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SARS-CoV-2 surrogate (*Phi6*) environmental persistence within free-living amoebae

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ABSTRACT

The reported persistence of SARS-CoV-2 virions in aquatic environments highlights the need to better understand potential mechanisms that may prolong its dissemination. We evaluated the possibility that amoebae might serve as transport hosts by studying the interaction of the enveloped bacteriophage Phi6, as a potential surrogated along with one of the most common amoebae in engineered aquatic environments, *Vermamoeba vermiformis*. Using microscopy, imaging flow cytometry and bacteriophage cell culture, our results imply that the SARS-CoV-2 surrogate triggers amoebic mitochondria and induced apoptosis to promote viral persistence in trophozoites. Furthermore, virus-infected amoebae were still infectious after 2 months within FLA cysts. These results suggest that amoebae could contribute to the environmental persistence of SARS-CoV-2, including disinfection processes. In addition, amoebae could be a successful model system for understanding respiratory virus-eukaryotic biology at the cellular and molecular levels.

Key words: apoptosis, bacteriophage Phi6, environmental persistence, free-living amoebae, mitochondria, SARS-CoV-2, transmission

HIGHLIGHTS

- Amoebic mitochondria serve as a support organelle platform for the SARS-CoV-2 surrogate persistence.
- Long-term stability of RNA enveloped viruses in amoeba-forming cysts.
- Free-living amoebae as a potential vector and environmental reservoir for RNA enveloped viruses.

INTRODUCTION

Free-living amoebae (FLA) are ubiquitous in natural and engineered aquatic and moist environments (Jadin 1973; Rodríguez-Zaragoza 1994; Lau & Ashbolt 2009; Samba-Louaka *et al.* 2019), and known natural environmental reservoirs for a plethora of respiratory pathogens and a vector of human water-associated infections (Rowbotham 1986; Essig *et al.* 1997; la Scola *et al.* 2003; Lamoth & Greub 2010; Bousbia *et al.* 2013; Santos-Montañez *et al.* 2015; Taylor-Mulneix *et al.* 2017). Increasing evidence suggests that FLA are potential environmental reservoirs for human viruses (enteric and respiratory) (Danes & Cerva 1981; la Scola *et al.* 2005; Mattana *et al.* 2006; Scheid & Schwarzenberger 2012; Boratto *et al.* 2014; Hsueh & Gibson 2015; Atanasova *et al.* 2018; Folkins *et al.* 2020), but how the virus infect and survive in amoeba hosts is unclear. To date, there is also no epidemiologic evidence for waterborne transmission of COVID-19, unlike SARS-CoV-1 demonstrated via wastewater aerosols (Yu *et al.* 2014). However, we have recently reported that enveloped human respiratory syncytial virus (RSV) persists within various FLA genera and could be transmitted via extracellular amoebal-vesicles in the respiratory size range (Dey *et al.* 2021).

While the environmental persistence underlying severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2-induced COVID-19) is subject to intense investigation (Chin *et al.* 2020; Fukuta *et al.* 2021; Sala-Comorera *et al.* 2021), any potential role played by FLA is unreported. Since viral RNA of SARS-CoV-2 have been detected in various waste/water systems (Ahmed *et al.* 2020; Bivins *et al.* 2020; Haramoto *et al.* 2020; la Rosa *et al.* 2020; Rimoldi *et al.* 2020; Wu *et al.* 2020a; de Oliveira *et al.* 2021), and the presence of biota increased the aquatic persistence of infectious SARS-CoV-2 (Sala-Comorera

et al. 2021), we considered the possibility that amoebae might serve as natural hosts and increase its persistence for environmental dispersal.

We tested the amoeba-host hypothesis by studying the interaction of the enveloped bacteriophage Phi6 surrogate, a dsRNA phage of the *Cystoviridae* family that has been a popular model for studying enveloped RNA viruses including coronaviruses SARS-CoV-1 and SARS-CoV-2 (Casanova & Weaver 2015; Aquino De Carvalho *et al.* 2017; Prussin *et al.* 2018; Silverman & Boehm 2020; di Novo *et al.* 2021; Ma *et al.* 2021; Pino *et al.* 2021; Vatter *et al.* 2021); and its association with one of the most common amoeba in human aquatic environments *Vermamoeba vermiformis* (Jadin 1973; de Moura *et al.* 1985; Bradbury 2014; Delafont *et al.* 2018).

