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Susceptibility of midge and mosquito vectors to SARS-CoV-2 by natural route of infection

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Abstract

1 SARS-CoV-2 is a recently emerged, highly contagious virus and the cause of the current
2 pandemic. It is a zoonotic virus, although its animal origin is not clear yet. Person-to-person
3 transmission occurs by inhalation of infected droplets and aerosols, or by direct contact with
4 contaminated fomites. Arthropods transmit numerous viral, parasitic, and bacterial diseases;
5 however, the potential role of arthropods in SARS-CoV-2 transmission is not fully understood.
6 Thus far, a few studies have demonstrated that SARS-CoV-2 replication is not supported in cells
7 from certain insect species nor in certain species of mosquitoes after intrathoracic inoculation. In
8 this study, we expanded the work of SARS-CoV-2 susceptibility to biting insects after ingesting
9 a SARS-CoV-2infected blood meal. Species tested included *Culicoides sonorensis* biting
10 midges, as well as *Culex tarsalis* and *Culex quinquefasciatus* mosquitoes, all known biological
11 vectors for numerous RNA viruses. Arthropods were allowed to feed on SARS-CoV-2 spiked
12 blood and at various time points post infection analyzed for the presence of viral RNA and
13 infectious virus. Additionally, cell lines derived from *C. sonorensis* (W8a), *Ae. aegypti* (C6/36),
14 *Cx. quinquefasciatus* (HSU), and *Cx. tarsalis* (CxTrR2) were tested for SARS-CoV-2
15 susceptibility. Our results indicate that none of the biting insects, nor the insect cell lines support
16 SARS-CoV-2 replication. We conclude, that biting insect do not pose a risk for transmission of
17 SARS-CoV-2 to humans or animals following a SARS-CoV-2 infected blood meal.

18 **Keywords**: SARS-CoV-2, susceptibility, midges, mosquitoes

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Introduction

22 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of the
23 2019 coronavirus disease (COVID-19) pandemic. SARS-CoV-2 belongs to the order
24 *Nidovirales*, family *coronaviridae*, and genus *betacoronavirus*. It is an enveloped virus with a
25 positive-sense, single-stranded RNA genome of approximately 30 kb in length (Chen et al.
26 2020). SARS-CoV-2 infects humans, and has the potential to infect various animal species (Chu
27 et al. 2020). Transmission from these animals to humans is not yet clearly understood. The virus
28 is mainly transmitted from person-to-person by inhalation of droplets and aerosols produced by
29 infected people (Chan et al. 2020), or through contact with contaminated surfaces (Goldman
30 2020, Sonja A. Rasmussen 2020; Kwon et al., 2020). Arthropods transmit numerous diseases to
31 humans and animals via biological and mechanical transmission (Leitner et al. 2015). Although
32 the SARS-CoV-2-related coronaviruses SARS-CoV-1 and MERS-CoV are not transmitted by
33 insects, concerns have been raised, by those in both public health and agricultural sectors, as to
34 their potential role in spreading SARS-CoV-2 among humans and animals. For arthropods to be
35 transmission-competent vectors, the respective pathogen must be acquired from a host during
36 blood feeding, then infect the midgut, escape the midgut barrier, disseminate to and infect the
37 salivary glands, and finally be transmitted to a susceptible host during subsequent blood feeding
38 (Franz et al. 2015). A recent report demonstrated that SARS-CoV-2 replication was not
39 supported in *Aedes aegypti*, *Ae. albopictus* and *Culex quinquefasciatus* mosquito species after an
40 intrathoracic route of infection (Huang et al. 2020). Another report showed that the SARS-CoV-
41 2 does not replicate in cells derived from *Aedes* mosquitoes, nor was it present in field-caught
42 *Culex* and *Anopheles* mosquitoes from Wuhan (Xia et al. 2020).

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43 Here, we report the first susceptibility study of SARS-CoV-2 infection using three critical insect
44 vectors following ingestion of a SARS-CoV-2 infected blood meal, including an agriculturally
45 important animal disease vector, *Culicoides sonorensis* biting midges, and two significant human
46 disease vector mosquito species, *Cx. tarsalis* and *Cx. quinquefasciatu*s. Additionally, four insect-
47 derived cell lines from *C. sonorensis* (W8a), *Ae. aegypti* (C6/36), *Cx. tarsalis* (CxTrR2), and *Cx.*
48 *quinquefasciatus* (HSU) were also evaluated for SARS-CoV-2 susceptibility.

