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Research Article

Spatial-temporal assessment of Norovirus contamination in mussels from Cherrat estuary, Morocco, by real-time Reverse Transcription-Polymerase Chain Reaction

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Abstract
Mussels filter large amounts of water to extract nutrients; therefore, they can concentrate and accumulate in their tissues infectious agents, and vectors of enteric diseases. The aim of this study was to assess mussel contamination by Norovirus genogroups I and II in the Cherrat estuary to determine the public health risk linked to their consumption. Mussels (*Mytilus galloprovincialis*) were collected (n=52 samples; 12 mussels/sample) at four sites (S1 and S2 located on the right rocky bank / S3 and S4, located on the rocky left bank) in the Cherrat estuary (Casa-Settat region), Morocco, during 13 months, from March 2019 to March 2020. Norovirus was detected and quantified by real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Norovirus genogroups I and II were detected in 17.30% and 94.23% of mussel samples, respectively. Contamination by Norovirus (genogroups I and II) was not correlated with seasonal factors (month and rainfall), and Norovirus prevalence was comparable among the four sampling sites. Consumption of raw or undercooked mussels contaminated with Norovirus can cause gastroenteritis, which represents a potential risk to human health. The present study would be helpful to control and manage the potential risk to the public health of the Moroccan population due to the degradation of water quality continuously impacted by runoff, the urban wastewater treatment system malfunctions, and overflows from nearby sewage systems.

Keywords: Cherrat estuary, mussels, Norovirus genogroups I and II, real-time RT-PCR, Viral contamination

INTRODUCTION

The Cherrat estuary is a transition zone between the Cherrat beach, classified as category A (i.e. excellent-quality bathing water) (Bazir *et al*., 2022; Ministry of Energy, Mines, Water and of the Environment in charge of the environment / Ministry of Equipment, Transport and Logistics, 2016), and Cherrat valley, labeled as Site of Biological and Ecological Interest (Bazir *et al*., 2022; Ministry of Territorial Development, Water and the Environment/Secretariat of State for the Environment, 2003).

Urban development and tourism at Cherrat estuary are important economic sources, and the related income is constantly increasing. However, they represent a danger to the marine environment and a public health risk because the water quality at the estuary is negatively affected by wastewater discharges. Indeed, wastewater can contaminate the beach directly in the form of illicit discharges evacuated through storm sewer outlets and due to malfunctioning of the local wastewater treatment systems, and indirectly through the pollution carried by rivers, runoff waters and marine currents (Bazir *et al*., 2022; Ministry of Energy, Mines, Water and of the Environment in charge of the environment / Ministry of Equipment, Transport and Logistics, 2016).

At Cherrat estuary, wild mussels (*Mytilus galloprovincialis*) are collected in an informal and traditional way, without any sanitary control. However, these bivalve mollusks play a very important role in the transmission of viruses because they filter large water volumes to extract nutrients. Therefore, they concentrate pathogen microorganisms and toxic substances (Oliveira *et al.,* 2011; Harsono *et al.,* 2017 and Zormati *et al.,* 2018).

Consumption of raw or undercooked shellfish causes human viral diseases (Schaeffer *et al.,* 2018), and bivalve mollusks can be one of the main vectors of enteric disease transmission (Bazzardi *et al.,* 2014). Contamination of bivalve shellfish, particularly oysters, by Norovirus is recognized as a food safety risk, with a considerable number of literature reports on outbreaks (Bellou *et al.,* 2013 and Lowther *et al.,* 2018). Worldwide, Noroviruses are the main cause of sporadic and acute viral gastroenteritis (Karangwa *et al.,* 2017), and they are the viral pathogens most associated with gastroenteritis outbreaks through consumption of contaminated food (De Andrade *et al.,* 2017), particularly bivalve mollusks (Kosek *et al.,* 2003). For instance, Norovirus RNA was detected in 68.7% of oyster samples tested in a study carried out in five European Union member states (Schaeffer *et al.,* 2018). In Morocco, Noroviruses have been detected in various mollusk types (Benabbes *et al.,* 2013 and El Moqri *et al.,* 2019).

Noroviruses belong to the *Calciviridae* family. This group of viruses is genetically divided into ten genogroups (GI to GX) that can infect humans (viruses from the GI, GII, GVIII and GIX groups) and animals (Chhabra *et al.,* 2019; Tatusov *et al.,* 2021; Ferla *et al.,* 2021 and Bonura *et al.,* 2021). The Norovirus genome is composed of ~7.5 kb positive-polarity, single-stranded RNA organized in three open reading frames (ORFs), except for murine noroviruses containing a fourth ORF (Chhabra *et al.,* 2019). This small, spherical, non-enveloped virus is stable and very resistant to the environment (Knight *et al.,* 2016), and can contaminate rivers and coastal waters: the areas where various types of bivalve mollusks are harvested (Schaeffer *et al.,* 2013).

