

# Escherichia coli, Salmonella spp., Hepatitis A Virus and Norovirus in bivalve molluscs in Southern Italy

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## Summary

European Legislation has fixed microbiological, chemical and biotoxicological limits for shellfish but no limits for viruses. In the present study we report the results of an investigation on *Salmonella* spp., *Escherichia coli*, Hepatitis A virus (HAV) and *Norovirus* (NoV) contamination in 59 bivalve shellfish collected during the years 2011-2012 in Southern Italy. All the samples of *Mytilus galloprovincialis* and of *Solen marginatus* were negative for HAV whereas 6.8% of them were positive for *Norovirus* GI (NoVGI) and 11.9% positive for *Norovirus* GII (NoVGII). Samples were also negative for *Salmonella* spp., while 16 of them (27%) were positive for *E. coli*. No correlation was found between *E. coli* and NoV contamination in bivalve molluscs. Moreover, the Competent Authorities are advised to take into serious consideration additional measures for the legislation in force in order to guarantee the consumer's health.

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## Introduction

Currently there is no specific legislation enforcing the control of enteric viruses in bivalve mussels. Only art. 11.5, letter B of Reg. CE 853/2004 allows the possibility of laying down additional health standards for live bivalve molluscs in cooperation with the Community Reference Laboratory, including virus testing procedures and virological standards.

Moreover, the Legislative Decree n. 191/2006 obliges the regions of the Italian territory and the Italian autonomous provinces to apply a surveillance plan according to the different epidemiological situations against the zoonotic agents listed in the Annex I, part B of the Regulation (e.g. *Calicivirus* and HAV). In Italy, notification of cases of gastroenteritis from HAV is still very common. Many of them are reported in the Campania region and are related to the consumption of bivalve molluscs.

Since both NoV and HAV are transmitted via the human stomach and intestines, shellfish can be indirectly affected from water contaminated with faeces of infected individuals. Moreover NoV and HAV are considered two of the main causes of gastroenteritis in humans due to their ability to give infection with very low doses (1-10/UFU) and by surviving in the external environment for a long time (7, 11, 18). In Italy, confirmed cases of foodborne disease from *Norovirus* are still few,

but they could be underestimated, since outside Italy a considerable number of episodes has been recorded. In 2006, in the E.U. food and feed alert system (RASFF), nine outbreaks were reported from raw oysters (12) and in 2008 two outbreaks (in France and the Netherlands) were notified together with six human cases in Norway. All the cases regarded oysters coming from Spain, France and the U.K., and the French outbreak, were related to genogroup I detected in *Crassostrea gigas*. In the same year five cases of HAV were reported in Spain from tellina clams (13). In 2009, 19 human cases from *Norovirus* were reported in Norway from eating *Gigas oysters* coming from Sweden. 32 persons from the Czech Republic have also contracted HAV from semi-dried tomatoes imported from Turkey (14). In 2010, a total of 13 cases of *Norovirus* were notified in Denmark, Norway, Ireland, France and Sweden from lettuce, raspberries and bivalve molluscs (15). In 2011, 16 outbreaks of *Norovirus* were reported by Denmark from consumption of oysters or mussels (10 notifications) and raspberries (5 cases originating from Serbia and one from China) (16).

The potential role that mussels have as vectors of bacterial and viral diseases is closely related to their water filtration activity. Consequently they are able to concentrate in their tissues not only chemical residues but also microorganisms as contaminants of the waters they are collected from.

We know that the main hygienic and sanitary parameters for correct production and commercialization of bivalve molluscs are laid down in the Reg. (CE) n. 2073/2005. Apart from *E. coli* and *Salmonella* spp. we advise taking into serious consideration the presence of enteric viral contaminants within filter-feeding lamellibranch molluscs, in particular HAV and NoV genogroups GI and GII, which are related to the most important causes of human gastroenteritis worldwide.

