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ORIGINAL PAPER

Bioaccumulation Dynamic by *Crassostrea gigas* Oysters of Viruses That Are Proposed as Surrogates for Enteric Virus Contamination in Environmental Samples

Vilaine Corrêa da Silva[1] · Mariana Elois[1] · Beatriz Pereira Savi[1] · Marília Miotto[2] · Juliano De Dea Lindner[2] · Gislaine Fongaro[1] · Doris Sobral Marques Souza[1,2]

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Abstract

Oysters are filter-feeders and retain sewage-derived pathogens in their organs or tissues. Since most enteric viruses involved in outbreaks cannot grow in cell culture, studies using viral surrogate models are essential. Some species are proposed as surrogates for enteric viruses in environmental samples, including in bivalve mollusk samples, such as murine norovirus type 1 (MNV-1) and somatic (as φX) or F-specific coliphages (as MS2) bacteriophages. This study evaluated the tissue distribution of viral surrogates for enteric virus contamination after their bioaccumulation by *Crassostrea gigas*. Oyster tissues were analyzed for the distribution of viral surrogates (MNV-1, φX-174, and MS2) in digestive tissue (DT), gills (GL), and mantle (MT) after 4, 6, and 24 h of experimental bioaccumulation. MNV-1 had higher counts at 6 h in DT (1.2×10^3 PFU/g), followed by GL and MT (9.5×10^2 and 3.8×10^2 PFU/g, respectively). The bacteriophage φX-174 had a higher concentration in the MT at 4 and 6 h (3.0×10^2 PFU/g, in both) and MS2 in the GL after 24 h (2.2×10^2 PFU/g). The bioaccumulation pattern of MNV-1 by oysters was similar to the other enteric viruses (more in DT), while that of phages followed distinct patterns from these. Since the MNV-1 is bioaccumulated by *C. gigas* and is adapted to grow in cell culture, it is an important tool for bioaccumulation and viral inactivation tests in oysters. Although bacteriophage bioaccumulation was not similar to enteric viruses, they can be indicated for viral bioaccumulation analysis, analyzing MT and GL, since they do not bioaccumulate in DT.

Keywords Viral surrogates · HNoV · Bacteriophages · MNV-1 · Bioaccumulation

Introduction

Oysters are the most frequent involved food in gastroenteritis outbreaks, as they are often eaten raw or lightly cooked, which increases the possibility of the presence of infectious viruses within them. In addition, some enteric viruses are resistant to an increase in temperature (hepatitis A virus, HAV) or may persist for long times in the oysters (human noroviruses, HNoV), even after depuration (Nappier et al., 2008; Romalde et al., 1994; Schwab et al., 1998). These particularities may require different characteristics of a viral surrogate for its use in bivalve mollusk analyses: as a viral control in concentration and qPCR processes for estimating the viral recovery (ISO 15216-1:2017), or evaluation of viral inactivation by physicochemical treatments (Croci et al., 2005; Pilotto et al., 2019), and in bioaccumulation or depuration tests (Drouaz et al., 2015; Nappier et al., 2008; Souza et al., 2013, 2018). Therefore, the choice of the best viral surrogate in shellfish may not be an easy thing to do.

Human norovirus (HNoV) and hepatitis A viruses are the main enteric viruses associated with foodborne illness after bivalve mollusks consumption. HNoV belongs to the *Calicivirus* family and genogroups I, II, IV, XVIII, and XIX of the *Norovirus* genus (Chhabra et al., 2019). They are responsible for the most significant cause of acute gastroenteritis, causing about 200,000 deaths worldwide (Cardemil & Hall, 2020). They are resistant to various conditions in the environment and disinfectants used in water treatment plants

✉ Doris Sobral Marques Souza
doris.sobral@gmail.com

[1] Applied Virology Laboratory, Federal University of Santa Catarina (UFSC), Florianópolis, SC, Brazil

[2] Department of Food Science and Technology, UFSC, Florianópolis, SC, Brazil

(Cook et al., 2016). Despite successful trials for the cultivation of the HNoVs from clinical samples (Jones et al., 2015), until now, checking their infectivity from environmental samples is hard. Studies using human intestinal enteroids as models have been recently published (Randazzo et al., 2020). For this reason, studies using viral surrogate models for HNoV are still essential.

