Spatial dispersal of porcine reproductive and respiratory syndrome virus-contaminated flies after contact with experimentally infected pigs

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Objective—To determine whether flies can acquire porcine reproductive and respiratory syndrome virus (PRRSV) and disperse the virus throughout a designated area.

Animals—60 four-month-old pigs.

Procedure—On day 0, 28 of 60 pigs were inoculated with PRRSV MN 30-100 (index variant). On the same day, 100,000 pupae of ochre-eyed houseflies and 100,000 pupae of red-eyed (wild-type) houseflies were placed in the swine facility for a release-recapture study. Flies were recaptured at 2 locations within the swine facility, 6 locations immediately outside the facility, and 30 locations 0.4, 0.8, 1.3, 1.7, 1.9, and 2.3 km from the facility. Traps were emptied on days 2, 7, 8, 10, and 14. Samples derived from flies were tested by use of a polymerase chain reaction assay, virus DNA was sequenced, and viruses were tested for infectivity by means of a swine bioassay.

Results—PRRSV RNA homologous to the index PRRSV was detected in trapped flies collected inside and immediately outside the facility and from 9 of 48 samples collected at 0.4 km, 8 of 24 samples collected at 0.8 km, 5 of 24 samples collected at 1.3 km, and 3 of 84 samples collected at > 1.7 km from the facility. Two samples collected at 0.8 km contained genetically diverse variants of PRRSV. Swine bioassays revealed the virus in flies was infectious.

Conclusions and Clinical Relevance—Flies appeared to become contaminated with PRRSV from infected pigs and transported the virus ≥ 1.7 km. Fly-born transmission may explain how PRRSV is seasonally transported between farms. (*Am J Vet Res* 2004;65:1284–1292)

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important pathogen of swine throughout the world.[1] Although multiple methods for controlling PRRSV have been proposed, the efficacy of those methods is not 100% across all farms.[2-5] Therefore, attempts to eliminate PRRSV from infected farms have been developed and have been quite successful.[6,7] Despite these efforts, reinfection of farms with new strains of PRRSV is a frequent event. Reported sources of PRRSV transmission include infected pigs, semen, contaminated fomites, aerosols, and avian species. However, evidence of local spread of PRRSV by other unknown routes has recently been described.[8-16]

Potential vectors that may be capable of transporting PRRSV may include nonbiting insects such as houseflies (*Musca domestica*). Houseflies are abundant during summer wherever livestock and poultry are raised.[17] Furthermore, the flight range of the housefly exceeds 5 km from known points of origin, with maximum dispersal distances of 33 km.[18] These nonbiting flies have scraping and sponging mouthparts that are used to ingest liquids from animal tissues, including blood, serum, saliva, mucus, and lachrymal secretions. They use the process of repeated ingestion (regurgitation and reingestion) to enhance the digestion of protein.[19] Houseflies are also capable of harboring a number of important swine pathogens, including transmissible gastroenteritis virus, pseudorabies virus, hog cholera virus, and *Streptococcus suis*[20-23]; however, their role in transmission of these pathogens is not known.

Recently, it was demonstrated that under experimental conditions that optimize transmission, houseflies mechanically transmit PRRSV from infected to naive pigs and that PRRSV is viable in the digestive tracts of houseflies for up to 12 hours after feeding.[24,25] However, the role of nonhematophagous insects such as houseflies in the transport of PRRSV under field conditions is not known at this time. Therefore, the objective of the study reported here was to test the hypothesis that houseflies can acquire PRRSV from an infected pig population and disperse into the surrounding landscape during periods of warm weather.

Materials and Methods

Source of animals and study site—The study was conducted at the University of Minnesota Swine Disease Eradication Center research farm and the surrounding 16.6-km^2 premises. The farm site, located in west-central Minnesota, is annually inspected by the University of Minnesota Institutional Animal Care and Use Committee. It is located 16.6 km from the nearest commercial swine farm. The 16.6-km^2 premises consisted of corn and soybean acreage, wetlands, pasture for grazing cattle, and meadow. The pigs used in the study were obtained from a PRRSV-naïve source that had documented this status throughout a 10-year

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period by the absence of clinical signs of PRRSV and monthly monitoring of clinical, diagnostic, and production data.[26] Throughout the study period, pigs were housed and cared for according to institutional animal care and use committee regulations. Following completion of the study and attainment of the proper weight, pigs were marketed.

Sixty 4-month-old female pigs were transported to the farm and rested for 14 days prior to initiation of the study. A representative sample (n = 30) was given blood tests on arrival to verify PRRSV-naïve status. The pigs were housed in a total-confinement finishing facility that was mechanically ventilated, with partially slatted concrete floors. The facility consisted of 11 pens with solid concrete walls and vertical-rod (open) gating between pens. Pigs were placed in pens (moving east to west within the barn) 1, 3, 5, 7, 9, and 11, which provided 1 m^2 of space per pig. Pens 4, 6, 8, and 10 remained empty throughout the study period, providing 2.5-m spaces between pens.

Infection of pigs and monitoring protocol—On day 0 **postinfection (pi)**, 28 of the 60 index pigs were experimentally infected with PRRSV MN 30-100 via the intranasal route[27]; 2×10^4 $TCID_{50}$ was administered per pig. Prior to the infection, 3 specific groups were organized to verify successful experimental infection and detect viremia and shedding throughout the study period. These groups were called the index group, the direct-contact control group, and the indirect-contact control group **(Figure 1)**. There were 2 indirect-contact control groups (n = 10 pigs/group), which were located in pens 1 and 11. In the remaining pens (pens 3, 5, 7, and 9), 10 pigs were housed. Within each of these pens, 7 pigs were designated as index pigs (n = 28), whereas the remaining 3 pigs in each of these 4 pens were designated as direct-contact controls (12). For the purpose of monitoring, 3 of the 7 index pigs in each pen were identified by use of numbered ear tags (total, n = 12 pigs). Different colored ear tags were applied to all 12 of the direct-contact contact control pigs and 20 of the indirect-control pigs. It was planned that each monitor group on day 0, 7, and 14 pi would receive blood tests.

