CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

Journal home page: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

SEASONAL IMPACT ON THE RISK ASSESSMENT RELATED TO THE SPATIAL PREVALENCE OF ENTEROVIRUS IN OYSTERS FROM OUALIDIA LAGOON IN MOROCCO

Rihabe Boussettine[1], Najwa Hassou[1], Nabil Abouchoaib[2], Hlima Bessi[1], Moulay Mustapha Ennaji[1]✉

[1]Team of Virology, Oncology, Total Quality and Medical Biotechnologies Laboratory of Virology, Microbiology, Quality, Biotechnologies/Ecotoxicoloy and Biodiversity (LVMQB-ETB), Faculty of Sciences & Technologies - Mohammedia (University Hassan II of Casablanca - Morocco).
[2]Casablanca Regional Research and Analysis Laboratory of National Food Safety Office (ONSSA), Province Nouaceur – Casablanca - Morocco.
✉m.ennaji@yahoo.fr

https://doi.org/10.34302/crpjfst/2020.12.2.7

Article history:
Received:
3 Febryuary 2020
Accepted:
2 June 2020

Keywords:
Enterovirus;
Oysters;
viral contamination;
real-time PCR;
water resources.

ABSTRACT
Enteroviruses are ubiquitous pathogens that are present worldwide and they are able to survive for long periods in a marine environment. They are transmitted through water and food contaminated with stools. The purpose of this study was to evaluate the prevalence of enteroviruses in oysters collected from Oualidia lagoon, using real-time RT-PCR. To achieve this objective, 624 oysters representing 52 batches were collected and tested for viral contamination using real-time polymerase chain reaction. The results show that 32.69% of the samples tested were positive for enteroviruses. The spatial distribution of the enteroviruses was statistically significant. Moreover, a prominent seasonal variation of enterovirus contamination was observed in this study. The consumption of oysters contaminated with this type of virus presents a major risk to human health, By causing serious illnesses such as gastroenteritis, hepatitis, and poliomyelitis, the presence of enteroviruses in oyster production areas represents a potential health risk.

1.Introduction

The surveillance systems implemented by the majority of the countries for assessment of the microbiological quality of shellfish and shellfish harvesting waters are mainly based on bacteriological parameters as indicators of fecal contamination (Anonymous, 2004, 2005). However, several studies have demonstrated the inadequacy of bacterial indicators for assessment of viral contamination in shellfish (Croci *et al*., 2000). In recent years, other putative indicators have been proposed, such as bacteriophages, in particular, the B40-8 phage of the Bacteroides fragilis (Havelaar, 1987). However, the use of these microorganisms as indicators of viral presence has been widely questioned (Xavier Abad *et al*., 1997).

Because shellfish are filter-feeding organisms, they can accumulate foodborne viruses, bacteria, and heavy metals in the digestive gland, the mucosa of the gills, and other tissues. As a consequence, they have frequently been implicated as major vectors in the transmission of many enteric diseases (Greening, 2006; Lees, 2000; Dong Joo Seo *et al*., 2014). Indeed, several viral outbreaks have been associated with the consumption of bivalve mollusks that had nonetheless met the bacteriological standards (Sugieda *et al*., 1996).

The frequency of foodborne viral infections associated with shellfish consumption is increasing worldwide. This is a public health issue that results in a lack of public confidence in shellfish and, therefore, in high economic losses for the seafood industry.

Human enteric viruses are present in large numbers in feces and sewage and they can be found in surface waters, groundwaters, and seawaters.

The viruses most often transmitted by contamination of the marine water are noroviruses (NoV), hepatitis A virus (HAV), hepatitis E virus (HEV), adenovirus (AdV), astrovirus (AV), rotavirus (RV), and enteroviruses (EV) (poliovirus, coxsackievirus, echovirus) (Griffen *et al*., 2003; Le Guyader *et al*., 2009). They are commonly transmitted by the fecal-oral route.

Enteroviruses are ubiquitous pathogens that are present worldwide and they are able to survive for long periods in the marine environment (OMS, 2013). Furthermore, the resistance of these viruses to conditions such as acidic pH and extreme temperatures facilitates their transmission (Bouseettine *et al*., 2020).

Enteroviruses are associated with a heterogeneous range of diseases. In addition to poliomyelitis, they can cause severe acute diseases such as hepatitis, gastroenteritis, meningitis, and non-specific febrile illness (Cristina and Costa-Mattioli, 2007; Gibson, 2014; Shulman *et al*., 2006).

Studies have shown the involvement of a panel of enteroviruses, such as enterovirus types 68-71, in acute flaccid paralysis (AFP) (Bahri *et al*., 2005; Delpeyroux *et al*., 2013; OMS, 2013).