Some peculiarities contribute to HNoV disease being the most frequent cause of non-bacterial gastroenteritis associated with oysters. HNoV can persist attachment to carbohydrate ligands (HBGA-like) present in cells into the digestive glands (midgut, main and secondary ducts, and tubules) of some species of oysters (Guyader et al., 2006). Maalouf (2010) showed a seasonal correlation between ligands and HNoV presences. These authors also demonstrated that in the winter, HBGA-*like* ligands increase in the digestive tissues of some oysters, allowing more viruses to remain attached to those cells. In addition, low temperatures favor HNoV viability persistence (Maalouf et al., 2010). This attachment allows HNoV persistence after depuration processes (McLeod et al., 2017). Some viruses have been used as HNoV surrogates in depuration experiments, however, they have no similar persistence in oysters (McLeod et al., 2017). Long persistence in digestive tissue (DT) of oysters is an important characteristic for surrogate selection for its uses as viral contamination control in tests that evaluate depuration efficiency.

Members of the *Calicivirus* family are used as enteric virus surrogates in experiments involving bivalve mollusk species. MNV-1, Tulane virus (TV), and Feline Calicivirus (FCV) are the most used viruses. All of them are adapted to grow through laboratory cell lines (Bae & Schwab, 2008; Farkas et al., 2010), making it easier to obtain viral inoculum with higher concentration for their use in tests that evaluate the viral decay in mollusks. MNV-1 belongs to genogroup V of the *Norovirus* genus, and it has been used in several studies involving bivalve mollusks that estimate the viral recovery efficiency in some steps of sample processing (Girard et al., 2013; Souza et al., 2018); in the evaluation of the viral accumulation, stability (during cooking, UV irradiance, and others) (Bae & Schwab., 2008; Croci et al., 2005; Nappier et al., 2008; Pilotto et al., 2019; Solis-Sanchez et al., 2020); and depuration (Provost et al., 2011; Souza et al., 2013). *Tulane* virus, genus *Recovirus*, has a histogroup linkage similar to that of HNoV, making it a compatible surrogate for depuration tests (Farkas et al., 2010). *Feline Calicivirus*, genus *Vesivirus* (ICTV, 2018), is also used as a substitute for HNoV in these experiments (Ohmine et al., 2018). However, Ueki et al. (2007) observed that it was rapidly depurated after 3 days, while HNoV persisted for more than one month (Nappier et al., 2008).

Some enteric viruses are adapted to grow in cell cultures, such as the HAV, human Adenovirus (HAdV), and enterovirus (EV). They are also used as surrogates for enteric virus contamination in tests with bivalve mollusks (Corrêa et al., 2012; Souza et al., 2013). The major disadvantage of their use is that they may come from natural contamination if mollusks grow in contaminated water. Even if a sample of oysters is tested before the artificial contamination process, it is impossible to affirm that the rest of the animals in the tank were not already contaminated.

Bacteriophages have been used as surrogates due to many characteristics similar to mammalian viral pathogens, such as size, shape, morphology, surface chemistry, and physical chemistry. In addition, they cannot replicate outside the host bacteria in water environments (Tufenkji & Emelko, 2011). F-specific MS2 and somatic coliphages ΦX are some examples of attractive alternatives as viral surrogates for enteric viruses. MS2 is an icosahedral, positive-sense single-stranded RNA virus that infects the bacterium *Escherichia coli* and other members of the *Enterobacteriaceae* (Van Duin & Tsareva, 2004). PhiX (ΦX) is a single-stranded DNA (ssDNA) virus that infects *E. coli* (Labrie et al., 2014). Besides their ubiquity in community sewage effluent and similarities to fecal viruses (Burkhardt et al., 1992; Doré et al., 2000; McMinn et al., 2017), the phage lysis technique offers an effective and low-cost surrogate for the detection of pathogenic viruses in shellfish. Olalemi (2015) demonstrated that the target phages (somatic coliphages, F-RNA coliphages, and GB124 phages) are effective surrogates of viral pathogens in two commonly harvested shellfish, *Mytilus edulis* and *C. gigas* species.