Flies, fly traps and preliminary study—Houseflies from 2 colonies were used to study dispersal of flies and associated PRRSV from the point-source study farm. A colony of red-eyed (wild-type) houseflies was established from specimens netted in St. Paul, Minn, in August 2002. **A second colony has been maintained continuously under laboratory conditions for at least 15 years.[28] This colony is fixed for a recessive allele that produces an ochre-eye phenotype that is readily distinguished from wild-type houseflies. The ochre-eye phenotype is absent in the field and is therefore a suitable marker for use in a release-recapture study.**

Adults of both colonies were housed in cages (30 cm^3) with a temperature of 25° to 32°C and provided with cubed sucrose, powdered milk, and water ad libitum. Eggs were collected weekly in small dishes of 1-day-old larval medium consisting of 1 part (by volume) rat diet,[a] 2 parts hardwood sawdust, and 2 parts tap water. Pupae were placed in cages to replenish adults. For field release, eggs were collected daily and resulting cohorts of pupae were stored at 10°C until needed for release. For release, pupae were packaged in screen-topped plastic containers, transported in coolers, and uncovered to allow adults to escape as they completed development at the study site.

Commercial baited jug traps[b] were used for collection of flies during the study.[29] The traps were filled with an attractant at a concentration of 30 mL/2 L of water per trap. A preliminary laboratory study was conducted to test the possibilities that PRRSV could transfer between contaminated and uncontaminated flies while the flies were immersed in a trap's attractant fluid and that a trap's fluid could interfere with diagnostic testing methods for PRRSV.

Adult houseflies of the 2 kinds were captured from their cages with individual, screen-bottomed vials. Ten red-eyed houseflies were exposed to PRRSV MN 30-100 and commingled with virus-free flies in a trap. To contaminate the flies, the 10 red-eyed houseflies were isolated for 24 hours without food and water in transparent, plastic, screw-cap vials (3 cm in diameter, 5 cm in height) with nylon mesh (sixty-four 1-mm diameter holes/cm^2) on their bottoms.[24,25] The flies were then provided access to a 10% sucrose solution spiked with PRRSV MN 30-100 at a dose of 2×10^4 $TCID_{50}$ in 5 mL of finished solution. The solution was poured onto a cotton gauze pad placed within a Petri dish, each plastic vial was set in an upright position on the gauze pad, and all houseflies were allowed to feed to repletion. In tandem with the red-eyed houseflies, 10 ochre-eyed houseflies were handled as described and allowed to feed on a 10% sucrose solution that was void of PRRSV. After cessation of feeding, both groups of flies were transferred into a trap where they commingled for 24 hours. Flies were then retrieved, separated by eye color, and tested for PRRSV. As positive controls, 2 more subsets (10 red-eyed houseflies and 10 ochre-eyed houseflies) were allowed to feed as described, but were not commingled in the traps prior to testing; these flies were held in vials after feeding for 24 hours. Negative control subsets of each group were tested as well.

To determine whether the attractant could interfere with selected PRRSV diagnostic tests, 10 mL of undiluted attractant and 10 mL of PRRSV MN 30-100 (dose, 2×10^4 $TCID_{50}$) were mixed together. Five 2-mL aliquots of the mixture were allowed to incubate at 20°C for 24 hours, another five 2-mL aliquots were incubated at 4°C for another 24 hours, and both were submitted for **polymerase chain reaction (PCR)** testing. Positive controls consisted of a 2-mL aliquot of undiluted PRRSV MN 30-100 and a 2-mL aliquot of PRRSV MN 30-100 mixed in equal volumes with **minimum essential medium (MEM)**. Negative controls consisted of two 2-mL volumes of insect attractant that was void of virus.

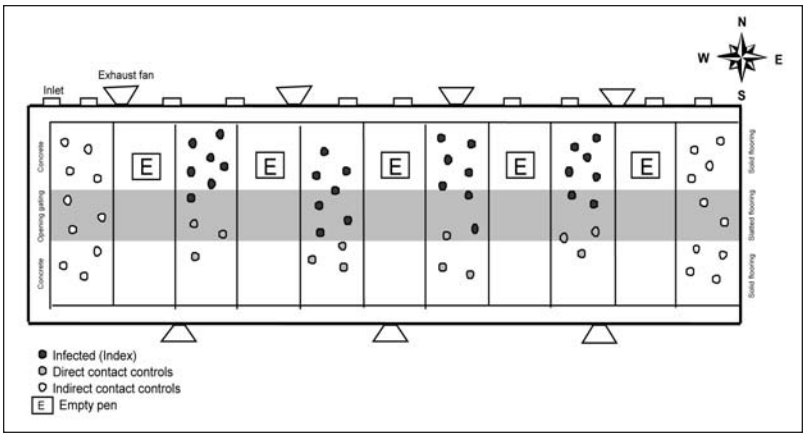

Figure 1—Schematic representation of a swine facility used to house experimentally infected and control pigs in a study of spatial dispersal of flies contaminated with porcine reproductive and respiratory syndrome virus (PRRSV).