Therefore, environmental monitoring can provide an additional tool to determine the different viruses present in a community (Pinto *et al*., 2007; Shulman *et al*., 2006) by examination of environmental samples, particularly from bivalve mollusks. Enteroviruses are one of the most frequently monitored viruses in environmental waterways and they are often used as a bio-indicator of viral contamination (Wurtzer *et al*., 2014).

In the past, the method for detecting enteroviruses has been based on virus infectivity of cell lines, but this method is expensive, time-consuming, and difficult to perform due to the absence of a susceptible cell line for each enteric virus.

Nucleic acid-based methods have proven to be highly specific and they can detect even very small amounts of viral particles in mussel homogenates (LeGuyader *et al*., 2001). However, successful application of molecular methods for enteric virus identification has been limited by the presence of RT-PCR inhibitors in shellfish.

Dissection of the digestive tract and diverticula (hepatopancreas) appears to reduce the presence of such inhibitors and it increases the sensitivity of molecular methods (Le Guyader *et al.*, 2006).

The purpose of this study was to evaluate the presence of enteroviruses in oysters collected for environmental monitoring and to analyze the seasonal variation of the contamination levels Additionally, our study aimed to illustrate the importance of including a routine virological analysis of shellfish in the monitoring of shellfish harvesting areas in Morocco.

2.Materials and Methods

2.1.Sample collection and processing

Oualidia lagoon (34°47'N - 6°13'W and 34°52'N - 6°14'W) is located on the Moroccan Atlantic coast in the province of Sidi Bennour, 168 km south of the city of Casablanca. It extends parallel to the coast for a distance of approximately 8 km and it is 0.5 km wide. Due to its location between two hills that constitute its watershed, Oualidia lagoon is a confluence of runoff water during rainfall in the region. On the other hand, fecal contamination (during the spreading of animal manure) and wastewater infiltration from septic tanks are the major mainland inputs to the lagoon (El Himer *et al*., 2013 ; Hassou *et al*., 2016). This lagoon is facing various environmental problems (liquid and agricultural practices) that will destroy the quality of the coast and threaten the collection

of various aquatic products for consumption (Hassou, 2014). Oyster (*Crassostrea gigas*) samples (n=12 individuals/sample) were collected bimonthly over a 13-month period from March 2018 to March 2019, amounting to a total of 52 samples. The sampling sites (S1 and S2) correspond to two oyster farms located in Oualidia lagoon (Figure 1).

