

# Further assessment of houseflies (*Musca domestica*) as vectors for the mechanical transport and transmission of porcine reproductive and respiratory syndrome virus under field conditions

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## Abstract

The purpose of this study was to evaluate the potential for houseflies (*Musca domestica*) to mechanically transport and transmit porcine reproductive and respiratory syndrome virus (PRRSV) between pig populations under controlled field conditions. The study employed swine housed in commercial livestock facilities and a release-recapture protocol involving marked (ochre-eyed) houseflies. To assess whether transport of PRRSV by insects occurred, ochre-eyed houseflies were released and collected from a facility housing an experimentally PRRSV-inoculated population of pigs (facility A) and collected from a neighboring facility located 120 m to the northwest that housed a naïve pig population (facility B). All samples were tested for PRRSV RNA by polymerase chain reaction (PCR). To assess transmission between the 2 populations, blood samples were collected from naïve pigs in facility B at designated intervals and tested by PCR. A total of 7 replicates were conducted. During 2 of 7 replicates (1 and 5), PCR-positive ochre-eyed houseflies were recovered in facility B and pigs in this facility became infected with PRRSV. Chi-squared analysis indicated that the presence of PRRSV in an insect sample was significantly ( $P = 0.0004$ ) associated with infection of facility B pigs. Porcine reproductive and respiratory syndrome virus was not recovered from other reported routes of transmission during the study period, including air, fomites, and personnel. In conclusion, while an insufficient number of replicates were conducted to predict the frequency of the event, houseflies may pose some level of risk for the transport and transmission of PRRSV between pig populations under field conditions.

## Résumé

Le but de la présente étude était d'évaluer le potentiel des mouches domestiques (*Musca domestica*) à transporter mécaniquement et transmettre le virus du syndrome reproducteur et respiratoire porcin (PRRSV) entre des populations de porcs dans des conditions de terrain contrôlées. Dans l'étude, on a utilisé des porcs logés dans des installations commerciales d'élevage et un protocole de relâche-recapture impliquant des mouches domestiques marquées (yeux ocrés). Afin d'évaluer si le transport du PRRSV par les insectes est survenu, les mouches domestiques marquées étaient relâchées et récoltées d'une installation où était logée une population de porcs inoculés avec le PRRSV (installation A) et récoltées d'une installation voisine, qui hébergeait une population de porcs naïfs (installation B), située à 120 m au nord-ouest. Tous les échantillons ont été testés pour détecter l'ARN du PRRSV par réaction d'amplification en chaîne par la polymérase (PCR). Afin d'évaluer la transmission entre les deux populations, des échantillons sanguins ont été prélevés des porcs naïfs dans l'installation B à des intervalles prescrits et testés par PCR. Un total de 7 réplifications a été effectué. Au cours de 2 des 7 réplifications (1 et 5), des mouches domestiques marquées positives par PCR ont été retrouvées dans l'installation B et des porcs dans cette bâtisse sont devenus infectés par le PRRSV. Des analyse de chi-carré ont indiqué que la présence de PRRSV dans un échantillonnage d'insectes était associée de manière significative ( $P = 0,0004$ ) avec l'infection des porcs de l'installation B. Au cours de la période d'essai, le PRRSV n'a pas été retrouvé à partir d'autres vecteurs de transmission, incluant l'air, les objets et le personnel. En conclusion, bien qu'un nombre insuffisant de réplifications ait été effectué pour prédire la fréquence de l'évènement, les mouches domestiques peuvent représenter un certain niveau de risque pour le transport et la transmission du PRRSV entre des populations de porcs dans des conditions de terrain.

(Traduit par Docteur Serge Messier)

## Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically significant disease of pigs, estimated to cost the US swine industry approximately 560 million dollars per year (1). The etiologic

agent of PRRS is porcine reproductive and respiratory syndrome virus (PRRSV), a single-stranded, positive-sense, enveloped RNA virus classified in the order *Nidovirales*, family *Arteriviridae*, and genus *Arterivirus* (2). Control of the disease via traditional methods such as vaccination and animal flow has not been successful in all

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cases; therefore, attempts to eradicate the disease have been made. While eradication has been successful at the individual farm level, re-infection due to local spread of the virus, defined as the introduction of an unrelated variant of PRRSV to a population of pigs via an indirect route, is a frequent and frustrating event (3–5).

