

Molecular characterization of rotavirus in oysters from Oualidia lagoon in Morocco

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ABSTRACT

Group A rotavirus (RVA) is the most important foodborne pathogen and is transmitted by oysters. The purpose of this study was to monitor and evaluate the prevalence of RVA in oysters collected in Oualidia Lagoon, Morocco from March 2018 to March 2019 and its relationship to watershed contribution. To achieve this, 312 oysters from 26 lots were collected and tested for viral contamination using real-time polymerase chain reaction. The results showed that 46.15% of the tested samples were positive for RVA. The seasonal distribution of RVA was statistically significant. This is a warning signal against the potential health risks in this oyster growing area, following the consumption of oysters contaminated with RVA, which presents a huge threat to human health resulting in serious illnesses of gastroenteritis.

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Introduction

Oualidia is the largest lagoon in the Atlantic Sea and the aquaculture sector plays an important role in the regional economy. The shellfish farming sector is the driving force of the region's economy which is a producer and purification center for bivalve molluscs either consumed locally or exported (1). The consumption of shellfish products, although recommended for their nutritional value, may pose a risk to human health due to their filtering activity and ability to bioaccumulation pathogens from surrounding waters (3). Among other pathogens, human rotaviruses are excreted through the feces of infected individuals and can enter coastal areas through inefficient wastewater treatment, or direct discharge of untreated wastewater (4,5). This factor is of particular concern in developing countries, where untreated sewage is discharged into water sources, causing environmental pollution (6). Therefore, shellfish have been proposed as bioindicators of the microbial quality of the aquatic environment. In addition, they have been suggested for estimating the prevalence of viral diseases in populations living in coastal areas, for hepatitis A virus (HAV) (7,8) and more recently SARS-CoV-2. Norovirus (NoV), rotavirus (RV), adenovirus (AdV), hepatitis A (HAV) and hepatitis E virus (HEV) are the causative agents of gastroenteritis, meningitis and hepatitis (9).

Rotavirus is one of the leading causes of infectious gastroenteritis worldwide (10, 11). Prior to the introduction of vaccines, it was estimated that approximately all children under the age of 5 had been infected with rotavirus; 352,000 to 592,000 children under the age of 5 are estimated to die each year from rotavirus infections, and 82% of these deaths occurred among children in low-income

countries (12). Rotavirus is transmitted from person to person via the fecal-oral route (11,13). It can also be transmitted through fecal-contaminated water, especially in developing countries (11,14). Eating shellfish can also be a source of rotavirus contamination. Oysters are considered important vectors for RVA transmission (15- 17). Rapid and sensitive assays are urgently needed to monitor rotavirus contamination in oysters (18).

Rotavirus detected in oysters is the cause of oyster-related food poisoning (16, 19). Oysters can accumulate gastroenteritis-causing viruses discharged into seawater through filter-feeding (20). In particular, norovirus accumulation in oysters has been actively studied by many researchers (21, 22).

In our study, the concentration of rotavirus A (RVA) in cultured oysters was investigated using quantitative real-time PCR (qPCR) to study the relationship between RVA in oysters and the seasonal variation. RVA concentrations in oysters were found to increase during winter. However, RVA in oysters was related to either RVA concentration in sewage or the number of rotavirus-associated gastroenteritis cases.

Materials and Methods

Sample collection and processing

Oualidia Lagoon (34°47'N - 6°13'W and 34°52'N - 6°14'W) in Morocco on the Atlantic coast of Sidi Benour, 168 km south of the city of Casablanca. It extends a distance parallel to the coast of about 8 kilometers long and 0.5 kilometers wide expired to its position between the two mountains that formed its watershed, Oualidiya lagoon is a confluence of Runoff water when it rains in

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the area. On the other hand, fecal contamination, in the application of animal manure and wastewater septic tank infiltration, is the most important Input from the mainland into the lagoon (23). This lagoon is Facing various environmental issues (liquids and agricultural practices) that will destroy them Coastal Quality and Threat Collection Oyster (*Crassostrea gigas*) samples (n = 12 individuals/sample) were collected every two months over 13 months from March 2018 to March 2019, for a total of 26 batch samples. The sampling points (S1 and S2) correspond to two oyster farms in the Oualidia lagoon (Figure 1).

