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

Rebecca L. Pedler[a], James O. Harris[a], Peter G. Speck[a]#

[a]College of Science and Engineering, Flinders University, Bedford Park, South Australia, Australia.

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

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

Mailing address:

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

GPO Box 2100

Adelaide 5001,

South Australia, Australia.

ABSTRACT

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

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

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

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

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

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

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

Huh-7 cells, obtained from M. Beard were grown in Dulbecco's modified eagle medium (DMEM) (Gibco #11965118) supplemented with 10% foetal bovine serum (FBS) (Gibco #10099141), according to standard methods (38). Twelve *C. gigas* oysters, grown in Coffin Bay, South Australia, were purchased from local seafood merchants. After opening, *C. gigas* hemolymph was extracted from the pericardial cavity using a sterile syringe and 27g needle. Hemolymph was pooled, sterilised using a 0.2μm filter and stored at -20°C until required. Cytotoxicity of *C. gigas* hemolymph was determined using a trypan blue exclusion assay (26). Huh-7 cells were seeded into a 24-well plate with medium as above and 0%, 2%, 5%, 10% or 20% (v/v) *C. gigas* hemolymph. Cells were incubated for two days at 37°C in 5% CO_2, before being stained *in situ* with 0.4% trypan blue (Gibco #15250061) (26). The number 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 750µg 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 ($TCID_{50}$) (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% CO_2 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 $TCID_{50}$ 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 $TCID_{50}$ 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,

from HSV-1 infection was observed when *C. gigas* hemolymph was added 0-2 hours after infection (26, 27), suggesting that the antiviral effect was likely exerted after virus attachment and entry. An intracellular mode of antiviral action has been observed for other molluscan compounds, including lipophilic extract of *H. laevigata* (17) and myticin C peptides from *M. galloprovincialis* (22). Here, Huh-7 cells were seeded into 24-well plates with DMEM and 10% FBS. A series of dilutions (10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) were prepared using HCoV-229E stock and DMEM and inoculated into plates. *C. gigas* hemolymph was added immediately or 60 minutes after HCoV-229E. There was little difference in *C. gigas* hemolymph antiviral activity when it was added to Huh-7 cells immediately (98.21%) or 60 minutes after HCoV-229E infection (96.11%) (Table 2). This suggests that *C. gigas* hemolymph most likely acts during an intracellular stage of HCoV-229E infection. Antiviral compounds which act during an intracellular stage of HCoV-229E infection have been identified (42-44). The macrolide and immunosuppressive compound, FK06 inhibits HCoV-229E replication in Huh-7 cells (44), while the antimalarial drug chloroquine inhibits HCoV-229E replication in epithelial lung cells (L132) by suppressing P38MAPK (43). Thapsigargin, from the Thapsia (*Thapsia garganica*) plant acts during an intracellular stage of HCoV-229E infection either by inhibiting replication or activating unknown antiviral effector systems in Huh-7 cells (42).

This study reveals that *C. gigas* hemolymph has *in vitro* antiviral activity against human coronavirus HCoV-229E. This finding is relevant in the current pandemic and reinforces that *C. gigas* hemolymph has broad-spectrum antiviral activity. Further research is required to identify and characterise the antiviral compound(s) produced by *C. gigas.*

REFERENCES

1. Woo PCY, Huang Y, Lau SKP, Yuen K-Y. 2010. Coronavirus genomics and bioinformatics analysis. Viruses 2:1805-1820.
2. Wang C, Horby PW, Hayden FG, Gao GF. 2020. A novel coronavirus outbreak of global health concern. Lancet 395:470-473.

