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Molecular detection of enteric viruses in black shellfish from Ecuador, 2021

Tesis en torno a una hipótesis o problema de investigación y su contrastación

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DEDICATORIA

A mi familia, pilar fundamental en mi vida

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RESUMEN

Las enfermedades trasmitidas por alimentos son un grave problema de salud en el mundo, atribuyéndose más de la quinta parte a virus entéricos como agente etiológico. Al menos el 60% de los brotes asociados al consumo de alimentos reportados en Europa son causados por el consumo de alimentos provenientes del mar; con los virus como protagonistas en el mayor número de casos. Los moluscos bivalvos son reconocidos reservorios y vectores de patógenos humanos gracias a su capacidad de concentrar patógenos y toxinas presentes en el agua circundante del medio en que viven, misma que puede contaminarse como consecuencia del mal manejo de desechos humanos y aguas residuales. En Ecuador, la contaminación de alimentos con virus es un área poco explorada, por tanto, este estudio descriptivo tuvo como objetivo determinar la presencia de seis virus entéricos en conchas negras expendidas en varios mercados del país durante el segundo semestre del año 2021. Se analizó 101 muestras de conchas negras mediante qRT-PCR para detectar material genético de NoVGI, NoVGII, HAdV, RV, HAstV y SaV. El 69.3% de las muestras de concha negra analizadas presentaron al menos uno de los seis virus estudiados; 38.6% mostró contaminación con un solo virus siendo HAdV el más común (49.5%), seguido de NoVGI (20.8%). En 19.8% de las muestras se detectó dos virus, siendo RV+HAdV la combinación más frecuente. El 8.9% de las muestras fueron positivas para tres y 1.9% para cuatro virus de los seis analizados. Se observó estacionalidad en la detección de HAdV, reportándose asociación con época seca. En conclusión, este estudio demuestra presencia de virus patógenos humanos en conchas comercializadas en el país, sugiriendo el riesgo de consumir este alimento crudo.

Palabras clave:

Virus entéricos, Conchas negras, Moluscos bivalvos, Enfermedades transmitidas por alimentos, Norovirus, Astrovirus, Sapovirus, Adenovirus, Rotavirus

ABSTRACT

Foodborne diseases (FBD) are a serious health problem in the world, and more than a fifth of FBD are attributed to enteric viruses as the etiological agent. At least 60% of the FBD outbreaks reported in Europe are caused by the consumption of seafood; with viruses as protagonists in most of the cases. Bivalve mollusks are recognized reservoirs and vectors of human pathogens thanks to their ability to concentrate pathogens and toxins present in the surrounding water of the environment in which they live, which can be contaminated because of the mismanagement of wastewater. In Ecuador, food contamination with viruses is a little explored area, therefore, this descriptive study aimed to determine the presence of six enteric viruses in black shells sold in local markets during the second semester of 2021. We analyzed 101 samples of black shells using qRT-PCR to detect NoVGI, NoVGII, HAdV, RV, HAstV, and SaV. At least one virus was detected in 69.3% of the black shell samples analyzed, and 38.6% showed contamination with a single virus, HAdV was the most common (49.5%), followed by NoVGI (20.8%). Two viruses were detected in 19.8% of the samples, being RV + HAdV the most common combination. Three viruses were detected in 8.9% of the samples, and four viruses were detected in 1.9%. Seasonality was observed in the detection of HAdV, reporting an association with the dry season. In conclusion, this study demonstrates the presence of human pathogenic viruses in shells marketed in the country, suggesting the risk of consuming this raw food.

Keywords:

Enteric viruses, Black shellfish, Bivalve shellfish, Foodborne disease, Norovirus, Astrovirus, Sapovirus, Adenovirus, Rotavirus

TABLA DE CONTENIDO

BLACK SHELLFISH

Black shellfish are bivalve mollusks belonging to the Mollusca phyla, the Bivalvia class, Arcidae family, and Anadara genus (Schoch CL, 2020) that inhabit mangrove ecosystems through Central and South America (Ramos, 2019). This genus includes several species, being *Anadara tuberculosa* and *Anadara similis* the most important species commercially (Prado et al., 2020) and gastronomically (Orden-Mejía et al., 2021) in Ecuador. Esmeraldas, El Oro, and Guayas provinces are the major producers of black shellfish, which are collected from San Lorenzo, Muisne, Jambelí, Puerto El Morro, Puerto Jeli, Puerto Hualtaco and Puerto Bolivar (Moreno, 2017).

ENTERIC PATHOGEN

Kolling defines “enteric pathogen” as any microbe able to cause enteric disease, and enteric disease is defined as ≥3 unformed stools per day and any documented intestinal infection associated with disrupted intestinal absorptive and/or barrier function (Kolling et al., 2012). Enteric pathogens include viral, bacterial, or protozoan organisms (Kolling et al., 2012) which are responsible for more than 90% of foodborne illnesses worldwide with NoV being the leading agent causing more than 20% of foodborne cases reported in 2010 (Choi et al., 2016).

Escherichia coli as fecal indicator

Escherichia coli (*E. coli*) is a gram-negative bacterium that belongs to the Enterobacterales order (Janda & Abbott, 2021). This bacterium is predominantly found in the gastrointestinal tract of humans and warm-blooded animals and has been widely used as an indicator organism in water and food to assess their food safety. According to Food and Drug Administration (FDA), high *E. coli* counts are associated with the presence of pathogens and it shows recent fecal contamination or unsanitary food processing (Peter Feng, Stephen D.

Weagant, Michael A. Grant, 2020). The Most probable Number technique (MPN) is the FDA-recommended method for *E. coli* enumeration in shellfish. This quantitative method estimates viable bacteria in food samples (Chandrapati & Williams, 2014). International (EU, 2004) and national criteria (NORMA PARA MOLUSCOS BIVALVOS VIVOS Y MOLUSCOS BIVALVOS CRUDOS, 2013) regarding *E. coli* counts for seafood describe <230MPN/100g as the cut-off value for bivalve shellfish commercialization.

Pathogenic strains of *E. coli* have been identified in bivalve shellfish (Ekici & Dümen, 2019; Miotto et al., 2019), but those strains cannot be detected by the conventional methods for *E. coli* count in food.

