**Monitoring and Genotyping of *Norovirus* in Bivalve Molluscan Shellfish from Northern Italian Seas (2018-2020)**

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**Abstract**

*Norovirus* (NoV) is an enteric virus with foodborne transmission. Bivalve shellfish are a main source of infections and outbreaks. In Italy a voluntary based monitoring plan to control the safety of bivalve shellfish was set up at provincial level. This study describes the occurrence and distribution of NoV in the Northern Adriatic Sea and in the Ligurian Sea.

From October 2018 to April 2020, 683 bivalve shellfish samples (n=194 oysters, n=149 mussels, n=280 clams, n=60 other bivalve shellfish) were tested by One-Step RT Real-time PCR for *Norovirus* GI and GII and quantified according to the ISO 15216:2017. Positive samples were further analyzed to determine genotype by sequencing of the ORF1/ORF2 junction of the viral genome.

A total of 124 (18%, CI95%: 15.4 - 21.3) samples were positive for NoV (clams 43%, mussels 27%, oysters 27%, other molluscs 4%) and positive samples were found mainly in the colder season. Of these samples, 88% (CI95%: 80.5 - 92.8) was NoV GII, 53% (CI95%: 44.1 - 62.2) was NoV GI and in 41% (CI95%: 32.5 - 50.3) of samples both genogroups were identified.

Thirty-seven samples were typeable (GI n=12 and GII n=25) with GI samples belonging to four genotypes and GII samples belonging to five genotypes. GII.3 genotype was the most prevalent, followed by GII.4, particularly Sydney 2012 subtype was found in three oysters’ and three clams’ samples and it is a leading cause of infections worldwide. The phylogenetic analysis reveals a high heterogeneity among the species that are scattered in the several clusters.

The overall prevalence of NoV in edible shellfish, particular those to be eaten raw or undercooked, is moderately high.

The presence of genotypes frequently involved in human infections strengthens the need for ongoing monitoring, which should be extended to all provinces involved in shellfish farming.

**Keywords**: *Norovirus*, bivalve molluscan shellfish, food safety, monitoring, genotyping

**1. Introduction**

*Noroviruses* (NoVs), members of the *Caliciviridae* family, are the major responsible of acute viral gastroenteritis (Goodgame, 2006; Pang and Lee, 2015; Savini et al., 2021) and the main causes of foodborne diseases (Koopmans & Duizer, 2004).

NoVs are icosahedral, non-enveloped viruses with a 7.5 kb single-stranded RNA genome with positive polarity and presenting three open reading frames (ORFs) (Dingle et al., 1995). NoV GI, NoV GII and NoV GIV are the main 3 genogroups, among the 10 identified, recognised as a possible cause of the disease in humans (Chhabra et al., 2019; de Graaf et al., 2016).

NoVs are highly infective due to their very low infectious dose and, due to the viral capsid, they can survive for long time in the environment (R. I. Glass et al., 2000). The main transmission route is faecal-oral, followed through ingestion of contaminated food or water; person-to-person transmission should also be considered, especially in semi-closed environments such as schools, cruise ships, hospitals and nursing homes due to the ability of the virus to be transmitted by the vomiting aerosol and fomites (Hall et al., 2012; Randazzo et al., 2018; Verhoef et al., 2015). NoVs infection is self-limiting and the main common symptoms are vomiting, fever, anorexia, and abdominal cramps with a 2 to 6 days convalescence; in rare cases and generally in vulnerable patients it can lead to severe dehydration or death (Calderwood et al., 2022; Glass et al., 2009; Kim et al., 2019; Parrón et al., 2021). Generally, NoVs cases are more common in the winter season (Jiang et al., 1990; Kojima et al., 2002).

The genogroup classification is based on the sequence of the viral protein VP1, the major capsid protein encoded by ORF2, while ORF1 encodes a polyprotein that is proteolytically cleaved into six non-structural proteins and ORF3 encodes the minor capsid protein VP2 (P. J. Glass et al., 2000). More than 40 NoVs genotypes can be typed due to the high nucleotide variability of the RNA-dependent RNA polymerase encoded by ORF1 (Chhabra et al., 2019b). Indeed, Desdouits and colleagues showed that the NoVs recombination occurs frequently at the junction between ORF1 and ORF2 (Desdouits et al., 2020).