3. Ghanbari R, Teimoori A, Sadeghi A, Mohamadkhani A, Rezasoltani S, Asadi E, Jouyban A, Sumner SCJ. 2021. Existing antiviral options against SARS-CoV-2 replication in COVID-19 patients. Future Microbiol 15.
4. Triggle CR, Bansal D, Ding H, Islam MM, Farag EABA, Hadi HA, Sultan AA. 2021. A comprehensive review of viral characteristics, transmission, pathophysiology, immune response, and management of SARS-CoV-2 and COVID-19 as a basis for controlling the pandemic. Front Immunol 12:631139.
5. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, Shi Z, Hu Z, Zhong W, Xiao G. 2020. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res 30:269-271.
6. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, Hohmann E, Chu HY, Luetkemeyer A, Kline S, Lopez de Castilla D, Finberg RW, Dierberg K, Tapson V, Hsieh L, Patterson TF, Paredes R, Sweeney DA, Short WR, Touloumi G, Lye DC, Ohmagari N, Oh M-d, Ruiz-Palacios GM, Benfield T, Fätkenheuer G, Kortepeter MG, Atmar RL, Creech CB, Lundgren J, Babiker AG, Pett S, Neaton JD, Burgess TH, Bonnett T, Green M, Makowski M, Osinusi A, Nayak S, Lane HC. 2020. Remdesivir for the treatment of Covid-19 — final report. N Engl J Med 383:1813-1826.
7. Indari O, Jakhmola S, Manivannan E, Jha HC. 2021. An update on antiviral therapy against SARS-CoV-2: How far have we come? Front Pharmacol 12:632677.
8. Benkendorff K. 2010. Molluscan biological and chemical diversity: secondary metabolites and medicinal resources produced by marine molluscs. Biol Rev Camb Philos Soc 85:757-775.
9. Summer K, Browne J, Liu L, Benkendorff K. 2020. Molluscan compounds provide drug leads for the treatment and prevention of respiratory disease. Mar Drugs 18:570.
10. Riccio G, Ruocco N, Mutalipassi M, Costantini M, Zupo V, Coppola D, de Pascale D, Lauritano C. 2020. Ten-year research update review: Antiviral activities from marine organisms. Biomolecules 10:1-36.
11. Sagar S, Kaur M, Minneman KP. 2010. Antiviral lead compounds from marine sponges. Mar Drugs 8:2619-2638.
12. Agius JR, Corbeil S, Helbig KJ. 2020. Immune control of herpesvirus infection in molluscs. Pathogens (Basel) 9:1-11.
13. Dang VT, Benkendorff K, Green T, Speck P. 2015. Marine snails and slugs: A great place to look for antiviral drugs. J Virol 89:8114-8118.
14. Tiscar PG, Mosca F. 2004. Defense mechanisms in farmed marine molluscs. Vet Res Commun 28:57-62.
15. Bratbak G, Heldal M, BØrsheim KY, Bergh Ø. 1989. High abundance of viruses found in aquatic environments. Nature 340:467-468.
16. Suttle CA. 2007. Marine viruses - major players in the global ecosystem. Nat Rev Microbiol 5:801-812.
17. Dang VT, Benkendorff K, Speck P. 2011. *In vitro* antiviral activity against herpes simplex virus in the abalone *Haliotis laevigata*. J Gen Virol 92:627-637.
18. Defer D, Bourgougnon N, Fleury Y. 2009. Screening for antibacterial and antiviral activities in three bivalve and two gastropod marine molluscs. Aquaculture 293:1-7.
19. Talaei Zanjani N, Miranda-Saksena M, Valtchev P, Diefenbach RJ, Hueston L, Diefenbach E, Sairi F, Gomes VG, Cunningham AL, Dehghani F. 2016. Abalone hemocyanin blocks the entry of herpes simplex virus 1 into cells: A potential new antiviral strategy. Antimicrob Agents Chemother 60:1003-1012.