Studies have reported that the enteric viruses usually accumulate in high concentrations in the DT of bivalves (Maalouf et al., 2010; Napier et al., 2008; Souza et al., 2018). Thus, it is important to know the behavior of the viral surrogate after bioaccumulation by mollusks, and whether it will be able to function as a positive control for viral contamination in that particular test. This study aimed to evaluate the tissue distribution of some proposed viral surrogates (MNV-1, somatic, and F-specific phages) for enteric viral contamination in environmental samples after MNV-1 and bacteriophages bioaccumulation by *C. gigas*.

Materials and Methods

Viruses Production

MNV-1 was produced in RAW 264.7 cells (a macrophage-like Abelson leukemia virus-transformed cell line derived from BALB/c and MNV-1 mice) and titrated by lysis plaque following protocols described by Bae and Schwab (2008). Both cells and MNV-1 were kindly donated by Dr. Rosina Gironès from the University of Barcelona, Spain.

For phage production, ΦX-174 and MS2 were inoculated into two cultures grown (optical density 0.6) of the host bacteria *E. coli* (ATCC 13,706) and *Salmonella enterica* serovar Typhimurium (ATCC 14,028), in Luria–Bertani (LB) broth (Kasvi, Espanha). The supernatants were filtered through a 0.22 μM filter (Millipore, Burlington, Massachusetts, USA), and phages were quantified in double-agar technique, as described by Adams (1995).

Animal Collection and Artificial Bioaccumulation Processes

Six dozen adult *C. gigas* oysters and seawater were supplied by the Marine Mollusks Cultivation Laboratory (LCMM) of the Federal University of Santa Catarina (UFSC), Florianópolis, Santa Catarina, Brazil, to carry out the experiments. The samples consisted of living, commercially sized oysters. The oysters were transported to the laboratory and remained in acclimation to the laboratory conditions for 12 h, in tanks. After this period of time, 12 oysters were collected and analyzed (as a pool) for the presence of MNV-1 and the phages (φX-174 and MS2), using the same protocols described above.

Oysters were distributed in aquaria (12 units per aquarium) containing 10 L of natural seawater, and one strain of the surrogate in each (7×10^8 PFU of MNV-1; 5×10^3 PFU of ΦX-174 or MS2). Another aquarium with seawater and oysters only was a negative control of viral contamination during the bioaccumulation process. Three oysters per sample were collected after 4, 6, and 24 h from each aquarium. They had their digestive tissue (DT), gills (GL), and mantles (MT) dissected, forming pools of tissues from 3 oysters each. Figure 1 presents the main structures of *C. gigas*. The surrogates were quantified in the tissues by RT-qPCR and by plaque assay for MNV-1 (Bae & Schwab, 2008; Baert et al., 2008), and only by plaque assay for the bacteriophages (Adams, 1995).

During acclimation and bioaccumulation assays, physicochemical parameters were monitored (water temperature, salinity, pH, and oxygen) using a colorimeter Acquacombo (Alfakit, Florianopolis, Brazil). Both acclimation and bioaccumulation processes happened in the laboratory at temperature of 20ºC ± 2, and oxygen pumps were added to the aquaria for ensure oxygen level around 8 mg L^{-1}. Three bioaccumulation assays were performed in different weeks from 2020 to 2021. Two grams of each sample (DT, GL, and MT) of oysters were analyzed for the presence and quantity of each surrogate, as follows.

MNV-1 Analysis

Each sample was homogenized separately with a tissue homogenizer (Nova Technique Industry and Commerce of Laboratory Equipment Ltda.). The samples were subjected to viral concentration by polyethylene glycol 6,000 (PEG 6000) according to the protocol described by Lewis and Metcalf (1988), with some modifications, as described by Rigotto et al. (2010). Two hundred microliters of these final concentrated were used for the nucleic acid extraction before the qPCR (Baert et al., 2008), as described below, and non-cytotoxic dilution of these final concentrated was tested for MNV-1 infectious (presence and quantification) by plaque assay, as described by Bae and Schwab (2008).