NoVs are difficult to isolate and only recently promising results regarding virus growth in human intestinal enteroid monolayer cell cultures have been obtained (Ettayebi et al., 2016). However, this method is not currently applicable routinely. The ISO/TS 15216-2:2013 and ISO 15216-1:2017 standard methods, instead, explain how to concentrate and detect the viral RNA in different food matrices including berries, broad-leaved vegetables, water and molluscs. In 2021, edible shellfish and products were more among the food vehicles implicated in food outbreaks caused by NoV (Anonymous, 2022). Among bivalve shellfish, oysters are a main source of NoVs infection due to their water-filtering activity which leads to the possible accumulation of different pathogens that can infect the consumer if raw eaten (Fusco et al., 2019; Lowther et al., 2018). Indeed, NoVs can survive in aquatic environments, determining the role of bivalve molluscs as one of the main sources of foodborne infection (Terio et al., 2017; Fusco et al., 2019; Purpari et al., 2019).

In Europe, in 2021, NoV were the third most frequently reported agent causing Foodborne outbreaks (Anonymous, 2022). It is known the difficult to estimate the proportion of NoVs contamination in food, in Europe, mainly due to the lack of mandatory surveillance systems and the inability of existing systems to determine the proportion of foodborne diseases. In Italy a voluntary based monitoring plan to control the hygiene of bivalve shellfish was set up to investigate the occurrence and distribution of NoVs in molluscs from different areas of northern Italian sea.

The aim of this study was to investigate the genotypes circulating in Italy, in the different species of molluscs analysed, exploiting the samples collected through the monitoring plan for the years 2018 to 2020.

**2. Materials and Methods**

*2.1. Sampling*

From October 2018 to April 2020, 683 bivalve shellfish samples were collected in the Northern Adriatic Sea and in the Ligurian Sea , according to a non-probabilistic sampling method. The analyzed molluscs were divided in the following categories: clams (*Tapes semidecussatus*, *Tapes philippinarum, Meretrix chione, Venus verrucosa,* and *Venus gallina*; n=280), mussels (*Mytilus galloprovincialis*; n=149), oysters (*Crassostrea gigas,* and *Ostrea edulis*; n=194) and other molluscs (*Donax trunculus*, *Ensis leei,* and *Solen marginatus*; n=60).

The collection was performed in coastal and off-shore farming sites and purification plants and included official (n=446) and self-monitoring (n=237) samples.

*2.2. Virus concentration and RNA extraction*

Virus concentration and RNA extraction from bivalve shellfish were carried out according to ISO/TS 15216-2:2013 and ISO 15216-1:2017 guidelines.

For the virus concentration, the digestive gland of a minimum of 10 individuals (at least 2 grams), were chopped. Afterwards, 10 µL of process control (recombinant Mengovirus MC0 strain, ATCC VR-1597™, 104 viral particle/μL) and 2 mL proteinase-K solution (0.1 mg/mL) were added to each sample to allow the release of the virus from the tissue (Le et al., 2018). Following a 3,000 × g for 5 minutes centrifugation, the supernatant was recovered and used for the subsequent viral nucleic acid extraction step.

The viral RNA was extracted and purified following the instructions of the NucliSENS® miniMAG® kit (bioMérieux, Marcy-l’Etoile - France). RNA extraction efficiencies were calculated according to the ISO/TS 15216-2:2013. The acceptability limit required was 1% of process control virus recovery. The eluted RNA was stored at -80 °C until use.

*2.3. One-step RT Real-time PCR amplification and quantification*

Viral detection was performed by One-step RT Real-time PCR using primer and probes for Mengovirus, NoV GI and NoV GII, as reported by the ISO/TS 15216-2:2013. Positive samples were quantified (detectable virus genome copies per RNA µL) using NoV GI and NoV GII dsDNA as standard curves according to the ISO 15216-1:2017. Reaction conditions were 60 minutes at 55° C, 5 minutes at 95° C, followed by 45 cycles of 15 seconds at 95° C, 1 minute at 60° C and 1 minute at 65° C. The analyses were performed on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA - USA). One µL of external inhibition control (EC RNA) for both NoV GI and NoV GII was used as positive amplification/inhibition control.

*2.4. Genotyping of positive samples*

Positive samples were further analyzed by Sanger sequencing the ORF1/ORF2 junction of the genome to determine the viral genotype or subtype.