MATERIALS AND METHODS

Propagation and purification of bacteriophages

Bacteriophages Phi6 (HER-102) and its host strain *Pseudomonas syringae* HB1OY#3 (HER-1102) were purchased from the Félix d'Hérelle Reference Center for Bacterial Viruses (Université Laval, QC, Canada). Bacteriophage Phi6 was propagated in the host strain; the host culture was grown in BBL Trypticase Soy Broth (BD, Ref# 211768) at 26 °C and 250 rpm. The overnight stock culture was added (20% v/v) to fresh Tryptic Soy Broth that also contained 5 mM of each $CaCl_2$ and $MgCl_2$ and incubated for 2 h. Then, the exponentially growing host was infected with the Phi6 bacteriophage, and the incubation was continued overnight without shaking. Overnight lysates were centrifuged at 4,000 *g* for 30 min to precipitate host cell debris and supernatant was filtered through a 0.22 mm syringe filter (Merck Millipore, REF # SLGS033SS) into a sterile Amicon Ultra 100 K centrifugal filter device (Merck Millipore, REF # UFC910024). The Ultra 100 K device was centrifuged again at 4,000 *g* for 20 min to remove growth media. The bacteriophage-containing retentate that remained on the filter part of the device (about 250 μL) was treated with DNAse I (Roche Diagnostics, cat # 10104159001) to remove residual host DNA. Twenty-five microliters of 10× DNAse I buffer (100 mM Tris HCl, pH 7.5, 25 mM $MgCl_2$, and 5 mM $CaCl_2$ in MQ water) and 2 μL of 2.5 mg/mL DNAse I, dissolved in storage buffer (20% glycerol in 75 mM NaCl), were added and the retentate was incubated for 45 min at 37 °C. All chemicals were purchased from Sigma unless stated otherwise. After the incubation, bacteriophage was rinsed with 10 mL of 1× HyClone PBS (HyClone Laboratories, REF #SH30256.02) and resuspended in PBS to the original volume.

Amoebae growth conditions

The amoeba used in this study was *V. vermiformis* (ATCC 50237) isolated from a hospital cooling tower drain. Amoebae were grown in tissue culture flasks in SCGYEM (Serum-Casein-Glucose-Yeast-Extract-Medium: ATCC medium 1021) at 25 °C in a 5% CO_2 incubator. The trophozoites were maintained in exponential growth phase by sub-culturing every 3–4 days in fresh SCGYEM, and then harvested by tapping the flasks to dislodge surface-adhered cells and washed three times with sterile distilled water with centrifugation to remove carried-over nutrients in the supernatants. Experiments were carried out in 15 mL screw-cap tubes, using 10 mL of medium.

Phi6 infectivity assays

V. vermiformis and Phi6 were co-cultured at a ratio of 1:100 in conical Falcon tubes, vortexed to favour phages interaction with amoebae and then transferred to 6-well culture plates (Fisher Scientific 130185). After 72 h incubation at 30 °C, Phi6 phages were released from amoeba trophozoites by lysing the FLA by passing the sample through a sterile 20-gauge syringe needles (BD, Franklin lakes, USA 304827) five to six times. To assess the number of internalized phages, the viral titer (plaque-forming units per mL, PFU mL^{-1}) was employed and determined by double-layer agar assay as described by Fedorenko *et al.* (2020) with minor modification. Briefly, solid and soft trypticase soy agar was prepared from BBL Trypticase Soy Broth with the addition of 1.5 and 0.6% agar, respectively. Then, 0.015% of Triphenyltetrazolium Chloride was added to the sloppy agar to increase the contrast of the plaques against the host lawn due to the formation of pink-coloured formazan by the actively growing bacteria. The concentration of internalized phages was compared with a control of Phi6-only (without *V. vermiformis*). The total number of Phi6 phage (with or without FLA) was established by performing serial 10-fold dilutions of the co-culture medium containing lysed amoebae and phages that were subsequently spread in triplicate.

