NOROVIRUS GENOTYPES INVOLVED IN THE OUTBREAKS OF GASTROENTERITIS IN CROATIA DURING THE WINTER SEASON 2004–2005

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Summary. – Seven outbreaks and four sporadic cases of the non-bacterial gastroenteritis caused by a norovirus (NoV) were detected in Croatia between November 2004 and February 2005. An enzyme immunoassay (EIA) and three different RT-PCRs for the viral polymerase (ORF1 RT-PCR) and genogroup I (GI) or II (GII) of capsid gene regions (GI-ORF2 RT-PCR; GII-ORF2 RT-PCR) were performed to detect NoV in 21 stool samples. To characterize NoVs, sequencing of the ORF1 region was performed on 12 RT-PCR positive samples, whereas the ORF2 region was sequenced for 5 cases. Four outbreaks were caused by the genotype GII.4 (Lordsdale) and one outbreak was caused by the genotype GI.1 (Norwalk). One of the outbreaks was characterized as potentially mixed GII.4 and GI.1 infection. In the monitored period, genotype GII.4 dominated as the cause of noroviral infections in adults.

Key words: norovirus; genotype; outbreak; gastroenteritis; Croatia

Introduction

Human NoV is a major cause of non-bacterial gastroenteritis occurring in the communities such as families, schools, camps, elderly homes, hospitals, and cruise ships (Koopmans *et al*., 2000; Reuter *et al*., 2002; Widdowson *et al*., 2004; Ike *et al*., 2006). In addition to the outbreaks, NoVs were detected in sporadic cases (Foley *et al*., 2001; Buesa *et al*., 2002) and represented the second most important cause of acute diarrhea in childhood worldwide (Lew *et al*., 1994; Bon *et al*., 1999; Pang *et al*., 2000).

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Abbreviations: EIA = enzyme immunoassay; FBVE = food-borne viruses in Europe; GI = genogroup I; GII = genogroup II; NoV = norovirus; ORF1 RT-PCR = RT-PCR specific for ORF1 polymerase gene region; ORF2 RT-PCR = RT-PCR specific for ORF2 capsid gene region

The genus *Norovirus* within the family *Caliciviridae* comprises small, unenveloped viruses with a single-stranded, positive-sense RNA genome containing three ORFs. ORF1 encodes non-structural viral proteins, including the RNA-dependent RNA polymerase, ORF 2 encodes major capsid protein, and ORF 3 encodes a minor structural protein (Vinjé *et al*., 2000, 2004; Kojima *et al*., 2002). It was found by the PCR that these viruses represented a highly heterogeneous group that was divided into five major genogroups GI, GII, GIII, GIV and GV. Genogroups GI and GII (Green *et al*., 1994; Wang *et al*., 1994; Kojima *et al*., 2002) encompassed most of the human strains, whereas GIII and GV included bovine and murine noroviruses (Ando *et al*., 2000; Vinjé *et al*., 2004; Wobus *et al*., 2006). Genogroup GIV contained a single virus strain infecting humans (Vinjé and Koopmans, 2000). The genogroups were further classified into 22 genetic clusters by Vinje *et al*. (2004), but Zheng *et al*. (2006) described many as 29 genetic clusters based on the genetic relationship within the ORF2 sequence.

Each cluster was given the Roman numeral of the genogroup followed by the cipher in the succession the "prototype strains" were published, e.g. GI.1 is a cluster belonging to the GI with Norwalk/1968/US virus as a prototype (Ando *et al.*, 2000).

Whereas many papers have described the circulation of different genogroups and genotypes of NoV in many countries (Noel *et al.*, 1999; Lindell *et al.*, 2005; Manuula *et al.*, 2005; Bull *et al.*, 2006; Ike *et al.*, 2006), the epidemiological data concerning the occurrence of outbreaks and the identification of NoV types circulating in Croatia have not been reported so far. The objectives of this study were to determine the presence of the virus in stool specimens from outbreaks of acute gastroenteritis in Croatia during the winter season 2004–2005 and to characterize the genotypes of the identified viruses.

Materials and Methods

Stool specimens were collected from the subjects involved in outbreaks of acute gastroenteritis in Croatia from November 2004 to February 2005. *Salmonella* spp., *Shigella* spp*., Campylobacter* spp*., Escherichia coli*, and *Yersinia enterocolitica* were detected by the standard cultivation methods at the county institutes of public health. The stool specimens were stored at -20°C and sent to the Department of Virology at the Croatian National Institute for Public Health for detailed diagnosis. The specimens were tested for rotaviruses, adenoviruses (latex agglutination, Orion Diagnostica and immunochromatography, BioMérieux), astroviruses and NoVs.

