

1 **Antiviral activity of Pacific oyster (*Crassostrea gigas*) hemolymph**
2 **against a human coronavirus.**

3 Rebecca L. Pedler^a, James O. Harris^a, Peter G. Speck^{a#}

4 ^aCollege of Science and Engineering, Flinders University, Bedford Park, South Australia,
5 Australia.

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7 Running Head: Antiviral activity of *C. gigas* against HCoV-229E

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9 #Address correspondence to Peter G. Speck, peter.speck@flinders.edu.au

10 Mailing address:

11 *College of Science and Engineering, Flinders University, Bedford Park,*

12 *GPO Box 2100*

13 *Adelaide 5001,*

14 *South Australia, Australia.*

15 **ABSTRACT**

16 Coronaviruses can cause severe respiratory infections in humans. This study aimed to assess
17 the antiviral activity of Pacific oyster (*Crassostrea gigas*) hemolymph against a human
18 coronavirus, HCoV-229E. An eight-fold reduction in infectivity of HCoV-229E on Huh-7
19 cells was observed in the presence of 10% *C. gigas* hemolymph. Antiviral activity of *C. gigas*
20 hemolymph positively correlated with its concentration and appears to be active during an
21 intracellular stage of HCoV-229E infection.

22 **KEYWORDS:** molluscan antivirals, HCoV-229E, marine invertebrates, *Crassostrea gigas*,
23 SARS-CoV-2

24 Human coronaviruses are enveloped, single stranded RNA viruses that are further classified
25 as alpha-coronaviruses (human coronavirus-229E (HCoV-229E) and HCoV-NL63) or beta-
26 coronaviruses (HCoV-OC43, HCoV-HKU1, middle eastern respiratory syndrome (MERS-
27 CoV), severe acute respiratory syndrome (SARS-CoV) and SARS-CoV-2) (1). SARS-CoV-2
28 is a novel human coronavirus which emerged in December 2019 as the causative agent of
29 coronavirus disease 2019 (Covid-19) (2-4). Safe and effective antiviral treatments for SARS-
30 CoV-2 are yet to be identified, despite multiple drug repurposing attempts (5-7).

31

32 Marine molluscs represent an unexploited source of medicinal compounds (Pedler and
33 Speck, Rev. Med. Virol, in press) (8-11). Marine molluscs lack an adaptive immune system
34 and exclusively elicit innate immune responses (12-14), while living in an environment
35 containing virus particles in the order of $>10^7$ per ml (15, 16). This demonstrates the success
36 of their strategies to prevent viral infection, which includes production of potent antiviral
37 compounds (11, 13). *In vitro* inhibition of HSV-1 has been observed using extracts from the
38 common cockle (*Cerastoderma edule*), greenlip abalone (*Haliotis laevis*) (17), Japanese
39 carpet shell (*Ruditapes philippinarum*), European flat oyster (*Ostrea edulis*), common whelk
40 (*Buccinum undatum*) (18), blacklip abalone (*Haliotis rubra*) (19, 20), veined rapa whelk
41 (*Rapanosa venosa*) (21) and Mediterranean mussel (*Mytilus galloprovincialis*) (22). Extract
42 from the flesh of the red abalone (*Haliotis rufescens*) protect mice against poliovirus and
43 influenza A (23, 24), while paolin II from the Eastern oyster (*Crassostrea virginica*) inhibits
44 poliovirus (25).

45

46 Hemolymph of the Pacific oyster (*Crassostrea gigas*) has *in vitro* antiviral activity against
47 HSV-1 and adenovirus respiratory strain 5 (AdV-5) (26-28). The major *C. gigas* hemolymph
48 protein, cavortin, exerts an antiviral effect against HSV-1 after entry into Vero cells (26).

49 Cavortin is a Mr 20,000 protein which acts as a metal chaperone (29). Intracellular zinc has
50 therapeutic potential for SARS-CoV-2 (30-32), and its efficacy is improved by coupling with
51 a metal chaperone (31, 33). *Crassostrea gigas* has a high zinc content (34) and given
52 cavortin's suggested role as a metal chaperone (29), it is possible that *C. gigas* cavortin has
53 potential antiviral activity against SARS-CoV-2 and may facilitate zinc transport into host
54 cells.