Data were analyzed by means of Student's *t*-test and are means ± SEM of three independent experiments.

Induction of cyst state in *V. vermiformis*

After incubating the phages with amoeba trophozoites for 24 h, amoebae cells were washed with PBS to remove extracellular and surface-bound phages and resuspended in 5 mL sterile distilled water in culture flasks and stored at 4 °C. After 2 months, aliquots from the amoeba cysts were collected for TEM analysis and the other aliquots from the same experimental samples were resuspended in tissue culture flasks in fresh SCGYEM medium at 25 °C for 72 h for a full trophozoites state recovery. The intracellular phage concentrations (PFU mL^{-1}) were estimated with the culture method as previously described.

Imaging flow cytometry analysis

Since staining with Acridine Orange (AO) is a reliable method for identifying RNA within cells by showing an intense red fluorescence (Keeble & Jay 1962; Doynikova & Vekshin 2019), Phi6 phages were pre-stained with AO (ThermoFisher A3568) for 10 min, after staining the phages were washed and filtered through an Amicon-Ultra-15 Ultracel™ 100 K centrifugal filter unit (EMD Millipore UFC910024) several times to remove residual AO. Unexposed amoebae to AO-Phi6 provided as a control to assess if any presence of autofluorescence in the AO channel (see Supplementary Material, Figure S1).

ImageStream® cytometry analysis and the instrument gating strategy for FLA was performed as previously described (Dey *et al.* 2019). Briefly, *V. vermiformis* trophozoites were infected for 24 h with AO-Phi6 at MOI of 100, washed and resuspended in PBS prior to processing through the ImageStream®X Mark II (Millipore Sigma). Cells were examined at 60× magnification. Analysis was performed using the IDEAS software (Amnis®, Seattle) and cells (fluorescent phages and amoebae) were identified based on bright-field morphology, size, and red fluorescence signal.

Transmission electron microscopy

V. vermiformis trophozoites were co-cultured with Phi6 at an MOI of 100 on Thermonax® cover slips (Thermo Fisher 174985). After decanting the medium, amoebae were fixed at room temperature with 2.5% glutaraldehyde and 0.1M sodium cacodylate buffer (Electron Microscopy Sciences 15960). The samples were submitted for processing at the imaging core at the University of Alberta, Faculty of Biological sciences. Sectioned and carbon-coated samples were observed with a Hitachi H-7650 transmission electron microscope.

RESULTS AND DISCUSSION

AO-Phi6 phages were shown by ImageStream® flow cytometry to be internalised within the amoeba (Figure 1(a)) and further examined by transmission electron microscopy (TEM). The phage particles were observed attachment at the amoeba cell surface and present within numerous *V. vermiformis* replicative phagosomes (Figure 1(b)). Like human RSV, an enveloped

