Human enteric viruses' detection in mussels (*Mytilus galloprovincialis*) farmed in the central Adriatic Sea

Gianluigi Ferri, Vincenzo Olivieri, Alberto Vergara

Department of Veterinary Medicine, Post-Graduate Specialization School in Food Inspection "G. Tiecco", University of Teramo, Piano d'Accio, Italy

Correspondence: Gianluigi Ferri, Department of Veterinary Medicine, Post-Graduate Specialization School in Food Inspection "G. Tiecco", University of Teramo, Strada Provinciale 18, 64100, Piano d'Accio, Teramo, Italy.
Tel.: +39 0861266886. E-mail: gferri@unite.it

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Abstract

Human enteric viruses, such as hepatitis A virus (HAV), hepatitis E virus (HEV), and norovirus genogroups I and II (NoVGI and NoVGII), cause infections, and it has been largely demonstrated that mussels play an important role if consumed as raw or undercooked food matrices. This study aimed to investigate, through qualitative and quantitative biomolecular assays, the detection of partial genomic regions belonging to the most relevant enteropathogenic viruses for humans (HAV, HEV, NoVGI and NoVGII) in mussels (*Mytilus galloprovincialis*) farmed along the coasts of two Italian regions on the central Adriatic Sea: Abruzzo (Casalbordino, Chieti) and Molise (Termoli, Campobasso). A total of 425 animals were sampled, and the respective georeferentiations were registered. A total of 85 pools, each composed of five subjects/aliquots, were formed (22 from Abruzzo and 63 from Molise regions). This step was followed by homogenization and RNA extraction, and then the biomolecular assays [nested reverse transcription polymerase chain reaction (PCR) and real-time reverse transcription-quantitative PCR] were performed. 1.17% of the pool was positive for HAV RNA detection (10^2 copies/mL), 9.41% for HEV (10^2-10^3 copies/µL), 2.35% for NoVGI (10^1 copies/µL), and no pool was positive for NoVGII. This study demonstrated the human enteric viruses' presence in mussels farmed in a low-investigated marine area. Based on a one-health point of view, this paper aims to enforce the importance of biomolecular and epidemiological screenings as surveillance systems to guarantee human, animal, and environmental health.

Introduction

Bivalve mussels, as *Mytilus galloprovincialis*, represent environmental sentinels for chemical (*i.e.*, polycyclic aromatic hydrocarbons, *etc*.) and microbiological parameters (*i.e*., viruses, bacteria, *etc*.) (Fiorito *et al.*, 2019). Human viral infections, caused by contaminated food ingestions (also including shellfish-borne enteric disease), are related to the bio-concentration phenomenon typical of lamellibranch tissues (Rowan, 2023). Different from the bacterial *noxae* (*i.e*., *Escherichia coli*, *etc*.), enteric viruses preserve high infectious titers among tissues for several days (Razafimahefa *et al.*, 2020). The European Food Safety Authority and the U.S. Food and Drug Administration classified the following enteric viruses as the main ones responsible for thousands of infectious outbreaks in the world: hepatitis A virus (HAV), hepatitis E virus (HEV), and norovirus (NoV) genogroups I and II (NoVGI and NoVGII) (Xu *et al.*, 2022). These pathogens are linked by many aspects: transmission route (orofecal pathway), ribonucleic acid as genome, and notable environmental resistance and persistence (Fenaux *et al.*, 2019; Farkas *et al.*, 2021; Pisano *et al.*, 2021). In the last 10 years, infections have increased, but viral pathogens are