## Materials and Methods

### Sampling

The survey was carried out on 59 samples of Italian bivalve molluscs. Fifty one samples were represented by *Mytilus galloprovincialis* collected from class A and B water production areas in Naples and Caserta provinces and 8 samples were represented by *Solen marginatus*, collected from natural beds in Caserta and Salerno provinces.

### Microbiological control

*Salmonella* spp. and *E. coli* were isolated using MPN standard methods as described in UNI EN ISO 6579:2004 and ISO TS 16649-3:2005.

### Virological control

A PCR protocol for detecting HAV, NoVGI and NoVGII used by the Italian National Reference Laboratory (Istituto Superiore di Sanità) for control of viral contaminations in bivalve molluscs has been adopted (3, 6, 8, 18). This protocol uses 2 mL of a K proteinase solution (0.1 mg/mL) (Sigma-Aldrich, Milan, Italy) in which 2 g of cut epatopancreas is added from each sample, incubated in agitation at 37°C for 60 min and then in a water bath at 65°C for 15 min, centrifuged at 3,000 g for 5 min. The supernatant was collected and stocked at -20°C.

RNA extraction was carried on with Kit Nucleospin RNA II (Macherey- Nagel GmbH & Co., Duren, Germany). The extraction started with 500 µL of sample and the RNA was eluted with 100 µL of elution buffer. The retro-transcription mix and the one-step PCR was prepared using the reagents of the Platinum qRT PCR ThermoScript one-step system (Invitrogen, Karlsruhe, Germany).

Primers and probes used for the detection of hepatitis A virus are: (FW) HAV68 5'-TCA CCG CCG TTT GCC TAG-3', (REV) HAV240 5'- GGA GAG CCC TGG AAG AAA G-3', probe HAV 150 FAM- CCT GAA CCT GCA GGA ATT AA-MGB (3). For *Norovirus* GI: (FW) QNIF4 5'-CGCTGG ATG CGN TTC CAT-3' (4), (REV) NV1LCR 5'-CCT TAG ACG CCA TCA TCA TTT AC-3', probe NGI FAM-TGG ACA GGA GAY CGC RAT CT-

TAMRA (18). For *Norovirus* GII: QNIF2 (FW) 5'-ATG TTC AGR TGG ATG AGR TTC TCW GA-3' (8), (REV) COG2R 5'- TCG ACG CCA TCT TCA TTC ACA-3' (6), probe NGII FAM-AGC ACG TGG GAG GGC GAT CG-TAMRA (8).

The retro-transcription and amplification were carried out using the following thermal scheme: 55°C for 60 min, 95°C for 5 min and 45 cycles at 95°C for 15 sec, 60°C for 1 min and 65°C for 1 min.

Each sample was considered positive when Ct was ≤ 44.

## Results

Samples were all negative for *Salmonella* spp. and HAV, while 16 of them (27%) were positive for *E. coli*. It is useful to underline that these samples were represented by 10 *Mytilus galloprovincialis* and 6 *Solen marginatus*. Nine samples (56%) showed *E. coli* counts higher than 230 MPN/100g, which is the value allowed by current legislation. Among the positive samples, 1 was collected from a class A water production area while all the others were from class B waters. *Norovirus* GI Viral RNA was detected in 4 samples and *Norovirus* GII RNA was detected in 7 samples. The details of the findings are reported in Table I.

As far as the co-presence of NoV and *E. coli* is concerned, the results are the following: among the 4 samples positive for *Norovirus* GI, only 1 sample of *Solen marginatus* was also positive for *E. coli* with a value of 78 MPN/100g, while in the 7 samples positive for NoVGII, *E. coli* was isolated in only 2 samples of *Solen marginatus* with values of 78 MPN/100g and 230 MPN/100g respectively (Table II).

Moreover, 2 samples were positive for NoVGI and NoVGII. 1 sample was *Mytilus galloprovincialis* and was negative for *E. coli*. The other was *Solen marginatus* and it was also positive for *E. coli* with a count of 78MPN/100 g.

