EXPERIMENTAL CONTAMINATION OF *CHAMELEA GALLINA* WITH MURINE NOROVIRUS AND EFFECTIVENESS OF DEPURATION

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ABSTRACT

Human Norovirus has been reported as the major non-bacterial cause of human gastroenteritis due to the consumption of contaminated bivalve mollusks. The European legislation established microbiological criteria only for bacteria (*Salmonella* spp. and *Escherichia coli*), while no viruses have still been considered. In this study, samples of *Chamelea gallina* were harvested along the Central Adriatic coasts (Italy) and artificially contaminated with Murine norovirus-1 (MNV-1) up to a final concentration of 10^3 $TCID_{50}$/ml in water. They were subject to a depuration process in a closed-circuit system using both ozone and ultraviolet light. Four experimental trials (100 specimens/trial) were performed and, at the end of depuration, the digestive glands of mollusks were examined by means of two methods – namely, RT-PCR and tissue culture. The results of RT-PCR ranged from $10^{3.17}$ to $10^{4.60}$ $TCID_{50}$/ml, and the constant presence of MNV-1 was confirmed by the tissue culture as well. In conclusion, no significant viral reduction was obtained, but the contaminated bivalve mollusks remained infectious until the end of the depuration treatment. The proper cooking of live bivalve mollusks could be considered the most important preventive measure against this sanitary risk.

Keywords: Norovirus, clams, depuration, tissue culture, RT-PCR

1. INTRODUCTION

Norovirus is a non-enveloped, single-stranded positive RNA virus, a member of the family *Caliciviridae*, and divided into six genogroups (GI-GVI). However, only the genogroups GI, GII and GIV were identified in humans (ILIC *et al*., 2017). Moreover, over 40 genotypes based on the capsid were identified (LEROUX-ROELS *et al*., 2017). A novel GII.17 variant emerged in Asia (China, Japan, Korea and Taiwan) in 2014 (CHENG *et al*., 2017; SUFFREDINI *et al*., 2017) and was also reported in other countries such as Canada, the United States (U.S.), New Zealand as well as some European States – i.e. Germany, Italy, Hungary and Slovenia (CHAN *et al*., 2017). According to the European Union Rapid Alert System for Food and Feed (EU RASFF), the majority of alert notifications involving Norovirus in food were reported by Denmark, France, Italy, the Netherlands and Norway as notifying countries, and France and Serbia as countries of origin, respectively. With regards to border rejection notifications, the main countries of origin were France and Serbia, whereas Italy and Spain submitted to the EU RASFF the majority of them (PAPAPANAGIOTOU, 2017).

Human Norovirus (HuNoV) is reported as the main non-bacterial cause of foodborne outbreaks due to the consumption of live bivalve mollusks. The main clinical symptoms of such an illness, with an incubation period of 10-51 hours, are nausea, sudden onset of vomiting and/or watery non-bloody diarrhea, abdominal or general muscle pain, headache and mild fever (HASSARD *et al*., 2017; JEON *et al*., 2017). In addition, it can lead to more severe conditions, such as dehydratation, hospitalization and potentially death in vulnerable individuals including children and elderly population (TRIVEDI *et al*., 2013; FUSCO *et al*., 2017).

Bivalve mollusks are filter-feeding organisms that can retain and concentrate in their own body not only nutrients but also suspended viruses or bacteria. However, the European Union (EU) Legislation (EC, 2004a) established that sanitary controls of live bivalve mollusks must be based only on the detection of *Escherichia coli* used as an indicator of faecal contamination for the classification of production areas, from which they can be collected. Moreover, Regulation EC No 2073/2005 (EC, 2005) and its amendments reported the absence of *Salmonella* spp. in 25 g of live bivalve mollusks and a range of 230 to 700 MPN/100 g of flesh and intravalvular liquid for *E. coli*. In the U.S. as well, the standards used for shellfish hygiene controls in both growing areas during primary production and for end-products are represented by faecal or total coliforms (CAMPOS *et al*., 2017). On the contrary, viruses are not investigated as vehicles for foodborne disease transmission according to the above mentioned legislations.