Potential routes of PRRSV transmission include infected pigs, contaminated semen, fomites, farm personnel, insects (houseflies and mosquitoes), transport vehicles, and aerosols (3,6–12). In regard to insects, previously published studies have demonstrated that while mosquitoes (*Aedes vexans*) and houseflies (*Musca domestica*) could serve as mechanical vectors of PRRSV, the virus did not replicate within these species and they could not act as biological vectors. (8–9,13). It has also been shown that the site of the virus in these species is the intestinal tract with retention dependent upon the quantity of virus ingested and the corresponding environmental temperature (14–15). Furthermore, it has been reported that houseflies are capable of transporting PRRSV for up to 2.4 km from an infected swine population (16).

However, while the results from these studies are interesting, they all possessed significant limitations, such as being conducted under laboratory conditions involving small numbers of pigs, using nonrealistic procedures to enhance contact between infected animals and insects (8–9), and focusing only on transport of the virus between farms (16). The purpose of this study, therefore, was to use a large population of PRRSV-pigs housed in commercial facilities to re-evaluate the role of houseflies in the transport and transmission of PRRSV between farms under controlled field conditions that were representative of the swine industry in many countries.

## Materials and methods

### Site description and source of animals

This study was conducted on the University of Minnesota Swine Disease Eradication Center (SDEC) research farm, a site that is separated from other swine farms by a distance of 16 km. The study was conducted in Minnesota during the summer (June to September) of 2007. Over this 16-week period, 7 two-week replicates were conducted. Two facilities on the SDEC site were used for the assessment: facility A and facility B, the latter being stationed 120 m northwest of facility A. Facility A was mechanically ventilated and housed 300 head of grow-finish pigs ranging in size from 25–120 kg on partially slotted flooring; facility B was naturally ventilated and housed 20, 25-kg pigs on solid concrete flooring. Approximately 27 to 28 pigs were placed in each of the 11 pens in facility A, and all 20 pigs were housed in one pen in facility B. The source of animals was the same for both facilities (Genetiporc, Alexandria, Minnesota, USA) and had been documented to be free of PRRSV for over 20 y, based on the lack of clinical signs along with a PRRSV-naïve diagnostic database. During the study, animals were cared for using protocols approved by the University of Minnesota Institutional Animal Care and Use Committee at all times.

### Source of PRRSV and inoculation procedures

To initiate the study, 100 of the 300 pigs in facility A were experimentally inoculated via the intra-nasal route with 2 mL of PRRSV

variant MN-184 (total dose =  $2 \times 10^4$  TCID<sub>50</sub>), a highly virulent isolate capable of producing high concentrations of virus in blood, tissues, and oro-nasal secretions of infected pigs (17).

### Source of flies

A laboratory-derived colony of ochre-eyed houseflies (*Musca domestica*) was used for the release-recapture phase of the study. This colony was fixed for a recessive allele that produced an ochre-eye phenotype allowing for differentiation of these flies from wild-type houseflies having a red-eye phenotype. In the laboratory, adult flies were housed in cages (30 cm<sup>3</sup>) at a temperature range of 25°C to 35°C and provided with cubed sucrose, powdered milk, and water ad libitum. Pupae were placed in cages to replenish adults. Eggs were collected daily and resulting cohorts of pupae were stored at 10°C in preparation for release. For release of ochre-eyed flies into facility A, 100 000 pupae were packaged in screen-topped plastic containers and shipped from the laboratory to the farm weekly via an overnight delivery service for a total of 2 shipments of 100 000 pupae per replicate. Upon receipt of pupae, containers were uncovered and placed on the top of each concrete pen divider (1 container for each of 11 pens dividers) in facility A to allow adult flies to emerge and contact pigs.