The sampling sites (S1 and S2) correspond to two oyster farms located in the Oualidia lagoon (Figure 1).

- S1, downstream of the lagoon (sandy sedimentary),
- S2 in the middle of the lagoon (muddy sedimentary),

Oysters are shipped to the laboratory within 24 hours of harvest at a controlled temperature (+4°C). They were dealt with immediately. Digestive Tissue (DT) was dissected, minced aliquoted at 1.5g and stored at -80 °C for further viral RNA extraction use. Meteorological data for 2018-2019 were obtained from local weather stations (Figure 2).

According to the rain gauge data, the survey period is divided into June 2018 and the dry period from July-August 2018 to September 2018 and the rainy period from October 2018 to May 2019.

Total RNA extraction

RNA extraction was performed from the Digestive Tissue (DT) of mussels by The GenElute™ Universal Total RNA Purification Kit (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions, and the qualities of the extracted RNAs were assessed using a NanoDrop 2000/200c spectrophotometer (Thermo Fisher Scientific, USA) at 260 and 280 nm.

Evaluation of extraction efficiency and detection limits

Once the extraction is performed, three separate subsamples of the nucleic acid suspension are analyzed. To one subsample a known concentration of the ssRNA control is added. With two of the subsamples, including the one with the ssRNA control, quantification of HAV is performed. With the third subsample, quantification of mengovirus is assessed, by adding a known quantity of Mengovirus to each sample before processing and comparing real-time RT-PCR amplification of the spiked sample to that of an equal quantity of purified Mengovirus. Accordingly, 10 µL Mengovirus (Mengovirus@ ceeramTools™ Kit, Ceeram,

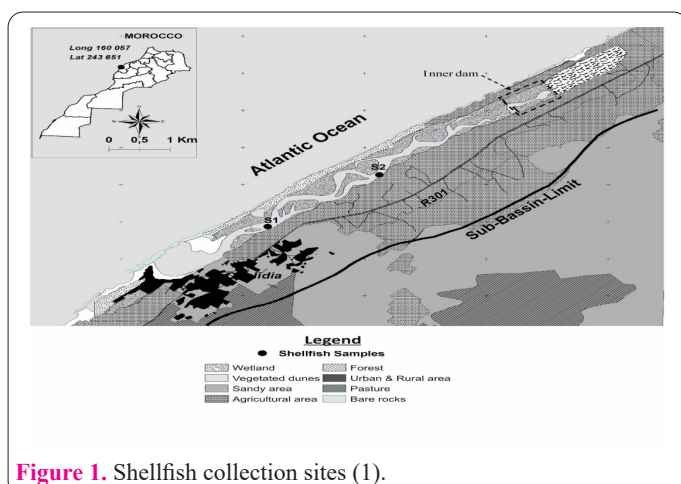


Figure 1. Shellfish collection sites (1).

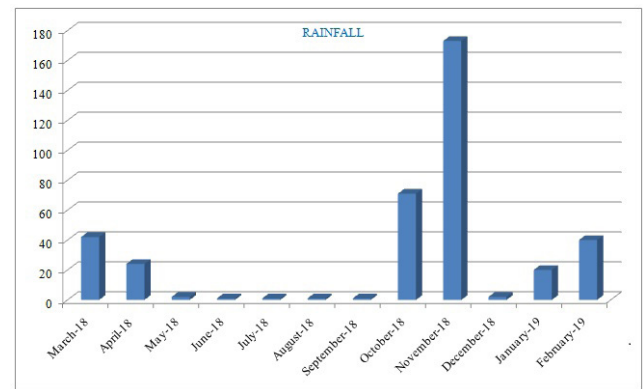


Figure 2. Average monthly rainfall in the Oualidia lagoon during the period March 2018-March 2019 (1).

France) was added to 1.5 ± 0.2 g artificially contaminated digestive tissues before extraction, and a 10-fold serial dilution of Mengovirus was used to establish a standard curve. The standard curve was then used to estimate the Ct value of Mengovirus RNA extracted from artificially contaminated samples and, thus, assess extraction efficiency. Extraction yields of >1% were considered valid.

