

*Full Length Research Paper*

# Typing of enterovirus identified from Moroccan mussels (*Mytilus galloprovincialis* Lamarck, 1819) by seroneutralization

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Received 9 October, 2023; Accepted 20 November, 2023

**Non-poliovirus enteroviruses (NPEV) are infectious agents which can determine various illness in human such as hand-foot-mouth syndrome, angina, respiratory diseases, acute or chronic heart disease, diarrhea, pancreatitis, acute hemorrhagic and conjunctivitis. These viruses are eliminated in the stool and thus contaminate the marine environment and shellfish. In Morocco, shellfish sanitary quality analysis does not include enteroviruses detection. Therefore, the objective of this study was to detect and to type enteroviruses in 288 mussel samples. These samples (*Mytilus galloprovincialis*) were collected between February 2014 and February 2015 from three wild populations (Bouregreg estuary, Yacoub Al Mansour, and Harhoura coast). 216 of 288 samples (75%) were revealed positive by the cell culture method, with 204 strains of NPEV (70.8%) and 12 strains of *Poliovirus* Type 1 (4.2%). The serotype of 204 NPEV strains has been determined a typable strains (64.7%) and non-typable strains (35.3%) in the marine environment. However, the proportion of untypable strains confirms the presence of new serotypes. The diseases caused by NPEV constitute an important public health problem. To fight against this human risk related to viral contamination, it is necessary to have a methodology for the control and virological monitoring of the marine ecosystem.**

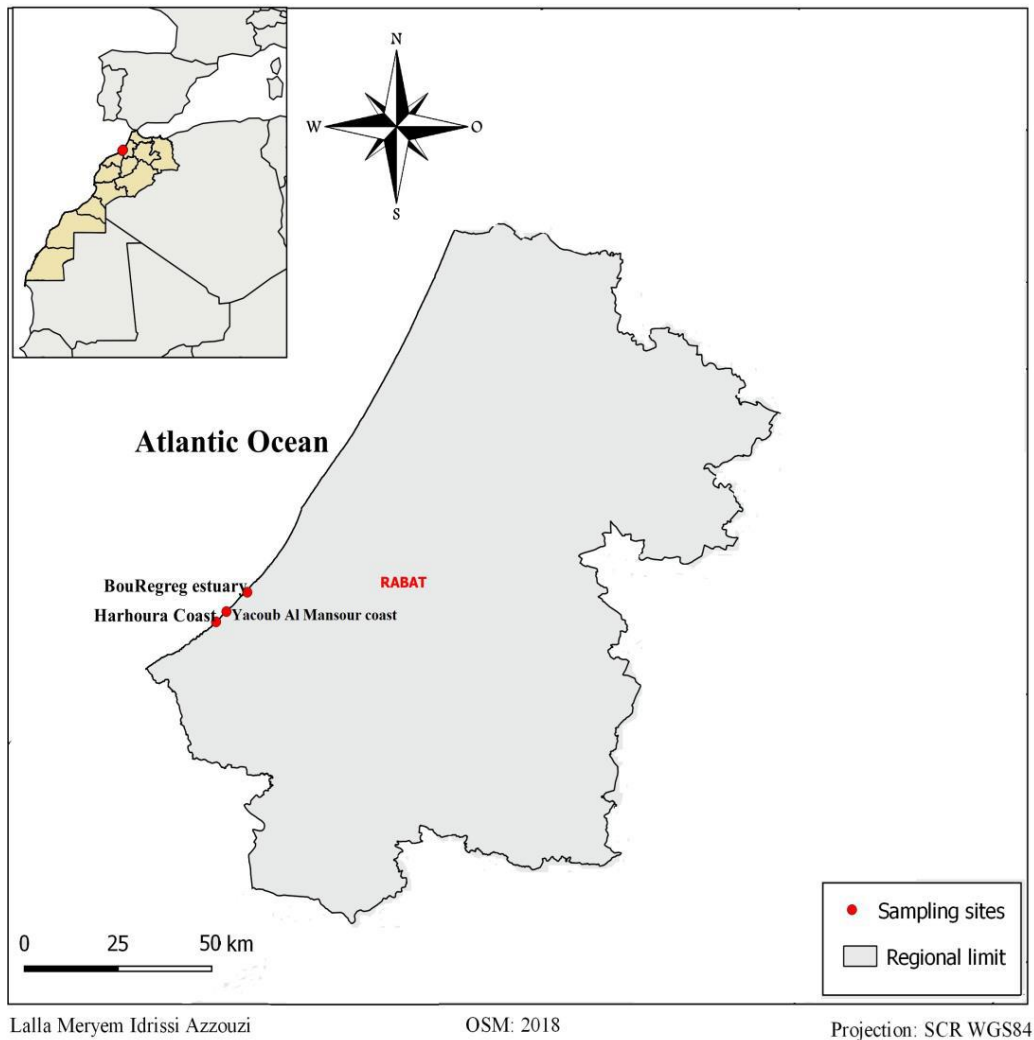
**Key words:** Non-poliovirus enteroviruses, *Mytilus galloprovincialis*, marine environment, shellfish, seroneutralization.

