Hepatitis A Virus PCR Analysis and *E. coli* Detection in Oysters at Oualidia Lagoon and Their Correlation

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Abstract

The present study aims to evaluate hepatitis A virus (HAV) prevalence and faecal contamination indicators *Escherichia coli* (*E. coli*) in oysters from Oualidia lagoon (Moroccan Atlantic coast) and to study the correlation between the two parameters. The survey was carried out on 87 samples of oysters (*Crassostrea gigas*) collected monthly between November 2015 and February 2017 from three sites corresponding to different oyster farms in the lagoon. Sanitary status of bivalve molluscs was assessed by *E. coli* enumeration using ISO 16649-3. Detection of hepatitis A virus, was carried out by real-time reverse transcriptase polymerase chain reaction (rRT-PCR) according to ISO 15216-2 method. The prevalence of samples for which *E. coli* contamination exceeds the threshold of 230 *E. coli*/100g of flesh and intravalvular fluid (FIF) is 43%. HAV RNA was detected in 2% of the samples analyzed. This RNA was even detected in a sample meeting the bacterial criteria. Viral health surveillance of bivalve molluscs is therefore necessary before their delivery for human consumption.

Keywords

Hepatitis A Virus, *E. coli*, Bivalves, Shellfish, RT-PCR, Oualidia Lagoon

1. Introduction

Contaminated shellfishes are frequently involved in foodborne diseases and infections. Coastal marine ecosystem is threatened by a wide range of pollutants that filter-feeding molluscs can accumulate in their tissues and are therefore likely

to cause a serious risk for consumer's health [1] [2] [3]. Viral persistense has been demonstrated in bivalve molluscs and as they are often consumed raw or slightly cooked, the microbial risk is therefore increased. [3] [4] [5] [6].

Enteric viruses of human origin are very common in seawater where they are rejected in large amount, shed by infected people [7].

They belong to a large panel of family and genus and among this diversity, HAV, ranked as priority hazards by WHO/FAO, is known since antiquity to be responsible for many epidemics, causing acute inflammation of the liver [8]. Nowadays it is still implicated in several outbreaks around world as 1.4 millions clinical cases occur annually [9].

This illness is strongly related to age: if it is a mild disease in children under five, protective antibodies bestow lifelong immunity, in older children and adults may have acute inflammation of the liver with icteric symptomatology, though without involving chronic liver disease [10]. Healing is current; however, in rare cases, it can lead to fulminant hepatitis that progresses to a fatal outcome. The economic burden is significant as recovery can be long and disabling [11].

HAV is a member of the Picornaviridae family, genus Hepatovirus [12]. Although known as limited to man and some species of non-human primates [13], presence of HAV has been recently detected in animals other than primates as seals [14], woodchuck [15] and marsupials [16]. A single serotype is present in human and strains are classified into six genotypes [17]. Very resistant under various conditions of environment, it can survive, and remain infectious, for a long period outside a host [18]. Epidemics occur in a cyclical manner by fecal-oral transmission and close contact with infected persons and contaminated food and water [19].

HAV outbreaks linked to consumption of bivalve molluscs represent 17% of total foodborne outbreaks [5]. These outbreaks occur as a consequence of the contamination of surrounding water by pathogens.

Currently, microbiological surveillance of bivalve molluscs is based solely on bacteriological parameters. Moroccan regulation (decision n˚ 1950-17 of 07/08/2017 of Moroccan Ministry of Agriculture, Rural Development and Maritime Fisheries) requires a sanitary control of harvesting shellfish areas by enumeration of *E. coli* as faecal indicator bacteria. Four classes are identified according to their microbiological quality: A (80% of samples < 230 *E. coli* per 100 g of shellfish flesh and intravalvular fluid (FIF) and the remaining 20% of samples < 700 *E. coli* per 100 g of FIF), B (90% of samples < 4600 *E. coli* per 100 g of FIF and the remaining 10% of samples 4600 ≤ *E. coli* ≤ 46,000 per 100 g of FIF), and C (100% of samples *E. coli* ≤ 46,000 per 100 g of shellfish FIF). However, several studies have shown that bacteria are not reliable indicators of viral contamination in shellfish [20] [21].