ENTERIC VIRUSES

Enteric viruses are pathogens found in the human gastrointestinal tract, excreted in human feces and transmitted by the fecal-oral route (Cook, 2016). These viruses transmitted by contaminated water and food, via person to person, via contact with contaminated surfaces and through aerosols (D'Souza & Joshi, 2015) cause gastroenteritis, hepatitis (enterically transmitted), and diseases that affect organs different to the digestive tract (eyes, respiratory system, central nervous system) (Cook, 2016). Caliciviruses, rotaviruses, adenoviruses, astroviruses, and coronaviruses are considered viral pathogens, and have been associated with foodborne disease outbreaks worldwide (Cook, 2016). WHO describes NoV as the major cause of foodborne diseases reported in 2010 (Choi et al., 2016).

NOROVIRUS

Norovirus (NoV), formerly known as the Norwalk-like virus, was first described in 1972 in stool samples from a school gastroenteritis outbreak in Ohio (Ludwig-Begall et al.,

2021). NoV is a member of the *Caliciviridae* family. It is a small, non-enveloped, linear, positive-sense, single-stranded RNA virus with a length of 7.3-7.5 kb and a 27-30 nm diameter. The NoV genome contains 3 ORFs; except for Murine Norovirus (MNV). ORF1 encodes a polyprotein containing RdRp (RNA-dependent RNA polymerase). ORF2 and ORF3 encode capsid proteins VP1 and VP2, respectively. A virulence factor is encoded by the extra ORF4 of MNV (Ludwig-Begall et al., 2021).

This virus is genetically and antigenically diverse. It includes 10 genogroups (GI-GX) and 49 genotypes according to the current classification system (Chhabra et al., 2019). NoV GI, GII, GIV, GVIII, and GIX have been described as human pathogens (Ludwig-Begall et al., 2021).

Currently, this virus is recognized worldwide as the leading cause of gastroenteritis (both, sporadic and epidemic) and is responsible for a significant economic ($4.2 billion annually) and health burden (219 000 deaths annually) (Ludwig-Begall et al., 2021). The global prevalence of NoV as the etiologic agent of acute gastroenteritis (AGE) is reported as 16% in the world, with a higher prevalence of 22% in South America (Liao et al., 2021). In Ecuador, NoV was described in 18% of children with and without diarrhea, and 30% of children were infected by this agent before the age of 3 years (Gastañaduy et al., 2015). Globally, NoV GII.4 is the predominant genotype associated with 55-85% of outbreaks in recent years (Ludwig-Begall et al., 2021).

Human NoV attaches to the host cell using ABH and Lewis histo-blood group antigens as ligands (A. Ford-Siltz et al., 2021). Likewise, human secretor status plays an important role in the susceptibility to NoV infection, being NoV GII.4 disease more prevalent

in secretors who have a FUT2 active allele (Currier et al., 2015; Frenck et al., 2012), and infection with NoV non-GII.4 associated with no secretors (B. A. Lopman et al., 2015)

Human NoV is transmitted through the consumption of contaminated food and water, or person-to-person contact (Ludwig-Begall et al., 2021). The infectious dose is <18 viral particles (Pringle et al., 2015; A. N. Williams et al., 2021). Although the disease is mostly self-limiting, it can be severe and persistent in elderly, malnourished, and immunocompromised patients (Cook, 2016; Ludwig-Begall et al., 2021). The incubation period is short: 24-48h, and the clinical symptoms (abdominal cramps, nausea, chills, mild fever, bloating, myalgia) last for 2-3 days with a high number of viral particles being shed in feces and vomit. Dehydration is the most common complication and may require rehydration therapy (Ludwig-Begall et al., 2021).

Shellfish have been identified as a vehicle for human NoV transmission in 61% of global outbreaks; with oysters as the most commonly implicated shellfish (89%) (Hardstaff et al., 2018). According to a systematic review, the prevalence of human NoV in shellfish worldwide was 29% during the last 20 years, with GII.4 identified as the most common genotype (Li et al., 2023).

ROTAVIRUS

Rotavirus (RV) was first described in 1973 from stool samples from children (Olaniran & Discipline, 2022). This virus belongs to the Reoviridae family and contains a non-enveloped, double-stranded RNA consisting of 3 concentric capsids surrounding a genome of 11 segments. The RV genome is 16-27 kb and its particle measures 60-80 nm in diameter. Six structural viral proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and six non-structural viral proteins (NSP1, NS2, NSP3, NSP4, NSP5 and NSP6) are encoded by the RV

genome (Olaniran & Discipline, 2022). Ten species of RV (A-J) have been described according to sequence and antigenic differences of VP6, with RVA being the most common cause of AGE in children. 32 G genotypes and 47 P genotypes have been assigned based on the sequence of segments VP7 and VP4 (Crawford et al., 2017).

Before the introduction of vaccination against RV, this virus was described as the most common cause of non-bacterial AGE and was responsible for more than 500 000 deaths in 2013, with 45% occurring in children <5 years (Clark et al., 2017). The global prevalence of RV in feces from people with diarrhea is 7.6% in people older than 5 years (Arakaki et al., 2021; Purpari et al., 2019). In Ecuador, the prevalence of RV in children's stool (determined by immunochromatographic technique) was described as around 16-19% (B. Lopman et al., 2013; Simaluiza-Masabanda et al., 2016).

RV is transmitted through the fecal-oral route, person-to-person contact, and through contaminated fomites (Crawford et al., 2017) with 10 viral particles being sufficient to cause human disease (Cook, 2016).

The virus requires HBGAs and sialic acids to attach to human enterocytes (Amimo et al., 2021) and the disease is characterized by diarrhea, vomiting, malaise, and fever that persists for 3-8 days with dehydration as the most important complication (Crawford et al., 2017). A high number of viral particles are shed on human feces (10^{12} per g) (Cook, 2016).

Shellfish has been analyzed worldwide to determine RV RNA, showing a 9% prevalence in bivalves in Italy (Fusco et al., 2019) and 25-60% in Brazil (Keller et al., 2019).

ASTROVIRUS

Human Astrovirus (HAstV) was first isolated in 1975 from stool samples from children. HAstV is a small, non-enveloped, positive-sense, single-stranded RNA virus. Viral particles are 28-30 nm in diameter, and the genome is 6.8-7.8 kb in length (Bosch et al., 2014). HAstV belongs to the family *Astroviridae* which includes two genera, Avastrovirus (avian virus with 3 genotypes) and Mamastrovirus (mammalian virus with 19 genotypes) (Cortez et al., 2017). AstV contains 3 ORFs; ORF1a encodes a serine protease and VPg; ORF1b encodes RdRp, and ORF2 encodes the capsid protein (Cortez et al., 2017). AstV is categorized into 8 classic types (HAstV1-8) and novel genotypes including MLB, VA, and HMO (Cortez et al., 2017).

