Survival of porcine reproductive and respiratory syndrome virus in houseflies

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Abstract

The objectives of the study were to determine the duration of porcine reproductive and respiratory syndrome virus (PRRSV) survival in houseflies (*Musca domestica* Linnaeus) following feeding on an infected pig, and to determine whether the virus was present on the exterior surface or within the internal viscera of the fly. A total of 210 laboratory-colonized houseflies were allowed to feed to repletion on a pig, experimentally infected with PRRSV on day 7 postinoculation, and then maintained alive under laboratory conditions (27°C). Two subsets (A and B) of 30 flies were collected at each of the following sampling points; 0, 6, and 12 hours post feeding (pf). Subset A contained an extra group of 30 flies collected at 24 hours pf due to the availability of extra flies. Flies in subset A were processed as whole fly homogenates, while the exterior surface washes and digestive organs were collected from flies in subset B. Whole fly homogenates, collected at 0, 6, and 12 hours pf, were positive by both polymerase chain reaction (PCR) and swine bioassay. Digestive organs, collected at 0 and 12 hours pf, were positive by PCR and swine bioassay. The PRRSV RNA was detected by PCR from the exterior surface wash of subset B flies collected at 0, 6, and 12 hours pf; however, only the subset collected at 0 hour pf was swine bioassay-positive. This study indicates that infectious PRRSV can survive within the intestinal tract of houseflies for up to 12 hours following feeding on an infected pig, but only for a short period on the exterior surface of the flies.

Résumé

*Cette étude avait comme objectifs de déterminer la survie du virus responsable du syndrome respiratoire et reproducteur porcin (PRRSV) chez la mouche domestique (*Musca domestica *Linnaeus) après contact avec un porc infecté, et de déterminer si le virus était présent sur la surface extérieure des mouches ou dans les viscères internes. Un total de 210 mouches domestiques élevées en laboratoire se sont nourries en étant mises en contact avec un porc, infecté expérimentalement avec le PRRSV au jour 7 post-inoculation, et ont été maintenues par la suite en vie dans des conditions de laboratoire (27°C). Deux sous-échantillonnages (A et B) de 30 mouches ont été effectués à chacun des temps suivants : 0, 6 et 12 h post-repas (pf). Le sous-échantillonnage A comportait un groupe supplémentaire de 30 mouches amassées 24 h pf. Les mouches du sous-échantillon A ont été traitées comme un homogénat de mouche entière, alors que les mouches du sous-échantillon B ont été traitées pour obtenir un lavage de la surface externe ainsi que les organes digestifs. Les homogénats de mouches entières prélevés aux temps 0, 6 et 12 h pf se sont avérés positifs par épreuve d'amplification en chaîne par la polymérase (PCR) ainsi que par une épreuve de bio-essai chez le porc. L'ARN du PRRSV a été détecté par PCR à partir du liquide de lavage de la surface externe des mouches aux temps 0, 6 et 12 h pf. Toutefois, seul le sous-échantillon prélevé au temps 0 h pf était positif dans l'épreuve de bio-essai chez le porc. Cette étude a permis de démontrer que le virus responsable du PRRS peut survivre dans le tractus intestinal des mouches domestiques jusqu'à 12 h suivant un repas sur un porc infecté, mais seulement pour une courte période sur la surface extérieure des mouches.*

(Traduit par Dr Serge Messier)

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically significant pathogen in the swine industry (1). Known possible routes of transmission of PRRSV are infected pigs, semen, needles, fomites (coveralls and boots), and mosquitoes (*Aedes vexans* Meigen) (2–6). In order to reduce the risk of PRRSV entry into naïve swine populations, swine producers utilize stringent measures to enhance the biosecurity of their farms; however, infection of naïve herds frequently occurs through unidentified routes. In contrast, aerosol transmission of PRRSV is still under debate at this point. Some studies have shown that PRRSV could be

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transmitted by aerosol only in short distances (within 1 m) under experimental conditions (7–9); however, it has been documented that airborne transmission of PRRSV over long distances was an infrequent event under field conditions (10,11).

