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Occurrence of Hepatitis A Virus in Mussels (*Mytilus Galloprovincialis*) In the Gulf of Shengjin, Albania

Egon Andoni[1*], Ani Vodica [2], Enkeleda Ozuni [1]

[1]*Faculty of Veterinary Medicine, Agricultural University of Tirana, Albania*
[2]*Institute of Veterinary and Food Safety, Albania*

[*]*(e.andoni@ubt.edu.al) Email of the corresponding author*

Abstract – Viral pollution deputizes a worldwide problem for human health causing illness due to consume of contaminated shellfish. Mussels can serve as reservoir of viral particles during the process of filter feeding in marine environment. Therefore, epidemiological survey is necessary to determine the level of viral pollution in coastal areas utilized for mussel's production. From January 2015 to December 2016, 52 samples of mussels (*Mytilus galloprovincialis*) were collected in harvesting areas from Shengjin in Albania and were analyzed for the presence of HAV utilizing TaqMan real-time RT-PCR. Hepatitis A virus was present in (38%) of mussels during the two years of the survey. Periodic evaluations of water quality in harvested mussel areas in Shengjin for the presence of HAV represent a useful instrument to evaluate risk assessment and prevent food borne illness.

Keywords – Hepatitis A Virus, Mussels, PCR, Albania, Survey

I. INTRODUCTION

Heavy Food-borne viruses deputize an emerging problem for public health due to an increase number of outbreaks diseases. According to a report by EFSA, in 2014 viruses were, for the first time, the most commonly detected (20.4%) causative agent in foodborne outbreaks [21]. Several viruses have the capacity to survive and remain infectious in the environment for long period. Viruses can survive on their surface once harvested [6] and can remain infectious for several days or weeks and even during commercial and household storage for periods of up to 5 weeks [3]. Hepatitis A virus (HAV) is classified within the *Picornaviridae* family representing a non-enveloped virus with a single RNA genome. HAV viruses cannot rise in food however it has the ability to survive in seawater for 10 months and less than 100 viruses are enough to cause illness in humans. During their natural feeding process, mussels are able to filter large quantities of water, retaining and concentrating in their bodies not only bacteria that are present in the environment but also viruses [5,19,27,30]. The filtration can lead to concentrations in shellfish 100–1000 times higher than that in the surrounding water [6]. Enteric viruses, particularly HAV, may persist in the organism even after they have been transferred into clean water to be subjected to purification treatments and fecal coliform bacteria, used as a fecal contamination index, have been eliminated [15,10].

Consume of mussels is related with different types of dangers which include, increase of microbial load, pollution from heavy metals and presence of viruses. Traditionally, bivalve mollusks shellfish such as oysters, mussels, clams and cockles have been considered as a principal source of food-borne virus that may subsequently be disseminated [28]. Approximately 1.4 million people worldwide become infected with HAV annually [18]. In Italy, consumption of shellfish, especially raw or lightly cooked, is a major risk factor reported in 62% of HAV cases [25]. Depuration deputies the most utilized method able to decrease the microbial load

in mussels. Nevertheless, these approaches as a legal requirement offer variable effectiveness for the lamination of bacteria. It is widely accepted that depuration is not a suitable intervention for enteric virus, due to the ability of enteric viruses to persist in shellfish tissues [2,12,13,20,24]. Therefor harvesting mussels from clean water where the fecal contamination is minimal is the best approaches to guaranties the quality of mollusks. In an effort to control shellfish-borne infections, the EU has proposed several control measures for shellfish safety, which culminated in the implementation of Regulations 853/2004, 854/2004 and 1021/2008 [7,8]. Despite all the efforts, periodic emergence of outbreaks associated with shellfish consumption with a presumed viral etiology continue to pose a real public health dilemma that result in substantial economic losses by the seafood industry and a lack of public confidence over shellfish [32]. The pathology affects all the people being asymptomatic in children under 6 years old whereas in older persons is more severe. Many cases of hepatitis A are considered to be unreported while many persons can be asymptomatic. Principal symptom may include nausea, abdominal pain, fever, anorexia, jaundice or enlargement of the liver. Symptoms may occur 1-2 weeks after and recovery is full after several weeks.

