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BRIEF COMMUNICATION

Norovirus Foodborne Outbreak Associated With the Consumption of Ice Pop, Southern Brazil, 2020

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Abstract

Norovirus is a major cause of foodborne-associated acute gastroenteritis (AGE) outbreaks worldwide. Usually, food products are contaminated either during harvesting or preparation, and the most common products associated to norovirus outbreaks are raw or undercooked bivalve shellfish, fruits (frozen berries) and ready-to-eat produce. In the present study, we investigated an AGE outbreak caused by norovirus associated with the consumption of ice pops in southern Brazil. Clinical stool samples from patients and ice pops samples were collected and analyzed for viruses' detection. By using RT-qPCR and sequencing, we detected the uncommon genotype GII.12[P16] in clinical samples and GII.12 in samples of ice pop. Strains shared identity of 100% at nucleotide level strongly suggesting the consumption of ice pops as the source of the outbreak.

Keywords Foodborne outbreak · Norovirus · Ice pop · Acute gastroenteritis · Brazil

Introduction

Every year, contaminated food results in 600 million cases of foodborne diseases and 420,000 deaths worldwide (WHO, 2015). Human noroviruses are recognized as important cause of foodborne illness outbreaks globally (FAO/WHO, 2012; WHO, 2015). The estimated proportion of norovirus-illnesses attributed to food ranges from 12 to 47% (FAO/WHO, 2012; Hardstaff et al., 2018) and in many countries norovirus causes the highest number of foodborne illness (Lee & Yoon, 2021). Worldwide, norovirus accounts for one-fifth of all cases of acute gastroenteritis (AGE) among all age groups, leading to an estimated $ 4.2 billion in direct health system costs each year and over 200,000 deaths, mostly in low-income countries (Ahmed et al., 2014; Atmar et al., 2018; Bányai et al., 2018).

Norovirus belongs to the genus *Norovirus* in the family *Caliciviridae*. Noroviruses are divided into ten genogroups and at least 48 genotypes based on amino acid identity of the major capsid protein (VP1) encoded by ORF2 (Chhabra et al., 2019; Green, 2013). Also, a dual classification system (P = polymerase and C = capsid) was created by including the typing of the polymerase region (located at the 3′ end of ORF1) and currently, at least 60 P-types have been identified (Chhabra et al., 2019). Although viruses belonging to GI, GII, GIV, GVIII and GIX can cause disease in humans, the majority of illnesses are associated to GI and GII genotypes (Chhabra et al., 2019; Vega et al., 2011). Norovirus disease affects all age groups and despite its broad genetic diversity, genotypes belonging to GII, specifically GII.4 and more recently GII.2 and GII.17, account for the majority of norovirus infection worldwide (Barclay et al., 2019; De Graaf et al., 2015; Niendorf et al., 2017).

In Brazil, several studies have described the role of norovirus as a major cause of AGE in outpatients and also in hospitalized patients. Fumian et al. (2013) detected norovirus in 34.1% in children hospitalized for AGE in Belém city, Northern Brazil. Another study performed in southern Brazil, between 2004 and 2011, revealed norovirus causing almost 50% of AGE outbreaks reported by the State Central

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Laboratory (Andrade et al., 2014). While norovirus prevalence and molecular epidemiology are well described in Brazil, foodborne viral outbreaks are rarely investigated, so there is a lack of knowledge of the burden of norovirus outbreaks associated with foodborne transmission. So far, only three studies have investigated norovirus foodborne outbreak in Brazil. Two of them have analyzed either food (Morillo et al., 2012) or clinical samples (Andrade et al., 2018) and just one study analyzed and detected norovirus from both type of samples to confirm the source of the outbreak (Morillo et al., 2017). Here, we describe an AGE outbreak caused by norovirus likely associated with the consumption of contaminated ice pops that affected more than two hundred people in at least four different districts in southern Brazil. We detected and characterized norovirus from both clinical stool samples and the food product using RT-qPCR and sequencing to elucidate the origin of an outbreak.