still not included in the European legislation for food safety, with special regard referring to the so-called food safety criteria and the hygiene processes ones, mentioned by Regulation No. 2073/2005 as amended (European Commission, 2005). The lack of established legal cut-offs has induced many researchers to perform biomolecular investigations trying to give scientific data and evidence to provide scientific barriers of knowledge able to control these sanitary concerns (da Silva *et al.*, 2023). On the European continent, epidemiological studies have amplified NoV, HAV, and HEV by different bivalve species, including *Mytilus* spp. (Tan *et al.*, 2021). The above-mentioned viral pathogens have been majorly studied from mussels (*M. galloprovincialis*) in the southern Italian regions describing different prevalence values regarding their circulation: *i.e.*, Campania (HAV: 8.9%, HEV: not detected, NoVGI: 10.8%, NoVGII: 39.7%) (Fusco *et al.*, 2019) and Sicily (HAV: 13.0%, HEV: 0.9%, NoVGI: 2.9%, NoVGII: 21.7%) (Purpari *et al.*, 2019; Macaluso *et al.*, 2021). Studies have been also performed in the northern Adriatic Sea detecting both NoVGI and NoVGII (prevalence data: 40.0%) by Mangeri *et al*. (2024), and 47.8% of NoVGI and NoVGII from *Crassostrea* spp. samples by Suffredini *et al*. (2014). This study aimed to screen, using qualitative and quantitative biomolecular methods, the circulation of human enteric viruses (HAV, HEV, NoVGI, and NoVGII) from mussels (belonging to the *M. galloprovincialis* species) farmed in the central Adriatic Sea: along the coasts of the Abruzzo and Molise regions (Italy). This marine area is not much investigated for the shellfish-borne enteric viral pathogen detection. Indeed, in the Molise region, Savini *et al.* (2009) observed prevalence values of NoVGI RNA detection as follows: 1.7% from mussels and 2.9% from calms. For this purpose, the present paper wants to contribute by providing original and recent epidemiological data on the circulation of genetic fragments (RNA) belonging to the human enteric viruses responsible for foodborne zoonotic infections (*i.e.*, HAV, HEV, NoVGI, and NoVGII). Although polymerase chain reaction (PCR) assays present limitations to the distinction between infectious and inactivated viruses, they still allow the monitoring of the main circulating genotypes and subtypes, characterized by different morbidity and mortality, in a specific geographical area, enforcing the strategic role of biomolecular screenings, which represent the substantial basis of studies for further disciplines such as risk analysis in public health.

Materials and Methods

Sample collection and processing

The sampling activities lasted from March to May 2023, and a total of 425 mussels, belonging to the *M. galloprovincialis* species, were collected from two marine farms, as illustrated in Figure 1. These are respectively located along the coasts of the Abruzzo region (Casalbordino, Chieti province) named F1, and the Molise region named F2 (Termoli, Campobasso province). The georeferencing data are schematically represented by Table 1.

All screened animals were collected from marine waters classified as A category, in agreement with the legal parameters reported by Regulation 853/2004 (European Parliament and Council of the European Union, 2004), and relative official controls (European Commission, 2019a). After collection from mussel farms, all mussels were directly conferred to the laboratories, and they were not involved in the purification processes. For these purposes, to avoid RNA molecule degradation, samples were stored at -80°C until their processing.

The gastrointestinal gland (or hepatopancreas) was the target organ, which was sterilely collected from all animals; indeed, the applied procedures followed the International Standards for viral pathogen detection: ISO 15216-1 (2017), as performed by La Rosa *et al.* (2018). More specifically, hepatopancreas tissues were sterilely collected using mono-usage scalpels (Monopec Scalpels, Thermo Fisher Scientific™, Waltham, MA, USA). The cited target organs were organized into pools composed by 5 animals/vial/farm. Therefore, starting from a total of 425 animals, the obtained pools were 85: 22 from Abruzzo and 63 from Molise regions. After sectioning procedures, pools were homogenized using the T18 digital Ultra-Turrax® (Staufen, Germany) for 2 minutes. This last step was followed by the centrifugation process: 4000 g for 20 minutes at a controlled temperature (+4°C); successively, pools were filtered, obtaining final volumes of 5 mL, stored at -80°C until RNA extraction and molecular assays, as methodically described by Szabo *et al.* (2015).

RNA extraction, real-time polymerase chain reaction, and nested reverse transcription polymerase chain reaction assays

After homogenization, the pools were used for the RNA extraction procedures, which were performed following the TRIzol

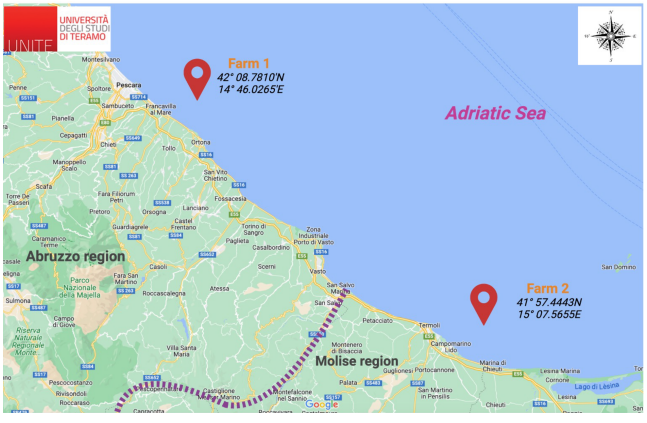

Figure 1. Geographical distribution of the screened farms. This map was reproduced using Google Maps.

