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Short Communication

Susceptibility of Midge and Mosquito Vectors to SARS-CoV-2

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Abstract

SARS-CoV-2 is a recently emerged, highly contagious virus and the cause of the current COVID-19 pandemic. It is a zoonotic virus, although its animal origin is not clear yet. Person-to-person transmission occurs by inhalation of infected droplets and aerosols, or by direct contact with contaminated fomites. Arthropods transmit numerous viral, parasitic, and bacterial diseases; however, the potential role of arthropods in SARS-CoV-2 transmission is not fully understood. Thus far, a few studies have demonstrated that SARS-CoV-2 replication is not supported in cells from certain insect species nor in certain species of mosquitoes after intrathoracic inoculation. In this study, we expanded the work of SARS-CoV-2 susceptibility to biting insects after ingesting a SARS-CoV-2-infected bloodmeal. Species tested included *Culicoides sonorensis* (Wirth & Jones) (Diptera: Ceratopogonidae) biting midges, as well as *Culex tarsalis* (Coquillett) and *Culex quinquefasciatus* (Say) mosquitoes (Diptera: Culicidae), all known biological vectors for numerous RNA viruses. Arthropods were allowed to feed on SARS-CoV-2-spiked blood and at a time point postinfection analyzed for the presence of viral RNA and infectious virus. Additionally, cell lines derived from *C. sonorensis* (W8a), *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) (C6/36), *Cx. quinquefasciatus* (HSU), and *Cx. tarsalis* (CxTrR2) were tested for SARS-CoV-2 susceptibility. Our results indicate that none of the biting insects, nor the insect cell lines evaluated support SARS-CoV-2 replication, suggesting that these species are unable to be biological vectors of SARS-CoV-2.

Key words: SARS-CoV-2, susceptibility, midges, mosquito

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of the 2019 coronavirus disease (COVID-19) pandemic (Chan et al. 2020). SARS-CoV-2 belongs to the order *Nidovirales*, family *coronaviridae*, and genus *betacoronavirus*. SARS-CoV-2 infects humans, and has the potential to infect various animal species (Chu et al. 2020, Shi et al. 2020). The predominant mode of SARS-CoV-2 transmission is by the respiratory route. However, SARS-CoV-2 viral RNA was identified in blood and serum samples from infected patients (Chen et al. 2020, Hogan et al. 2020, Young et al. 2020). This suggests that virus may be present in the blood of infected humans. The potential circulation of SARS-CoV-2 in the bloodstream of COVID-19 patients justifies the need for studies on the susceptibility of hematophagous insects to SARS-CoV-2. Arthropods transmit numerous pathogens to humans and animals via biological and mechanical transmission (Leitner et al. 2015). A recent report demonstrated that SARS-CoV-2 replication was not supported in *Aedes aegypti* (Linnaeus), *Aedes albopictus* (Skuse), and *Culex quinquefasciatus* (Say) mosquito (Diptera: Culicidae) species after an intrathoracic route of infection (Huang et al. 2020). Another report showed that the SARS-CoV-2 does not replicate in cells derived from *Aedes* mosquitoes, nor was it present in field-caught *Culex* and *Anopheles* mosquitoes from Wuhan (Xia

et al. 2020). The previous studies have examined SARS-CoV-2 replication in a few species of mosquitoes. Analysis of other species and arthropods important for pathogen transmission is needed due to the variation of vector competency within the same species and among different arthropods.

Here, we report the first susceptibility study of SARS-CoV-2 infection using three critical insect vectors following ingestion of a SARS-CoV-2 infected bloodmeal, including an agriculturally important animal disease vector, *Culicoides sonorensis* (Wirth & Jones) (Diptera: Ceratopogonidae) biting midges, and two significant human disease vector mosquito species, *Culex tarsalis* (Coquillett) (Diptera: Culicidae) and *Cx. quinquefasciatus*.