### Movement of personnel between facilities

During the study period, designated personnel ( $n = 3$ ) visited facility A and B daily to conduct protocols of animal inspection common to those practiced on commercial swine operations. Facility B was always visited before facility A. Personnel donned farm-specific boots, coveralls, and gloves upon entry to the pens in facility A and the single pen in facility B. They then visually inspected the pigs, adjusted feeders, checked water flow rates, treated sick animals with injectable medications, etc. Following completion of the daily inspection of facility B, study personnel removed boots and coveralls, walked the 120 m to facility A and cared for animals as described. Following completion of these daily tasks, personnel took a shower in the farm house and left for the day. At the end of each replicate, the 20 pigs from facility B were moved into facility A and facility B was sanitized using a 0.8% concentration of 7% glutaraldehyde and 26% quaternary ammonium chloride (Synergize; Preserve International, Atlanta, Georgia) which was applied via a foamer and allowed to dry overnight (18). No other personnel entered facility B during the project period and doors remained locked at all times.

### Sampling procedures

#### Pigs

To confirm the PRRSV status of the facility A pig population following the experimental infection, sera from 10 inoculated pigs were collected on day 7 post-inoculation. To monitor the PRRSV status in the facility B pig population, serum from all 20 pigs was collected on days 2, 5, 7, 9, and 12 of each replicate.

#### Flies

For collection of flies, two 1% niathiazine strips (Quick Strike; Wellmark International, Schaumburg, Illinois, USA) were hung from the ceiling inside of both facilities, approximately 25 cm from the floor. Directly underneath each strip, an aluminum pan (29.4 cm

in diameter and 7.6 cm in depth) was placed to collect fallen flies following contact with the insecticide. On days 2, 5, 7, 9, and 12 of each replicate, flies were removed from traps and processed using previously published techniques (15,16). Traps were first processed from facility B and then from facility A. Flies were counted and placed in a plastic bag marked with the collection date and trap location. Flies were then pooled by collection day with 30 flies included in each pool (15,16). To process each pool, a 10.2 × 10.2-cm sterile gauze sponge (Johnson & Johnson Industries, Skillman, New Jersey, USA) was placed over the top of disposable foam beverage cup (Dart Container, Mason, Michigan, USA) (15,16). Personnel donned vinyl examination gloves (Medicine Industries, Mundelein, Illinois, USA), placed the 30 flies onto the gauze and rinsed them with 10 mL of minimum essential medium (MEM) (Difco, Detroit, Michigan, USA). The gauze containing the pooled sample was then manually compressed several times, allowing the exudates from flies to filter through the gauze and into the cup until further seepage of exudates was not observed (15,16). Filtrates were then stored at -20°C until testing was initiated. Personnel changed gloves and used a new sponge and cup for each sample.

### Assessment of additional routes of PRRSV transmission

To assess the potential of other reported routes of PRRSV introduction to facility B, additional samples were collected during each replicate. To evaluate the potential for fomites and personnel to introduce the virus, swab samples were collected daily before personnel entered the animal holding area in facility B (7,11). Specifically, sterile cotton swabs (Fisher Scientific, Hanover Park, Illinois, USA) were drawn over both sides of gloved hands, the front and back sides of coveralls, the soles and sides of boots, as well as the surfaces of all incoming fomites including blood testing equipment, cable snare, and feed bags. Swabs were first moistened with MEM and then applied to the described surfaces using a zig-zag pattern. Swabs were then stored in sterile plastic tubes (Falcon, Becton-Dickinson, Franklin Park, New Jersey, USA) containing 3 mL of MEM and stored at -20°C until being tested at the conclusion of each replicate. To determine whether virus could have entered facility B via the aerosol route, air samples were collected at 10 AM CST for a 30-minute period on days 1, 2, 3, 5, 6, 7, 8, 9, 10, and 12 of each replicate using a cyclonic collector capable of collecting 400 L of air per minute (Midwest MikroTech, Brookings, South Dakota, USA) (12). During the collection process, the instrument was placed at the facility B inlet, inspired air was continuously washed with 10 mL of MEM supplemented with 3% fetal calf serum, and a 5-mL aliquot was removed for testing. Finally, upon completion of each replicate, the floor and the walls of the facility B pig pen were sanitized as previously described and then swabbed to document the absence of residual PRRSV using 27 × 21 cm polyester pads (Swiffer sweepers; Proctor and Gamble, Cincinnati, Ohio, USA). Following sampling, pads were rinsed in MEM and a 5-mL aliquot was submitted for diagnostic evaluation.

