ARTICLE / INVESTIGACIÓN

Development of enhanced primer sets for detection of Norovirus and Hepatitis A in food samples from Guayaquil (Ecuador) by reverse transcriptase-heminested PCR

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Abstract: Norovirus (NV) is an infectious biological agent that causes gastrointestinal problems of the original nonbacterial appearance of foodborne illnesses. The genotype of NV responsible for the most frequent NV disease outbreaks is GII, accounting for 60–80% of the cases. Moreover, original and new NV variants are continuously emerging, concurrent with the recent global increase in NV infections. Hepatitis A virus (HAV) is another foodborne pathogen frequently implicated in acute gastroenteritis cases around the world. The virus is transmitted among humans via the fecal-oral route, and infection by HAV causes the most severe form of viral illness acquired from foods. In this study, we implemented primer sets to detect NV genotypes I and II. We also developed primer sets for the detection of HAV. The primers were used in a heminested reverse transcriptase PCR (hnRT-PCR) protocol that was rapid and sensitive for detecting NVG1, NVGII and HAV virus in food. The hnRT-PCR was applied successfully to strawberries and spinach obtained from a local fresh-food market, where we could see NVGI, NVGII and HAV.

Key words: Norovirus[1], Hepatitis A[2], gastroenteritis[3], genotypes[4], NVG1[5], NVGII[6], hnRT-PCR[7].

Introduction

Norovirus (NV), family Caliciviridae, is a major cause of acute viral gastroenteritis in humans[1]. Disease onset typically appears between 12 and 48 h after infection, and although symptoms are usually mild and self-limited, they can be severe in immunocompromised patients and the elderly[2]. NV disease is considered a foodborne illness, but person-to-person transmission and waterborne outbreaks are also important sources of infection[3]. The NV genome is composed of a single-stranded positive-sense RNA (+ssRNA), with approximately 7.7 kb and three open reading frames (ORFs): ORF1, ORF2, and ORF3[4]. ORF1 encodes six nonstructural proteins, including the RNA-dependent RNA polymerase (RdRp)[5]. ORF2 and ORF3 encode major (VP1) and minor (VP2) structural capsid proteins, respectively[6]. VP1 consists of a folded domain (S) and two prominent domains (P)[7]. The P1 domain, a central region of the flexible loop, is located between the S and P2 domains[5]. The P2 domain is a hypervariable region that binds to the host cell[8].

The stability of VP1 is increased by VP2, which prevents its degradation[5]. NV is classified into six groups, genogroups I to VI (GI to GVI), based on the amino acid sequences of the RdRp and VP1[9]. The genogroups GI, GII, and GIV, are found in humans[10], and outbreaks appear more frequently within these genogroups[11]. GII continuously evolved in an evolutionary pattern every 2 to 3 years[12]. This genotype is equivalent to 87% of the occurrence of noroviruses worldwide[13]. NV GII.4 Sydney type emerged in Korea between 2012 and 2013, where 60.4% of NV diagnoses showed GII[13]. The NV GII strain is difficult to identify the existing RT-PCR because of the continuous variation of the strain. In addition, the primer set does not always have sufficient specificity to detect NV and false positive detection frequently occurs[14].

Hepatitis A virus (HAV) is a hepatotropic virus that belongs to the family Picornaviridae[1,15]. The HAV has a single-stranded positive-sense RNA genome of 7.5-kilobase (kb). HAV genome is wrapped up by an icosahedral capsid, formed by up to 60 copies of three surface proteins, VP1, VP2, and VP3 (named 1D, 1B, and 1C), transcribed into one open reading frame (ORF)[16]. The single ORF of 2217 to 2280 amino acids of HAVs is divided into P1, P2, and P3 regions. The P1 part contains the capsid polypeptides VP1 to VP4, whereas P2 and P3 regions include the nonstructural polypeptides[1]. Flanking the HAV genome, there are regulatory 5' and 3'UTRs areas with a small poly-A tail[17]. HAV immunogenic neutralization sites are located on the structural surface of proteins[18].

Primates are the only natural host of HAV, and infection confers lifelong protection[19,20]. After ingestion, absorption from the gastrointestinal tract, and replication in the liver, HAV is excreted in the bile in high concentrations of viral particles that can be found in stool specimens. HAV transmission occurs by the fecal-oral through direct contact with

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an HAV-infected person or by consuming HAV-contaminated food or water. The median latency period (i.e., time from exposure to onset of symptoms) is 28 days (range 15 to 50 days)[19] infectivity peaks two weeks before the onset of jaundice and declines one week after the start. Elevating serum alanine aminotransferase [ALT] levels can lead to maximal infectivity in people without jaundice. Viremia can be detected before ALT levels rise, and HAV RNA levels often remain detectable even after ALT levels normalize and symptoms resolve[21].

Hepatitis A begins with symptoms such as fever, loss of appetite, nausea, vomiting, diarrhea, muscle aches, and fatigue. Jaundice, dark-colored urine, or light-colored stools may be present initially or followed by systemic symptoms within a few days if the physical findings include abdominal tenderness, hepatomegaly or splenomegaly[22]. In most cases, hepatitis A lasts for several weeks. Recurrence of symptoms with new increases in serum aminotransferase levels occurs in 10% of patients, and relapses can last up to 6 months[12]. All-cause mortality is 0.3%, compared to 1.8% among people around age 50. People with underlying chronic liver disease are at increased risk of death[23].

HAV contamination of food can occur at any stage of growing, harvesting, processing, distribution, or preparation. Foodborne infections can be challenging to detect from routine surveillance data because case patients may have difficulty recalling their dietary history 2 to 6 weeks before illness[15,19], cases may accrue gradually or not be reported[24], a food item may be focally contaminated[21], some exposed persons may have unrecognized HAV infection[25], some susceptible persons may have preexisting immunity from a previous infection or vaccination[26], persons who acquire infection through contaminated food are not recognized amid an ongoing high incidence in the community. Cases are geographically scattered over several public health jurisdictions[27].

This study aimed to develop a rapid and sensitive detection method for Norovirus genotypes I and II and Hepatitis A in food matrices of high demand and consumption. The current problem in public health in Ecuador is that many diseases associated with gastrointestinal issues are associated with bacteria and not with viruses; in this context, the identification of Norovirus, specifically genogroup II, which is associated with high cases of foodborne infection, allows to identify the type of disease in relation to the infectious biological agent correctly. Concerning the Hepatitis A virus, many farmers in our country do not carry out good agricultural practices concerning the sanitary quality of the irrigation water used for fruit and vegetable crops, specifically strawberries and spinach, being a route of infection by cross-contamination. Since these foods are not heat-treated in most cases, the risk to public health is considerable.