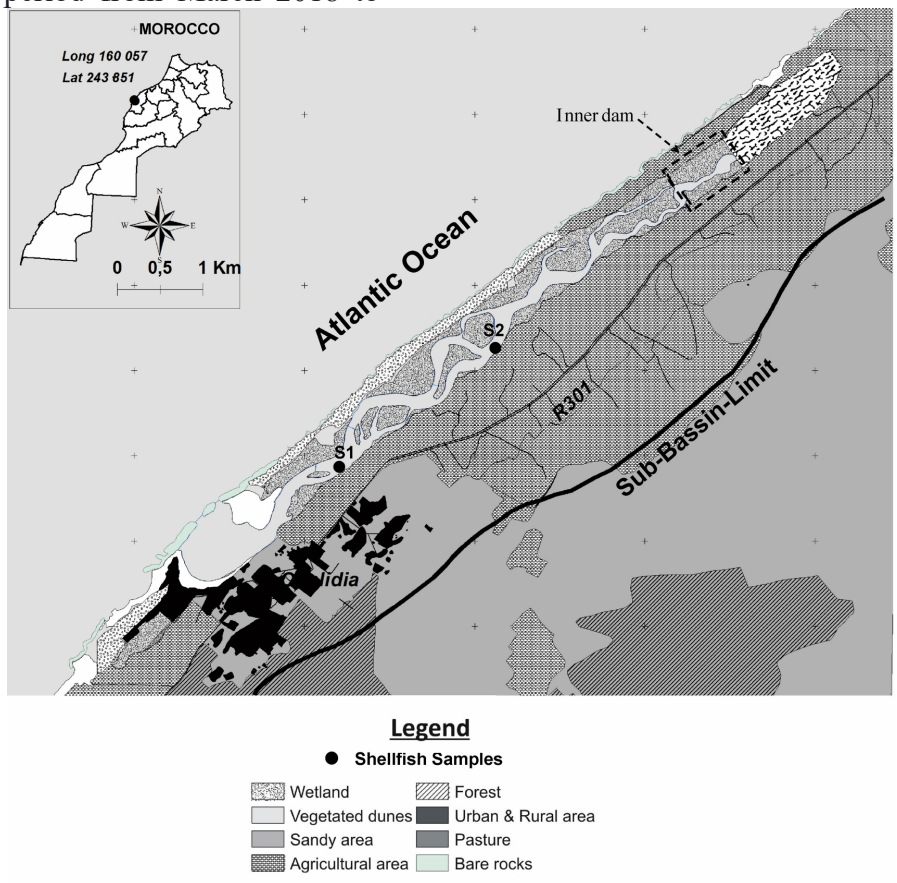

Figure 1. Shellfish collection sites

• S1, downstream of the lagoon (sandy sedimentary),

• S2 in the middle of the lagoon (muddy sedimentary),

Bivalve mollusks were transported to the laboratory under controlled temperature (+4 °C) within 24 hours of being harvested. They were processed immediately. The digestive tissues were dissected, finely chopped, and stored at -80 °C for further use. Aliquots of 2 grams were prepared for extraction of viral RNA. The pluviometry data for 2018-2019 were obtained from the local weather station. Based on the pluviometry data, the study period was divided into a dry period comprising June 2018 and July-August - September 2018 and a rainy period from October 2018 to May 2019.

2.2.Viral RNA extraction:

The tissues were homogenized with TRIzol reagent (1/1 w/v), and the homogenates were incubated at room temperature with mild agitation (200 rpm) for 20 minutes. After centrifugation at 3000 x g for 10 minutes, the supernatants were collected and mixed with 4 µl of the internal extraction control RNA. RNA was extracted from 500 µl of each supernatant using a PureLink™ Viral RNA/DNA Mini Kit (Invitrogen), according to the manufacturer's protocol. The RNA was recovered in DNase/RNase-free sterile water from the kit.

The RNA quality (ratio of absorbance at 260 and 280 nm) was assessed (30 µl of each eluate) using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA).

2.3.One-Step qRT-PCR

qRT-PCR assays for detection and quantification of enteroviruses were performed in a 7500 Fast Real-time PCR System (Applied Biosystems) using specific One-Step System kit Primerdesign™ Ltd enterovirus (5' non-coding region Genesig® Advanced Kit). Five microliters of RNA sample was amplified in a total volume of 20 µl with one-step reaction mix. Five microliters of each RNA sample was amplified in 20 µl of reaction mix that contained 1 X reaction mix, 10 µL of oasig® OneStep 2X RT-qPCR Master Mix, 1 µl of enterovirus primer/probe mix, 1 µl of internal extraction control primer/probe mix, and 3 µl of RNase/DNase-free water). The primers have previously been described by the manufacturer in the Genesig® Advanced Kit handbook. The amplification protocol was as follows: reverse transcription at 55 °C for 10 min was followed by 50 cycles of 2 min at 95 °C, 10 s at 95 °C, then 60 s at 60 °C for data collection, and then cooling at 40 °C for 30 seconds. Fluorescent signals were acquired during this step through the FAM and VIC channels. All of the samples were analyzed in duplicate. Two negative controls (only RNase/DNase-free sterile water) were included in each RT-PCR series. Five standards (from 2 copy numbers/µl to $2x10^4$ copy numbers/µl) were prepared by dilution of a positive control ($2x10^5$ copy numbers/µl). Sample purity (the absence of inhibitors that limit PCR efficiency) was tested using internal controls (IC) from the RT-PCR kits that were spiked in the samples. Briefly, 10 µl of the IC was added to each RNA sample and the Ct values were compared with the Ct value of the negative control (RNase/DNase-free sterile water) spiked with the IC. A threshold Ct value of 30 was used to determine sample purity.

The interpretation of the results was as follows:

Positive control: A positive result indicates that the primers and the probes for detecting the target enterovirus gene worked properly. In case of a negative result, the test results are invalid and must be repeated.

Negative control: A negative result indicates that the reagents had not become contaminated while setting up the run.

Internal RNA extraction control: The internal control is detected through the VIC channel and yielded a Cq value of 28 ± 3 depending on the level of sample dilution.

Sample results:

A positive quantitative result is thought to be the case when:

The negative control was verified as being negative,

The positive control was positive with a CT value between 16 and 23,

The internal control was positive with a CT value within the range of 28 ± 3

The sample could be amplified by 50 cycles of the OneStep RT-qPCR amplification protocol.

The positive control template is expected to amplify between CT 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

2.4.Statistical analysis

Spearman's rank correlation analysis was employed to correlate the results of positive samples pooled by month and pluviometry. These correlations were performed with the total accumulated rain of the previous month, assuming that each oyster harvesting area was mainly affected by the rains of the preceding month. All of the statistical analyses were performed using the statistical package SPSS Statistics 17.0. Statistical significance was determined by a P-value < 0.05.