HAstV is an emerging cause of sporadic and epidemic AGE showing a global prevalence of 4.2% in children with AGE; with HAstV1 being the most prevalent genotype (Razizadeh, Pourrostami, et al., 2022). This virus is described as the third most common agent associated with viral AGE after NoV and RV, causing 15-20% of non-bacterial gastroenteritis in humans (Cook, 2016).

The cellular receptors that promote HAstV infection in human cells are currently unknown (Bosch et al., 2014; Cortez et al., 2017) as the infectious dose, however, it is supposed to be very low (Pexara & Govaris, 2020). The incubation period appears to be 24-36 hours (Fu et al., 2023).

Viral transmission takes place through the fecal-oral route with food and water acting as vehicles (Bosch et al., 2014). In the last 20 years, 32 outbreaks associated with HAstV have been documented around the world, the largest one (4 700 people affected) occurred in Japan in 1991 and was associated with contaminated food (Fu et al., 2023).

The disease is characterized by self-limiting diarrhea (lasting 1-4 days), vomiting, fever, and abdominal pain (Cook, 2016; Cortez et al., 2017). In immunocompromised patients, the infection can become systemic (Bosch et al., 2014). A high number of viral particles are shed on stool (10^{10}-10^{11} per g) (Bosch et al., 2014).

SAPOVIRUS

Sapovirus (SaV), formerly called the Sapporo-like virus, was first described in 1976 from stool samples of children in Sapporo, Japan (Razizadeh, Khatami, et al., 2022). SaV are non-enveloped viruses of 7.1-7.7 kb and 41-46 nm in diameter belonging to the family *Caliciviridae* (Diez Valcarce et al., 2021). Its positive-sense, single-stranded RNA genome contains 3 ORFs; the RdRp and the major capsid protein (VP1) are encoded by ORF1, a small protein is encoded by ORF2, and ORF3 encodes for a protein whose function is unknown (D'Souza & Joshi, 2015; Razizadeh, Khatami, et al., 2022). This virus is classified into 5 genogroups (GI-GV) and 19 genotypes; SaV GI, GII, GIV, and GV are responsible for human infections (Razizadeh, Khatami, et al., 2022).

SaV has been described as the etiologic agent of 3.4% of AGE according to a systematic review conducted by Diez in 2021, with children less than 5 years of age being the most affected (4.4%), and genogroup GI as the most commonly detected among AGE cases (Diez Valcarce et al., 2021). However, Rohuani found higher frequency considering only 8 countries; showing a prevalence of 24.7% in diarrheal stool samples with 60% of children infected during the first 2 years of life (Rouhani et al., 2022). Viral transmission is associated with the fecal-oral route, person-to-person contact, and contaminated water or food (Xuefeng Chen, et al et al., 2011). Shellfish are recognized as an important source of SaV infection

(Xuefeng Chen, et al et al., 2011), Spain has reported a prevalence of 21,8% of SaV in mollusks over 20 years (Romalde et al., 2018).

Symptoms include fever, diarrhea, and vomiting. The infectious dose is not well established but high levels of the virus can be excreted in the stool ($l0^6$-10^{11} per g) (Cook, 2016; Oka et al., 2015). SaV immunology has not been clearly described, however, bile acids seem to have a play role in porcine SaV entry and replication (Oka et al., 2015).

ADENOVIRUS

HAdV was first isolated in 1953 (Shieh, 2022) from human adenoids. They belong to the *Adenoviridae* family and the Mastadenovirus genus. HAdV is classified into seven species (A-G) and organized into more than 100 types (do Nascimento, Fialho, et al., 2022). This virus has a double-stranded linear DNA genome that is 70-100 nm in diameter and 26-45 kb in size (do Nascimento, Fialho, et al., 2022; Greber & Flatt, 2019). A wide variety of diseases can be caused by HAdV according to its tissue tropism: respiratory, gastrointestinal, or ocular (Shieh, 2022). HAdV type F40 and F41 are recognized worldwide as agents of acute gastroenteritis in children (do Nascimento, Fialho, et al., 2022) reaching a 15-25% prevalence in AGE cases (do Nascimento, Fialho, et al., 2022; Shieh, 2022). Symptoms of the HAdV AGE include acute abdominal pain, diarrhea, nausea, vomiting, and fever. The disease is often self-limited lasting 2-3 days (Shieh, 2022) with a high number of viral particles shed on feces (10^{11} per g) (do Nascimento, Fialho, et al., 2022). This virus is transmitted by direct contact, fecal-oral route, or, even respiratory and environmental route (Cook, 2016; Shieh, 2022).

Using a mathematical model, the mortality due to HAdV was estimated and HAdV40/41 were described as responsible for 53 613 deaths in children under 5 years of age

with AGE during the year 2016 (Troeger et al., 2018). A wide range of receptors has been described for HAdV infection regardless of its cellular tropism. Enteric HAdV seems to use CAR (Coxsackievirus AdV receptor) as an important human receptor (Greber & Flatt, 2019). The presence of HAdV in water and shellfish is considered a potential indicator of viral contamination and/or fecal pollution (Nagarajan et al., 2022; Rodriguez-Manzano et al., 2014).

DIAGNOSIS OF ENTERIC VIRUS

For clinical diagnosis, several methods have been used including electron microscopy, serological or molecular assays, and cell culture techniques. Cell culture is the gold standard, however it is laborious and time-consuming. Electron microscopy has low sensitivity and requires expensive equipment and trained personnel. Serological assays include ELISA and immunochromatography while RT-PCR is the most widely used molecular assay (Malik et al., 2019).

When food matrices are analyzed, viral detection becomes more difficult due to the low viral load and the complexity of the matrix, which can have an inhibitory effect. Molecular methods are commonly used for the detection of viruses from food, mainly PCR-based methods which are sensitive and quick (Malik et al., 2019). This technique requires a virus process control to determine the efficiency of the virus extraction, and an RNA control to evaluate the inhibition or the amplification as described in the ISO 15216-2017 (International Organization for Standardization, 2017).