Methods

49 The SARS-CoV-2 USA-WA1/2020 strain was acquired from Biodefense and Emerging
50 Infection Research Resources Repository (BEI Resources, Manassas, VA, USA) and was
51 passaged three times on VeroE6 cells (ATCC, VA, USA) with a final titer of 2.5 x 10^6
52 $TCID_{50}$/ml. Arthropod cell cultures were derived from *C. sonorensis* embryos (W8a; McHolland
53 and Mecham 2003), *Cx. tarsalis* embryos (CxTrR2; Arthropod-Borne Animal Diseases Unit;
54 ABADRU, Manhattan, KS, USA), *Cx. quinquefasciatus* ovaries (HSU; Hsu et al. 1970), and *Ae.*
55 *albopictus* larva (C6/36). The W8a, CxTrR2, HSU, and C6/36 cells were maintained in CuVa
56 medium, L-15 medium (with 10% tryptose phosphate broth) and Medium 199H, respectively.
57 All media (Sigma-Aldrich, St. Louis, MO, USA) was supplemented with 10-20% FBS (ITFBS;
58 Sigma). Cells were maintained at 27°C in closed T-flasks and inoculated with SARS-CoV-2 at
59 approximately 0.1 multiplicity of infection (MOI) for 1h before the inoculum was replaced with
60 fresh culture media. Cell cultures were monitored for cytopathic effect (CPE) by light
61 microscopy and culture supernatants were collected at 0, 2, 4, and 8 days post infection (dpi) for
62 subsequent titration by $TCID_{50}$-CPE assay on VeroE6 cells.

63 *Cx. tarsalis*, *Cx. quinquefasciatus*, and the ABADRU *C. sonorensis* colonies were reared and
64 maintained in the ABADRU insectary. Arthropods were transported to Kansas State University,

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65 Biosecurity Research Institute (BRI) for infection studies under Arthropod Containment Level-3
66 (ACL-3) conditions.

67 Adult female *C. sonorensis* (n=200) midges were allowed to feed on defibrinated sheep blood
68 mixed 1:1 (v/v) with SARS-CoV-2 (2.0 x 10^6 $TCID_{50}$/ml). Negative control unfed midges
69 (n=100) were maintained in adjacent cages. For mosquitoes, 8-day old *Cx. tarsalis* (n=100) or
70 10-day old *Cx. quinquefasciatus* (n=100) were allowed to feed on SARS-CoV-2 spiked sheep
71 blood as described above. Negative control mock-infected blood-fed *Cx. tarsalis* (n=50) were
72 maintained in adjacent cages. After an hour of feeding, midges or fully engorged mosquitoes
73 were held at 28°C for 10 days. Surviving midges and mosquitoes at day 10 were pooled (n=5-10)
74 in 1 ml virus transport media (199E media supplemented with antibiotic-antimycotics; Sigma),
75 and stored at -80°C until processed for virus isolation (VI) and RNA extractions.

76 Pooled arthropods were homogenized by a Tissuelyser II (Qiagen, Germantown, MD, USA)
77 using tungsten carbide beads (Qiagen). An aliquot (140 µl) of homogenate was used for RNA
78 extraction with the remaining homogenate filtered through a 0.22 µm PES membrane filter
79 (MIDSCI, St. Louis, MO, USA) before subsequent VI.

80 RNA extraction was performed using the QIAamp viral RNA mini kit (Qiagen) as per
81 manufacturer's instructions. RT-qPCR assay was performed according to the Center for Disease
82 Control (CDC) protocol for detection of SARS-CoV-2 nucleocapsid (N)-specific RNA
83 (https://www.fda.gov/media/134922/download) using Script XLT One-Step RT-qPCR Tough
84 Mix (Quanta Biosciences, Beverly, MA, USA) on a CFX96 Real-time thermocycler (BioRad,
85 Hercules, CA, USA). Plate controls included a quantitated SARS-CoV-2 N-specific qPCR
86 positive control, diluted 1:10 (Integrated DNA Technologies, IA, USA), and a non-template

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87 control (NTC). Results were analyzed using the Bio-Rad CFX Manager 3.1 with samples below
88 40 Ct considered positive.