*2.4.1. Reverse-trascription and PCR amplifications*

Viral RNA was reverse transcribed (RT) to cDNA. RT was performed on the GeneAmp™ PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA - USA) in a total volume of 40 μL, containing 4.5 μL of viral RNA and 35.5 μL of reaction mixture. The reaction mixture contained 16 μL of dNTPs pool (10 mM), 8 μL of MgCl2 (25mM), 8 μL of 5X Transcriptase Buffer (Invitrogen, Carlsbad, CA - USA), 2 μL of primer (50 μM), 1 μL of RNAse Inhibitor (40 U/μL) (Invitrogen, Carlsbad, CA - USA) and 0.5 μL of M-MLV Reverse Transcriptase (200 U/μL, Promega Corporation, Madison, WI - USA). The primers were respectively G1SKR for NoV GI RT and G2SKR for NoV GII RT Table 1. Each run included a negative and a positive reaction control. RT was carried out at 50 °C for 60 minutes, followed by a denaturation step at 94 °C for 15 minutes.

A first PCR for each viral genogroup was performed in a total volume of 30 µL per sample, containing 15 µL of 2X QuantiNova Probe PCR Master Mix (Qiagen GmbH, Hilden - Germany), 0.3 µL of each specific primer (50 µM) Table 1 and 11.4 µL of DNAse-RNAse-free water (Sigma-Aldrich, St. Louis, Missouri - USA). Three (3) µL of cDNA template were added to the reaction mix.

The PCR reactions were performed on the GeneAmp® PCR System 9700 Thermal Cycler with the following thermal profile: 5 minutes at 95 °C, followed by 40 cycles of 30 seconds at 94 °C, 30 seconds at 50 °C and 1 minute at 72 °C, and a final extension at 72 °C for 5 minutes.

Finally, a semi-nested PCR for each viral genogroup was performed in a total volume of 25 µL, containing 12.5 µL of 2X QuantiNova Probe PCR Master Mix, 0.25 µL of each primer (50 µM) Table 1 and 9.5 µL of DNAse-RNAse-free water. A quantity of 2.5 µL of the first PCR DNA was added to the reaction mix for a total volume of 15 µL. Each run was performed using the same amplification conditions of the first PCR.

The semi-nested PCR products were loaded into 3% agarose gel (Agarose MP, Roche, Basel - Switzerland), stained with EuroSafe Nucleic Acid Stain (EuroClone, Pero, Milan - Italy) in 1X TAE buffer. For the electrophoretic run a 100 bp marker (Invitrogen, Carlsbad, CA - USA) was used. The agarose gel was then observed on the FireReader V10 transilluminator (UVITEC Cambridge, Rugby - UK) to detect the presence of 329 bp fragments for NoV GI and 343 bp fragments for NoV GII.

*2.4.2. Sequencing*

Amplified products were enzymatically purified using the Thermo Scientific™ FastAP™ Thermosensitive Alkaline Phosphatase (1 U/µL; 1 µL/reaction) and the Thermo Scientific Exonuclease I (20 U/µL; 0.5 µL/reaction) (Thermo Fisher Scientific, [Waltham](https://www.google.com/search?sxsrf=ALeKk01YCfEFdK7VS7fg8Jr_Vfy6Ep2Wdw:1598446853648&q=Waltham&stick=H4sIAAAAAAAAAOPgE-LSz9U3MCooMTBJU-IAsTOqjE21tLKTrfTzi9IT8zKrEksy8_NQOFYZqYkphaWJRSWpRcWLWNnDE3NKMhJzd7AyAgDThZNCUQAAAA&sa=X&ved=2ahUKEwjDrZ3H9rjrAhVE3KQKHRmQBqoQmxMoATATegQIDhAD), MA - USA). The samples were incubated at 37 °C for 15 minutes and at 85 °C for 15 minutes and then sequenced in both directions: the forward and reverse sequence reactions were prepared using the primers shown in Table 1 for semi-nested PCR on the GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA - USA). Each reaction was performed in a total volume of 10 µL, containing 2 µL of 2.5 X Big Dye Terminator Reaction Mix, 1 µL of 5X Big Dye Terminator Sequencing Buffer, 2 µL of 1.6 µM primer and 4 µL of DNAse-RNAse-free water. One µL of purified product was added to each reaction. The samples were then incubated at 96 °C for 90 seconds and then amplified for 25 cycles at 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes.

