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# Detection of enterovirus in mussels from Morocco by cell culture and real-time PCR

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**The bivalve shellfish are filter feeders and they act as natural bio-filters in seawater and can thus efficiently bio-concentrate and bio-accumulate enteric viruses in their digestive tissue. In Morocco, shellfish sanitary quality analysis does not currently include enteric virus detection. Therefore, the objective of this study was to detect the presence of enterovirus in mussels (*Mytilus galloprovincialis*) collected from three wild populations (Bouregreg estuary, Yacoub Al Mansour coast and Harhoura coast) in order to get an overview on the viral contamination in the aquatic environment. Between February 2014 and February 2015, two hundred and eighty-eight samples were collected and tested for viral contamination using cell culture and real-time polymerase chain reaction (real-time PCR) for intratypic differentiation (ITD). The results by cell culture and real-time PCR showed that the consumption of mussels originated from a contaminated area revealed a clear risk of infection. For this reason, the presence of enterovirus in shellfish production area represents a potential health risk by causing serious illnesses (gastroenteritis, hepatitis and poliomyelitis).**

**Key words:** Enterovirus, shellfish, viral contamination, cell culture, real-time polymerase chain reaction (real-time PCR).

## INTRODUCTION

The impact of environmental pollution, especially in marine environment, by the transmission of viral infections was suspected in the beginning of the 20th century. However, the propagation of viral diseases has been demonstrated earlier throughout the period from 1940 to 1945 during epidemics of poliomyelitis (Le Guyader et al., 2014). The viruses most often transmitted by contamination of the marine water were noroviruses (NoV), hepatitis A virus (HAV), hepatitis E virus (HEV), adenovirus (AdV), astrovirus (AV), rotavirus (RV) and the

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enteroviruses (EV) (poliovirus, coxsackievirus, echovirus) (Griffen et al., 2003; Le Guyader et al., 2009). The diseases associated with enteric viruses are heterogeneous. In addition to poliomyelitis, enteric viruses in the human stool 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). Therefore, the environmental monitoring can provide an added tool to determine the different viruses circulating in a community (Pinto et al., 2007; Shulman et al., 2006). This is a way to monitor viral transmission in human populations by examining environmental samples in particular from bivalve molluscs (mussels). The mussels were chosen for their wide geographical distribution from temperate to subarctic regions and also because they are filter feeders. These characteristics make them useful bio-indicators (bio-accumulators) to evaluate and monitor the contamination level in aquatic environment (Formiga-Cruz et al., 2003).

The enteroviruses are one of the most frequently monitored viruses in environmental waterways and are often used as a bio-indicator of viral contamination (Wurtzer et al., 2014). For this reason, this kind of analysis demonstrates the importance of environmental monitoring by the assessment of enteric virus contamination in selfish. In Morocco, the viral pollution of the environment was the subject of several studies, but no study has been done to search the poliovirus in aquatic environment. The lack of a national monitoring program of enteric viruses was one of the reasons to do this study in order to evaluate the contamination of enteroviruses in mussels collected from potentially polluted areas. The target of this study was to supplement the Moroccan databases available on environmental contamination by enterovirus and illustrate the importance of including routine virological analysis of shellfish in Morocco.

## MATERIALS AND METHODS

### Sample collection and processing

A total of 288 mussels samples (*Mytilus galloprovincialis*) were collected between February 2014 and February 2015, from three wild population sites that receive domestic waste without previous treatment (Bouregreg estuary, Yacoub Al Mansour coast and Harhoura coast). The sampling sites were situated mainly in Rabat-Sale-Kenitra region in Morocco (Figure 1 and Table 1). This region covers an area of 18,194 $km^2$, with population nearing 4,581,000. This area belongs to the Mediterranean climate marked by two main seasons softened by oceanic influences. The average temperatures are around 12°C for the colder months (December and January) and 22°C for the warmer months (July to September). The average annual rainfall is more than 550 mm/year.

The coastal zone of the region faces various environmental problems (liquid and solid waste) that will destroy coastal quality and threaten the collection of various aquatic products for consumption. The samples were shipped to laboratory on the same day, in chilled condition. Therefore, they were processed before being stored at -20°C until virological analysis.

### Extraction-concentration of virus from samples

The technical laboratory method described by El-Senousy et al. (2013) was adopted, which is based on the adsorption of the viruses with acid pH and their elution with basic pH, according to the following protocol. The shells were opened in an aseptic way; the digestive system was dissected with a sterile shucking knife, making elimination of inhibitors tissues (polysaccharides, sexual gonads) possible while analyzing a larger number of individuals (1.5 g of hepatopancreas, weight corresponding to an analysis, represent on average 12 mussels). To extract the viruses, 1.5 g of hepatopancreas were homogenized for 5 to 10 min in a blender. The ground material was subjected to stirring for 15 min at room temperature in the presence of 10 mL of buffer (0.1 M glycine: 0.3 M NaCl) at a pH of 9.