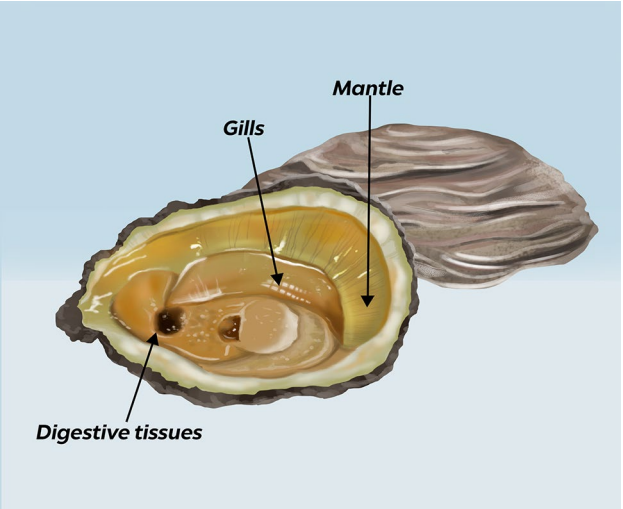

Fig. 1 Schematic drawing of the anatomy of the oyster *Crassostrea gigas,* showing the location of the mantle, gills, and digestive organs

Molecular Detection of MNV-1 in the Oysters

The nucleic acids from samples were extracted using Purelink® viral RNA/DNA Kit (Invitrogen, Massachusetts, United States), according to the manufacturer's guidelines. The nucleic acids were analyzed by qPCR tenfold diluted and undiluted. All qPCR run in duplicate in the StepOne™ Plus Real-Time PCR System (Applied Biosystems, CA, USA), using standard curves with the plasmids with cloned sequences of the MNV-1. The same protocols of temperatures and time were used in qPCR, with the same sequences/concentration of primers and probes as described by Baert et al. (2008). Non-template controls were included in each run. Only Cq ≤ 38 was accepted.

Plaque Assay for Infectious MNV-1 Analysis in Oysters

Non-cytotoxic dilutions of oyster samples were inoculated in a 6-well plate with RAW 264.7 cells to quantify the infectious MNV-1 in DT, GL, and MT samples, as described by Bae and Schwab, (2008). Briefly, after the

viral concentration by PEG 6000, samples were previously treated with an antibiotic/antifungal (100 U ml penicillin G, 100 µg/ml 180 streptomycin, and amphotericin B 0.25 mg/ml), diluted, and inoculated to 6-well plates with confluent RAW 264.7 cell monolayer and were incubated for 1 h at 37ºC/CO_2 for cell infection. The diluted samples were removed, and the cells were overlaid with 2 ml of 3% of low melting point agarose (SeaPlaque, Lonza, CA, USA), diluted in 2X concentrated medium (DMEM, Gibco), supplemented with antibiotic and antifungal in the same concentration used described before. The plates were incubated again at 37 °C/CO_2 for 48 h, corresponding to the viral replication cycle. A second layer of the same agarose (2 ml) was added, prepared according to the same protocol described above, but with the addition of a 0.01% solution of neutral red (Sigma, Switzerland) to allow visualization of the formed lysis plaques, which were monitored during 8 h. The resultant lysis plaques were counted, and the number of viral titers, PFU/ml, was estimated.

Phages Analysis

Two grams of DT, or GL, and or MT per sample/time were homogenized separately with a tissue homogenizer. These samples were diluted 5 times serially and plated for phage quantification using the double-agar technique (Adams, 1995). Briefly, 1 ml of the samples was mixed with 1 ml of log-phase *E. coli* (ATCC 13,706) and *S. enterica* serovar Typhimurium (ATCC 14,028) culture (Optical Density 0.6), and the samples were each added to 1 ml of solid BHI agar. The plates were incubated again at 37ºC/16 h to allow the appearance of plaques. The phage titer was determined and expressed in PFU.

Statistical Analysis

Statistical analyses were performed by GraphPad Prism 8 using Two-Way ANOVA. The value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Bioaccumulation of MNV-1 in Different Tissues

Oyster tissues were analyzed for the viral surrogates distribution (MNV-1, φX-174, and MS2) in DT, GL, and MT after 4, 6, and 24 h of experimental bioaccumulation. The infectious particles of MNV-1 were distributed among the three tissues evaluated over 24 h, with counts higher after 6 h, and in the DT (1.2×10^3 PFU/g), followed by GL and MT (9.5×10^2 and 3.8×10^2 PFU/g, respectively) (Fig. 2). Despite MNV-1 showing higher concentration in DT, like other enteric viruses, there was no statistically significant difference between the results of virus concentrations in tissues over time ($P > 0.05$).