## INTRODUCTION

The microbial pollution in the marine environments is a key determinant for the evaluation of the level of viral contamination, with major impacts on the control of the faecal risk for human health. For evaluating the latter risk, different markers have been proposed, including enteroviruses and adenoviruses (Hot et al., 2003; Rajtar et al., 2008; Fong and Lipp, 2005; Jung et al., 2014; Warish et al., 2018).

The advantage of enteroviruses as a marker of viral contamination is that certain genotypes are relatively easy to cultivate in cell culture, which is still the reference method for environmental monitoring (Ehlers et al., 2005; Hematian et al., 2016; Itani et al., 2023). Indeed, typing enteroviruses strains existent in the marine environment may be an important objective, especially to detect the presence of non-poliovirus enteroviruses strains and

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**Figure 1.** Map of Morocco coast showing the sampling sites.

Poliovirus strains in areas where these agents are still circulating (Hovi et al., 2005; Klapsa et al., 2022).

Non-poliovirus enteroviruses circulate in all populations and infection can be associated with a vast range of presentations. In this study, the serotype identification of non-poliovirus enteroviruses was done according to the procedures recommended by the World Health Organisation (WHO, 2004).

The identification of newly isolated strains by specific neutralization becomes increasingly difficult, as many types of enteroviruses exist in the environment. Seroneutralization tests with composite antiserum pools are very economical in tissue culture and time that the use of pooled antiserum initially is advantageous. The reference method for the laboratory diagnosis of enteroviruses is isolation of the cell culture, followed by serotype identification (Oberste and Pallansch, 2005; Hambling et al., 2009).

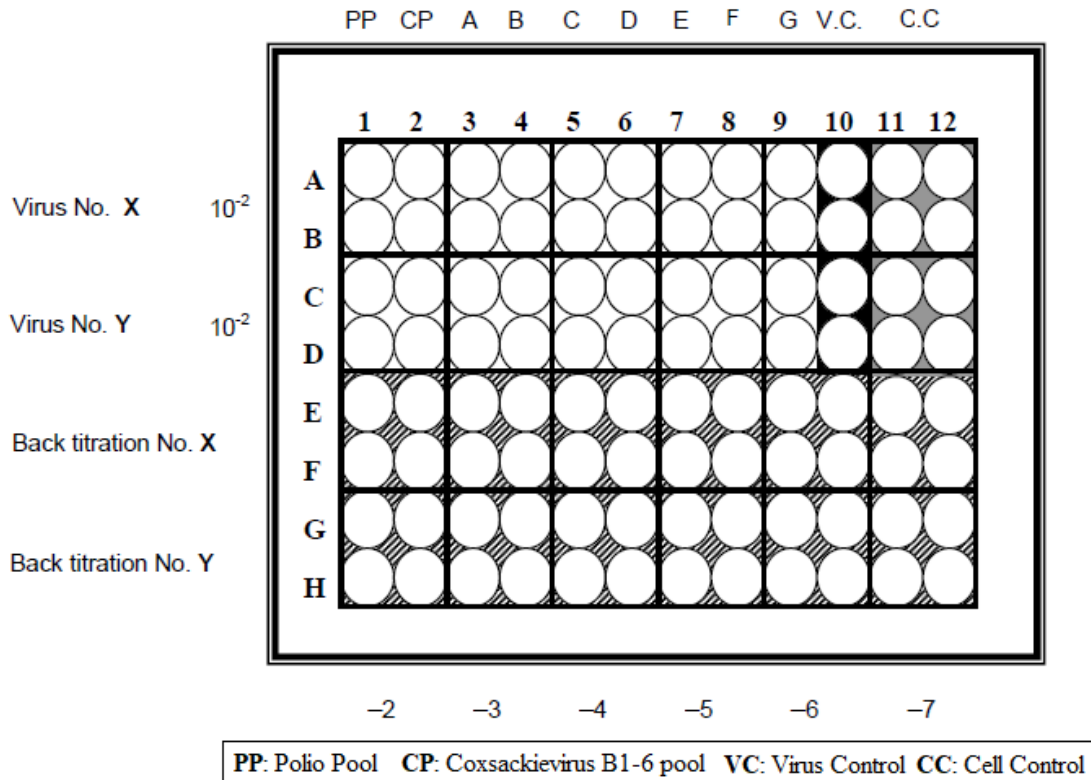
The lack of a national monitoring program of

