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Detection and molecular characterization of enteric viruses in bivalve molluscan shellfish and stool samples between July 2022 and June 2023 in Quito, Ecuador.

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Detection and molecular characterization of enteric viruses in bivalve molluscan shellfish and stool samples between July 2022 and June 2023 in Quito, Ecuador.

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DEDICATORIA

A todos mis profesores que me inspiraron a iniciar una carrera en el campo de la investigación.

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RESUMEN

Las enfermedades transmitidas por alimentos siguen representando un importante problema de salud pública y están relacionadas con pérdidas económicas significativas a nivel global. Los moluscos bivalvos han sido implicados en casos esporádicos y brotes de gastroenteritis mundialmente. Son importantes reservorios de patógenos bacterianos y virales, por lo tanto, consumirlos crudos o poco cocinados suponen un riesgo para los humanos. El presente estudio tuvo como objetivo determinar la frecuencia y estacionalidad de cinco virus entéricos (norovirus, rotavirus, adenovirus, sapovirus, y astrovirus) en conchas negras (*Anadara tuberculosa* y *Anadara similis*) tomadas desde julio 2022 hasta junio 2023 en un mercado local de mariscos en Quito. Adicionalmente, para investigar la diversidad genética en conchas y humanos, muestras de heces de pacientes con gastroenteritis viral aguda fueron recibidas de un hospital terciario durante el mismo periodo. En total, 55 de 120 muestras de conchas negras fueron positivas para uno o varios virus entéricos. Rotavirus fue el patógeno más frecuente seguido de norovirus y adenovirus. Se demostró que las conchas contaminadas con adenovirus poseen un comportamiento estacional presentando un mayor número de muestras positivas durante la época seca. En las muestras clínicas, rotavirus y norovirus también fueron los agentes virales más comunes, por lo tanto, los ensayos de genotipificación se llevaron a cabo para las muestras de conchas y clínicas positivas para estos dos patógenos. A pesar de que no fue posible detectar algún genotipo de norovirus en conchas, las muestras clínicas revelaron una gran variabilidad genética de las cepas de norovirus. Notablemente, en estas muestras el genotipo más común fue la variante recientemente reportada GII.4 San Francisco [P31]. Por otro lado, el genotipo G3 de rotavirus fue el más frecuentemente detectado en muestras de heces y conchas negras, a pesar de que solo un pequeño grupo de muestras de conchas pudo ser genotipificado. Esta investigación sugiere que las conchas negras no deberían

consumirse crudas debido a la presencia de virus entéricos, y resalta la necesidad de implementar programas de depuración de moluscos bivalvos previo a su comercialización.

Palabras clave: virus entéricos, moluscos bivalvos, *Anadara tuberculosa, Anadara similis,* genotipificación, rotavirus, norovirus.

ABSTRACT

Foodborne diseases still represent a major public health issue and are related with significant economic losses globally. Bivalve molluscan shellfish have been implicated in gastroenteritis sporadic cases and outbreaks worldwide. They are important reservoirs of bacterial and viral pathogens, so consuming them raw or undercooked poses a risk for humans. The present study aimed at determining the frequency and seasonality of five enteric viruses (norovirus, rotavirus, adenovirus, sapovirus, and astrovirus) in black cockles (*Anadara tuberculosa* and *Anadara similis*) sampled from July 2022 to June 2023 at a local seafood market in Quito. Additionally, to investigate viral genetic diversity in shellfish and humans, stool specimens from patients with acute viral gastroenteritis were received from a tertiary hospital during the same period. In total, 55 out of 120 black cockle samples were positive for one or more enteric viruses. Rotavirus was the most frequent pathogen followed by norovirus and adenovirus. Our results demonstrated that adenovirus shellfish contamination exhibited a seasonal behavior with a higher number of positive samples during the dry season. In clinical samples, rotavirus and norovirus were also the most common viral agents, so genotyping assays were performed for shellfish and clinical samples positive for these two pathogens. Although, we were not capable to detect any norovirus genotype in shellfish, clinical specimens revealed high genetic variability of noroviruses strains. Remarkably, in these samples the most common genotype was the recently reported variant GII.4 San Francisco [P31]. On the other hand, rotavirus genotype G3 was the most frequently detected in fecal samples and black cockles, even though only a small subset of shellfish could be genotyped. This investigation suggests that black cockles should not be consumed raw due to the presence of enteric viruses and highlights the need to implement depuration programs prior to their commercialization.

Key words: enteric viruses; bivalve molluscan shellfish; *Anadara tuberculosa*, *Anadara similis*, genotyping, rotavirus, norovirus.

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LITERATURE REVIEW

Viral gastroenteritis

Worldwide, most of acute gastroenteritis diseases are caused by viral agents. Enteric viruses replicate in the intestinal mucosa and are transmitted by the fecal-oral route. Viral gastroenteritis is characterized by several clinical manifestations including nausea, vomiting, fever, anorexia, abdominal cramps, watery diarrhea, and dehydration (Bishop & Kirkwood, 2014). Large quantities of viruses are shed in the stool of infected people, even several days after the onset of clinical symptoms (Bosch et al., 2018). Although viral gastroenteritis is usually a self-limited disease which lasts from 2 to 5 days, for susceptible population like infants, elderly people and immunocompromised patients, more severe and prolonged symptoms have been observed, especially in developing countries (Stuempfig & Seroy, 2023). Viral gastroenteritis is associated with both sporadic cases and epidemic outbreaks in enclosed environments such as childcare facilities, nursing homes, military populations, and cruises (Page et al., 2019). Infections with enteric viruses occur throughout the year; however, some viruses display a more seasonal behavior with a peak during the winter months (Stuempfig & Seroy, 2023) .

Viruses associated with acute gastroenteritis.

The enteric viruses group is constituted by different viral genera, and there are two subsets that should be mentioned. Some viruses cause localized infection mainly in the small intestinal mucosa leading to acute gastroenteritis, while others cause a more disseminated infection of the intestinal tract and produce disease when they spread to other parts of the body, for example, polioviruses, coxsackieviruses, and hepatitis E and A viruses (Bishop & Kirkwood, 2014). The scope of the present review is the group that produces localized intestinal infections in humans such as adenovirus (F40/41, A and G), norovirus, sapovirus, astrovirus

and rotavirus (Lockhart et al., 2022). A common feature is that they are non-enveloped viruses with icosahedral capsids, highly resistant to environmental conditions, for example, detergents, acidic pH, drying, and disinfection methods like ethanol and chlorine (Murray et al., 2020; Stuempfig & Seroy, 2023). The main characteristics of these viruses are summarized in Table 1 (Bányai et al., 2018; Benkő et al., 2022; Bishop & Kirkwood, 2014; Vinjé et al., 2019).

Table 1. Characteristics of the main enteric viruses that cause acute gastroenteritis in humans.

Viral genera	Family	Virion size	Capsid organization	Genome
Rotavirus	*Sedoreoviridae*	70-75 nm	- Icosahedral - 3 concentric layers: **Inner** (VP2) I**ntermediate** (VP6), T=13 **Outer** (VP4, VP7)	- 11-segment double-stranded RNA (dsRNA) - Size 18,5 kb pairs - Encodes for 11 or 12 proteins: 6 structural (VP1-VP4, VP6, and VP7) and 5 or 6 nonstructural (NSP1-NSP5 or NSP6)
Norovirus *Sapovirus*	*Caliciviridae*	27–40 nm	- T=3 icosahedral symmetry - 90 dimers of VP1 protein	- Linear positive-sense single- stranded RNA with 5′-end VPg and 3′-end poly(A) - Size 7,4–7,7 kb - Norovirus genome: 3 ORFs - Sapovirus genome: 2 ORFs
Mastadenovirus	*Adenoviridae*	90 nm	- pseudo *T*=25 icosahedral symmetry - 240 non-vertex capsomers (hexon) – 12 vertex capsomers (penton bases) - fiber	- Linear double-stranded DNA with inverted terminal repeats (ITRs) - A terminal protein is linked to the 5’ end of each strand. - Size 25–48 kb pairs - Encodes for 40 different proteins

Mamastrovirus	*Astroviridae*	28–30 nm	- T=3 icosahedral symmetry - Precursor protein cleaved into several proteins	- Linear positive-sense single-stranded RNA - Size 6,4 -7,7 kb - 3'-end has a poly(A) tract. - 3 ORFs **ORF1a** and **ORF1b**: nonstructural proteins **ORF2**: capsid protein precursor

Rotavirus is a leading cause of acute diarrhea in children worldwide, even though rotavirus vaccines have been introduced in the national immunization programs of 122 countries (International Vaccine Access Center (IVAC), 2024). Rotavirus causes more severe gastroenteritis than other enteric disease agents. For instance, Rotavirus A accounts for most diarrhea-associated hospitalizations and deaths in children under five years old globally. Around 90% of rotavirus associated deaths occur in developing countries where rehydration therapy is not widely available, whereas in developed countries morbidity rates are still significant and represent a high burden for public health systems (Bányai et al., 2018; Esona et al., 2021). Worldwide, norovirus is recognized as the leading cause of acute gastroenteritis within all age groups, accounting for 16% of all cases (Li et al., 2023). Additionally, noroviruses cause the majority of acute gastroenteritis outbreaks which have a peak during cooler months in developed countries. Approximately 50% of all foodborne outbreaks are caused by norovirus and are associated mainly with the consumption of shellfish and fresh produce like salads and berries (Page et al., 2019; Stuempfig & Seroy, 2023). Genetic and antigenic analyses demonstrate that noroviruses are highly variable and exhibit constant evolution resulting in the emergence of new variants periodically (Bányai et al., 2018).

In addition to *Rotavirus* and *Norovirus,* other relevant viral causative agents of gastroenteritis are adenoviruses, sapoviruses and astroviruses. Each of these viruses accounts for about 2% to 9% of viral gastroenteritis cases (Stuempfig & Seroy, 2023). Human

adenoviruses belong to the genus *Mastadenovirus* (family *Adenoviridae*) and could be divided into 7 groups (A-G), 56 serotypes and more than 100 genotypes (Lei et al., 2023). According to tissue tropism of HAdV species, they could cause a wide range of clinical manifestations in humans including gastroenteritis, conjunctivitis, respiratory illness, hemorrhagic cystitis, among others. The enteric adenoviruses F40/F41 are most associated with acute gastroenteritis and are recognized as a major cause of infantile viral diarrhea, after rotavirus (Benkő et al., 2022). Another member of the *Caliciviridae* family that is an important gastrointestinal pathogen is the *Sapovirus* genus. Based on phylogenetic analyses of complete VP1 amino acid sequences, at least 19 genogroups have been described, of which GI, GII, GIV and GV are known to cause infections in humans (Vinjé et al., 2019). Although, sapovirus gastrointestinal infections have been observed in adults, they are more common in children under five years old. Interestingly, there is evidence supporting that genogroup or genotype-specific immunity could not provide cross protection, so symptomatic reinfections have been reported (Zhuo et al., 2021). Finally, astroviruses have a characteristic star-like morphology as revealed by electron microscopy and have been identified as an important cause of acute gastroenteritis in sporadic cases and large outbreaks. Human astroviruses (genus *Mamastrovirus*) are classified into 8 genotypes (HAstV1-HAstV8) (Fu et al., 2023).