55

56 The discovery of antiviral agents for SARS-CoV-2 is challenged by the limited number of
57 laboratories with the appropriate biosafety containment level (35, 36). HCoV-229E can be
58 handled in lesser-rated laboratories making it more accessible for research on human
59 coronaviruses (37) and this virus could be used for initial screening for anti-coronavirus
60 activity. This study is the first we are aware of that assesses antiviral activity of *C. gigas*
61 hemolymph against HCoV-229E.

62

63 Huh-7 cells, obtained from M. Beard were grown in Dulbecco's modified eagle medium
64 (DMEM) (Gibco #11965118) supplemented with 10% foetal bovine serum (FBS) (Gibco
65 #10099141), according to standard methods (38). Twelve *C. gigas* oysters, grown in Coffin
66 Bay, South Australia, were purchased from local seafood merchants. After opening, *C. gigas*
67 hemolymph was extracted from the pericardial cavity using a sterile syringe and 27g needle.
68 Hemolymph was pooled, sterilised using a 0.2 μ m filter and stored at -20°C until required.
69 Cytotoxicity of *C. gigas* hemolymph was determined using a trypan blue exclusion assay
70 (26). Huh-7 cells were seeded into a 24-well plate with medium as above and 0%, 2%, 5%,
71 10% or 20% (v/v) *C. gigas* hemolymph. Cells were incubated for two days at 37°C in 5%
72 CO₂, before being stained *in situ* with 0.4% trypan blue (Gibco #15250061) (26). The number
73 of non-viable cells in three different fields of view were counted using an Olympus CK2

74 microscope at 40x magnification (39). The mean number of non-viable cells was lowest for
75 0% (1.00 ± 0.00 cells) and 2% (1.00 ± 0.47 cells) (Fig. 1). Huh-7 cell death sharply increased
76 as hemolymph concentration exceeded 10% (Fig. 1), therefore 10% was considered an
77 appropriate concentration for use in anti-HCoV-229E assays. In Vero cells, *C. gigas*
78 hemolymph can cause 10% cell death at a concentration of 13% (v/v) (26) or 50% cell death
79 at $750\mu\text{g ml}^{-1}$ (27). The acellular and cellular fractions of *C. gigas* hemolymph can cause
80 50% cell death in Hep-2 cells at concentrations of 0.32mg ml^{-1} and 0.19mg ml^{-1} respectively
81 (28).

82

83 HCoV-229E was obtained from H. Whiley . Virus titres were determined as 50% tissue
84 culture infective doses (TCID₅₀) (40). Huh-7 cells were seeded into 96-well plates contain
85 either 0 or 10% *C. gigas* hemolymph. Three ten-fold dilutions, followed by eight two-fold
86 dilutions were prepared using HCoV-229E stock and DMEM and inoculated into 96-well
87 plates. Cells were incubated at 37°C in 5% CO₂ for five days before being fixed with 10%
88 formaldehyde and stained with 0.5% crystal violet (Thermo #S25275B). Wells illustrating
89 cytopathic effect were counted, allowing TCID₅₀ to be calculated using the Reed-Muench
90 method (41). When Huh-7 cells were assayed with 10% *C. gigas* hemolymph, an eight-fold
91 reduction in the HCoV-229E titre (4.00×10^5 TCID₅₀ ml⁻¹) which is an antiviral activity of
92 87.5%, was observed (Table 1). A dose-response curve was generated using 0%, 2%, 5%,
93 10% and 15% *C. gigas* hemolymph which revealed that antiviral activity positively correlated
94 with its concentration (Table 1, Fig. 3). This is consistent with the dose-dependent antiviral
95 activity of *C. gigas* hemolymph protein, cavortin, against HSV-1 (26).