The amplification of the mengovirus gene was carried out using the primers described by (Pinto et al., 2009) (24), (sensMengo110: GCGGGTCCTGCCGAAAGT), and (anti-senseMengo210: GAAGTAACATATAGACAGACGCACA), targeting a non-coding conserved region (5' region) of mengovirus viral RNA and probe (Mengo147: ATCACATTACTGGCCGAAGC), labelled at the 5' end with the fluorophore 6-carboxy fluorescein (FAM), and a quencher at the 3' end by adding a minor groove binder (MGB). The extraction efficiency calculation was performed in the 26 TD samples, which were tested diluted at (1:10), by QiagenOneStepRT-PCR for the presence of mengovirus RNA.

Identification of false negatives

To assess the frequency of false negative PCR results resulting from the presence of inhibitors, 1 µL titrated internal control (IC) was added to all real-time RT-PCR reactions.

Molecular characterization of RVA by RT-PCR One Step

RT-PCR molecular analysis was performed using the Primer design genesig Advanced Kit for Human Rotavirus A, which targets the NSP5 gene. The 20-µL reactions included 5 µL of sample RNA (1:100 to 1:1000 dilution, depending on sample concentration) and 15 µL of master mix and were amplified using the SaCycler-96 (Sacace Biotechnologies), following the manufacturer's instructions: reverse transcription at 55°C for 10 min, enzyme activation at 95°C for 2 min, 50 denaturing cycles at 95°C for 10 s, and then FAM and VIC quantification at 60°C for 60 s. All samples were analyzed in duplicate, and two negative controls (RNase-free water) were included, as well as five standard concentrations (2 to 2×10^4 copies/µL) of positive control (2×10^5 copies/µL).

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.

Results

Limit of detection of the Primer design test

The nucleic acid extraction efficiency for all shellfish samples was evaluated. The detection limit of the Primer design genesig Advanced Kit for Human Rotavirus A, which targets the NSP5 gene was established using a 10-fold serial dilution of RVA stock solution (concentration of 2×10^5 copies/ μ L), an amplification efficiency of 96% was obtained. The evaluation of real-time PCR detection limits, which were performed using 10-fold dilutions of positive control, revealed a sensitivity of 10 genomic copies per reaction. The Ct value of 32.2 relatives to 10 copies/reaction corresponds to the detection limit (Table 1).

Rotavirus prevalence and quantity

Extraction efficiency was calculated using the difference between the Ct value of the sample and the Ct value of the mengovirus used as process control in the assay. Therefore, 12 samples (46.15%) of the 26 were positive Primer design genesig for Rotavirus A, with viral loads ranging from 0.421×10^3 to 1.7603×10^4 PFU/g digestive tissues (Table 2). In terms of seasonal variation, Rotavirus A was very abundant during the rainy season and absent during the dry season. Regarding the sampling sites, 38.46% of the samples collected from station S1 are positive for RVA while 53.85% of the samples collected from station S2 are positive for RVA.

Discussion

Human and animal rotavirus have been previously reported as contaminants in water and shellfish and, also associated with food-borne outbreaks (15, 18, 19).

This study was conducted to assess the presence of RVA in oysters harvested from two oyster farming sites in the Oualidia lagoon, classified as sampling zone "B" (decision n° 1950-17 of 07/08/2017 of the Moroccan Ministry of Agriculture, rural development and maritime fishing). We found that 46.15% of the samples collected during the period from March 2018 until March 2019 are contaminated with this virus. These Positive samples correspond to batches harvested during the rainy period. This result is explained by the overflow of septic that had filled during the summer (1, 2). 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). 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.

These results can also be explained by the fact that viruses are more stable during the winter, when, UV radiation is less intense. In several studies, enteric viruses have been reported to survive for longer and occur more frequently during the winter months in natural environments (25, 26). Contamination by RVA is higher at station S2 (53.85%) compared to station S1 (38.46%), this difference is explained by the nature of the sediments which are muddy sediments at station S2 and therefore have strong retention compared to the sedimentary sand of the S1 station.

An extensive study of Florida Keys shows widespread bacterial and viral contamination of near-shore surface

Table 1. Detection limits of real-time PCR tests and within-test dilution accuracy results of 10 times positive control tested in three specimens.