Detection of enteric viruses.

Several diagnostic methods have been described for the detection of enteric viruses, such as cell culture, electron microscopy, immunological methods, and molecular methods (Bosch et al., 2018; Vinjé, 2015). The gold standard for the isolation and propagation of enteric viruses is the culture in permissive cells (Malik et al., 2019). Even though it allows a direct measure of viral infectious titer in contaminated shellfish, there are some limitations that impede the regular use of cell culture-based techniques including high detection thresholds;

slow viral replication; low viral load in naturally contaminated shellfish; laborious procedures that require expensive reagents and trained staff; inability to replicate *in vitro* of some viral strains, among others (Desdouits et al., 2023). Currently, procedures for cultivating most of the enteric viruses that contaminate shellfish have been developed. For example, norovirus was considered not cultivable until a few years ago; however, culture in human intestinal enteroids and B cells have been reported (Bosch et al., 2018; Ghosh et al., 2022; Malik et al., 2019). Restricted access to specialized cell lines and reagents, technique complexity, failure to sustain virus passaging, and overall low efficiencies represent some of the major challenges for these norovirus culture systems which are not suitable for routine use yet (Hasing & Pang, 2021).

Electron microscopy can detect multiple viral pathogens, though the equipment is very expensive and qualified personnel is necessary for the operation, so it is available mainly in reference laboratories (Vinjé, 2015). Immunological methods, such as enzyme-linked immunosorbent assay (ELISA) and lateral flow immunochromatographic assay (LFIA), are based on the detection of viral antigens or antibodies. They are used in public health facilities, clinical laboratories, and points of care, and allow to obtain results in less time than other techniques like molecular methods (Vinjé, 2015). There are several immunochromatography tests commercially available characterized by high specificity and variable levels of sensitivity (Ye et al., 2015). For example, the *OnSite Rotavirus Ag Rapid Test* and the *OnSite Rota/Adeno Ag Rapid Test* developed by CTK Biotech, qualitatively detect rotavirus and rotavirus/adenovirus antigens in fecal samples, respectively. Immunochromatography is based on the migration of the sample by capillary flow through a membrane that can be nitrocellulose or filter paper. The test cassette contains the sample pad and the conjugation pad where free gold- or latex-labeled antibodies are present. If the sample contains the antigen of interest, an immunocomplex is formed, and subsequently detected when the antigen-antibody complex is

captured by the fixed antibodies in the test line. Therefore, a colored band appears and indicates a positive result (Dixit & Twyman, 2018).

Molecular tests are based on the detection of nucleic acids, and present high sensitivity and specificity, so they are used by many laboratories for the reliable diagnostic of enteric viruses. First, the isolation of viral genetic material takes place, followed by the amplification with polymerase chain reaction (PCR) or other related techniques like qPCR and loop-mediated isothermal amplification (LAMP) (Malik et al., 2019). Some samples could have a complex composition and carry high concentration of inhibitors, such as feces, wastewater, and food (Hrdy & Vasickova, 2022). Additionally, in food matrices the viral load could be very small, even below the limit of detection of some methods that are used for clinical samples, *e.g.* immunoassays. Inhibitors could affect some steps during sample preparation, for example, viral particle concentration, sample lysis, and degradation of nucleic acids, but also during the amplification reaction. In fact, PCR inhibitors may bind to the target DNA, compete for the DNA polymerase, and form a complex with Mg^{2+}, the cofactor of DNA polymerase (Acharya et al., 2017; Hrdy & Vasickova, 2022).

At present, RT-qPCR is the preferred option for the diagnostic of enteric viruses in clinical and food samples. Among the advantages offered by qPCR are: (a) simultaneous amplification and detection, so electrophoresis analysis is not required; (b) multiplexing capability allowing the detection of multiple targets simultaneously; (c) fluorescent-labeled probes that make the reaction highly specific; and (d) high-throughput formats that permit to test lots of samples at the same time. The amplicons obtained with qPCR are approximately 100 bp in length, so genotyping studies are based on conventional PCR followed by sequencing preferably in genomic regions prone to recombination (Bosch et al., 2018; Vinjé, 2015). Even though these methods are very useful, they only indicate the presence of viral genome without a relation with the infectivity of viral particles (Desdouits et al., 2023). Within food industry

and foodborne outbreak investigations, a standard method developed by ISO (2017) for the quantitative detection of Norovirus (NoV) and Hepatitis A Virus (HAV) in different food matrices, have permitted to associate an increasing number of infections with food consumption (Bosch et al., 2018).

In the clinical field, molecular diagnostic tests based on the simultaneous detection of several gastrointestinal pathogens (viruses, bacteria, and parasites) are commercially available, for example, FilmArray Gastrointestinal (GI) Panel (BioFire Diagnostics Inc.), xTAG GPP (Luminex Corporation), and Verigene Enteric Pathogens test (Nanosphere Inc.). These rapid diagnostic tools contribute to proper treatment of infectious diarrheas in clinical settings. The FilmArray GI Panel can detect 22 enteric pathogens, including norovirus GI/GII, rotavirus A, adenovirus F40/41, sapovirus, and astrovirus, 13 bacterial species, and 4 parasite species. The sample processing is highly automated and involves nucleic acid extraction, reverse transcription, amplification, and further analysis, obtaining results in about an hour. The principle of detection consists of nested multiplex RT-PCR followed by endpoint melting curve analysis. Some disadvantages of this diagnostic platform are: (a) requirement of expensive instrument and reagents, (b) processing one sample at a time, and (c) lack of quantitative data that allow better interpretation of results (Malik et al., 2019; Vinjé, 2015; Zhang et al., 2015).

Next generation sequencing (NGS) is a valuable tool for studying molecular epidemiology of enteric viruses in stool specimens and in samples with low level of viral contamination, *e.g.* food matrices. For instance, Oxford Nanopore Technologies (ONT) can be useful to identify viral recombinant strains, discover emerging genotypes, and determine the source of contamination and transmission pathways during an outbreak investigation (Ollivier et al., 2022). On the other hand, metabarcoding approach using the Illumina technology is useful for genotype identification and genetic diversity studies including the low abundance strains. NGS technologies have demonstrated that several norovirus strains can be

simultaneously detected in bivalve molluscan shellfish and human population allowing for a better characterization of Norovirus diversity (Ollivier et al., 2022).

Pathogens associated to bivalve molluscan shellfish (BMS).

Viruses implicated in foodborne diseases are transmitted by food contaminated early in the food production chain, such as shellfish, berries and leafy greens, or lack of hygiene of food handlers. Sewage contamination of drinking water and recreational activities in contaminated water bodies are also risk factors for infection with enteric viruses (O'Shea et al., 2019). Shellfish are important vehicles for the transmission of human pathogens due to fecal anthropogenic discharges of bacteria and viruses into the marine environment (Li et al., 2023). For instance, body waters at Esmeraldas province, *i.e.* river mouths, estuaries, and mangroves, contain fecal coliforms at concentrations greater than $2x10^2$ NMP/100mL, the microbiological criterion established for the use of aquatic species (fishing and aquaculture) from marine and estuary waters. Additionally, the presence of *Escherichia coli, Vibrio parahaemolyticus* and *Aeromonas hydrophila* has also been reported (Pernia et al., 2019; TULSMA, 2015).

Although in some countries there are regulations for assuring shellfish microbiological quality, they are frequently involved in foodborne outbreaks. Worldwide, the presence of enteric (adenovirus, rotavirus, sapovirus, aichivirus, astrovirus, enterovirus, hepatitis A virus, hepatitis E virus, and norovirus) and non-enteric (SARS-CoV) viruses have been reported in these foods (O'Shea et al., 2019). Improvements in cell culture-based methods allowed to determine that naturally contaminated shellfish possess high viral infectious titers during long periods of time. Together with the low infectious doses for enteric viruses, eating raw or undercooked shellfish could be a potential risk for consumers. In addition to viral pathogens, some bacterial agents that have been detected in bivalve mollusks belong to the following genera: *Vibrio, Escherichia, Salmonella, Shigella, Klebsiella, Campylobacter, Arcobacter,*

Helicobacter, Clostridium, and *Enterococcus*. Some of these species represent a public health risk as they are capable to spread antimicrobial resistance genes (*e.g.* extended-spectrum β-lactamase (ESBL)), possess virulence factors (*e.g.* toxins, hemolysins, adhesins, type III secretion systems) that can cause disease in humans, and have the capacity to form biofilms (Desdouits et al., 2023).

The feeding mechanism of bivalve shellfish, *e.g.* mussels, oysters, clams, and cockles, involves pumping water through their gills and capturing suspended particles like plankton and microscopic organisms (Li et al., 2023). Viral particles present in coastal waters could be also filtered by bivalve shellfish and accumulated in their digestive tract by binding to specific ligands present in gastrointestinal epithelial cells, for example, carbohydrates similar to histo-blood group antigens (HBGAs) for the case of noroviruses (Maalouf et al., 2011). As reviewed by Yang et al. (2022), accumulation efficiency depends on the distribution of HBGA-like carbohydrates on shellfish tissues. In mantle, gills, and the digestive gland of oysters, types A and H1 HBGA-like structures are expressed, whereas Lewis b type is mainly found in the gills. Consequently, different viral strains could preferentially bind to a particular tissue. As an example, NoV GI.1 and GI.3 could be found mainly in the digestive gland, while GII.3 and GII.4 in the mantle, gills, and digestive gland (Yang et al., 2022). Bioaccumulation and persistence are also affected by environmental conditions such as global warming and water temperature. There is evidence that cooler temperatures contribute to the preservation of noroviruses in oysters for longer periods (Desdouits et al., 2023; Yang et al., 2022).

Monitoring harvesting areas is a useful approach to determine if shellfish are suitable for human consumption, and the level of post-harvest treatment required for improving its quality. For instance, in the European Union the standard procedure ISO 16649-3 is used for the detection and quantification of *Escherichia coli* in shellfish as a marker of fecal contamination (Desdouits et al., 2023). In order to remove microorganisms from shellfish,

several approaches have been developed, for example, depuration with purified seawater by the means of chlorine, UV irradiation and ozone treatment. These methods can eliminate bacterial contamination successfully; however, they are not effective for virus eradication (Li et al., 2023). Monitoring programs should include other enteric pathogens such as norovirus due to its association with most of the foodborne illness outbreaks worldwide (Desdouits et al., 2023).

DETECTION AND MOLECULAR CHARACTERIZATION OF ENTERIC VIRUSES IN BIVALVE MOLLUSCAN SHELLFISH AND STOOL SAMPLES BETWEEN JULY 2022 AND JUNE 2023 IN QUITO, ECUADOR.

