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ORIGINAL PAPER

Molecular Detection of human Noroviruses in Influent and Effluent Samples From Two Biological Sewage Treatment Plants in the Region of Monastir, Tunisia

Mouna Hassine-Zaafrane · Khira Sdiri-Loulizi · Jérôme Kaplon · Imen Ben Salem · Pierre Pothier · Mahjoub Aouni · Katia Ambert-Balay

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Abstract Noroviruses (NoVs) are responsible for numerous cases of waterborne and foodborne gastroenteritis every year. They are released in the sewage and their detection in this environment can reflect the epidemiology of the viral strains circulating in the community. A three-year (2007–2010) survey was conducted in order to evaluate the presence of human NoVs using RT-PCR in 518 sewage samples collected at the entrance and exit of two biological sewage treatment plants located in Monastir region, Tunisia. In this study, we aimed to genetically characterize the most prevalent GI and GII NoV strains, in order to obtain a rough estimate of the efficacy of disinfection treatments and to compare the results with clinical data documented in the same area during the same period. This work confirms the wide circulation and the genetic diversity of NoVs in Tunisia and the widespread distribution of NoV variants in both raw and treated wastewater. Indeed, NoV was detected in 192 (37.1 %) sewage samples, among them mixed infections with group A rotavirus were detected in 125 (65.1 %) cases. The genotypes of the GI NoVs were GI.1, GI.2, GI.4, GI.5, and GI of unassigned genotype (GI.UA), and the genotypes of the GII NoVs were all GII.12. This study enhances the currently poor environmental virological data gathered in Tunisia, demonstrates the benefit of environmental surveillance as a tool to determine the epidemiology of NoVs circulating in a given community, and underlines the need for the design and support of similar long-term studies in our country, in order to compensate for the absence of a national surveillance system for gastroenteric viruses.

Keywords Norovirus · Sewage · RT-PCR · Sequencing · Phylogenetic analysis · Tunisia

M. Hassine-Zaafrane · K. Sdiri-Loulizi · I. B. Salem · M. Aouni
Laboratory of Infectious Diseases and Biological Agents, Faculty of Pharmacy, University of Monastir, 5000 Monastir, Tunisia

M. Hassine-Zaafrane (✉) · K. Sdiri-Loulizi · J. Kaplon · P. Pothier · K. Ambert-Balay
National Reference Center for Enteric Viruses, Laboratory of Virology, CHU of Dijon, University of Bourgogne, 2 Rue Angélique Ducoudray, 21000 Dijon, France
e-mail: mouna20781@yahoo.fr

Introduction

Noroviruses (NoVs) are among the most common viral agents that cause gastroenteritis in humans of all ages worldwide. They spread through the fecal-oral route as well as by aerosolized vomitus and fomite contamination. NoVs are shed in feces at high concentrations of 10^5–10^9 virus particles per gram during the symptomatic phase (Mori et al. 2005; Ozawa et al. 2007), but they are also shed from patients after cessation of the symptoms (Marshall et al. 2001; Rockx et al. 2002; Ozawa et al. 2007). In addition, recent reports showed relatively high levels of shedding from asymptomatic individuals (Goller et al. 2004; Mori et al. 2005; García et al. 2006; Ozawa et al. 2007). NoVs can persist for long periods in the environment, and be introduced into environmental waters through the discharge of sewage. In addition to contact with contaminated surfaces or via close contact with an ill person, NoVs can, therefore, be transmitted by drinking or accidentally swallowing contaminated water, as well as by eating contaminated foods.

Diagnosis of NoV infection is difficult because the virus is uncultivable, and electron microscopy (EM) is insensitive. The development of a broadly reactive reverse

transcription polymerase chain reaction (RT-PCR) has facilitated the detection of the virus in both clinical and environmental samples.