### Diagnostic testing

All samples collected during each replicate were tested for the presence of PRRSV RNA using the qualitative *TaqMan* polymerase

chain reaction (PCR) assay (Perkin Elmer Applied Biosystems, Foster City, California, USA) performed at the Minnesota Veterinary Diagnostic Laboratory (19). Prior to initiation of the study, the minimum detection limit of the PCR assay for the detection of PRRSV in insect pools, swabs, and air samples had been consistently calculated to be  $1 \times 10^1$  TCID<sub>50</sub>/mL (20). If a positive PCR result was obtained from a facility B sample, its open reading frame (ORF) 5 region was nucleic acid sequenced and compared to the similar region of a PRRSV isolate recovered from the infected swine population in facility A (21). Once PCR-positive serum samples were detected on 2 of the sampling days within each replicate, the facility B pig population was considered infected, the replicate was terminated, and no further sampling was conducted.

### Swine bioassay

Swine bioassays were performed to determine if infectious PRRSV was present in PCR-positive pooled fly samples collected from facility B (22). For the purpose of this assay, PCR-positive insect filtrates were injected via the intra-muscular route into PRRSV-naïve pigs housed at the study site. These assays were performed after the study had been completed and bioassay pigs were housed in isolated, sanitized facilities. Each pig was housed in an individual room, and study personnel changed boots, coveralls, gloves, and hairnets between rooms. For the purpose of a negative control, a PRRSV naïve pig was inoculated with a pool of fly samples collected on day 0 post-inoculation. Blood was collected from all pigs on days 7 and 14 post-inoculation, and sera were tested for PRRSV RNA by PCR.