Table 1. Mariculture farms: georeferencing and distance between sampling points.

Farms	Referring city for samples collections	Farms georeferencing	Distribution along coasts
F1	Casalbordino	42° 08.7810' N 14° 46.0265' E	35 Km
F2	Termoli	41° 57.4443' N 15° 07.5655' E	25 Km

F1, Casalbordino (CH) farm; F2, Termoli (CB) farm.

LS method (Invitrogen, Ltd., Paisley, UK). The obtained final pellets were resuspended in 50 µL of RNase free water (Invitrogen UltraPure DNase/RNase-Free Distilled Water, ThermoFisher™, Waltham, MA, USA) and stored at -80°C till biomolecular screenings. The one-step real-time PCR was performed using the GENE UP® System (bioMérieux, Paris, France). HAV, HEV, NoVGI, and NoVGII detections were performed using specific reaction kits (ceeramTools, Thermo Fisher Scientific™, Waltham, MA, USA). According to the manufacturer's instructions, the final reaction volumes were performed in 25 µL, which were composed of 20 µL of reagents and 5 µL of extracted RNA. Positive and negative controls were also included in the commercial kits. The standard curves, for each screened viral pathogen, were realized by performing ten-fold dilutions starting from an initial concentration of 10^6 copies/µL (ceeramTools, Thermo Fisher Scientific™, Waltham, MA, USA) to 10^1 copies/µL. The fluorescence channel parameters associated with each well were 520 nm. Thermocycler setting GENE UP® System (bioMérieux, Paris, France) was the same for HAV, HEV, NoVGI, and NoVGII, as indicated by manufacturers (ceeramTools, Thermo Fisher Scientific™, Waltham, MA, USA). The amplification program was divided into three steps: the first part was RNA reverse transcription performed at 45°C for 10 minutes, the second one was 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. After quantitative biomolecular assays, the nested reverse transcription PCR (RT-PCR) tests were performed. The reverse transcription (step one) included the usage of the Qiagen® OneStep RT-PCR Kit (Hilden, Germany), and the second one (step two) was done with the Green Master Mix Promega® (Madison, WI, USA), as described by the manufacturer instructions. The final reaction volumes, in the two reactions, were 25 µL. For each pathogen, primers and thermocycler settings were performed in accordance with the respective references: HAV (Taffon *et al.*, 2011), HEV (Wang *et al.*, 1999; Johne *et al.*, 2010), NoVGI and NoVGII (Yan *et al.*, 2003). At the end of each qualitative PCR assay, amplicons were loaded onto wells of agarose gels (at different concentrations 1.5-2-0% depending on their sizes), and their respective seizures were compared with specific DNA ladders (Genetics, FastGene^{Ò} 50 bp or 100 bp DNA Marker). The suspected positive amplified bands were successively purified using commercial kits Qiagen QIAquick® PCR Purification Kit (Hilden, Germany), and the Sanger sequencing was performed in collaboration with BioFab Research (Rome, Italy). The BLAST system (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch) was used for the nucleotide similarity evaluations. The suspected positive HEV RNA sequences were also loaded and analyzed using the HEV-typing tool (https://www.rivm.nl/mpf/typingtool/hev/).

Statistical analysis

The IBM® SPSS 20.0 Software (SPSS, Chicago, IL, USA) was used to perform the statistical analysis. The dependent variables "HAV", "HEV", "NoVGI", and "NoVGII" were analyzed by performing a two-tailed paired *t*-test, comparing the discovered pathogen amounts (copies/µL)/geographical area. The Shapiro-Wilk test was used to evaluate the normality assumption, and the α was <0.05. For each calculated percentage, when applicable, the confidential intervals (CI) at 95% were calculated.

Results

The molecular biology results showed a general viral RNA detection of 11/85 or 12.94% (CI 95%: 5.81-20.07%) among the screened pools. It is mandatory to affirm that pools were positive for the detection of one of the screened pathogens; no coexisting viral genome fragments, belonging to the different above-mentioned pathogens, were parallelly amplified in the same specimen.