Release-recapture study of dispersal—Thirty-eight baited jug traps were placed inside and around the outside of the study facility to capture houseflies and other species that were associated with the study facility and the sampling premises during

the course of the experimental PRRSV infection. On the day before pigs were to be infected (day 1 pi), 8 traps were placed at 2 points within the barn and at 6 points immediately surrounding the barn. An additional 30 traps were set up at more distant points on the next day (day 0). These traps were arranged radially in 8 directions at 0.4 km (8 traps), 0.8 km (4 traps), 1.3 km (4 traps), 1.7 km (4 traps), 1.9 km (6 traps), and 2.3 km (4 traps) from the center point of the barn. A global positioning system[c] was used to determine the center point of the swine facility, place the traps at the desired locations throughout the sampling region, and record their locations. A minimum of 5 satellites was used for all readings, and the readings were recorded as degrees, minutes, and seconds. The global positioning system was accurate to within 6 m. Coordinates recorded during the placement of traps were used to construct a map of the sampling premises **(Figure 2)** by use of computerized software.[d]

On the day of infection (day 0), approximately 100,000 pupae of ochre-eyed houseflies and 100,000 pupae of red-eyed houseflies were transported to the farm and placed in the empty pens of the swine facility. Adults began emerging the day after placement. When contact between newly hatched flies and infected pigs was first observed, the wall fans were turned off and the inlets opened to allow some of the flies to escape from the facility. This procedure was carried out for 2 hours between 8:00 AM and 10:00 AM during each day of the 15-day collection period. During this period, specific environmental parameters were recorded, including temperature, wind speed, and the presence or absence of rainfall and overcast skies.

The traps that had been placed throughout the 16.6 km^2 sampling premises were emptied of flies between 7:00 AM and 4:00 PM on days 0, 2, 7, 8, 10, and 14 pi. On each day, traps were processed from the most distant trap inward to reduce the risk of personnel spreading PRRSV outward from the facility. To empty each trap, a plastic funnel with a screen placed over the 2.5-cm orifice was placed upright in a plastic 4-L pail. A 15-cm hole had been cut in the cover of the pail to stabilize the funnel. Trap contents were poured from the trap into the 20-cm orifice of the funnel, allowing separation of the attractant liquid from the captured flies through the screened 2.5-cm orifice. Flies from each trap were placed in a plastic bag that was marked with the collection date and trap location. The bag was placed on ice during the collection period and stored at –20°C until testing was initiated. The trap was then refilled with fresh attractant. Between trappings, the collection equipment was washed with 10% bleach and rinsed with water, and study personnel changed disposable gloves between each trap. To verify the absence of trap-to-trap contamination, the interior of the funnel was swabbed with sterile polyethylene fiber-tipped plastic applicator swabs,[e] on each collection day after equipment had been sanitized. Swabs were then placed into sterile plastic tubes[f] that contained 2 mL of MEM.

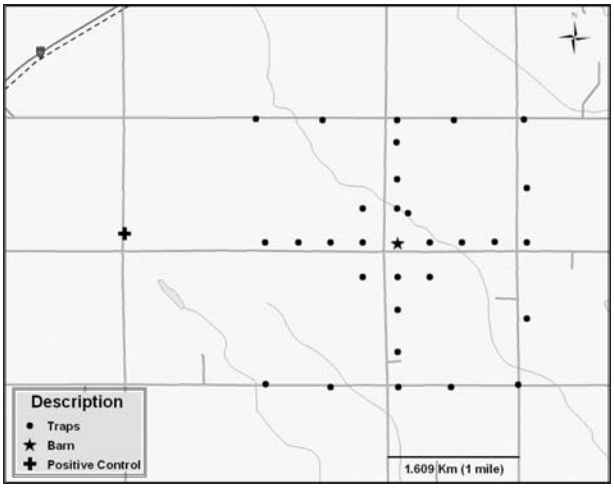

Figure 2—Illustration of a sampling area in a study of spatial dispersal of flies contaminated with PRRSV. Star indicates swine facility, solid circles indicate locations of fly traps, and cross indicates location of carbohydrate fly food source with modified-live PRRSV vaccine.

Controls—A PRRSV vaccine control was used to validate the trapping and testing methods under field conditions and verify transport of PRRSV by the resident (wild-type) fly population that were found in the area surrounding the farm. For the purpose of the vaccine control, an ad libitum carbohydrate source (10% sucrose and water solution) was spiked with 100 mL of a modified-live PRRSV vaccine.[g] The mixture was poured into a rubber basin and placed on the perimeter of the 16.6 m^2 sampling area, 3.3 km directly west of the swine facility (Figure 2). The solution was replenished every third day of the study. The vaccine was selected on the basis of an 11.3% divergence of the **open reading frame (ORF)** 5′ region of the PRRSV used to prepare the vaccine (VR-2332), compared with the same region in the index PRRSV used to inoculate the pigs. As a negative control, houseflies were collected from the 2 traps within the barn and the 6 traps immediately outside of the barn prior to the initiation of the experimental infection in the pig population.

Sample handling and analysis—Flies from each trap were sorted to count the number of ochre-eyed and red-eyed houseflies. Counts of the remaining calyptrate (*Muscidae* and *Calliphoridae*) species were estimated by measuring volume of flies collected. This involved counting randomly selected sets of flies, determining mean resulting counts per milliliter (1 mL contained 20 flies), and multiplying this figure by the total volume of captured flies in each trap to estimate the total number of flies. Flies of all species from each trap were then recombined into 1 pooled sample, and the sample was processed for PCR testing. To process each individual pooled sample, a 10.2 × 10.2-cm sterile gauze sponge[h] was placed over the top of a disposable, foam beverage cup.[i] Personnel used vinyl examination gloves,[j] and pooled flies were poured onto the suspended gauze sponge and rinsed with 10 mL of MEM. The sponge containing the pooled sample was removed from the cup and repeatedly compressed manually, filtering the exudate through the gauze sponge and into the cup, until seepage of exudate was not observed. Filtrates were stored at –20°C until testing was initiated. Personnel changed gloves and used a new sponge and cup for every sample.