PREVENTION OF ENTERIC VIRAL DISEASES

Regarding preventive measures to reduce enteric viral diseases in humans, a wide range of actions have been described. To prevent contamination of food and water,

reinforcing the use of clean water during the growth, washing, and preparation of produce is recommended (Ezzatpanah et al., 2022). Likewise, the improvement in hygiene and sanitation is an effective measure for reducing the risk of viral FBD (Crawford et al., 2017).

Timely detection of viral contamination in water and food (D'Souza & Joshi, 2015) and the use of physical (heat), chemical, and purification processes to reduce the viral load on food (D'Souza & Joshi, 2015) contribute to prevention. Consumers are encouraged to avoid the consumption of raw or undercooked bivalves (Guix et al., 2019).

RV vaccines are available worldwide; however, vaccines against NoV, HAstV, and SaV are not currently available. Some promising candidates for NoV vaccination are in the clinical phases (Santos-Ferreira et al., 2021).

PART II: ORIGINAL ARTICLE

MOLECULAR DETECTION OF ENTERIC VIRUSES IN BLACK SHELLFISH FROM ECUADOR, 2021

INTRODUCTION

According to the World Health Organization (WHO), "Foodborne illnesses are usually infectious or toxic in nature and caused by bacteria, viruses, parasites or chemical substances entering the body through contaminated food"(World Health Organization, 2022). Foodborne diseases (FBD) resulted in 600 million cases and 420 000 deaths in 2010, with NoV being the leading cause of gastroenteritis (120 million cases) and accounting for a high mortality burden globally (35 000 deaths) (Choi et al., 2016). Children aged <5 years old account for 40% of the disease burden (Choi et al., 2016), and diarrheal disease is one of the leading causes of death in this age range (Abbafati et al., 2020; World Health Organization, 2017).

In addition to NoV, HAdV, HAstV, SaV, and RV have also been described as agents of food and waterborne diseases (Cook, 2016). Except for HAdV, the other four enteric viruses are included in the list of 31 major foodborne pathogens identified by the CDC (Scallan et al., 2011). Contamination of water and food, which contributes to the fecal-oral transmission of enteric viruses, is facilitated by the low number of viral particles capable of causing human infection (18 for NoV), the high number of viruses shed in feces (up to 10^{10} per gram), and the viral stability in the environment (Bosch et al., 2018). Viral contamination of food can occur at two different stages of production: preharvest or postharvest. Preharvest contamination results from environmental contamination associated with wastewater and is

common for seafood and vegetables. Postharvest contamination is common for ready-to-eat foods and is associated with bad hygiene practices (Bosch et al., 2018).

Bivalve shellfish have been widely described as reservoirs and vectors of human pathogens and are responsible for more than 60% of foodborne outbreaks worldwide (EFSA, 2015; Hardstaff et al., 2018). This fact can be attributed to their feeding mechanism through filtration which allows them to concentrate pathogens from the surrounding water (Yang et al., 2021). A long list of microorganisms such as viruses (including human calicivirus NoV and HAstV, HAV, HEV, RV, HAdV, EV (Lees, 2000); bacteria (*Vibrio* spp., *Salmonella* spp., *E. coli* Shigellae, *Aeromonas* spp. *Plesiomonas* spp.) (Adeel A Butt, Kenneth E Aldridge, 2004) and parasites (both protozoa and helminths) (Butt et al., 2004), are transmitted to humans by ingesting contaminated raw shellfish; which can be an important issue in Ecuador because raw or undercooked shellfish are consumed frequently (Orden-Mejía et al., 2021).

E coli count has been widely used as a marker for food safety, however, viruses have been shown to be cleared from shellfish tissue less efficiently than bacteria. Therefore, bacteria counts could not always reflect the viral risk of shellfish for human consumption (Sharp et al., 2021).

Viral FBDs are at the top of the food safety priorities established in the EU (Rowe & Bolger, 2017). In addition, the development of policies and programs to strengthen food safety is encouraged by the WHO (Choi et al., 2016), and assessment and control of foodborne pathogens is one of the key issues (Bosch et al., 2018). In Ecuador, SIVE-Alerta regularly reports the agents associated with FBDs but focuses on limited pathogens considering only *Salmonella* spp., *E. coli* Shigellae, and Hepatitis A virus (HAV) for

mandatory reporting, even though these agents represent only a quarter of the FBD cases identified (Subsistema de Vigilancia SIVE-ALERTA MSP, 2023). In the first two months of 2023, 1 868 cases of FBD were documented; and 1 377 were categorized as “other food poisoning” without specifying the etiological agent (Subsistema de Vigilancia SIVE-ALERTA MSP, 2023). This illustrates the limited characterizing of FBD agents in Ecuador, where only a few studies have described viruses in food, including the detection of noroviruses and HAV in strawberries and spinach (Salazar et al., 2023). This highlights the need to strengthen surveillance and tools to identify the specific pathogens circulating in our country responsible for this important burden, allowing us to better characterize the local epidemiology.

Our study aimed to describe fecal contamination and estimate the detection rate of enteric viruses in black shellfish commercialized in local Ecuadorian markets using molecular methods (qRT-PCR).

MATERIALS AND METHODS

Sampling

A total of 101 bivalve shellfish samples were collected from markets in different Ecuadorian cities (Quito, Cuenca, Esmeraldas, Huaquillas, Guayaquil, Machala, San Cristobal Island, and Santa Cruz Island) during the year 2021 (from July to December) as shown in table 1. Convenience sampling was performed during the rainy and dry seasons, and collection time points occurred weekly or between two weeks. Each sample consisted of ten raw black shellfish comprising two species, *Anadara tuberculosa* and *Anadara similis* (except for Galapagos islands, where oysters were collected). Bivalve samples were collected directly from market stalls using sterile bags, kept at 4°C, and transported immediately to the

Food Microbiology Laboratory at Universidad San Francisco de Quito (USFQ) for microbiological and molecular analysis.

***Count of E. coli* (Most Probable Number technique)**

Only live, not frozen shellfish were analyzed (19 samples out of 101 were excluded). The Most Probable Number technique (MPN) was used to determine fecal contamination of 82 samples according to the five tubes-three dilution method described by The Centre for Environment, Fisheries, and Aquaculture Science-CEFAS (Stockley, 2019). The method for *E. coli* count was based on β-glucuronidase production as established by ISO 16649-3.