In Tunisia, routine laboratory diagnosis of NoVs in stool samples of cases of acute gastroenteritis is not performed; therefore, little is known about the prevalence of NoV in endemic and epidemic disease (Sdiri-Loulizi et al. 2009; Hassine-Zaafrane et al. 2013). Since NoVs are released in the sewage, environmental surveillance could provide valuable supplementary information about the epidemiology of circulating strains in the community.

To the best of our knowledge, only our previous study initiated in January 2003 and documenting epidemiological data for NoVs (Sdiri-Loulizi et al. 2010) in Tunisian sewage has been conducted. In the present work, sewage samples collected from two biological sewage treatment plants (STPs) in Monastir region, Tunisia were analyzed in order to continue the evaluation of NoV contamination and the identification of their genetic and evolutionary characteristics in the environment, as a reflection of the diversity of these viruses in the population. Comparison of these strains with those found in the pediatric population was also performed.

The understanding of the circulation of NoVs in the environment and in the community should help improving guidelines for better prevention of infection by this widespread viral pathogen.

Materials and Methods

Sewage and Stool Samples

Environmental samples were collected regularly during a period of 3 years from April 2007 to April 2010. Two hundred fifty nine samples each of 500 ml of raw and treated sewage were collected upstream (inlet) and downstream (outlet), respectively, from two STPs located in the Monastir region: El Frina (STP-A) and Sayada-Lamta-Bouhjar (STP-B), for a total of 518 samples. At these plants activated sludge and aeration were applied for sewage treatment. STP-A was charged with 71,546 population equivalents (pe) up to a total capacity of 13,500 m^3 of urban sewage per day. STP-B has a total capacity of 6 044 m^3 of urban sewage per day and was polluted with 22 947 pe.

The samples were transferred to the laboratory into a cool box and they were stored at +4 °C upon arrival.

Stool samples were collected during the same period and in the same region, between April 2007 and April 2010 in Monastir, from children with symptoms of acute gastroenteritis. Their characterization was reported in Hassine-Zaafrane et al. 2013.

Sample Concentration, Viral Extraction, and Molecular Analysis

Viruses were extracted and concentrated from sewage samples according to a previously published protocol (Sdiri-Loulizi et al. 2010) with minor modifications, i.e., the second centrifugation was done at 10,000 g for 45 min, and the final resuspension was made in 3 mL phosphate-buffered saline (PBS) pH 7.

One milliliter of each environmental concentrate was then used for the extraction of viral RNA using the NucliSENS® EasyMAG™ platform (bioMérieux, Marcy L'Etoile, France), according to the manufacturer's instructions. RNA was eluted in a final volume of 110 µl before the amplification procedure by RT-PCR. NoV detection was done using two previously described RT-PCR that target a conserved region of the viral RNA-dependent RNA polymerase (RdRp) gene and a conserved region of the ORF2 gene (Sdiri-Loulizi et al. 2009).

Sequence Analysis

To determine the genotypes of NoVs detected in the sewage samples, the PCR products were directly applied for sequence analysis and sequences were compared with those of reference strains in the GenBank, as previously described (Hassine-Zaafrane et al. 2013). The nucleotide sequences in this study were submitted to the GenBank database under nucleotide accession numbers *JX455837-JX455895*.

Statistical Analyses

Statistical analyses were performed with SPSS® software, version 19 as previously described (Hassine-Zaafrane et al. 2011).

Results

Prevalence and Phylogenetic Analysis of NoVs Detected From Sewage

NoV was detected in 192 (37.1 %) sewage samples with 100 (52.1 %) and 92 (47.9 %) positive samples in raw and treated sewage, respectively ($P = 0.467$).

The distribution of NoV strains before and after treatment showed no significant difference in STP-A ($P = 0.374$) and STP-B ($P = 0.639$) (Table 1).