All samples from pigs, pooled fly samples, and collection-instrument swab specimens were tested for PRRSV RNA by use of a PCR assay.[30] The ORF 5′ region of PCR-positive samples from pigs and pooled fly samples was sequenced to determine nucleic acids.[31]

Bioassays—Swine bioassays were conducted to assess whether infectious PRRSV was in selected PCR-positive pooled fly samples.[32] After nucleic acid sequencing of pooled samples, filtrates containing PRRSV RNA homologous to the index virus and the vaccine virus collected at different distances from the facility were injected via the IM route into PRRSV-naïve pigs housed at the research farm. A positive swine bioassay result was based on positive results of PCR and ELISA tests in each pig; RNA from sera from bioassay pigs with positive results of the PCR assay was evaluated via nucleic acid sequencing. Each pig was housed in an individ-

ual room, and study personnel changed boots, coveralls, gloves, and hairnets between rooms. A negative control pig was inoculated with a pool of fly samples collected on day 0 pi. Blood was collected from all pigs on days 7 and 14 pi, and sera were tested for PRRSV RNA by use of PCR assay and for PRRSV-antibodies by use of an ELISA.

Statistical analyses—Rates of contamination with PRRSV RNA were calculated from pooled samples of flies of all species combined from individual traps, grouped by date of collection, and whether they were < or > 1.2 km from the study facility. Rates were calculated with both the **minimum infection rate (MIR)** and **maximum likelihood estimation (MLE)** methods for pooled samples of unequal sizes.[33] The MIR method assumed only 1 fly was contaminated in each positive pool and was calculated as the number of PCR-positive pooled samples divided by the number of specimens in the positive and negative pooled samples combined. The MLE was to find a value of , the estimated proportion of specimens that had positive results that balanced the following equation:

$$N = \sum_{m} m x_m \left(1-[1-P]^n\right)$$

where N was the total number of flies in all pooled samples, m was the number of flies in a pool, and x_m was the number of pooled samples of size m that had positive results. Contamination rates determined by use of both methods were expressed per 10,000 flies. The more widely used MIR method is known to underestimate true contamination rates, whereas MLE is unbiased. Similarly, rates of contamination with infectious PRRSV, as determined by swine bioassay, were estimated from 6 pooled fly samples containing the index strain of virus and 3 pooled samples containing the vaccine control strain.

Results

Preliminary study—Results from the preliminary laboratory study indicated cross-contamination of PRRSV was likely to occur between flies commingled in baited jug traps. After the 24-hour contact period in the trap, the 10 red-eyed houseflies (PRRSV-exposed) and the 10 ochre-eyed houseflies (nonexposed) yielded positive results for PRRSV RNA by use of PCR assay. When the 2 groups were not commingled, cross-contamination was not observed and both groups of negative control flies yielded PRRSV PCR negative results.

The insect attractant did not appear to interfere with the PCR reaction. All 5 aliquots of the PRRSV-attractant mixture incubated at 20°C, and all 5 aliquots incubated at 4°C yielded PCR-positive results. Positive controls (undiluted PRRSV) yielded PCR-positive results, and negative controls (MEM) yielded negative results.

Verification of experimental PRRSV infection of swine and contact with flies—All swine tested yielded PCR-negative results upon arrival at the farm. On day 7 pi, 12 of 12 index pigs from the monitor group, 5 of 12 pigs from the direct-contact group, and 1 of 20 pigs from the indirect-contact group yielded positive results from serum tested via PCR assay. On day 14 pi, 12 of 12 index pigs, 12 of 12 direct contacts, and 13 of 20 indirect contacts yielded positive results from serum tested via PCR assay. Clinical signs observed in infected pigs consisted of fever (40° to 41°C), anorexia, signs of depression, periocular edema, and nasal discharge.[34] Nucleic acid sequencing of 10 randomly selected PRRSV isolates recovered from swine sera collected throughout the 14-day monitoring period indicated 100% homology with the index virus.

Throughout the study period, extensive contact between flies and pigs was observed. Flies were seen feeding upon nasal, lachrymal, and salivary secretions of the infected pigs.[35] Furthermore, flies were also observed to be consuming blood from skin abrasions, secondary to trauma caused by fighting.

Environmental parameters recorded during the study period—Throughout the 15-day study period, the maximum daily environmental temperature ranged from 22° to 33°C (mean, 27.5°C), whereas the mini-

Table 1—Trapping rates (No. of flies per day) of ochre-eyed and red-eyed houseflies collected in baited jug traps located inside, outside, and at various distances from a swine facility during a study of fly-associated dispersal of porcine reproductive and respiratory syndrome virus (PRRSV).

	Trap location (No. of traps)							
Trapping interval (d*)	**Inside (2)**	**Outside (6)**	**0.4 km (8)**	**0.8 km (4)**	**1.3 km (4)**	**1.7 km (4)**	**1.9 km (6)**	**2.3 km (4)**
Ochre-eyed flies								
–1–0	0.0	0.0	—	—	—	—	—	—
> 0–2	11.0	14.7	0.0	7.0	0.0	0.0	0.0	0.0
> 2–7	7.3	18.3	1.2	1.9	1.2	0.0	0.0	0.0
> 7–8	13.0	18.1	3.8	0.0	10.5	0.0	0.0	0.0
> 8–10	13.8	50.0	0.8	0.1	0.0	0.0	0.1	0.0
> 10–14	1.1	1.3	0.0	0.0	0.0	0.0	0.0	0.0
Mean	**7.7**	**17.1**	**1.0**	**1.5**	**2.0**	**0.0**	**0.0**	**0.0**
Red-eyed flies								
–1–0	1.0	3.5	—	—	—	—	—	—
> 0–2	24.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
> 2–7	11.0	22.2	1.0	2.9	0.4	0.0	0.2	0.4
> 7–8	34.5	34.2	0.0	0.0	5.3	0.0	0.5	0.0
> 8–10	80.0	41.0	0.4	0.1	0.4	1.0	0.3	0.0
> 10–14	15.5	3.0	0.3	0.0	0.0	0.1	0.3	0.5
Mean	**27.7**	**17.3**	**0.3**	**0.5**	**1.2**	**0.2**	**0.2**	**0.2**

*Trapping interval in days in relation to inoculation of pigs with PRRSV on day 0. — = Traps not initially set at these locations.

mum daily temperature ranged from 8º to 19ºC (mean, 14.5ºC). Wind speed ranged from 16.7 to 57.4 km/h (mean, 37.9 km/h). Rainfall and overcast skies were observed during 7 of the 15 days. On the 6 days in which collection of insects occurred, the maximum daily environmental temperature ranged from 22º to 31º C (mean, 26ºC) and mean daily minimum temperature ranged from 11º to 19ºC (mean, 15ºC). Wind speed during these 6 days ranged from 27.8 to 50 km/h (mean, 39.8 km/h), and rainfall and overcast skies were observed on 3 of the 6 days.