After centrifugation of mixture at 10,000 g for 10 min at +4°C, 5 mL of phosphate buffered saline (PBS) at a pH = 7.2 was added to the supernatant, to which polyethylene glycol (PEG 6000 at 50%) was added to a quarter of the final volume to increase the concentration. The pH value was adjusted to 7.2 and the mixture was incubated overnight. The following day, the precipitate formed is recovered by centrifugation at 10 000 g for 30 min at +4°C. The final pellet was resuspended in 5 mL of PBS at pH 7.2.

To prevent the contamination of the extracts, it was necessary to add to the mixture 30 µL of antibiotics (Penicillin 10.000 U/mL and Streptomycin 10.000 µg/mL) and 20 µL of Fungizone (250 µg/mL). The viral concentrate was stored at -20°C until used in one of the cell culture or iRNA viral extraction (El-Senousy et al., 2013).

### Cell culture

Two cell lines cell culture were used according to the new algorithm (Figure 2) (WHO, 2004); the first one was the RD cells line, with $5 \times 10^4$ cells/mL, the passage was 255/3; the cells originated from Center of Disease Control (CDC) in Atlanta; they were derived from Human Rabdomyosarcom, sensitive to poliovirus and other non-polio enteroviruses (Figure 3) and the second one was the L20B cells line, with $5 \times 10^4$ cells/mL, the passage was 18/3; cells originated from CDC; cells line results from mouse and have specific receptors to poliovirus and some other non-polio enteroviruses such as coxsackievirus (Figure 4) (Bahri et al., 2005). Both cell lines were grown in the Minimum Essential Medium (MEM) (Invitrogen, USA) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen, USA) in 75 $cm^2$ plastic flasks. The inoculation of 100 µl of the extract on the two cells line was done in 96 well plates, which were then incubated at 36°C. The reading was made in inverted microscope with LCD display (Life biotechnology, USA) every day to search the appearance of a cytopathogenic effect (CPE) evocative of enteroviruses for 10 days (Sdiri et al., 2004).

### Real-time PCR

#### *Extraction and purification of the viral RNA*

The RNA of the viruses for all the positive analyzed cultured samples was extracted using a commercial kit (Magmax™ total of the nucleic acids isolation kit, part number AM1840, Termofisher Scientific, USA) according to the supplier instructions.