Introduction

Foodborne diseases have a large impact in public health burden and economic losses worldwide producing 600 million cases and 420,000 deaths every year (World Health Organization, 2015). Among the agents that cause foodborne illnesses, enteric viruses have received special attention. The most common viruses associated with acute gastroenteritis (AGE) are norovirus, sapovirus, rotavirus, astrovirus, adenovirus, and enterovirus. They are characterized by intestinal replication and fecal-oral transmission which includes the consumption of contaminated food and water, and the contact with fomites or an infected person (Bosch et al., 2018). A recent publication demonstrated that enteric viruses like norovirus, rotavirus and astrovirus were capable to replicate in salivary glands of mice and be transmitted by the oral-oral route through saliva of infected hosts (Ghosh et al., 2022). These viruses do not possess an envelope, so they resist the harsh conditions of the gastrointestinal tract and persist in the environment for long periods. Additionally, low doses could cause disease in humans, for instance, norovirus infective dose is between 1 and 10 viral particles. Rotaviruses and adenoviruses F40/41 mainly affect infants, whereas caliciviruses and astroviruses can cause disease among all age groups (Bosch et al., 2018; Murray et al., 2020).

Globally, *Rotavirus* is a leading cause of acute gastroenteritis that belongs to the family *Sedoreoviridae* and possesses a triple-layered capsid enclosing an 11-segment double-stranded RNA (dsRNA) genome. Importantly, reassortment could happen during co-infection events resulting in great rotaviruses diversity. Structurally, the inner capsid is composed of the VP2 protein, whereas the intermediate capsid is composed of the VP6 protein. The outer capsid is

formed by the protease-sensitive protein VP4, and the glycoprotein VP7, which function as cell-attachment proteins and independently elicit neutralizing antibodies (Murray et al., 2020). Based on the antigenicity of the VP6 protein, *Rotavirus* is classified in 10 different groups (A to J). Rotavirus A, B, C and H could cause disease in humans; however, group A is the most relevant at clinical level (Bányai et al., 2018). Furthermore, genotype classification is based on the proteins VP7 (G-type) and VP4 (P-type). At present, RVA viruses are categorized in 42 G-types and 58 P-types (Rotavirus Classification Working Group: RCWG, 2024).

Four oral, live-attenuated vaccines have been prequalified by the World Health Organization (WHO): Rotarix™, a monovalent vaccine consisting of the G1P[8] human strain; RotaTeq™, a pentavalent vaccine of bovine-human reassortant rotaviruses that contain G1, G2, G3, G4, and P[8] antigens; Rotavac™, a naturally occurring bovine-human reassortant strain G9P[11] known as 116E; and RotaSiil™, a bovine-human reassortant with G1, G2, G3, G4, and G9 strains (Amin et al., 2023; Skansberg et al., 2021). Although the implementation of vaccines has decreased severe gastrointestinal diseases, a total of 128,500 deaths related to Rotavirus infection in children <5 years was reported worldwide in 2016 (Troeger et al., 2018). Ecuador introduced Rotarix™ vaccine in 2007 (International Vaccine Access Center (IVAC), 2024) which is included in the National Vaccination Scheme. However, RotaTeq™ is available in private health centers (Ministerio de Salud Pública del Ecuador, 2019).

Worldwide Norovirus is a leading cause of foodborne outbreaks, with contaminated oysters being an important vehicle for its transmission (Desdouits et al., 2023). Norovirus is part of the *Caliciviridae* family. The virion is non-enveloped with icosahedral capsid and contains single-stranded positive-sense RNA genome of approximately 7.5 kb, that is structured in three open reading frames. ORF1 encodes a polyprotein that is proteolytically cleaved after transcription; ORF2 encodes the major capsid protein VP1 which consists of two domains: Shell (S) and Protruding (P); and ORF3 encodes the minor capsid protein VP2 (Chhabra et al.,

2019). The P-domain of VP1 protein is divided into P1 and P2 sub-domains. Viral-cellular attachment involves the interaction of highly variable motifs present in P2 sub-domain and histo-blood group antigens (HBGA). Additionally, P2 displays antigenic sites to neutralizing antibodies (Ford-Siltz et al., 2021). Based on the complete amino acid sequences of VP1 protein, noroviruses are divided in ten stablished genogroups (GI-GX) and two tentative genogroups, of which GI, GII, GIV, GVIII and GIX are capable to infect humans. Furthermore, classification into genotypes and P-types could be performed with a dual typing system based on the amplification of the junction of 3'-end ORF1 (P-type) and 5'-end ORF2 (genotype) for GI and GII noroviruses. Currently, there are at least 9 GI and 26 GII genotypes, and 14 GI.P types and 37 GII.P types (Tatusov et al., 2021).

There are some kind of foods that are closely related with viral infections, for example, raw or undercooked shellfish, fresh fruit, and leafy greens (Bosch et al., 2018). The correlation between bivalve molluscan shellfish consumption and foodborne diseases has been well documented in many countries as a significant public health issue (Desdouits et al., 2023). For centuries, black cockles or mangrove cockles (*Anadara tuberculosa* and *Anadara similis*) have been important food and economic resources for mangrove communities that are located on the Ecuadorian coast. However, their consumption has spread to the entire country and has become very popular since the 1980s (Treviño, 2022). In Esmeraldas province there is a large area of mangrove forest where the main species are *Rhizophora mangle*, *Rhizophora harrisonii*, and *Pelliciera rhizophorae*. Black cockles live buried in the mud between mangrove roots at a depth from 10 to 30 cm. Most of the specimens sold at the markets in Quito come from Esmeraldas Province. No post-harvest depuration treatment or microbiological control is carried out before the commercialization phase (Caicedo, 2014). Black cockles contamination with enteric viruses was reported previously by Ulloa (2023) with high positivity rates. For instance, almost 70% of the shellfish samples acquired at local markets in several Ecuadorian cities were positive for

at least one virus. Adenovirus, norovirus, and rotavirus were the most prevalent in Ulloa's investigation. Furthermore, 85.4% of samples contained concentrations of *E. coli* that exceeded the maximum permissible limit for human consumption (230 MPN/100g) according to international food standards (CODEX STAN 292-2008). Other studies have provided evidence of *Anadara* species contamination with mercury in samples collected at markets in Quito (Nasevilla et al., 2022), and with cadmium, chromium, and lead, in samples from Santa Rosa Island at Esmeraldas Province (Romero-Estévez et al., 2020).

Taking this background into account, the current investigation aimed to determine the presence of enteric viruses norovirus, rotavirus, adenovirus, sapovirus, and astrovirus, in black cockles collected in one of the main seafood markets in Quito for one year period (July 2022 – June 2023). Another objective was to study the association between viral detection frequency and seasonality (dry season versus rainy season) in shellfish harvesting area. To compare genotype distribution in shellfish and human population, fecal specimens from patients with gastroenteritis who attended a tertiary referral hospital during the sampling period, were collected, and tested with the same methodology used for shellfish. Genotyping was performed for norovirus and rotavirus, the most common viral pathogens in both sample types. Finally, statistical differences between Cq values in clinical and shellfish samples were analyzed.

Materials and methods

Shellfish sampling and viral extraction procedure

Shellfish specimens (*Anadara tuberculosa* and *Anadara similis*) were acquired at one of the main seafood markets (Mercado América) in Quito-Ecuador. In total, 120 samples were collected three times a month from July 2022 to June 2023. Each sample was composed by 10-12 bivalve molluscan shellfish (BMS). Viral extractions were performed according to ISO 15216-1:2017. In brief, shellfish were scrubbed with a brush to remove any dirt. Then, digestive

glands were isolated and finely chopped until paste consistency, and 2,0 ±0,2 g was transferred to a centrifuge tube. Next, 2 mL of 0.1 mg/mL Proteinase K solution was added, and samples were thoroughly mixed by pipetting. After that, samples were incubated at 37 °C with agitation for 60 min, and then at 60 °C for 15 min. Finally, tubes were centrifuged at 3000 xg for 5 min, and supernatants were transferred to microcentrifuge tubes and stored at -80°C for further analysis (ISO, 2017)

Stool samples and ethical clearance

Study protocol was approved by the research ethics committee of Universidad San Francisco de Quito USFQ. Stool samples proceeded from patients that were diagnosed with viral gastroenteritis at a tertiary referral hospital in Quito (Hospital de los Valles-HDLV) from July 2022 to June 2023. Viral pathogens were detected by the BioFire® FilmArray® Gastrointestinal Panel (GIP) multiplex PCR system or by the CTK Biotech Lateral Flow Immunochromatographic Assay (LFIA). Samples were anonymized and assigned an internal code, and then a portion was transported at 4 °C to Instituto de Microbiología at USFQ. Next, stool samples were resuspended in phosphate-buffered saline (PBS) to a dilution 1:5 for aqueous samples, or 1:10 for solid samples, followed by centrifugation at 4000 rpm for 5 min. Finally, 250 µL of supernatant was transferred to a microcentrifuge tube for nucleic acid extraction.

Nucleic acid extraction and cDNA synthesis

Nucleic acid extractions were performed from 250 µL supernatants (shellfish or stool) utilizing the *ReliaPrep™ RNA Cell Miniprep System* (Promega, Madison, USA) according to manufacturer´s guidelines; except that the *DNase I* treatment was omitted. For nucleic acid elution, 30 µL of nuclease-free water was used, then samples were aliquoted and stored at -80

°C. Concentration and quality of the samples were analyzed by NanoDrop One spectrophotometer (Thermo Scientific™, Madison, WI, USA).

For cDNA synthesis, SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was used. The reverse transcription master mix consisted of 1x first-strand buffer, 5 mM DTT, RnaseOut™ (1 U), random primers (30 ng), dNTPs (0.375 mM each), SuperScript™ III Reverse transcriptase (5U), and ultrapure distilled water per final reaction. 5 µL of sample was incubated for 5 min at 97 °C followed by cooling for 2 min, and then 15 µL of the master mix was added and mixed by pipetting. Thermocycler protocol consisted of the following steps: 25 °C for 10 min, 42 °C for 50 min, 70 °C for 15 min, and 4 °C for 10 min. cDNA was stored at -20 °C for subsequent experiments.

Viral detection by qPCR

Norovirus GI (NoV GI), Norovirus GII (NoV GII), Rotavirus A (RVA), Adenovirus F40/41 (AdV), Sapovirus I, II, IV, and V (SaV), and Astrovirus (AstV) were identified in shellfish and clinical specimens by qPCR with TaqMan™ Fast Universal PCR Master Mix (2X) (Applied Biosystems, Vilnius, Lithuania) using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Singapore). The protocol for the Gastroenteritis Viral Panel (GVP) assay was kindly provided by the Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, which consisted in three duplex qPCRs: 1) NoV GI and GII, 2) RVA and AdV, and 3) SaV and AstV. Primers and probes used in the present research were described previously and are listed in

Table 2. Probes were labeled at the 5’ end with the reporter dyes 6-carboxyfluorescein (FAM), or 2’-chloro-7’-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC), and at the 3’ end with the quenchers 5-carboxytetramethylrhodamine (TAMRA), or minor groove binder-nonfluorescent quencher (MGB-NFQ).

Table 2. List of primers and probes used in the present study.