Release-recapture study of dispersal—Overall, 4,210 houseflies were obtained from the 38 traps during the 15 days (day 0 to 14 pi) of the study. Among them were 1,802 ochre-eyed houseflies and 2,408 red-eyed wild type houseflies. Besides houseflies, 135,600 additional calyptrate flies were obtained; therefore, 139,810 flies of all species were trapped during the study period. Calyptrate flies consisted of blow flies (at least 4 species of *Calliphoridae)* and muscid flies (black garbage fly [*Hydrotaea ignava*] and stable fly [*Stomoxys calcitrans*]), in descending order of abundance.

Counts of ochre-eyed and red-eyed houseflies were converted to daily catch rates to standardize for uneven numbers of days among trapping intervals and different numbers of traps at the different distances from the barn (**Table 1**). At the beginning of the study, no ochre-eyed houseflies were captured anywhere, confirming their initial absence at the site. Thereafter, catch rates increased during days 0 to 10 pi and then declined during days 10 to 14 pi. Catch rates also varied with distance from the barn.[18] During each trapping interval, catch rates were consistently greatest with traps inside or immediately outside the barn. Furthermore, catch rates diminished with distance from the barn, with many flies caught at 1.3 km on days 7 and 8 pi, and 1 ochre-eyed housefly recovered at 1.9 km on day 8 to 10 pi. This pattern of abundance with increasing time and distance after release was consistent with diffusion outward from the barn on day 0 pi. A similar pattern in catch rates of red-eyed houseflies was also observed, although a few were initially present during days –1 to 0 and 0 to 2 pi, and more specimens were obtained beyond 1.3 km, compared with ochre-eyed flies.

Diagnostic analysis of insect samples—All control swab specimens taken from collection instruments during the 15-day study period yielded negative results for PRRSV RNA by PCR assay, indicating the procedures were not transferring the virus among traps. In addition, 8 pooled samples of flies obtained from the 2 traps inside the barn and the 6 traps immediately outside (total, 968 flies) on days –1 and 0 pi also had negative results. Thus, PRRSV was most likely absent from the premise and the resident fly population before experimental infection.

During days 0 to 14 pi, 66 (36%) of 182 pooled fly samples obtained from all locations yielded positive results for PRRSV RNA by use of the PCR assay (**Table 2**). The first positive result was obtained 0 to 2 days pi from 1 trap located 0.8 km south of the facility, which contained 28 red-eyed houseflies and 1,200 other species of flies. Thereafter, as demonstrated by the MIR and MLE calculations, the number of pooled samples with positive results increased substantially and was consistently greater among pooled samples from traps < 1.2 km than from farther locations. Species composition of the pooled samples indicated that flies of all species may have harbored PRRSV RNA. Fourteen of the 66 pooled samples with positive results contained only calliphorid flies and muscid flies, but lacked houseflies; the remaining 52 pooled samples contained mixtures of all species.

Estimated rates of contamination per 10,000 flies were initially low among flies trapped on days 0 to 2 pi. Thereafter, rates increased during all subsequent

Table 2—Rates of contamination of houseflies and other fly species with PRRSV RNA at various distances from a facility housing PRRSV-inoculated pigs.

Trapping interval (d*)	Distance (km)†	No. flies	Pooled fly samples	No. pooled samples with positive results	Contamination rate			
					MIR	SE	MLE	SE
–1–0	< 1.2	968	8	0	0.0	NA	NA	NA
	> 1.2	—	—	—	—	—	—	—
>0–2	< 1.2	13,153	20	1	0.8	0.8	0.8	0.8
	> 1.2	7,542	12	0	0.0	—	—	—
>2–7	< 1.2	20,217	20	17	8.4	2.3	67.7	26.4
	> 1.2	39,044	18	4	1.0	3.4	1.2	0.7
>7–8	< 1.2	4,544	19	13	28.6	24.3	215.8	106.6
	> 1.2	5,881	18	2	3.4	2.7	3.5	2.9
>8–10	< 1.2	11,743	20	12	10.2	33.4	26.9	10.1
	> 1.2	12,621	18	3	2.4	2.6	6.3	4.3
>10–14	< 1.2	6,430	19	9	14.0	8.8	30.2	11.2
	> 1.2	18,635	18	5	2.7	2.2	4.1	1.9
0–14	< 1.2	56,087	98	52	9.3	1.7	18.8	3.2
	> 1.2	83,723	84	14	1.7	1.3	2.2	0.7
Total		**139,810**	**182**	**66**	**4.7**	**0.8**	**7.2**	**1.1**

*Trapping interval in days in relation to inoculation of pigs with PRRSV on day 0. †20 fly traps < 1.2 km from barn, 18 fly traps > 1.2 km from barn. Pooled samples = Flies of all species combined in each trap.

MIR = Minimum infection rate (No. pooled samples with positive results/No. of flies)/10,000 flies. MLE = Maximum likelihood estimate.

NA = Not applicable.