3.Results and discussions:

Enteroviruses (EV) were detected by real-time RT-PCR in 36.54% of the oysters (Table 1). Based on the statistical analysis, the

detection rate of EV was significantly high in the oysters ($P < 0.05$).

Table 1. Prevalence of EV from bivalve molluscs collected in Oualidia Lagoon.

Stations / Month	S1		S2	
March 2018	+	+	-	+
April 2018	+	+	+	+
Mai 2018	-	-	-	-
June 2018	-	-	-	-
July 2018	-	-	-	-
August 2018	-	-	-	-
September 2018	-	-	-	-
October 2018	+	+	-	-
November 2018	+	+	+	+
December 2018	+	+	+	+
Junuary 2019	-	-	-	-
February 2019	+	+	-	-
March 2019	-	-	-	-
	12/26* (46.16%)		**7/26* (26.92%)**	
	19/52* (36.54%)			

*Significant difference of EV detection rate in S1 and S2 ($P < 0.05$).

Of the 26 batches of oysters collected at site 1, 46.16% were positive. Of the 26 batches of oysters from site 2, 26.92% were positive for these foodborne viruses. In terms of the seasonal variation, the detection rate of EV in oysters was 0.0% in the dry periods and 32.69% in the winter/rainy period (Table 2).

Table 2. Seasonal prevalence of EV detected in bivalve molluscs collected from Oualidia Lagoon.

Season	EV
Dry periode	0/52* (0.0)
Rainy periode	17/52* (32.69)

*Detection rates of EV were statistically significant in rainy season ($P < 0.05$).

These properties ensure that enteroviruses are very well-dispersed on the water surface or the wastewater from sewage treatment. Human enteroviruses are not inactivated in the water environment and will, therefore, often be caught and activated by the filter feeders of such mussels (Benabbes et al., 2013b). Most of the positive samples ranged from 4.31 to 2E+05 RNAc/g (Table 3).

Table 3. Monthly geometric mean quantification numbers of positive samples EV along the study period.

		CT value	Log Enteroviral Quantification	Enteroviral Quantification
S1	**November-18**	19	5,301	2,00E+05
	October-18	23	3,968	9,28E+03
	December-18	26	2,968	9,28E+02
	April-18	28	2,301	2,00E+02
	February-19	28	2,301	2,00E+02
	March-18	29	1,968	9,28E+01
S2	**November-18**	22	4,301	2,00E+04
	October-18	-	-	-
	December-18	30	1,634	4,31E+01
	April-18	32	0,968	9,28E+00
	February-19	-	-	-
	March-18	33	0,634	4,31E+00

EV yielded the highest average viral levels (2×10^5 RNAc/g DT) at site 1 in November. The results of the quantification show that the concentration of the enteroviruses had a spatio-temporal variation. These concentrations increased with the first heavy rains during October, November, and December, which caused septic tanks that had filled during the summer to overflow. The concentrations after the rains in March and April were lower due to the effect of the dilution caused by the rains of the winter months (Figure 2).

The results obtained for the two sampling sites were grouped to evaluate their seasonal prevalences. EV was detected in 6 out of 13 months in S1. EV was detected in 4 months, in March and April and between October and December in 2018, and in February of 2019. The average quantification level percentages of EV-positive samples over the study period are shown in Table. 3. Fisher's exact test showed highly significant differences in the virus prevalence between the dry period (May to September) and the rainy period (October to April) ($P < 0.0001$). A significant positive correlation between the number of positive samples and the average rainfall was observed.

Shellfish are known to accumulate human pathogens such as human enteric viruses, as demonstrated for rotavirus in mussels (Bosch *et al*., 1995) and enterovirus (McLeod *et al*., 2009), hepatitis A virus (Kingsley and Richards, 2003), and norovirus (Maalouf *et al*., 2010) in oysters. Shellfish contamination has been investigated in many countries (Benabbes *et al*., 2013; Coelho *et al*., 2003a,b; Croci *et al*., 2000; DePaola *et al*., 2010; Diez-Valcarce *et al*., 2012; Formiga-Cruz *et al*., 2002; Gallimore *et al*., 2005; Hansman *et al*., 2008; Kittigul *et al*., 2011; Mesquita *et al*., 2011; Namsai *et al*., 2011; Nishida *et al*., 2003; Romalde *et al*., 2002; Vilariño *et al*., 2009; Wang *et al*., 2008; Yilmaz *et al*., 2010). Although the elution concentrations and detection methods were different, the contamination level of foodborne viruses varied by country, the season, and the type of shellfish. In Morocco, the measurement of enterovirus levels in shellfish has been the subject of several studies in different lagoon regions or coastal areas and using different methods and techniques.