Virus	Primer or probe	Function	Sequence (5'-3')	Target	Reference	Molecular method
Norovirus GI	MON432	Forward	TGG ACI CGY GGI CCY AAY CA	3'-end of ORF1 and 5'-end of ORF2	(Chhabra et al., 2021)	RT-PCR
	G1SKR	Reverse	CCA ACC CAR CCA TTR TAC A			
	NoV1-Fb	Forward	TYC GYT GGA TGC GNT TYC ATG A	Junction of RdRp and capsid	(Kageyama et al., 2003)	Norovirus GI/GII duplex qPCR
	NoV1-R	Reverse	CTT AGA CGC CAT CAT CAT TYA C			
	NoVG1c-Probe	Probe	VIC-AGA TYG CGR TCY CCT GTC CA-TAMRA			
Norovirus GII	MON431	Forward	TGG ACI AGR GGI CCY AAY CA	3'-end of ORF1 and 5'-end of ORF2	(Chhabra et al., 2021)	RT-PCR
	G2SKR	Reverse	CCR CCN GCA TRH CCR TTR TAC AT			
	NoV2-F	Forward	CAR GAR BCN ATG TTY AGR TGG ATG AG	Junction of RdRp and capsid	(Kageyama et al., 2003)	Norovirus GI/GII duplex qPCR
	NoV2-R	Reverse	TCG ACG CCA TCT TCA TTC ACA			
	NoV2-Probe	Probe	FAM-TGG GAG GGY GAT CGC AAT CT-TAMRA			
Rotavirus A	Rota-F	Forward	ACC ATC TAC ACA TGA CCC TC	Nonstructural Protein NSP3	(X. Pang et al., 2011; X. L. Pang et al., 2004)	Rotavirus/ Adenovirus duplex qPCR
	Rota-F-GII	Forward	ACC ATC TTC ACG TAA CCC TC			
	Rota-R	Reverse	GGT CAC ATA ACG CCC C			
	Rota-Probe	Probe	FAM-ATG AGC ACA ATA GTT AAA AGC TAA CAC TGT CAA -TAMRA			
	9con1/F	Forward	TAG CTC CTT TTA ATG TAT GG	First-amplification consensus primers	(World Health Organization, 2009)	Rotavirus G Genotyping
	9con2/R	Reverse	GTA TAA AAT ACT TGC CAC CA			
	9T-1	Reverse	TCT TGT CAA AGC AAA TAA TG	Second amplification VP7 genotype-specific Primers		
	9T-2	Reverse	GTT AGA AAT GAT TCT CCA CT			
	9T-3	Reverse	GTC CAG TTG CAG TGT AGC			
	9T-4	Reverse	GGG TCG ATG GAA AAT TCT			
	9T-9	Reverse	TAT AAA GTC CAT TGC AC			
	G12B	Forward	CCG ATG GAC GTA ACG TTG TA			
	con3/F	Forward	TGG CTT CGC TCA TTT ATA GAC A	First amplification consensus primers		Rotavirus P Genotyping
	con3/R	Reverse	ATT TCG GAC CAT TTA TAA CC			
	1T-1	Reverse	TCT ACT TGG ATA ACG TGC	Second amplification VP4 genotype-specific Primers		
	2T-1	Reverse	CTA TTG TTA GAG GTT AGA GTC			
	3T-1	Reverse	TGT TGA TTA GTT GGA TTC AA			
	4T-1	Reverse	TGA GAC ATG CAA TTG GAC			

	5T-1	Reverse	ATC ATA GTT AGT AGT CGG			
	ND2	Reverse	AGC GAA CTC ACC AAT CTG			
	Beg9	Forward	GGC TTT AAA AGA GAG AAT TTC CGT CTG G	VP7 gene fragment		RT-PCR
	End9	Reverse	GGT CAC ATC ATA CAA TTC TAA TCT AAG			
Adenovirus F40/41	Ad2-F	Forward	CCA GGA CGC CTC GGA GTA	Hexon	(Wong et al., 2008)	Rotavirus/ Adenovirus duplex qPCR
	Ad2-R	Reverse	AAA CTT GTT ATT CAG GCT GAA GTA CGT			
	Ad2-P	Probe	VIC-AGT TTG CCC GCG CCA CCG-TAMRA			
	Ad4-F	Forward	GGA CAG GAC GCT TCG GAG TA			
	Ad4-R	Reverse	CTT GTT CCC CAG ACT GAA GTA GGT			
	Ad4-P	Probe	VIC-CAG TTC GCC CGY GCM ACA G-TAMRA			
Sapovirus (I, II, IV, and V)	SV-F13	Forward	GAY YWG GCY CTC GCY ACC TAC	Junction of RdRp and capsid	(Okada et al., 2006)	Outer PCR
	SV-R13	Reverse	GGT GAN AYN CCA TTK TCC AT			
	SV-F14	Forward	GAA CAA GCT GTG GCA TGC TAC			
	SV-R14	Reverse	GGT GAG MMY CCA TTC TCC AT			
	SV-F22	Forward	SMW AWT AGT GTT TGA RAT G	Partial capsid region		Nested PCR
	SV-R2	Reverse	GWG GGR TCA ACM CCW GGT GG			
	SaV124F	Forward	GAY CAS GCT CTC GCY ACC TAC	Partial RdRp region	(X. L. Pang et al., 2014)	Sapovirus/ Astrovirus duplex qPCR
	SaV1F	Forward	TTG GCC CTC GCC ACC TAC			
	SaV5F	Forward	TTT GAA CAA GCT GTG GCA TGC TAC			
	SaV1245R	Reverse	CCC TCC ATY TCA AAC ACT A			
	SaV124TP	Probe	FAM-CCR CCT ATR AAC CA-MGB-NFQ			
	SaV5TP	Probe	FAM-TGC CAC CAA TGT ACC A-MGB-NFQ			
Astrovirus	Astr-1F	Forward	CTT AAT CGC CAT GTA CTT CTA CCA TC	Partial capsid region	(X. L. Pang et al., 2014)	Sapovirus/ Astrovirus duplex qPCR
	Astr-1R	Reverse	TGT TGT TGA AAA CTG CCC AGA T			
	Astr-1P	Probe	VIC-AAG TCA CCT TGC AGA CAC GAG GTA ATC-TAMRA			
	Astr-2F	Forward	GGC ACT AAT CAA ATG CCT AAT GTT T			
	Astr-2R	Reverse	GGA GAC TGT ACC CTC GAT CCT ACT C			
	Astr-2P	Probe	VIC-TGG AGA CCG CGG CCA CGC-TAMRA			

Samples with a cycle threshold (Cq) < 43 were considered positive. Appropriate controls, *i.e.,* negative, positive, and non-template control (NTC), were included in each run.

The positive control was a 10^{-6} dilution of a gBlock Gene Fragment (Integrated DNA Technologies, Coralville, IA, USA), a 614 bp double-stranded DNA fragment that contains the viral target sequences. Prior to sample analysis, amplification efficiency and correlation coefficient (R^2) were determined for each virus (Table 3). In brief, 10-fold serial dilutions ($1.55x10^7$ to $1.55x10^2$ copies/µL) of the positive control were prepared, and each point was assessed by triplicate. Standard curves were constructed with Bio-Rad CFX Manager 3.1 software.

Table 3. Standard curves and amplification efficiencies for qPCR assays.

qPCR assay	Standard curve	Amplification efficiency (%)	R^2
NoV GI	y=-3.406x+35.464	96.6%	0.993
NoV GII	y=-3.701x+37.266	86.3%	1.00
RVA	y=-3.262x+33.676	102.6%	0.996
AdV	y=-3.303x+34.160	100.8%	0.995
SaV	y=-3.539x+32.283	91.7%	0.987
AstV	y=-3.484x+37.255	93.7%	0.999

For the Sapovirus qPCR assay, several samples produced unusual shape curves. To determine if they were true positive samples, nested PCR with universal primers to amplify a region of the capsid was assessed as described elsewhere (Okada et al., 2006). The experiments were carried out using GoTaq® DNA Polymerase (Promega, Madison, WI, USA) with final primer concentration of 0.4 µM. A clinical sample positive for Sapovirus by both Filmarray GIP and GVP was used as positive control.

Clinical samples agreement analysis

Fifty percent of clinical specimens were analyzed by Filmarray GIP, and the remaining was assessed by LFIA. The first test can identify the same viruses as GVP, whereas the second one only detects Rotavirus and Adenovirus. These results were compared with GVP findings by classifying samples in two categories: concordant and discordant. A sample was concordant

when the same virus(es) was detected by both the GIP and the GVP, or the LFIA and the GVP assays. If only one test could detect the viral pathogen, or different viruses were detected, the samples were discordant. For discordant specimens, nucleic acid isolation and cDNA synthesis were repeated and then samples were re-assessed by GVP to confirm results.

Finally, NoV discordant samples were further analyzed by a conventional RT-PCR that targets the junction of ORF1 and ORF2 in *Norovirus* genome (Chhabra et al., 2021). Briefly, cDNA was synthesized as described above, and then PCRs were performed in 10 μL of volume reaction using GoTaq® DNA Polymerase with 0.5 μM of each primer. Concordant samples for NoV GI and GII were utilized as positive controls resulting in 579 bp and 570 bp products, respectively. In order to evaluate the level of agreement between the diagnostic methods used by HDLV (Filmarray GIP or LFIA) and GVP, the Cohen's kappa statistic, κ, was calculated for each viral target with Stata version 13.0 (Stata Statistical Software, College Station, TX, USA).

Norovirus and Rotavirus Genotyping

For *Norovirus* molecular characterization, a dual genotyping assay (polymerase and capsid) was carried out as described by Chhabra et al. (2021). Conventional RT-PCR that targets the 3' end of ORF1 and 5' end of ORF2 (polymerase-capsid junction) in Norovirus genome was performed with SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). For genogroup I typing, MON432 and G1SKR primers were used, and MON431 and G2SKR for genogroup II (

Table 2) at a final concentration of 0.3 μM each. Thermocycling conditions consisted of reverse transcription at 45 °C for 30 min, Taq polymerase activation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 68 °C for 1 min.

Final extension took place at 68 °C for 10 min. PCR products were visualized on 1X TBE 2% agarose gels (Promega, Madison, WI, USA) to confirm specific products of 579 bp and 570 bp for genogroup I and genogroup II, respectively. Next, samples were sequenced using Sanger technology by Macrogen. Consensus sequences were generated with Pregap4 and Gap4 tools present in the Staden package (Staden et al., 2000), and genotypes were assigned using the Human Calicivirus Typing Tool (https://calicivirustypingtool.cdc.gov/bctyping.html) (Tatusov et al., 2021). Finally, homology sequence analyses were performed using the *Basic Local Alignment Search Tool* (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 19 December 2023).

For Rotavirus molecular characterization, the protocol described by the World Health Organization (2009) was used with minor modifications. First amplification was performed with SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase with consensus primers for VP7 and VP4 fragments. In brief, oligonucleotides at final concentration of 0.4 µM were pre-mixed with each RNA sample and UltraPure distilled water (Invitrogen, Grand Island, NY, USA), and incubated at 97 °C for 5 min. Then, tubes were placed in a cooling rack for 2 min, and briefly centrifuged. The other master mix, consisting of 2X Reaction Mix and SuperScript III RT/Taq Enzyme Mix, was added to each tube to a final volume of 12.5 µL per reaction. Thermocycler was programmed under the following steps: reverse transcription at 45 °C for 30 min, Taq activation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 42 °C for 30 s, and extension at 68 °C for 1 min, 1 cycle of final extension at 68 °C for 7 min.