Methods

The SARS-CoV-2 USA-WA1/2020 strain was acquired from the Biodefense and Emerging Infection Research Resources Repository (BEI Resources, Manassas, VA) and was passaged three times on African green monkey kidney Vero E6 cells (ATCC CRL-1586, Virginia) with a final titer of 2.5×10^6 $TCID_{50}$/ml, determined by $TCID_{50}$-CPE assay. The virus was sequenced by next-generation sequencing (NGS) and its consensus sequence was found to be homologous to the USA-WA1/2020 strain (GenBank accession: MN985325.1). Arthropod cell cultures were derived from *C. sonorensis* embryos (W8a; McHolland and Mecham 2003), *Cx. tarsalis* embryos (CxTrR2; Arthropod-Borne Animal Diseases Unit; ABADRU, Manhattan, KS), *Cx. quinquefasciatus* ovaries (HSU; Hsu et al. 1970), and *Ae. albopictus* larva (C6/36). The W8a, CxTrR2, HSU, and C6/36 cells were maintained in CuVa medium, L-15 medium (with 10% tryptose phosphate broth), and Medium 199H, respectively. All media (Sigma–Aldrich, St. Louis, MO) was supplemented with 10% fetal bovine serum (FBS; Sigma) except for CuVa media, which was supplemented with 20% fetal bovine serum. Cells were maintained at 27°C in sealed T-25 flasks and inoculated with SARS-CoV-2 at approximately 0.1 multiplicity of infection for 1 h before the inoculum was replaced with fresh culture media. Cell cultures were monitored for cytopathic effect (CPE) by light microscopy and culture supernatants were collected at 0, 2, 4, and 8 d postinfection (dpi) for subsequent titration by $TCID_{50}$-CPE assay on Vero E6 cells.

Culex tarsalis, *Cx. quinquefasciatus*, and the ABADRU *C. sonorensis* colonies were reared and maintained in the ABADRU insectary. Arthropods were transported to Kansas State University, Biosecurity Research Institute (BRI) for infection studies under Arthropod Containment Level-3 (ACL-3) conditions.

Adult female *C. sonorensis* (n = 200) midges were allowed to feed on defibrinated sheep blood mixed 1:1 (v/v) with SARS-CoV-2 (2.0×10^6 $TCID_{50}$/ml) using a bell jar method with parafilm membrane. Negative control unfed midges (n = 100) were maintained in adjacent cages. For mosquitoes, 8-d-old *Cx. tarsalis* (n = 100) or 10-d-old *Cx. quinquefasciatus* (n = 100) were allowed to feed on SARS-CoV-2 spiked sheep blood, as described above. Negative control mock-infected blood-fed *Cx. tarsalis* (n = 50) were maintained in adjacent cages. The purpose of the mock-infected mosquitoes was to determine if there was excess mortality in mosquitoes fed infectious blood versus noninfectious blood. Midges and mosquitoes were allowed to feed for an hour. Following feeding, mosquitoes were anesthetized and individuals with full blood meals were selected and held at 28°C for 10 d. We were not able to select blood-fed midges due to known mortality from anesthesia. Nevertheless, more than 90% of midges were blood fed. Surviving midges and mosquitoes at day 10 were pooled (n = 2–10) in 1 ml virus transport media (199E media supplemented with antibiotic-antimycotics; Sigma), and stored at –80°C until processed for virus isolation (VI) and RNA extractions.

Pooled arthropods were homogenized by a Tissuelyser II (Qiagen, Germantown, MD) using tungsten carbide beads (Qiagen). An aliquot (140 µl) of unfiltered homogenate was used for RNA extraction and the remaining homogenate was filtered through a 0.22-µm membrane filter (PES filters, MIDSCI, St. Louis, MO) before being used for VI. RNA extraction was performed using the QIAamp viral RNA mini kit (Qiagen) as per the manufacturer's instructions. RT-qPCR assay was performed according to the Center for Disease Control (CDC) protocol for detection of SARS-CoV-2 nucleocapsid (N)-specific RNA (https://www.fda.gov/media/134922/download) using Script XLT One-Step RT-qPCR Tough Mix (Quanta Biosciences, Beverly, MA) on a CFX96 Real-time thermocycler (BioRad, Hercules, CA). Plate controls included a quantitated SARS-CoV-2 N-specific qPCR positive control, diluted 1:10 (Integrated DNA Technologies, Iowa), and a non-template control. Results were analyzed using the Bio-Rad CFX Manager 3.1 with samples below 40 Ct considered positive. We generated a standard curve using the above RT-qPCR assay on RNAs extracted from defined SARS-CoV-2 titers. A reference standard curve was created by plotting the obtained Ct values against the SARS-CoV-2 titers. Then the approximate SARS-CoV-2 titer of each sample was estimated from the standard curve (data not shown).