Briefly, 10 live black shellfish were washed and scrubbed under tap water to remove soil, mud, or sand. Shells were opened using sterile instruments (globes, knives, clamps, and scalpels). The shellfish meat and intervalvular liquid were kept in a sterile container, and 2 g ± 0.2 of digestive tissue were aseptically removed and used for further viral extraction.

Twenty-five g of the remaining flesh was used for *E. coli* enumeration. The meat was placed into a sterile stomacher bag and 100 mL of 0.1% peptone solution (P) were added. The bag was homogenized for 3 minutes at medium speed, then 125 mL of P were added and mixed thoroughly to get a master 10^{-1} dilution.

A further 10-fold dilution (10^{-2}) was made by adding 1 mL of the master dilution to 9 mL of P. Five tubes containing 10 mL of double strength fluorocult medium were each inoculated with 10 mL of master dilution (equivalent to 1 g of molluscan shellfish tissue per tube). Five tubes containing 10 mL of single strength fluorocult (MERCK) medium were each inoculated with 1 mL of master dilution (equivalent to 0.1 g of molluscan shellfish tissue per tube). An additional set of five tubes of single strength fluorocult medium was inoculated with 1 mL of the 10^{-2} dilution (equivalent to 0.01 g of molluscan shellfish tissue

per tube). Positive control tubes inoculated with *E. coli* strain ATCC 25922 and negative control (uninoculated tubes) were also included in the analysis. Tubes were incubated at 37 °C for 24 ±2 hours. After incubation, each tube was examined to detect a color change from yellow to blue or green, indicating coliform presence. Tubes showing color change were analyzed under UV light and fluorescence detection at 366 nm was considered suggestive of *E. coli*. A positive indole test was used to confirm the presence of *E. coli*.

Additionally, subcultures onto Chromocult coliform agar (MERCK) plates were performed to confirm positivity from each tube. Dark blue to violet colonies were identified as *E. coli*, and pink to red colonies as other coliform bacteria. The number of positive tubes for each dilution was registered and results were interpreted according to tables given by CEFAS (Stockley, 2019).

Viral Extraction

Based on ISO 15216-1 (International Organization for Standardization, 2017), 2 g ± 0.2 of digestive tissue (DT) were used for viral extraction. DT was finely chopped using sterile scalpels to get a paste. The homogenized sample was placed into falcon tubes and 2 mL of proteinase K solution (Invitrogen, 100µg/mL) was added to the tube. Samples were vortexed (30 seconds) and placed in a shaking incubator at 37 °C for 60 min and 300 rpm. A second incubation step was performed at 60°C for 15 min. Samples were then centrifuged at 3 000 g for 5 min and the supernatant was collected and used for RNA extraction. Extraction of viral RNA (on 250 µL aliquot of the supernatant) was performed using ReliaPrep RNA Cell Miniprep System according to the manufacturer's instructions with modifications (we omitted the DNase treatment step to allow posterior detection of HAdV). Extracted

DNA/RNA was eluted on 50 µL of free nuclease water and kept at -80°C immediately after extraction for further analysis.

Reverse Transcription PCR

Aliquots of 5 µl of RNA from each sample were subjected to reverse transcription (RT). Extracted RNA was pipetted into a PCR tube and placed in a Bio-Rad thermocycler at 97°C for 5 min. The PCR tube was placed in an ice bath for 5 minutes, spun for 10 seconds, and returned to the ice bath. A 15 µL mix containing 1X first strand buffer, 5 mM of DTT, 0.375 mM of dNTPs, 30 ng of random primers, 1 U of RNase out, and 5 U of Super Script II was used for the RT reaction. Cycling conditions included 60 min at 42°C, 70 °C for 15 min, and hold at 4 °C. cDNA was used immediately for qPCR, otherwise, it was kept at -20°C until testing.

The multiplex qPCR method EVPrtPCR described elsewhere (Pang, Xiaoli. Preiksaitis, Jutta. Lee, 2014), was used to assess the presence or absence of any of six enteric viruses: Norovirus GI (NoVGI), Norovirus GII (NoVGII), Astrovirus (HAstV), Sapovirus (SaV), Adenovirus (HAdV) and/or Rotavirus (RV). Primers and probes used for TaqMan Real-Time PCR are described in Table 2.

The qPCR amplification was performed in a 10 µL volume reaction containing 5 µL of FUMM (TaqMan™ Fast Universal PCR Master Mix -Applied biosystems by Thermo Fisher Scientific) and 3 µl of cDNA. For NoVGI, NoVGII, RV, and HAdV primers and probes, the final concentration was 4.5 µM, and 1.25 µM, respectively. For SaV and HAstV primers and probes, the concentration was 2.25 µM and 0.625 µM, respectively.

Cycling conditions included initial denaturation for 20 sec at 95 °C, followed by 45 cycles of 3 sec at 95 °C and 3 sec at 60 °C. The reaction was performed using a Bio-Rad CFX96 Touch Real-Time PCR Detection System. Synthetic positive controls (gBlocks gene fragment-Integrated DNA Technologies) and PCR water were used as positive and negative controls, respectively. Samples showing a Cq ≤38 were considered positive.

Statistical Analysis

The association between each virus identification and dichotomous variables was evaluated using Fisher´s exact test because of the lack of assumptions for the Chi-square test. Those associations showing statistical association in the descriptive analysis were later explored for association measure (odds ratio) to estimate the effect of seasonality (dry or rain), and geographical origin source (San Lorenzo, Puerto El Morrro, Galapagos, Jambelí, Muisne, Puerto Bolivar, Puerto Jeli and Puerto Hualtaco) on viral identification. To analyzing geographical origin and because most of the samples were from San Lorenzo whereas the rest of the locations each had a small number of samples, we categorized samples as from “San Lorenzo” or as “regions other than San Lorenzo”. Odds ratios were calculated to estimate the effect of seasonality and geographical origin on viral positivity using a logistic regression model. An association was considered statistically significant if the p-value was equal to or lower than 0.05. Statistical analyses were performed using the software SPSS 28.0.1 (IBM), and Excel.

RESULTS

Seventy samples of black shellfish (85.4 %) showed *E. coli* counts above the permissible limit (≤ 230 MPN) established by the Ecuadorian regulation INEN 2729 (NORMA PARA MOLUSCOS BIVALVOS VIVOS Y MOLUSCOS BIVALVOS

CRUDOS, 2013), and Codex Alimentarius 315-2014 (Norma Para Los Moluscos Bivalvos Vivos y Moluscos Bivalvos Crudos, 2015) as shown in table 1.