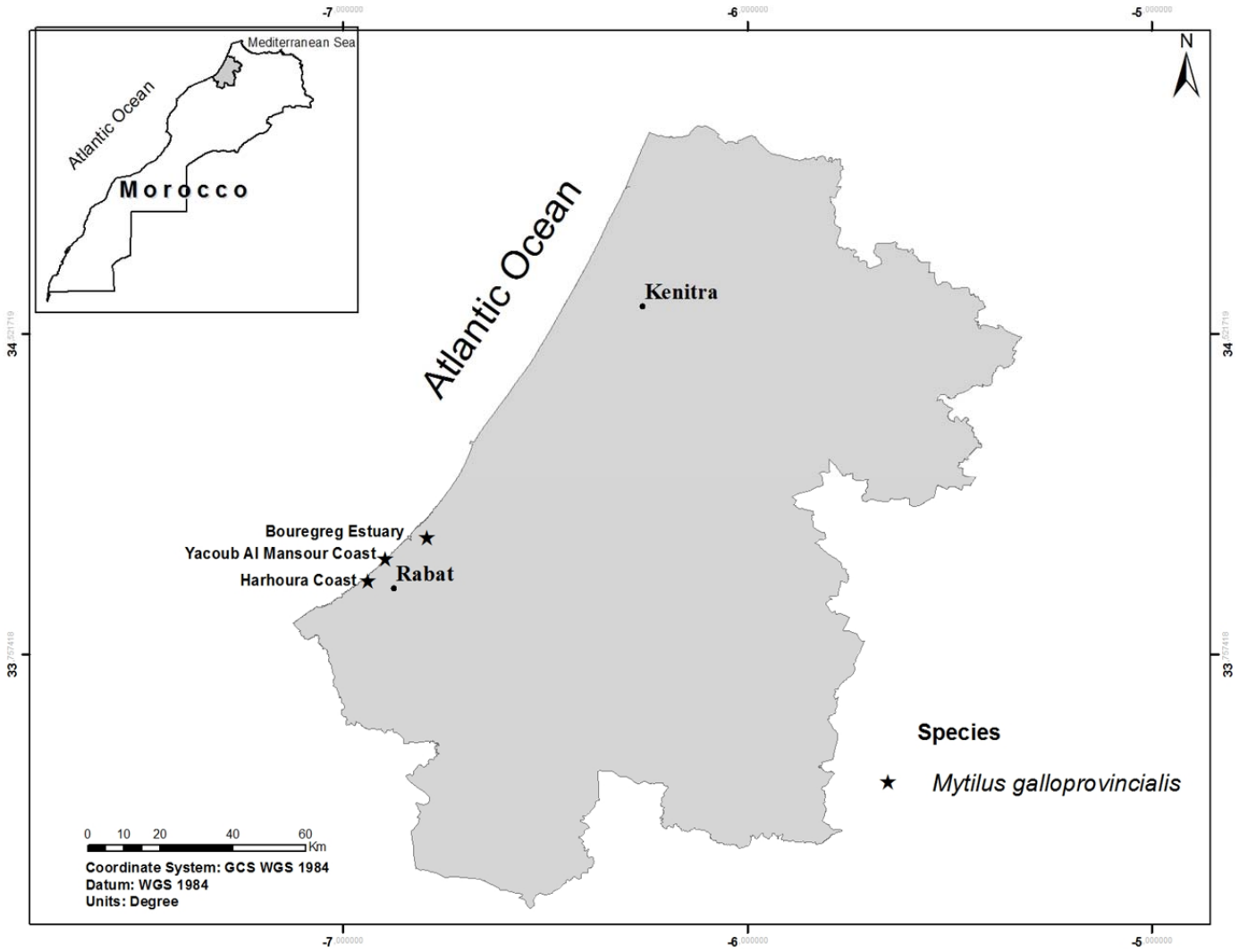


**Figure 1.** Map of Morocco coast showing locations of sampling.

**Table 1.** Mussels (*Mytilus galloprovincialis*) samples from three wild population of Morocco including geographic coordinates of the location, the size of the samples during the same period.

| Specie | Sampling site | Coordinates | Sample number | Sample date |
|---|---|---|---|---|
| *M. galloprovincialis* | BouRegreg estuary | N 34°2'8"; W 6°50'7" | 96 | February 2014 to February 2015 |
| | Yacoub Al Mansour coast | N 33°59'3"; W 6°53'41" | 96 | |
| | Harhoura coast | N 33°57'24"; W 6°55'28" | 96 | |

The RNA viral extraction was carried out using guanidinium thiocyanate, which makes it possible to quickly release the nucleic acid by a chemical lysis and simultaneously inactivate the nucleases in the matrix of the sample. Afterward, the nucleic acids of the viruses were insulated by purification step using the microspheric magnetic beads, to eliminate substances which could interfere with the real-time PCR reaction. These beads used for the complete binding of the nucleic acid, were washed with absolute isopropanol to eliminate proteins and other contaminants (Karamoko et al., 2006b). Finally, the elution buffer used (PBS) made it possible to recover the viral particles that adhered to the surface of the magnetic beads.

***Detection of the viral genome***

After extraction and purification of the viral RNA, the enterovirus was detected according to World Health Organization