— = No traps set. *See* Table 1 for remainder of key.

intervals and in all locations, but were greatest among flies in traps nearest the barn at days 7 to 8 pi.[18] Rates determined by use of the MIR method were numerically smaller than matching MLE estimates; however, the MIR method assumes that only 1 specimen in each pool actually yielded positive results, which is known to underestimate true infection rate.[33]

The ORF 5′ regions of 41 of the 66 pooled fly samples that yielded positive results of PCR assay were evaluated via nucleic acid sequencing. Six of the pooled samples originated from traps within or surrounding the barn or both, whereas 35 were obtained from the outlying traps. All 6 samples from within and surrounding the barn were 99.7 to 100% similar to the index virus used to inoculate the pigs, as were 25 of the remaining 35 pooled samples. Nine of 25 were located 0.4 km from the barn, 8 at 0.8 km, 5 at 1.3 km, 0 at 1.7 km, 2 at 1.9 km, and 1 at 2.3 km from the barn (**Figure 3**).

In contrast, 8 of the 35 more distant pooled samples with positive results of PCR assay contained PRRSV RNA that was 99.7% to 100% similar to the vaccine virus. These samples were recovered from traps ranging from 2.3 to 3.7 km from the source of the vaccine virus (**Figure 4**). The final 2 pooled samples (**Figure 5**) contained PRRSV RNA that was genetically distinct from both the index and vaccine viruses. For convenience, these variants were designated as PRRSV-A and PRRSV-B. The PRRSV-A was recovered on day 2 from a trap located 0.8 km directly west of the swine facility. The ORF 5′ region of PRRSV-A was found to have 9.7% divergence from the index virus and 12.3% divergence from the vaccine virus. The second of these variants (PRRSV-B) was recovered on day 8 in a trap located 0.8 km to the south of the facility. Its ORF 5′ region was determined to have 7.9% and 12.1% divergence, compared with the index and vaccine viruses, respectively. When PRRSV-A and -B were compared, their ORF 5′ regions differed by 11.3%.

Swine bioassay—Swine bioassay was used to detect the presence of infectious PRRSV in filtrates.[32] Bioassay samples were stratified on the basis of PRRSV strain identified in the filtrate by PCR assay and trap location. Twelve pigs were used in the bioassay. One of the 12 pigs was inoculated with 1 pooled sample consisting of 1 mL from each of the 9 filtrates that yielded positive results of PCR assay for the index virus and were obtained from a site located within 0.4 km of the facility. Another pig was inoculated with 1 pooled sample consisting of 1 mL from each of the 8 filtrates that yielded PCR assay positive results for the index virus and were obtained from sites located within 0.8 km of the facility. Another pig was inoculated with a pooled sample consisting of 1 mL from each of the 5 filtrates that yielded PCR-assay positive results for the index virus and were obtained from sites that were located

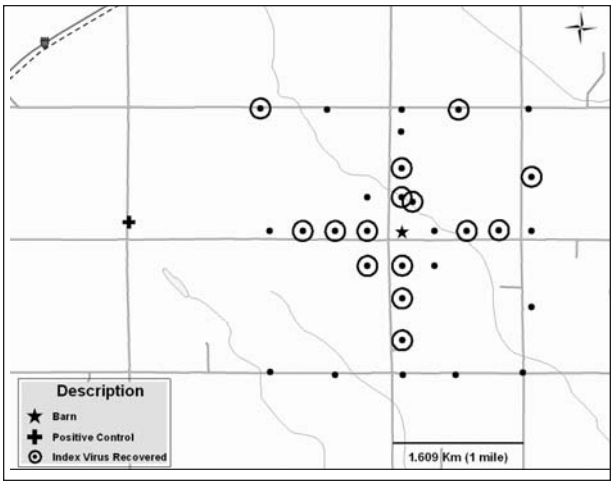

Figure 3—Illustration of a sampling area in a study of spatial dispersal of flies contaminated with PRRSV. Star indicates swine facility, solid circles indicate locations of fly traps, solid circles with surrounding open circles indicate traps in which index PRRSV was recovered, and cross indicates locating of carbohydrate fly food source with modified-live PRRSV vaccine.

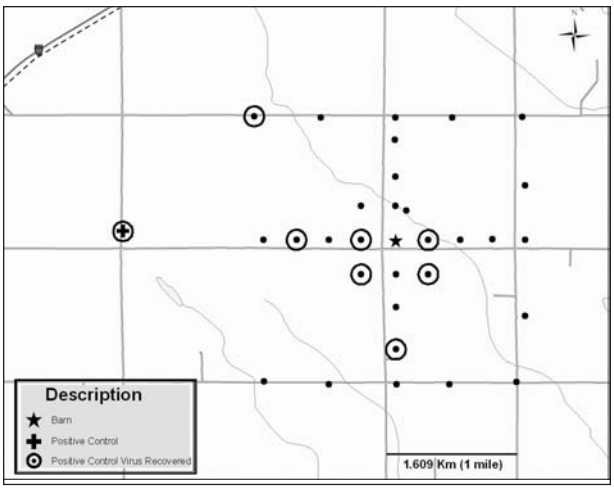

Figure 4—Illustration of sampling area in a study of spatial dispersal of flies contaminated with PRRSV. Star indicates swine facility, solid circles indicate locations of fly traps, solid circles with surrounding open circles indicate traps in which modified-live PRRSV vaccine was recovered, and cross indicates locating of carbohydrate fly food source with modified-live PRRSV vaccine.