EIAs. A commercial EIA kit for detecting astroviral antigen (DakoCytomation) was used (McIver *et al*., 2000). Noroviral antigens in the stool specimens were also detected using the commercial EIA (DakoCytomation). The EIA utilized GI and GII specific monoclonal antibodies immobilized on the microtitration plate to capture the NoV antigen present in the specimen what allowed detection and differentiation of NoV GI and GII genogroups. The captured antigen was detected by the enzyme-conjugated antibody against NoV. The specimens positive in EIA were sent to the Istituto Superiore di Sanità Roma, to perform the molecular analysis.

RT-PCRs. Total RNA was extracted with Qiamp Viral extraction kit (Qiagen). The extracted RNAs were subjected to the three types of RT-PCRs targeting the polymerase gene (ORF1 RT-PCR) and GI and GII of the capsid gene region (GI-ORF2 RT-PCR and GII-ORF2 RT-PCR).

ORF1 RT-PCR was performed using the SuperScript™ One-Step RT-PCR with Platinum® Taq (Invitrogen) and primers JV12-JV13 (Vinjé *et al*., 1997) that amplify a 327 bp DNA fragment. Four µl of RNA were added to 2x Buffer RT-PCR Master Mix (Invitrogen), dNTP mix (0.2 mmol/l each nucleotide) and 0.3 µmol/l of primers JV12 and JV13 in a final volume of 15 µl. The mixture was subjected to the RT-PCR under the following conditions: one cycle (reverse transcription) at 45°C for 30 mins followed by denaturation at 94°C for 2 mins, 40 cycles at 94°C for 45 secs, 37°C for 1 min, 72°C for 1 min, followed by a final elongation step of 72°C for 7 mins.

The RNAs were also examined by two distinct ORF2 RT-PCRs (315 bp fragment) of GI and GII using primer pairs G1SKF/G1SKR and G2SKF/G2SKR, resp. (Kojima *et al*., 2002). The ORF2 RT-PCRs were performed essentially as described for the ORF1 RT-PCR protocol except for increased annealing temperature to 52°C.

Sequencing. To confirm the diagnosis and to characterize the NoVs, the RT-PCR products from either ORF1 or ORF2 were sequenced using the dideoxy method by cycle sequencing with the ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit, version 3.1, (PE Applied Biosystems) and an automated sequencer (ABI Prism 310 DNA sequencer, Applied Biosystems). The sequence reactions were performed with the same primer of the RT-PCRs, i.e. JV12 and JV13 for ORF1 and G1SKF/G1SKR or G2SKF/G2SKR for ORF2. The obtained sequences were aligned with those of the Food-borne viruses in Europe (FBVE, https://hypocrates.rivm.nl/bnwww/) and NCBI databases and analyzed using the DNASIS Max software (Hitachisoft).

Results

From November 2004 to February 2005, seven outbreaks of acute gastroenteritis and four sporadic cases were detected in Croatia. The exact number of cases in a particular outbreak was not recorded, but the local medical staff indicated that each outbreak counted between 10 and 50 cases. Clinical manifestations included diarrhea with or without vomiting and nausea, sometimes accompanied with fever up to 38°C. The symptoms persisted from 24 to 48 hrs. The geographic locations of the outbreaks are shown in Fig. 1.

Fig. 1

Location of the gastroenteritis outbreaks in Croatia represented as dots

Numbers close to the dots indicate outbreak sign.

Table 1. Epidemiological data of NoV outbreaks of gastroenteritis in the winter 2004–2005

Outbreak No.	Facility	Cases	Number of specimens		
			Tested samples (N = 40)	EIA-positive (N = 26)	ORF1 or ORF2 RT-PCR-positive (N = 21)
1	Hospital	Patients	7	5	4
2	Hospital	Patients	3	1	1
3	Nursery	Children and staff	11	8	6
4	Hospital	Patients	2	2	2
5	Hospital	Patients and staff	3	2	1
6	Hospital	Patients and staff	1	1	1
7	Military	Military recruits	9	3	2
–	Sporadic cases	Patients	4	4	4

The full amount of 40 stool specimens sampled from 7 outbreaks and 4 sporadic cases were tested for common bacterial enteropathogens with negative results. Similarly, the presence of rotavirus, adenovirus, and astrovirus was negative by the specific diagnostic assays. The NoV was detected in 26 out of the 40 stool specimens using the EIA and a total of 21 samples (comprising of 17 samples from outbreaks and 4 samples from sporadic cases) were confirmed positive by the RT-PCR (Table 1).