89 Arthropod homogenates (100 µl) were added on to VeroE6 cells in 24 well plates and incubated
90 at 37°C and 5% CO_2 for 3 days. Culture supernatents were blind-passaged three times on VeroE6
91 cells, and at the first and third passage, cells were examined by an indirect immunofluorescence
92 assay (IFA) for the presence of SARS-CoV-2 antigen. Briefly, 3 dpi cells were fixed with ice
93 cold 100% methanol for 10 mins at -80°C and washed three times with 1 x PBS Tween 20
94 (0.05%). Mouse monoclonal antibodies (in house) specific for the Receptor Binding Domain
95 (RBD) of spike protein of SARS-CoV-2 was diluted 1:5 in 1x PBS containing 1% BSA and 150
96 µl was added to each well and incubated at room temperature (RT) for 1 h. The cells were
97 washed three times as described above, and then incubated with 150 µl of FITC-conjugated goat
98 anti-mouse IgG (Abcam, Cambridge, MA, USA), diluted 1:500 in 1x PBS with BSA, for 1 h at
99 RT. After washing and drying, cell monolayers were examined by an EVOS fluorescent
100 microscope (ThermoFisher Scientific, Waltham, MA, USA) for the presence of FITC positive
101 cells. Mock infected and SARS-CoV-2 infected VeroE6 cells were used as negative and positive
102 controls, respectively.

Results and discussion

103 The goal of this study was to determine whether arthropods are susceptible to SARS-CoV-2 by a
104 natural route of infection which has not yet been evaluated. In addition to important mosquito
105 vectors, *Culicoides* midges were also evaluated in this study. Initial infection studies were
106 performed *in vitro* with the insect cell lines W8a, C6/36, CxTrR2, and HSU. Two independent
107 experiments showed no obvious sign of CPE for any of the SARS-CoV-2-infected arthropod-

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108 derived cell cultures, nor for any of the insect culture supernatants collected at 2, 4 or 8 dpi and
109 passaged on VeroE6 cells.

110 Next, susceptibility of insects after an infectious blood meal was investigated. Of 200 midges
111 allowed to feed on the SARS-CoV-2-spiked blood meal, 140 survived until 10 days post blood
112 meal and were further analyzed. The majority (85%) of virus-fed midge pools had detectable
113 SARS-CoV- 2-specific RNA with an average Ct value of 34.84±2.6; the day 10 control unfed
114 midges were negative for SARS-CoV-2 RNA (Table 1). Among the *Cx. tarsalis* mosquitoes
115 allowed to feed on SARS-CoV-2-spiked blood (n=100), only 48 virus-fed mosquitoes survived
116 until day 10. One out of 6 (17%) virus-fed *Cx. tarsalis* mosquitoes had detectable SARS-CoV-2-
117 specific RNA with an average Ct value of 31.3, and none of the 30 mock-infected blood-fed *Cx.*
118 *tarsalis* mosquitoes were SARS-CoV-2 RNA positive (Table 1). Similarly, of 100 *Cx.*
119 *quinquefasciatus* mosquitoes, 47 virus-fed survived until day 10. Viral RNA was detected in
120 50% of the SARS-CoV-2-fed mosquitoes with an average Ct value of 34.17 (Table 1).

121 To determine the presence of infectious virus, serial passages of pooled arthropod homogenates
122 were performed on VeroE6 cells. No CPE was observed after three passages of virus-fed
123 *Culicoides* midge homogenates, and IFA analysis of passage one and three of inoculated VeroE6
124 cells confirmed the absence of SARS-CoV-2 (Table 2; Figure 1). SARS-CoV-2-infected VeroE6
125 cells were used as an IFA positive control and showed a clear positive staining pattern (Fig.1).
126 Unfed control midge samples were negative by VI and IFA (Table 2). Similarly, no infectious
127 virus was detected from any of the six homogenate pools of SARS-CoV-2-fed *Cx. tarsalis*
128 mosquitoes that were passaged on VeroE6 cells and analyzed for CPE and by IFA; the control
129 *Cx. tarsalis* homogenate pools were also negative by both methods (Table 2). The six virus-fed

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130 *Cx. quinquefasciatus* mosquito homogenates tested for infectivity by VI and IFA were also
131 negative for SARS-CoV-2 (Table 2).