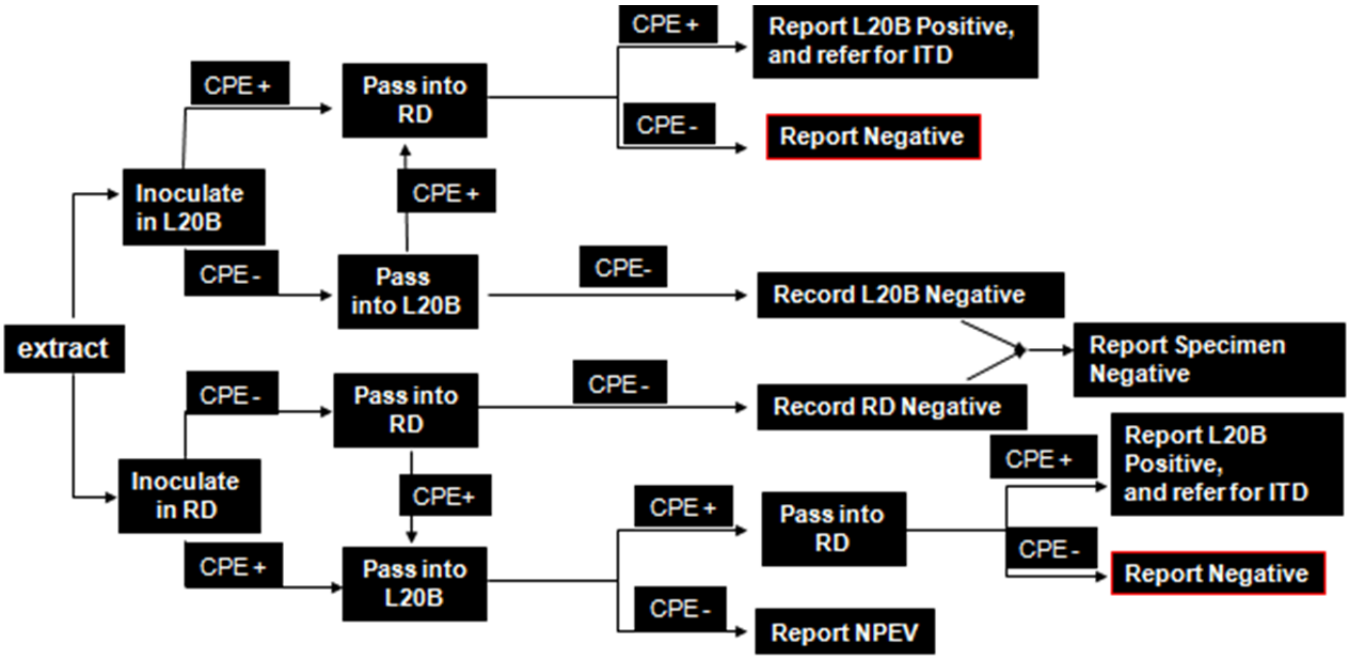


**Figure 2.** New algorithm for the isolation of enteroviruses (WHO, 2004).

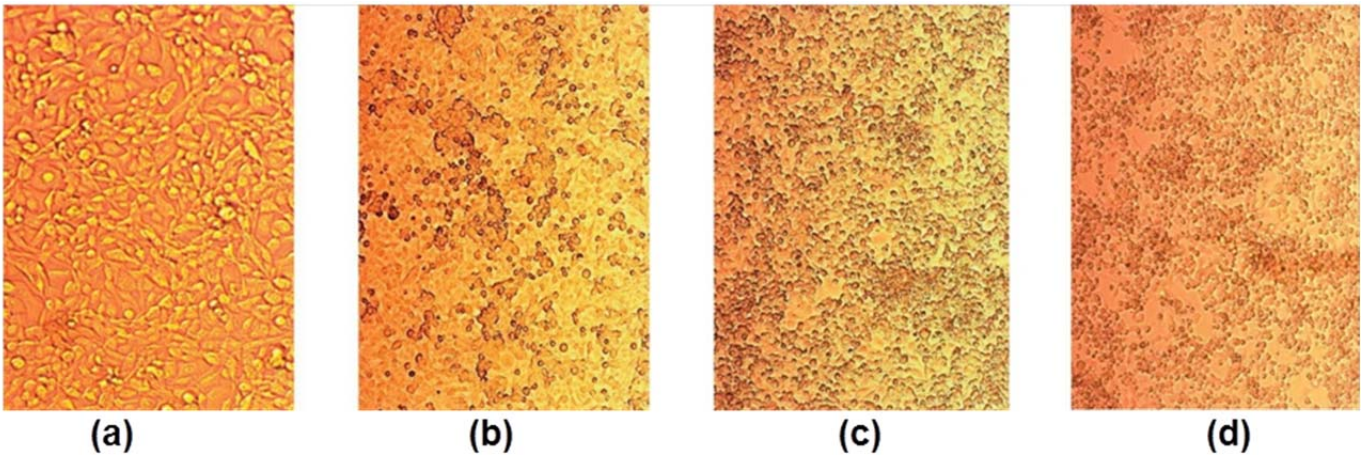


**Figure 3.** Appearance of inoculated and uninoculated RD cells in inverted microscope with LCD display. (a) RD: Uninoculated; (b) RD: CPE = 2+; (c) RD: CPE = 3+; (d) RD: CPE = 4+.

recommended protocols (WHO, 2004), by amplification of the region 5'NTR-3'NTR genome *in vitro*, using specific enterovirus primers (Pan Enterovirus (Pan EV), Pan Poliovirus (Pan PV), Poliovirus Serotype 1 (PV1), Poliovirus Serotype 2 (PV2), Poliovirus Serotype 3 (PV3), Sabin Multiplex) (Table 2) and Sabin primers (Sabin 1 Vaccine-Derived Poliovirus (S1 VDVP), Sabin 2 VDVP (S2 VDVP), Sabin 3 VDVP (S3 VDVP) (Table 3) and the technique of quantitative RT-qPCR (Applied Biosystems, Termofisher Scientific, USA) . The detection of amplicons generated at each amplification cycle required the use of fluorescent probes (TaqMan®, CDC) hybridizing on specifically amplicon. Quantification of DNA was achieved by the Ct value (Cycle threshold), which corresponds to the number of PCR cycles required for the fluorescence in the sample. This Ct value can be related to an amount of DNA through the use of a standard range or be compared with that of a reference gene. The sample was considered positive if the Ct is less than 30.

The real-time PCR reactions were performed in ABI 7500 fast real-time PCR System (Applied Biosystems, Termofisher Scientific, USA) as follows: Reverse transcription reaction at 42°C for 45 min, inactivation at 95°C for 3 min followed by 40 cycles of PCR at 95°C for 24 s and 47°C for 30 s, then a 25% speed ramp at 60°C for 24 s. The end point fluorescent data was collected at the end of the 47°C anneal step and the data were captured and analyzed by using the SDS Software v2.0.5 instrument.

