DETECTION OF SMALL ROUND STRUCTURED VIRUSES IN STOOL SPECIMENS FROM OUTBREAKS OF GASTROENTERITIS BY ELECTRON MICROSCOPY AND REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

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Received October 26, 1999; accepted December 16, 1999

Summary. – In testing 60 stool specimens small round structured virus (SRSV) particles were detected in 35 (58%) specimens from all 17 outbreaks of gastroenteritis by electron microscopy (EM), while SRSV genes were found in 36 (60%) specimens from 15 outbreaks by reverse transcription–polymerase chain reaction (RT-PCR) by use of 2 primer pairs. Specimens from 2 outbreaks were found SRSV particles-positive by EM but SRSV genes-negative by RT-PCR. EM remains the basic examination method for diagnosis of SRSV agents.

Key words: small round structured virus; gastroenteritis; detection; electron microscopy; RT-PCR

Introduction

Norwalk virus (NV) is one of the causative agents of gastroenteritis (Kapikian *et al.,* 1972) and a prototype of SRSVs. A large part of SVSRs were still unknown because their culture systems had not yet been established. By EM, distinction between NV and a typical calicivirus among SRSVs was possible, but classification of other SRSVs was difficult. On the other hand, the nucleotide sequence of the NV genome was elucidated (Jiang *et al.,* 1990), and SRSVs such as NV or Snow Mountain virus (SMV) are generally considered members of the *Caliciviridae* family (Gree *et al.,* 1994). Nucleotide sequence analysis of caliciviruses showed the presence of two kinds of viruses, one of which was related to an animal calicivirus, and the other to SRSVs (Jiang *et al.*, 1994; Matson *et al.*, 1995). The NV genome has three open reading frames (ORFs). With advances in studies on SRSVs at the gene level, various primers for RT-PCR have been developed (Jiang *et al.*, 1992). In this study, the sensitivity of detection of SRSVs by EM and RT-PCR was investigated.

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Abbreviations: ELISA = enzyme-linked immunosorbent assay; EM = electron microscopy; NV = Norwalk virus; ORF = open reading frame; RT-PCR = reverse transciption–polymerase chain reaction; SMV = Snow Mountain virus; SRSV = small round structured virus

Materials and Methods

Specimens consisted of 60 stools from 17 outbreaks of acute gastroenteritis in Kyushu, Japan from 1987 to 1995 kept at –80°C. These stool specimens were negative for a typical rotavirus and an adenovirus causing enteritis using commercial Latex (Rota-Adeno Dry, Daiichi Pure Chemicals Co.) or enzyme-linked immunosorbent assay (ELISA) (Rota, Adeno Clone, T.F.B. Inc.) kits.

EM examination of stool specimens was carried out in a standard manner (Otsu, 1998).

RNA was extracted from 300 µl aliquots of 10% suspensions of stool specimens using cetyltrimethyl-ammonium bromide (Sigma) (Jiang *et al.*, 1992). The extracted RNA was dissolved in 30 µl of 0.1% diethyl pyrocarbonate (Sigma) and subjected to RT-PCR (Yamazaki *et al.*, 1996).

RT-PCR. Two primer pairs, namely one from the polymerase (Pol) region (ORF-1, nt 4475–4944) of NV genome (sense primer

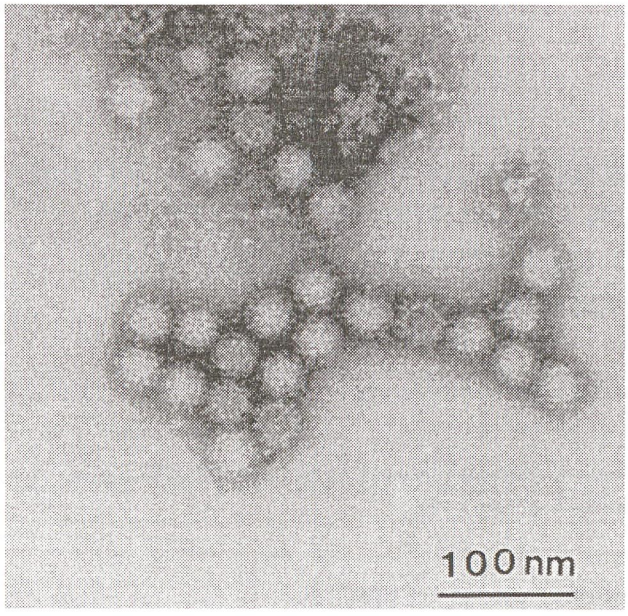

Fig. 1
Electron micrograph of SRSVs detected from outbreak No. 3

Table 1. Detection of SRSVs by RT-PCR and EM from 1987 to 1995

Outbreak No.	Date	No. of specimens				Total examined
		EM-positive	RT-PCR-positive			
			Primer pairs		Total	
			NV36/35	S9/10		
1	Dec. 1987	2	3	2	3	6
2	Feb. 1988	3	3	3	3	6
3	May 1988	3	0	0	0	5
4	Nov. 1988	3	4	4	4	6
5	Dec. 1988	2	2	2	2	3
6	Jan. 1989	2	2	2	2	2
7	Dec. 1989	2	2	1	2	2
8	Dec. 1989	1	1	1	1	1
9	Feb. 1991	1	2	2	2	3
10	April 1991	1	0	2	2	3
11	Nov. 1991	3	4	0	4	6
12	Jan. 1992	4	1	4	4	4
13	Dec. 1992	1	0	1	1	1
14	Feb. 1993	2	0	3	3	3
15	Dec. 1993	2	1	1	2	3
16	Nov. 1995	2	0	1	1	3
17	Dec. 1995	1	0	0	0	3
Total		35	28	26	36	60