Material and Methods

Outbreak Description

On 19th October 2020, the Food- and Waterborne Disease State Program of Rio Grande do Sul State, Brazil, became aware of an outbreak of AGE occurring in a municipality located in the coast of the state (https://g1.globo.com/rs/rio-grande-do-sul/noticia/2020/10/21/secretaria-da-saude-do-rs-emite-alerta-sobre-marca-de-picoles-apos-surto-de-intoxicacao-no-litoral-norte.ghtml). Initially, public health authorities notified at least 50 outpatients, children and adults, showing symptoms of vomiting, diarrhea and nausea. By the end of October 2020, the number of outpatients increased to 176. Other cases in nine neighboring municipalities with clinically compatible illness (vomiting and diarrhea) were also notified by the State Surveillance Health Secretariat. Through epidemiological investigations conducted through face-to-face interviews during emergency medical visits or by telephone, most of the cases informed the consumption of a local brand of ice pop. The factory is located in one of these municipalities with a restricted local chain of distribution within the state. Patients reported the onset of AGE symptoms after 12–24 h of the suspect ice pop consumption.

Clinical and Food Samples

Clinical stool samples ($n=6$) were collected from symptomatic cases from three municipalities, being four from one municipality and two others from two neighboring municipalities. Samples were collected between 20th and 22nd October 2020, from patients aged between 3 and 36 years old, and forwarded to Laboratory of Comparative and Environmental Virology (LVCA) for viral investigation. The LVCA houses the Regional Rotavirus Reference Laboratory (RRRL) at Oswaldo Cruz Institute and is part of the ongoing national viral AGE surveillance program coordinated by General Coordination of Public Health Laboratories, Brazilian Ministry of Health. Patient's data are maintained anonymously and the study is approved by the Ethics Committee of the Oswaldo Cruz Foundation (FIOCRUZ), number CAAE: 94144918.3.0000.5248.

Ice pop were collected from cases' homes in four municipalities that recorded AGE cases after the consumption of the products. Frozen ice pop samples (unopened package) were sent to LVCA to investigate for viruses' presence. Ice pops were separated in batches according to collection site. The batches were thawed, mixed in a sterile beaker and 42 mL of each batch was collected for viral concentration using the ultracentrifugation method (Pina et al., 1998). Briefly, samples were centrifuged at 100,000 × g for 1 h at 4 °C. After supernatant discharge, pellet was re-suspended and mixed in 4 mL of 0.25 N glycine buffer (pH 9.5) and incubated at 4 °C for 30 min. The solution was then neutralized by adding 4 mL of 2 × phosphate-buffered saline (PBS, pH 7.2), and clarified by centrifugation at 12,000 × g for 20 min. Finally, supernatant samples (~ 8 mL) were centrifuged at 100,000 × g for 1 h at 4 °C, viral particles were re-suspended in 400 µL of 1 × PBS (pH 7.2) and processed immediately for nucleic acid extraction or stored at − 80 °C until use.

Nucleic Acid Extraction

Viral nucleic acids were purified from 140 µL of clarified stool suspension (10% *w/v*) prepared with Tris-calcium buffer (pH = 7.2) and from 140 µL of concentrated ice pop samples. Samples were subjected to an automatic nucleic acid extraction using a QIAamp® Viral RNA Mini kit and a QIAcube® automated system (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions and eluted in 60 µL of AVE elution buffer. The isolated nucleic acid was immediately tested for virus presence and the remaining was stored at − 80 °C for further molecular analysis. In each extraction procedure, RNAse/DNAse-free water was used as negative control.

Virus Detection and Quantification

Norovirus GI and GII and rotavirus were screened using TaqMan®-based RT-qPCR with primers and probes as previously described by Kageyama et al. (2003) and Zeng et al. (2008), respectively. Primers (COG1F and R; COG2F and R; final concentration of 600 nM) and probes (RING1C and RING2; final concentration of 300 nM) targeting ORF1-2 junction region were used to detect norovirus GI and GII,