Second semi-nested amplification was performed with the same forward primers used in the first amplification, and specific reverse primer mixes for G genotypes (G1, G2, G3, G4, G9), and P genotypes (P[4], P[6], P[8], P[9], P[10], P[11]), respectively. For the detection of G12 genotype, second amplification was carried out with G12B and 9con2/R primers. Each

12.5 µL of reaction mix consisted of 2.5 U of AmpliTaq™ DNA Polymerase with Buffer II (Applied Biosystems, Carlsbad, CA, USA), 1X PCR Buffer II, 0.4 mM of dNTPs mix, 0.4 µM of each primer, 2 mM of $MgCl_2$, 1 µL of dilution 1/20 of first amplification product, and UltraPure distilled water. Thermocycling conditions consisted of initial denaturation at 95 °C for 2 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 42 °C, and 60 s at 72 °C for G genotyping or 45 s at 72 °C for P genotyping, with a final extension of 7 min at 72 °C. Then, PCR products were visualized on 1X TBE 2.5% agarose gels.

Genotypes were assigned according to fragment size. For G genotyping, PCR products of 159 bp, 245 bp, 465 bp, 404 bp, 111 bp, and 395 bp were designated for G1, G2, G3, G4, G9, and G12, respectively. For P genotyping, 346 bp, 484 bp, 268 bp, 392 bp, 584 bp, and 122 bp fragment sizes corresponded to P[8], P[4], P[6], P[9], P[10], and P[11], respectively.

Data Analysis

Statistical analyses were carried out using Stata version 13.0 (Stata Statistical Software, College Station, TX, USA) and GraphPad Prism version 10.1.0 (GraphPad Software, Boston, MA, USA). Viral frequencies (%) were calculated for shellfish and fecal samples with 95% confidence intervals (CI). Rate of viral co-infections were estimated for both sample types. Mann Whitney two-sample tests were performed to analyze differences between Cq values in shellfish vs. clinical samples, and in typeable vs. non-typeable samples, respectively. To evaluate the correlation between seasonality and viral positivity rates, precipitation levels in Esmeraldas, shellfish harvesting site, were kindly provided by INAMHI (Instituto Nacional de Meteorología e Hidrología). Chi-square test was used for assessing significant differences between number of positive samples in dry and rainy seasons. Significance level, α, was set at 0.05.

Results

Enteric virus frequencies in bivalve molluscan shellfish (BMS)

During the study period, 120 BMS (*Anadara tuberculosa* and *Anadara similis*) were tested for the presence of enteric viruses by RT-qPCR GVP assay. RVA was the most frequent virus detected in shellfish samples with a positivity rate of 36.7%, followed by NoV GI and AdV with 10% each, and less than 1% for NoV GII, and SaV, respectively. AstV was not found in any specimen. A set of samples producing flat curves for SaV qPCR tests were analyzed again by conventional PCR with universal primers. SaV positive control generated a 420 bp fragment; however, no amplification was observed for the tested samples which were reported as negative for SaV.

Table 4. Frequency of viral detection in Anadara tuberculosa and Anadara similis.

	***n*=120**	
	Positive *n* (%)	**95% CI**
Norovirus GI (NoV GI)	12 (10)	5.2 - 16.8
Norovirus GII (NoV GII)	1 (0.83)	0.02 - 4.6
Rotavirus (RVA)	44 (36.7)	28 - 45.9
Adenovirus (AdV)	12 (10)	5.2 - 16.8
Sapovirus (SaV)	1 (0.83)	0.02 - 4.6
Astrovirus (AstV)	0	--

Almost 75% of positive results accounted for a unique viral pathogen (41/55), whereas co-infections with two or three viruses represented 23.6% (13/55) and 1.8% (1/55), respectively. In particular, RVA and AdV was the most commonly occurring co-infection, followed by RVA and NoV GI. RVA – SaV and NoV GI – AdV contamination were found in one sample each. RVA-NoV GI-AdV triple detection was identified in one sample.

Monthly positivity rates (%) for enteric viruses did not remain constant during the sampling period (Figure 1, right y-axis). Concerning RVA detection, positive results were observed each month except for November 2022, and positivity rates ranged from 15.4% to 77.8%. Detection rates for NoV GI were estimated to fluctuate between 7.7% and 40%, and for AdV between 8.3% and 60%. Interestingly, over 4 consecutive months, February 2023 to May 2023, no positive AdV results were reported. SaV was only co-detected with RVA in one sample in August 2022, and NoV GII was found in a unique sample in February 2023.

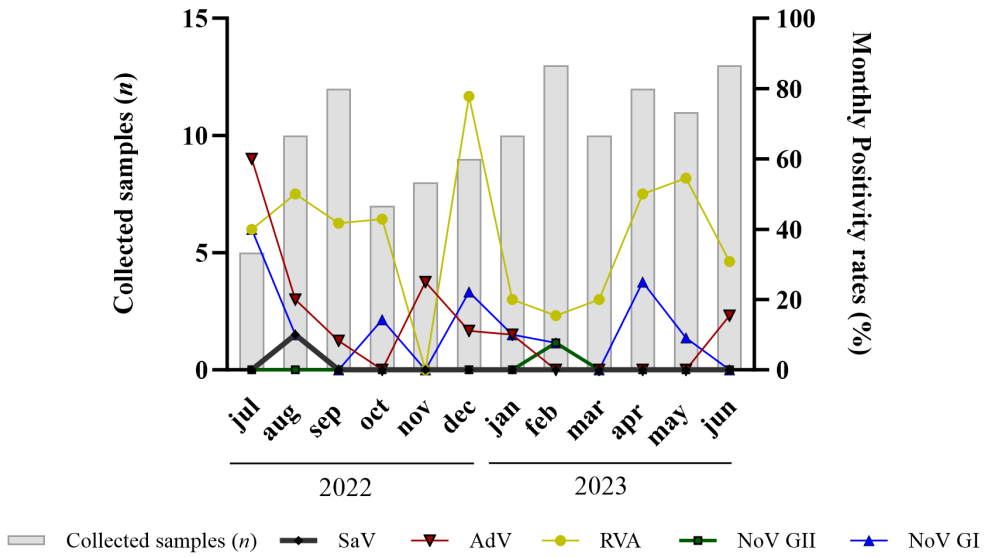

Figure 1. Monthly positivity rate of shellfish samples. As shown by right y-axis, monthly positivity rates for each viral pathogen fluctuated notably in mangrove cockles (*Anadara tuberculosa* and *Anadara similis*). While RVA and NoV GI were detected almost all year round, AdV exhibited four consecutive months with no positive samples. SaV and NoV GII were identified only one time. Left y-axis represents the amount of mangrove cockle samples (*n*) collected each month at one of the main seafood markets in Quito (each sample consisted of the digestive glands pooled from 10-12 specimens).

In addition, the correlation between viral frequency detection and precipitation levels in shellfish harvesting area (Esmeraldas) was analyzed using Chi-square test. The dry season took place between July and December 2022, and in June 2023, and showed average precipitation of 30.7 mm (95% CI 18.5-43.0), while the rainy season extended from January 2023 to May 2023 with higher average precipitation level (130.3mm, 95% CI 89.6-170.9). In the graph

below, the correlation between number of positive samples and season (dry vs. rainy) is presented. The only virus that exhibited a seasonal pattern was AdV. For instance, the number of positive samples were 90% higher during the dry season in comparison with the rainy one (*Chi-square test, $p<0.05$*). There were no statistical differences in the frequency of viral detection by season in the other viruses (Figure 2).

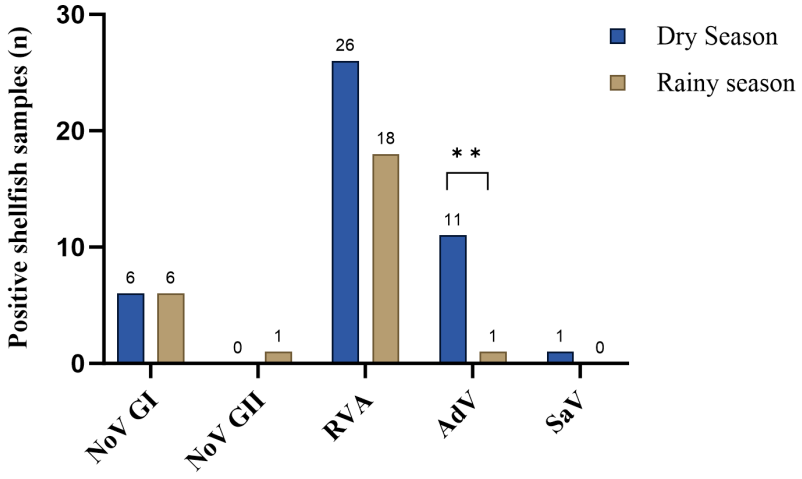

Figure 2. Distribution of positive samples during dry and rainy seasons. The number of mangrove cockles (*n*) positive for each viral pathogen in dry and rainy seasons is presented. Although RVA and AdV exhibited a higher number of positive samples in the dry season, statistical significance was only observed for AdV detection (*$p<0.05$*).

Enteric virus frequencies in clinical samples

During the sampling period, 80 stool clinical samples positive for one or more enteric viruses were received from HDLV. Approximately, 26% of samples (21/80) were co-infected with one or more (up to four) non-viral gastrointestinal pathogens. Co-infection rates were 12.5% for enteropathogenic *E. coli* (EPEC), 10% for enteroaggregative *E. coli* (EAEC), 3.75% for Shiga toxin–producing *E. coli* (STEC), 3.75% for *Clostridioides difficile* (A/B toxin), 2.5% for enterotoxigenic *E. coli* (ETEC), 2.5% for enteroinvasive *E. coli* (EIEC), 1.25% for *Campylobacter spp.,* 1.25% for *Giardia lamblia*, 1.25% for *Entamoeba coli*, and 1.25% for *Endolimax nana*. Remarkably, one sample presented three bacterial agents (ETEC, STEC, and

Clostridioides difficile), and another specimen four *E. coli* pathotypes (EAEC, EPEC, ETEC, EIEC).

Viral frequencies were determined using the GVP assay. By large, RVA was the most frequently viral agent detected in stool samples at HDLV during July 2022 and June 2023, with a positivity rate of 76.3% (61/80). NoV GII (20%) and AdV (13.8%) were detected at lower rates, and the rest of the viruses accounted for less than 10% of clinical samples (Table 5). Four of 80 samples were negative for all viruses, even though they tested positive for enteric viruses by Filmarray GIP at HDLV. Approximately two-thirds of the specimens (66.3%) were infected with one viral pathogen and viral co-infections were detected in 23 out of 80 samples. Indeed, 26.3% of samples (21/80) were positive for two enteric viruses. Most cases were combinations of RVA with NoV GII, AdV, or SaV. AstV – RVA and AdV – NoV GII detection were less common. Three viral agents were co-detected in 2.5% (2/80) of samples (RVA-NoV GII-AstV and RVA-NoV GI-SaV).