Generally, viruses show a higher environmental resistance than bacteria, and depuration is poorly effective on decontamination of live bivalve mollusks (VARELA *et al*., 2016). The most common depuration systems are based on the use of chlorine, ultraviolet light (UV) and ozone. While chlorine can have organoleptic effects in mollusks and cause the formation of chlorinated by-products, UV and ozone have gained popularity in recent years but both of them can be limited because the first is effective in high flow rates (POLO *et al*., 2014a) and ozone is influenced by some parameters such as temperature, salinity, pH and dissolved oxygen (ILIC *et al*., 2017).

The aim of this study is the evaluation of a depuration process in a closed-circuit system using both ozone and UV in clams (*Chamelea gallina*), experimentally contaminated with Murine Norovirus-1 (MNV-1), because it has genetic and pathological features similar to HuNoV and therefore it can be used as surrogate (PREDMORE *et al*., 2015; KIM *et al*., 2017).

2. MATERIALS AND METHODS

2.1. Samples' collection and depuration process

Samples of *C. gallina* (25-32 mm) were harvested along the Central Adriatic coast of Molise region, Italy, in 4 different periods (named A to D) of the year 2015 (from January to October) and put into aquariums containing seawater for acclimation and evaluation of their viability. Then, they were transferred into tanks filled with artificial marine water (Ocean Fish, Prodac International, Padova, Italy) and artificially contaminated for 72 hours with the MNV-1 provided by the Istituto Zooprofilattico Sperimentale delle Venezie (Italy), up to a final concentration of 10^3 $TCID_{50}$/mL in water.
The depuration process was carried out for 72 hours in a closed-circuit system (Tecno Impianti International s.r.l., Riccione, Italy) equipped with UV and ozone. It consisted of 197x72x45 cm tanks with a perlon wool prefilter and hyperactive carbon filter, an active biological filter using *Lithothamnium calcareum* algae and an UV sterilization plant. With regards to the sterilization unit and ozonator, power was 230V and power consumption was 16W and 0.5 A, respectively.
Aliquots of 100 specimens were analyzed at time 0 as negative control, before being placed in the depuration tanks, and further 3 aliquots were examined at intervals of 24, 48 and 72 hours. The clams were washed, opened and their digestive glands were pooled together and homogenized. Then they were analyzed by both tissue culture and RT-PCR according to BAERT *et al*. (2008).

2.2. Preparation of viral stocks

MNV-1 was propagated in monolayers of RAW 264.7 (Mouse Macrophage) purchased from American Type Culture Collection (ATCC-LGC Standards, Milano, Italy) and cultured in 75 cm^2 tissue culture flasks. The cells were maintaned in Dulbecco's Modified Eagle Medium, DMEM (Gibco, New York, USA) supplemented with sterile phosphate buffered saline (PBS, pH 7.4), 1% antibiotics (penicillin, nystatin, gentamicin, streptomycin) and 10% fetal bovine serum (Merck, Darmstadt, Germany). They were incubated at 37°C in a humidified atmosphere of 5% CO_2. For the preparation of viral stocks, the growth medium was removed, the cells were infected with MNV-1 with a virus titer of 10^3 $TCID_{50}$/mL and incubated for 48 hours. When significant cytopathic effects were observed, the supernatant was centrifuged at 500 *g* at 4°C for 30 min. MNV-1 titer was assessed by both RT-PCR assay and traditional virus end point titration according to REED and MÜENCH (1938).

2.3. Tissue culture assay

An aliquot of pooled hepatopancreas (1±0.2 g) was homogenized with sterile quartz sand and diluted 1:10 (w/v) in Antibiotics Antimycotic Solution (100X). The sample was stored at 4°C for 1 hour and centrifuged at 5000 *g* for 10 min. Twenty-four well microplate monolayers of RAW 264.7 were infected with 200 µL of the diluted sample (1:10 and 1:100) in DMEM and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 4-6 days. The presence of MNV-1 was evaluated by means of RT-PCR.