respectively. For rotavirus, primers (NSP3F and R, final concentration of 400 nM) and probe (NSP3p, final concentration of 200 nM) targeting the conserved NSP3 gene were used. All qPCR reactions, performed in duplicate, were carried out in an ABI PRISM 7500® Real-Time System v2.0 (Applied Biosystems, Foster City, CA, USA) using 12.5 µL of SuperScript™ III Platinum® One-Step Quantitative RT-PCR kit (Invitrogen, CA, USA) and 5 µL of extracted RNA for a final volume of 25 µL. For RT-qPCR protocols, positive, negative and non-template controls (NTC) were included. Norovirus quantification was performed using a standard curve with serial dilutions (10^6–10^1) of a double-stranded DNA fragments (gBlock® Gene Fragment, Integrated DNA Technologies, Iowa, USA) containing the qPCR target region (ORF1-2 junction) of norovirus genome. Samples with Ct value equal to or less than 38 showing a characteristic sigmoid curve were considered positive.

Norovirus Molecular Characterization

For molecular characterization of norovirus GII, it was used primers of Mon 431 and G2SKR targeting the 3′-end of ORF1 and 5′-end of ORF2 generating a ~557 bp amplicon (Cannon et al., 2017). For ice pop samples, a semi-nested PCR using primers COG2F (Kageyama et al., 2003) and G2SKR was used to achieve enough DNA for sequencing reaction. Sanger sequencing was performed using both forward and reverse primers with the BigDye™ Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) and reactions were run at FIOCRUZ Institutional Sequencing Platform (PDTIS) on an ABI Prism 3730*xl* genetic analyser (Applied Biosystems). Consensus sequences were obtained using Geneious prime (Biomatters Ltd, Auckland, New Zealand). Norovirus genotypes were firstly assigned using the two norovirus typing tools (https://www.rivm.nl/mpf/typingtool/norovirus and https://norovirus.ng.philab.cdc.gov). Phylogenetic trees were constructed using the maximum-likelihood method and the Kimura two-parameter model (2000 bootstrap replications for branch support) in MEGA X (Kumar et al., 2018) with norovirus reference sequences obtained from the National Center for Biotechnology Information (NCBI) database. Norovirus GII nucleotide sequences generated in this study were submitted to GenBank and assigned the following accession numbers: MW676032 to MW676035.

Results and Discussion

Here we describe an AGE outbreak caused by norovirus to be associated with the consumption of ice pop occurred in southern Brazil. Five clinical samples (5/6) tested positive for norovirus GII. Three of these samples were detected with high viral loads per gram of stool, demonstrated by the low Ct values (13.3, 17 and 18.2) and the other two samples were detected at Ct values of 28.7 and 29.1. We successfully sequenced three samples and the recombinant GII.12[P16] norovirus was identified. Identity among the three sequenced strains were 100% at nucleotide level (Fig. 1). All samples tested negative for rotavirus and norovirus GI.

Recently, the polymerase type P16 has been detected associated with two emergent and widespread genotypes: GII.4 Sydney and GII.2 (Ao et al., 2016; Bidalot et al., 2017; Lun et al., 2018; Cheung et al., 2019). In Australia and New Zealand, norovirus clinical and wastewater-based molecular surveillance demonstrated the cocirculation and increased prevalence of GII.4 Sydney and GII.2 in 2016 and 2017, both of which include the P16 polymerase type (Lun et al., 2018). In Brazil, P16 type combined with GII.4 and GII.2 genotypes have been detected in AGE stool samples since 2016 (Barreira et al., 2017; Cantelli et al., 2019; Hernandez et al., 2020) and also from bivalve shellfish samples (Sarmento et al., 2020). Norovirus GII.12 is an uncommon genotype that is sporadically detected (van Beek et al., 2018). It is usually detected as a recombinant strain, commonly associated with Pg type, as GII.12[Pg] (Giammanco et al., 2012; Mans et al., 2014; Vega et al., 2011).

Our study describes for the first time in Brazil the detection of the recombinant strain GII.12[P16]. In Canada, during March 2018 and February 2019, the Alberta Molecular Surveillance Program detected this novel recombinant genotype GII.12[P16], and it was the second most prevalent strain identified in AGE outbreaks and sporadic cases after November 2018 (Pabbaraju et al., 2019). Sequences, within the ORF1-2 amplified region, from clinical samples detected in our study shared nucleotide identity varying from 99% to 99.4% with GII.12[P16] strains from Canada (MK355721), USA (MK754447), Spain (MT501819) and Japan (LC579431), within the ORF1-2 amplified region. Polymerase type P16 has been identified associated with multiple capsid genotypes (Barclay et al., 2019), and studies have suggested that, at least for GII.4 viruses, genetic changes in the GII.P16 RNA-dependent RNA polymerase may provide an increased transmissibility and an advantage of infection (Choi et al., 2017; Ruis et al., 2017; Tohma et al., 2017).