Materials and methods

Analysis of HAV genomic sequences

The genomic sequences of NV GI, GII, and HAV strains were collected from the National Center for Biotechnical Information (NCBI) genetic sequence database and analyzed using Geneious Prime® 2021.0.3. The current study's sequences are listed in Tables 1, 2 and 3. Genetic sequences were aligned with Muscle to identify around 500 nucleotides as the target for RT-PCR diagnoses and positive controls. Selected sequences were cloned into a T7-driven vector for in vitro transcription of the favorable rules. Genetic sequences of the gene fragments and primers are shown in Tables 4 and 5.

Accession number (GenBank)	Genotype	Strain
FJ515294	GI.2	Leuven/2003/BEL
JN183159	GI.9	S48/2008/Lilla
JN603244	GI.3	S29/2008/Lilla Edet/Sweden
JQ388274	GI.6	Kingston/ACT160D/2010/AU
JQ911594	GI	10360/2010/VNM
KF039725	GI.1	CHA7A009/2010/USA
KF039726	GI.1	/CHA6A003 20091031/2009/USA
KF039727	GI.1	CHA2A014/2008/USA
KF039728	GI.1	CHA2A014/2008/USA
KF039729	GI.1	CHA6A007/2010/USA
KF306212	GI.2	Jingzhou/2013401/CHN
KF429761	GI.1	8MoIIIL/1972/USA
KF429765	GI.1	8W/1968/USA
KF429770	GI.1	8McIII/1973/USA
KF429773	GI.1	8CKIIIc/1974/USA
KF429774	GI.1	8UIIIf/1973/USA
KF429783	GI.1	8K/1979/USA
KF429789	GI.1	8MC/1978/USA
KM246914	GI.14	Nanning/2011/CHN
HQ637267	GI	Vancouver730/2004/CAN

Table 1. Description of NV GI sequences for genetic analysis.

Accession number (GenBank)	Genotype	Strain
AB447433	GII.4	Aormori/2006/JP
AB541321	GII.4	Osaka2/2007/JP
AB662873	GII.2	OC09104/2009/JP
AY485642	GII.4	Langen 1061/2002/GER
AY502023	GII.4	Farmington Hills/2002/USA
DQ658413	GII.4	MD-2004/2004USA
DQ078814	GII.4	Hunter504D/2004/AU
EF202588	GII.4	Toronto SK/2005/CAN
EU310927	GII.4	TCH186 2002 US
EU373815	GII	Luckenwalde591/2002/DE
EF684915	GII.4	Shellharbour NSW696T/2006/AUS
GQ845369	GII.4	Armidale NSW3901/2008/AU
GU134965	GII.7	1738/2009/USA
GU969058	GII.13	8679/Maizuru/2008 JPN
GU980585	GII.3	CBNU1/2006/KOR
HQ664990	GII.12	HS206/2010/USA
JN400623	GII.4	CGMH25/2010/TW
JN595867	GII.4	Ascension208/2010

Table 2. Description of NV GII sequences for genetic analysis.

Accession number (GenBank)	Genotype	Strain
JQ425480.1	HAV A	Hepatovirus A
KP879216.1	HAV A	Hepatovirus A
KP879217.1	HAV A	Hepatovirus A
KT891985.1	HAV A	Hepatovirus A
M16632.1	HAV A	Hepatovirus A
M59286.1	HAV A	Hepatovirus A
M59808.1	HAV A	Hepatovirus A
NC_001489	HAV A	Hepatovirus A
NC_008250.2	HAV A	Duck hepatitis A virus 1
NM_012206	HAV A	Homo sapiens

Table 3. Description of HAV sequences for genetic analysis.

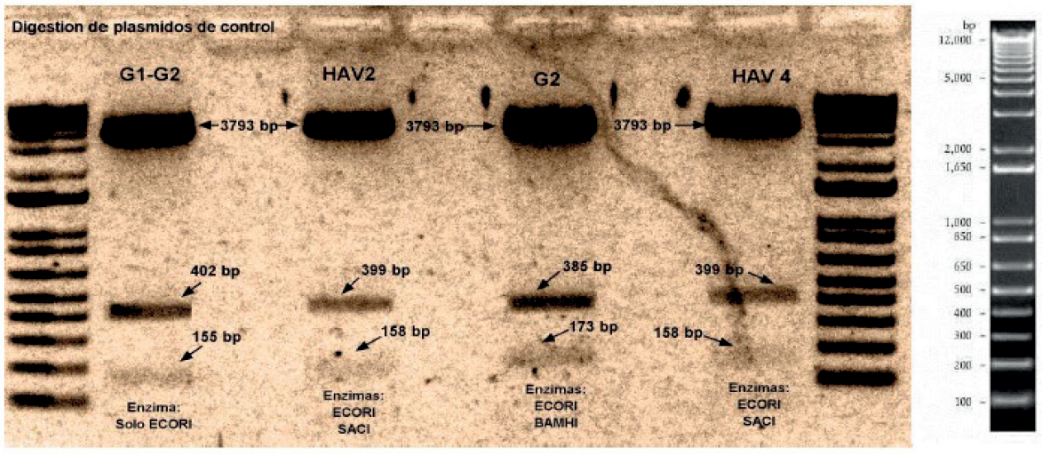

Figure 1. Digestion of plasmid pTop Blunt V2 with an inserted sequence of NVGI, NVGII, and HAV gene.

Norovirus GII	TAATACGACTCACTATAGGGATTTCTGAGGATCTGGATGGTTTAACCTTTCTGCGGAGA ACTGTAACCCGTGATCCAGCTGGTTGGTTTGGTAAATTAGAACAGAGCTCAATACTTAG GCAGATGTACTGGACTAGGGGCCCCAACCATGAGGATCCATCTGAAACAATGATACCA CATTCCCAAAGGCCCATACAGTTGATGTCTCTGCTAGGTGAAGCTGCATTGCACGGTCC AGCATTCTACAGCAAAATCAGTAAACTAGTCATTGCAGAGTTGAAGGAAGGTGGCATG GACTTTTACGTGCCCAGGCAAGAGCCGATGTTCAGATGGATGAGATTCTCAGACCTGAG CACGTGGGAGGGCGATCGCAATCTGGCTCCCAGTTTTGTGAATGAAGATGGCGTCGAAT GACGCCGCCCCATCTAATGATGGTGCAGCCGGTCTCGTACCAGAGGTCAACAACGAGAC GATGGCCCTCGAACCGGTGGCTGGGGCTTCTATAGCCGCCCCTCTAACCG	521
HAV_Hepatitis A	TAATACGACTCACTATAGGGAGATTCTACATTTGGATTGTTTCTATTCGAGATTGCAAAT TACAATCATTCTGATGAATATTTGTCCTTCAGTTGTTATTTGTCTGTCACAGAGCAATCA GAGTTCTATTTTCCTAGAGCTCCATTAAATTCAAATGCTATGTTGTCCACTGAATCCATG ATGAGTAGAATTGCAGCTGGAGACTTGGAGTCATCAGTGGATGATCCCAGATCAGAGG AGGATAGAAGATTTGAGAGTCATATAGAATGTAGGAAACCATACAAAGAATTGAGACT GGAGGTTGGGAAACAAAGACTCAAATATGCTCAGGAAGAGTTATCAAATGAAGTGCTT CCACCTCCTAGGAAAATGAAGGGGTTATTTTCACAAGCTAAAATTTCTCTTTTTTATACT GAGGAGCATGAAATAATGAAGTTTTCTTGGAGAGGAGTGACTGCTGATACTAGGGCTTT GAGAAGATTTGGATTCTCTCTGGCTGCTGGTAGAAGTGTGTGGACT	520

Table 4. Sequence of genes synthesized for positive control and primer design Norovirus (GI And GII); Hepatitis A (HAV) obtained by alignment from strains the genetic sequence database at the National Center for Biotechnical Information (NCBI).