20. Dang VT, Speck P, Doroudi M, Smith B, Benkendorff K. 2011. Variation in the antiviral and antibacterial activity of abalone *Haliotis laevigata*, *H. rubra* and their hybrid in South Australia. Aquaculture 315:242-249.
21. Nesterova NV, Zagorodnya SD, Moshtanska V, Dolashka P, Baranova GV, Golovan AV, Kurova AO. 2011. Antiviral activity of hemocyanin isolated from marine snail *Rapana venosa*. Antiviral Res 90:A38-A38.
22. Novoa B, Romero A, Álvarez ÁL, Moreira R, Pereiro P, Costa MM, Dios S, Estepa A, Parra F, Figueras A. 2016. Antiviral activity of myticin C peptide from mussel: An ancient defense against herpesviruses. J Virol 90:7692-7702.
23. Li C, Precott B, Jahnes W. 1962. Antiviral activity of a fraction of abalone juice. Proc Soc Exo Biol Med 109:534-538.
24. Li C. 1960. Antimicrobial effect of abalone juice. Proc Soc Exo Biol Med 103:522-524.
25. Prescott B, Li C, Caldes G, Martino E. Chemical studies of Paolin. II. An antiviral substance from oysters. Proc Soc Exo Biol Med 123:460-464.
26. Green TJ, Robinson N, Chataway T, Benkendorff K, O'Connor W, Speck P. 2014. Evidence that the major hemolymph protein of the Pacific oyster, *Crassostrea gigas*, has antiviral activity against herpesviruses. Antiviral Res 110:168-174.
27. Olicard C, Renault T, Torhy C, Benmansour A, Bourgougnon N. 2005. Putative antiviral activity in hemolymph from adult Pacific oysters, *Crassostrea gigas*. Antiviral Res 66:147-152.
28. Carriel-Gomes MC, Kratz JM, Muller VDM, Barardi CRM, Simues CMO. 2006. Evaluation of antiviral activity in hemolymph from oysters *Crassostrea rhizophorae* and *Crassostrea gigas*. Aquat Living Resour 19:189-193.
29. Scotti PD, Dearing SC, Greenwood DR. 2007. Characterisation of cavortin, the major haemolymph protein of the Pacific oyster (*Crassostrea gigas*). N Z J Mar Freshwater Res 41:91-101.
30. Carlucci PM, Ahuja T, Petrilli C, Rajagopalan H, Jones S, Rahimian J. 2020. Zinc sulfate in combination with a zinc ionophore may improve outcomes in hospitalized COVID-19 patients. J Med Microbiol 69:1228-1234.
31. Brewer J, Gomez Marti JL, Brufsky A. 2021. Potential interventions for SARS-CoV-2 infections: Zinc showing promise. J Med Virol 93:1201-1203.
32. Asl SH, Nikfarjam S, Majidi Zolbanin N, Nassiri R, Jafari R. 2021. Immunopharmacological perspective on zinc in SARS-CoV-2 infection. Int Immunopharmacol 96:107630.
33. te Velthuis AJW, van den Worml SHE, Sims AC, Baric RS, Snijder EJ, van Hemert MJ. 2010. Zn2+ inhibits coronavirus and arterivirus RNA polymerase activity in vitro and zinc ionophores block the replication of these viruses in cell culture. PLoS pathogens 6:e1001176.
34. Kong N, Zhao Q, Liu C, Li J, Liu Z, Gao L, Wang L, Song L. 2020. The involvement of zinc transporters in the zinc accumulation in the Pacific oyster *Crassostrea gigas*. Gene 750:144759-144759.
35. Kaufer AM, Theis T, Lau KA, Gray JL, Rawlinson WD. 2020. Laboratory biosafety measures involving SARS-CoV-2 and the classification as a Risk Group 3 biological agent. Pathology 52:790-795.
36. Hall MD, Anderson JM, Anderson A, Baker D, Bradner J, Brimacombe KR, Campbell EA, Corbett KS, Carter K, Cherry S, Chiang L, Cihlar T, de Wit E, Denison M, Disney M, Fletcher CV, Ford-Scheimer SL, Götte M, Grossman AC, Hayden FG, Hazuda DJ,

Lanteri CA, Marston H, Mesecar AD, Moore S, Nwankwo JO, O'Rear J, Painter G, Saikatendu KS, Schiffer CA, Sheahan TP, Shi P-Y, Smyth HD, Sofia MJ, Weetall M, Weller SK, Whitley R, Fauci AS, Austin CP, Collins FS, Conley AJ, Davis MI. 2021. Report of the national institutes of health SARS-CoV-2 antiviral therapeutics summit. J Infect Dis 224:S1-S21.