In Europe, to protect consumers, the sanitary control of bivalve mollusks is regulated by legislation from the European Union (EU) and the European Commission (EC). Specifically, the EC Regulation n°2073/05 (2005a) states the microbiological criteria that are based on the determination of specific bacteriological parameters (*Salmonella spp.* and *Escherichia coli*). However, the same regulation specifies that the determination of faecal indicators does not reliably demonstrate the presence or absence of viral contamination (Bazzardi *et al.,* 2014). In agreement, many epidemics of gastroenteritis are linked to the consumption of bivalve mollusks from areas that conform to such food security criteria (El Moqri *et al.,* 2019). In addition, a systematic review of viral gastroenteritis epidemics linked to the consumption of bivalve mollusks found that Noroviruses were the most frequently involved pathogens (~84% of outbreaks) (Bellou *et al.,* 2013).

In this context, the present environmental and virological study evaluated the risk factors of virological contamination of mussels collected at the Oued Cherrat estuary (Casa-Settat region) in Morocco. The objectives of the study were i) to determine the level of contamination by Norovirus genogroup I (NoV GI) and Norovirus genogroup II (NoV GII) in wild mussels collected at four different sites in the Cherrat estuary; ii) to study the correlation between environmental/seasonal factors and the rate of mussel contamination by NoV GI and NoV GII.

MATERIALS AND METHODS

Study area

Cherrat estuary is downstream of a valley that constitutes a geographical and administrative limit between the prefecture of Skhirate-Témara (Rabat-Salé-Kénitra region) and the province of Benslimane (Casa-Settat region), and is located on the Moroccan Atlantic coast, 53 Km north of Casablanca (33°49'52.71" N - 7° 07'23.33" W). Cherrat estuary is part of a beach classified as category A for bathing water on the basis of microbiological parameters- faecal coliforms (*Escherichia coli*) and enterococci (*faecal streptococci*)] according to the relevant National Standard (NM 03. 7. 200), transposed from the European Directive (76/160/EEC) and the Directives WHO/UNEP, applicable to the health surveillance of marine bathing waters. Despite the ecological importance of this valley, the watershed area suffers from urban pressure and industrial pollution. This has a negative impact on this ecosystem, especially downstream (where the sampling areas were located), due to the natural resource overexploitation and the water quality degradation because of runoffs, urban wastewater treatment system malfunctions and overflows of nearby sewerage systems (Bazir *et al*., 2022; Ministry of Energy, Mines, Water and of the Envi-

ronment in charge of the environment / Ministry of Equipment, Transport and Logistics, 2016).

Sample collection

Naturally growing mussels (*Mytilus galloprovincialis*) (n=52 samples; ~600 mussels in total), were collected at four sites (S1 and S2 located on the right rocky bank / S3 and S4, located on the rocky left bank) at the Cherrat estuary (Casa-Settat region) (Fig. 1) from March 2019 to March 2020 (13 months in total). Each sample included 12 individual mussels. All samples were shipped to the laboratory in a refrigerated box within 24 h after collection. These sampling sites corresponded to wild mussel harvesting areas overexploited by the local population. The collected mussels are sold locally in an informal and traditional way by the local population, without any sanitary control.

Sample processing and RNA extraction

Shellfish samples were processed according to the ISO/TS 15216-1:2017 protocol for NoV GI and NoV GII detection in food samples (Microbiology of the food chain-Horizontal method for determination of Hepatitis A Virus and Norovirus using real-time RT-PCR - Part 1: Method for quantification). Mussels were rapidly rinsed, shucked and dissected. The digestive glands were removed and pooled to have a final weight of 2±0.2g for each sample.

Digestive tissues were mixed with 10 µL of Mengovirus (CeeramTOOLS®) as a control of nucleic acid extraction. Then, 2.0±0.2 mL of Proteinase K solution was added for tissue digestion at 37±1.0 °C with shaking at 320 rpm for 60±5 min. This was followed by a second incubation at 60±2.0 °C for 15±1 min in a water bath and centrifugation at 3000g at room temperature for 5.0±0.5 min. Then, supernatants were collected in clean tubes for immediate analysis or storage at -70°C until testing.