Verification of positive controls

Gene fragments were received in a pTop Blunt V2 vector and transformed in DH5alpha Escherichia coli competent cells. Gene fragments were verified by restriction digestion with the following sizes: NV GI 557bp; NV GII 558bp; and HAV 557 bp (Fig. 1).

Primer Design

Specific primers for NVand HAV were designed from conserved regions of ORF1 and ORF2. The expected size for NVGI in the first PCR is 288 bp, and using heminested PCR it is 213 bp, for NVGII the anticipated size of the initial amplification is 286 bp and using heminested PCR the predicted size is 215 bp while for HAV the anticipated length of the initial PCR is 395 bp and using the heminested PCR 160 bp.

Collection of food Samples

This study's test sample sizes were 25 g (fruit and vegetables like strawberry and spinach), 330 ml (bottled water) and 25 g of sausages. All samples were tested using the relevant matrix-specific test protocols[28]. All food samples were tested for NV GI and GII, and HAV. Samples were obtained at the wholesale food market of Mount Sinai, Guayaquil, during October and November of 2020.

Validation of hnRT-PCR

Thoroughly washed strawberries, spinach and sausages were inoculated with in vitro transcribed NV GI, GII and HAV RNA-positive controls. Food samples without inoculation were used as negative controls. The weight of strawberry, spinach and sausage was 25 grams and 300 ml for bottled water[28].

Food sample washed with water (except bottled water sample). It was established as a protocol to inoculate the food with NVGI (600 ng/ul), NVGII (500 ng/ul) and HAV (450 ng/ul), established as a positive control. Subsequently, a process was established by inoculating viral load without food NVGI (600 ng/ul), NVGII (500 ng/ul) and HAV (450 ng/ul) established as the positive control. Positive control was established within the process based on synthetic RNA obtained by reverse transcription of the PCR product of the plasmids.

Experimental protocol

Locally purchased strawberries, spinaches, sausages, and bottled water were used in this study. Food samples (25g) were mixed with 60 ml of elution buffer (50 mM glycine, 100 mM Tris, 1% [wt/vol] beef extract [pH 9.5]) for 15 min at room temperature with constantly shacking (Fig. 2). The elution buffer was then filtered through a 40-m pore size nylon cell strainer (BD Falcon, Basel, Switzerland). The recovered elution buffer was adjusted to pH 7.0 with 9.5 M HCl and centrifuged at 3,500 g for 15 min. The supernatant was transferred to a new tube, and the volume was brought up to 25 ml with phosphate buffer saline and left at 4C overnight. The next day the extract was centrifuged at 3,500 g for 15 min, and the supernatant was then transferred to a Centricon Plus-70 centrifugal filter device (100K NMWL; Millipore, Molsheim, France) and centrifuged at 3,500 g to concentrate viral particles into a volume of ca. 400 ul. The filter device was subsequently rinsed with 200 ul of elution buffer to improve virus recovery[29] (Fig. 2).

Viral RNA Extraction

Total RNA was extracted with TRI Reagent according to the manufacturer protocol (12). Total RNA was resuspended in 30 μL of elution buffer and stored at −80°C until use in the RT-hnPCR assay.

RT-PCR and Heminested PCR

Extracted RNA was reverse-transcribed and amplified using SuperScript III One-Step RT-PCR Kit (Thermo Fisher, USA) following the manufacturer's instructions. The reaction mixture consisted of 10 ul of reaction buffer, 0.4 ul of Triton x100, 0.2 µM of each primer (NVGI-F/R, NVGII-F/R, HAV-F/R), 0.5 ul of enzyme mix (SuperScript III One-Step RT PCR with Platinum Taq DNA Polymerase), 2 ul of the RNA template, and 11.1 ul and RNase-free water.

The thermocycling protocol was as follows

RT at 50 C for 30 min; initial denaturation at 94 C for 2 min; 40X cycles of 94 C for 30s, annealing at 60 C (NV GI), 55 C (NV GII), and 45 C (HAV), for 30 s, extension at 72 C for 40 s, and a final extension at 72 C for 5 min.

For the heminested PCR step, 1 ul of the RT-PCR reaction was mixed with 2 ul of 10X reaction buffer, 1 ul of 50mM

(a) NVGI NVGI primer set	
	NVGI (forward)
Primer	TGGACYCGHGGSCCCAAYCA
Promotor T7 + aligned sequence NVGI	C C G T
	NVGI (reverse)
Primer	GCGTCCTTAGACGCCATCATC
Promotor T7 + aligned sequence NVGI	TAAG
	NVGI (reverse heminested)
Primer	GAAVCGCATCCAGCGGAACATGG
Promotor T7 + aligned sequence NVGI	CC T A G
(b) NVGII NVGII primer set	
	NVGII(forward)
Primer	TGGACNAGRGGNCCYAAYCA
Promotor T7 + aligned sequence NVGII	T G C C C
	NVGII (reverse)
Primer	GTCATTCGACGCCATCTTCATTC
Promotor T7 + aligned sequence NVGII	T GC C G A
	NVGII (reverse heminested)
Primer	GARAAYCTCATCCAYCTRAAC
Promotor T7 + aligned sequence NVGII	GG T G T G A
(c) HAV HAV primer set	
	HAV(forward)
Primer	CTATTCAGATTGCAAATTAYAAT
Promotor T7 + aligned sequence NVGII	C
	HAV (reverse)
Primer	AAYTTCATYATTTCATGCTCCT
Promotor T7 + aligned sequence NVGII	A G A C
	HAV (reverse heminested)
Primer	CTCCAGCTGCAATTCTACTCATC
Promotor T7 + aligned sequence NVGII	T T T G T

Table 5. The sequence of genes synthesized of NV GI (a) NV GII (b) and HAV (c) obtained by alignment from strains in the genetic sequence database at the National Center for Biotechnical Information (NCBI) were compared to determine consensus identity with sequences of designed primer sets.