The aim of our study was to evaluate hepatitis A virus prevalance in oysters harvested at Oualidia lagoon, using real-time RT-PCR analysis according to the ISO 15216-2 method as well as the enumeration of *E. coli* performed in accordance with ISO/TC 16649-3.

2. Materials and Methods

2.1. Description of Study Area

Oualidia is a lagoon without outlet, located on the Atlantic coast, 168 km south of Casablanca, at (32˚46'N, 09˚01'W). It is listed as RAMSAR site (wetlands of international importance).

It communicates with the ocean through a major inlet of 150 m wide and a secondary one of 50 m. The sedimentation is sandy near the passes and silty upstream where turbidity is higher in the rainy season, between October and April [22]. An artificial dam contributes to trap sedimentary organic matters and protects this ecosystem from confinement. Anthropic pressure on this ecosystem is linked to agriculture, tourism, oyster farming and urbanization. Even if some residential on the shoreline only have septic tanks, the commissioning of the treatment plant in 2012 helped reduce this pressure.

Renowned for its oysters with a production close to 36 tons annualy, Oualidia lagoon is classified as a "B" harvesting area. Therefore, depuration processing is a statutory requirement before marketing for human consumption.

2.2. Shellfish Sampling

Oyster samples (n = 12 individuals/sample) were collected, over 16 months between November 2015 and February 2017, twice per month except for May and June where the sample has been taken only once, giving a total of 87 samples. Sampling sites (S1, S2 and S3) correspond to three oyster farms located in Oualidia Lagoon. S1 is located downstream of the lagoon near the principal pass with a sandy sedimentary facies. S2 and S3 are further upstream, before inner dam location, and are characterized by muddy sediment (**Figure 1**).

Bivalve molluscs were transported to the laboratory under controlled temperature (+4˚C) within 24 hours. The samples were processed rapidly for *Escherichia coli* and for HAV or stored at −80˚C.

2.3. Viral RNA Extraction

To evaluate the presence of HAV, RNA extraction method included the use of guanidine isothiocyanate as chaotropic agent. Shellfish were thawed, cleaned and shukked in sterile conditions, the hepatopancreas was removed and chopped using a razor blade. 2.0 ± 0.2 g subsample of digestive tissues was transferred to clean tube and mixed with 10 µl of mengovirus as a process control of nucleic acid extraction (Ceeramtools®). 2 ml of Protéinase K solution (100 µl/ml) was added to digest the tissues that were incubated with shaking (37˚C ± 1.0˚C/320 ± 20 rpm) for 60 min followed by a second incubation (60˚C ± 2.0˚C/15min). Then samples were centrifuged at 3000 g for 5 min and the supernatant collected in a clean tube. RNA was extracted from 500 µl and eluted in 100 µl of elution buffer using commercial Nucleospin RNA virus kit (Macherey Nagel, Germany) following the manufacturer's instruction. RNA was tested for HAV immediately or stored at −20˚C until use.