More in detail, HAV RNA fragments were amplified from 1/85 pools (1.17% CI 95%: 0.02-3.45%) collected from F2. The real-time RT-quantitative PCR discovered 10^2 copies/µL. Concerning HEV, its sequence fragments were amplified from 8/85 pools representing 9.41% (CI 95%: 3.21-15.61%). From a geographical perspective, 6/22 or 27.27% (CI 95%: 8.66-45.88%) were discovered from F1 showing 10^2 copies/µL/positive pool in the Abruzzo region. Two out of 63 specimens (3.17% CI 95%: 0.02-7.49%), belonging to the F2, showed an average amount of 10^3 copies/µL/positive pool. Comparing the results obtained from the two screened areas and the amplified HEV RNA copies/µL, a statistically significant difference was observed with $p<0.001$.

Two out of 85 pools, which represent 2.35% (CI 95%: 0.07-4.63%), harbored NoVGI nucleotide fragments. More specifically, both positive ones were collected from F2 being 2/63 pools or 3.17% (CI 95%: 0.02-7.49%), presenting an amount of 10^1 copies/µL/positive pool. The F1 samples were all negative for NoVGI RNA detection. Concerning the NoVGII, all samples, coming from F1 and F2, were negative for their detections.

From a geographical perspective, F2 resulted significantly and statistically different from F1 ($p<0.001$) if compared based on the genetic copy detections, expressed as copies/µL. Indeed, three of the screened viral pathogens (HAV, HEV, and NoVGI) were discovered from F2, and in F1, only HEV RNA was amplified.

The nucleotide similarities, performed using the BLASTN system, demonstrated high identities (nt. identity over 98.0%) with the screened pathogens HAV, HEV, NoVGI and NoVGII. More in detail, the HEV-typing tool permitted to identify the genotype 3c as the unique involved.

Discussion

The human enteric viruses represent consistent one-health concerns with high repercussions on human, animal, and environmental medicines. In the last decades, HAV, HEV, NoVGI and NoVGII RNA fragments have been largely amplified from many animal productions, with special regard to the mussels (*Mytilus* spp.) (Farkas *et al.*, 2021). The bioaccumulation process, of chemical and microbiological particles observed in bivalve lamellibranch species, allows us to consider them as environmental sentinels useful for monitoring activities of marine ecosystems (Chahouri *et al.*, 2022). In the European continent, and more specifically in Italy, the viral pathogen circulations in mussels (*Mytilus* spp.) have been lowly investigated. In the scientific literature, few studies were performed on amplified genetic fragments belonging to NoVGI and NoVGII in the Terranean Sea, with the following prevalence data: 18% and 43%, respectively (Fiorito *et al.*, 2019). Fusco *et al.* (2019) observed the following percentages: HAV in 8.9% of screened shellfish, NoVGI in 10.8%, and NoVGII in 39.7%. In the Mediterranean Sea, and more specifically along the Sicilian coasts, the following prevalence was reported by many authors: La Rosa *et al.* (2018) amplified part of the HEV genome from 2.6% of collected mussels; Purpari *et al.* (2019) observed HAV (13%), NoVGI

(18.5%) and HEV (0.9%); and finally Macaluso *et al.* (2021) reported an average value (5.56%) of parallel detection of two or more viral pathogens (HAV, HEV, NoVGI, NoVGII, adenovirus, rotavirus, and mengovirus). The Adriatic Sea was also screened with special regard to the coasts located in front of the Apulia region, presenting the following prevalence data: 1.6% NoVGI, 12.2% NoVGII, and HAV not detected (La Bella *et al*., 2017). In the northern Adriatic Sea and Ligurian one, NoVGI and NoVGII were observed in 40.0% of the screened mussel samples (Mangeri *et al*., 2024) and a similar result (47.8%) of NoVGI and NoVGII (amplified from *Crassostrea* spp.) was previously reported by Suffredini *et al*. (2014).

The present study wanted to screen, performing qualitative and quantitative molecular assays, the viral nucleotide fragment detection belonging to the genomes of the most frequently amplified entero-viruses: HAV, HEV, and NoVGI-GII. The sampled species, *M. galloprovincialis*, was collected from two marine farms (named F1 and F2) located respectively in the Abruzzo and Molise regions. The tested organs were 425 gastro-enteric glands (hepatopancreas) organized into 85 pools.