We detected one, two, three, and four viruses in 39 (38.6%), 20 (19.8%), 9 (8.9%), and 2 (2%) samples, respectively (Table 3). HAdV + RV was the most common combination detected (7 samples, 6.9%).

When we evaluated the effect of seasonality on viral detection, we observed an association between the dry season and HAdV molecular detection, (p=0.001, Fisher Exact Test) (Table 4). Samples collected during the rainy season showed a 75% lower probability to be positive for HAdV than those samples collected during the dry season (OR:0.25, CI 95% 0.11-0.58).

When we analyzed the *E. coli* counts and detected viruses by sample origin, we found that samples collected from regions other than San Lorenzo had a 4.7 higher probability of showing positivity for NoVGI than samples collected from San Lorenzo (CI 95%= 1.34-16.7). Conversely, samples collected from regions other than San Lorenzo showed a 90% lower probability to get impermissible *E. coli* counts than samples collected from San Lorenzo (OR:0.1, 95% CI= 0.02-0.38).

No significant association was identified between fecal contamination (*E. coli* counts) and molecular detection of each enteric virus (p >0.05, Fisher Exact Test) (Table 5).

DISCUSSION

In this study, we assessed the presence of six viral human pathogens and one fecal indicator bacteria in black shellfish collected in Ecuador. Black shellfish constitutes an

important food resource in Ecuadorian gastronomy and is frequently consumed raw (Orden-Mejía et al., 2021); therefore, there is a strong need to assess and implement pathogen surveillance in commercialized bivalve mollusks to determine their quality and safety for human consumption.

We detected at least one of six viruses (NoVGI, NoVGII, HAdV, RV, SaV, and HAstV) in 69.3% of 101 samples collected in the second semester of the year 2021. Importantly, most of the shellfish samples analyzed in this study had high *E. coli* counts (85,4%) and did not meet the national and international microbiological criteria set, respectively, by INEN 2729 (NORMA PARA MOLUSCOS BIVALVOS VIVOS Y MOLUSCOS BIVALVOS CRUDOS, 2013) and CODEX STAN 292-2008 (Norma Para Los Moluscos Bivalvos Vivos y Moluscos Bivalvos Crudos, 2015). High *E. coli* counts ($4x10^3$ to $2x10^7$ CFU/g) in black shellfish have been previously described in Puerto El Morro, and the implementation of purification treatment before commercialization of this bivalve shellfish suggested (Delgado Madeleyne, 2017).

Fecal contamination of oysters, mussels, clams, and other bivalves has been documented at varying frequencies around the world. In India, *E. coli* has been reported in 100% of samples tested (Das et al., 2020). In South America, this indicator of fecal contamination has been described in 51% of shellfish samples analyzed in Brazil (Miotto et al., 2019); these data are consistent with our findings; but contrast with the 11.1% found in Argentina (Cammarata et al., 2021).

E. coli as an indicator of fecal contamination allows the prediction of the presence of other enteric pathogens such as viruses or parasites in food (Ekici & Dümen, 2019) and determines its safety for human consumption. Despite the high levels of fecal contamination

found, we did not observe a correlation between the presence of *E. coli* and the presence of any of the viruses analyzed. This is consistent with the study by Sharp and colleagues who found that *E. coli* is not a good indicator of viral contamination of shellfish or water since viruses persist in shellfish tissue longer than bacteria, even when purification measures are applied (Sharp et al., 2021). This different clearence rate between bacteria and viruses may explain the association found between non-impermissible *E. coli* counts in samples collected from San Lorenzo, despite their lower NoVGI positive rate. Typical *E. coli* assessment does not include pathogenic strains, therefore we cannot exclude the presence of *E. coli* pathotypes in black shellfish, as described in Brazil (Miotto et al., 2019).

When we analyzed the *E. coli* counts and detected viruses by sample origin, we found that samples collected from regions other than San Lorenzo had a 4.7 higher probability of showing positivity for NoVGI than samples collected from San Lorenzo (CI 95%= 1.34-16.7). Conversely, samples collected from regions other than San Lorenzo showed a 90% lower probability to get impermissible *E. coli* counts than samples collected from San Lorenzo (OR:0.1, 95% CI= 0.02-0.38).

Among the viruses analyzed, HAdV was more frequently detected (49.5% of the samples). Detection of this virus has been reported in different classes of shellfish: 6.3%-11.7% in Taiwan (Nagarajan et al., 2022); 21.27% in India (Ghosh et al., 2019); 43.3% in Spain (Rodriguez-Manzano et al., 2014), and 24.7%-75% in Brazil (do Nascimento, Sarmento, et al., 2022; Keller et al., 2019). Not many studies have been conducted on food in Latin America. However, Brazil (Keller et al., 2019) reported a high frequency of detection of HAdV in mollusks from mangroves (75%), with higher frequencies in mussels. Both Ghosh (Ghosh et al., 2019)and Keller (Keller et al., 2019) reported a species-dependent frequency; a higher positivity was attributed to clams or mussels compared to oysters or

shrimps. This suggests that shellfish may concentrate or retain viruses differently depending on the species, as proposed for NoV (Franoise S. Le Guyader et al., 2012). This would explain the high HAdV recovery from black shellfish in our study.

The most common non-bacterial agents described in gastroenteritis globally are NoVGI and NoVGII (WHO, 2015). They were detected in 20.8% and 6.9% of the analyzed samples, respectively. NoVGII (GII.4) leads the worldwide list of noroviruses responsible for human gastroenteritis outbreaks (S. . Williams & O'Brien, 2019). In Ecuador, NoVGII was described in 2015 as the most frequent genogroup found in stool samples from children (Gastañaduy et al., 2015; B. A. Lopman et al., 2015). Globally this genogroup was found as the most prevalent in shellfish (Li et al., 2023). Interestingly, we found a higher prevalence of NoVGI in black shellfish. Some studies have demonstrated the in vitro ability of mollusks to concentrate NoVGI over NoVGII due to specific carbohydrates (including blood group antigens) in shellfish tissue acting as ligands, promoting viral strain-specific accumulation (Françoise S. Le Guyader et al., 2008; Franoise S. Le Guyader et al., 2012; Maalouf et al., 2011). Furthermore, NoVGI has been widely associated with outbreaks caused by shellfish (Keller et al., 2019; Kittigul, L., Thamjaroen, A., Chiawchan, S., Chavalitshewinkoon-Petmitr, P., Pombubpa, K., & Diraphat, 2016; Moon et al., 2011), due to its ability to bioaccumulate NoVGI even in the presence of low levels of the virus in the surrounding environment (Yang et al., 2021).