Sequence reaction products were purified using the BigDye XTerminator® Purification Kit (Thermo Fisher Scientific, [Waltham](https://www.google.com/search?sxsrf=ALeKk01YCfEFdK7VS7fg8Jr_Vfy6Ep2Wdw:1598446853648&q=Waltham&stick=H4sIAAAAAAAAAOPgE-LSz9U3MCooMTBJU-IAsTOqjE21tLKTrfTzi9IT8zKrEksy8_NQOFYZqYkphaWJRSWpRcWLWNnDE3NKMhJzd7AyAgDThZNCUQAAAA&sa=X&ved=2ahUKEwjDrZ3H9rjrAhVE3KQKHRmQBqoQmxMoATATegQIDhAD), MA - USA), according to the manufacturer’s instructions. Samples were finally typed on SeqStudio Genetic Analyzer (Applied Biosystem Inc., Foster City, CA - USA).

The sequence alignments were generated with the MEGA6 software (Tamura et al., 2013). The phylogenetic tree was inferred using the Neighbor-Joining method (Saitou & Nei, 1987) and evolutionary analyses were conducted in MEGA6 software.

The obtained sequences were compared with reference sequences from the NoroNET database (Chhabra et al., 2019b) and submitted to NCBI GenBank with the following accession numbers: OP587228 to OP587239 for NoV GI, and OP588720 to OP58744 for NoV GII.

*2.5. Statistical analysis*

Considering the non-probabilistic nature of sampling in this study, a data analysis based on standard errors (such as p-values and confidence intervals) was not considered appropriate. In addition, the significant/non-significant dichotomy based on a predetermined p-value cut-off for the interpretation of results is under reconsideration by the biomedical scientific community, as also expressed by the American Statistical Association (ASA), which has cautioned the use and interpretation of the p-value.

In light of this, we focused on the extent of the estimation uncertainty according to a Beta-Binomial Bayesian Model :

Where **Y** , the number of positive sample in a fixed number ***n*** of independent sample follow a Binomial distribution with probability **π** of being positive to NoV. **π** follow a Beta distribution with α and β shape hyperparameters.

Given the observed data ( y positive in n sample) , according to Bayes’ theorem, the posterior distribution of **π** is a conjugate prior for corresponding Bin(n, π) data model:

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where π is the probability of being NoV-positive; x is the number of positive samples; n is the number of tested samples and α and β are the hyperparameters of the a priori beta distribution of π.

Considering that from previous similar studies the probability of NoV positive in shellfish samples range from 0.083 to 91.87 in which most of studies have small sample size and wide uncertainty (fig.x) ( except for Green et al. study that sampled 2500 oysters), we choose the uninformative Jeffreys prior as Pior model distribution of π, with α and β = 0.5 .

Credible intervals was then constructed from the posterior distribution, which collected the highest probability of density corresponding to 95% of the estimated probability (π) values. The point and interval estimates of the NoV prevalence were calculated using the function “binom.bayes()” of the binom package in R language. The forestplot package in R was used to obtain a forest plot, where for each year period ( cold and mild) , the results for season and category of samples are presented.

Moreover, the number of NoV positive shelllife from similar studies were retrieved, and consequently, the respective point and interval estimates of probability with 95% credibility interval were with same Beta binomial model to better describe and contextualise our results.

~~Statistical analysis was carried out using R software (3.5.2. version).~~

~~The prevalence was calculated as proportion between positive samples on total samples and was expressed in percentage values.~~

~~The prevalence and the 95% confidence interval (95%CI) were calculated with the One Sample Proportions test with continuity correction.~~

~~Pearson’s Chi-squared test with Yates’ continuity correction was used to test differences between positive samples found in different seasons and between positive samples based on the category.~~

~~Moreover, Fisher’s Exact test was used to assess the prevalence of positive samples collected in different sampling areas.~~

**3. Results and discussion**

A total of 124 out of 684 samples (18%, CI95%: 15.4 - were positive for NoV.

Within the specific tested categories, clams, oysters, and mussels had 19% (53/280, CI95%: 14.6 - 24.1),

17% (33/194, CI95%: 12.1 - 23.2) and

22% (33/149, CI95%: 15.9 - 29.8) positivity respectively,

while the other grouped molluscs species revealed a lower observed contamination with 8% (5/60, CI95%: 3.1 - 19.1) prevalence.