Genogroup GI was detected in 185 samples (96.4 %), genogroup GII in four samples (2 %), and both GI and GII in 3 samples (1.6 %). The genotyping showed a great diversity in the genogroup I, with five different genotypes

Table 1 Distribution of NoV strains in raw and treated sewage in the two biological STPs

Strains	*El Frina (STP-A)* ($n = 260$)		*SLB (STP-B)* ($n = 258$)	
	Raw sewage	Treated sewage	Raw sewage	Treated sewage
Musgrove GI.5 ($n = 13$)	1	5	2	5
GI. unassigned ($n = 108$)	33	25	27	23
Norwalk GI.1 ($n = 26$)	8	6	6	6
Chiba GI.4 ($n = 32$)	9	5	8	10
Southampton GI.2 ($n = 5$)	0	1	2	2
Wortley GII.12 ($n = 4$)	0	0	2	2
Chiba GI.4/Wortley GII.12 ($n = 3$)	1	0	0	2
Norwalk GI.1/Musgrove GI.5 ($n = 1$)	1	0	0	0
Total	53	42	47	50
*P**	0.374		0.639	

* χ^2 Test and Fisher's exact test, $P \leq 0.05$ when there is a significant difference in the distribution of NoV strains between the raw and treated sewage of each STPs

isolated, while only one genotype in genogroup II was characterized (Table 1).

A mixed contamination by two different genotypes of genogroup GI was found: the genotype GI.1 was detected by amplification of the polymerase gene and the genotype GI.5 identified by amplification of the capsid gene.

The presence of both genogroups GI and GII in the same sewage sample was observed in three samples involving both strains GI.4 and GII.12.

The sequence and phylogenetic analyses of the capsid region of NoV strains (Fig. 1) showed that the 26 GI.1 strains were homologous to each other (87.9–100 % nucleotide identity) and that they showed high identity to the reference strain Norwalk/1968/US (GenBank accession number M87661) (89.6-100 % nucleotide identity).

GI.unassigned (GI.UA) strains detected in raw and treated sewage at STP-A and STP-B were homologous to each other (95–100 % nucleotide identity) and presented high identity to Singaporean strain Hu/GGI/UE/270607-1/2007/SGP (GenBank accession number GQ925160) with 96.2–97.9 % nucleotide identities.

The fourteen GI.5 strains shared 99.5–100 % nucleotide identity with each other and presented 91.5–93.4 % nucleotide identity in the capsid region with the reference strain Musgrove/1989/UK (GenBank accession number AJ277614).

The GI.4 strains displayed nucleotide identities ranging from 93.6 to 96.4 % in the capsid gene with reference strain Chiba407/1987/JP (GenBank accession number AB042808). All these strains shared nucleotide identities ranging from 92.4 to 100 % with each other.

The five GI.2 strains shared nucleotide identities ranging from 94.8 to 99.7 % with each other, and presented 96.9–98.3 % nucleotide identities with the reference strain Southampton/1991/UK (GenBank accession number L07418). They also showed high nucleotide identities with the Tunisian clinical strain Hu/GGI.2/11054/TUN/2009 (GenBank accession number JQ692916) ranging from 96.1 to 99.6 %.

Molecular analysis based on partial capsid sequences revealed that the GII.12 strains shared 97.7–100 % nucleotide identities with each other and 92.1–94.6 % nucleotide identities with the reference strain Wortley/1990/UK (GenBank accession number AJ277618).

Correlation Between the Environmental and Human Strains

Phylogenetic analysis (Fig. 1) was used to compare the sequences of NoV strains detected in sewage with those detected in pediatric clinical cases in order to identify any relationship between environmental contamination and clinical isolates. Human NoVs were isolated during the same period and in the same region, between April 2007 and April 2010 in Monastir, in stool samples from children with symptoms of acute gastroenteritis. Their characterization was reported in Hassine-Zaafrane et al. 2013.

Nucleotide sequences and phylogenetic analysis of the clinical strains showed a predominance of GII compared to GI and these strains were grouped into eight different genotypes: GI.2, GIb/I.6, GIIb/II.3, GIIb/II.13, GII.4, GII.6, GII.7/II.6, and GII.8 (Hassine-Zaafrane et al. 2013). On the contrary, there was a higher proportion of GI NoVs found in the environment.