A potential route of PRRSV that has not been well explored is through the housefly (*Musca domestica* Linnaeus). Although it is not certain at this point if houseflies can actually transmit swine pathogens between commercial pig farms, several studies have shown that they can serve as mechanical vectors of several swine pathogens under experimental conditions, including hog cholera virus, transmissible gastroenteritis virus, *Streptococcus suis*, and pseudorabies virus (12–15). Current biosecurity methods, such as shower-in and shower-out, personnel downtime (no swine contact for 48 to 72 h prior to entry to the farm), quarantine and testing of incoming stock, and bird proofing of facilities do not prevent the entry of insects into swine herds. Recently, we have documented mechanical transmission of PRRSV by houseflies under experimental conditions, and that PRRSV can survive in housefly populations for up to 6 h following feeding on an infected pig (16). However, limitations of the survival phase of that study were that only a single replicate was conducted, the duration of virus viability was not conclusively determined, and whether infectious PRRSV was located within the internal viscera or on the exterior surface of the flies was not proven. This information was considered to be important for assessing the potential significance of houseflies as vectors of PRRSV under field conditions. Therefore, the objectives of this study were to reconfirm the duration of PRRSV survivability in houseflies, as well as to determine whether infectious PRRSV was present within the internal viscera or on the exterior surface of houseflies following feeding on a PRRSV-viremic pig.

Materials and methods

Infection model

A total of 2, 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. The pigs were each housed in separate rooms 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 between rooms. Upon arrival to the isolation facility, both animals were blood-tested to insure a PRRSV-naïve status. During the study period, both pigs were cared for under the guidelines of University of Minnesota Institutional Animal Care policies. To initiate the study, 1 pig was intranasally inoculated with 5 mL of a PRRSV field isolate (MN-30100) (17) at a concentration of 10^4 $TCID_{50}$/mL, while the remaining pig served as a negative control.

Source of houseflies

A laboratory colony of houseflies (*Musca domestica* Linnaeus) was established at the Department of Entomology, University of Minnesota. Three- to 4-day-old adult flies were placed into incubation cages (27°C) and starved for 24 h prior to contact with pigs.

Insect contact protocols

Fly-to-pig contact took place on day 7 postinoculation (pi) of the experimentally infected pig. 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 postinfection (4–6,16,18). To allow flies to feed on the infected pig, a manual vector transmission protocol was applied. This method had been previously used to study transmission of bovine leukosis virus from infected to susceptible cattle by stable flies (*Stomoxys calcitrans*), transmission of PRRSV from infected to naïve pigs by mosquitoes (*Aedes vexans*), and houseflies (6,16,19). The pig was consciously restrained in a Panepinto sling (Asset Inventory project, University of Minnesota, Minnesota, USA), and transparent plastic screw-cap vials (3 cm diameter, 5 cm height) containing an individual fly were adhered with masking tape (Highland 2600 tape; 3M, St. Paul, Minnesota, USA) over 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 fly to access to the pig skin. To enhance the ability of flies to access blood, a slight hemorrhage was visible on the dorsal surface of each pig. The pig was scarified once on 3 spots and each scarification spot was approximately 1.5 cm^2 in size. These scarified lesions were observed during 14-day studying period to insure the absence of chronic or secondary bacterial infection of the skin.

On day 7 pi, a total of 210 flies were allowed to feed to repletion on the experimentally infected pig. For each individual fly, contact of the mouthparts to the scarified regions of the pig skin, ingestion of blood, and abdominal reddening were observed. Following completion of feeding (3 to 5 min), all flies remained in their individual transfer vials in order to prevent PRRSV contamination secondary to fly-to-fly contact post-feeding (pf), and were incubated at a temperature of 27°C 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. Two subsets (subset A and B) of 30 blood-fed flies (total 60 flies per sampling period) were randomly collected at each of the following times: 0, 6, and 12 h pf). The subset collected at 0 h pf was placed on dry ice immediately following feeding on an infected pig. Flies in subset A were designated as whole fly homogenates. Due to the availability of 30 extra flies, subset A contained an extra group of 30 flies collected at 24 h pf. To construct whole fly homogenates, the 30 flies of the subset A were pooled at each of the 4 sampling times into sterile tubes (Falcon, Franklin Lakes, New Jersey, USA) containing 2 mL of minimum essential medium (MEM). Pooled insects were compressed against the tube wall using sterile swabs (Dacron fiber tipped plastic applicator swabs; Fisher Science Laboratory, Hanover Park, Illinois, USA), centrifuged at 4200 × *g* for 5 min, and the supernatant tested for PRRSV.