Among the screened pathogens, HEV RNA was mostly amplified, if compared with the other ones, in both marine farms, representing 9.41% (CI 95%: 3.21-15.61%) of the screened specimens. The obtained prevalence was higher than the 2.60% observed in Sicily by La Rosa *et al.* (2018) and the 0.89% observed in Apulia by La Bella *et al.* (2021). The virus was not detected in Campania (Fusco *et al.*, 2019). In Apulia, Campania, and Sicily, mussels were farmed in mariculture farms with waters classified as B category. The scientific explanation of the obtained geographical epidemiological data in the present study was related to the marine environmental characteristics of the Adriatic Sea, *i.e*., different marine water microbiological quality (classified as A quality in the Abruzzo and Molise regions, in agreement with Regulation 853/2004) (European Parliament and Council of the European Union, 2004; Croci *et al.*, 2007; Scarponi *et al.*, 2022). Furthermore, the peculiarity of marine currents, in this part of the central Adriatic Sea, provides favorable conditions for microbial accumulation (Croci *et al*., 2007; Errani *et al*., 2021). The consistent anthropization impact (industrialization processes and tourism) determines the crucial environmental release of viral and bacterial pathogens (Stagličić *et al.*, 2021; Scarponi *et al.*, 2022). Different from these studies, the present analyses were performed in the spring season rather than the winter one, as described in many papers (La Bella *et al.*, 2017; La Rosa *et al.*, 2018; Fusco *et al.*, 2019). In agreement with the previous-cited studies (La Bella *et al.*, 2017; La Rosa *et al.*, 2018; Fusco *et al.*, 2019), in all cases, genotype 3 was involved. The sequences' analyses discovered high nucleotide similarities (nt. id. 100%) with the last ones amplified from wild boars in the Marche region, and more specifically to the MN20202101 registered on GenBank by Ferri *et al.* (2023). Based on its structural characteristics, HEV, being a quasi-enveloped single-strand RNA virus, has demonstrated prolonged environmental resistance, maintaining its infectivity for up to 30 days at 20°C in the marine water environment too (Purdy *et al.*, 2017). Following these reasonings, it has been observed that lamellibranch mussels can concentrate virions, in agreement with the bioaccumulation phenomenon, from the water filtration physiological process (Fusco *et al.*, 2019).

In the present study, the real-time assays amplified an average value of 10^2 copies/µL in Abruzzo and 10^3 copies/µL in Molise. Although it has been discovered that the infectious dose that can induce hepatitis corresponds to the infectious dose (ID50) by the oral-fecal route in humans, which would seem to be at least $10^{5.5}$ copies/µL(quantified by the real-time PCR assays) (La Rosa *et al.*, 2018; Fusco *et al.*, 2019; Macaluso *et al.*, 2021), in the present scientific investigation, this limit was not gained. However, immune-depressed human patients can be easily infected by low dosages (10^2 copies/mL) showing the typical clinical symptoms (*i.e*., nausea, diarrhea, *etc*.) and, in any severe cases, multifocal necrotic lesions, as reported by Van der Poel *et al.* (2018). Based on these observations, more specific and restrictive cut-offs should be introduced by the European legislator to guarantee safe products for all final consumers (Purdy *et al.*, 2017).

Concerning HAV, a fragment belonging to its genome was amplified from 1/85 pools, presenting a percentage value of 1.17% (CI 95%: 0.02-3.45%), which were collected from F2. The quantitative assay showed an amount of 10^2 copies/µL. Comparing the obtained data to analogous studies, this result was lower than the other ones, performed in the Adriatic Sea, 6.67% (Errani *et al.*, 2021) and 6.0% (Croci *et al.*, 2007) along the coasts of the Emilia Romagna region. This mentioned difference was mainly justified by the different microbiological water quality where in Emilia Romagna, marine farm waters were classified as B category, following the European directive (Council of the European Communities, 1991), instead of A category which characterized the screened farms (F1 and F2) of the present investigation. Differently from the northern-east Adriatic Sea, HAV was not discovered in a recent study performed in the Apulia region, located in the southern-east Adriatic Sea, by La Bella *et al.* (2017). Their findings resulted approximatively in line with our described prevalence (1.17%, CI 95%: 0.02-3.45%), and these partial similarities have been justified by the water quality as A category.