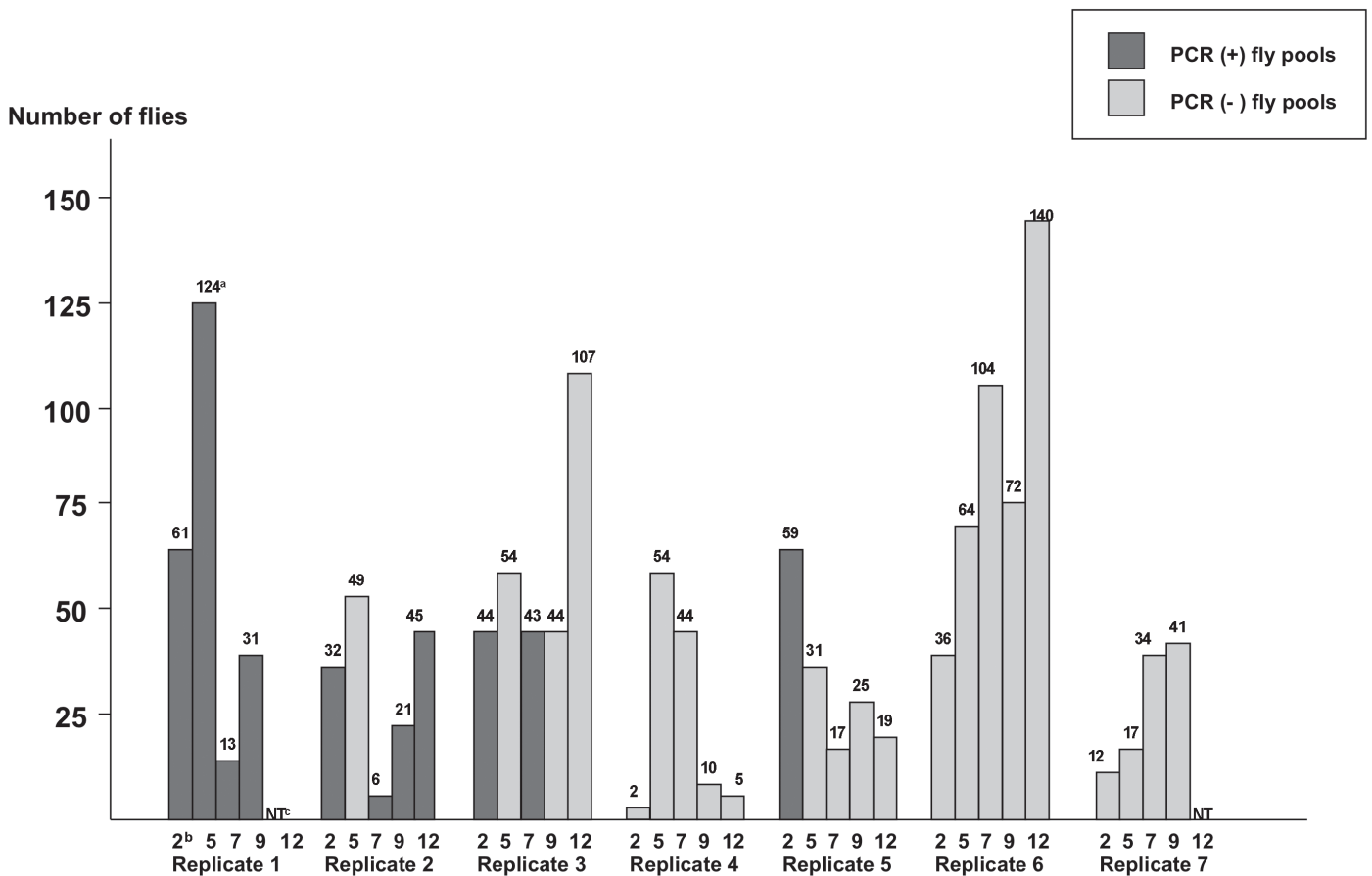
### Data analysis

The association between the recovery of a PRRSV-positive ochre-eyed fly sample in facility B and the subsequent infection of the facility B pig population was tested for statistical significance by Chi-squared analysis using computer software (Statistix 8.0, Analytical Software, Tallahassee, Florida, USA).

## Results

Throughout the 14-week study period, clinical signs of PRRS (dyspnea, hyperthermia, anorexia, and weight loss) were observed throughout the facility A pig population and 12% of these animals died. The PRRSV-infection in facility A was confirmed by PCR-positive results in serum collected from 10/10 experimentally inoculated pigs on day 7 post-inoculation. Throughout the study, extensive contact between flies and pigs was observed, with flies seen feeding upon feces and urine, as well as upon skin abrasions, nasal, lachrymal, and salivary secretions of sick pigs.

A total of 49 ochre-eyed fly pools (1459 total flies) were submitted for testing from facility A. Sixteen of these pools (479 total flies) were PCR-positive with positive pools distributed across 11 sampling days throughout replicates 1, 2, 3, and 5 (Figure 1). In contrast, PRRSV RNA was not detected in pools collected during replicates 4, 6, and 7. In facility B, a total of 12 pools (365 flies) were submitted for testing; PCR-positive pools were found on days 5 and 9 of replicate 1, and on day 2 of replicate 5 (Figure 2). Most of the ochre-eyed flies in facility B were recovered during replicates 1 and 5 (218 and 120 flies, respectively) with limited numbers (0 to 14) of flies being recovered



**Figure 1.** Summary of the number of ochre-eyed flies collected per sampling day in facility A and PRRSV status of fly pools by sampling day across replicates.