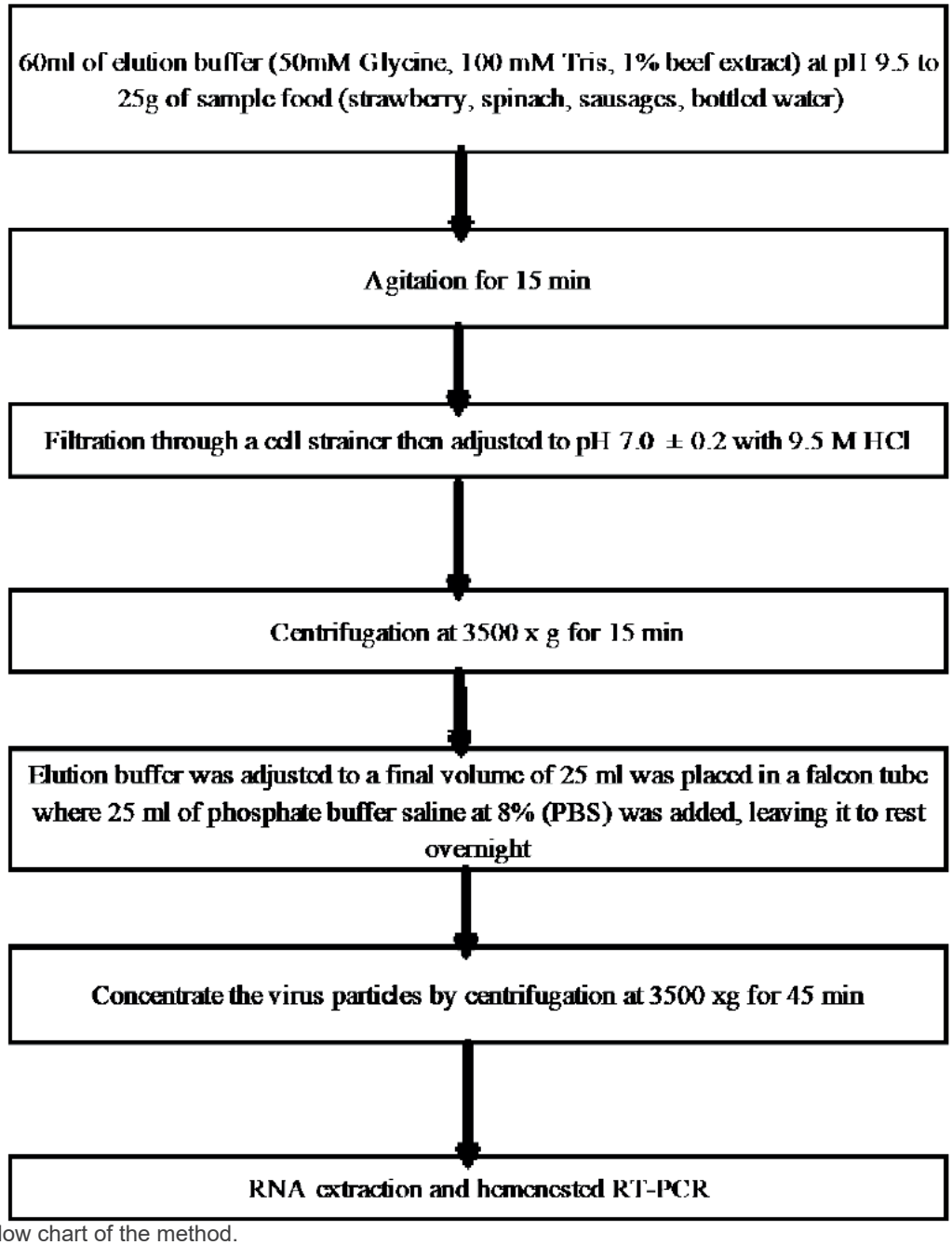

Figure 2. Flow chart of the method.

MgCl2, 0.4 ul of 10uM dNTPs, 1 ul of 0.2uM primers, 0.08 ul Platinum Taq DNA Polymerase (Invitrogen) and 14.52 ul of RNase-free water. PCR conditions were as follows: initial denaturation at 94°C for 2min, 40 cycles of denaturation at 94°C for 30s, annealing at each optimal annealing temperature (Tables 6, 7 and 8) for 30 s, extension at 72°C for 40 s, and a final extension at 72°C for 5min.

Analysis of PCR Products

The RT-PCR and heminested PCR products were analyzed using 2% agarose gel electrophoresis in TAE buffer. Bands were visualized with Sbyr Safe (Invitrogen, USA).

Results

To evaluate the efficiency of new primer sets (NV-GI-F/R, NVGII-F/R and HAV-F/R) to detect NV and HAV, we used RT-PCR followed by a heminested PCR to improve the sensitivity of the current diagnostic. To this end, four food samples were collected in the local wholesale food market to obtain contaminating viral RNA and diagnose for NV GI, GII and HAV. The presence of RT-PCR inhibitors was evaluated as previously described[29].

The RNA extraction method was also evaluated in conjunction with the food sample treatment process to avoid inhibiting viral RNA by substances present in them (phenols, vitamins, proteins, carbohydrates). This was evidenced since when amplifying the genetic material, amplification of the bands was observed in the food samples that were intentionally inoculated with the virus (NVGI / GII and HAV). The method of inhibiting substances inherent to the food[29] does not influence the extraction of genetic material; therefore, the process is validated concerning the results obtained.

NVGI was detected in the strawberry and spinaches

Primers	Sequence (5'- 3')[a]	Location[b]	Region	Annealing temperature (°C)	Polarity	Reference
NVGI-F	TGGACYCGHGGSCCCAAYCA	5092-5112	RdRP	60	(+)	this study
NVGI-R	GCGTCCTTAGACGCCATCATC	5359- 5380	Capsid	60	(-)	this study
NVGI-RH	GAAVCGCATCCAGCGGAACATGG	5282-5305	RdRP	60	(-)	this study
a. The means of alphabet sequence are the following: H= A, C, T; S = C, G; V=A, C, G; Y = C, T.						
b. Location is based on accession number M87661 (Norwalk virus).						

Table 6. Information of NV GI primer sets.

Primers	Sequence (5'- 3') a	Location[b]	Region	Annealing temperature (°C)	Polarity	Reference
NVGII-F	TGGACNAGRGGNCCYAAYCA	4820-4839	RdRP	55	(+)	this study
NVGII-R	GTCATTCGACGCCATCTTCATTC	5083-5106	Capsid	55	(-)	this study
NVGII-RH	GARAAYCTCATCCAYCTRAAC	5014-5035	RdRP	55	(-)	this study
a. The alphabet sequence means: N= A, C, G, T; R =A, G; Y = C, T.						
b. Location is based on accession number X86557 (Lordsdale virus).						

Table 7. Information of NV GII primer sets.

Primers	Sequence (5'- 3') a	Location[b]	Region	Annealing temperature (°C)	Polarity	Reference
HAV-F	CTATTCAGATTGCAAATTAYAAT	2896-2918	Capsid	45	(+)	this study
HAV-R	AAYTTCATYATTTCATGCTCCT	3269-3291	Capsid	45	(-)	this study
HAV-RH	CTCCAGCTGCAATTCTACTCATC	3033-3056	Capsid	45	(-)	this study
a. The means of alphabet sequence are the following: Y= C, T.						
b. Location is based on accession number M14707 (Hepatitis A virus).						