96

97 Time of addition assays were used to determine the stage of HCoV-229E infection targeted
98 by *C. gigas* hemolymph. In previous studies, the greatest antiviral protection of Vero cells,

99 from HSV-1 infection was observed when *C. gigas* hemolymph was added 0-2 hours after
100 infection (26, 27), suggesting that the antiviral effect was likely exerted after virus attachment
101 and entry. An intracellular mode of antiviral action has been observed for other molluscan
102 compounds, including lipophilic extract of *H. laevigata* (17) and myticin C peptides from *M.*
103 *galloprovincialis* (22). Here, Huh-7 cells were seeded into 24-well plates with DMEM and
104 10% FBS. A series of dilutions (10^{-4} , 10^{-5} , 10^{-6} and 10^{-7}) were prepared using HCoV-229E
105 stock and DMEM and inoculated into plates. *C. gigas* hemolymph was added immediately or
106 60 minutes after HCoV-229E. There was little difference in *C. gigas* hemolymph antiviral
107 activity when it was added to Huh-7 cells immediately (98.21%) or 60 minutes after HCoV-
108 229E infection (96.11%) (Table 2). This suggests that *C. gigas* hemolymph most likely acts
109 during an intracellular stage of HCoV-229E infection. Antiviral compounds which act during
110 an intracellular stage of HCoV-229E infection have been identified (42-44). The macrolide
111 and immunosuppressive compound, FK06 inhibits HCoV-229E replication in Huh-7 cells
112 (44), while the antimalarial drug chloroquine inhibits HCoV-229E replication in epithelial
113 lung cells (L132) by suppressing P38MAPK (43). Thapsigargin, from the Thapsia (*Thapsia*
114 *garganica*) plant acts during an intracellular stage of HCoV-229E infection either by
115 inhibiting replication or activating unknown antiviral effector systems in Huh-7 cells (42).
116
117 This study reveals that *C. gigas* hemolymph has *in vitro* antiviral activity against human
118 coronavirus HCoV-229E. This finding is relevant in the current pandemic and reinforces that
119 *C. gigas* hemolymph has broad-spectrum antiviral activity. Further research is required to
120 identify and characterise the antiviral compound(s) produced by *C. gigas*.