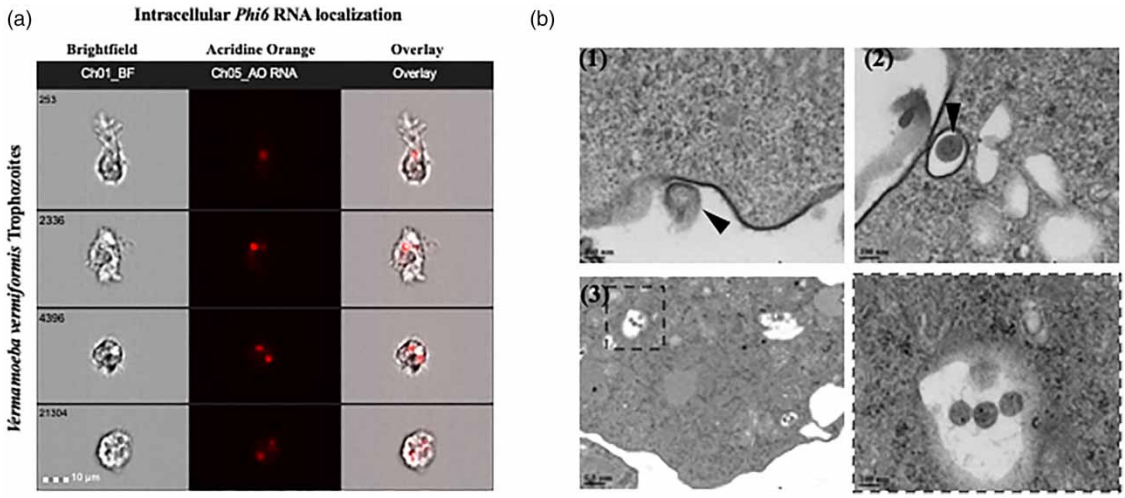

Figure 1 | Bacteriophage Phi6 internalization. (a) Localization of internalized AO-Phi6 RNA particles in live amoebae using ImageStream® flow cytometry. (b) Transmission electron micrographs of thin sections of *V. vermiformis* cells incubated with Phi6 phages for 6 h. Phi6 particles (black arrowheads) are seen at the cell surface (1), within cytoplasmic vesicle (2), and within food vacuoles (3).

human pathogen we have shown to subvert amoebal innate immunity (Dey *et al.* 2021), Phi6 altered the host mitochondrial ultrastructure at 24 h post-infection (Figure 2(a)), with swollen mitochondria and cristae disappearance by 48 h (Figure 2(b)). At 72 h post-infection, amoebae cells showed depletion of all organelles with characteristics of apoptotic cells (Figure 2(c)). Also present in Figure 2(c), are intracellular clusters of phages within apoptotic amoeba cytosol. Of note, similar results were obtained with the FLA *Willaertia magna* and *Acanthamoeba polyphaga*, indicating that the observed phenomenon was not limited to a single amoebic species (see Supplementary Material, Figure S2). To determine if internalized phages remained infectious, we performed a quantitative analysis of the intracellular phage population at 72 h post-infection. The intracellular phages were released by lysing the amoebae and then used to infect their host bacteria for quantification of the recovered phages by titration analysis. The results revealed that the phages retained their infectious capacity during the 72 h incubation period with titres peaking at ~10^6 PFU mL^{-1}, at a similar infectivity to Phi6-only controls (Figure 2(d)).

Hence, the surrogate Phi6 appears to rapidly induce amoeba apoptosis by targeting their mitochondria in order to remain infectious within 'ghost' host cells. Given the bacterial origin of mitochondria, the high mutation rates and expanded host range of the RNA-Phi6 phage (Duffy *et al.* 2006; Ferris *et al.* 2007; Ford *et al.* 2014); our results imply that the amoebic mitochondria served as a support structure for Phi6 phage persistence. Mitochondria are dynamic multifunctional organelles involved in a variety of cellular and metabolic processes including ATP production, Ca^{2+} homeostasis, and programmed cell death (Twig *et al.* 2008). An increasing number of pathogens, including viruses and bacteria, have been shown to directly or indirectly alter mitochondrial function and dynamics leading to cell death (James & Martinou 2008; Ohta & Nishiyama 2011; Kim *et al.* 2013; Ding *et al.* 2017). Interestingly and like Phi6 phage, SARS-CoV-1 and SARS-CoV-2 also target mitochondrial functions to proliferate and subsequently disseminate by killing host cells (Yan *et al.* 2004; Bordi *et al.* 2006; Shi *et al.* 2014; Gordon *et al.* 2020; Singh *et al.* 2020; Wu *et al.* 2020b; Zhu *et al.* 2020; Flynn *et al.* 2021). Additionally, evidence of evolutionary affinity between cystoviruses (*Pseudomonas* bacteriophages) and the dsRNA viruses infecting diverse