Table 8. Information of HAV primer sets.

samples. NVGII was detected in spinaches, while HAV was detected in bottled water. Each virus showed a clear amplification product at 213pb, 215 and 160pb, respectively (Fig 3, 4, 5, 6, 7, 8).

To evaluate the primers (NVGI-F/R, NVGII-F/R and HAV-F/R), 57 samples were amplified from the 4 samples selected in the Mount Sinai market and duplicated to the schematization of the proposed design. Of the 57 samples, 12 (21 %) were positive for the NVGI presence. In the case of NVGII, their presence was detected in 11 (19.3 %) of the analyzed samples. In 12 samples (21 %) HAV was detected with primers designed for HAV detection.

Except for the negative control, that is, they do not contain the genetic material; the rest were amplified, demonstrating the validation of the NV GI, NVGII, and HAV detection method through the design of the primers of the present study.

Discussion

In the USA, an estimated 570–800 people die annually from diseases associated with NV infection. Outbreaks of the virus occur regularly, and new variants are identified worldwide every 2 to 3 years[30].

The widespread use of RT-PCR and Heminested PCR for NV and HAV detection can be attributed to its superior sensitivity compared to other methods[31]. Virus detection is susceptible in food because, in most cases, the viral load is low; the virus does not infect food and therefore does not replicate its genetic material. Before extracting RNA from the food, we recommend inhibiting any substance that is part of the food such as phenols, vitamins, proteins, and carbohydrates, using the method described as an experimental protocol[29].

Norovirus GI was detected in strawberries and spinach, Norovirus GII in spinach, and HAV in bottled water. The virus's origin or source of contamination of the food is unknown. Contaminated water for plant irrigation or cross-contamination by sick food handlers can be a possible source of viral infection.

The food handler is an essential link in NV transmission, being epidemiologically involved in 80 % of the NV outbreaks in Belgium[32].

Contamination of food with HAV can occur anywhere in the food chain, from farm to fork. Contact with incorrectly treated sewage or sewage-polluted water, infected food handlers and, to a lesser extent, contaminated surfaces represent the most common routes of HAV contamination in food. Approximately 2–7% of all HAV outbreaks worldwide can be attributed to contaminated food.

In the present study, we developed specific primers to

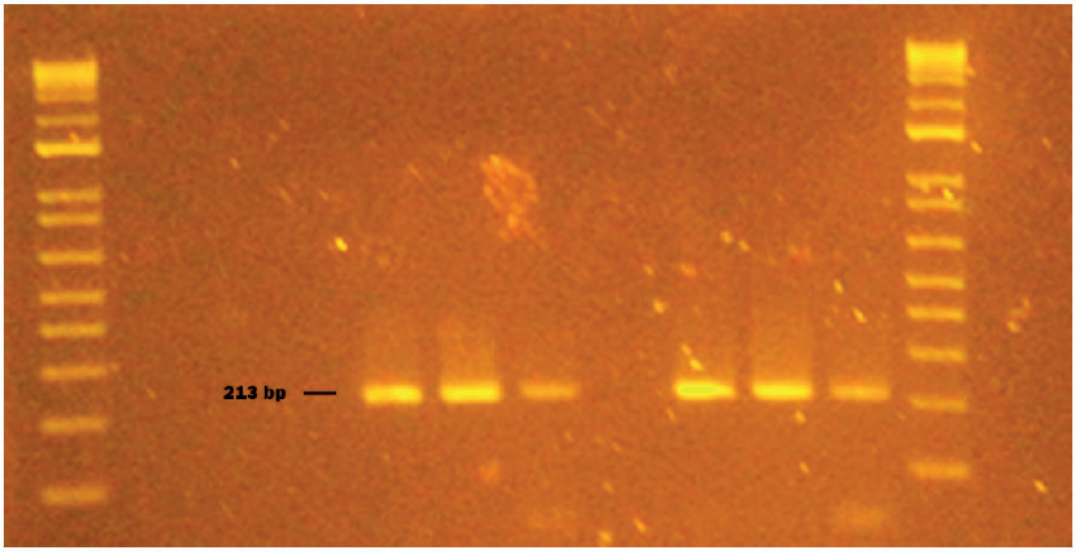

Figure 3. Genogroup GI NV obtained from naturally contaminated food during sampling from October to November 2020 in Monte Sinai, Guayaquil- Ecuador. Lanes 1 and 14: DNA ladder 12000 pb; lane 2 negative control PCR; lane 3 extraction control; lane 4 NVGI identified in strawberry (213pb); lane 5 washed strawberry sample; lane 6 strawberry inoculated with NVGI (Positive Control); lane 7 NVGI virus inoculated without strawberry (Positive Control); lane 8 sausage sample; lane 9 washed sausage sample; lane 10 sausages inoculated with NVGI (Positive Control); lane 11 NVGI virus inoculated without sausage (Positive Control); lane 12 bottled water sample; lane 13 NVGI Positive Control (Synthetic RNA). Samples were analyzed in 2 % agarose gels and stained with cyanine dye.

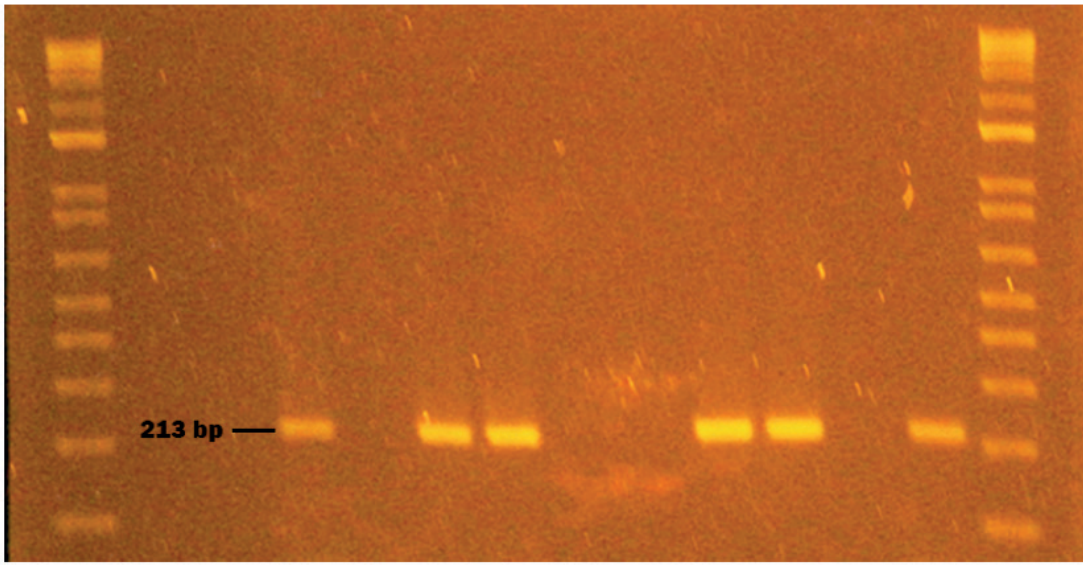

Figure 4. Genogroup GI NV obtained from naturally contaminated food during sampling from October to November 2020 in Monte Sinai, Guayaquil- Ecuador. Lanes 1 and 12: DNA ladder 12000 pb; lane 2 negative control PCR; lane 3 extraction control; lane 4 bottled water sample; lane 5 bottled water sample inoculated with NVGI (Positive Control); lane 6; NVGI virus inoculated without bottled water (Positive Control); lane 7 NVGI identified in spinach (213pb); lane 8 washed spinach; lane 9 spinach inoculated with NVGI (Positive Control); lane 10 NVGI virus inoculated without spinach(Positive Control); lane 11 NVGI Positive Control (Synthetic RNA). Samples were analyzed in 2 % agarose gels and stained with cyanine dye.