An immunofluorescence assay was used to determine absence/presence of infectious virus in arthropod samples. Arthropod homogenates (100 µl) were added on to Vero E6 cells in 24-well plates and incubated at 37°C and 5% CO_2 for 3 d. Following incubation, the supernatants were blind-passaged three times on Vero E6 cells, and at the first and third passage, cells were examined by an indirect immunofluorescence assay (IFA) for the presence of SARS-CoV-2 antigen. Briefly, 3 dpi cells were fixed with ice cold 100% methanol for 10 min at –80°C and washed three times with 1× PBS Tween 20 (0.05%). Mouse monoclonal antibodies (in house) specific for the receptor binding domain (RBD) of the spike protein of SARS-CoV-2 was diluted 1:5 in 1× PBS containing 1% BSA and 150 µl was added to each well and incubated at room temperature (RT) for 1 h. The cells were washed three times as described above, and then incubated with 150 µl of FITC-conjugated goat anti-mouse IgG (Abcam, Cambridge, MA), diluted 1:500 in 1× PBS with BSA, for 1 h at RT. After washing and drying, cell monolayers were examined by an EVOS fluorescent microscope (ThermoFisher Scientific, Waltham, MA) for the presence of FITC-positive cells. Mock-infected and SARS-CoV-2-infected Vero E6 cells were used as negative and positive controls, respectively.

Results and Discussion

The goal of this study was to determine whether *Culicoides* midges, *Cx. tarsalis*, and *Cx. quinquefaciatus* mosquitoes are susceptible to SARS-CoV-2 by an oral route of infection, which was not previously evaluated. Initial infection studies were performed in vitro with the insect cell lines W8a, C6/36, CxTrR2, and HSU. Two independent experiments showed no obvious sign of CPE for any of the SARS-CoV-2-infected arthropod-derived cell cultures, nor for any of the insect culture supernatants collected at 2, 4, or 8 dpi and titered on Vero E6 cells.

Next, susceptibility of insects after an infectious bloodmeal was investigated. One hundred forty blood-fed midges survived to day 10 post-bloodmeal and were divided into 14 pools with 10 midges each for analysis. The majority (85%) of virus-fed midge pools had detectable SARS-CoV-2-specific RNA with an average Ct value of 34.8 ± 2.6; the day 10 control unfed midges were negative for SARS-CoV-2 RNA (Table 1). Forty-eight blood-fed *Cx. tarsalis* mosquitoes survived to day 10 and were divided into six pools for analysis. One out of six (17%) virus-fed *Cx. tarsalis* mosquito pools had detectable

Table 1. Detection of SARS-CoV-2 viral RNA in various arthropods by RT-qPCR

Treatment group	No. of insect tested	No. of pools (numbers of insects per pool)	Positive homogenate pools out of total (% positive)	Mean Ct ± SD (estimated SARS-CoV-2 titer/ml of sample)	95% confidence interval (adjusted mean Ct value)
Unfed *Culicoides sonorensis*	40	4 (10)	0/4 (0)	ND[a]	None[c]
SARS-CoV-2 fed *Culicoides sonorensis*	140	14 (10)	12/14 (85)	34.8 ± 2.6 ($<5 \times 10^{-1}$)	34.0–37.4(35.7)
Mock-infected fed *Culex tarsalis*	30	3 (10)	0/3 (0)	ND[a]	None[c]
SARS-CoV-2 fed *Culex tarsalis*	48	6[b] (10,10,10,10,5,3)	1/6 (17)	31.3 ($<5 \times 10^{-1}$)	34.8–42.3(38.6)
SARS-CoV-2 fed *Culex quinquefasciatus*	47	6[b] (10,10,10,10,5,2)	3/6 (50)	34.2 ± 3.3 ($<5 \times 10^{-1}$)	32.9–41.3 (37.1)

[a]ND = not detected.