Enteroviruses was one of the reasons for this study in order to evaluate the viral contamination in mussels collected from potentially polluted areas. The target of this research was to study circulating strains of enteroviruses in the marine environment, in order to supplement the Moroccan databases available on environmental contamination by enteroviruses and to illustrate the importance of including routine virological analysis of shellfish in Morocco.

## MATERIALS AND METHODS

### Study area

Between February 2014 and February 2015, three sampling sites (Bouregreg estuary, Yacoub Al Mansour and Harhoura coast) located in the Rabat Region of Morocco (Figure 1) were chosen for the collection of 288 mussel samples (*Mytilus galloprovincialis*) from wild population sites that receive domestic waste without



**Figure 2.** Plate set-up for identification of Enterovirus isolates using seroneutralization.

previous treatment. This region covers an area of 18.194 km<sup>2</sup>, with a population of about 4.581.000. This area belongs to the Mediterranean climate characterized by two main seasons softened by oceanic influences. The average temperatures are approximately 22°C for the warmer months (July to September) and 12°C for the colder months (December and January). Relating to the annual rainfall is in average more than 550 mm/year (Idrissi Azzouzi et al., 2017a, b).

### Samples preparation

The shells were opened aseptically; the digestive system was dissected with a sterile knife allowing the elimination of inhibitors tissues (polysaccharides, sexual gonads). To analyse a larger number of individuals 1.5 g of hepatopancreas were used with a weight corresponding to an analysis, representing on average 12 mussels.

### Virus recovery from mussels' samples

Two hundred and eighty-eight samples of mussels were collected from three stations of Rabat Region in Morocco. Mussels samples (1.5 g of hepatopancreas) were added to 10 mL of buffer (0.1 M glycine: 0.3 M NaCL) at a pH = 9. The mixture was homogenized for 15 min then centrifuged at 10 000 g for 10 min at 4°C. The pellet was resuspended in 5 mL of phosphate buffered saline (PBS) and adjusted to pH = 7.

The mixture was homogenized again and centrifuged at 10 000 g for 30 min at 4°C. The supernatant was used for virus detection.

### Concentration of virus suspensions

Virus particles recovered from mussel samples by precipitation with polyethylene glycol (PEG) 6000 at 50% as previously described (El-Senousy et al., 2013; Idrissi Azzouzi et al., 2017a). In brief, suspensions were mixed with 25% (V/V) PEG 6000 and incubated at 4°C overnight. The mixtures were then centrifuged at 10 000 g for 30 min. The final pellet was resuspended in 5 ml of 0.1 M phosphate buffer pH 7.2 and then filtered through a 0.22 µm Millex-GS membrane. To prevent the contamination of the concentrate, 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 suspension was either treated immediately or stored at -20°C until use.