detect NV GI, GII and HAV, targeting ORF1, ORF2 and capsid genes, respectively. We used a one-step RT-PCR and heminested PCR protocol to enhance further our ability to detect human enteric food viruses. The study carried out is unprecedented in Ecuador because the presence of the virus has not been evaluated in food in our country. The results obtained in samples such as strawberries, spinach and bottled water in a popular sector of the city of Guayaquil suggest that gastrointestinal diseases in the city may have been crucial viral etiology besides bacterial contamination.

Our study showed that the NVGI-F/R, NVGII-F/R and HAV-F/R primer sets could be necessary for the epidemiological diagnosis of NVGI, NVGII and HAV considering the method of extraction and inhibition of food-specific substances that could inhibit or degrade viral RNA in food matrices.

The Biplot shows that only bottled water has a strong relationship with the presence of HA. Other relationships are observed, although not as strong, such as spinach with the presence of N.GI and N.GII. Sausages and strawberries show connections with the absence of pathogens.

The Biplot obtained through the multiple correspondence analysis (Fig.9) shows the significant relationship be-

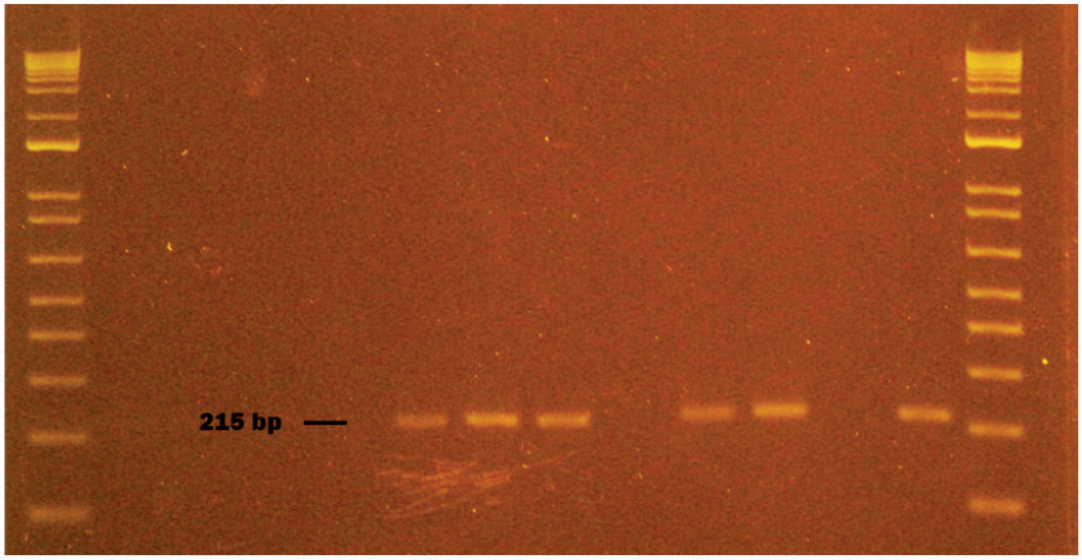

Figure 5. Genogroup GII NV obtained from naturally contaminated food during sampling from October to November 2020 in Monte Sinai, Guayaquil- Ecuador. Lanes 1 and 14: DNA ladder 12000 pb; lane 2 negative control PCR; lane 3 extraction control; lane 4 sausage sample; lane 5 washed sausage sample; lane 6 sausage inoculated with NVGII (Positive Control); lane 7 NVGII virus inoculated without sausage (Positive Control); lane 8 NVGII identified in spinach (215pb); lane 9 washed spinach sample; lane 10 spinach inoculated with NVGII (Positive Control); lane 11 NVGII virus inoculated without spinach (Positive Control); lane 12 strawberry sample; lane 13 NVGII Positive Control (Synthetic RNA). Samples were analyzed in 2 % agarose gels and stained with cyanine dye.

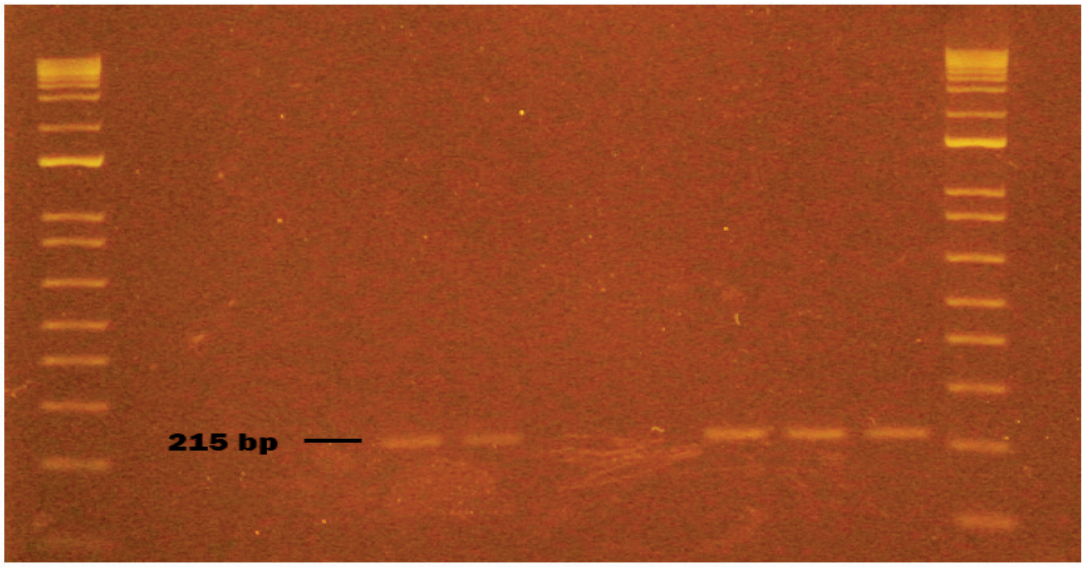

Figure 6. Genogroup GII NV obtained from naturally contaminated food during sampling from October to November 2020 in Monte Sinai, Guayaquil- Ecuador. Lanes 1 and 12: DNA ladder 12000 pb; lane 2 negative control PCR; lane 3 extraction control; lane 4 washed strawberry sample; lane 5 strawberry sample inoculated with NVGII (Positive Control); lane 6 NVGII virus inoculated without strawberry (Positive Control); lane 7 bottled water sample; lane 8 bottled water sample (duplicate); lane 9 bottled water inoculated with NVGII (Positive Control); lane 10 NVGII virus inoculated without bottled water (Positive Control); lane 11 NVGII Positive Control (Synthetic RNA). Samples were analyzed in 2 % agarose gels and stained with cyanine dye.

tween bottled water and the presence of Hepatitis A (Val-p = 0.004). It also shows a relationship (not significant) between spinach and the presence of Norovirus GI and GII (p-value > 0.05). Fisher's (Table 9) test was developed to determine the relationship between the presence of viruses and selected food matrices.