[b]The numbers for each pool is written out in parenthesis. For the confidence interval (95%) evaluation, negative samples were considered to have a Ct value of 40 (as per limit of detection) for calculating the adjusted mean.

[c]None = not calculated.

SARS-CoV-2-specific RNA with an average Ct value of 31.3, and none of the three pools of mock-infected blood-fed *Cx. tarsalis* mosquitoes were SARS-CoV-2 RNA positive (Table 1). Similarly, 47 blood-fed *Cx. quinquefasciatus* survived to day 10 and were divided into six pools for analysis. Viral RNA was detected in 50% of the SARS-CoV-2-fed mosquito pools with an average Ct value of 34.2 (Table 1). Additionally, the approximate Ct values of day 0 insect pools was estimated to be between 16 and 18 based on a standard curve of SARS-CoV-2 titer versus Ct values (data no shown). The Ct values of all the groups were also evaluated for 95% confidence interval (Table 1).

To determine the presence of infectious virus, serial passages of pooled arthropod homogenates were performed on Vero E6 cells. No CPE was observed after three passages of virus-fed *Culicoides* midge homogenates, and IFA analysis of passage one and three of inoculated Vero E6 cells confirmed the absence of SARS-CoV-2. SARS-CoV-2-infected Vero E6 cells were used as an IFA positive control and showed a clear positive staining pattern (data not shown). Unfed control midge samples were negative by CPE and IFA. Similarly, no infectious virus was detected from any of the six homogenate pools of SARS-CoV-2-fed *Cx. tarsalis* mosquitoes that were passaged on Vero E6 cells and analyzed for CPE and by IFA; the control *Cx. tarsalis* homogenate pools were also negative by both methods. The six virus-fed *Cx. quinquefasciatus* mosquito homogenate pools tested for infectivity by CPE and IFA were also negative for SARS-CoV-2.

Overall, our results agree with previously published findings that *Aedes* mosquito-derived cells do not support SARS-CoV-2 replication (Xia et al. 2020). Additionally, we could show that two different *Culex*-derived cell lines and one *Culicoides*-derived cell line are also refractory to SARS-CoV-2 infection. In a previously published SARS-CoV-2 susceptibility study in mosquitoes, intrathoracic injection of SARS-CoV-2 grown in Vero 76 cells was used to determine the susceptibility of *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* mosquitoes to the virus (Huang et al. 2020). They found that none of these mosquitoes species were susceptible to SARS-CoV-2.

Our in vivo studies of midge and mosquito susceptibility to SARS-CoV-2 infection following an oral route of exposure showed that viral RNA remained in virus-fed arthropods for up to 10 d postvirus-spiked blood feeding. However, no infectious virus was recovered from these RNA-positive arthropods, even after three passages on highly susceptible Vero E6 cells. The absence of infectious virus in these insects could be due to poor or no replication of SARS-CoV-2. In this context it should be mentioned that the limit of detection of the VI assay is approximately 10 TCID50/ml.

Our study has some technical limitations; we did not examine the mechanical transmission of SARS-CoV-2 by these insects. Reports have shown that SARS-CoV-2 RNA was detected in low levels in the blood of the COVID-19-infected humans (Chen et al. 2020, Hogan et al. 2020, Young et al. 2020) and in insects of this study. Moreover, the efficiency of mechanical transmission is dose dependent (Boullis et al. 2019). Therefore, the likelihood for transmission of SARS-CoV-2 by these insects is extremely low. Our study did not examine the dissemination of SARS-CoV-2 infection within individual insects. However, the absence of viral infection in the insects studied here and by others negates the need for such an examination.

In conclusion, the insect vector species known to transmit animal and human pathogens used in this study are refractory to SARS-CoV-2 infection under experimental conditions and, therefore, most likely do not play a role in transmission of SARS-CoV-2.

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