132 A key factor crucial for arthropod-mediated transmission is that the infected host, either person
133 or animal, is viremic at the time of feeding. Thus far, SARS-CoV-2 is known to cause viremia in
134 some cases of infected people (Young et al. 2020). Susceptible animal models tested so far
135 appear to be aviremic (Shi et al. 2020) except for hamsters which regularly show viremia (Chan
136 et al. 2019). Therefore, controlled studies to rule out arthropod transmission of this RNA virus
137 are critical for determining risk, as well as developing accurate epidemiological modeling and
138 control strategies.

139 Overall, our results agree with previously published findings that *Aedes* mosquito derived cells
140 do not support SARS-CoV-2 replication (Xia et al. 2020). Additionally, we have shown that two
141 different *Culex* species derived cell lines and one *Culicoides* midge derived cell line are also
142 refractory to SARS-CoV-2 infection. In a previously published SARS-CoV-2 susceptibility
143 study in mosquitoes, intrathoracic injection of SARS-CoV-2 grown in Vero76 was used to
144 determine the susceptibility of *Ae. aegypti*, *Ae. Albopictus*, and *Cx. quinquefasciatus* to the virus
145 (Huang et al. 2020); however, intrathoracic inoculation bypasses the natural route of infection
146 via the ingestion of a virus-infected blood meal (Franz et al. 2015). Therefore, in the present
147 study, testing of arthropods for SARS-CoV-2 susceptibility was performed following the
148 ingestion of a virus-spiked blood meal; this is important not only because it is the most relevant
149 route of infection for arthropod vectors, but also because of the quasi-species nature of an RNA
150 virus inoculum such as for SARS-CoV-2 (Jary et al. 2020). Considerable genetic bottle necks
151 and natural selection processes exist for viruses in arthropod replication and arthropod-borne
152 transmission. Depending on the insect vector, the number of virus particles ingested via a blood

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153 meal depends on the level of viremia in the host and the volume of the meal. When virus
154 particles ingested via the blood meal enter the midgut, few will be able to infect the midgut
155 epithelium. After midgut replication, progeny viruses escape the midgut barrier and disseminate
156 in the insect's hemocoel, and then next they infect and replicate in the salivary gland. This is
157 critical for subsequent virus transmission to a susceptible host during a blood meal. The quasi-
158 species nature of RNA viruses combined with the natural selection for defined viral genotypes
159 enables RNA viruses to infect and replicate in arthropods and results in a defined virus
160 population selected for increased fitness for the arthropod environment. These viral genotypic
161 changes could lead to viral biotypes with a higher ability of salivary gland infection and bite
162 transmission, when compared to a virus which is artificially injected into the insect's hemocoel.

163 Our *in vitro* and *in vivo* studies of midge and mosquito susceptibility to SARS-CoV-2 infection
164 following a natural ingestion route of exposure showed that viral RNA remained in virus-fed
165 arthropods for up to 10 days post virus-spiked blood feeding. However, no infectious virus was
166 recovered from these RNA-positive arthropods, even after three passages on highly susceptible
167 VeroE6 cells. Our in vitro studies using various insect cells support these results since no
168 infectious virus was detected in supernatants of SARS-CoV-2 inoculated insect cell cultures. In
169 conclusion, the insect vector species known to transmit animal and human pathogens used in this
170 study are refractory to SARS-CoV-2 infection under experimental conditions and, therefore,
171 most likely do not play a role in transmission of SARS-CoV-2.

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176 NR-52281). C6/36 cells were kindly provided by Robert B Tesh, UTMB, Galveston, TX.*Cx.*
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179 **Conflict of interest**

180 All the authors declare no conflict of interest.

181 **Disclaimer**

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

Treatment Group	Positive homogenate pools out of total (% positive)	Mean Ct ± SD
Unfed *C. sonorensis*	0/4 (0)	ND*
SARS-CoV-2 fed *C. sonorensis*	12/14 (85)	34.84±2.6
Mock-infected fed C*x. tarsalis*	0/3 (0)	ND*
SARS-CoV-2 fed C*x. tarsalis*	1/6 (17)	31.3
SARS-CoV-2 fed C*x. quinquefasciatus*	3/6 (50)	34.17±3.3

*ND=not detected

Table 2: Detection of infectious SARS-CoV-2 in arthropod homogenate pools on VeroE6 cells by virus isolation and an indirect immunofluorescence assay

Species	Control arthropods (positive/N) VI	Control arthropods (positive/N) IFA	SARS-CoV-2 fed arthropods (positive/N) VI	SARS-CoV-2 fed arthropods (positive/N) IFA
C. sonorensis	0/4	0/4	0/14	0/14
Cx. tarsalis	0/3	0/3	0/6	0/6
Cx. quinquefasciatus	N/A	N/A	0/6	0/6

N= total number of day 10 homogenate pools. 0= no virus positive or FITC positive cell cultures; N/A = not tested

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Fig. 1.