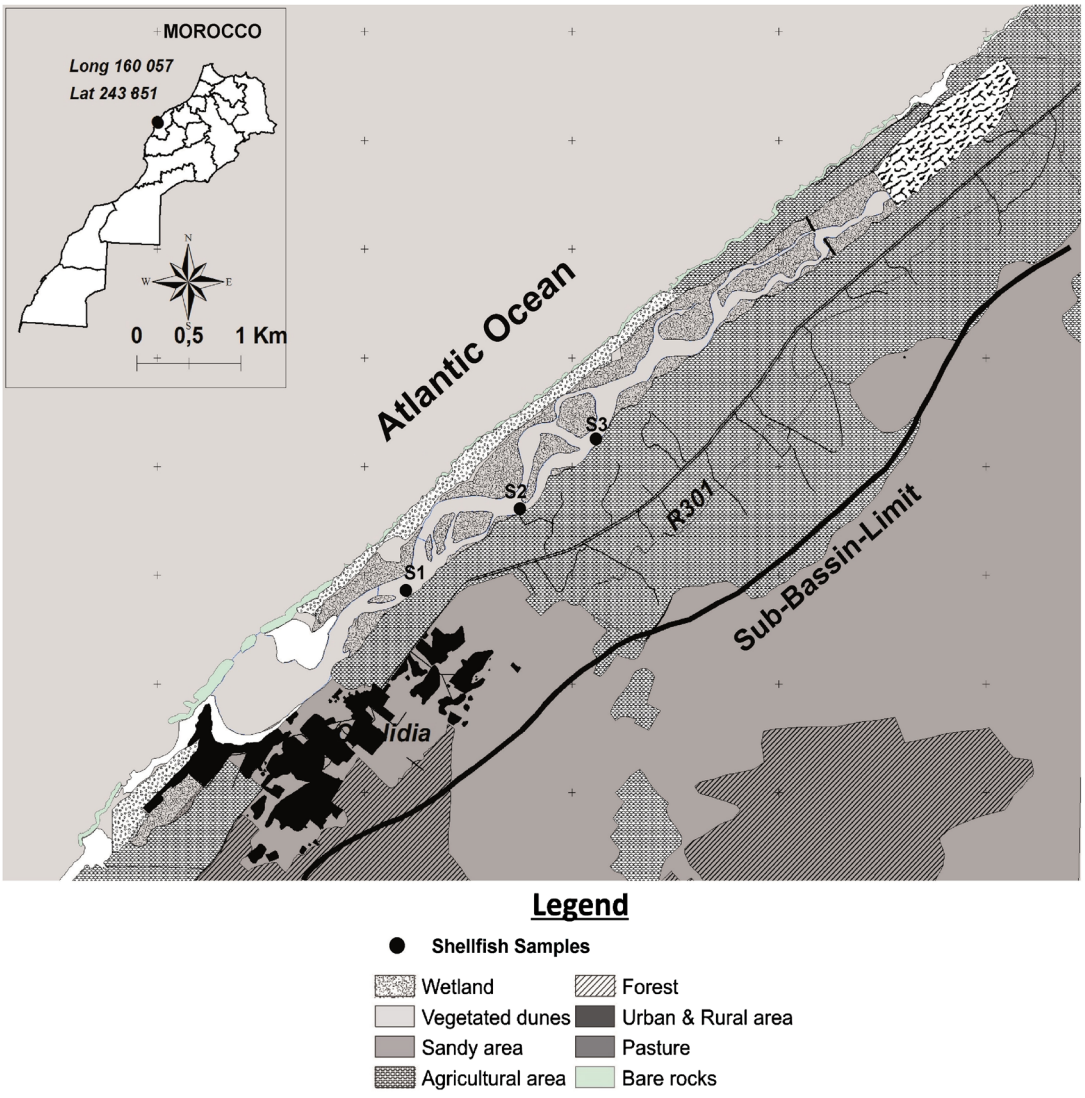

Figure 1. Shellfish collection sites.

2.4. One-Step qRT-PCR

HAV, HAV68 and HAV240 primers and HAV150 probe [23], and Mengovirus vMCO strain (Ceeramtools®) were used. 5 µl of each RNA sample was amplified in 25 µl total volume with one-step reaction mix prepared using the SuperScript® III Platinum® One-Step Quantitative RT-PCR Kit (Invitrogen) that contained 1× reaction mix, 0.5 pmol/µl of forward primer, 0.9 pmol/µl of reverse primer and 0.25 pmol/µl of probe, 1× ROX Reference Dye and 1.25 µl of SuperScript® III RT/Platinum® Taq mix. The set of primers and probes and their reference are listed in Table 1. Amplification was performed in a 7500 Fast Real-time PCR System (Applied Biosystems) using the following cycling parameters: reverse transcription at 50˚C for 60 min, denaturation at 95˚C for 5 min, followed by 40 cycles of amplification with a denaturation at 95˚C for 15 s and annealing and extension at 60˚C for 1 min.