The comparison of clinical and environmental data showed that only the GI.2 strain was detected in both types of samples. The five GI.2 strains (capsid gene) detected in sewage (two in raw sewage and three in treated sewage) have 96.1–99.6 % nucleotide identities with the clinical strain GI.2 (11054/21-04-2009/TUN) found in a stool of a child with gastroenteritis. It should be noted that the environmental strain SLBE98/16-04-2009/TUN and the human strain 11054/21-04-2009/TUN were detected at the same period and cluster together on the phylogenetic tree (99.6 % nucleotide identity).

Discussion

During the last years, more attention has been focused on the sewage virological quality, because of the risk of virus-associated waterborne illness, with the need for routine monitoring of viral contamination and the environmental surveillance through the analysis of sewage (Morace et al.

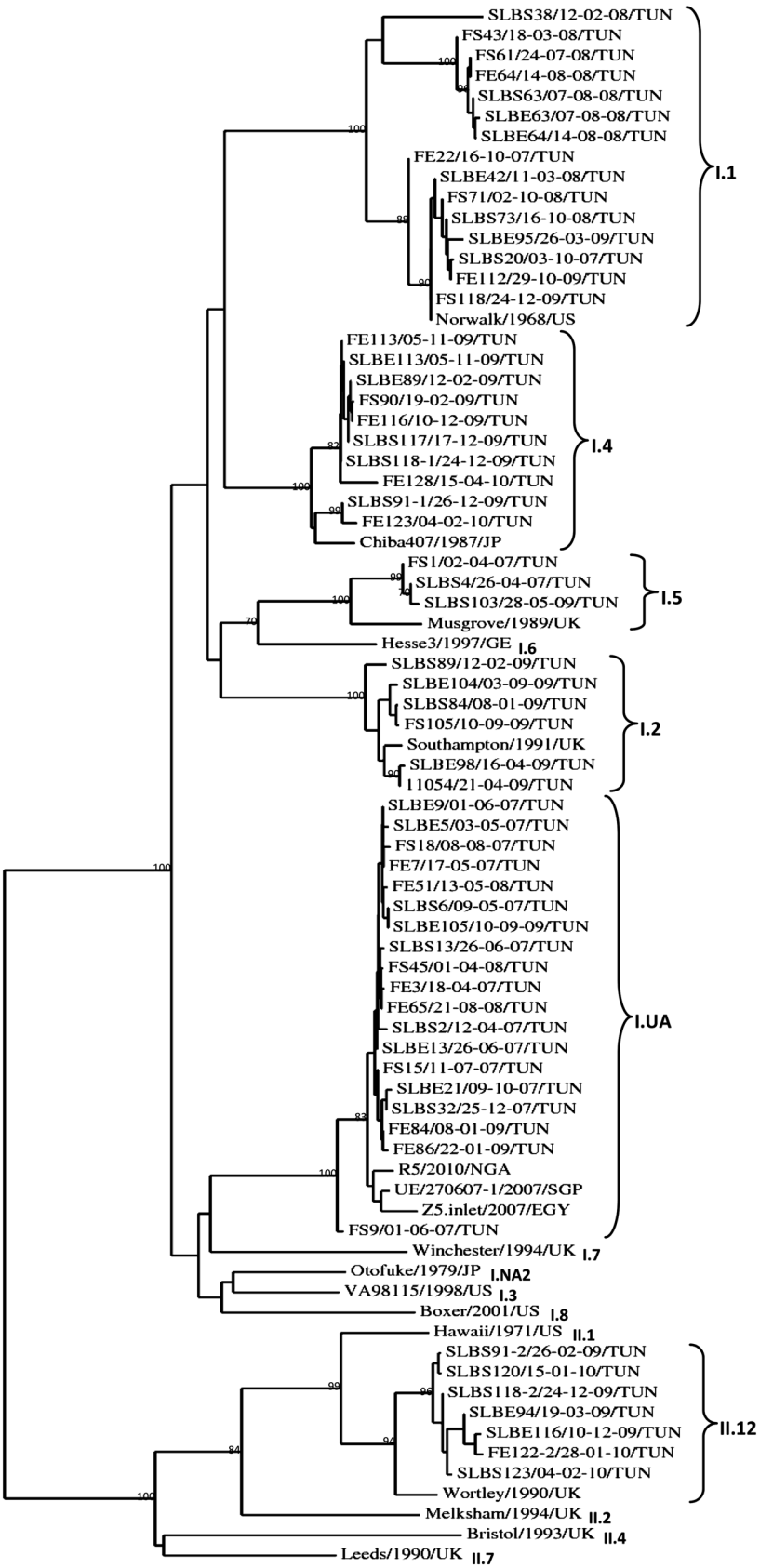

Fig. 1 Phylogenetic analysis based on the partial nucleotide sequences of the region coding the capsid gene of NoV strains recovered from sewage in Monastir region, Tunisia, between April 2007 and April 2010. GenBank accession numbers for reference strains in this figure are as follows: Norwalk/1968/US : M87661; Chiba407/1987/JP : AB042808; Musgrove/1989/UK : AJ277614; Hesse3/1997/GE : AF093797; Southampton/1991/UK : L07418; Hu/GGI.2/11054/TUN/2009 : JQ692916; Hu/GGI/UE/270607-1/2007/SGP : GQ925160; Hu/GGI/Z5.inlet/2007/EGY : GQ265898; Winchester/1994/UK : AJ277609; Otofuke/1979/JP : AB187514; VA98115/1998 : AY038598; Boxer/2001/US : AF538679; Hawaï/1971/US : U07611; Wortley/1990/UK : AJ277618; Melksham/1994/UK : X81819; Bristol/1993/UK : X76716; Leeds/1990/UK : AJ277608. Capsid sequences determined in this study were deposited in GenBank under accession numbers JX455837-JX455895. For sequences of NoV with 100 % nucleotide identity, one representative sequence was sent to GenBank