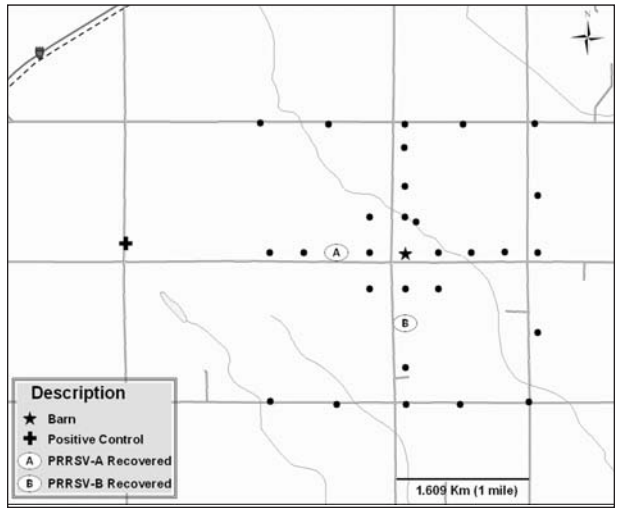

Figure 5—Illustration of a sampling area in a study of spatial dispersal of flies contaminated with PRRSV. Star indicates swine facility, solid circles indicate locations of fly traps, and cross indicates locating of carbohydrate fly food source with modified-live PRRSV vaccine. A = Trap in which PRRSV-A was detected. B = Trap in which PRRSV-B was detected.

Table 3—Results of a swine bioassay for infective PRRSV from selected fly samples.

PRRSV	Distance from source (km)	No. traps	No. flies	Result
Index	0.4	9	371	Pos
Index	0.8	8	130	Pos
Index	1.3	5	299	Pos
Index	1.9	1	452	Pos
Index	1.9	1	22	Neg
Index	2.3	1	312	Pos
Vaccine	2.3	1	151	Pos
Vaccine	3.7	1	13	Neg
Vaccine	3.7	1	14	Neg
PRRSV-A	0.8	1	88	Pos
PRRSV-B	0.8	1	2	Neg

Index = PRRSV MN-30100 used to inoculate pigs. Vaccine = Vaccine virus in carbohydrate basin located 3.3 km from swine facility for feeding flies. Distance from source = Location of sample in relation to swine facility or vaccine virus source. Source of reference for PRRSV-A and -B was the swine barn. No. traps = Number of traps pooled to produce tested sample. No. flies = Number of flies contained in the sample used to inoculate bioassay pigs. Pos = Bioassay pig inoculated with sample became infected with PRRSV (positive results of polymerase chain reaction [PCR] assay and ELISA). Neg = Bioassay pig inoculated with sample did not become infected.

within 1.3 km of the facility. Six pigs were individually inoculated with either 1 of the 3 filtrates that yielded PCR-assay positive results for the index virus or 1 of the 3 filtrates that yielded PCR-assay positive results for the vaccine virus and were obtained from sites located > 1.7 km from the facility. Two pigs were inoculated either with filtrate containing PRRSV-A or PRRSV-B. A twelfth pig (negative control) was inoculated with a pool of samples collected on day 0 prior to inoculation of the pigs.

Bioassay results were tabulated (**Table 3**). Infectious PRRSV that was 99.7% to 100% similar to the index virus was detected in all 3 bioassay pigs inoculated with pooled samples recovered from traps located 0.4 km, 0.8 km, and 1.3 km from the facility. The MIR for flies contaminated with the index virus among these flies was 37 of 10,000 flies. An infectious PRRSV variant with 100% identity to the index virus was also detected in 1 sample collected at 2.3 km and 1 sample collected at 1.9 km from the facility. The third sample, collected at 2.3 km, yielded negative results. The MIR for the index virus in these more distant pooled samples was estimated to be 25 of 10,000 flies, somewhat lower than among flies closer to the barn. Only 1 of 3 samples that contained the vaccine virus yielded positive results for the bioassay. This sample was collected 2.3 km from the carbohydrate basin. The other 2 samples, collected 3.8 km from the facility, yielded negative bioassay results. The rate of contamination by the vaccine virus was estimated to be 56 of 10,000 flies. Infectious PRRSV was detected in the sample that contained PRRSV-A, but not in the sample that contained PRRSV-B. All PRRSV isolates recovered from bioassay pigs were sequenced and found to have 100% homology with the PRRSV in the original bioassay sample inoculated into the naïve pigs. The pig inoculated with the negative control insect pool yielded negative results throughout the testing period.

Discussion

The objective of this study was to assess whether flies could acquire PRRSV via contact with experimentally infected pigs and transport it into the surrounding environment. This was the first experimental attempt to evaluate dispersal of PRRSV by a known nonporcine vector under field conditions. It was hoped that if the dispersion of PRRSV by flies throughout an agricultural area could be proven, some insight into a potential mechanism of transport of the virus could be gained. Under the conditions of this study, it appeared that houseflies and other fly species were able to harbor PRRSV after contact with experimentally infected pigs and transport it at least 2.3 km into the surrounding environment.

These results must be interpreted with caution because the role of insects in the transmission of swine pathogens is unclear at this time. Although a small number of swine pathogens, such as pseudorabies virus and hog cholera virus, might be transmitted by houseflies,[36-38] other agents (rotavirus, transmissible gastroenteritis virus, and *S suis*) have only been detected on the interior or exterior surfaces of the flies. Therefore, no attempt to evaluate vector competency has been made.[20-23] This also is an acknowledged limitation of our study because, although the results suggest that flies can acquire PRRSV and disperse it into the surrounding environment, transmission of the virus to susceptible pigs outside of the facility by flies was not proven, possibly because of the lack of a susceptible pig population within the sampling area. However, transmission of PRRSV by houseflies from infected to susceptible pigs has been demonstrated under laboratory conditions.[24] Also, because only a single replication of the study was conducted and a limited sampling area was used, it is not known whether the observed dispersion would have been replicated or whether transport of PRRSV by flies could have occurred over even greater distances. Furthermore, because of budgetary constraints, it was not possible to quantify the amount of PRRSV RNA in pooled fly samples. However, because catch rates of ochre-eyed and red-eyed houseflies and pool contamination rates all decreased with time and increasing distance from the barn, the risk of PRRSV transport by flies may decrease as distance between farms increases.[18]

Another limitation was that because of cross-contamination of virus between the different species of flies within the traps, it was not possible to determine which species of flies actually harbored the virus. Because PRRSV RNA was detected in traps containing both types of houseflies, as well as in traps that contained just other fly species, it appears that transmission of PRRSV by flies may be attributable to general mechanical transmission and is not species-specific.

