosquito homogenate was homologous (100%) to the strain of PRRSV found on the farm, and the homogenate contained infectious PRRSV (swine bioassay). Furthermore, we recently demonstrated transmission of PRRSV by mosquitoes from infected to naïve pigs under experimental conditions. This indicates that mosquitoes can serve as mechanical vectors of PRRSV; however, whether the virus can replicate in the mosquito (biological vector) is not known.

Objectives: To determine whether mosquitoes can serve as biological vectors of PRRSV.

Hypothesis: Mosquitoes are very unlikely to serve as biological vectors of PRRSV.

Materials and Methods:
Specific aims:
1. Evaluate if PRRSV antigen is present in mosquito tissues following feeding on infected pigs.
2. Determine the fate of PRRSV in mosquitoes following feeding on infected pigs.

Experiment 1: Location of PRRSV in mosquitoes
Objective: To evaluate if PRRSV antigen is present in specific sites within mosquitoes following feeding on infected pigs.

Hypothesis: PRRSV antigen will not be detected intracellularly in mosquitoes.

Materials and methods:
Source of pigs: Four-week-old pigs will originate from a documented PRRSV-naïve source farm, based on 5 years of diagnostic and production data, clinical observations. Pigs will be tested by ELISA to insure PRRSV-naïve status.

Source of mosquitoes: Mosquitoes will be trapped at a PRRSV-naïve commercial farm. Prior to the initiation of the experiments, randomly sampled mosquitoes will be classified by genus and species, and tested by TaqMan polymerase chain reaction (PCR) and virus isolation (VI) to insure the absence of PRRSV.
Source of virus: As described in Experiment 1.

Infection model: This phase will consist of 3 replicates. Two 4-week-old pigs, housed in the UMN CVM isolation facilities, will be inoculated with 5 ml of PRRSV (10⁴ TCID₅₀/total dose). Another pig will serve as a negative control, housed in a separate room. Positive controls will consist of mosquitoes placed in direct contact with the study PRRSV isolate.

Collection of blood-fed mosquitoes: The peak of viremia of infected pigs will be day 5, 6, and 7 post inoculation (pi). Therefore, mosquito-to-pig contact will take place at this time. To collect blood-fed mosquitoes, a manual vector transmission protocol will be used. During the mosquito feeding periods, pigs will be consciously restrained in a Panepinto sling, and small plastic vials containing an individual mosquito will be loosely adhered with masking tape over the dorsal surface of the pig. These vials contain nylon net on the bottom, allowing the mosquito to access to pig skin. Mosquitoes will be observed to insure completion of feeding. A total of 200 blood-fed mosquitoes will be collected during each exposure day (day 5-7 pi), for a total of 600 insects/replicate. Fifty mosquitoes will be collected following feeding on the negative control pig. Mosquitoes will be placed into a humidified (27 °C) incubation cage, labeled by exposure day, and kept alive with sucrose solution. Within the incubator, mosquitoes will remain in individual vials to prevent cross-contamination. Negative control mosquitoes will occupy a separate incubator.

Sampling and analysis: From each incubator, a total of 10 blood-fed mosquitoes will be randomly sampled at the following times: 0 min, 2 hr, 4 hr, 8 hr, 12 hr and 24 hr post-feeding (pf), and 2-7 days pf (total: 120 insects/exposure day). Five negative control mosquitoes will be included at each sampling time. Mosquitoes will be fixed in 10% formalin, paraffin embedded, immunohistochemical stains applied, and examined for PRRSV antigen. Anatomical sites with evidence of PRRSV antigen will be recorded. Swine tissues (lymphoid and lung) infected with PRRSV will serve as positive controls for the staining procedure.

Experiment 2: Fate of PRRSV in mosquitoes

Objective: To determine if PRRSV is present on the exterior surface of the mosquitoes or can replicate in the mosquitoes over time following feeding on infected pigs.

Hypothesis: PRRSV will be detected on the exterior and within internal viscera of mosquitoes only immediately following feeding.

Source of pigs, mosquitoes, and virus: As described in Experiment 2.

Infection model and collection of blood-fed mosquitoes: As described in Experiment 2. For positive controls, 30 mosquitoes (anesthetized at 4 °C for 15 minutes) will be stored in 5 ml aliquots (1 aliquot/sampling time) of stock solution of the study isolate (10⁴ TCID₅₀/5 ml) and frozen at −70 °C until testing is initiated.