## Discussion

Some of the results of this study have been presented at the 2<sup>nd</sup> Annual World Congress of Virus and Infection (WCVI) in Beijing, China, in 2011 (1).

**Table I.** *Norovirus*, hepatitis A virus, *E. coli*, and *Salmonella* spp. in samples of bivalve molluscs (2011-2012).

Samples	N	NoV GI	NoV GII	HAV	<i>E. coli</i>	<i>Salmonella</i> spp.
<i>Mytilus galloprovincialis</i>	51	3	5	0	10	0
<i>Solen marginatus</i>	8	1	2	0	6	0
Total	59	4	7	0	16	0

**Table II.** Correlation between positive samples for norovirus detection and *E. coli* count (MPN/100g).

Positive samples for norovirus/ <i>E. coli</i>	NoV GI	NoV GII	<i>E. coli</i>
<i>Mytilus galloprovincialis</i>	+	-	-
	+	-	-
	-	+	-
	-	+	-
	-	+	-
	-	+	-
	+	+	-
<i>Solen marginatus</i>	+	+	78 MPN/100g
	-	+	230 MPN/100g

They showed some interesting findings, even if related to a relatively small number of samples and require some general considerations.

The totality of *Mytilus galloprovincialis* samples were collected by the Italian public veterinary service from class A and B waters according to the Reg. CE 854/2004.

Since the results of our research have shown no correlation between the concentration of *E. coli* and NoV, we think that the detection of the pathogen *E. coli* is insufficient if used as the only parameter to assure the health quality of water and molluscs. That is why it is important to integrate the legislation in force with an adequate viral marker, which must be able to survive in the environment and must be easily detectable by conventional laboratory techniques.

The authors are aware that when a microbial indicator is chosen it has to have biological and biochemical characteristics similar to the target that needs to be evaluated in samples. Moreover the laboratory test used for detection must be sensitive, fast, validated and standardized. Viruses are incapable of replicating in food and they have biological and biochemical features completely different from bacteria. For these reasons, foodborne viruses must be directly detected or indirectly investigated by identifying other viruses with very similar characteristics in the samples (2, 9, 17).

In our study most of the samples positive for NoV detection were negative for *E. coli*, if considering the legal limits required for live edible lamellibranch molluscs produced in class A and B waters. This

result clearly highlights the absence of correlation between bacterial and viral faecal contaminants.

It is interesting to underline that *E. coli* was present in six of the eight samples of *Solen marginatus* tested, and two of them showed counts higher than the legislative limits. We think that this edible mollusc deserves particular attention because it lives wildly in the sand and it is not subjected to any depuration system. For this reason it can present hygienic-sanitary features that may differ from species collected from controlled waters.

Moreover it is useful to highlight that bacterial indicators, unlike viruses, present a higher sensitivity to environmental stress factors and to depuration activity, which leads to much better depuration results when compared to viruses (5, 19).

## Conclusions

No provisions for viral contaminants in edible live lamellibranch molluscs are currently included in the legislation. Moreover, because of the absence of correlation between faecal bacterial indicators and foodborne viruses, it is not possible to evaluate NoV and HAV presence indirectly through *Salmonella* spp. and *E. coli* counts, as shown with the results of our findings. For these reasons we propose the Competent Authorities urgently consider additional measures to update the legislation in force in order to fully control and guarantee the consumer's health from consumption of bivalve molluscs, especially those originating in class A water production areas. In fact, the efficacy of public controls must be a priority for every Sanitarian System, Italian or European, since consumer's health is their common concern. International food safety politics is essentially based on risk analysis, hazard identification and application of strategies to reduce food contamination, and consequently consumers' exposure. The microbiological control of water and bivalve molluscs through bioindicators represents the only instrument we have to achieve the main goal of public health, which is 'safe food'. For this purpose Official Laboratories can be supported by the use of molecular biology techniques that are rapid and efficient screening tests. In fact, according to the European guidelines, real-time PCR is considered by the Italian Ministry of Health the most sensitive method, and it is recommended for virus detection in food (10).

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