Viral RNA was extracted from 500µL of each supernatant and eluted in 100µL of DNase/RNase-free sterile water using the NucleoSpin RNA virus Kit (Macherey Nagel Germany) according to the manufacturer's instructions.

RT-PCR assays

The Luna® Universal Probe One-Step RT-qPCR Kit (New England BioLabs) was used for NoV GI, NoV GII, and Mengovirus MC_0 strain detection by real-time RT-PCR. Briefly, 4µL of each RNA sample was amplified in 21µL of reaction mix that contained 1X reaction mix, 10µL of Luna Universal Probe One-Step Reaction Mix (2X), 0.5 pmol/µL of forward primer, 0.9 pmol/µL of reverse primer, 0.25 pmol/µL of the probe, and 1.25 µL of Luna WarmStart® RT Enzyme Mix (20X). Primers and probes and their respective references are listed in Table 1.

RT-PCR assays to detect NoV GI and NoV GII were performed on an AriaMx 96-well plate real-time PCR instrument (Agilent Technologies, Santa Clara, CA) as follows: reverse transcription reaction at 55°C for

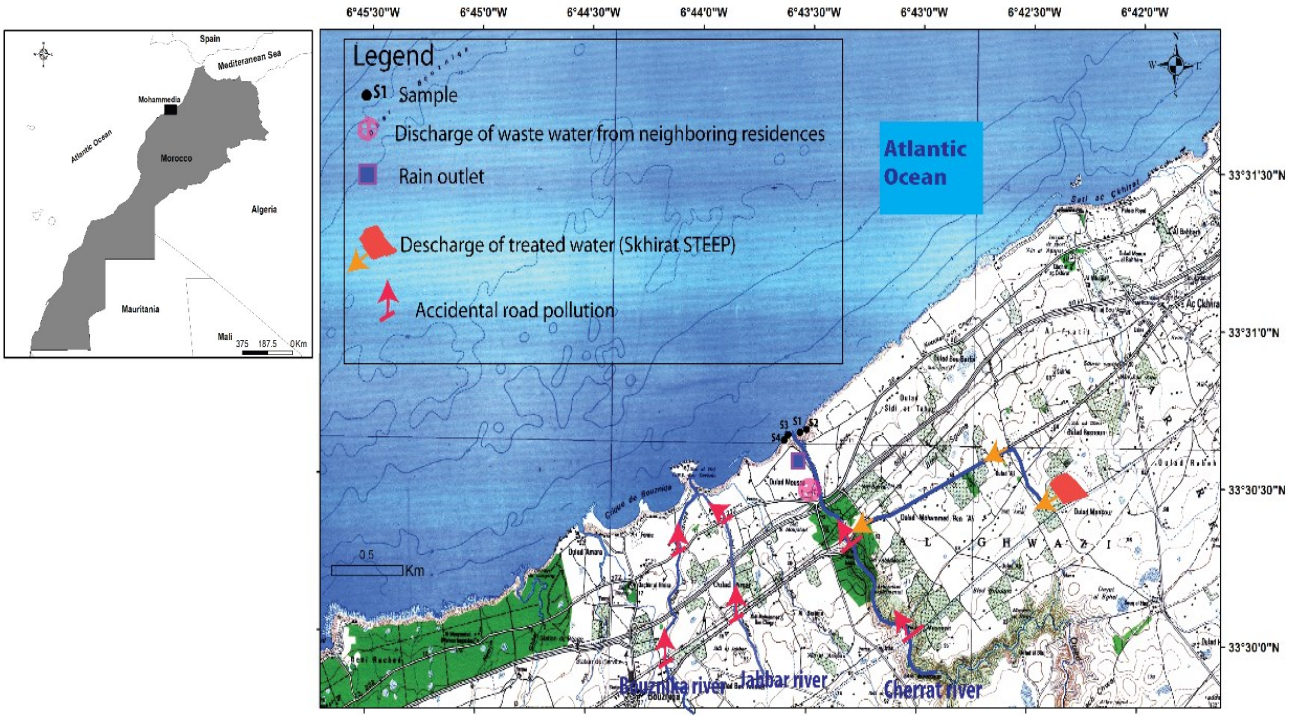

Fig. 1. *Localization of the four mussel sampling areas (S1 to S4) at Cherrat estuary. S1 and S2 are located on the right rocky side and S3 and S4 are on the rocky left side of Cherrat estuary, Morocco*

10min, initial denaturation at 95°C for 1min, followed by 45 cycles of denaturation at 95°C for 10s and extension at 60°C for 1min. The endpoint fluorescence signal was measured at the end of the 60°C annealing step, and data were captured and analyzed using the Agilent Aria software, v 1.71.