37. Bracci N, Pan H-C, Lehman C, Kehn-Hall K, Lin S-C. 2020. Improved plaque assay for human coronaviruses 229E and OC43. PeerJ 8:e10639-e10639.
38. Thermofisher. 2021. Gibco Cell Culture Basics. https://www.thermofisher.com/au/en/home/references/gibco-cell-culture-basics.html. Accessed October 24th.
39. Strober W. 2019. Trypan blue exclusion test of cell viability. Curr Protoc Immunol 111.
40. Smither SJ, Lear-Rooney C, Biggins J, Pettitt J, Lever MS, Olinger GG. 2013. Comparison of the plaque assay and 50% tissue culture infectious dose assay as methods for measuring filovirus infectivity. J Virol Methods 193:565-571.
41. Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. Am J Epidemiol 27.
42. Shaban MS, Müller C, Mayr-Buro C, Weiser H, Meier-Soelch J, Albert BV, Weber A, Linne U, Hain T, Babayev I, Karl N, Hofmann N, Becker S, Herold S, Schmitz ML, Ziebuhr J, Kracht M. 2021. Multi-level inhibition of coronavirus replication by chemical ER stress. Nature communications 12:5536-5536.
43. Kono M, Tatsumi K, Imai AM, Saito K, Kuriyama T, Shirasawa H. 2008. Inhibition of human coronavirus 229E infection in human epithelial lung cells (L132) by chloroquine: Involvement of p38 MAPK and ERK. Antiviral Res 77:150-152.
44. Carbajo-Lozoya J, Müller MA, Kallies S, Thiel V, Drosten C, von Brunn A. 2012. Replication of human coronaviruses SARS-CoV, HCoV-NL63 and HCoV-229E is inhibited by the drug FK506. Virus Res 165:112-117.