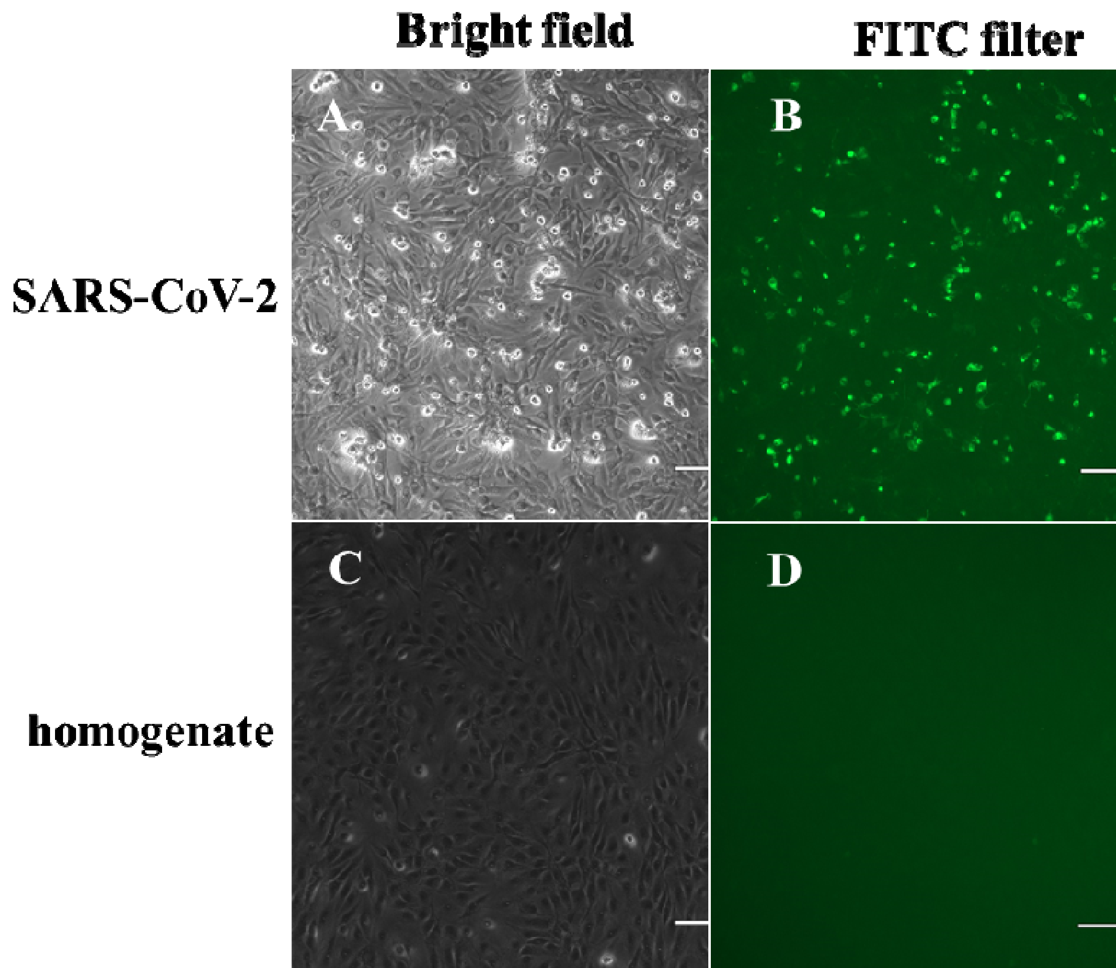

Figure 1. Indirect immunofluorescence assay for the detection of SARS-CoV-2 infected cells. SARS-CoV-2 and a representative passage 3 arthropod homogenate were incubated 3 days on VeroE6 cells. A) Bright field; B) Positive SARS-CoV-2 infected VeroE6 cells (positive control cells); and C) Bright field; D) VeroE6 cells inoculated with passage three of a RT-qPCR+ *Culicoides sonorensis* day 10 homogenate. (Magnification 10x)