Each sample was amplified in duplicate (undiluted and 1/10 diluted) in each run. The presence of PCR inhibitors was evaluated, by amplifying the Mengovirus RNA, according to the manufacturer's instructions, and by comparing the Ct (cycle threshold) values of the pure and diluted RNA samples. A Ct value difference <3.3 indicated the presence of inhibitors. The extraction efficiency was evaluated by comparing the Ct values of the Mengovirus RNA extracted from the samples and those obtained for the standard curve. Results ≥1% were considered valid.

NoV GI and GII RNA samples were used as positive controls. Two negative controls were included in each RT-PCR run: negative control of the extraction (i.e. nuclease-free water) with the PCR mixture and negative control of amplification (i.e. nuclease-free water) with the PCR mixture.

Statistical analysis

The Spearman's rank correlation coefficient was used to correlate NoV-positive sample rates with sampling month and rainfall data. The pluviometry data for 2019-2020 were obtained from the local weather station. These correlations were performed with the total accumulated rain of the previous month, assuming that each mussel harvesting area was mainly affected by the rain of the preceding month. All statistical analyses were done with the IBM SPSS 17.0 software.

RESULTS

Viral detection

NoV GII was detected in 94.23% and NoV GI in 17.30% of all mussel samples analyzed (Table 2). All sampling points (S1–S4) showed similar viral detection frequencies.

Seasonality of viral detection

Results obtained for the four sampling sites were grouped to evaluate the monthly prevalence of NoV contamination. NoV GII was the most frequently detected virus throughout the collection period. NoV GI was detected from March to June 2019 and then in December, January and March 2020. The monthly percentages of NoV-positive samples during the study period are shown in Table 2.

NoV detection rate was not significantly different (Fisher's exact test) between the warm months (April to September) and the cold months (October to March). NoV contamination, especially NoV GII, was found throughout the year. No correlation was found between the number of positive samples and the total rainfall in the previous month.

DISCUSSION

At the four study sites, 94.23% of mussel samples were contaminated by NoV GII and 17.30% by NoV GI. NoV presence in mussels collected at the Cherrat estuary is explained by contamination with human fecal matter brought by wastewater treatment effluents, storm overflows, sewage overflows, and discharges from septic tanks of houses close to the Cherrat estuary (Fig. 1). Moreover, the watershed of Cherrat River drains exclusively impermeable land and covers an area of 620.8 km². Its flow decreases from May and disappears in August (Ministry of Agriculture, Maritime Fisheries, Rural Development, Water and Forests). Viral particles can persist in the marine environment and be transported (>10 km), representing a possible contamination risk for shellfish production areas (Flannery *et al.,* 2013; Winterbourn *et al.,* 2016; Hassard *et al.,* 2017).

Based on our data, NoV detection rate in mussel samples was different between the GI and GII genogroups

Table 1. Sequences of the primers and probes used for NoV GI and NoV GII detection

Virus	Sequence	Reference
NoV GI	Forward primer QNIF4 5′-CGC TGG ATG CGN TTC CAT-3′	(Da Silva *et al.,* 2007)
	Reverse primer NV1LCR 5′- CCT TAG ACG CCA TCA TCA TTT AC-3′	(Svraka *at al.,* 2007)
	Probe NVGG1p FAM 5′-TGG ACA GGA GAY CGC RAT CT-3′ TAMRA	(Svraka *at al.,* 2007)
NoV GII	Forward primer QNIF2 5′-ATG TTC AGR TGG ATG AGR TTC TCW GA-3′	(Loisy *at al.,* 2005)
	Reverse primer COG2R 5′-TCG ACG CCA TCT TCA TTC ACA-3′	(Kageyama *at al.,* 2003)
	Probe QNIFs FAM 5′-AGC ACG TGG GAG GGC GAT CG-3′ TAMRA	(Loisy *at al.,* 2005)

Table 2. Detection rate of NoV GI and GII virus in mussels collected at four sampling sites at Oued Cherrat estuary (obtained from Bouregreg and Chaouia hydraulic basin agency), Morocco