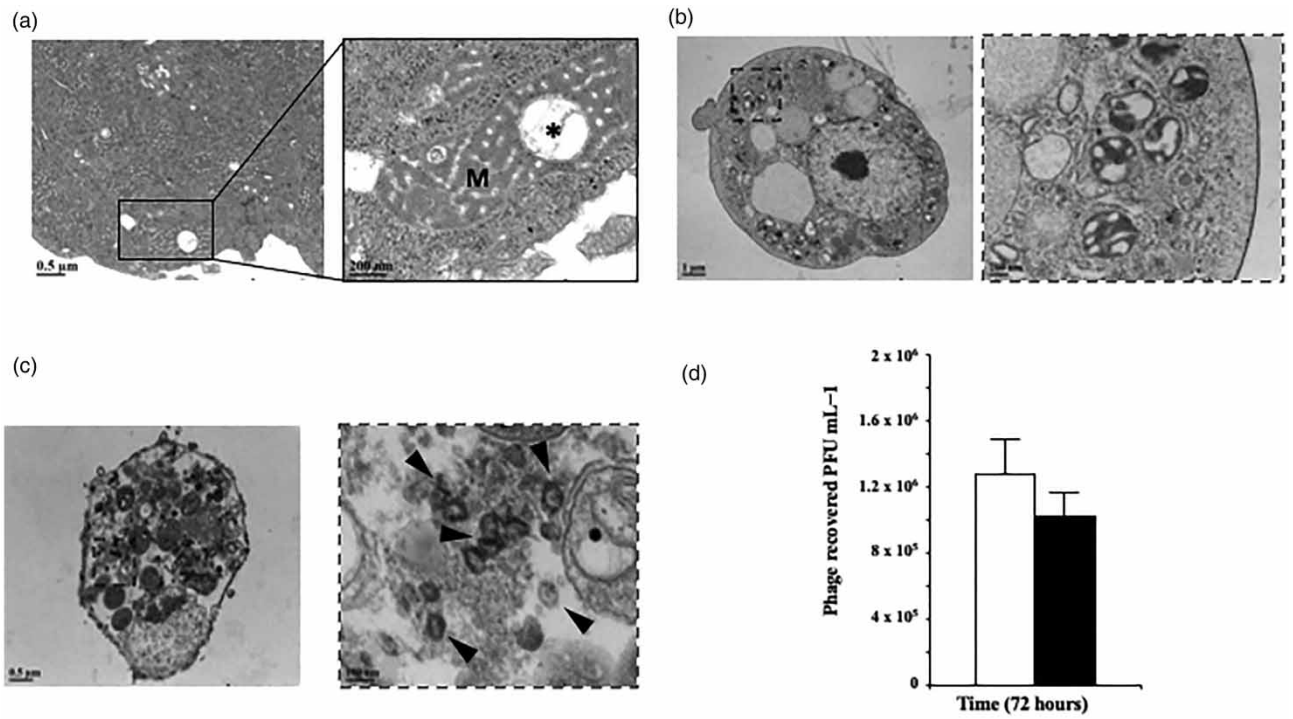

Figure 2 | *Phi6* infection induces mitochondrial alteration and cell apoptosis. Ultrastructure analysis of Phi6-infected *V. vermiformis* cells showing: (a) at 24 h post-infection, TEM of altered mitochondria (M) with dilated cristae and separation of the mitochondrial outer membrane (star). (b) The ultrastructure of various altered mitochondria and abnormal organelles after 48 h post-infection. (c) At 72 h post-infection, TEM showing apoptotic amoeba with depletion of all organelles. In the zoomed images, altered and swollen mitochondria with loss of cristae in infected cells are shown in (a and b). Phi6 particles (black arrowheads) within apoptotic amoeba cell is also shown in (c). (d) Quantification of internalized Phi6 phages from *V. vermiformis* trophozoites. Amoebae were incubated with phages for 72 h. Control Phi6 alone (white bar) and Phi6-containing amoeba trophozoites (black bar) analyzed by plaque assay.