Table 5. Frequency of viral detection in clinical samples by GVP assay.

	***n*=80**	
	Positive n (%)	**95% CI**
Norovirus GI (NoV GI)	2 (2.5)	0.3 - 8.7
Norovirus GII (NoV GII)	16 (20)	11.9 - 30.4
Rotavirus (RVA)	61 (76.3)	65.4 - 85.1
Adenovirus (AdV)	11 (13.8)	7.1 - 23.2
Sapovirus (SaV)	6 (7.5)	2.8 - 15.6
Astrovirus (AstV)	5 (6.3)	2.1 - 13.9

Agreement between Filmarray GIP/LFIA and RT-qPCR GVP assay

There were 10 discordant samples that tested positive for NoV GI/GII by Filmarray GIP and NoV GI/GII negative by GVP. In 7 of these samples, other pathogens were found with GVP (6 RVA and 1 AdV). These discordant samples were re-tested for NoV by the

conventional PCR method described elsewhere (Chhabra et al., 2021); however, no amplification was observed, confirming the GVP negative results.

On the other hand, RVA testing generated 13 discordant samples, reported negative by GIP test and positive by GVP. Interestingly, one EPEC positive sample by GIP, was Rotavirus positive by GVP. The median Cq value (32.99, 95% CI 30.84-34.19) obtained for these discordant samples was higher than the median Cq of the 11 positive concordant samples (13.08, 95% CI 11.87-34.63) (Mann Whitney test, $p<0.05$).

Regarding AdV detection, 3 positive concordant samples were identified (Cq median 28.19, 95% CI 12.64-36.83), and 4 discordant samples that tested negative by GIP and positive by GVP (Cq median 35.99, 95% CI 17.74-36.91) with no statistically significant differences between Cq medians (Mann Whitney test, $p>0.05$). AstV had one discordant sample testing positive by GIP and negative by GVP which was co-infected with EAEC. Finally, SaV showed only concordant results.

To measure the level of agreement between both methods, kappa coefficients (κ) were calculated. In table 6, the results for the concordance analysis between Filmarray GIP and GVP are presented. For the detection of RVA, NoV GI/GII and AdV in clinical samples, moderate agreement was observed (κ coefficients: 0.40, 0.52, and 0.55, respectively). There was substantial agreement for AstV detection using both techniques (κ=0.79), and there was perfect agreement for SaV detection (κ=1). The results of this analysis allowed the rejection of the null hypothesis, so the amount of agreement is not due to chance ($p<0.05$).

Table 6. Agreement for viral detection between Filmarray GIP and RT-qPCR GVP tests.

	GVP					
	Negative	Positive	Agreement	Expected agreement	Kappa	*p*-value
GIP-NoV GI/GII						
Negative	17	0				
Positive	10	13	75%	47.4%	0.52	**0.0001**
GIP-RVA						

Negative	16	13				
Positive	0	11	67.5%	45.5%	0.40	**0.0007**
GIP-AdV						
Negative	33	4				
Positive	0	3	90%	77.63%	0.55	**<0.0001**
GIP-SaV						
Negative	37	0				
Positive	0	3	100%	86.13%	1.0	**<0.0001**
GIP-AstV						
Negative	37	0				
Positive	1	2	97.5%	88.25%	0.79	**<0.0001**

The agreement analysis between LFIA and GVP methods can be found in Table 7. Two discordant samples were observed for RVA detection, and both samples tested positive by LFIA but negative by GVP. In the case of AdV, there were three discordant samples which tested positive by GVP and negative by LFIA. The median Cq value of 12.66 (95% CI 11.96-13.71) obtained for positive RVA concordant samples indicates high viral load. For AdV, the three discordant results were reported with a median Cq value of 35.21 (95% CI 34.83-41.88). For RVA and AdV identification, moderate (κ=0.48) and fair (κ=0.38) agreement rates were obtained between the two methods, respectively. The hypothesis that the agreement between both techniques is due to chance can be rejected ($p<0.05$).

Table 7. Agreement for Rotavirus and Adenovirus detection between LFIA and RT-qPCR GVP tests.

	GVP					
	Negative	Positive	Agreement	Expected agreement	Kappa	*p*-value
LFIA-RVA						
Negative	1	0				
Positive	2	37	95%	90.38%	0.48	**0.0002**
LFIA-AdV						
Negative	36	3				
Positive	0	1	92.5%	88%	0.38	**0.001**

Enteric viral concentration in shellfish and clinical specimens

The values of quantification cycle (Cq) obtained during the detection of enteric viruses in shellfish and stool samples were analyzed for significant differences between them (

Figure 3). In general, it was observed that median Cq values for clinical samples were lower than shellfish Cq values with statistical significance only for RVA detection (Mann-Whitney test, $p<0.0001$). These results suggest that amplification in stool specimens was achieved in earlier cycles due to a higher viral load. Among all the viruses studied, RVA was characterized for the lowest median Cq values in both sample types with 13.44 (95% CI 12.68-15.40) in stool samples, and 35.09 (95% CI 34.30-35.99) in mangrove cockle samples.

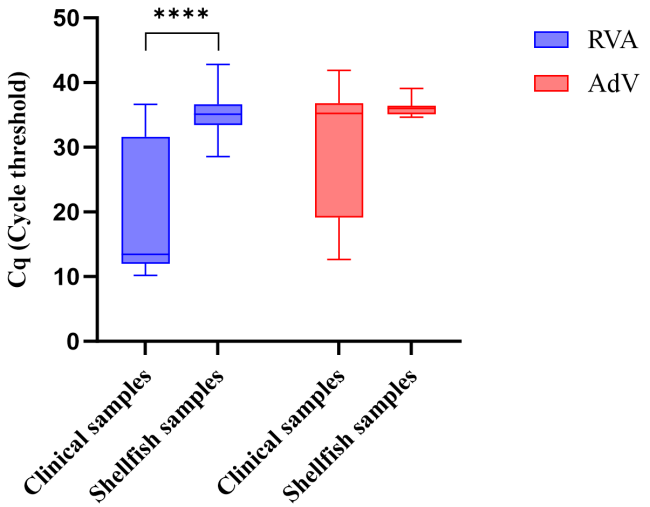

Figure 3. Cq values by sample type. Cq values were lower in fecal specimens than in shellfish samples with statistically significant differences for RVA (Mann-Whitney test, p<0.0001)

Rotavirus and Norovirus Genotyping

In total, 61 clinical specimens and 44 shellfish samples were reported positive for RVA by the GVP assay and were further analyzed by semi-nested RT-PCR to determine G and P-types. A high rate of fecal samples could be genotyped (51/61), while in shellfish only 18% (8/44) of the samples could be assigned a G-type. In clinical samples, Cq values were significantly lower in typeable samples than in non-typeable ones (Median 13.08; 95% CI

12.46-13.79 vs. Median 33.90; 95% IC 32.66-34.63, respectively, Mann Whitney test, *p<0.0001*), as shown in Figure 4. In contrast, no significant differences were reported in typeable vs. non-typeable shellfish samples characterized by median Cq values of 36.84 and 35.03, respectively.

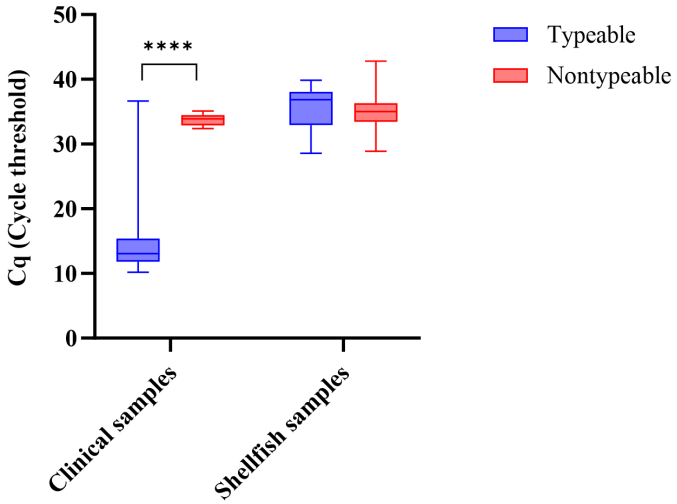

Figure 4. Cq values for RVA typeable and nontypeable samples according to specimen type (bivalve molluscan shellfish vs. fecal samples). It was observed that median Cq value of typeable clinical samples was significantly lower than median Cq value of the samples that could not be assigned a genotype (Mann Whitney test, $p<0.0001$).

Genotype frequencies according to sample type are presented in Table 8. The most common RVA genotype identified in fecal specimens over the study period was G3P[8] accounting for almost 50% of samples genotyped. Nine out of 61 samples were assigned to G3 genotype but were non-typeable for P-type. Three samples were characterized with mixed G-types, G2G12P[8], and one sample with G2G12 genotypes but non-typeable for P-type. G3P[8]P[11] was identified in seven samples, and P[10] in one sample. Only G3 type was found in shellfish specimens (8/44). A total of 46 samples were non-typeable strains for both P and G genotypes.

Table 8. Rotavirus G and P genotyping frequencies by sample type.

		Strains classification *n* (%)						
Sample type	**Number of samples examined in genotyping analysis**	**G3P[8]**	**G3P[8]P[11]**	**G3P[NT]**	**G2G12P[8]**	**G2G12P[NT]**	**G[NT]P[10]**	**NT**
Clinical samples	61	30 (49)	7 (11)	9 (15)	3 (5)	1 (2)	1 (2)	10 (16)
Shellfish samples	44	0 (0)	0 (0)	8 (18)	0 (0)	0 (0)	0 (0)	36 (82)

On the other hand, NoV GI and NoV GII positive samples detected by GVP assay were subjected to dual genotyping (polymerase-capsid) by conventional RT-PCR followed by Sanger sequencing. For clinical samples, median Cq values were 31.22 (95% CI 26.39-36.04) and 28.63 (95% CI 19.31-36.76) for GI and GII, respectively. In total, 14 out of 18 clinical samples yielded PCR products of 579 bp and 570 bp, for GI and GII, respectively, for which genotypes could be assigned (

Table 9). No PCR products were obtained for any shellfish specimens, for which the median Cq value was 40.85 (95% CI 39.53-42.09). Three fecal samples showed non-specific PCR products of different size than expected; consequently, genotypes could not be determined.

Six capsid-based genotypes were found in fecal samples obtained from HDLV as follows: GI.2 (1/14), GII.3 (1/14), GII.4 (6/14), GII.8 (1/14), GII.13 (1/14) and GII.17 (4/14). The majority of C-types accounted for GII.4, variants San Francisco and Sydney, followed by GII.17. Analysis of RdRp gene identified the next P-types: GII.P3, GII.P8, GII.P16, GII.P17, GII.P25, and GII.P31. Although the *Calicivirus typing tool* did not assign a P-type to GI.2 strain, BLAST analysis found high identity (>98%) with sequences from USA (MZ223426.1, MZ223425.1) and France (MK956176.1) classified as GI.2[P2]. Overall, recombinant strains carrying polymerase and capsid genes of different types, were observed during the study period, of which the novel variant GII.4 San Francisco with a GII.P31 polymerase was the most

common (22%). High nucleotide identities (>97%) with GII.4 sequences from China (KY580759.1), USA (OR262325.1, MT028542.1), United Kingdom (OR262327.1, OR262326.1), India (MW405903.1, LC769681.1), and Botswana (MW045406.1) were revealed by BLAST analysis.