Most viral testing in shellfish focuses on detecting NoV or HAV, but other viruses including RV are also found in these samples. Kittigul reported up to 8% shellfish contamination with RV using nested PCR in Thailand (Kittigul et al., 2015), which is lower than what we observed (15.8%). However, the shellfish species analyzed in Thailand's study were different (cockles, oysters, and mussels); and detection frequencies varied among them,

again suggesting that the accumulation may be species-dependent. Panamá reported a higher rate of RV (60%) in *A. tuberculosa* analyzed through ELISA technique (Bourdett-Stanziola et al., 2022). However, the high positivity rate was attributed to the sampling area, which was characterized by high tourist activity and subsequent human wastewater in the mangrove (Bourdett-Stanziola et al., 2022).

HAstV and SaV have also been associated with foodborne outbreaks, although in a lesser extent (Diez Valcarce et al., 2021; Razizadeh, Khatami, et al., 2022; Xuefeng Chen, et al et al., 2011). We found each, SaV and HAstV, in 10% of tested samples, which is in line with the results reported in Europe, where virus surveillance in food and shellfish is well developed. In Galicia, Spain, human SaV was detected more frequently in seafood, ranging from 17.9% to 37.5% (Varela, Hooper, et al., 2016; Varela, Polo, et al., 2016), which is similar to reports from Italy (18.8%) (Fusco et al., 2019). Regarding HAstV, up to 20.8% of samples (mussels and clams) in Italy showed contamination with this agent (Fusco et al., 2019)

The presence of multiple viruses in shellfish is not uncommon (Françoise S. Le Guyader et al., 2008). We describe the coexistence of NoVGI and NoVGII in 3.9% of the samples analyzed. We also identified up to four viruses in the same sample; suggesting that the coexistence of different species, genotypes, and genogroups is common in our country, as described in oyster samples in Brazil (do Nascimento, Sarmento, et al., 2022).

Despite the reports describing NoV disease peaks in the rainy or winter season (Ludwig-Begall et al., 2021), we did not identify any seasonality for NoVGI, NoVGII, RV, SaV, or HAstV. In contrast to previous reports (Tao et al., 2016), we found an association between HAdV and dry season. While a lack of HAdV seasonality was reported in shellfish

in Brazil (do Nascimento, Sarmento, et al., 2022), a seasonality has been described based on stool sample analysis in the Northern region of this country with a higher prevalence of HAdV during summer and spring (do Nascimento, Fialho, et al., 2022).

The concentration of human pathogens (viruses and bacteria) in shellfish is generally the result of contamination of the environment in which they live, and this may be caused by sewage (Hassard et al., 2017). Mangrove represents a special ecosystem and contamination assessment studies have documented fecal contamination in Ecuadorian mangroves (Pernia et al., 2019) and rivers (Vinueza et al., 2021). Our study supports these previous reports and provides evidence of fecal contamination of black shellfish; suggesting that efforts to ensure mangrove-safe water quality should be reinforced.

Black shellfish are commonly consumed raw in typical Ecuadorian dishes such as ceviche, posing a risk to the consumer, as described by Orden-Mejia (Orden-Mejía et al., 2021). Post-harvest purification methods, including placing shellfish in clean tanks before commercialization, are commonly used to remove pathogens or reduce their concentration in shellfish (Yang et al., 2021). However, this process is not as effective at removing viruses as it is at removing bacteria (Sharp et al., 2021). Proper cooking of seafood (90 degrees Celsius over 90 seconds) is a better strategy to reduce the risk (Bozkurt et al., 2015), as high temperatures are effective at inactivating viruses (Bozkurt et al., 2015)

To the best of our knowledge, this study is the first to identify viral genetic material from black shellfish sold in local markets. Our findings highlight the need to strengthen assessment tools for the timely identification of viral pathogens in commercial shellfish for risk assessment of food-transmitted diseases. In the future, larger sampling, and including

additional sampling areas, as well as genotyping analyses will be needed to better characterize the prevalence and genetic diversity of human pathogens among black shellfish.

TABLAS

Table 1. Frequency of detection of enteric viruses and *E. coli* in black shellfish by geographical origin

ORIGIN	Sample N°/*n* (%)	NoVGI Positive (%)	NoVGII Positive (%)	RV Positive (%)	HAdV Positive (%)	HAstV Positive (%)	SaV Positive (%)	*E. coli* (%)
San Lorenzo	86/101 (85)	15 (14.85)	4 (3.96)	15 (14.85)	42 (41.58)	10 (10)	10 (10)	64 (78.05)
Puerto El Morro	2/101 (2)	2 (1.98)	1 (0.99)		1 (0.99)			1 (1.22)
Galápagos	3/101 (3)				1 (0.99)			1 (1.22)
Jambelí	1/101 (1)				1(0.99)			
Muisne	3/101 (3)	1 (0.99)	1 (0.99)	1 (0.99)	3 (2.97)			2 (2.44)
Puerto Bolívar	2/101 (2)	1 (0.99)	1 (0.99)		1 (0.99)			1 (1.22)
Puerto Jeli	1/101 (1)	1 (0.99)						1 (1.22)
San Vicente	2/101 (2)	1 (0.99)						
Puerto Hualtaco	1/101 (1)				1 (0.99)			
TOTAL	101/101 (100)	21/101 (20.8)	7/101 (6.9)	16/101 (15.8)	50/101 (49.5)	10/100 (10)	10/100 (10)	70/82 (85.4)