Both HEV and HAV have demonstrated a consistent ability to be bioaccumulated in mussel tissues (Fusco *et al.*, 2019), and the high titers of copies/µL are mainly associated with recent human-related environmental pollutions. Generally, the initial viral loads decrease by 89% after 24-48 hours post-infection; if they were farmed in A category waters, they could gain a final residual decreasing of 1% per day, as observed in an *in-vitro* study performed by Amoroso *et al.* (2021). However, these last-mentioned fascinating ecological aspects can be influenced by any environmental and host variables, *i.e*., water temperature (increased viral loads in the winter season), animal gender, host immune response, *etc*., as supposed by Polo *et al.* (2014).

In the Terranean Sea, the prevalence values were higher than the ones reported in the Adriatic sea (8.9%) (Fusco *et al.*, 2019), and in (13.67%) (La Rosa *et al.*, 2020), but the viral genetic fragments were never discovered in the Sardinia (Bazzoni *et al.*, 2019).

Among the screened animals, 2.35% (CI 95%: 0.07-4.63%) resulted positive to NoVGI genome fragment detection. The positive pools were all collected from F2. They presented average amounts of 10^1 copies/µL which can be marginally dangerous and responsible for human infections (La Rosa *et al.*, 2020). The results, obtained in the present study, were higher than the 1.6% reported in Apulia (La Bella *et al.*, 2017) and the 1.7% observed by Savini *et al*. (2009) along the coasts of the Molise region, but lower than the 10.82% reported in the Campania region (Fusco *et al.*, 2019) and the 79.7%, in the same region discovered in a previous investigation (Suffredini *et al*., 2014). The discrepancy emerging from this analysis can be hypothetically justified by the water temperature variable. The sampling season, as previously described, can influence the viral loads. More specifically for NoVGI, winter has been designed as the most favorable condition for viral environmental persistence (Choi and Kingsley, 2016). Indeed, our sampling activities were performed starting in March (defined as the spring period) and not in January as described by Bazzoni *et al.*

(2019). This physical parameter has been demonstrated to play a crucial role in viral load detections in fish products, with special regard to mussels (Amoroso *et al.*, 2021).

Finally, NoVGII was not discovered in the present molecular investigation; on the contrary, other authors amplified parts of its genome in Campania [39.74% discovered by Fusco *et al.* (2019)], Apulia [12.21% reported by La Bella *et al.* (2017)], and Sicily [38.28% (Macaluso *et al.*, 2021)] regions. As described above, the observed differences, also for this pathogen, can be justified by the different sampling seasons; consequently, water temperatures could be involved in the absence of the viral genome. As the last aspects, the sampled marine areas (Abruzzo and Molise regions) were categorized as A, instead of B, for the Apulia (La Bella *et al.*, 2017; La Bella et al., 2021), and Campania (Fusco *et al.*, 2019) regions.

The present biomolecular screening, which involved the main human enteric viral pathogens, produced preliminary and epidemiological data concerning HAV, HEV, and NoVGI circulation in the central Adriatic Sea. These specific marine areas were never investigated before.

Although, the F1 and F2 were located, as marine farms, in A category waters [in agreement with Regulation 625/2017 (European Parliament and Council of the European Union, 2017) and Regulation 626/2019 (European Commission, 2019b)] viruses have been amplified. This screening also wants to contribute by further enforcing the importance of biomolecular assays as a means of preventive medicine. Based on the obtained results, the aim was also to stimulate national and European legislators to produce *ad hoc* guidelines or laws to guarantee human, animal, and environmental health. The environmental variable covers the strategic role of alimentary safety and guarantee. Anthropic activities have heavy repercussions on viral circulation. This last aspect, coupled with the viral structural characteristics (as non-enveloped strains), has provided ideal evolutionary conditions to survive in many organic hosts.

Conclusions

The main human enteric viral pathogens are involved in the occurrence of many foodborne outbreaks on different continents (Purdy *et al.*, 2017). Their widespread use has attracted more attention from the scientific community, which has highlighted a consistent legislative gap in the European Union. The severity of gastroenteric symptoms varies depending on the viral loads and host immune conditions. For these reasons, the so-called food safety criteria (mentioned in Regulation 2073/2005) could be implemented by the introduction of viral pathogens (*i.e.*, NoV, HAV, HEV, *etc.*) as referring parameters (Errani *et al.*, 2021). Based on a one-health approach, the inclusion of foodborne viruses is mandatory to guarantee human, animal, and environmental health.

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