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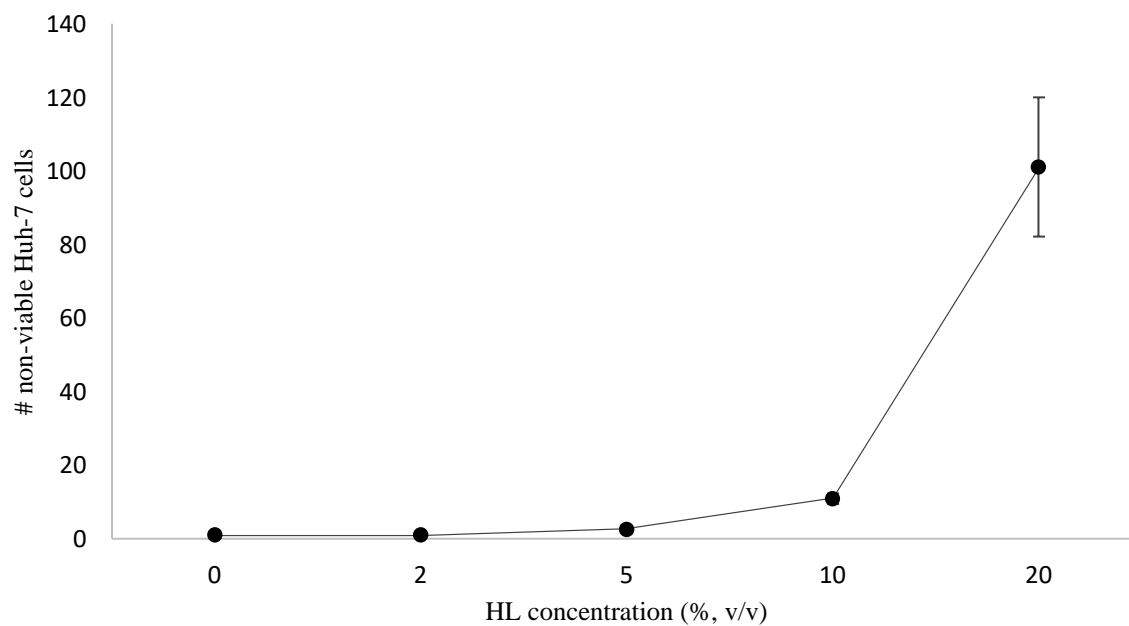
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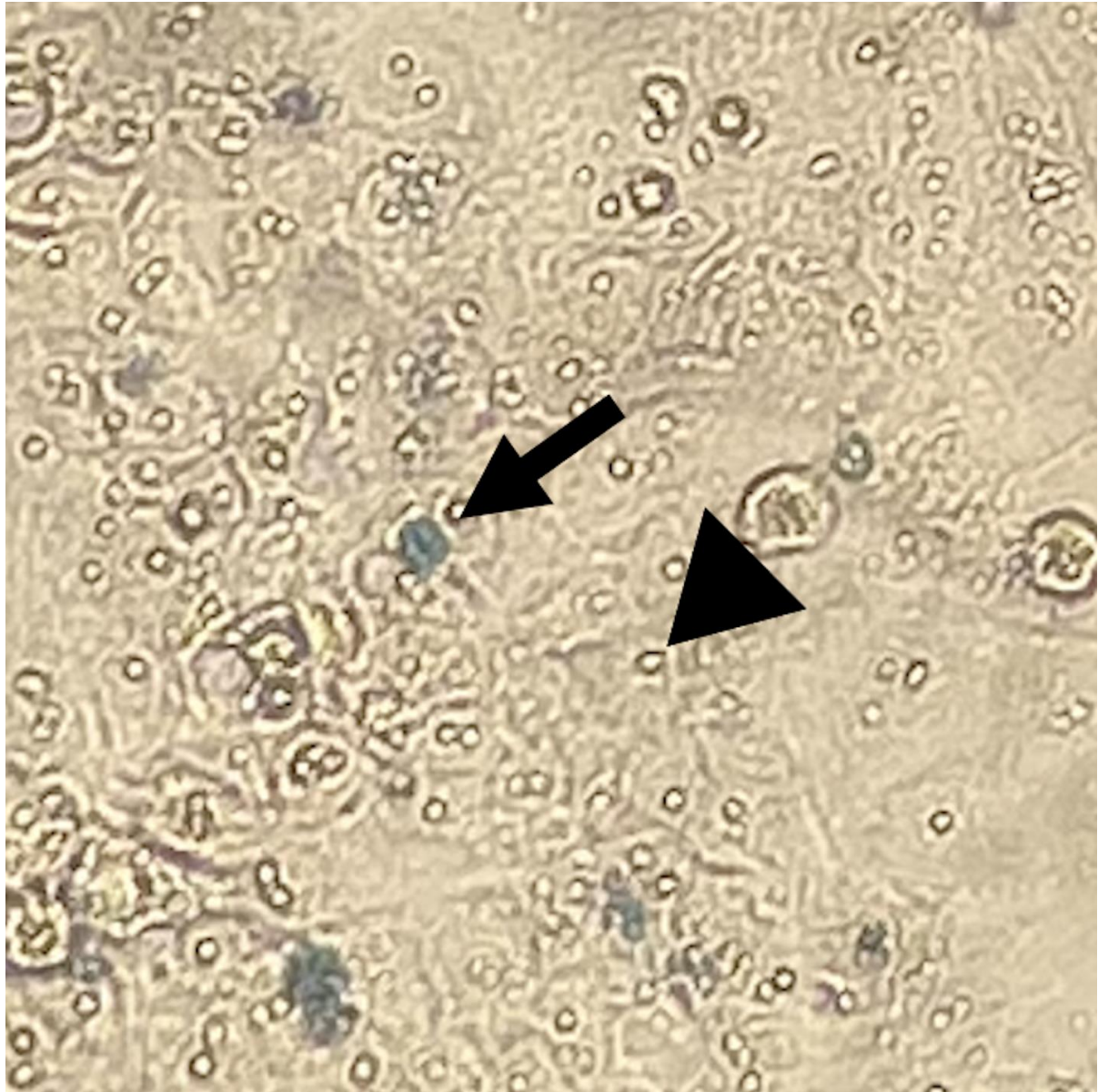
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250 FIG. 1. Mean (\pm standard deviation) number of non-viable Huh-7 cells treated with varying
251 concentrations of Pacific oyster (*Crassostrea gigas*) hemolymph (0, 2, 5, 10, 20% v/v).