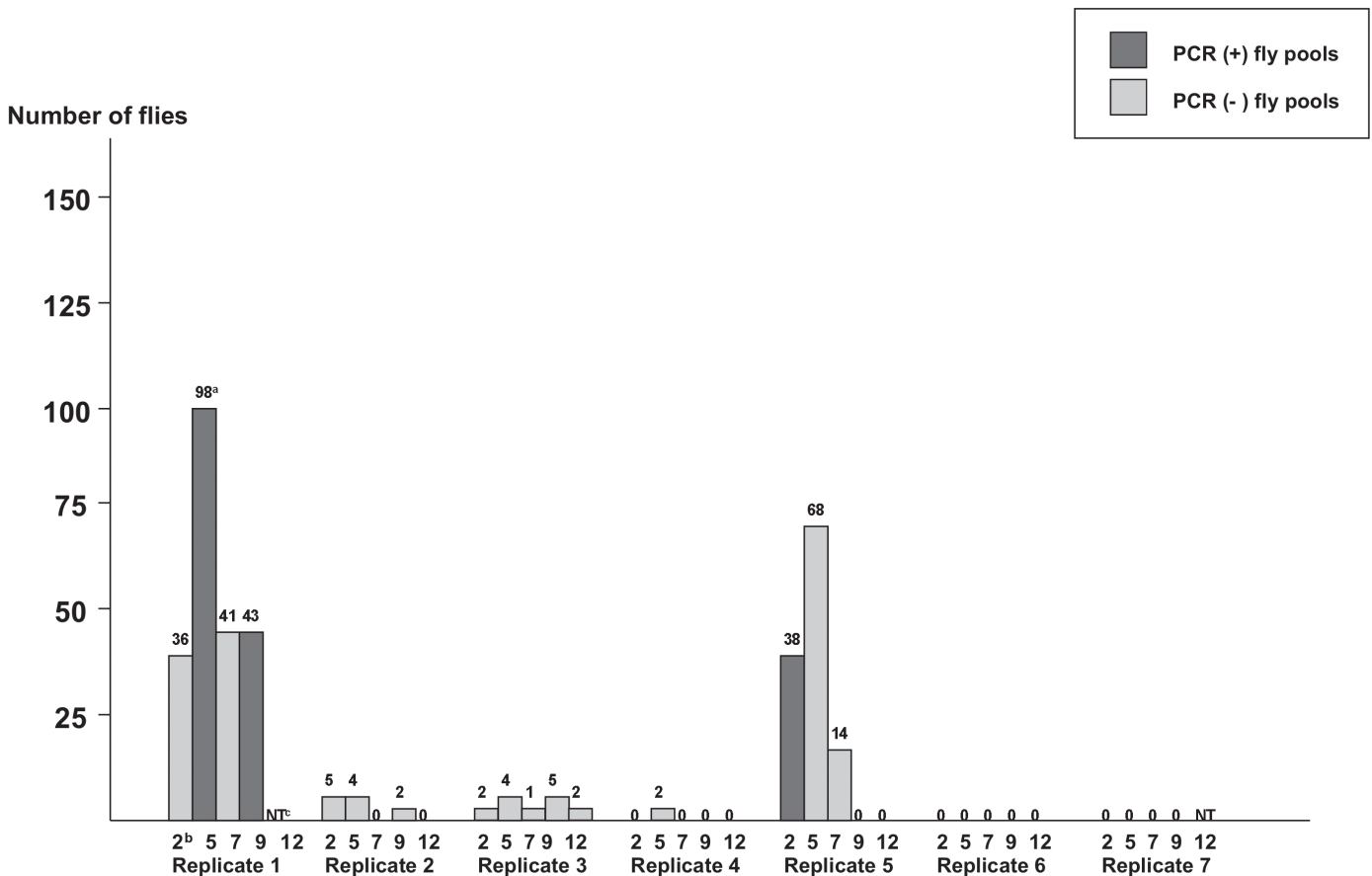
- <sup>a</sup> Number of ochre-eyed flies collected on each sampling day.
- <sup>b</sup> Days of fly collection in each replicate.
- <sup>c</sup> Not tested.

across the other replicates. Nucleic acid sequencing of the ORF 5 region of fly samples recovered from both facilities indicated a high degree (99.9–100%) of homology.

Infection of pigs in facility B was also observed in replicates 1 and 5, based on the detection of PRRSV RNA in sera collected on days 7 and 9 of replicate 1, and on days 9 and 12 of replicate 5. The ORF 5 region of these samples was homologous (99.9–100%) to PRRSV sequences present in fly pools from both facilities and from clinically affected animals in facility A. The presence of infectious PRRSV in insect filtrates was confirmed by swine bioassay. Specifically, 3 filtrates prepared from ochre-eye fly pools collected on days 5 and 9 during replicate 1 and day 2 during replicate 5 were tested and found to be positive for viable virus. In contrast, the negative control bioassay pig did not become infected. In addition, PRRSV RNA was not detected in any of the air samples ( $n = 70$ ) or from swabs from fomites and personnel ( $n = 1176$ ), and all 7 swab samples collected from sanitized facilities were PCR-negative as well. Finally, Chi-squared analysis indicated a significant association between the presence of PRRSV RNA in ochre-eyed fly samples during a replicate and subsequent infection of facility B pigs ( $P = 0.0004$ ).