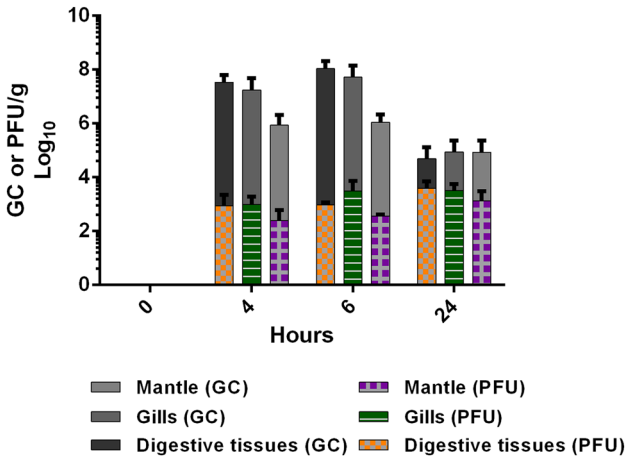

Fig. 2 MNV-1 distribution in oyster tissues up to 24 h subjected to experimental contamination. GC: genome copies; PFU: plaque forming units

MNV-1 was distributed among the three tissues analyzed. Schwab et al. (1998) observed that HNoV was more concentrated in the digestive tissues (digestive gland and stomach) of *Crassostrea virginica*, regardless of the quantity added to the tank (10^3 to 10^7 PFU) when compared with adductor muscle and hemolymph cells. Maalouf et al. (2010) compared HNoV GI.1 and GII.4 VLPs (Virus-Like Particles) distribution among DT, GL, and MT of *C. gigas*. They concluded that while GI.1 was accumulated almost exclusively in DT, GII.4 was equally distributed among DT, GL, and MT of oysters. Studies that evaluated enteric viruses bioaccumulation and distribution among oysters tissues (*C. gigas* and others) focused mainly in HNoV genogroups (Lowmoung et al., 2017; Maalouf et al., 2010; Schwab et al., 1998; Souza et al., 2018) and used molecular techniques in the analyses. Few studies have evaluated the tissue distribution of other enteric viruses, such as HAV (Romalde et al., 1994) and poliovirus (Mcleod et al., 2009) in *C. gigas* or other species. When comparing the results of MNV-1 bioaccumulation to those obtained with HNoV GII.4 VLPs (Maalouf et al., 2010), the results obtained in the present study were similar to those of these authors, with GII.4 being distributed among the three tissues of *C. gigas*. However, when comparing with the results obtained by the same authors but with the GI, it was observed that MNV-1 accumulated in greater concentration the DT, but differed from that genogroup, since MNV-1 also accumulated in the GL and MT of the oysters.

Despite the molecular similarities between HNoV and MNV-1, some points must be considered when evaluating their bioaccumulation pattern in oysters. While the first (HNoV) is usually added to oyster's tanks inside an

inoculum composed by fecal or sewage matrices, the second (MNV-1) is part of a viral suspension in a medium from cell culture. It is known that when viruses are in liquid matrices, they tend to bind or not bind to various particles, depending on environment pH. Fecal and sewage have several organic flocs that may promote viral binding (Gerba & Betancourt, 2017), which may affect the virus bioaccumulation. Beninger et al. (2008) stated that there is a selection of the particles ingested by the oysters during the filter-feeding process. They observed that the shape, size, and chemical constitution of the particles are some of the characteristics that influence the intake. Then, depending on what kind of particles viruses are attached to, their bioaccumulation by oysters may also be affected.

Bioaccumulation of Phages in Different Tissues

The results showed a higher concentration of φX in MT at 4 and 6 h (3.0×10^2 PFU/g, in both) and in GL of MS2 after 24 h (2.2×10^2 PFU/g) (Fig. 3). However, there was no statistically significant difference between the results of φX and MS2 concentrations in tissues over time ($P > 0.05$).