2002; Villar et al. 2007; Carducci et al. 2009). Sewage could contain NoVs shed from affected and healthy individuals, and therefore, detectable viruses in sewage would reflect the actual state of the circulating viruses in the area. The purpose of this work was to continue our study initiated in January 2003 (Sdiri-Loulizi et al. 2010) in order to evaluate the role of environmental contamination as a possible vehicle for transmission of NoV genogroups I and II. In this study, we investigated NoVs in sewage from April 2007 to April 2010 in Monastir region, Tunisia and compared them with the viruses detected from clinical cases at the same period and in the same region, to get an overview of the prevalence and the diversity of these viruses in the community.

In our study, NoVs were detected in 37.1 % of the sewage samples. Several other authors have reported the contamination of sewage by NoV with frequencies ranging from 18 to 100 % (Lodder et al. 1999; Laverick et al. 2004; Van den Berg et al. 2005; Nordgren et al. 2009; Kamel et al. 2010; La Rosa et al. 2010; Blanco Fernández et al. 2011). Our results showed a widespread distribution of NoV particles in both raw (52.1 %) and treated (47.9 %) wastewaters. The detection of NoVs in treated sewage samples proves their resistance to the processes used in the STPs. The NoV virion is robust, and well adapted for survival in the environment outside the host, and to withstand different treatment processes (Duizer et al. 2004; Rzezutka and Cook 2004). NoVs are not removed efficiently by sewage treatment. Discharge of treated sewage onto surface waters may lead to exposure of possible hosts to multiple NoV strains by the use of sewage-contaminated surface water for recreation, shellfish culture, or drinking water production. Numerous environmental problems linked to sewage discharges persist because of the lack of investment in wastewater treatment systems or the failure of existing ones. Furthermore, the presence of multiple NoV strains in raw and treated sewage water may contribute to the occurrence of new recombinants which may be more virulent and pathogenic than the NoV strains already circulating in the population (Van den Berg et al. 2005).