### Typing of non-poliovirus enteroviruses with antiserum pools

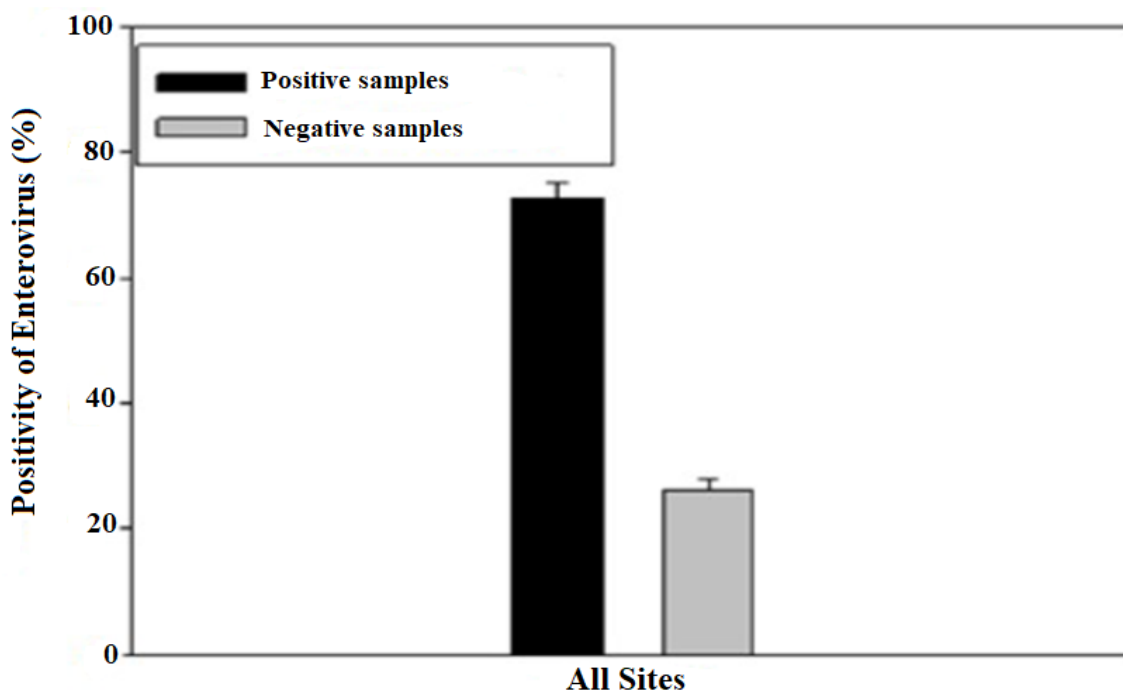
Poliovirus strains have been identified by molecular method (real-time PCR) according to the procedures recommended by WHO (Idrissi Azzouzi et al., 2017a).

The identification of non-poliovirus enteroviruses serotypes (serotyping of NPEV) by the seroneutralization test was done using pools of antiserum prepared and provided by the National Institute of Public Health and the Environment (RIVM) (WHO, 2011).

Each box of RIVM enteroviruses typing antiserum contains anti-enterovirus pools A, B, C, D, E, F and G, anti-Coxsackievirus B pool and a trivalent anti-Poliovirus pool (Figure 2). These pools must be diluted before use. The recommended dilution for all pools is 0.5 mL, of which each pool is added to 9.5 mL of maintenance medium (minimum essential medium MEM with HEPES and 2% FBS).