~~However, statistical analyses did not show significant differences between categories positivity.~~

The consumption of oysters could however lead to an increased risk of NoV infection, respect to the other molluscs; indeed, even if the virus would not resist proper cooking, oysters are often eaten raw or undercooked, and this, especially in certain European regions (i.e. France) (Le Guyader et al., 2012).

~~Focusing on positive samples (124 samples), 88% (CI95%: 80.5 -92.8) was NoV GII (n=109), 53% (CI95%: 44.1 – 62.2) was NoV GI (n=66) and in 51 samples both genogroups were identified (41%, CI95%: 32.5 – 50.3).~~

The identification of NoV GII as the prevalent genotype is in accordance to the global distribution of NoV GII amongst the human population (Qi et al., 2018) and to an Italian survey from 2014 to 2019 on different food matrices in Italy (Pavoni et al., 2022).

The mean contamination level for NoV GI was 3×103 gc/µL and ranged 101-105 gc/µL; the mean contamination for NoV GII was 2×103 gc/µL and ranged 100 - 104 gc/µL.

The contamination levels calculated, are in agreement with a previous study, in which NoV GI is more efficiently concentrated than NoV GII and high viral titer is often found in oyster and mussel samples (Dirks et al., 2021).

The positive samples were found mainly in the colder seasons with 52% (CI95%: 43.3 - 61.4) of them found in winter and 40% (CI95%: 30.9 - 48.7) in autumn as also reported in other studies (M. S. Kim et al., 2016; Pavoni et al., 2022; Verhoef et al., 2008); while 5% (CI95%: 1.9 - 10.7) were found in summer and 3% (CI95%: 1.0 - 8.5) in spring.

This positive rate difference ?? in cold seasons (autumn and winter) respect to mild seasons (spring and summer) was statistically significant (*p*=2.24×e-11). ????

Indeed, the positive samples’ prevalence, compared to the total of analyzed samples,

was 29% (CI95%: 22.8 - 34.9) in winter and

22% (CI95%: 17.0 - 28.3) in autumn,

while only 5% (CI95%: 1.7 - 13.8) in spring and

4% (CI95%: 1.5 - 8.4) in summer. ????

The odds ratio was 7.62% (CI95%: 3.9 - 14.9) in the cold seasons compared to the mild ones Table 2.

Indeed, clear seasonal peaks occur usually in cooler winter months. The majority of all outbreaks, in particular, occur from November to April in the boreal emisphere, and from May to September in the austral one. However, in tropical countries, *Norovirus* may be less seasonal (Organization et al., 2008).

Despite having incomplete data available on the distance of the farms from the coast, it was observed a higher prevalence (*p*=0.049) in molluscs harvested from farms located 3-6 nautical miles offshore (73%, CI95%: 39.3 - 92.6), respect to those collected closer to the coast (0-3 nautical miles) (38%, CI95%: 28.6 - 48.9).

These findings suggest that the higher percentage of positive samples collected 3-6 miles from the coast may be due to the fitness of NoVs to survive at the lower temperatures of the open sea compared to the estuaries’ water with higher temperature. Indeed, it seems that virions in coastal water are more likely to be degraded when filtered by molluscs (Kauppinen & Miettinen, 2017). Moreover, the low solar irradiation in deep water, may avoid the virion to be damaged (Lopman et al., 2009).

Thirty-seven (37) samples were genotyped (NoV GI n=12 and NoV GII n=25), with NoV GI samples belonging to four genotypes, GI.1 n=6 (OP587230, OP587231, OP587235, OP587236, OP587238, OP587239), GI.3 n=1 (OP587232), GI.4 n=3 (OP58728, OP587233, OP587237), and GI.5 n=2 (OP587229, OP587234). NoV GII samples were grouped into five genotypes: GII.2 n=3 (OP588725, OP588743, OP588724), GII.3 n=8 (OP588723, OP588727, OP588728, OP588729, OP588730, OP588732, OP588734, OP588744) GII.4 n=7 (OP588726, OP588733, OP588735, OP588737, OP588739, OP588740, OP588741) GII.6 n=3 (OP588720, OP588721, OP588738 and GII.13 n=4 (OP588722, OP588731, OP588736, OP588742) Table 3. GII.3 was the most prevalent genogroup (8/25), followed by GII.4 (7/25), in particular Sydney 2012 subtype (found in 3 oysters and 3 clams).