Nb of RNA copies per reaction	Detected samples	Average Ct
10^4	3/3	23.5
10^3	3/3	26.7
10^2	2/3	29.6
10^1	2/3	32.2
1	1/3	36.3

Table 2. Quantification of RVA in positive samples during the study period.

Sampling periods	Station 1			Station 2		
	RT-PCR Result	CT	Viral Load UFP/g	RT-PCR Result	CT	Viral Load UFP/g
03-2018	+	28.2	11283	+	30.5	1790
04-2018	-	34.3	<LD	+	29.8	2438
05-2018	-	-	-	+	33.3	421
06-2018	-	-	-	-	-	-
07-2018	-	-	-	-	-	-
08-2018	-	-	-	-	-	-
09-2018	-	-	-	-	-	-
10-2018	+	27.6	16049	+	32.7	632
11-2018	+	27.5	17603	+	31.2	1620
12-2018	+	30.8	1970	+	32.9	624
01-2019	+	30.7	2221	-	-	-
02-2019	-	-	-	+	32.6	667
03-2019	-	-	-	-	-	-

bodies of water commonly associated with sewage septic systems deal with, a total of 17 canal locations and 2 coastal waters, 79% of sample sites were positive for enterovirus, 63% were HAV positive and 11% were Norwalk virus positive when examining samples by RT-PCR (27). Inside Southern California coastal waters near US-Mexico Boundary, Jiang et al. (2001) (28) found that 33% (4 out of 12) of the ocean sample was positive for adenovirus. Interestingly in these marine locations beyond the river, Jiang et al. (2001) pointed out bacterial indicators and the presence of the virus.

The moderate number of RVA genomes detected indicates water contamination from human sewage. RVA is excreted in high concentrations (up to 10^{10} gc/g) in the feces of infected individuals and remains intact for long periods in the environment (27). Fumian et al (2011) (29) tested RVA in human wastewater and found a 100% positive rate between 10^4 and 10^7 gc/L. A recent study conducted in Morocco by Hatib et al (2021b) (4), the aim of which was to assess the prevalence of RVA in mussel specimens collected from the Oued El Maleh estuary, revealed the presence of rotavirus RNA in 37.5% of the batches of samples analyzed, this allows us to distinguish that this virus is already in circulation in Morocco.

To our knowledge, this is the first study that describes the presence of RVA in shellfish cultured in the coastal zone of the Oualidia lagoon, although the presence of other human enteropathogenic viruses, such as norovirus, hepatitis A and enterovirus, has already been reported (1, 2, 30, 31). It should also be noted that our results correlate with other similar studies, in particular the work of Boussettine et al, (2020) (1) who worked during the same period (from March 2018 to March 2019) of our studies and showed the presence of the enterovirus genome in 32.69% of the batches of shellfish tested.

Mozgovoj et al (2022) (32) showed that virus particles remain infective in oysters and that consumers may be at risk of infection. These results constitute a warning and justify the need to strengthen the surveillance of foodborne viruses in order to better understand their circulation in our country and to improve the control protocols for seafood products. Especially Moroccan regulations (decision n°1950-17 of 07/08/2017 of the Moroccan Ministry of Agriculture, Rural Development and Maritime Fisheries) define the microbiological criteria for bivalve molluscs by measuring the concentration of *Escherichia coli* (230 per 100 g) without taking into account the presence of enteric viruses.

Conclusion

Our study showed that the prevalence of the RVA of oysters from the lagoon of Oualidia was quite high, and it was significantly associated with seasonal variation. The Oualidia lagoon has been affected by human faecal pollution, and the levels of pathogens detected in this study indicate that they could pose a risk to the population. It is therefore particularly important to develop water quality indicators for human wastewater contamination to address the risk of viral infections.

Author's contribution

A.H: Conceived the project design, experimental analysis and drafted the manuscript; R.B: Collection of samples, participation in the drafting and review of the manuscript;

N.H: statistical analysis, drafting and review of the manuscript; A.B: participated in the experimental analysis and review of the final manuscript; M.M.E: Conceived the design and coordination of the project and review of the final manuscript, and coordinate the whole project. All authors have reviewed and approved the manuscript.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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