Oyster-related outbreaks: Nos. 1, 2, 5, 6, 7, 8, 13, and 14.

NV36, 5′-ATAAAAGTTGGCATGAACA-3′; antisense primer NV35, 5′-CTTGTTGGTTTGAGGCCATA-3′) (Jiang *et al.*, 1992)), and another designed from the ORF-2 region of the NV genome encoding the capsid region (nt 5346–5800), (sense primer S9, 5′-ATGATGATGGCGTCTAAGGAC-3′; antisense primer S10, 5′ACATCAGCAATCACATGTGG-3′, (Yamazaki *et al.*, 1996)) were used. The PCR products were of 470 bp and 455 bp, respectively.

Results

By EM, SRSVs of about 30 nm in diameter could be detected in 35 (58%) of 60 stool specimens from 17 outbreaks (Table 1), and no marked morphologica differences were observed among the particles detected (Fig. 1).

In examining the 60 specimens by the RT-PCR, the viral sequences were detected with the primer pair N36/35 in 28 (47%) specimens from 11 outbreaks and with the primer pair S9/10 in 26 (43%) specimens from 14 outbreaks. The viral sequences were detected (1) with both primer pairs (in two PCRs) in 18 (30%) specimens from 9 outbreaks, (2) with NV36/35 but not with S9/10 in 10 (17%) specimens from 5 outbreaks, (3) with S9/10 but not with NV36/35 in 8 (13%) specimens from 5 outbreaks, and (4) with neither primer pair in 24 (40%) specimens from 11 outbreaks (Table 1). When both primer pairs used (in one PCR), the viral sequences could be detected in 36 (60%) specimens from 15 outbreaks.

Specimens from outbreaks Nos. 3 and 17 (Table 1), which were SRSV-negative by RT-PCR (with both primer pairs), were tested further. A PCR using different primer pairs, namely NV1/4 and NV3/51 (Jiang *et al.*, 1992), gave negative results for all these specimens (data not shown). A nested PCR using another three primer pairs, namely NV, SM82/NV81, and NV39/69 (Jiang *et al.*, 1992), gave positive results for 2 specimens from the outbreak No. 17 with just one primer pair (NV82/81) (data not shown).

Discussion

By use of the EM method, SRSVs were detected in all 17 outbreaks from which stools could be collected, and the detection rate was 58%, which was higher than that of about 35%, the rate at the time of onset in ordinary cases (Otsu, 1999). These results account for selective preservation of the specimens that were positive by EM at the onset.

The EM method demonstrated the presence of a considerable number of complete particles in some stool specimens from the outbreak No. 3 which no viral sequences could be detected by the RT-PCR method. The reason why the viral sequences could not be detected by the RT-PCR method is unknown, but (1) the involvement of an inhibitor,

(2) the use of SRSVs-related primers (Saito *et al.*, 1999) other than those used in this study, (3) the relationships between the number of particles, the degree of their denaturation and the sensitivity of the RT-PCR method, and (4) execution of the 2nd (nested) PCR should be evaluated. As a result of evaluation of the RT-PCR methods for detection of SRSVs, the use of multiple primers was shown to increase the detection rate of viral sequences. However, in the outbreak No. 3, the result of the 2nd PCR was also negative. The characteristic of this SRSV should be made and changes in the prevalence in this type of SRSV will need careful monitoring. Concerning the results of the RT-PCR method in individual cases, the detection pattern differed among specimens from the same case in 4 outbreaks. Two of them (Nos. 1 and 7) were related to oysters, while the other two (Nos. 12 and 15) occurred in an influenza-like major epidemic. This difference in the detection pattern is not clear partly because of small number of cases examined, but testing errors and co-presence of two kinds of SRSV are possible (Sugieda *et al.*, 1996). Viral sequences were detected more often with S9/10 primer pair than with NV36/35 one since 1991, which suggests a change in the dominant epidemic genotype (Ando *et al.*, 1996) in the Kyushu region. Additional investigation (nucleotide sequence analysis) pertaining to the results and specimens referred to in this study, are currently in progress.

In conclusion, compared with the EM method, the RT-PCR method has a higher detection sensitivity. However, as no widely applicable primers are available today, the EM method, which is conventional and standard, should be performed simultaneously to avoid false diagnostic results.

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