**Table 1.** Association of Antiserum pools (A-G) for non-poliovirus enteroviruses typing by seroneutralization.

Antiserum pool	A	B	C	D	E	F
A		<i>Echovirus 4</i>	<i>Echovirus 7</i>	<i>Echovirus 11</i>	<i>Echovirus 14</i>	<i>Echovirus 9</i>
B	<i>Echovirus 4</i>		<i>Coxsackievirus A9</i>	<i>Echovirus 1</i>	<i>Echovirus 27</i>	<i>Echovirus 3</i>
C	<i>Echovirus 7</i>	<i>Coxsackievirus A9</i>		<i>Echovirus 21</i>	<i>Echovirus 22</i>	<i>Echovirus 2</i>
D	<i>Echovirus 11</i>	<i>Echovirus 1</i>	<i>Echovirus 21</i>		<i>Echovirus 20</i>	<i>Echovirus 12</i>
E	<i>Echovirus 14</i>	<i>Echovirus 27</i>	<i>Echovirus 22</i>	<i>Echovirus 20</i>		<i>Echovirus 33</i>
F	<i>Echovirus 9</i>	<i>Echovirus 3</i>	<i>Echovirus 2</i>	<i>Echovirus 12</i>	<i>Echovirus 33</i>	
G	<i>Echovirus 6</i>	<i>Echovirus 25</i>	<i>Echovirus 5</i>	<i>Echovirus 30</i>	<i>Echovirus 29</i>	<i>Echovirus 13</i>

**Figure 3.** Percentage of positivity for enteroviruses in mussels.

Aliquot pools into clearly labelled cryovials in 1 mL volumes and store at -20°C. Each unknown virus was tested in duplicate against a trivalent-pooled poliovirus antiserum (PP), a Coxsackievirus B1 to B6 pool (CP), and seven pools against Coxsackievirus A9 and 20 echoviruses (A-G) (Figure 2 and Table 1). Non-poliovirus enteroviruses that fail to be identified using this antiserum may be in an aggregated form that interferes with the complete neutralization by specific antiserum. Isolates can be retested after emulsification with chloroform (approximately 10% by volume) and separation of the supernatant.

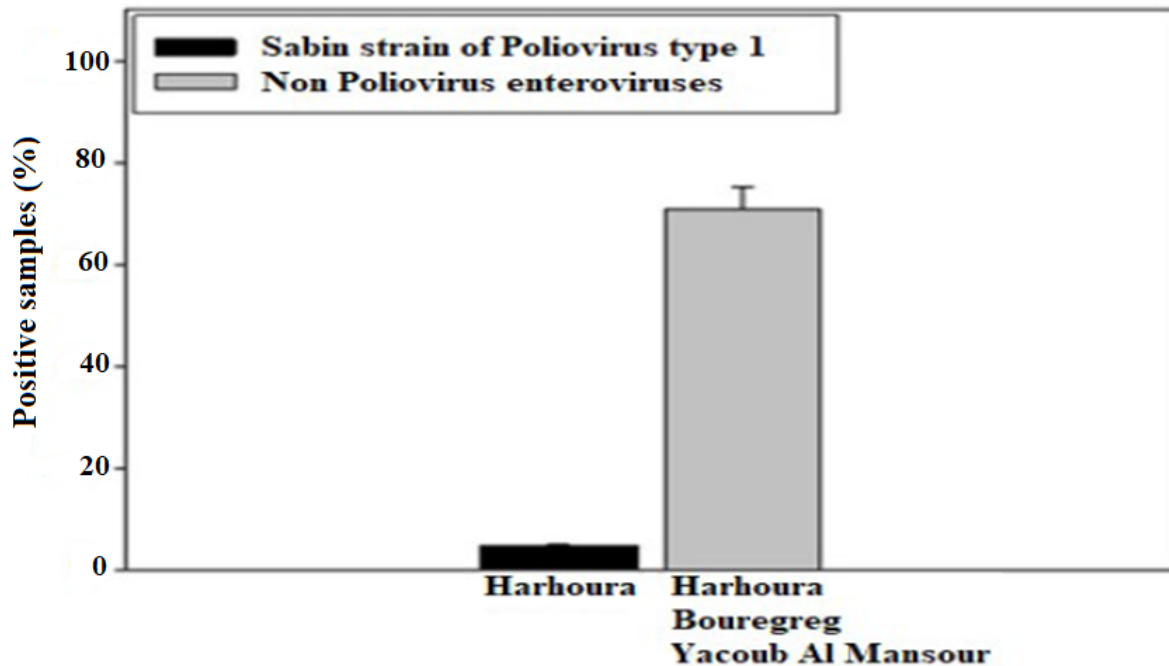
The virus suspension to be used in the seroneutralization tests was prepared by the inoculation of cultures of the specified cells. After inoculation, the cultures were examined daily for cytopathic effect (CPE). Complete destruction of the cells within 3 days is preferable, and if this was not obtained initially, a further passage should be made. When destruction is complete, the cultures are frozen, then rapidly thawed, and harvested. This harvest forms the stock virus suspension for all the seroneutralization tests and is stored at -20°C until required.