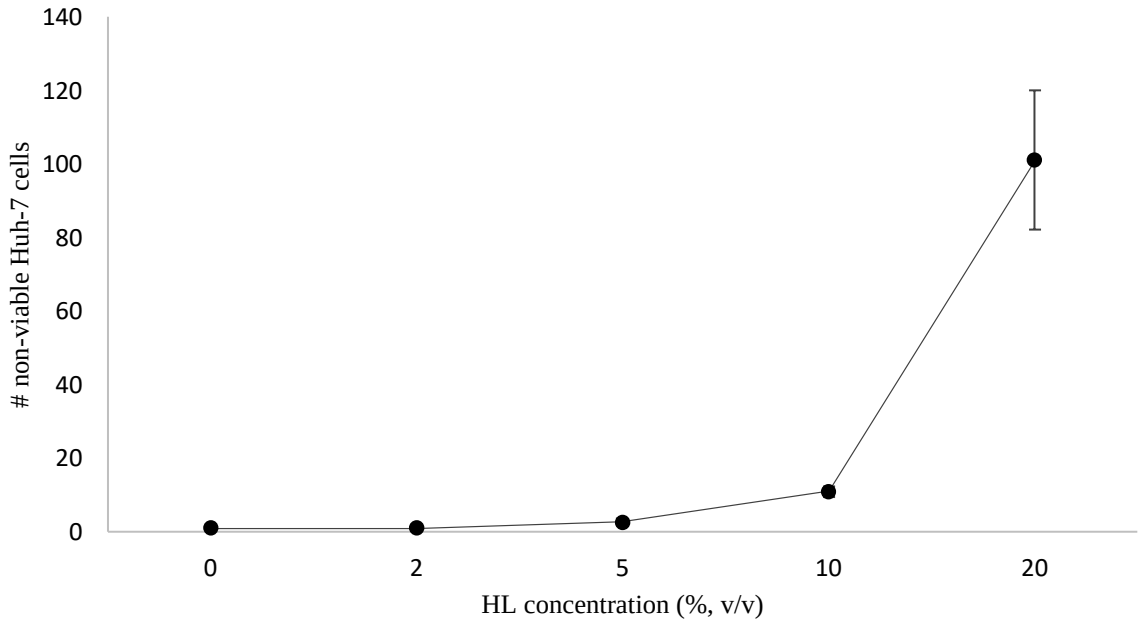


FIG. 1. Mean (± standard deviation) number of non-viable Huh-7 cells treated with varying concentrations of Pacific oyster (*Crassostrea gigas*) hemolymph (0, 2, 5, 10, 20% v/v).

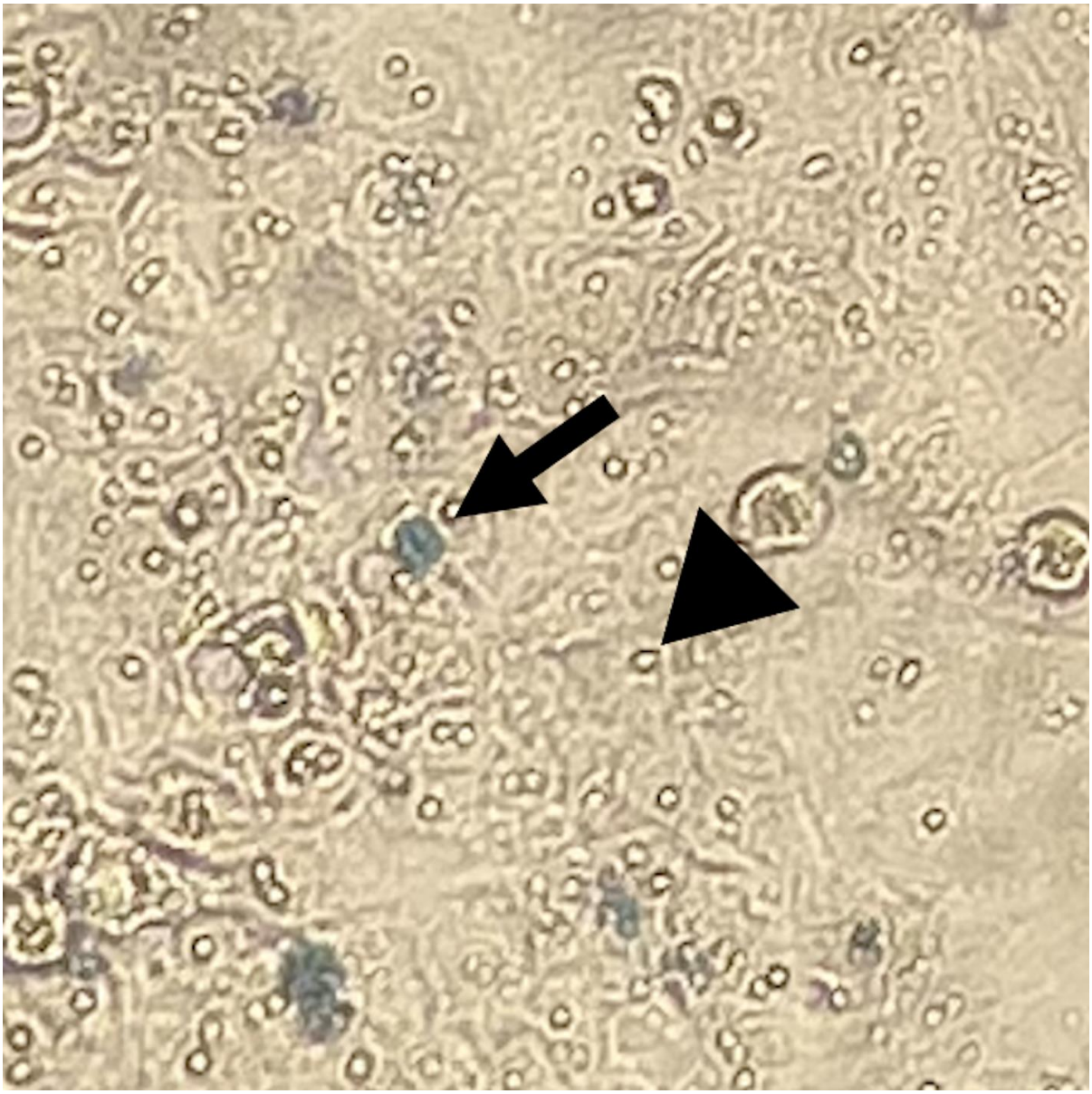
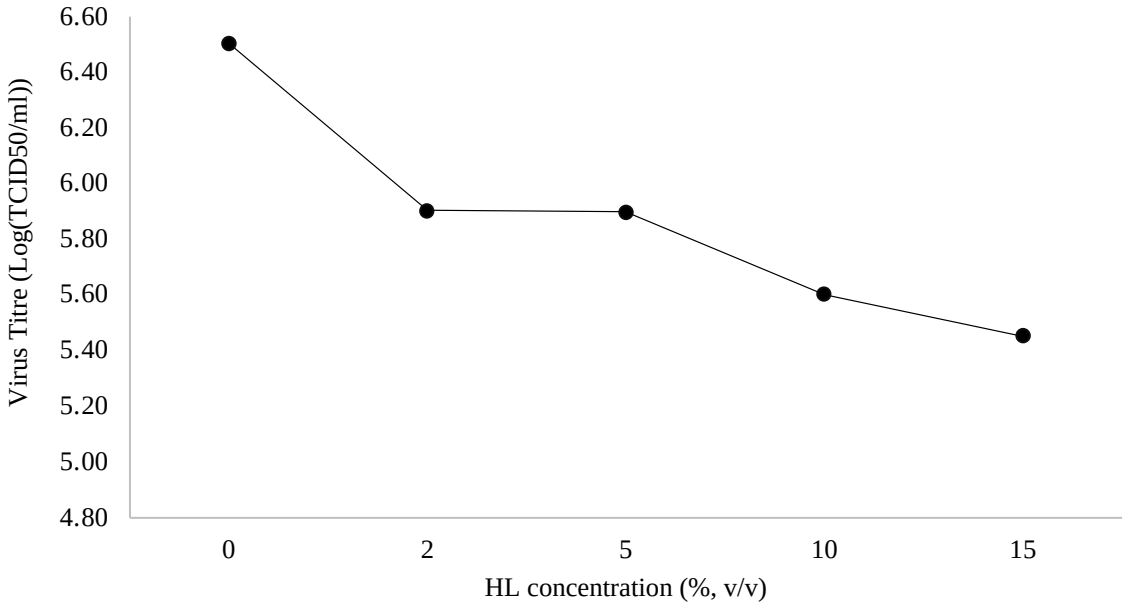


FIG. 2. non-viable (arrow) and viable (arrowhead) Huh-7 cells treated with 20% Pacific oyster (*Crassostrea gigas*) hemolymph.

255 TABLE 1. Virus titre values ($TCID_{50}\ ml^{-1}$) 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% *C. gigas* hemolymph	8.00×10^5	75.00
5% *C. gigas* hemolymph	7.92×10^5	75.24
10% *C. gigas* hemolymph	4.00×10^5	87.50
15% *C. gigas* hemolymph	2.83×10^5	91.16

258



259

260 FIG. 3. Virus titre values (Log($TCID_{50}$ 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 ($TCID_{50}\,ml^{-1}$) 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% *C. gigas* hemolymph	Immediately after infection	5.00×10^6	98.21
10% *C. gigas* hemolymph	60minutes after infection	1.08×10^7	96.11

266