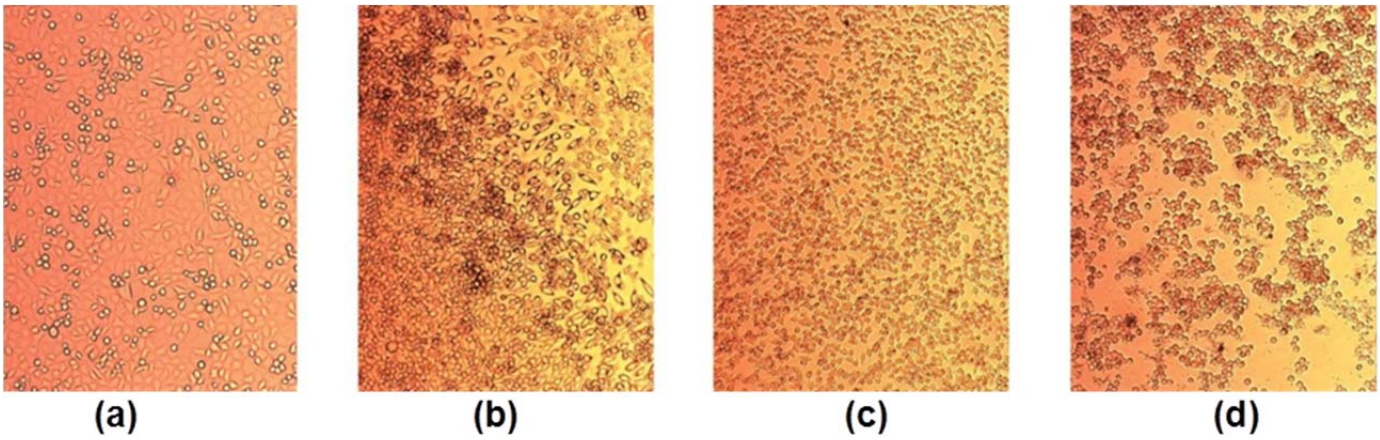


**Figure 4.** Appearance of inoculated and uninoculated L2B cells in inverted microscope with LCD display. (a) L20B: Uninoculated; (b) L20B: CPE = 2+; (c) L20B: CPE = 3+; (d) L20B: CPE = 4+.

**Statistical analysis**

The statistical analysis using the $\chi^2$ test for proportions was performed using SigmaPlot (version 12) to evaluate the level of pollution between different groups of wild sites. The *P* value superior of 0.05 was considered as non-significant.

## RESULTS AND DISCUSSION

From cell culture method of two cell lines RD 255/3 and L20B 18/3, 75% (216/288) of samples showed cyto-pathogenic effect suggestive of enterovirus (Figure 5). Therefore, the wild mussel samples were collected from areas with high faecal pollution by domestic wastewater.

The virological analysis (cell culture) of these samples displayed that 75% of mussels were contaminated by enteroviruses highlighted, with the predominance in 70.8% (204/288) of non-polio enteroviruses (NPEV) from Bouregreg estuary, Yacoub Al Mansour coast and Harhoura coast, whilst 4.2% (12/288) represented the Sabin strain of Poliovirus type 1 (PS1) from Harhoura coast, which was confirmed by real-time PCR for intratypic differentiation (ITD) (Figure 6 and Table 4).

The positivity of enteroviruses by cell culture in wild mussels from Bouregreg estuary was 87.5% (84/96), 75% (72/96) of Yacoub Al Mansour coast and 62.5% (60/96) of Harhoura coast (Figure 7 and Table 4). Consequently, the statistical analysis using the $\chi^2$ test for proportions revealed that differences in these three wild sites were not significant (*P* value > 0.05).

The enteroviruses are among the infectious agents associated with waterborne diseases faecal-oral transmission reflecting defective hygienic conditions. They can detect asymptomatic infections and multifaceted clinical variable diagnosis depending on the serotype involved (Avellon et al., 2003; Sutter et al., 2014). Several studies have shown the involvement of a panel of enteroviruses in the similar events that enhance the acute flaccid paralysis (AFP), in this case, related to different kind of enteroviruses which caused paralysis such as the enteroviruses types 68-71 (Bahri et al., 2005; Delpeyroux et al., 2013; OMS, 2013) and suggest a potential threat. In Morocco, the prevalence of circulating NPEV is unknown, which indicates the need for a deeper investigation of the dissemination in the environment in order to identify them and associate them with clinical manifestations in the country. Thus, the results reported in this study showed a potential health risk to the population. Indeed, the presence of this strain vaccine in the environment could be a source of infection for humans.