Sampling and analysis: This phase will consist of 3 replicates. Groups of 30
blood-fed mosquitoes (along with 1 positive control aliquot) will be randomly collected at each sampling time described in Experiment 2 (360 insects/replicate). Thirty negative control mosquitoes will be randomly sampled at 0-min pf. Each group will be divided into 2 subgroups. One subgroup will be sampled for attached virus on the exterior surface. Each mosquito will have its proboscis and anus tied off (to avoid contamination following regurgitation), be placed in a sterile tube containing MEM and surfactant, shaken vigorously for 30 seconds, insect removed, and the fluid assayed for PRRSV. The second subgroup will be used to test for PRRSV within internal structures. Each mosquito will have its proboscis and anus tied off (to enhance viability of internalized virus), washed with 70% ethanol to kill exterior virus, rinsed 25 times in sterile saline solution, and allowed to dry. Insects will be dissected, gastrointestinal tracts, salivary glands, brain, and fat body removed, tissues homogenized, and assayed for PRRSV. Quantitative PCR will be applied to determine the amount of PRRSV in external surface wash-fluid and visceral homogenates per sampling time.

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. 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 following 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. PRRSV 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 (Aedes 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 days. The first experiment in the study clearly demonstrated that infectious PRRSV could survive in the intestinal tract of mosquitoes for up to 6 hours following feeding on an infected pig.
However, PRRSV was not detected from salivary glands at any sampling points, and from thorax carcasses no later than 6 hours following feeding on an infected pig. These findings suggest that PRRSV was not disseminated in the body of the mosquito during 14-day incubation period. Moreover, inability of the mosquitoes that had fed on PRRSV-viremic pig to transmit to a susceptible pig following 7 to 14-day incubation period in the second experiment indicates that PRRSV is not capable to replicate within mosquitoes to establish a sufficient concentration of the virus to infect a naïve animal.

Author’s Note: While the original proposal called for an assessment of PRRSV replication in insect cell lines, this aim was changed after the mid term progress report was filed. The primary reason for the change was the loss of accessibility to insect cell lines. The professor who had originally agreed to assist with this aim changed her mind for reasons I was never able to figure out. In substitution, we conducted a transmission study from infected to naive pigs using the manual vector transmission protocol we have previously published. To better understand if mosquitoes could serve as biological vectors and transmit PRRSV to pigs, the transfer of female *Aedes vexans* that had been fed to repletion on viremic pigs. The transfer to recipients took place following a 7 or 14 day incubation period. All results were negative indicating the inability of PRRSV to replicate and disseminate in mosquitoes.

Lay Interpretation: This information is important because it would 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 collect blood meals multiple times in their life. Furthermore, mosquitoes frequently inhabit the interior of transport vehicles and livestock trailers, enhancing contact with pigs that are potentially infected with PRRSV, and allowing them to travel over greater distances during shorter period of time. All this information, along with previous evidence of the ability of mosquitoes to mechanically transmit PRRSV from infected to naïve pigs under experimental conditions, suggests that while mosquitoes may play a role in area spread of PRRSV during warm weather, they serve strictly as mechanical vectors and not as biological vectors. Their inability to serve as biological vectors may limit the significance of the mosquitoes in 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. Mosquitoes used in the study were identified for their genus and species, and the majority (95.6%) of the insect population was *Aedes vexans*. It has been reported that *Aedes vexans* is generally distributed over the whole North America including Canada, United States, and Mexico. However, this species is specially abundant in Midwest areas in United States, and is a majority of wild mosquito populations observed in
Minnesota (R Moon; personal communication 2002). Therefore, we believe that insect population used in the study was reliable to represent the wild mosquito population in Midwest areas in United States.

In conclusion, PRRSV can survive within the intestinal tract of mosquitoes for up to 6 hours following feeding on an infected pig; however, the infectious PRRSV is restricted to the intestinal tract and does not replicate or disseminate systemically within mosquitoes during a 14-day incubation period. This study suggests that while mechanical transmission of PRRSV by mosquitoes is possible, mosquitoes (Aedes vexans) are not likely to serve as biological vectors of PRRSV.

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