GI NoVs were frequently found in the environment throughout the period of study. This finding, suggesting a significant presence of GI in human populations, has already been described by others, both in France (da Silva et al. 2007) and in Sweden (Nordgren et al. 2009). In contrast to this, previous studies have reported NoV GII to be more prevalent than NoV GI in wastewater (Lodder and de Roda Husman 2005; Haramoto et al. 2006; Katayama et al. 2008).

It is important to note that, while GI NoVs were frequently found in the environment, most gastroenteritis cases in the pediatric population were due to genogroup II NoV strains (Hassine-Zaafrane et al. 2013). Although there is no clear explanation for this discrepancy, it is possible that adults are infected with various genotypes of viruses that differ from the prevalent ones causing gastroenteritis in children. Also, the NoV GI strains may give rise to less severe or asymptomatic infections as compared to the NoV GII strains, then going unnoticed. Moreover, it has been previously hypothesized that GI NoV might be more resistant in the environment compared to GII, especially to sewage treatment (da Silva et al. 2007). Differences in biological properties such as virulence, routes of transmission, or stability of the virus in the environment are possible explanation (Buesa et al. 2002). Additionally, their ability to evade the human immune system might also explain the constant release of these strains into the environment (La Rosa et al. 2010). However, these viruses have the potential to be a source of an endemic or epidemic. Also the rare detection of genogroup II strains in sewage samples may suggest that this strain, although circulating in the water was present at titers below the detection limit of the assay. This result can be due to a technical bias, facing a possible mixture of viruses in sewage samples, with only the most represented strain being characterized. The prevalence of GI NoVs in sewage is also in agreement with the results of many epidemiological studies showing that GI was often detected from shellfish associated with gastroenteritis outbreaks, after contamination of shellfish by soiled water (Le Guyader et al. 1996; Le Guyader et al. 2003; Jothikumar et al. 2005; Widdowson et al. 2005).

The role of NoVs as a cause of gastroenteritis in Monastir region was investigated by a 6-year study of stool samples from children suffering from acute gastroenteritis (Sdiri-Loulizi et al. 2009; Hassine-Zaafrane et al. 2013). NoVs were confirmed as the second most common viral agent after RV among these children. Our present results indicate that the occurrence of NoV gastroenteritis, especially that of NoV GI, may in fact be higher than suggested by epidemiological report (Hassine-Zaafrane et al. 2013). This underscores the importance of wastewater monitoring for gathering data on the occurrence, concentration, and genetic diversity of NoVs.

Although other genotypes of NoVs in sewage samples did not correlate with those from clinical cases, the genotype GI.2 of NoVs detected from sewage showed a close relationship with this from human case. Phylogenetic analysis showed that the nucleotide sequences of NoV GI.2 strains in environmental and clinical samples were closely related to each other.

Generally, the evaluation of water quality only includes measurements of the bacterial indicators, fecal coliform, and *Escherichia coli*. These methods do not include an estimation of virus contamination. Thus, even if water samples pass such inspections, water supplies could still be contaminated with viruses, including NoV.

This work confirms the frequent occurrence of NoV genomes in inflows and outflows of the two STPs, which reflect their circulation in the inhabitants, regardless of symptoms, suggesting that treated sewage may represent a source of environmental contamination with potentially infectious viruses. Our study enriches the poor available data on sewage virological quality, and demonstrates the advantages of environmental surveillance as a tool to elucidate the molecular epidemiology of community circulating viruses. We underline the need of environmental surveillance programs in countries such as Tunisia with limited epidemiological surveillance systems for viral gastroenteritis and no environmental surveillance system currently in action and we propose that similar long-term studies might be useful and offer a valuable and complementary tool to epidemiological surveillance systems.

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Conflict of interest There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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