The enteroviruses are ubiquitous pathogens present in all regions of the world and able to survive for long periods in the marine environment (OMS, 2013). Furthermore, the resistance of these viruses such as acid pH and extreme temperature facilitate their transmission. These properties ensure that enteroviruses are very well dispersed in water surface or wastewater from sewage treatment. The human enteroviruses are not inactivated in the water environment and will therefore often be caught and activated by the filter feeders such mussels (Benabbes et al., 2013b). In conclusion, this work gives subsidies to explain the high prevalence of non-polio enteroviruses in the aquatic environment for the countries with low socio-economic and hygienic status.

In front of this viral risk, it is necessary to have quick and reliable techniques in order to detect enteric virus from food matrices. Some studies have revealed the presence of hepatitis A virus (Karamoko et al., 2006a), adenovirus (Karamoko et al., 2005) and enteroviruses (Karamoko et al., 2006b) respectively in 37.5, 15 and10% of mussel samples in the costal media. Other studies

**Table 2.** List of primers and probes for intratypic differentiation (ITD) real-time PCR (WHO, 2004).

| Specificity | Primer or probe (Polarity) | Primer or probe sequence (5′→3′) |
|---|---|---|
| Pan enterovirus | PCR-1 (A) | GCGATTGTCACCATWAGCAGYCA |
| | PCR-2 (S) | GGCCCCTGAATGCGGCTAATCC |
| | PanEV Probe (S) | **FAM-**CCGACTACTTTGGGWGTCCGTGT-**BHQ1** |
| Pan poliovirus | PanPV/PCR-1 (A) | AYRTACATIATYTGRTAIAC |
| | PanPV/PCR-2 (S) | CITAITCIMGITTYGAYATG |
| | PanPV Probe21A (A) | **FAM**-TGRTTNARIGCRTGICCRTTRTT-**BHQ1** |
| Poliovirus serotype 1 | SeroPV1A (A) | ATCATIYTPTCIARPATYTG |
| | SeroPV1,2S (S) | TGCGIGAYACIACICAYAT |
| | SeroPV1 Probe16A (A) | **FAM**-TGICCYAVICCYTGIGMIADYGC-**BHQ1** |
| Poliovirus serotype 2 | SeroPV2A (A) | AYICCYTCIACIRCICCYTC |
| | SeroPV1,2S (S) | TGCGIGAYACIACICAYAT |
| | SeroPV2 Probe5S (S) | **FAM**-CARGARGCIATGCCICARGGIATNGG-**BHQ1** |
| Poliovirus serotype 3 | SeroPV3A (A) | CCCCIAIPTGRTCRTTIKPRTC |
| | SeroPV3S (S) | AAYCCITCIRTITTYTAYAC |
| | SeroPV3 Probe11S (S) | **FAM**-CCRTAYGTNGGITTRGCVAAYGC-**BHQ1** |
| Sabin 1 | Sab1/PCR-1 (A) | CCACTGGCTTCAGTGTTT |
| | Sab1/PCR-2 (S) | AGGTCAGATGCTTGAAAGC |
| | Sab1/Probe (A) | **CY5**-TTGCCGCCCCCACCGTTTCACGGA-**BHQ3** |
| Sabin 2 | Sab2/PCR-1 (A) | CGGCTTTGTGTCAGGCA |
| | Sab2/PCR-2 (S) | CCGTTGAAGGGATTACTAAA |
| | Sab2/Probe (S) | **FAM**-ATTGGTTCCCCCGACTTCCACCAAT-**BHQ1** |
| Sabin 3 | Sab3/PCR-1 (A) | TTAGTATCAGGTAAGCTATC |
| | Sab3/PCR-2 (S) | AGGGCGCCCTAACTTT |
| | Sab3/Probe (S) | **ROX**-TCACTCCCGAAGCAACAG-**BHQ2** |

**Table 3.** List of primers and probes for Sabin Vaccine-Derived Poliovirus (VDVP) real-time PCR (WHO, 2004).

| Primer specificity | Primer and probe sequences 5'-3' | |
|---|---|---|
| Sabin 1 VDPV VP1 | Sense | CATGCGTGGCCATTATA |
| | Anti-sense | CAAATTCCATATCAAATCTA |
| | VP1 probe | **FAM**-CACCAAGAATAAGGATAAGC-**BHQ1** |
| Sabin 1 VDPV 3D | Sense | GACACTAAGGAAATGCAAAAACTGC |
| | Anti-sense | ATCGCACCCTACTGCTGA |
| | 3D probe | **ROX**-TCAGTGGCAATGAGAATGGCTTTTGGG-**BHQ2** |
| Sabin 2 VDPV VP1 | Sense | GACATGGAGTTCACTTTTG |
| | Anti-sense | CTCCGGGTGGTATATAC |
| | VP1 probe | **FAM**-CATTGATGCAAATAAC-**BHQ1** |
| Sabin 2 VDPV 3D | Sense | AGGAAATGCGGAGACTCTTA |
| | Anti-sense | GGATCACAACCAACTGCACT |
| | 3D probe | **ROX**-CTTACCGCTTGTAACATATGT-**BHQ2** |
| Sabin 3 VDPV VP1 | Sense | CATTTACATGAAACCCAAAC |
| | Anti-sense | TGGTCAAACCTTTCTCAGA |
| | VP1 probe | **FAM**-TAGGAACAACTTGGAC-**BHQ1** |