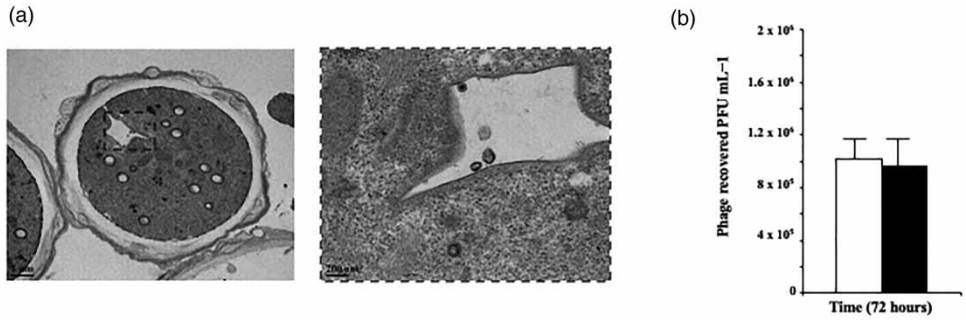

Figure 3 | Ultrastructure visualization and persistence of Phi6-containing amoeba cysts. (a) Transmission electron microscopy (TEM) of Phi6 particles contained within *V. vermiformis* cyst phagosome after 2 months of co-culture. (b) Infectivity assay of Phi6 phages from *V. vermiformis* cysts. Amoebae were incubated with phages for 2 months. Control Phi6 alone (white bar) and Phi6-containing amoeba cysts (black bar).

eukaryotic hosts has been presented (Poranen & Bamford 2012; el Omari *et al.* 2013; Pflug *et al.* 2014; Koonin *et al.* 2015; Wolf *et al.* 2018). Thus, complementary phylogenic and comparative genomic analysis with human coronaviruses against their persistence within amoebae, and in particular, SARS-CoV-2 is needed. This subversion of amoebae innate immunity by Phi6 may have important implications for a better understanding of RNA enveloped viruses disease pathogenesis, which could make amoebae a suitable model system for understanding respiratory virus-eukaryotic biology at the cellular and molecular levels. Further studies are required to elucidate the precise mechanisms by which RNA viruses affect amoebic mitochondrial dynamics, initiate and regulate apoptotic cell death.

Since amoebic cysts are highly resistant to various harsh environmental stressors, thereby protecting their intracellular microbes and readily aerosolized to enhance dispersal from engineered water systems (Aksozek *et al.* 2002; Lambrecht *et al.* 2017; Borges *et al.* 2019), we also investigated the stability of Phi6 phage inside *V. vermiformis* cysts. TEM analysis showed that at 2 months after the infection vacuoles still contained infectious viruses in *V. vermiformis* cysts (Figure 3(a) and 3(b)). It has been shown that during viral respiratory diseases investigations, FLA cysts were isolated from the air where patients being nursed and from patients' upper respiratory tract (Wang & Feldman 1967; Kingston & Warhurst 1969). Consequently, it is reasonable to hypostatize that amoeba cysts may also be a vehicle and an intermediate environmental host for SARS-CoV-2 transmission through inhalation, and possibly also within the nasal-phalangeal track where similar amoeba have long been known to reside (Skocil *et al.* 1972; Cabello-Vílchez *et al.* 2014). It is, therefore, conceivable that amoebae cysts containing infectious RNA viruses could be protected against disinfecting agents/conditions and easily dispersed via aerosols. Thus, future studies are necessary to explore these possibilities.

CONCLUSION

Here, we successfully demonstrate the persistence of an infectious SARS-CoV-2 surrogate (Phi6 phage) within active amoebal trophozoites and dormant cysts. Prospectively, viral respiratory pathogens persistence and stability could be maintained by colonizing amoebae in water systems. However, further research is required to confirm the comparability of the fate of Phi6 within amoebae with the viruses of interest (i.e., SARS-CoV-1 and SARS-CoV-2).

It is noteworthy that our work sheds light on the environmental fate of FLA-associated enveloped viruses and raises new questions regarding the persistence of human-pathogenic RNA viruses in nature. Our study also exemplifies the utility of including amoebae as an environmental reservoir when conducting risk assessment for emerging RNA enveloped viruses in aquatic environments.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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