This study is very relevant in our country in the field of public health and food safety because the presence of these pathogens in food shows that the gastrointestinal problems associated with the population are not exclusively caused by bacteria, which a later study raises in determining in which other types of foods it is likely to detect viral load by Nv and HAV concerning a risk matrix.

In the case of viruses such as Hepatitis A and Norovirus present in spinach, strawberries and water samples, they show that many gastrointestinal problems that occur in Ecuador may not necessarily be caused by bacteria but also by viruses.

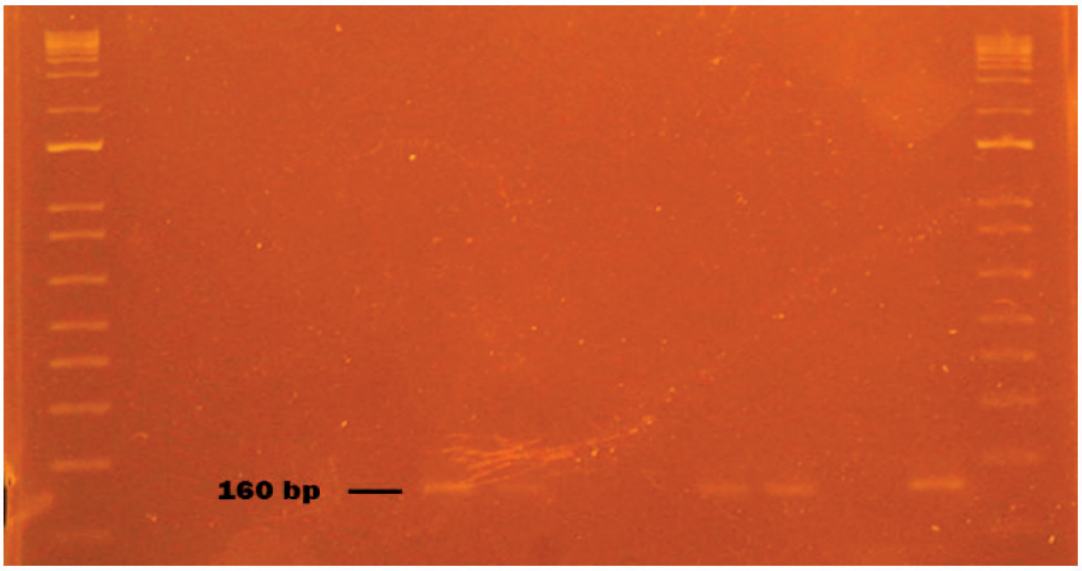

Figure 7. HAV virus obtained from naturally contaminated food during sampling from October to November 2020 in Monte Sinai, Guayaquil- Ecuador. Lanes 1 and 14: DNA ladder 12000 pb; lane 2 negative control PCR; lane 3 extraction control; lane 4 sausage sample; lane 5 washed sausage samp2le; lane 6 sausage inoculated with HAV (Positive Control); lane 7 HAV virus inoculated without sausage (Positive Control); lane 8 spinach sample; lane 9 washed spinach sample; lane 10 spinach inoculated with HAV (Positive Control); lane 11 HAV virus inoculated without spinach (Positive Control); lane 12 strawberry sample; lane 13 NVGII Positive Control (Synthetic RNA). Samples were analyzed in 2 % agarose gels and stained with cyanine dye.

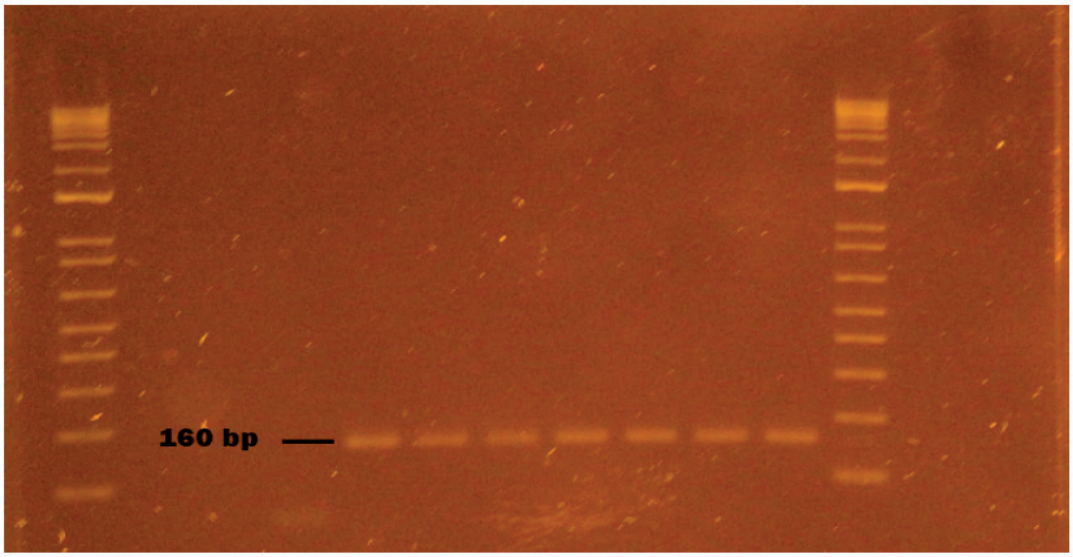

Figure 8. HAV virus obtained from naturally contaminated food during sampling from October to November 2020 in Monte Sinai, Guayaquil- Ecuador. Lanes 1 and 12: DNA ladder 12000 pb; lane 2 negative control PCR; lane 3 extraction control; lane 4 washed strawberry sample; lane 5 strawberry sample inoculated with HAV (Positive Control); lane 6 HAV virus inoculated without strawberry (Positive Control); lane 7 HAV identified in a bottled water sample (160pb); lane 8 HAV identified in bottled water sample duplicate (160pb); lane 9 bottled water inoculated with HAV (Positive Control); lane 10 HAV virus inoculated without bottled water (Positive Control); lane 11 HAV Positive Control (Synthetic RNA). Samples were analyzed in 2 % agarose gels and stained with cyanine dye.