Table 2. Multiplex qPCR primers and probes

Primers and probes used for Norovirus GI and GII detection			
NAME	TARGET	SENSE	Sequence 5´-3´
NoV1-Fb	Norovirus genogroup I	Forward primer	TYC GYT GGA TGC GNT TYC ATG A
NoV1-R	Norovirus genogroup I	Reverse primer	CTT AGA CGC CAT CAT CAT TYA C
NoV2-F	Norovirus genogroup II	Forward primer	CAR GAR BCN ATG TTY AGR TGG ATG AG
NoV2-R	Norovirus genogroup II	Reverse primer	TCG ACG CCA TCT TCA TTC ACA
NoVG1c-Probe	Norovirus genogroup I	Probe	VIC-AGA TYG CGR TCY CCT GTC CA-TAMRA
NoV2-Probe	Norovirus genogroup II	Probe	FAM-TGG GAG GGY GAT CGC AAT CT-TAMRA
Primers and probes used for Rotavirus and Adenovirus detection			
Rota-F	Non-structure protein 3	Forward primer	ACC ATC TAC ACA TGA CCC TC
Rota-F-GII	Non-structure protein 3	Forward primer	ACC ATC TTC ACG TAA CCC TC
Rota-R	Non-structure protein 3	Reverse primer	GGT CAC ATA ACG CCC C
Rota-P robe (FAM/ TAMRA)	Non-structure protein 3	Probe	FAM-ATG AGC ACA ATA GTT AAA AGC TAA CAC TGT CAA -TAMRA
Ad2-F	Non-structure protein 3	Forward primer	CCA GGA CGC CTC GGA GTA
Ad2-R	Hexon	Reverse primer	AAA CTT GTT ATT CAG GCT GAA GTA CGT
Ad2-P (VIC/TAMRA)	Hexon	Probe	VIC-AGT TTG CCC GCG CCA CCG-TAMRA
Ad4-F	Hexon	Forward primer	GGA CAG GAC GCT TCG GAG TA
Ad4-R	Hexon	Reverse primer	CTT GTT CCC CAG ACT GAA GTA GGT
Ad4-P (VIC/TAMRA)	Hexon	Probe	VIC-CAGTTCGCCCGYGCMACAG-TAMRA
Primers and probes used for Sapovirus and Astrovirus detection			
SaV124F	Junction of polymerase and capsid	Forward primer	GAY CAS GCT CTC GCY ACC TAC
SaV1F	Junction of polymerase and capsid	Forward primer	TTG GCC CTC GCC ACC TAC
SaV5F	Junction of polymerase and capsid	Forward primer	TTT GAA CAA GCT GTG GCA TGC TAC
SaV1245R	Junction of polymerase and capsid	Reverse primer	CCC TCC ATY TCA AAC ACT A
SaV124TP (FAM/ MGB)	Junction of polymerase and capsid	Probe	FAM-CCR CCT ATR AAC CA-MGB-NQF

SaV5TP (FAM/MGB)	Junction of polymerase and capsid	Probe	FAM-TGC CAC CAA TGT ACC A-MGB-NQF
Astr-1F	Polymerase	Forward primer	CTT AAT CGC CAT GTA CTT CTA CCA TC
Astr-1R	Polymerase	Reverse primer	TGT TGT TGA AAA CTG CCC AGA T
Astr-1P (VIC/TAMRA)	Polymerase	Probe	VIC-AAG TCA CCT TGC AGA CAC GAG GTA ATC-TAMRA
Astr-2F	Capsid	Forward primer	GGC ACT AAT CAA ATG CCT AAT GTT T
Astr-2R	Capsid	Reverse primer	GGA GAC TGT ACC CTC GAT CCT ACT C
Astr-2P (VIC/TAMRA)	Capsid	Probe	VIC-TGG AGA CCG CGG CCA CGC-TAMRA

Table 3. Co-detection of enteric viruses in black shellfish samples

DOUBLE DETECTION		TRIPLE DETECTION		QUADRUPLE DETECTION	
Combination	Positive (%)	Combination	Positive (%)	Combination	Positive (%)
NoVGI+NoVGII	4 (20)	RV +HAdV+HAstV	2 (22.2)	NoVGI+NoVGII+HAdV +HAstV	1 (50)
NoVGI+HAdV	2 (10)	HAdV+HAstV +SaV	1 (11.1)	NoVGI+HAdV +HAstV+ SaV	1 (50)
NoVGI + HAstV	1 (5)	NoVGI, NoVGII, HAdV	1 (11.1)		
RV +HAdV	7 (35)	NoVGII, RV +HAdV	1 (11.1)		
SaV +HAdV	1 (5)	NoVGI, HAdV + SaV	2 (22.2)		
SaV +HAstV	1 (5)	NoVGI, RV +HAdV	1 (11.1)		
SaV +RV	2 (10)	RV+HAstV+SaV	1 (11.1)		
HAdV +HAstV	2 (10)				
Total	20 (100)		9 (100)		2(100)

Table 4. Association between seasonality and samples´ origin with viral identification or E. coli count

	Virus	**NoVGI**	**NoVGII**	**RV**	**HAdV**	**HAstV**	**SaV**	***E. coli***
		n (%)	***n*** (%)	***n*** (%)	***n*** (%)	***n*** (%)	***n*** (%)	***n*** (%)
SEASON	Dry	8 (16.3%)	5 (10.2%)	9 (18.4%)	33 (67.4%)	6 (12.5%)	3 (6.3%)	28 (80%)
	Rainy	13 (27.7%)	2 (4.3%)	6 (12.8%)	**16 (34.1%) §**	3 (6.4%)	6 (12.8%)	42 (89.4%)
	p-value	0.22	0.43	0.58	**0.001§**	0.49	0.32	0.34
ORIGIN	San Lorenzo	15 (17.4 %)	4 (4.7 %)	15 (17.4 %)	42 (42.8 %)	10 (11.8 %)	10 (11.8 %)	64 (91.4 %)
	Galápagos	0	0	0	1 (33.3 %)	0	0	NA
	Other		3 (25 %)	1 (8.3 %)	7 (58.3 %)	0	0	**6 (50 %) §**
	p-value	0.035	0.07	0.81	0.74	0.53	0.53	**0.002§**

§ p-value <0.05

An association between Adenovirus molecular detection and dry season was found (*p*-value 0.00, Fisher´s exact test 1).

Table 5. Association between *E. coli* count and detected viruses

***E. coli* limit**	**NoVGI**	**NoVGII**	**RV**	**HAdV**	**HAstV**	**SaV**
	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***N* (%)**
Permissible	5 (41.7 %)	2 (16.7 %)	0	5 (41.7 %)	0	0
No-permissible	14 (20 %)	4 (5.7 %)	11 (15.7 %)	34 (48.6 %)	7 (10.1 %)	8 (11.6 %)

Fisher´s exact test showed no association between no permissible *E. coli* count and positivity for any virus $p>0.05$.

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