Flies in subset B were used to determine whether infectious PRRSV could be detected within the intestinal viscera or on the exterior surface of the house fly following feeding on an infected pig. For assessment of PRRSV on the exterior surface of flies, 30 flies were collected and pooled at each of the 3 sampling times into sterile tubes

containing 2 mL of MEM. Pooled insects were washed with the MEM by vortexing (Vortex-Genie Mixer; Scientific Industries, Bohemia, New York, USA) at 8000 × *g* for 10 s at room temperature (27°C). The washing fluid from each of the sampling times were transferred into separate sterile tubes and tested for the presence of PRRSV on the exterior surface of the flies. These samples were designated as exterior surface wash. For assessment of PRRSV within the internal viscera of flies, the washed flies were then disinfected with 70% ethanol, rinsed twice with MEM for 5 s, and dissected. Digestive organs including diverticulum, midgut, and hindgut were removed from each fly and pooled at each of the 3 sampling times into sterile tubes containing 2 mL of MEM. Pooled intestinal viscera were compressed against the tube wall using sterile swabs, centrifuged at 4200 × *g* for 5 min, and the supernatant tested for PRRSV. These samples were designated as gut homogenates.

Diagnostic analysis

All samples from pigs and insects 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) (20). In order for samples to be considered positive, PRRSV nucleic acid had to be detected by both tests. Samples with only 1 positive reaction were considered suspect. All samples were tested for viable PRRSV by virus isolation (VI) on both MARC-145 cells and porcine alveolar macrophages (21). The samples positive for PRRSV by PCR or VI from flies were nucleic acid-sequenced to compare the percent homology of the open reading frame (ORF) 5 region of the virus used to infect the pig (22). Serum from the pig was tested for PRRSV-antibodies using an enzyme linked immunosorbent assay (ELISA) (IDEXX ELISA; IDEXX Laboratories, Westbrook, Maine, USA) (23). Additionally, all samples from the flies were tested for the presence of infectious PRRSV by swine bioassay (24). For this procedure, a total of 11, 4-week-old pigs were obtained from the PRRSV-negative farm previously described, and housed in separate room of the isolation facility of the University of Minnesota College of Veterinary Medicine. Ten pigs were intramuscularly inoculated with 1.5 mL of either whole fly homogenates, gut homogenates, or exterior surface washes that had been collected at each of the sampling times, while the remaining pig served as a negative control. All of the pigs were bled weekly, and the PRRSV status of all pigs was monitored for 21 d by PCR, VI, and ELISA.

Laboratory detection of PRRSV on exterior surface of houseflies

As a positive control, the ability of PCR and VI to detect PRRSV on the exterior surface of houseflies was tested under laboratory setting. A total of 120 houseflies were obtained from the source previously described. Ninety of the flies were immersed into an aliquot of the PRRSV inoculum used for the study (approximately 25 µl per insect). They remained under laboratory conditions (27°C), and subsets of 30 flies were collected at the following sampling times: 0, 6, and 12 h following immersion. Flies were pooled and processed as previously described. Washing fluid was transferred into separate sterile tubes and tested by PCR and VI. As a negative control, a subset of remaining 30 flies were immersed into an aliquot of MEM, processed, and tested as previously described.

Results

Pigs

Following arrival, all animals were determined to be negative for PRRSV by PCR, VI, and ELISA. The pig inoculated with the index virus was PCR and VI-positive when fly-to-pig contact took place (day 7 pi). Antibodies against PRRSV were detected by ELISA on day 14 pi. A negative control pig remained negative by PCR, VI, and ELISA during the entire study.

Whole fly homogenates

Diagnostic results from whole fly homogenates are summarized in Table I. The PRRSV nucleic acid was detected by PCR from whole fly homogenates collected at 0, 6, 12, and 24 h pf. The ORF 5 region of representative PRRSV detected from whole fly homogenates was found to be 100% homologous to the virus used to infect the pig. Infectious PRRSV was detected by swine bioassay from whole fly homogenates collected at 0, 6, and 12 h pf, but not at 24 h pf. All samples were VI-negative.