2.4. RT-PCR assay

The glands were pooled and 2±0.2 g were homogenized with 2±0.2 mL of 0.1 mg/mL proteinase K solution (Qiagen, Hilden, Germany), incubated at 37°C with shaking (320 rpm/1 hour) and centrifuged at 3000 *g* for 5 min. Nucleic acids (100 µL of supernatant) were extracted using the Biosprint 96 authomatic system (Qiagen) with the Biosprint 96 One for all vet Kit (Qiagen) according to the manufacturers' instructions. Ten µL of Armored RNA West Nile Virus (HNY1999) (Asuragen, Santa Clara, CA, USA) diluted 1:100 were added to each sample as an internal control to check for any RT-PCR inhibition phenomena. Monolayers of RAW 264.7 infected with10-fold serial dilutions of MNV-1 were used for the devolopment of the RT-PCR assay and the C_t value was < 40. The master mix was prepared by using RNA Ultrasense One-step qRT-PCR system (Invitrogen, Carlsbad, CA, USA) as reported in Table 1.

Table 1. Composition of master mix.

Reagent	C1 [a]	C2 [b]	Vol (µL)
H_2O	5x		1.100
5x Ultrasense reaction mix	50x	1x	4.000
ROX Reference dye (50x)	4.0 µM	1x	0.500
MNV-F	4.0 µM	200 nM	1.000
MNV-R	4.0 µM	200 nM	1.000
MNV-P	20 µM	200 nM	1.000
NS5-2	50 µM	80 nM	0.188
NS5-2F	50 µM	150 nM	0.100
NS5-2R		150 nM	0.100
RNA Ultrasense enzyme mix			1.000
Total			10.0

[a] C1: initial concentration
[b] C2: final concentration

A primer and probe set was selected according to BAERT *et al*. (2008). The sequence of primer pairs and probes was as follows: for MNV-1, the probe was 5'FAM-CGC TTT GGA ACA ATG-3'MGB (MNV-P), the primer Fw was 5'-CAC GCC ACC GAT CTG TTC TG-3' (MNV-F) and the primer Rev was 5'-GCG CTG CGC CAT CAC TC-3' (MNV-R); for HNY1999, the probe was 5'VIC-CCA ACG CCA TTT GCT CCG CTG-3'TAM (NS5-2), the primer Fw was 5'-GAA GAG ACC TGC GGC TCA TG-3' (NS5-2F) and the primer Rev was 5'-CGG TAG GGA CCC AAT TCA CA-3' (NS5-2R). All the primers and probes were purchased from Eurofins MWG Operon (Louisville, USA).
Ten µL of master mix and 10 µL of viral RNA were used for RT-PCR. The assay was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) at the following thermal conditions: 50°C for 15 min – 95°C for 2 min – 40 cycles (95°C for 15 sec – 60°C for 1 min).
The analytical sensitivity of RT-PCR was tested analyzing the serial $\log_{10}$ dilutions of the MNV-1 tissue culture $10^{5.9}$ $TCID_{50}$/mL.

2.5. Statistical analysis

The Pearson correlation coefficient with confidence intervals of 95% was used to measure the association between time and viral titer.