Viral RNA extracted from each sample was tested undiluted and at a tenfold

dilution to evaluate the presence of inhibitors. A Ct value difference < 3.3 indicated the presence of inhibitors. In each operation, a Mengovirus standard curve was generated by a 10-fold serial dilution. Extraction efficiency was evaluated by comparing the Ct value of RNA of Mengovirus extracted from the samples to those obtained for the standard curve. Results ≥ 1% were considered valid. Wells containing nuclease free water as negative control and HM 175 strain of HAV as positive control were tested in parralel with each batch of samples.

2.5. *E. coli* Enumeration

Samples were analyzed for *E. coli* enumeration using most probable number (MPN) method in accordance with ISO/TS 16649-3: horizontal method for enumeration of beta-glucuronidase-positive *Escherichia coli*. The most probable number (MPN) of *E. coli* is calculated using the tables of ISO 7218: 2007). Whole oysters (flesh and intravalvular fluid) were homogenized by stomacher in peptone saline diluent followed by decimal dilution. A series of five tubes were inoculated, containing double strength minerals modified glutamate medium (MMGM), single strength MMGM, and 10-1, 10-2 and 10-3 dilutions of single strength MMGM. They were incubated at 37˚C ± 1˚C for 24 ± 2 H. Thereafter, tubes showing trace of acid production are subcultured in tryptone bile glucuronide agar (TBX) and incubated at 44˚C ± 1˚C for 22 H ± 2 H. The plates yielding growth of blue-green colony indicate *Escherichia coli* β-glucuronidase-positive.

3. Results

3.1. Evaluation of Viral Contamination in Shellfish Samples

Among the 87 samples analysed, 2% were HAV positive. The HAV-positive samples were collected during the winter after a rainy-period, in November 2015 and January 2016 (Table 2). Regarding the sampling sites, the two HAV-positive samples were from S3 located the most upstream.

Table 1. Sequences of the primers used for HAV detection.

Virus	Primer sequence	Reference
HAV	**Forward primer** VHA68 5'-TCA CCG CCG TTT GCC TAG-3'	[23]
	Reverse primer VHA240 5'-GGA GAG CCC TGG AAG AAA G-3'	[23]
	Probe VHA150 FAM 5'-CCT GAA CCT GCA GGA ATT AA-3'MGBNFQ	[23]

Table 2. Seasonal distribution of VHA-positive samples.

Date	HAV Positive Oysters
November 2015	1
January 2016	1

3.2. *E. coli* Bacterial Contamination and Correlation with Viral Contamination

Analysis of oyster samples showed 43% are contaminated by *E. coli* and the legislation in force defines Oualidia lagoon as a "B" harvesting area (90% of results ≤ 4600 *E. coli* FIF and 100% ≤ 46,000 *E. coli*/100g of (FIF)) (Figure 2). However, contamination with hepatitis A virus is variable as a sample HAV positive showed a low contamination with *E. coli* (not exceeding 230 *E. coli*/100g FIF) while the other HAV positive sample showed a high rate of contamination by *E. coli*. Samples contaminated by HAV come from (S3) while RNA-HAV was not detected in samples from (S1) and (S2). Thus, our results showed no correlation between HAV and *E. coli* contamination $p > 0.05$.

4. Discussion

Shellfish viral contamination data are scarce in Morocco. This study was conducted to evaluate the presence of HAV in oysters collected from different sites of the Oualidia lagoon, classified as "B" harvesting area. We found that 2% of the samples are contaminated with this virus.

The prevalence of contaminated oysters found in our study is low given Moroccan epidemiological context. Thus the year 2016 when most of the samples were taken, was a year od drought which reduced the incidence of soil leaching, but similar finding was reported previously [24]. Yet in the mediterranean rim countries, incidence rates of HAV are quite different between regions. It was detected in 26% of shelfish samples in Tunisia [25], 10.1% in Spain [26] whereas all samples were HAV negative in south Italy [27]. The results obtained can be the consequence of the water renewal influenced by currents and other hydrographic variables [28] [29].