NoVs belonging to GII were found in five outbreaks and two sporadic cases examined and GI NoV was detected in one outbreak and two sporadic cases. In one outbreak, the EIA showed the presence of both GI and GII NoVs (Table 2). To confirm these results, collected stool specimens were subjected to the RT-PCR with primers against the polymerase gene region and 15 samples were analyzed with two different pairs of primers annealing to the capsid region of GI or GII, respectively. The result of ORF1 RT-PCR in the case No. 16 could not confirm the EIA-positive result that was confirmed by the ORF2 RT-PCR. Out of the 15 specimens tested by the ORF2 RT-PCR, nine samples proved to contain GII-specific sequences and seven samples contained GI-specific sequences. The two specimens No. 15 and 16 appeared to contain NoVs of both genogroups,

Table 2. Molecular analysis of NoVs from outbreaks and sporadic cases of gastroenteritis in the winter 2004–2005

Case No.	Outbreak No.	NoV EIA	ORF1 RT-PCR	GI-ORF 2 RT-PCR	GII-ORF2 RT-PCR	ORF1 sequencing	ORF2 sequencing
1	1	II	+	ND	+	GII.4	ND
2	1	II	+	ND	ND	ND	ND
3	1	II	+	ND	+	GII.4	GII.4
4	1	II	+	ND	ND	GII.4	ND
5	2	II	+	ND	+	ND	GII.4
6	SPI	I	+	ND	ND	GI.1	ND
7	SPI	I	+	+	ND	GI.1	ND
8	SPI	II	+	ND	+	GII.4	GII.4
9	3	I	+	ND	ND	ND	ND
10	3	I	+	+	ND	GI.1	ND
11	3	I	+	+	ND	GI.1	ND
12	3	I	+	+	ND	GI.1	ND
13	3	I	+	ND	ND	GI.1	ND
14	3	I	+	+	ND	GI.1	ND
15	4	I&II	+	+	+	ND	ND
16	4	I&II	–	+	+	ND	GII.4
17	5	II	+	ND	+	GII.4	ND
18	6	II	+	ND	+	ND	ND
19	7	II	+	ND	ND	ND	ND
20	7	II	+	ND	–	ND	ND
21	SPZ	II	+	ND	+	ND	GII.6

ND = not done; SPI = sporadic case from Istria County; SPZ = sporadic case from Zagreb County; GI.1 = genetic cluster of Norwalk/1968/US prototype; GII.4 = genetic cluster of Lordsdale/1993/UK prototype; GII.6 = genetic cluster of Seacroft/1990/UK prototype.

whereas in the specimen No. 20 no amplification in the ORF2 area was detected. However, in this latter case the presence of NoV was confirmed by the EIA and ORF1 RT-PCR. Sequencing of an amplified ORF1 region was performed in 12 samples detecting an identical GII.4 strain in five patients from two outbreaks No. 1 and 5 and one sporadic case No. 8. The outbreak No. 3 (Istria county) was linked to a GI.1 genotype that was identical with the NoV found in the sporadic cases in the same county suggesting that this strain circulated in the community during the monitored interval. In addition to the GII.4 ORF1 sequences, the presence of GII.4 ORF2 sequences was confirmed in two samples (the outbreak No. 1 and sporadic case No. 8). ORF 2 sequencing detected the presence of GII.4 NoVs in two additional outbreaks No. 2 and 4, which had given negative results in ORF1 sequencing. The sporadic case No. 21 was confirmed as a GII.6 NoV by ORF2 sequencing.

The four outbreaks found to be associated with a GII.4 NoV occurred in hospital wards with patients' mean age of 75.4, 82.0, 46.5, and 45.0 years, respectively. The outbreak No. 6 also occurred in a hospital, but we were unable to identify the virus genotype in the only specimen available. Nevertheless, this one was characterized as a GII NoV by the EIA and GII-ORF2 RT-PCR. Conversely, the outbreak No. 3, related to a GI.1 NoV, occurred in a nursery. The outbreak No. 7 occurred in a military installation among recruits aged 20 years on average and was caused by a GII virus.