Because a large number of viruses makes it impractical to

perform individual neutralization tests, these have been pooled in an overlapping scheme that allows many viruses to be identified using as few as nine tests. Interpretation of the results was done with the assistance of a list of the neutralization patterns of individual viruses (Table 1).

## RESULTS AND DISCUSSION

The virological analysis (cell culture) of 288 samples collected from Bouregreg estuary, Yacoub Al Mansour and Harhoura coast, showed that 75% of mussels (*Mytilus galloprovincialis*) were contaminated by enteroviruses (Figure 3), with 204 strains (70.8%) of non-poliovirus enteroviruses (NPEV) and 12 strains (4.2%) of *Poliovirus* Type 1 (SL1) which was confirmed by real-time PCR using intratypic differentiation (ITD) method (Figure 4).



**Figure 4.** Percentage of positivity for Sabin strain of poliovirus type 1 and non-poliovirus enteroviruses in mussels.

From the isolates of NPEV were obtained RD cell lines, 204 of these strains were serotyped by seroneutralization using pools of antiserum, however only 132 strains (64.7%) could be identified, against 72 non-typable strains (35.3%) (Figure 5). Among these typable strains, 72.7% (96/132) have been determined as *Coxsackievirus* B and 27.3% (36/132) as *Echovirus* (Figure 6). The different serotypes of *Coxsackievirus* B and *Echovirus* could be identified as 62.5% of *Coxsackievirus* B5 (60 strains), 37.5% of *Coxsackievirus* B3 (36 strains), and 100% of *Echovirus* 6 (36 strains) (Figure 7).

This study revealed the circulation of an important number of typable strains (64.7%) and non-typable strains (35.3%) in the marine environment. However, non-typable strains confirm the presence of new serotypes. A seroneutralization test was used for the identification of enteroviruses in tissue culture with composite antiserum pools. This antiserum with twenty-seven enteroviruses was included in the pools that were used to examine 204 of non-poliovirus enteroviruses that consist of typed and untyped strains. The results indicate that this method provides a useful screening method for identifying enteroviruses. It has proved to be practicable, time-saving, and very economical in tissue culture.

The analysis of viruses in environmental samples is complex. There are a number of issues to consider. Primary isolates of many viruses of interest grow poorly, if at all, in cell culture systems (Duizer et al., 2004; Straub et al., 2007; Tanaka et al., 2007; Cromeans et al., 2008; Gerba and Betancourt, 2019). This is further compounded by the fact that major enteric viruses are present in low

numbers in the environment, and have been shown to have an infectious dose ranging from 1 to 100 particles. Therefore, a method must concentrate low levels of viral particles and eliminate any inhibitory substances that could interfere with the analytical process (Brundage and Fitzpatrick, 2006; Teunis et al., 2008). For this reason, it is important to develop methods sensitive enough to detect a single viral particle per sample. In addition, some of the important enteroviruses have a high degree of genetic and antigenic variability (Kageyama et al., 2004; Matthijnsens et al., 2008; Zheng et al., 2006). Therefore, to monitor viral contamination in marine environments, the use of molecular techniques targeting certain regions of the genome and the phylogenetic analysis of nucleotide sequences are recommended. These techniques will make it possible to identify new serotypes while ensuring the characterization of non-typable strains. Molecular tests will also, by the determination of recombinant strains explain the genetic evolution of NPEV strains.

The prevention of diseases caused by non-poliovirus enteroviruses such as *Coxsackievirus* B and *Echovirus* requires the identification of viral contamination sources and the development of effective intervention strategies and decontamination procedures for shellfish and aquatic ecosystem (Morley, 2010; Esposito et al., 2022).