Cases of hepatitis A are reported throughout the year and seasonal pattern of HAV have not been clearly observed [30]. Although, positive samples in our study were taken during the winter period. Similar data were reported in Tunisia, Hong Kong and USA [31] [32] [33]. This result can be linked to rainfalls,

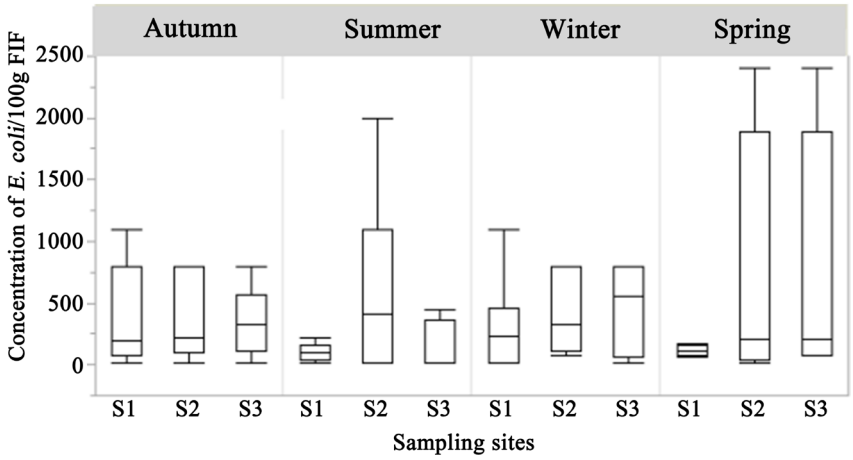

Figure 2. *E. coli* counts.

which would have caused the overflow of the septic tanks. However, as the two HAV positive samples having been obtained from the same site at quite close intervals of time (november 2015 and january 2016), this would be more in favor of spot contamination linked to feces discharge of infected persons.

Hepatitis A infection is reported to have intermediate endemicity in Morroco and episodes of epidemics are scarce as most people acquire long lasting immunity at a young age when the illness is benign or asymptomatic most of the time. Improving sanitary measures leads to shifting primary infection progressively to older ages but with more severe symptoms. Thereby decreased immunity will lead to more frequent outbreaks [34]. However, the long incubation period that separates the contamination and the onset of symptoms makes it difficult to identify the source of the infectious agent. Thus, as prospective virological analyzes of shellfish are not routinely conducted, the involvement of oysters as a vector of HAV is not known.

Physical, chemical and biological factors play a role in most microorganisms stability. However HAV is outstandingly resistant to environmental conditions and may persist for a long time in food, on fomites and in water. Adsorption to solid particles is one of most relevant factor involved in its survival and retention by shellfishs [35] [36] [37] [38]. Depuration is intended to eliminate shelfish pathogens, however effectiveness of this process has not been demonstrated in HAV [39].

Indeed, the monitoring of shellfish and shellfish waters is based solely on the enumeration of *Escherichia coli* as an indicator of faecal contamination; the viral hazard is not taken into account. According to several studies the analyzes of fecal coliforms and *E. coli*. give limited predictive value for pathogenic viruses such as enteroviruses [40], noroviruses and infectious hepatitis caused by hepatitis A virus [41]. Thus, average *E. coli* levels in HAV positive samples correspond to a class A (220 *E. coli*/100g FIF for the sample taken in november 2015) as well as a class B (780 *E. coli*/100g FIF for the sample analyzed in January 2016). Our findings are consistent with those obtained in various studies [20] [32] [40] [42]-[49].

For a while, the search for viruses stumbled on the lack of an appropriate method. HAV barely grows in cell cultures and often fails to produce cytopathic effect [50] [51]. Appropriate method for identifying HAV in oysters, available recently, overcame this difficulty. Fast and sensitive, RT-qPCR remains the most appropriate method for detecting a low concentration of viral particles. However, this analytical technique does not differentiate between infectious and non-infectious virus particles. Thereby, presence of HAV genome might balanced by a linked-risk analysis.

5. Conclusion

The absence of correlation between faecal bacteria as indicators of microbiological contamination and food-borne viruses highlights the need of additional con-

trol measures implementation. Updating existing legislation in force to include viral hazards in the monitoring of bivalve shellfish harvesting areas is an urgent matter to which the competent authorities should respond. Indeed, monitoring of foodborne viruses in bivalve molluscs may contribute to the prevention of viral food poisoning and the promotion of public health.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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