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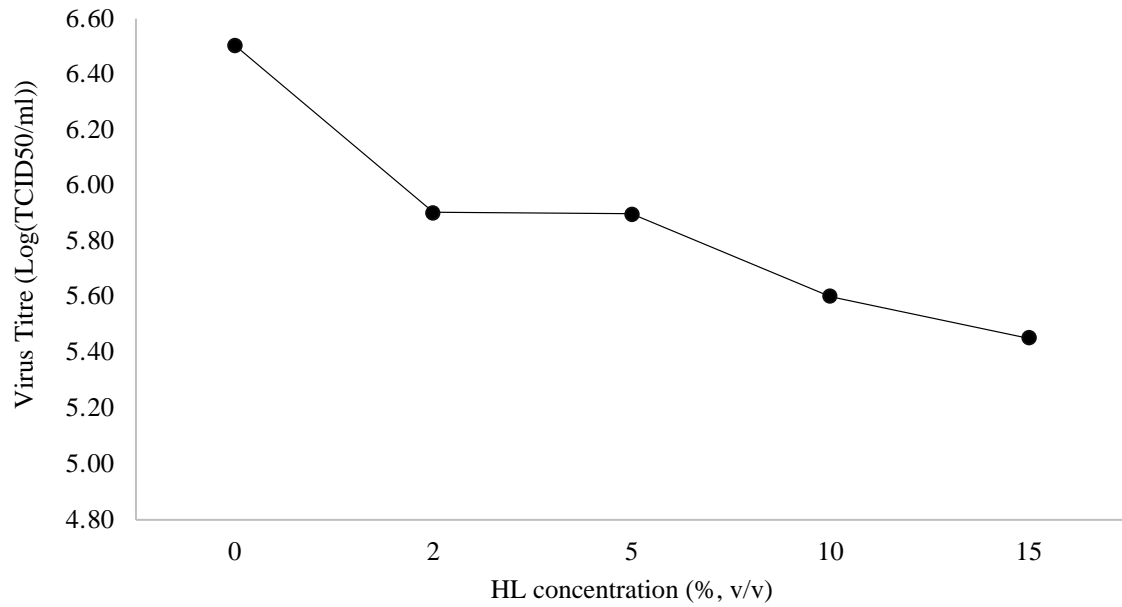
253 FIG. 2. non-viable (arrow) and viable (arrowhead) Huh-7 cells treated with 20% Pacific

254 oyster (*Crassostrea gigas*) hemolymph.

255 TABLE 1. Virus titre values (TCID₅₀ ml⁻¹) for human coronavirus 229E (HCoV-229E) in
256 Huh-7 cells treated with varying concentrations (0, 2, 5, 10, 15% v/v) of Pacific oyster
257 (*Crassostrea gigas*) hemolymph.

Extract/control	Virus titre (TCID ₅₀ ml ⁻¹)	Antiviral activity: % Reduction in virus titre
Negative control (DMEM)	3.20 × 10 ⁶	0.00
2% <i>C. gigas</i> hemolymph	8.00 × 10 ⁵	75.00
5% <i>C. gigas</i> hemolymph	7.92 × 10 ⁵	75.24
10% <i>C. gigas</i> hemolymph	4.00 × 10 ⁵	87.50
15% <i>C. gigas</i> hemolymph	2.83 × 10 ⁵	91.16

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260 FIG. 3. Virus titre values ($\text{Log}(\text{TCID}_{50} \text{ ml}^{-1})$) for human coronavirus 229E (HCoV-229E) in
261 Huh-7 cells treated with varying concentrations (0, 2, 5, 10, 15% v/v) of Pacific oyster
262 (*Crassostrea gigas*) hemolymph (HL).

263 TABLE 2. Virus titres (TCID₅₀ ml⁻¹) and antiviral activity (% reduction in virus titre) for
264 human coronavirus 229E (HCoV-229E) in Huh-7 cells treated with either 0% (negative
265 control) or 10% Pacific oyster (*Crassostrea gigas*).

Extract/control	Time of extract addition	Virus titre (TCID ₅₀ ml ⁻¹)	Antiviral activity: % reduction in virus titre
Negative control (DMEM)	-	2.79×10^8	0.00
10% <i>C. gigas</i> hemolymph	Immediately after infection	5.00×10^6	98.21
10% <i>C. gigas</i> hemolymph	60minutes after infection	1.08×10^7	96.11

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