Comparing the occurrence of viral pathogens in shellfish is difficult since few data are available in the literature and conditions are always different including site conditions, sampling, and detection methods. However, this study can be compared with a previous study conducted to analyse viral contamination in mussel

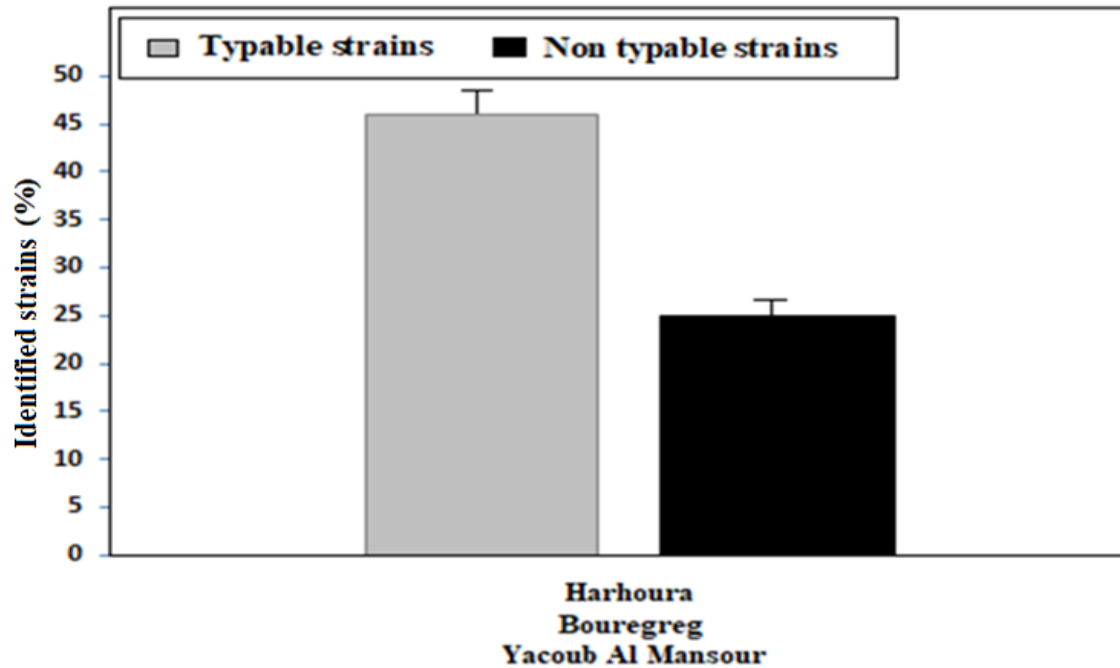


Figure 5. Percentage of typable and non typable strains of non-poliovirus enteroviruses using seroneutralization.