Month	Rainfall (mm)	NoV GI virus				NoV GII virus			
		Sampling sites				Sampling sites			
		S1	S2	S3	S4	S1	S2	S3	S4
March 2019	26.8	–	+ (Ct 32.89)	–	–	-	+ (Ct 25.82)	+ (Ct 30.88)	+ (Ct 31.09)
April 2019	34.5	–	–	+ (Ct 33.66)	–	+ (Ct 28.54)	+ (Ct 30.25)	+ (Ct 30.21)	+ (Ct 31.59)
Mai 2019	45.6	–	–	–	+ (Ct 30.66)	+ (Ct 29.66)	+ (Ct 29.87)	+ (Ct 32.06)	+ (Ct 28.49)
June 2019	10.9	–	–	+ (Ct 33.32)	–	-	+ (Ct 29.83)	+ (Ct 30.52)	+ (Ct 31.97)
July 2019	0	–	–	–	–	+ (Ct 29.90)	+ (Ct 30.04)	+ (Ct 30.29)	+ (Ct 31.08)
August 2019	0	–	–	–	–	+ (Ct 31.71)	+ (Ct 31.97)	+ (Ct 30.19)	+ (Ct 32.09)
September 2019	5	–	–	–	–	+ (Ct 30.20)	-	+ (Ct 31.68)	+ (Ct 31.67)
October 2019	10.1	–	–	–	–	+ (Ct 30.74)	+ (Ct 31.93)	+ (Ct 31.73)	+ (Ct 31.07)
November 2019	32.6	–	–	–	–	+ (Ct 31.14)	+ (Ct 31.03)	+ (Ct 30.94)	+ (Ct 30.98)
December 2019	74.8	+ (Ct 31.22)	+ (Ct 30.7)	–	–	+ (Ct 28.75)	+ (Ct 28.87)	+ (Ct 30.68)	+ (Ct 29.57)
January 2020	270	+ (Ct 33.57)	–	–	–	+ (Ct 30.45)	+ (Ct 30.90)	+ (Ct 31.77)	+ (Ct 31.66)
February 2020	58.4	–	–	–	–	+ (Ct 32.69)	+ (Ct 32.46)	+ (Ct 31.09)	+ (Ct 31.04)
March 2020	18.5	+ (Ct 32.87)	+ (Ct 31.5)	–	–	+ (Ct 29.83)	+ (Ct 29.01)	+ (Ct 28.50)	+ (Ct 29.54)
		3/13 (23.07%)	3/13 (23.07%)	2/13 (15.38%)	1/13 (7.69%)	11/13 (84.62%)	12/13 (92.32%)	13/13 (100%)	13/13 (100%)
		17.30%				**94.23%**			

S1 and S2, located on the right rocky side of Cherrat estuary; S3 and S4, located on the rocky left side of Cherrat estuary; ct- Cycle threshold

(17.30% and 94.23%) but similar among the four sampling areas. The higher detection rate of GII over GI observed in our study could be related to higher GII prevalence in viral load excreted by the infected population. The present results are in agreement with other studies demonstrating a higher detection of NoV GII than NoV GI in India (Das *et al.,* 2020), Italy (Fusco *et al.,* 2019), Brazil (Cantelli *et al.,* 2019; Reymão *et al.,* 2018), New Zealand (Hewitt *et al.,* 2013), and France (Zakhour *et al.,* 2010), but not with studies carried out in Brazil where NoV GI infection rate was higher than that of NoV GII (Victoria *et al.,* 2010a, 2010b).

In present study, no seasonal variation in NoV contamination rate was observed for all mussel samples collected at the Cherrat estuary (Fisher's exact test; $p > 0.05$).

Indeed, a high rate of contamination by Norovirus has been observed both in rainy and dry periods, contrary to other studies conducted in Morocco on shellfish (Tarek *et al.,* 2019; El Moqri *et al.,* 2019). This year-round presence of this enteric virus may be due to the reception of this estuarine ecosystem from permanent fecal pollution.

Conclusion

Norovirus's contamination of bivalve shellfish, particularly mussels, is a recognized food safety risk and a potential contributor to the overall burden of gastroenteritis in human communities. The present study is the first to evaluate contamination by Norovirus in wild mussels that are informally collected by the riverside population at the Cherrat estuary, Morocco. It showed that the contamination rate by Nov GII was higher than that by NoV GI in mussels from Cherrat estuary, and that it was not correlated with seasonal factors (temperature and rainfall). The higher detection rate of GII over GI observed in present study could be related to higher GII prevalence in viral load excreted by the infected population. This qualitative study highlights that mussels can play a potential role in spreading viral pathogens and in the risk of enteric diseases in the Moroccan population and provides more information on NoV circulation in the aquatic environment. It also highlights the importance of including routine virological analyses when checking the safety of bivalve mollusks.

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Conflict of interest

The authors declare that they have no conflict of interest.

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