Conclusions

Foodborne diseases are a public health problem worldwide. In the case of biological agents responsible for foodborne illnesses, it is common but largely underdiagnosed.

Norovirus and Hepatitis A are two classes of viruses that have been diagnosed within this group as the main ones responsible for foodborne illnesses, even according to reports documented by the United States Center for Disease Control and Prevention (CDC), 50% of Gastrointestinal disease in the American population is due to Norovirus, which can infect with a relatively low dose.

The great challenge of this research work was to develop a method that allows determining the presence of Norovirus and Hepatitis A in food, considering how complex it is to detect viruses in them because the food as such does not provide the requirements that the obligate intracellular parasite required to carry out the process of replication and increase in viral load.

To detect the viral load in food matrices such as strawberries, spinach, water and sausages, it was previously ne-

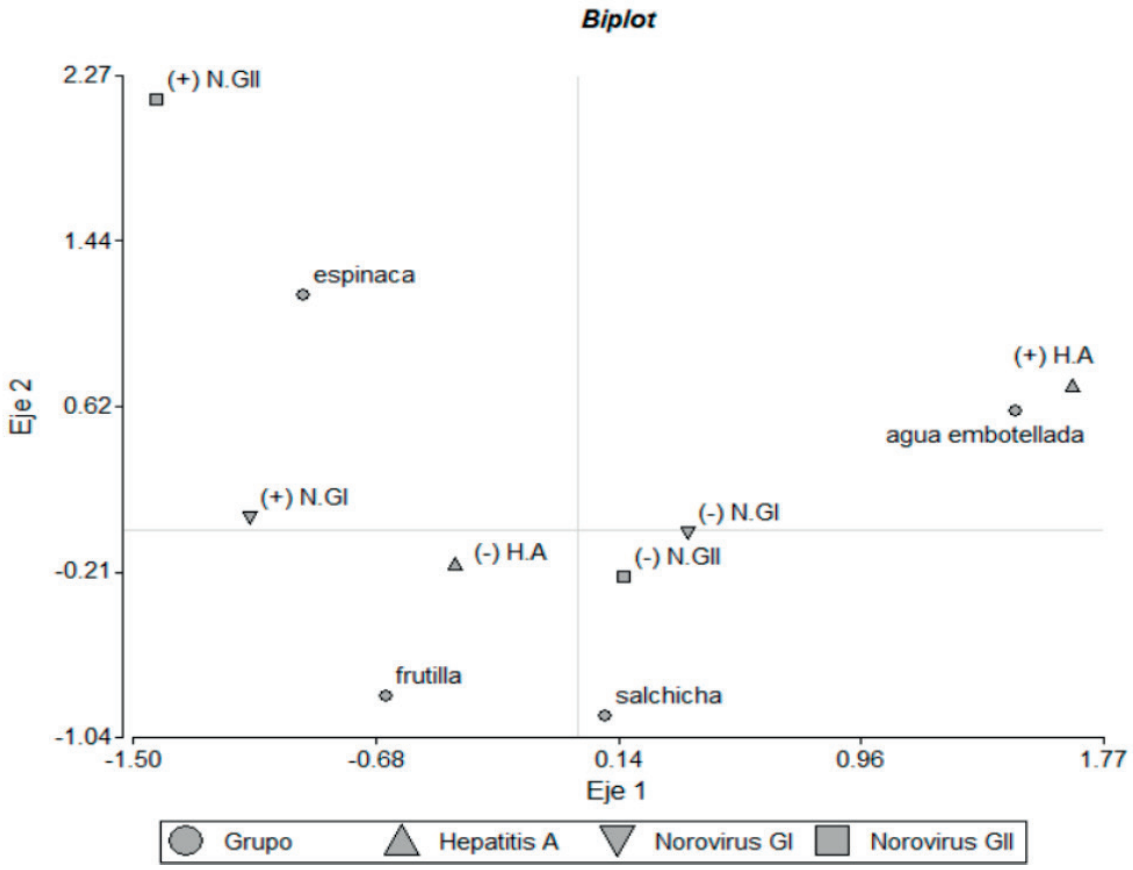

Figure 9. Biplot obtained through the A. Multiple Correspondence, which shows the relationships between pathogens and food groups.

cessary to inhibit compounds that are part of the chemical composition of the food, such as phenols, vitamins, proteins and carbohydrates, to avoid inhibition of the genetic material of the virus present. on the surface of the food.

The importance of this work was that through a good approach in developing the technique used to detect Hepatitis A and Norovirus in food matrices sampled in the city of Guayaquil, it has identified the presence of these infectious biological agents and probably the association of diseases. Significant foodborne infections associated with transmission by food handlers and sewage-contaminated food for these viruses. According to reports from the Ministry of Public Health in Ecuador, there are no reports related to gastrointestinal problems due to viral origin (Norovirus), which entails a greater challenge in properly identifying the origin of the disease and the palliative treatments applied to sick or dying people. recovery process. It is important to note that complex mixtures of human and animal viruses and other pathogens can be present in a single food, creating the potential for recombination or genetic rearrangement and thus further expansion of the diversity of these pathogens. Bringing together expertise from veterinary, food and clinical microbiology can help unravel these complexities and identify areas for intervention and prevention.

Author Contributions

"Conceptualization, E. J. Salazar and M.J. Guerrero.; methodology, E. J. Salazar.; software, M.J. Guerrero.; validation E. J Salazar, K.S. Suárez. and J.A. Villaquiran.; formal analysis M.J.Guerrero.; investigation, E.J. Salazar.; resources, J.M. Cevallos.; data curation M. J. Guerrero.; writing—original draft preparation, E.J. Salazar.; writing—review and editing, J.M. Cevallos.; visualization, E.J.Salazar.; supervision, J.M.Cevallos.; project administration, M.J. Guerrero.; funding acquisition, J.M.Cevallos.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

"Not applicable." for studies not involving humans.

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Conflicts of Interest

There are no conflicts of interest.

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Frequency table:
Hepatitis A
Group Absence HA Presence HA
bottled water 1 4
spinach 5 0
strawberry 5 0
sausage 5 0
Fisher's Exact Test for Count Data
p-value = 0.004128
alternative hypothesis: two. Sided
Frequency table:
Norovirus.GI
Group Absence N.GI Presence N.GI
bottled water 5 0
spinach 3 2
strawberry 2 3
sausage 5 0
Fisher's Exact Test for Count Data
p-value = 0.09701
alternative hypothesis: two. sided
Frequency table:
Norovirus.GII
Group Absence N.GII Presence N.GII
bottled water 5 0
spinach 3 2
strawberry 5 0
sausage 5 0
Fisher's Exact Test for Count Data
p-value = 0.2105
Alternative hypothesis: two. Sided

Table 9. Statistical support Fisher's exact test, for Annexes or complementary material.

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