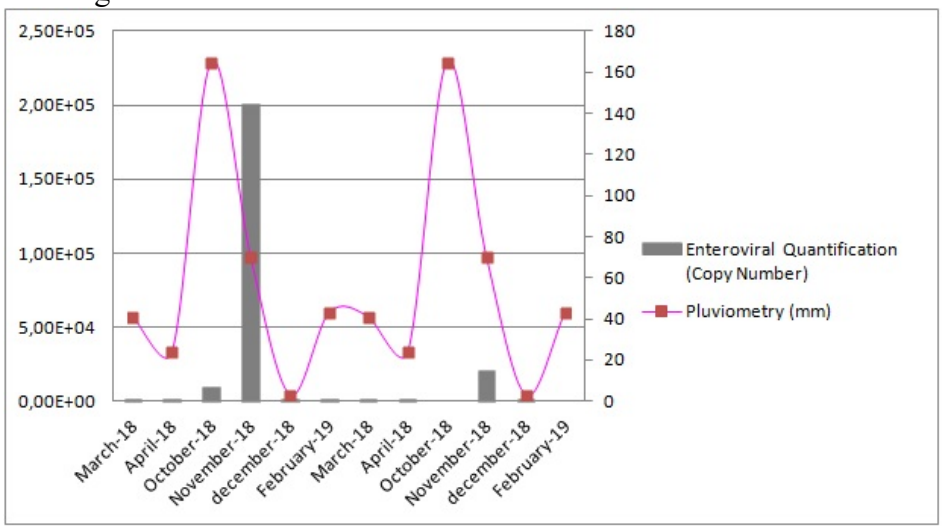

Figure 2. Spatio-temporal variation of enteroviruses concentration

As the technology for analytical methods develops, these viruses are being detected more and more. Indeed Karamoko *et al* in (2006) detected this virus in 10% of the samples tested from the Mohammedia area. Laila Benabbes *et al* in (2013b) found viral contamination of 36.3% of the samples collected from two shellfish production areas along the Moroccan Mediterranean coast, whereas in 2017 Lalla Meryem Idrissi Azzouzi

et al found that 75% of mussels were contaminated by enterovirus collected in three wild populations; Bouregreg estuary and Yaacoub Al Mansour In the present study, EV was detected significantly more frequently than NoV as found by (El Moqri *et al*., 2019). Moreover, the monthly prevalence was observed to be different, and many studies have reported a seasonal distribution of NoV in shellfish, water, and sewage samples, mainly in the cold months (Iwai *et al*., 2009; Le Guyader *et al*., 2000; Lowther *et al*., 2012b; Suffredini *et al*., 2012). Pluviometry is widely recognized to be one of the main environmental parameters that can influence viral detection in estuaries and shellfish due to the runoff or sewage treatment plant failures. Here, a correspondence between the number of detections and the pluviometry was statistically significant. Increases in pluviometry are more often associated with cold months; however, in the Oualidia region, heavy rainfalls typically occur in autumn and winter. The total rainfalls for the cold and the warm months during the study period were 237 mm and 64 mm, respectively. The relationship between the detection rate of EV and the sampling location showed that the prevalence of EV was significantly associated with the sampling site ($P < 0.05$). Several sites exhibited high EV contamination rates in shellfish. Indeed, Coelho *et al*. (2003b) found that oysters were readily contaminated in production areas that were close to human sewage (Rigotto *et al*., 2010). Thus, it is very important to control sewage or fecal contamination near shellfish production sites. In our study, monitoring of enterovirus detection in oysters was associated with seasonal variations. Indeed, a high EV contamination rate was found in winter. In several studies, seasonal variation of foodborne viruses has been explained by the fact that viruses are removed less effectively from shellfish in winter and that they can survive better in winter than in summer (Greening, 2006 ; Lees, 2000).

4.Conclusions

Our study showed that the prevalence of EV in oysters from Oualidia lagoon was quite high, and it was significantly associated with seasonal variation. Therefore, monitoring of foodborne viruses in oysters may contribute to the prevention of viral food poisoning and the promotion of public health. As a secondary conclusion, our study showed the importance of including routine virological analysis in the monitoring of shellfish harvesting areas in Morocco.

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Acknowledgments

The authors wish to thank the Virology, Oncology and Medical Biotechnology laboratory team members at FSTM-Hassan II University of Casablanca for all their efforts to support us during all of the stages of the work for this research study.