An interesting and unexpected observation was recovery of the 2 unrelated variants of PRRSV in the sampling area that were designated PRRSV-A and PRRSV-B. To ensure that laboratory error was not an issue, each variant was sequenced twice; it was noted that the laboratory equipment used to process and sequence those samples had not been exposed to similar viruses on the days that these specific samples were

analyzed. The origin of the 2 novel variants is not known. According to the Veterinary Diagnostic Laboratory database of more than 2,000 PRRSV isolates sequenced from Minnesota farms, isolates with 99.7% homology to PRRSV-A have been recovered from a number of farms in the west-central region of the state. These farms are located approximately 40 to 50 km to the north-northwest of the research site. In contrast, the only recorded sequences with a similar ORF 5′ sequence (97% to 98% identity) to PRRSV-B were from farms located more than 200 km from the site. Nevertheless, these sites may not have been the direct source of either virus. Vehicles may have transported flies into the sampling area, or there may have been unidentified sites in which small numbers of swine were raised in the sampling area. Furthermore, not every PRRSV isolate submitted to the laboratory is sequenced; therefore, there could be other farms in the state with unreported viruses that have close homology. Finally, whether other PRRSV variants were present in or around the facility or throughout the sampling area was not known because only a subset of the samples that yielded positive results of the PCR assay was sequenced because of budgetary constraints.

The presence of infectious PRRSV in pooled fly samples as detected by use of the swine bioassay was also interesting; this should not be overinterpreted because it was not possible to detect viable PRRSV in a single fly. However, the type of traps used and the schedule of sample collection may have impacted the results. Collection of insects did not occur on a daily basis, resulting in the storage of samples in transparent, plastic jug traps for extended periods in conditions known to be detrimental to viability of PRRSV, such as high temperatures (22 to 33ºC) and UV light.[39] Therefore, had samples been collected more frequently or stored in opaque containers between collection days, a greater percentage of positive-PCR assay results and positive bioassay results may have been recorded. In contrast, these factors may have had lesser impact on the viability of PRRSV within the body of the fly. Infectious PRRSV has been recovered for up to 12 hours from the intestinal tract of houseflies incubated at 28ºC, and the presence of the virus within the body of the fly would protect it from the detrimental effects of UV light from the sun.[25]

The environmental conditions observed during the study period should not have negatively impacted fly behavior. Muscid flies are known to rest in sunny sites when the environmental temperature is < 20ºC and in shady sites when the temperature exceeds 30ºC.[40] During the collection period, the daytime temperature exceeded 30ºC on only 3 of the 15 study days (days 5 to 7) and daytime temperatures did not decrease below 20ºC at any time; therefore, we speculated that normal fly behavior occurred. However, 1 factor that may have influenced the overall population of flies available during the study period was the initiation of the study in early June because the breeding season of muscid flies peaks during the warmer wetter months of the late summer and fall.[40] With this in mind, attempts were made to supplement the housefly population through the addition of the 100,000 pupae each of the ochre-eyed and red-eyed houseflies.

Following release of laboratory cultivated flies, it was observed that the temporal and geographic distribution of red-eyed flies was consistent with spread from the barn. However, the larger numbers of red-eyed flies captured early after release suggested that the laboratory-cultivated red-eyed houseflies had been augmented by a low-level resident population of unknown origin. Catch rates of blow flies and other species of muscid flies indicated that throughout the study, those species were distributed more uniformly, compared with ochre-eyed or red-eyed houseflies, including inside the barn, elsewhere on the study premise, and throughout the surrounding sampling region. Black garbage flies could have originated inside the study barn, but the remaining species were likely to have come from outside sources. The presence of all of the flies inside the study facility meant that in addition to the released houseflies, the other species could also have become contaminated with PRRSV through contact with the infected pigs.

Despite the acknowledged limitations, an important strength of this field study was its use of controls. Use of the vaccine virus verified that the trapping methods were effective, and the environmental factors encountered under field conditions did not adversely affect the PCR test. The study also detected the ability of the resident fly population to transport a variant PRRSV 2.3 to 3.7 km from its point source. The negative controls (flies collected on day 0) verified that other variants of PRRSV were either not present or not detectable prior to the introduction of PRRSV MN-30-100 to the pig population, and the swab specimen controls from the collection instruments verified that accidental contamination between traps did not occur. However, 1 control that was lacking in the design was a negative-control pig population and collection of insects from that population throughout the entire study period.

Results of the study indicated clearly that flies are able to transport PRRSV after contact with an infected swine population for extended distances into the surrounding environment and can also harbor variants of PRRSV that originate from unknown sources. These results provide insight into how farms may become infected with PRRSV during periods of warm weather. Transport of PRRSV by houseflies and other species should be evaluated further under commercial farm conditions to better understand the epidemiologic importance of fly-borne spread in relation to other routes of spread. If these observations prove to be valid, swine producers and veterinarians may need to improve biosecurity protocols to reduce the risk of fly-borne spread of PRRSV, perhaps by improving fly control in and around swine barns and by preventing movement of flies among source and recipient premises.

[a] #5012, PMI International, St Louis, Mo.
[b] Terminator, Farnam Companies Inc, Phoenix, Ariz.
[c] Garmin Corp, Olathe, Kan.
[d] ArcMap, version 8.2, ESRI, Redlands, Calif.
[e] Fisher Scientific, Hanover Park, Ill.
[f] Falcon, Franklin Lakes, NJ.
[g] Ingel Vac PRRS MLV, Boehringer Ingelheim Vetmedica, St Joseph, Mo.
[h] Johnson & Johnson Industries, Skillman, NJ.
[i] Dart Container Corp, Mason Mich.
[j] Medline Industries, Mundelein, Ill.

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