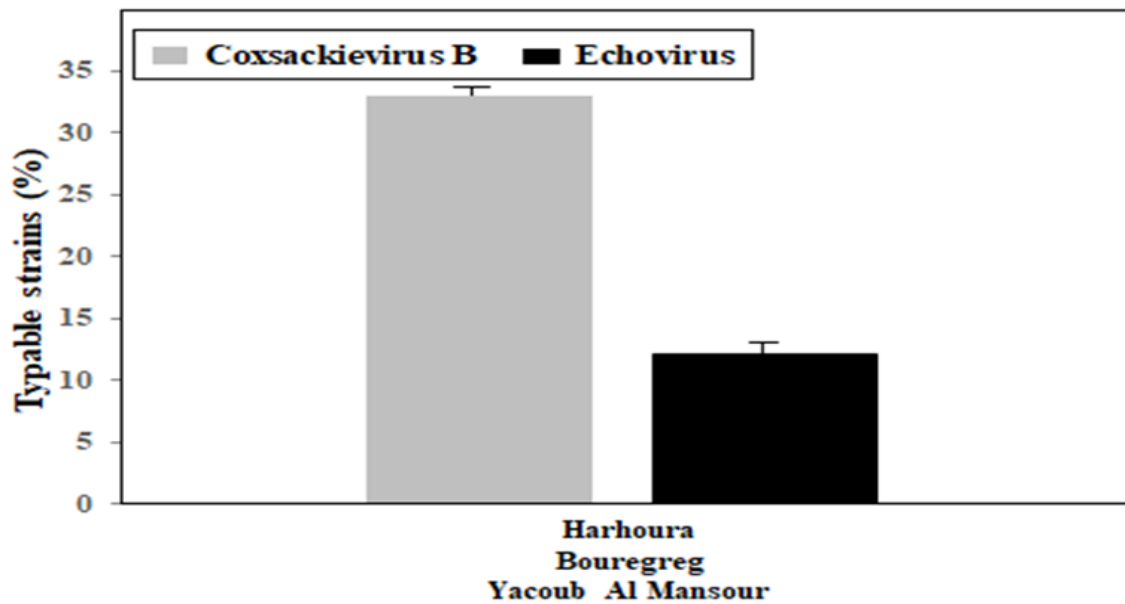


Figure 6. Typing of non-poliovirus enteroviruses strains identified from mussels.

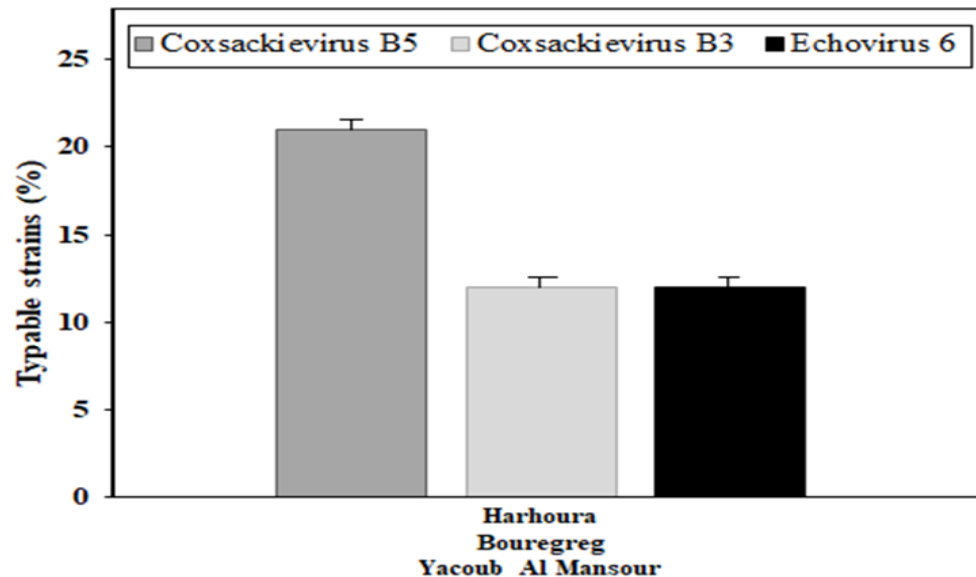
samples collected from sites occasionally impacted with sewage (Sdiri et al., 2004, 2006; Elamri and Aouni, 2005; Elamri et al., 2006; Karamoko et al., 2005, 2006a, b; Gharbi-Khelif et al., 2007; Bosch et al., 2008; Benabbes et al., 2013a, b; Bou M'handi and Laachir, 2015; Idrissi Azzouzi et al., 2017a). These results allow some conclusions and comments, as many of the collected

samples have been found contaminated with human enteric virus particles.

### Conclusions

The identification of isolated non-poliovirus enteroviruses





**Figure 7.** Percentage of different serotypes of *Coxsackievirus* B and *Echovirus* 6 typing by seroneutralization.

becomes indispensable, as it is important to know the new serotypes associated with diseases. Thus, the surveillance of enteroviruses circulation cannot be limited to the only surveillance of interhuman circulation and should include monitoring of enteroviruses in the marine environment.

The mean number of positive samples in this study is in accordance with data found in the literature, indicating that viral contamination of molluscs is similar among countries investigated which reflects the epidemiological status of the population. The results confirm that mussels in Morocco were contaminated with several enteroviruses. Therefore, to protect human health worldwide, research should be dedicated to better understand virus circulation and to develop appropriate monitoring in all shellfish producing countries. This will be helpful to understand virus circulation and to improve seafood safety

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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