The phylogenetic analysis of NoV GI and NoV GII was performed on sequences obtained from the 37 positive samples, highlighted the presence of several NoV genotypes in shellfish Table 3***.***

GII.3 genotype was the most prevalent in this study (eight samples) followed by GII.4 (seven samples), with six Sydney 2012 subtypes: both these genotypes are involved in human infections globally (Cannon et al., 2017; Ennuschat et al., 2021; Zhou et al., 2020). These samples come from Adriatic Sea and were mainly (12/15) collected in 2019, in particular during winter months. The phylogenetic trees in Figure 1 and 2 reveals a high heterogeneity among the species that are scattered in the several clusters.

The NoVs typing allows to obtain essential information for epidemiological purposes, and the comparison of sequences permits to determine the evolutionary relationship between genomes. However, the relatively low concentrations found in this study, did not allow for the genotyping of many samples (87/124). This data could be explained by the inhibition given by the complex matrix or by the low starting concentration in the samples. Moreover, currently, there are no rapid enrichment techniques to be exploited in the virus concentration phase; therefore, there is a potential loss of epidemiological information.

**5. Conclusions**

The overall prevalence of NoV in edible shellfish was 18% (CI95%: 15.4 - 21.3).

Given the high proportion of positive samples (27%, CI95%: 15.0 - 30.3), oysters, which are often eaten raw, represent the main risk of infection regardless the origin. ?? il rischio è circa il 5% ( 0.28\*0.17)

No difference in contamination was highlighted among the different species or different sampling sites.

Subtype GII.4 Sydney2012 is one of the most prevalent variants found as a leading cause of human infections worldwide, despite it does not the most prevalent in this study.

The presence of genotypes frequently involved in human infections strengthens the need for ongoing monitoring, in order to evaluate which are the shellfish most involved in *Norovirus* infections with the aim of ensuring a greater food safety, supporting by the European data of foodborne outbreaks caused by NoV, that increased in 2021 compared with 2020(Anonymous, 2022).

Further, the Next Generation Sequencing (NGS) approach would be essential for the characterization of complete genome of microorganism and to identify emerging variants and to understand NoVs evolution, involved in human diseases.

**Author Contributions**

**Author 1**: Data curation (equal); formal analysis (equal); investigation (equal); writing—original draft (lead); writing—review and editing (equal). **Author 2**: Data curation (equal); formal analysis (equal); investigation (equal); writing—review and editing (equal). **Author 3**: Data curation (equal); formal analysis (equal); investigation (equal); supervision (equal) writing—review and editing (equal). **Author 4**: writing—review and editing (equal). **Author 5**: Data curation (equal); formal analysis (equal); investigation (equal); writing—review and editing (equal). **Author 6**: Data curation (equal); formal analysis (equal); investigation (equal); writing—review and editing (equal). **Author 7**: Resources (equal); writing—review and editing (equal). **Author 8**: Conceptualization (lead); methodology (equal); project administration (lead); resources (equal); supervision (equal); writing—review and editing (equal). **Author 9**: Conceptualization (supporting); Data curation (equal); methodology (equal); supervision (equal); writing—review and editing (equal).

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**Table 1.** Primers set used for the Norovirus GI and Norovirus GII reverse-trascription and amplification. The reaction in which primers were used is specified in brackets.

|  |  |  |
| --- | --- | --- |
|  | **Direction and reaction** | **Sequence (5’ - 3’)** |
| ***Norovirus* GI** |  |  |
| COG1F | Forward-outer (first PCR) | CGYTGGATGCGNTTYCATGA (Kageyama et al., 2003) |
| G1SKR | Reverse (RT, first PCR and semi-nested PCR) | CCAACCCARCCATTRTACA (Kojima et al., 2002) |
| G1SKF | Forward-inner (semi-nested PCR) | CTGCCCGAATTYGTAAATGA (Kojima et al., 2002) |
| ***Norovirus* GII** |  |  |
| COG2F | Forward-outer (first PCR) | CARGARBCNATGTTYAGRTGGATGAG (Kageyama et al., 2003) |
| G2SKR | Reverse (RT, first PCR and semi-nested PCR) | CCRCCNGCATRHCCRTTRTACAT (Kojima et al., 2002) |
| G2SKF | Forward-inner (semi-nested PCR) | CNTGGGAGGGCGATCGCAA (Kojima et al., 2002) |