**Table 3.** Contd.

| | | |
|---|---|---|
| Sabin 3 VDPV 3D | Sense | CACCAAAGAAATGCAAAGACTTT |
| | Anti-sense | GGATCGCATCCAACTGCACT |
| | 3D probe | **ROX-**CCTACCATTAGTGACATATGT-**BHQ2** |

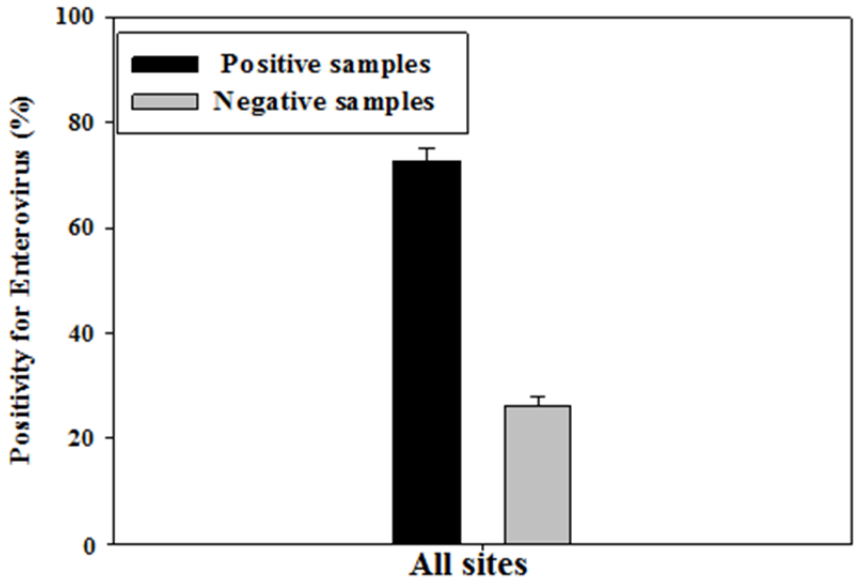


**Figure 5.** Percentage of positivity for enteroviruses in mussels.

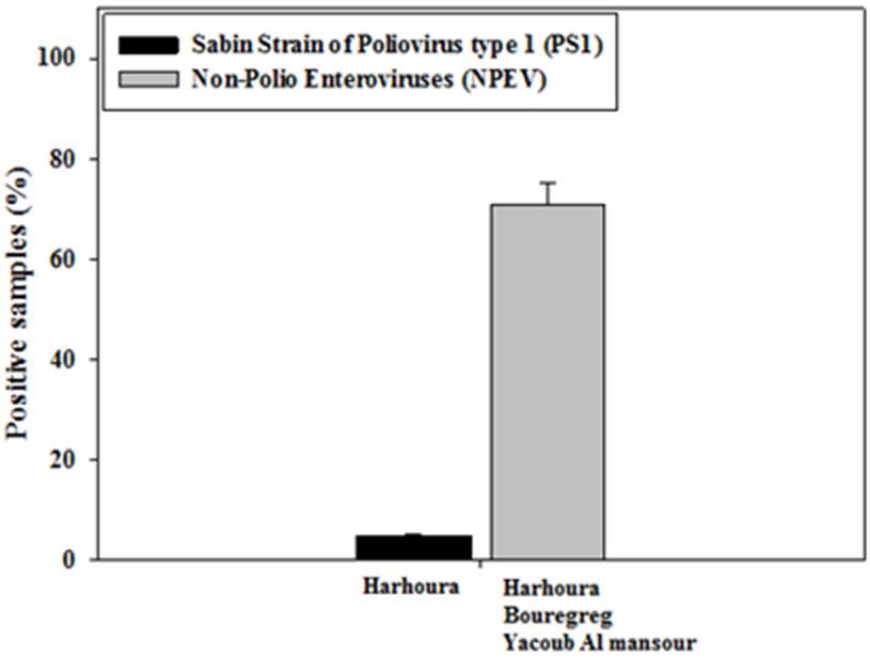


**Figure 6.** Percentage of positivity for Sabin strain of poliovirus type 1 and non-polio enteroviruses in mussels.

**Table 4.** Positive and negative samples described by sampling site and number.

| Sampling site | Sample | Sample number | Positivity of enteroviruses | Positive samples | Negative sample |
|---|---|---|---|---|---|
| Harhoura coast | M1 to M8 | 8 × 12 = 96 | 62.5% (60/96) of enterovirus | M1, M2, M3, M4, M5, M8 | M6, M7 |
| BouRegreg estuary | M9 to M16 | 8 × 12 = 96 | 87.5% (84/96) of enterovirus | M9, M10, M12, M13, M14, M15, M16 | M11 |
| Yacoub Al Mansour coast | M19 to M26 | 8 × 12 = 96 | 75 % (72/96) of enterovirus | M19, M20, M22, M24, M25, M26 | M21, M23 |
| All sites | M1 to M26 | 96 × 3 = 288 | 70.8% (204/288) of non-polio enteroviruses (NPEV) | M2, M3, M4, M5, M8, M9, M10, M12, M13, M14, M15, M16, M19, M20, M22, M24, M25, M26 | M6, M7, M11, M21, M23 |
| Harhoura coast | M1 to M8 | 8 × 12 = 96 | 4.2% (12/288) of Sabin strain of poliovirus type 1 (PS1) | M1 | M6, M7 |

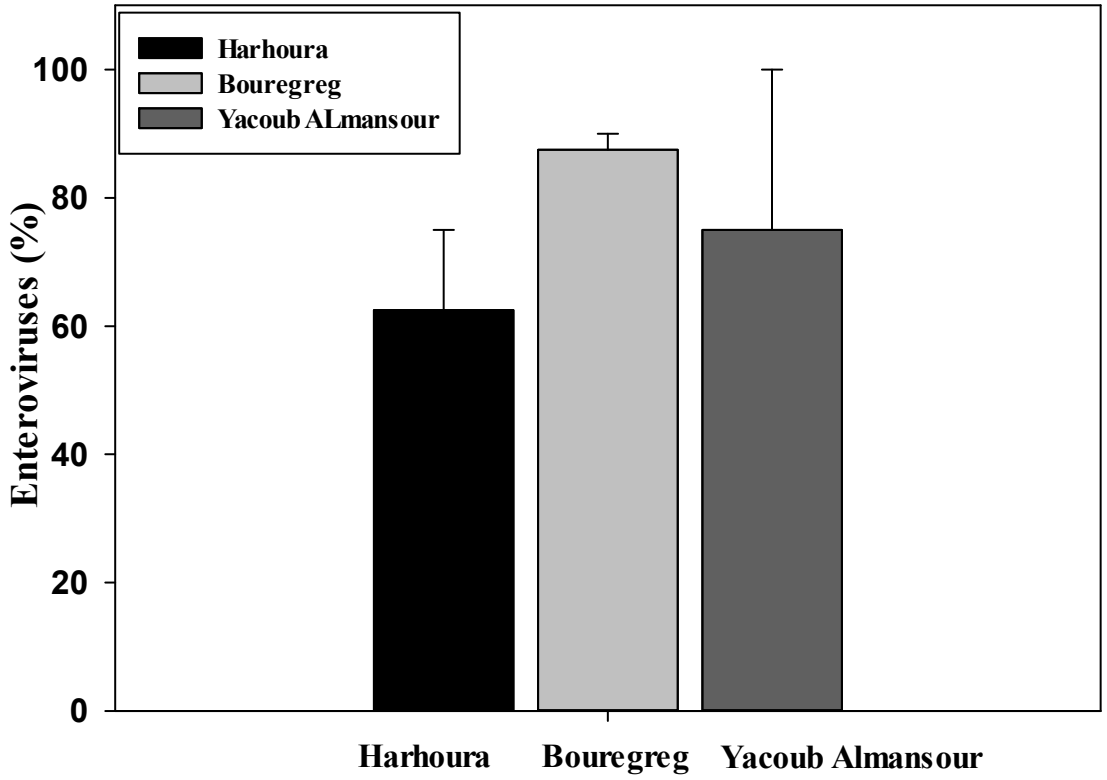