Regarding the ice pop samples, we tested seven batches and three tested positive for norovirus GII. Samples Ct values were 28.4, 34.1 and 37.8, corresponding to 7.6×10^3, 1.7×10^2 and 1.4×10^1 genome copies/ml of ice pop. Considering the low infectious dose of norovirus (≥ 18 viral particles) (Teunis et al., 2008), our results demonstrates that the viral load found in the samples was sufficient to start an infection, even if part of the detected viruses was represented by non-infectious particles. By semi-nested RT-PCR, we successfully obtained enough DNA for sequencing the

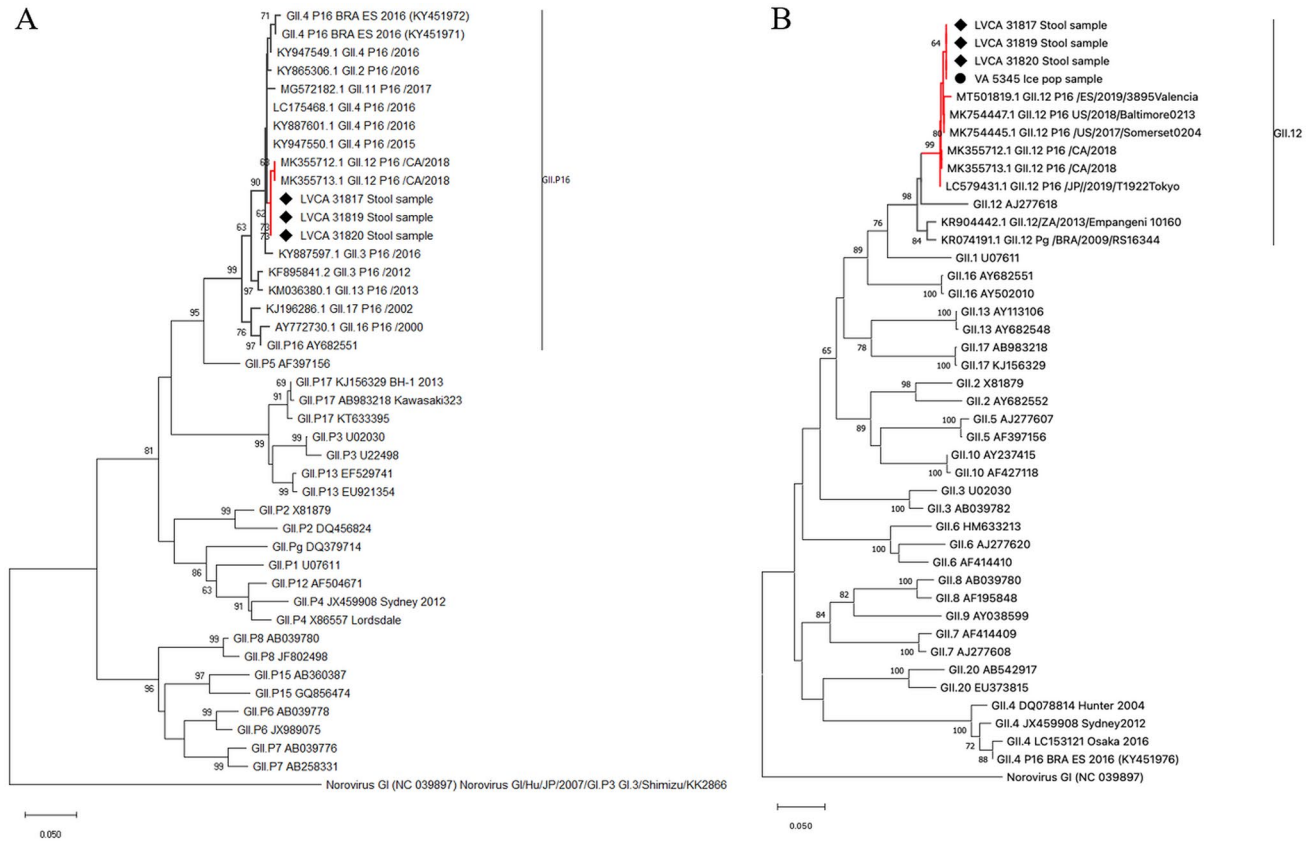

Fig. 1 Phylogenetic trees based on polymerase (**A**) and capsid (**B**) regions of GII norovirus. Norovirus GII strains isolated from clinical and ice pop samples in this study are shown in the phylogenetic analysis and are marked with a filled diamond. Reference strains were downloaded from GenBank and labeled with their accession number followed by genotype, year and country of isolation. Maximum likelihood phylogenetic trees were constructed with MEGA X software and bootstrap tests (2000 replicates) based on the Kimura two-parameter model. The bootstrap percentage values of ≥ 70% are shown at each branch point

sample with the lowest Ct value. After editing, we obtained a 366-nucleotide fragment of the 5`-end ORF2, that was genotyped as GII.12, with 100% of nucleotide identity with strains isolated from clinical samples (Fig. 1). It is worth mentioning that clinical and food samples were processed, analyzed and sequenced in different days and using separated rooms, ruling out the possibility of cross-contamination of samples within the laboratory.

It is well described the role of norovirus as a major pathogen associated with foodborne AGE and contamination usually occurs during food production or preparation (Hardstaff et al., 2018). Several foodborne norovirus-linked AGE outbreaks have been associated with the consumption of contaminated oysters and other raw shellfish (Alfano-Sobsey et al., 2012; Bellou et al., 2013; Doyle et al., 2004; Fouillet et al., 2020; Woods et al., 2016). Moreover, other types of food products had also been implicated with foodborne norovirus AGE outbreaks. Saupe et al. (2020) described an outbreak of norovirus AGE in Minnesota, USA, linked to the consumption of ice cream. Molecular analysis identified norovirus GII.17[P17] in clinical stool samples from patients and norovirus P17 was also detected in raspberry samples used during the ice cream production suggesting the