**Table 2.** Norovirus prevalence and 95% confidence intervals (95% CI) according to sampling seasons

|  |  |  |  |
| --- | --- | --- | --- |
| **Season** | **N° samples** | **N° positive samples** | **Prevalence** |
| *Autumn* | 221 | 49 | 22% (CI95%: 17.0 - 28.3) |
| *Winter* | 228 | 65 | 29% (CI95%: 22.8 - 34.9) |
| *Spring* | 75 | 4 | 5% (CI95%: 1.7 - 13.8) |
| *Summer* | 159 | 6 | 4% (CI95%: 1.5 - 8.4) |
| Total | 683 | 124 |  |

**Table 3.** Norovirus genotypes (n=37) and subtypes detected by species.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Species** | **Sampling year** | **Sampling area** | ***Norovirus* GI genotype** | ***Norovirus* GII genotype** | ***Norovirus* GII**  **subtype** |
| Tapes semidecussatus | 2018 | Adriatic Sea | GI.4 |  |  |
| Tapes semidecussatus | 2018 | Adriatic Sea |  | GII.6 |  |
| Tapes semidecussatus | 2018 | Adriatic Sea | GI.5 |  |  |
| Tapes semidecussatus | 2018 | Adriatic Sea | GI.1 | GII.3 |  |
| Tapes semidecussatus | 2019 | Adriatic Sea |  | GII.3 |  |
| Tapes semidecussatus | 2019 | Adriatic Sea |  | GII.4 | Sidney\_2012 |
| Tapes semidecussatus | 2019 | Adriatic Sea |  | GII.4 | Sidney\_2012 |
| Tapes semidecussatus | 2019 | Adriatic Sea |  | GII.3 |  |
| Tapes semidecussatus | 2019 | Adriatic Sea |  | GII.3 |  |
| Tapes semidecussatus | 2019 | Adriatic Sea |  | GII.4 | Sidney\_2012 |
| Tapes semidecussatus | 2020 | Adriatic Sea | GI.1 |  |  |
| Tapes semidecussatus | 2020 | Adriatic Sea |  | GII.3 |  |
| Tapes semidecussatus | 2020 | Adriatic Sea |  | GII.2 |  |
| Crassostrea gigas | 2018 | Adriatic Sea |  | GII.6 |  |
| Crassostrea gigas | 2019 | Adriatic Sea | GI.5 |  |  |
| Crassostrea gigas | 2019 | Adriatic Sea |  | GII.4 | Sidney\_2012 |
| Crassostrea gigas | 2019 | Adriatic Sea |  | GII.13 |  |
| Crassostrea gigas | 2019 | Adriatic Sea | GI.1 | GII.4 | Sidney\_2012 |
| Crassostrea gigas | 2019 | Adriatic Sea | GI.1 | GII.6 |  |
| Crassostrea gigas | 2019 | Adriatic Sea | GI.4 | GII.4 | Sidney\_2012 |
| Crassostrea gigas | 2019 | Adriatic Sea |  | GII.3 |  |
| Crassostrea gigas | 2019 | Adriatic Sea |  | GII.3 |  |
| Crassostrea gigas | 2020 | Adriatic Sea | GI.1 |  |  |
| Mytilus galloprovincialis | 2018 | Adriatic Sea |  | GII.13 |  |
| Mytilus galloprovincialis | 2018 | Adriatic Sea | GI.1 | GII.2 |  |
| Mytilus galloprovincialis | 2018 | Adriatic Sea |  | GII.2 |  |
| Mytilus galloprovincialis | 2018 | Adriatic Sea |  | GII.4 |  |
| Mytilus galloprovincialis | 2019 | Ligurian Sea |  | GII.13 |  |
| Mytilus galloprovincialis | 2019 | Ligurian Sea | GI.3 | GII.3 |  |
| Mytilus galloprovincialis | 2020 | Adriatic Sea |  | GII.13 |  |
| Other molluscs | 2019 | Adriatic Sea | GI.4 |  |  |