**Figure 7.** Comparison of positivity percentages for enteroviruses between three wild population sites of mussels.

have shown that 88.6% of all mussel analyzed samples were contaminated by adenovirus and enteroviruses respectively in 52.3 and 36.3% of the samples from two production areas in Moroccan Mediterranean coast (Benabbes et al., 2013b). Others highlighted the noroviruses contamination in 30% of samples collected in the Mediterranean Sea and the Atlantic Coast (Benabbes et al., 2013a; Vaillant et al., 2012). Overall, to control a sanitary quality of bivalve molluscs, the need to evaluate the viral contamination of the aquatic environment during the whole year was suggested. It was concluded that the results of this research underscore the importance of strengthening the virological quality of bivalve molluscs before their commercialization.

## Conclusion

The present study highlighted the circulation of a significant number of enteroviruses, with the predominance of non-polio enteroviruses (NPEV) and the presence of Sabin strain of poliovirus type 1 (PS1). The study also confirmed the absence of statistically significant difference between three wild populations. Therefore, the virological monitoring system of the environment should be reinforced by extending the virological investigation serotyping of NPEV strains, the reinforcement of the prevention against the propagation of enteroviruses (poliovirus and non-polio enteroviruses) and the establishment of a monitoring system of these viruses in the aquatic environment.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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