3. RESULTS AND DISCUSSION

The presence of MNV-1 in the artificially contaminated clams was observed by tissue culture assay just after 24 hours of exposure, even if the RT-PCR results showed that an interval of 72 hours was the optimum for the viral contamination because the values increased from $10^{3.60}$ (24 hours) to $10^{6.60}$ $TCID_{50}$/mL (72 hours). The analytical sensitivity of RT-PCR resulted $10^{0.9}$ $TCID_{50}$/mL corresponding to 38 C_t value (data not shown).
The results of the different experiments of the depuration process carried out by the tissue culture assay showed values of $3.98x10^4$ $TCID_{50}$/mL for trial A and $1.48x10^4$ $TCID_{50}$/mL for the remaining trials. The viral titer did not vary among the 4 trials, because MNV-1 resulted always vital.
The results of RT-PCR ranged from $10^{3.17}$ to $10^{4.60}$ TCID50/mL (data not shown).
The Pearson correlation coefficient was -0.15 (lower limit = -0.55 and upper limit = 0.29) and therefore not significant.
A similar study was carried out by POLO *et al*. (2014b) in samples of clams (*Venerupis philippinarum*) and mussels (*Mytilus galloprovincialis*) contaminated with MNV-1 and then depurated for 7 days by means of ozone and UV-C radiation for water sterilization. The average reductions compared with the initial levels of MNV-1 were 60.5% for clams and 91.6% for mussels, but they remained still infectious at the end of the process. POLO *et al*. (2014a) as well found the presence of Norovirus in clams and mussels after a depuration process based on water treatment by chlorination. The efficacy of depuration using traditional or closed-circuit system with disinfection by UV was evaluated by SAVINI *et al*. (2009), which reported no statistically significant differences between depurated and non-depurated samples (i.e. *M. galloprovincialis*, *Tapes decussatus* and *Crassostrea gigas*) and indicated that the process was not able to remove Norovirus.
Other studies (LEAL DIEGO *et al*., 2013; IMAMURA *et al*., 2016) showed the failure of depuration process applied on oyster samples using a system based on UV. These results demonstrated that the water exchange could be low and the initial contamination was too high (LE MENNEC *et al*., 2017). Therefore, some measures such as the increasing of depuration time and water circulation as well as the continuous exposure to UV treatment could be able to improve the effectiveness of the process. SOUZA *et al*. (2013) detected MNV-1 in oysters until 96 hours of depuration in a closed system using different UV doses.
The presence of MNV-1 after the depuration process described in the present study demonstrated that these viruses can survive and accumulate in live bivalve mollusks, and therefore they represent a source of foodborne disease for consumers. Recent studies showed that Norovirus strains can selectively accumulate in mollusks due to viral carbohydrate ligands of histo-blood group such as antigens in various tissues of clams, mussels and oysters (POLO *et al*., 2014a; MCLEOD *et al*., 2017). Therefore, the elimination of Norovirus can be difficult using traditional decontamination treatments, because the virus is internalized within the cells of the digestive organs and other tissues of mollusks (KIM *et al*., 2017).

According to the report of EFSA and ECDC (2017), during 2010-2016 the number of reported foodborne outbreaks linked to Calicivirus (including Norovirus) was quite stable, with some differences among the EU member states. In particular, a statistically significant increasing trend was described in Belgium, France, Portugal and the Netherlands, while Austria, Denmark, Estonia and Hungary reported a decrease. A national information system, called SINZoo was developed in Italy aiming at the collection of data regarding food contamination and related zoonoses occurrence (COLANGELI *et al*., 2013). Such a system highlighted 12 positive out of 176 mollusk samples, even if no outbreak linked to Norovirus was described in the year 2017.
The monitoring of viral contamination of mollusks represents an important tool for public health, especially because no legislative standards have been established for viruses. However, according to Regulation EC No 853/2004 (EC, 2004b), each EU member state may adopt national measures in order to amend non essential elements such as additional health standards for live bivalve mollusks in cooperation with the relevant Community Reference Laboratory, including virus testing procedures and virological standards. The multi-annual regional control plan for both Abruzzo and Molise regions (PPRIC 2015-2018) established sampling of mollusks every 2 months for *E. coli* and *Salmonella* spp., and every 6 months for viruses.

4. CONCLUSIONS

The consumption of live bivalve mollusks collected from seawater contaminated with sewage pollution, due to malfunctioning of sewerage system, represents an important risk for human health. In this study, no significant viral reduction was observed, the closed-circuit depuration system was not able to reduce the level of MNV-1 and clams remained still infectious until the end of the experimental design. Therefore, the ingestion of raw or undercooked mollusks should be avoided expecially by some population categories, such as immunocompromised individuals.

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