## Discussion

The purpose of this study was to assess the ability of houseflies to transport and transmit PRRSV between pig populations using conditions representative of commercial swine production. Under the conditions employed, we observed the transport of PRRSV RNA from facility A to facility B in 2 of 7 replicates. Porcine reproductive and respiratory syndrome virus RNA and infectious virus was detected in ochre-eyed fly pools recovered from facility B and the infected animals housed in this facility in both of these replicates (1 and 5). Based on the high degree of homology between swine and insect samples collected in facilities A and B, along with the inability to detect the presence of virus in all other samples collected, it is logical to conclude that the ochre-eyed houseflies which originated from facility A may have played a role in the transport of PRRSV between the facilities and may have introduced the virus to the pigs housed in facility B. However, one could justifiably argue that the transport and transmission of virus to facility B could have occurred via aerosols during a period when air sampling was not underway, or that virus may have been present in air samples at levels below the calculated sensitivity of the collection device. We acknowledge



**Figure 2. Summary of the number of ochre-eyed flies collected per sampling day in facility B and PRRSV status of fly pools by sampling day across replicates.**

- <sup>a</sup> Number of ochre-eyed flies collected on each sampling day.
- <sup>b</sup> Days of fly collection in each replicate.
- <sup>c</sup> Not tested.

the limitation that we were not able to collect air samples 24 h/d, as well as the fact that air samples were not collected every day of the entire study period. Unfortunately, we were unable to access equipment possessing the capability for 24-hour collection and are unsure if it even exists. Therefore, air was only tested for a maximum total of 5 h (1.5%) across each 14-day replicate.

It was surprising that the recovery of PRRSV RNA from ochre-eyed flies and pigs in facility B occurred in only 2 replicates and reasons for this are unknown. One possible explanation is that there may have been specific weather patterns present during these replicates, such as a predominant wind moving in a specific direction which may have influenced the movement of flies between facilities. However, while a southeasterly wind present during replicate 1 may have influenced the movement of flies from facility A to B, review of historical weather data did not indicate the presence of this consistent weather pattern. If one evaluates the number of PCR-positive flies collected across replicates in facility A, a marked absence of positive samples can be seen during the latter part of the study (replicates 4, 6, and 7), and the inability of flies to acquire virus from the source population would definitely reduce the risk of transport of PRRSV to facility B. A possible explanation for this observation

could have been a reduction in the number of viremic animals and/or lower quantities of virus per infected animal, secondary to the generation of a protective immune response over time following the experimental inoculation. While we attempted to maintain virus circulation in the facility A population through the addition of naïve or recently infected animals at the end of each replicate, the small numbers of animals introduced may not have been sufficient to maintain the dynamics of viral infections in an acute population, as was experienced in replicate 1. In addition, virus load in animals was not quantified and the immune response was not measured in the source population. Other possibilities include the inability of the laboratory-derived flies to survive in a natural environment once they escaped from facility A, or the desire of flies to remain in facility A and utilize existing feedstuffs, reducing the necessity to travel in search of alternative sources of nutrition.

In conclusion, despite the acknowledged limitations, based on these findings along with data from other investigators (15,16) it appears that under the proper conditions houseflies can acquire PRRSV from infected pigs, harbor the virus internally and transport it in a viable state over distances representative of building and/or farm separation representative of the commercial swine industries

of many countries. What role flies play in transmission of the virus to naïve pigs is still up for debate and most likely is an infrequent event that is highly dependent upon the quantities of virus ingested and the corresponding environmental temperature (15). Furthermore, even if flies were the source of virus to the facility B population, due to the limitations of the study design we still do not know if the transmission of PRRSV from flies to pigs can occur beyond 120 m. However, while further studies involving larger numbers of replicates must be conducted in an effort to measure their true risk and the frequency of the event, these results suggest that a protocol of insect control should be a component of a comprehensive PRRSV biosecurity program that also includes intervention strategies to reduce the risk of infection by contaminated transport, fomites, personnel, and aerosols. Therefore, farm owners and managers should work closely with veterinarians to apply scientifically validated intervention strategies for the control of insects, such as the use of screen on the sidewall inlets of facilities along with the proper use of insecticides to reduce this risk (23).

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