Albania is located in the Balkan Peninsula with coastline of 418 km composed from Adriatic and Ionian seas. Mussel's production is located throughout the Albanian costal in two different settlements. Butrint in the south represent the most important site which produces 1500 tons in a year. The Shengjin mussel farm represents a new enterprise created in 2007 into a protected bay in the north-west of Albania. The production area of 40.000 m2 and the capacity is 500 tons in a year. Nevertheless, no available data regarding the presence of HAV of viruses is Albania coastline in Shengjin bay. The aim of this research was to determine the presence of HAV in mussels in harvesting areas of Shengjin areas coastline. Indeed, these study provide all the results discovered from a two-year surveillance utilizing TaqMan real-time RT-PCR for HAV.

II. Materials and Method

A. *Mussel sampling*

During the years 2015-2016, fifty-two samples of mussels were collected from Shengjin coast to detect the presence of HAV. During the transportation to the laboratory samples were kept in closed box in temperature of 4-8^0 C and stored in -20^0 C, whereas analyzation was performed within 24 h after the collection. Separation of digestive tissue from each mussel was performed under sterile conditions, whereas 2 g of dissected hepatopancreas was utilized for RNA extraction.

B. *PCR for the detection of HAV*

The method used for the TaqMan real-time RT-PCR for HAV [9]. TaqMan real-time RT-PCR utilizes a short probe with a fluorescence label (report) and absorber (quencher) linked to opposite ends of the probe. The TaqMan probe was labeled with 6-carboxyfluorescein at the 5 end and was modified with a minor-groove binder at the 3 end [9]. Fluorescence can be measured at any stage of amplification. The amplification procedure and temperature in each step is strongly related with the types of reagents utilized. The amplification program consisted of preheating for 2 min at 95°C and 40 cycles of 10 s at 95°C for denaturation and 1 min at 60°C for annealing-extension. The fluorescence was read at the end of each cycle [9].

Figure 1: Sample zone: Shengjin 41^{0}47'04.8"N 19^{0}35'02.0"E

C. *RNA extraction*

For the extraction of RNA commercial kit (BioMerieux) were utilized, whereas positive

sample considered lower than Ct ≤ 44.0 cycles. To test for the presence of RT-PCR inhibitors and to calculate rRT-PCR efficiency in both dilutions of each examined sample, amplification using 2.5 ll of each extracted RNA and 2.5 µL of internal controls containing 10^3 genome copies of the respective virus type were evaluated in separate experiments [9,22]. Primers for the search of HAV should search on the region 5 of the genome and a summary of the sequencing is given on the Table 1.

Table 1. Primers utilized in this study

Primers	Sequences 5'	Target sequences	Reference
HAV68 (F)	TCACCGCCGTTTGCCTAG	68–85[a]	[9]
HAV240 (R)	GGAGAGCCCTGGAAGAAAG	241–223	
HAV150 (P)	FAM-CCTGAACCTGCAGGAATTAA	150-169	

III. Results

Over a period of 24 months in 2015-2015, 52 samples of mussel during the two years of the survey. All samples were tested for the presence of HAV utilizing TaqMan real-time RT-PCR at the Zooprophilactic Institute of Teramo, Italy. The highest number of positive sample with HAV were observed in the first year 15 sample or 43% whereas in the second year 35% of the sample resulted positive. All the results obtained for detecting HAV in mussels in the laboratory analysis are summarize in the table 2.