The φ-X-174 and MS2 phages were concentrated in MT (φ-X-174) and GL (MS2) for 24 h. Doré and Lees (1995) reported that *C. gigas* naturally contaminated with MS2 for one to three weeks (growing area near sewage outfall) retained more phages in the animals DT, than GL or MT, and other tissues. The contamination protocol performed by them was different in terms of inoculum constitution, natural contamination, and exposure time compared with the results obtained here. In our experiment, MS2 phages were from supernatants of the host bacteria culture. These differences may have affected the results. Other authors reported the persistence of MS2 in oysters, however, they processed total meat without analyzing separated tissues (Kingsley et al., 2018), as performed in this study.

Thus, the presence of bacteriophages in oysters did not follow the same bioaccumulation behavior observed for MNV-1 and human enteric viruses. Although bacteriophages are proposed as indicators of enteric viral contamination in bivalve mollusk samples (Flannery et al., 2009; Lowther et al., 2019), because their presence may be correlated with some enteric viruses, in the case of bioaccumulation assay use of MNV-1 as viral control may offer pattern more like that of enteric viruses.

Although MNV-1 showed better results than MS2 and ΦX-174 in the viral bioaccumulation tests in *C. gigas*, other information must be taken into account, such as what question need to be answered with the experiment performed, and evaluate if that surrogate choice will provide a reliable result. Richards (2012) highlighted some limitations in the use of viral surrogates instead of enteric viruses in some trials.

Conclusion

Comparing the tissue distribution pattern of MNV-1 in bioaccumulated *C. gigas*, MNV-1 proved to be an alternative viral model to HNoV, and other human enteric viruses, predominantly in DT but distributed in GL and MT corroborating the bioaccumulation profile of HNoV GII.4 reported in other studies. The same was not confirmed with the HNoV GI.1, which predominantly occurs only in DT. On the other hand, the bioaccumulation profile of the bacteriophages MS2 and ΦX showed no similarity with the profile of HNoV GI since they were found in MT and GL, not being accumulated in DT. Thus, it is concluded that MNV-1 is an alternative viral model to HNoV in DT bioaccumulation analysis in *C. gigas* oysters. At the same time, since bacteriophages MS2 and ΦX were not bioaccumulated in DT, they are indicated for viral analysis in C. gigas, if MT and GL are investigated as well.

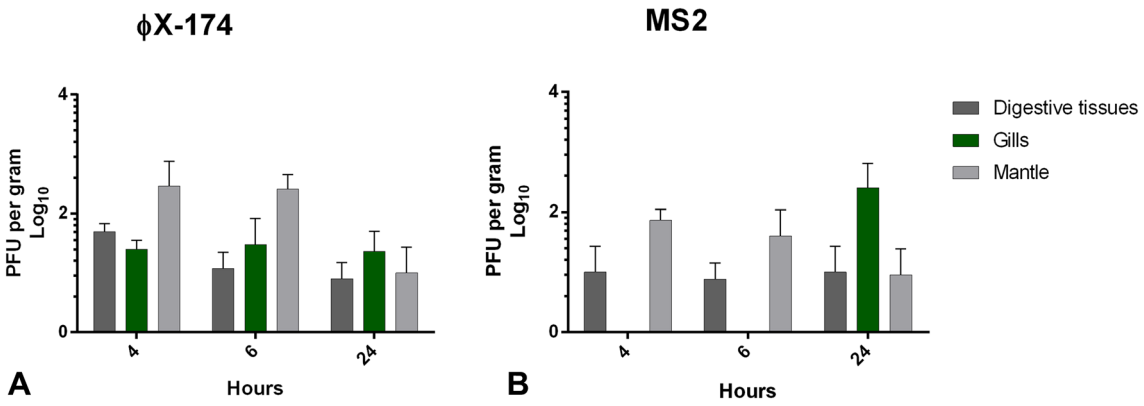

Fig. 3 ΦX-174 and MS2 distribution in oyster tissues up to 24 h subjected to experimental contamination. PFU: plaque forming units

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Author Contributions DSMS and GF contributed to conceptualization, methodology, formal analysis, writing—editing draft, and investigation. VCS; ME; BPS; DSMS; and GF contributed to methodology, investigation, and project administration. MG contributed to formal analysis, writing—original draft, conceptualization, methodology, investigation, and resources. VCS; ME; BPS; MM; JDDL; DSMS; and GF contributed to project administration, investigation, validation, and conceptualization. VCS; DSMS; and GF contributed to project administration, investigation, resources, and data curation.

Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

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