Exterior surface wash

Diagnostic results from the exterior surface wash of flies are summarized in Table I. The PRRSV nucleic acid was detected by PCR from the exterior surface washes of flies collected at 0, 6, and 12 h pf; however, only the sample collected at 0 h pf was determined to be infectious by swine bioassay. The ORF 5 region of representative PRRSV detected from the exterior surface washes of flies was found to be 100% homologous to the index virus. All samples were VI-negative.

Gut homogenates

Diagnostic results from gut homogenates from flies are summarized in Table I. The PRRSV nucleic acid was detected by PCR from gut homogenates collected at 0 and 12 h pf, while the sample collected at 6 h pf was PCR-suspect. The ORF 5 region of representative PRRSV detected from gut homogenates was found to be 100% homologous to the index virus. Swine bioassay was positive at 0 and 12 h pf; however, the PCR-suspect sample collected at 6 h pf was swine bioassay-negative. All samples were VI-negative.

Laboratory detection of PRRSV on exterior surface of houseflies

The PRRSV RNA was detected by PCR from the exterior surface washing fluid from flies collected at 0, 6 and 12 h following immersion, while all samples were VI-negative. Exterior surface wash from the flies immersed into MEM (negative control), was PCR and VI-negative.

Discussion

This study demonstrated that infectious PRRSV could remain viable within the intestinal viscera of houseflies for up to 12 h following feeding on an infected pig. In contrast to a previous study (16), all flies were housed individually, in separate transfer vials until they were processed for diagnostic testing in the current

Table I. Diagnostic results from whole fly homogenates, gut homogenates, and exterior surface wash of house flies

Sampling time	Fly samples	PCR	VI	Bioassay
0 h pf	Whole	+	−	+
	Gut	+	−	+
	Wash	+	−	+
6 h pf	Whole	+	−	+
	Gut	suspect	−	−
	Wash	+	−	−
12 h pf	Whole	+	−	+
	Gut	+	−	+
	Wash	+	−	−
24 h pf	Whole	+	−	−

PCR: polymerase chain reaction
VI: virus isolation
pf: post feeding on an infected pig
Whole: whole fly homogenates
Gut: gut homogenates of flies
Wash: exterior surface wash of flies
Suspect: a sample that demonstrated a positive results in 1 of 2 PCR tests
PCR (+): a sample that demonstrated a positive results in 2 of 2 PCR tests
VI (−): Negative for virus isolation
Bioassay (+): a sample demonstrating that the pig inoculated with an insect sample showed viremia and seroconversion during a 21-day monitoring period

study. This allowed us to rule out the possibility of contamination of PRRSV due to fly-to-fly contact during the incubation time. The current study also demonstrated that viable PRRSV was present for longer periods within the digestive organs of the fly, compared to what was detected on the exterior surface. This finding is important since the ability of PRRSV to reside within the body of a housefly may protect it against certain environmental factors known to be detrimental to PRRSV survivability outside of the host, such as ultraviolet light and drying (25). These findings, along with the documented ability of house flies to mechanically transmit PRRSV (16) and their capacity for traveling the distances of 5 km or more (26), support the hypothesis that houseflies may serve as a source of PRRSV-transmission within pig populations and, potentially, even between commercial swine farms. Furthermore, houseflies frequently inhabit the interior of transport vehicles and livestock trailers. This may enhance exposure of the insects to PRRSV infected animals, and allow for movement of the insects over greater geographical distances during time limited periods in commercial swine production settings. Since the usual feeding behavior of houseflies involves regurgitation and re-ingestion, the ability of PRRSV to survive in the digestive tract of the houseflies could result in mechanical transmission of PRRSV from pig to pig (27). However, whether houseflies can also serve as biological vectors of PRRSV is still not certain. It is unlikely that members of the Arterivirus group, such as PRRSV, are capable of replicating within insects (28); however, attempts to assess PRRSV survival in blood-fed houseflies over a longer period of time, and a quantitative analysis of the virus in each individual housefly would be needed to make a final conclusion regarding this issue.