Discussion

The described outbreaks occurred in the different parts of Croatia during the winter season (November 2004 through February 2005) suggesting that NoV was an important cause of non-bacterial gastroenteritis in Croatia. Similar outbreaks were described in most industrialized areas of the USA and Europe (Koopmans *et al*., 2000; Reuter *et al*., 2002; Fankauser *et al*., 2002). In spite of the low number of cases collected and tested, the presence of one or two NoVs was detected in specimens from all seven outbreaks, what allowed us to establish an etiological role for this pathogen.

A good correlation was found between the EIA results and the results of molecular methods used for detection of NoV. Our data suggested that the EIA could be a good diagnostic method for the first rapid testing. Subsequently, the EIA results could be confirmed by more sensitive and specific molecular assays. In addition, the EIA kit used in our trial was able to detect the NoVs belonging to at least three distinct genotypes within both GI and GII. In the outbreak No. 4, the EIA revealed the presence of two different NoV genogroups in both patients examined. Later on, these findings were confirmed by the ORF2 RT-PCRs. The sequence analysis of the ORF2 amplicon identified a GII.4 NoV in only the specimen No. 16, whereas ORF2 sequencing yielded ambiguous results in the other specimen No.15 not allowing the identification of the specific NoV genotype. The molecular methods allow sensitive diagnosis and may give a detailed viral characterization up to the genotype assignment and phylogenesis, but frequently the amount of amplified DNA is not sufficient for the sequencing. Consequently, no single RT-PCR appears to be sensitive enough to detect any NoVs in stool samples (de Bruin *et al*., 2006), due to the extreme variability of the NoV genome. The adoption of both ORF1- and ORF2-specific protocols may be needed to increase reliability of viral detection during outbreaks. Likewise, Vinjé *et al*. (2003) have compared several RT-PCRs for NoVs and found that no single procedure could confirm all positive samples detected by other RT-PCRs or electron microscopy. As possible explanations, the authors (Vinjé *et al*., 2003) considered the co-purification of RT-PCR inhibitors during the RNA extraction and the lack of primer specificity.

ORF1 RT-PCR and ORF2 RT-PCRs yielded consistent results in all but two cases No. 16 and 20, which positivity had been determined by the EIA. The results suggested that these RT-PCRs had similar sensitivity and specificity. However, the presence of questionable results, although limited in number, indicated that more detection methods might be needed to successfully establish a reliable diagnostic procedure for a higher quantity of samples in outbreaks. In addition, the availability of sensitive diagnostic tools may prove useful to understand the transmission routes of NoV that may likely involve subjects with mild or no gastroenteric symptoms, where a minimal amount of infectious virus may be expected to be shed for relatively long periods. This may also be of particular significance in the case of food handlers in the first weeks after recovery from NoV gastroenteritis.

A GII NoV was found in five outbreaks and in two sporadic cases, whereas one outbreak and additional two sporadic cases were related to a GI NoV. A mixed infection of GI and GII NoVs was detected in both patients from the outbreak No. 4, where both the EIA and RT-PCRs confirmed the simultaneous presence of both genogroups. Only the involvement of a genotype GII.4 NoV was confirmed by the sequencing.

In a study of 148 outbreaks, Ike *et al*. (2006) proved the involvement of 12 different genotypes with a predominant role of GII NoVs that accounted for 95% of the cases. The same study also found that the most prevalent genotype in epidemic cases in 2002 and 2003 was GII.4. Studies from different parts of the world have also confirmed the predominance of GII NoVs (Bon *et al*., 1999; Maguire *et al*., 1999; Koopmans *et al*., 2000; Kirkwood and Bishop, 2001; Fankhauser *et al*., 2002; Vipond *et al*., 2004).

Recent findings from different European countries show that GII.4 NoV outbreaks are most frequent in elderly homes, care facilities and hospitals (Ike *et al.*, 2006; Reuter *et al.*, 2005), suggesting that the elderly may be more susceptible to these particular genotype of NoV. Similarly, our study detected the relatively high patients' mean age in four hospital wards outbreaks associated with GII.4 NoV.

The GII.4 viruses characterized in our study appeared to be closely related to the Lordsdale-like NoV predominating in the EU countries. The sequences from distinct samples and outbreaks proved to be identical, suggesting that this genotype was circulating in Croatia during the winter period 2004–2005. Likewise, the GI.1 virus from the outbreak in Istria County and two sporadic cases found in the same area shared identical sequences with the strain that belonged to the Norwalk cluster.

Despite the low number of cases tested, NoVs circulation may be important in Croatia as in other European countries and deserves further investigation.

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