Table 2. Detection of HAV in sample tissue of *Mytillus galloprovicialis* collected from Shengjin

Year	***Sample size***	***Negative samples***	***Positive samples***
2015	26	15	11
2016	26	17	9
Mean value	26	16	10

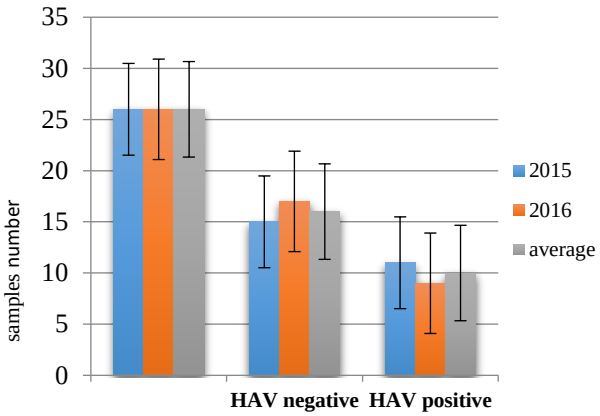

Figure 2: Comparison of average mean value and (SE) of HEV negative and positive sample cases during 2015 -2016 in *Mytillus galloprovincialis*

IV. DISCUSSION

Bivalve mollusks *(Mytilus galloprovincialis*) represent highly nutritive seafood rich in proteins, omega-3 Polyunsaturated Fatty Acids and low lipid values. Mussels contain a range of vitamins and minerals found in other meat-based sources of protein such as B-vitamins and trace minerals [34]. However, mussel can serve as source of contamination with heavy metals, pesticides, viruses and bacteria. The incidence of outbreaks of foodborne viral disease has increased considerably during the last decades, possibly due to the rapid globalization of the food market, the increase in personal travel and food transportation, and the profound changes in food consumption habits [31]. Mussels have been described as the bivalves which are most contaminated with human enteric viruses [17]. At the same time there is still uncertainty regarding the exact temperature able to guarantees the elimination of the HAV from mussels. Resistance of HAV to heating is strongly related with virus strain, initial level of contamination and food matrix. However, based on a research from [10] it was necessary to prolong the treatment at 100 °C for 2 min to completely inactivate the virus in contaminated mussel at 10^5. Another study reports that internal heat treatment of shellfish at 85–90°C for 1 min is sufficient to completely inactivate the virus [26]. Replication of HAV is very difficult consequently most of the laboratories are focused on in real time PCR (RT-PCR) for the identification of the viruses in mussel. In spite of this limitation, molecular methods like PCR are considering as the

most suitable tool to detect the presence of viruses in bivalve shellfish [32].

Shengjin represent the second most important location as mussel producer in Albania therefore, the is important to evaluate the virological quality of mussel in this geographic area. Many researchers have demonstrated the presence of HAV in several European countries with different percentages. Detection of HAV was performed in shellfish collected from five European countries – Greece, Spain and United Kingdom – and HAV was detected in 4, 3 and 1 sample, respectively, but not in Sweden [16]. In Turkey these percentage was (3.3 %), [35] in Poland, HAV was detected in 9 (7.5%) of the shellfish [1], while in Spain these percentages range from 10.1–75% of samples was positive for HAV [4,28,23, 29]. In our study 39% of the mussels resulted positive with HAV in the costline of Shengjin. These results are near with the finding from [33] conducted at Butrinti Lagoon in Albania, where 32,5% of samples in mussels were positive with HAV. Similar percentage was also observed in a research from [14] in mussels (*Mytilus galloprovincialis*) in the Gulf of Izmir, Turkey were analyzed samples were positive for HAV and prevalence was (30%).

V. CONCLUSION

Higher risk is observed in Mediterranean areas due to cooking behavior whereas mussels are often consumed raw. The evaluation of water quality can be utilized as u useful tool for the classification of the harvesting areas and the prediction viral load in mussels. Therefore, assessment of water quality and presence of *E. coli* or fecal coliform is necessary to determine the level of pollution in the harvested areas. National plan of risk assessment should also evaluate the viral load not only for HAV but moreover different types of viruses such as (NoV) Norovirus, RV (rotavirus), HEV (hepatitis E virus), AiV (aichivirus) in *Mytilus gallopronvincialis* (Mediterranean mussels). These measures are necessary not only to understand the epidemiological situation in Shengjin coastline but also to improve public health.

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