The second most frequent genotypes were GII.4 Sydney[P16] and GII.17[P3] (11% each). Finally, the rare strains GII.3[P25] and GII.13[P16] were identified in one sample each. GII.3[P25] exhibited more than 98% of identity with sequences assigned the same genotype from China (OL451532.1, OL444828.1, OL444826.1) and USA (OP690505.1).

Table 9. Norovirus polymerase and capsid dual types.

		C-type		P-type	
Sample	**Length (nt)**	**% Nucleotide identity**	**Genotype**	**% Nucleotide identity**	**Polymerase type**
VE008	533	98.8	GII.4 San Francisco	97.6	GII.P31
VE009	545	97.7	GI.2	75.0	GI.P untypeable
VE010	533	98.8	GII.4 San Francisco	97.0	GII.P31
VE015	532	98.8	GII.17	96.5	GII.P17
VE017	533	98.0	GII.4 Sydney	97.0	GII.P16
VE034	532	98.8	GII.4 San Francisco	97.6	GII.P31
VE035	534	98.8	GII.4 San Francisco	97.6	GII.P31
VE047	534	98.8	GII.4 Sydney	97.6	GII.P16
VE054	534	100.0	GII.17	97.0	GII.P17
VE060	534	91.6	GII.17	93.6	GII.P3
VE061	534	94.8	GII.8	94.7	GII.P8
VE062	532	96.0	GII.3	93.6	GII.P25
VE063	534	91.6	GII.17	94.1	GII.P3
VE068	534	96.8	GII.13	94.1	GII.P16

Discussion

In the present study, it has been demonstrated the presence of multiple enteric viruses in black cockles, *Anadara tuberculosa* and *Anadara similis,* at the commercialization stage for

human consumption in Quito-Ecuador from July 2022 to June 2023. Preliminary work reported that cockles collected at markets in several Ecuadorian cities in the period July to December 2021, were contaminated with Adenovirus (49.5%), Norovirus GI (20.8%), Rotavirus (15.8%), Astrovirus (10%), Sapovirus (10%), and Norovirus GII (6.9%) (Ulloa, 2023). In our study, Rotavirus was the most frequently detected viral agent in shellfish accounting for almost 40% of positive samples, followed by Norovirus GI (10%) and Adenovirus (10%). With very low frequencies, Sapovirus and Norovirus GII were detected. In contrast to earlier findings, Astrovirus was not found. Interestingly, positivity rates for the same viral agent differ between consecutive years, suggesting that shellfish could be reflecting temporal variations in the circulation levels of enteric viruses in human population (Desdouits et al., 2023). Coastal water quality could be affected by sewage discharges from human and animal origin that contaminate molluscan shellfish because they filter large amount of water for feeding and respiration processes (Desdouits et al., 2023).

A recent review compared enteric virus prevalence in bivalve molluscan shellfish (oysters, clams, mussels, and cockles) of investigations conducted in various countries at different time periods (2000-2018). In summary, percentage of positive samples for each viral target varied within different countries and years, but Norovirus GI/GII was the most or second most found, whereas Rotavirus, Sapovirus and Astrovirus presented lower detection rates. Finally, Adenovirus prevalence fluctuated between 1.90% and 85% (Desdouits et al., 2023). High RVA positivity rates have been reported in oyster and sewage samples collected from 2014 to 2016 in Japan. Interestingly, 54% of oyster specimens were contaminated with wild RVA, whereas Rotarix and RotaTeq strains were also found in 14% and 31% of the samples, respectively, suggesting that vaccine strains could efficiently bioaccumulate in bivalve molluscan shellfish (Ito et al., 2021). Regarding Norovirus detection, the European Union baseline survey found that oysters were more likely to be contaminated with NoV in production

areas in comparison to dispatch centers reporting prevalences of 34.5% and 10.8%, respectively. Remarkably, some production areas were susceptible to higher contamination in terms of frequency and viral loads than others. Depending on the production area, oysters should be treated (by depuration or relaying) after harvesting to reach microbiological criteria in order to be placed on markets for human consumption (European Food Safety Authority (EFSA), 2019). Post-harvest depuration is not mandatory for bivalve mollusks in Ecuador, so further studies are needed to understand if enteric viruses prevalence differ significantly in the harvest sites and markets. Apart from microbiological contamination, coastal areas, *i.e.,* mangrove forests, are subjected to different pollutants coming from land and sea, such as domestic and industrial wastewaters, runoff, garbage, plastics, oil spills, fuel, heavy metals, chloramines, and detergents, among others (Pernia et al., 2019), so shellfish could also accumulate these contaminants and reflect water quality.

Some authors observed that NoV contamination of molluscan bivalve products occurred mainly in spring and winter seasons (Desdouits et al., 2023; Li et al., 2023), as well as outbreaks, that usually take place in winter months; however, in countries closer to the equator no clear seasonal distribution is expected (CDC, 2023). In Ecuador, precipitation levels give rise to two seasons characterized by fluctuation in different geographical regions in terms of annual amount and seasonal distribution. In general, in Coast region, the rainy season (winter) extends from the end of December to April-May, and the dry season (summer) takes place the rest of the year. Annual temperature maintains relatively constant, with a mean value of 25 °C, though temperature is warmer during the rainy season (Jørgensen & León-Yánez, 1999). Our results demonstrated an association between prevalence and seasonality only for Adenovirus*,* that was found more frequently in mangrove cockles during the dry season. Surveillance of enteric viruses in shellfish (mussels and oysters) collected in coastal areas at Southeastern Brazil have reported that adenovirus was more prevalent in winter (Keller et al., 2019) and

summer (do Nascimento et al., 2022), but no statistical differences were observed. Another investigation in South Korea showed that the amount of rainfall was significantly associated with the prevalence of adenovirus, norovirus GII, and F^+ coliphages in water samples from irrigation reservoirs. For instance, higher viral frequencies were obtained for moderate precipitation levels, between 20 and 60 mm, in comparison with precipitation levels greater than 60 mm (Wang et al., 2020). This is consistent with our results that suggest that lower precipitation levels promote adenovirus accumulation in mangrove cockles.

In order to compare the enteric virus strains circulating in BMS and human population, stool samples from patients with symptomatic gastroenteritis, collected in the same period as mangrove cockles, were analyzed. Clinical specimens tested positive for viral pathogen(s) by one of two methods, FilmArray Gastrointestinal Panel (GIP) or immunochromatographic assay (LFIA). With the purpose of using the same methodology for evaluating viral frequencies in both sample types, clinical specimens were re-assessed with the in-house RT-qPCR method (GVP). This approach revealed acceptable levels of agreement between HDLV methods and ours, but some discordant results were observed. Likewise, a study conducted by Piralla et al. (2017) obtained around 7.7% of discordant results when compared FilmArray GI Panel with the methodologies used by two laboratories to diagnose gastrointestinal pathogens, *i.e.*, molecular and immunochromatographic assays, culture, and microscopic observation.

Zhang et al. (2019) evaluated sensitivity and specificity of FilmArray Gastrointestinal Panel and singleplex RT-qPCR for the detection of pathogens in clinical diarrhea samples. When discordant results were obtained, additional confirmatory tests (Luminex xTAG GPP for Rotavirus and Norovirus GI/GII, and qPCR for the other viruses) were carried out. Overall, sensitivity was better for FilmArray GI than for RT-qPCR (95.6% vs 88.4%), while specificity was higher for RT-qPCR (99.9% vs 98.4%). Like our results, several samples tested positive for NoV GI/GII by FilmArray GI and negative by qPCR. In our study, these samples were

confirmed to be negative for NoV GI/GII by other PCR method described elsewhere (Chhabra et al., 2021). A total of 13 samples were RVA negative by Filmarray GI and positive by GVP with Cq values > 30. This suggested low viral load that could be below the limit of detection of FilmArray GI. In 7 of these samples, RVA genotypes could be assigned. For Adenovirus, there were 4 discordant samples between Filmarray GI and GVP. It is possible that serotypes different than F40/41 could be present in these samples as GVP can detect a broad range of serotypes (Wong et al., 2008). Sapovirus detection exhibited perfect agreement between both methods, and Astrovirus detection caused one discordant sample.

The comparison between LFIA and RT-qPCR (GVP) also revealed discordant samples as reported by others (Ye et al., 2015). In fact, for RVA detection, 2 samples were positive by LFIA and negative by GVP, and for AdV, 3 samples were negative by LFIA and positive by GVP. It is well known that sensitivity for qPCR (or PCR) is better in comparison with lateral flow immunochromatographic assays (Simo-Fouda et al., 2021); however, comparable rates of specificity have also been reported (Ye et al., 2015). A limitation of our study was the lack of an additional confirmatory test (gold standard) for each viral target that would allow us to estimate sensitivity and specificity. Furthermore, a small number of samples were analyzed, and nucleic acid quality could have been affected as we worked with thawed clinical specimens. Although the performance of the GVP, LFIA and Filmarray against one another was not formally assessed as such comparison was out of the scope of this study, the level of agreement between GVP and Filmarray GIP/LFIA methods was significantly acceptable, so we reported viral frequencies with our test and believe that the GVP would not have significantly impacted or biased virus detection in shellfish vs clinical samples.

Co-detection of viral pathogens in stool samples from patients with gastroenteritis has been reported previously. Among viral combinations, the most frequent were RVA-AdV, RVA-NoV and NoV-AdV, whereas other co-detections such as RVA-AstV and RVA-SaV were less

prevalent (do Socorro Fôro Ramos et al., 2021). We also found these virus-virus interactions with some differences, for example, NoV-AdV co-detection was less common, while RVA-SaV was a frequent combination. As stated by Makimaa et al. (2020), co-infections could happen with viruses from different families as described above, or multiple strains of the same viral genus. Likewise, we found mixed infections with different genotypes of Rotavirus, *i.e.,* G2+G12, and P[8]+P[11]. In shellfish samples, RVA-AdV and RVA-NoV were the most common mixed infections as in stool samples. We did not find NoV genogroup co-infections in any sample type; nevertheless, they have been reported elsewhere (Li et al., 2023; Ollivier et al., 2022)

In our study, NoV genogroup frequencies differed among shellfish and clinical samples. Ollivier and co-workers (2022) evaluated next generation sequencing (NGS) platforms for studying NoV genetic diversity in positive oyster samples collected between 2016 and 2018 as part of the European Union baseline survey. The authors compared these findings with human NoV sequences uploaded to the NoroNet network corresponding to gastroenteritis outbreaks in several European countries within the same period. Differences in NoV genogroup prevalence according to sample type were also observed. In fact, GII was detected in 88.5% of fecal samples and 58% of oysters, whereas GI was found in 11.5% of stool samples and 42% of oysters. Likewise, a recent meta-analysis developed by Li et al. (2023) estimates that the global prevalence of NoV GII in shellfish (oysters, clams, and mussels) is higher than GI prevalence (13% vs. 4%). Our results and Ulloa (2023) findings differ from these studies as GI was more prevalent than GII in black cockles (*Anadara tuberculosa* and *Anadara similis*). The expression of specific glycan ligands in shellfish tissues affects bioaccumulation and distribution of NoV strains and might explain the higher prevalence of GI in black cockles obtained in our study. In fact, Maalouf et al. (2011) showed that GI.1 viruses tend to concentrate preferably in digestive tissues through binding type A HBGA-like structures, while GII.4 strains attach to sialic acid-

containing ligands present in mantle and gills that could inhibit the progress to digestive tissues and subsequent bioaccumulation. In clinical samples higher prevalence of GII is consistent with Ollivier et al. (2022). These results suggest that in our case black cockles might be the vehicle for transmission of other enteric viruses different that noroviruses, as we found NoV GI in shellfish and NoV GII in patients with gastroenteritis. However, a limitation of our research is that we did not collect information about the food that could cause the disease in each participant.