source of the outbreak. More recently, a rare norovirus strain (GIV) was detected during an AGE foodborne outbreak in USA, linked to the consumption of fruit salad (Barclay et al., 2021). In our study, we detected an uncommon norovirus genotype GII.12 in ice pop samples sharing 100% identity to norovirus sequences from cases stool specimens suggesting the consumption of ice pops as the source of the outbreak.

Excepted shellfish samples, methods used for viral recovery from food product are still experimental and challenging (Saupe et al., 2020), especially considering the broad types of foods that potentially could be implicated as transmission vehicle, such as fruits, salads, juices and sandwiches. For viral recovery, we used the ultracentrifugation method and successfully detected norovirus GII from ice pop samples. This method has been mainly used to concentrate viruses from small-volume sewage samples (Lun et al., 2018; Prado et al., 2021). In the absence

of strong epidemiological records, food testing is critical to confirm the outbreak vehicle. Unfortunately, it was not possible to point when the contamination occurred throughout the ice pop chain of production, neither of food-handler manipulation nor of contaminated ingredients. Another limitation of our study is the small number of food and clinical samples analyzed that precludes to assert the real source of the outbreak. In addition, the small volume of ice pop used for viral concentration could be pointed as one reason for the negative results among the analyzed samples.

In conclusion, we described an AGE outbreak affecting more than two-hundred people in nine municipalities in southern Brazil, possible associated with norovirus-contaminated ice pops. Norovirus GII.12[P16] was detected and characterized from clinical stool samples and from ice pop products collected from patients' home. In many countries including Brazil, viral foodborne outbreaks are common but rarely investigated. Therefore, efforts from different areas, such as local health authorities, epidemiologists and virologists are necessary to investigate and elucidate the cause of the outbreak and track its origin.

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Data Availability The data generated during and/or analyzed during the current study are included in this published article and additional information are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare they have no actual or potential competing financial interests.

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