As with all research, there were some inherent limitations and unanswerable questions that came about, and this was particularly true in the area of diagnostic analysis. Despite the detection of infectious PRRSV in gut homogenates collected at 0 and 12 h pf, a sample collected at 6 h pf was PCR-suspect and swine bioassay-negative. An explanation for this observation may be that these flies were truly PRRSV-negative, or the inability of the tests used in the study to detect a very low level of PRRSV present in this sample. Because we did not quantitatively assess PRRSV in each individual fly, it is possible that the variation in the amount of blood that each individual fly obtained may have altered the outcome of the study, even though the use of transparent plastic vials enhanced our ability to observe the entire feeding process of each individual fly.

Regarding the results from the exterior surface washes of flies, PRRSV nucleic acid was detected by PCR for up to 12 h pf; however, only a sample collected immediately following feeding on an infected pig (0 h pf) was found to be infectious by swine bioassay. Possible explanations for this would be either that a sufficient quantity of the virus was not present on flies to infect a naïve pig, or that virus residing on the exterior surface of houseflies was inactivated and lost its infectivity following prolonged exposure to certain environmental factors, such as ultraviolet light or drying (25). Results from laboratory detection of PRRSV on the exterior surface washes from houseflies immersed into an aliquot of PRRSV (positive controls) basically support the ability of our method to qualitatively detect PRRSV on the exterior surface of flies following feeding on an infected pig at a temperature of 27°C. The use of the incubation temperature of 27°C was representative of environmental temperature of the warm weather in the United States and year-round conditions in tropical countries. However, no conclusion can be made regarding the amount of the virus present in fly samples because, again, the diagnostic methods in this study were not quantitative.

Finally, VI results from whole fly homogenates, gut homogenates, and the exterior surface washes of flies were all negative. Similar findings also have been observed in previous studies showing that VI was all negative from mosquito (*Aedes vexans*) and housefly homogenates following feeding on PRRSV-infected pigs (6,16). A possible explanation for this finding could be the relative lower sensitivity of VI as compared to PCR (20). However, RNA detected from fly samples were nucleic-acid sequenced and found to be 100% homologous (ORF 5 region) to the index virus, and swine bioassay was conducted for all fly samples to determine whether the virus detected by PCR was infectious. Therefore, through the use of multiple diagnostic testing, we feel confident that we were able to overcome the limitations of VI regarding the detection of PRRSV from insect samples.

An important consideration when designing and conducting this study was the welfare of pig during the time that the pig was in contact with houseflies. During this period, pig was consciously restrained for approximately 2.5 h in Panepinto slings specifically designed for surgical procedures. The pig was held in an upright position and the leg opening in the slings were lined with soft cloth to avoid irritation, and was frequently removed for exercise purposes throughout the fly contact period. The pig was provided treats and fruit juice, and it appeared to be very comfortable in the slings since it frequently fell asleep during the procedure. Small areas of scarification were made on the pig skin due to the fact that houseflies require the presence of open wounds to access blood. Skin abrasions and lacerations are frequently observed in commercial swine farms, especially during the suckling period or immediately post-weaning when pen mates fight to establish a hierarchy, and these lesions frequently attract houseflies in commercial swine farms (S. Dee, personal communication, 2002). The pig did not demonstrate evidence of pain during the scarification process, and lesions healed within 24 h. Finally, clinical evidence of dermatitis, secondary to staphylococcal infections was not observed in the pig, and it did not require supplemental medication or anti-inflammatory agents throughout the 14-day period of the study.

In conclusion, the present study indicates that infectious PRRSV can survive within internal viscera of houseflies for a longer period of time (up to 12 h) than it can on the exterior surface of the flies. This new information, along with the results from a previous study demonstrating mechanical transmission of PRRSV by houseflies from infected to naïve pigs (16), raise the potential significance of houseflies as mechanical vectors of PRRSV and support the hypothesis that houseflies may contribute to horizontal transmission of PRRSV among pigs within infected commercial farms. However, further epidemiological studies are needed before a final conclusion can be drawn regarding the significance of houseflies in transmission of PRRSV throughout commercial swine producing areas.

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