As mentioned before, one objective of this investigation was to compare the Cq values in shellfish and stool specimens. Enteric viruses propagate in the intestinal tract mucosa, so they are present in large numbers in the stool of infected people (up to 10^{13} viral particles/g). On the other hand, in food matrices viral concentrations are low, near the limit of detection of molecular methods (Bosch et al., 2018). Accordingly, we observed lower Cq values in clinical samples which suggest a higher viral load in these samples. However, we only had enough data to perform these comparisons for Rotavirus and Adenovirus. Our findings revealed that Rotavirus had the highest viral loads in shellfish and fecal specimens, in contrast with previous results that showed greater concentration of Norovirus in shellfish samples in comparison with other enteric viruses (do Nascimento et al., 2022).

Prior to the implementation of Rotavirus vaccination in Ecuador, G9P[8] was the most frequent genotype during the period 2005-2006 (Endara et al., 2007; Naranjo et al., 2008), whereas by 2007, the substitution of G9 by G1 and G2 genotypes was observed in rural and urban settings (Hasing et al., 2009). Another study indicated that in stool samples from symptomatic cases collected from 2011 to 2012, post-vaccine implementation, G2P[4] genotype was the most prevalent followed by G9P[8] (B. Lopman et al., 2013). Changes in predominant genotypes have also been observed in other countries (Mijatovic-Rustempasic et al., 2022; Zhuo et al., 2023). Globally, six human genotype combinations (G1P[8], G2P[4],

G3P[8], G4P[8], G9P[8] and G12P[8]) cause the majority of Rotavirus infections (Amin et al., 2023). Although the most widely used vaccines, Rotarix™ and RotaTeq™, could offer protection against the six most common genotypes, lesser effectiveness has been observed against non-vaccine genotypes such as G12P[8] and G2P[4] (Amin et al., 2023; Esona et al., 2021).

Regarding Rotavirus genotyping, we found that the most common genotype in fecal samples collected in the 2022-2023 period was G3P[8]. A research in Colombia conducted by Martinez-Gutierrez et al. (2019) also showed this genotype as the most prevalent in children under 5 years old followed by G3P[9], which was not found in the present investigation. Our methodology could not detect the equine-like G3P[8], a human-animal reassortant strain that emerged in 2013 in Australia and Thailand which has been detected globally with different frequencies (Tacharoenmuang et al., 2020). As shown in a previous study by Ide et al. (2016), our results revealed mixed infection G2+G12 in 4 out of 61 stool samples, of which 3 were combined with P[8] type. In recent years, G12P[8] has been the predominant genotype in countries with vaccine implementation like USA and Canada, as well as in places where vaccines are not used yet (Mijatovic-Rustempasic et al., 2022; Zhuo et al., 2023). Finally, rare genotypes such as P[10] and P[8] + P[11] were also identified in our samples. P[10] has been detected sporadically around the world in combination with different G types, *i.e.,* G8 and G4 (Ghosh et al., 2013); however, in this study, a G type could not be assign. P[11] is a predominant genotype in bovines in combination with the G-types G6 and G10 (Shin et al., 2023; Varshney et al., 2002), whereas in a stool sample from alpaca, the G3P[11] combination has been detected (Garmendia et al., 2015). Remarkably, we did not find G1P[8], the strain present in Rotarix™ vaccine that has been used in Ecuador since 2007 (International Vaccine Access Center (IVAC), 2024). On the other hand, the large proportion of G3P[8] in our samples suggest that

RotaTeq™ should be implemented in the National Immunization Program as it offers protection against G3P[8] (Amin et al., 2023).

In this investigation, G3 was the only genotype reported in mangrove cockles sampled from July 2022 to June 2023. Kittigul et al. (2015) have found G1, G3, G9, and G12 Rotavirus strains in shellfish samples (oysters, mussels, and cockles) collected from 2011 to 2012 at local markets in Bangkok, Thailand. In that study, G3 was the most frequent genotype with sequences from human and animal origin (equine, canid, and porcine). Recent evidence suggests that the distribution of genotypes in shellfish and stool samples could be similar. For instance, the most common Rotavirus genotypes in shellfish were also present in clinical samples, even though some genotypes were more abundant in each sample set (Hoque et al., 2022). We were not capable to know much about genotype distribution in mangrove cockles because only a low proportion of specimens could be genotyped in the case of Rotavirus, and no shellfish sample was genotyped in the case of Norovirus. Almost all samples had Cq values greater than 30, so the viral loads could be below the limit of detection of the RT-PCR used for genotyping assays (Mijatovic-Rustempasic et al., 2016).

Norovirus genotypes can exhibit great variability in different parts of the world (Ollivier et al., 2022), so molecular epidemiology surveillance at a local level is highly recommended. Norovirus genotype distribution has been reported previously in Ecuador in children with diarrhea and in healthy controls. For instance, a study carried out by Lopman et al. (2015) with samples collected from 2006 to 2009, found GII.4, GII.1, GI.3, GII.2, and GII.23 genotypes in children ≤ 3 years old. GII.4 viruses were the most prevalent with the following variants: New Orleans, Den Haag, Sydney, and Osaka. Another investigation reported that GII.6 was the most frequent genotype, followed by GI.3 and GII.16 in stool specimens from children under five years old sampled between 2011 and 2012 (Gastañaduy et al., 2015). In agreement with Lopman et al. (2015), GII.4 were the most common genotype in the present investigation. Since

2002, the predominant circulating norovirus genotype has been GII.4 worldwide. New GII.4 emergent variants have replaced the predominance of the previous one every two to four years. However, since 2012 GII.4 Sydney has been the most prevalent strain (CDC, 2023). Recently, a new variant named GII.4 San Francisco was reported in stool samples collected from 2017 to 2022 in USA, United Kingdom, Gabon, and South Africa (Chhabra et al., 2024). Remarkably, the present study identified GII.4 San Francisco variant with GII.P31 P-type as the most prevalent in stool samples collected during 2022-2023 from symptomatic patients at HDLV, suggesting a high circulation of this strain within South America.

GII.P31 polymerase was associated with the emergence of the epidemic GII.4 Sydney variant in 2012 (Tohma et al., 2021). Then, in 2015 GII.4 Sydney with a novel GII.P16 polymerase emerged, and in recent years this recombinant strain became commonly detected in some countries such as USA, France, Australia, and New Zealand replacing the predominance of previous GII.4 Sydney[P31] (Ao et al., 2023; Barclay et al., 2019). In the present investigation, 2 out of 14 samples were assigned to GII.4 Sydney[P16]. In addition, GII.P16 polymerase with GII.13 capsid were found in one sample. Barclay et al. (2019), reported GII.13 association with an extant GII.P16 that has been circulating in the USA since 2010 at very low frequencies. Another rare recombinant genotype identified in the present study was GII.3 [P25], which has also been reported in China and USA in 2021 and 2022, respectively (Zhang et al., 2023).

We also identified the uncommon genotypes GII.8[P8] and GI.2 in one sample each. GII.8[P8] is an uncommon strain that rarely undergoes intergenotypic recombination (Chuchaona et al., 2024). It has caused gastroenteritis outbreaks and sporadic cases with low prevalence. In fact, GII.8[P8] has been detected previously with a low positivity rate (0.5%) in stool samples from Brazilian children younger than 10 years (Hernandez et al., 2018), and in one sample in a large outbreak of acute diarrhea disease (ADD) probably caused by sewage-

contaminated water in Southern Brazil (Fumian et al., 2023). GI.2 capsid was found with an untypeable P-type; however, BLAST analysis revealed high identity (>98%) with PII.2 polymerase type. A global surveillance network reported norovirus genotype distribution in children with acute gastroenteritis during 2016-2020, showing a very low frequency of GI.2[P2] strain (Cannon et al., 2021), in agreement with our results.

The second most prevalent capsid type we found was GII.17. This norovirus strain, known as Kawasaki 308–like 2014, emerged in China and Japan in 2014-2015 during the winter season and became a common cause of gastroenteritis outbreaks in those countries, coming to replace GII.4 predominance. GII.17 strain exhibited 2 amino acid insertions in the most surface-exposed region (P2 subdomain) of VP1 protein which is associated with altered antigenic properties and host susceptibility (Chan et al., 2017a). This strain was identified with a new polymerase type, assigned as GII.P17, that is genetically related to GII.P3 and GII.P13 (Tohma et al., 2021). Interestingly, previous GII.17 strains with GII.P13, GII.P16, GII.P3, and GII.P4 polymerases did not became prevalent (Chan et al., 2017b). In the present study, GII.17[P17] and GII.17[P3] strains were found. The circulation of GII.17[P17] within South America has been reported by previous investigations (Degiuseppe et al., 2020; Sarmento et al., 2023). To conclude, norovirus genotype distribution exhibited great variability as we found 6 capsid genotypes and 6 polymerase types in a small set of samples.

Conclusions

The current study identified enteric viruses in black cockles (*Anadara tuberculosa* and *Anadara similis*) from mangrove forests located at San Lorenzo-Esmeraldas and commercialized at seafood markets in Quito. Although the presence of viral genome in shellfish is not an estimation of the actual infectious risk, these findings support the necessity to implement surveillance systems by Ecuadorian regulatory agencies in order to improve the

microbial safety of shellfish and other kind of foods associated with foodborne illnesses. Black cockles should not be consumed raw or undercooked, and some measures such as depuration after harvesting should be applied by policymakers to avoid the dissemination of gastrointestinal pathogens. Black cockles, as other bivalve mollusks, represent a good tool to investigate the level of pollution in the environment. We found that the most prevalent enteric viruses were rotavirus and norovirus in shellfish and stool samples from patients with gastroenteritis. Fecal clinical specimens demonstrated high genotype diversity especially for norovirus. Additionally, the distribution of genotypes in shellfish and clinical samples was similar as we found G3 as the most common Rotavirus G-type in both sample types. A low proportion of shellfish samples could be genotyped, so future research should apply NGS technology to study genotype distribution of enteric viruses in shellfish. Our study revealed the importance of continuous monitoring systems not only in food and stool specimens, but also in environmental samples (One Health approach).

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