Original Article

Detection of Enteric Viruses in Fecal Specimens from Nonbacterial Foodborne Gastroenteritis Outbreaks in Tokyo, Japan between 1966 and 1983

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SUMMARY: We investigated the prevalence of 5 enteric viruses (norovirus [NoV], sapovirus, rotavirus, astrovirus, and adenovirus) in archived stool specimens collected from 70 foodborne gastroenteritis outbreaks in Tokyo, Japan, which occurred from 1966 to 1983, and genetically characterized these viruses. NoV was detected in 48 (68.6%) outbreaks, while SaV, group C rotavirus (RVC), and astrovirus were detected in 1 (1.4%) outbreak each. Based on the partial capsid sequences, the detected NoVs were classified into the following genotypes: 9 in genogroup I (GI; GI.1-6, GI.8, GI.9, and GI.NA), 13 GII (GII.1-9, GII.13, GII.16, GII.17, and GII.22), and one in GIV. The oldest NoV outbreaks occurred in 1966. No predominant genotype was found. One strain, classified as GI. NA based on the N/S region sequence, was subsequently classified as GI.8 based on the complete VP1 sequence. Nine types of recombinant NoV sequences, including 7 unreported combinations, were identified. Further genetic characterization of NoV GII.17 and GII.4 demonstrated that the NoV GII.17 strains detected from 1970 to 1982 clustered independently from previously reported NoV GII.17 strains. Phylogenetic analysis, using the complete VP1 region and the P2 domain, demonstrated that NoV GII.4 strains collected between 1975 and 1980 clustered with archival strains collected in the USA in the mid-1970s. In contrast, a NoV GII.4 strain collected in 1983 formed an independent branch from reference strains collected in the mid-1970s to 2012.

INTRODUCTION

Norovirus (NoV), sapovirus (SaV), rotavirus (RV), astrovirus (AstV), and enteric adenovirus (AdV) are viruses that cause gastroenteritis (1–3). These enteric viruses were first detected by electron microscopy in stool specimens from patients with acute gastroenteritis in the 1970s (4–8). Subsequently, highly sensitive molecular biology techniques were developed to detect these viruses. NoV is the leading cause of gastroenteritis outbreaks (9). NoV gastroenteritis can occur through the consumption of contaminated foods or water, and it can spread from person to person via contaminated utensils or facilities (1–2). Human NoVs are classified into 3 genogroups based on the capsid region sequences: I, II, and IV. Genogroup I (GI) includes 9 genotypes, while GII includes 22 genotypes (10). The NoV genome is a single-stranded positive sense RNA, and encodes 3 open reading frames (ORFs) (11). ORF1 encodes nonstructural proteins, while ORF2 and ORF3 encode the structural proteins VP1 and VP2, respectively. Recombination at the ORF1-ORF2 junction has been reported (12). Amino acid substitutions in the P2 domain, located within the VP1 of NoVs, have also been reported, especially in NoV GII.4 (13). The P2 domain is located on the outermost exterior surface of the capsid (14), and contains epitopes for neutralizing antibodies (15). Since the mid-1990s, NoV GII.4 has been the major cause of NoV-related gastroenteritis outbreaks (16). However, a new variant of NoV GII.17 was frequently detected in the 2014/2015 winter season (17). We investigated the prevalence of NoV, SaV, RV, AstV, and AdV in archived specimens collected from outbreaks of nonbacterial foodborne gastroenteritis in Tokyo, Japan between 1966 and 1983, prior to the introduction of viral testing. We genetically characterized these viruses and compared them with previously reported NoV GII.4 and GII.17 strains.

MATERIALS AND METHODS

Samples: Stool specimens were collected from 307 patients with nonbacterial gastroenteritis from 70 foodborne outbreaks in Tokyo, Japan from 1966 to 1983. Specimens were stored at −20°C until analysis.

Nucleic acid extraction and reverse transcription: Freshly prepared 10% fecal suspensions in phosphate-buffered saline were centrifuged at 1,300 × g for 5 min at 4°C, followed by centrifugation at 14,000 × g for 30 min at 4°C. Nucleic acids were extracted from 140 μL of supernatant using the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

The reverse transcription reaction mix contained 12 μL of the extracted nucleic acid and 18 μL of a mixture containing 0.5 mM deoxynucleoside triphosphate (dNTPs), 5 mM dithiothreitol (DTT), 16 units of
RNase inhibitor (Nacalai Tesque, Kyoto, Japan), 1 µg of random hexamers, 300 units of SuperScript II reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), and buffer, and was incubated at 42°C for 1 h.

**Real-time polymerase chain reaction (PCR):** NoV was detected by real-time PCR using the COG1F/1R primer pair and RING1TPa probe to detect GI strains and the COG2F/2R primer pair and RING2TP probe to detect GII strains (18). Following reverse transcription, real-time PCR was performed using 5 µL cDNA and 20 µL TaqMan Universal Master Mix (Thermo Fisher Scientific), 1 µM of each primer pair, and 0.15 µM of each probe. Real-time PCR was carried out using the ABI PRISM 7900HT Sequence Detection System (Thermo Fisher Scientific) with the following reaction conditions: a 10-min denaturation step at 96°C, followed by 45 cycles at 96°C for 15 s and 56°C for 1 min. The presence of SaV, RVA, RVC, AstV, and AdV, was assessed in all samples by real-time PCR as described previously (19–21). For real-time PCR assays, 5 µL of cDNA was used for SaV, RVA, RVC, and AstV, while 5 µL of nucleic acid was used for AdV.

**Conventional PCR:** Conventional PCR assays, using primer pairs COG1F/G1SKR for GI and COG2F/G2SKR for GII, were conducted to sequence the N-terminus and the (N/S) region of NoV-positive samples by real-time PCR (22). Semi-nested PCR was performed for samples that were negative in the first round of PCR with G1SKF/G1SKR and G2SKF/G2SKR primer pairs (22). For samples that were also positive for other viruses by real-time PCR, conventional PCR was performed to identify the virus. The primer sets F22 and R2, Beg9 and End9, Beg and End, Mon244 and Mon245, and hex1885 and hex1913 were used to amplify the partial SaV capsid, RVA VP7, RVC VP7, AstV capsid, and AdV hexon genes, respectively, as previously described (23–27).

**Analysis of NoV recombination:** Recombination between ORF1 and ORF2 in the detected NoVs was investigated. To amplify a portion of the RNA-dependent RNA polymerase (RdRp) region and the capsid N/S region in a single PCR product, the following primer pairs were used: NV82/G1SKR (1,117 base pair (bp) product) for GI and LV82/G2SKR (1,108 bp product), LV4282-99F/G2SKR (1,108 bp product), and NVGIIuni4769S19/G2SKR (621 bp product) for GII (28–30).

**Analysis of the complete VP1 region of NoV:** To determine sequence of the complete VP1 region of GI.4 and the untypable GI strains, reverse transcription was conducted using 50 pmol of Tx30SXN primer (31), 10 µL RNA, and 10 µL of a mixture containing 10 mM dNTPs, 100 mM DTT, 16 units of RNase inhibitor (Nacalai Tesque), 200 units SuperScript II RT (Thermo Fisher Scientific), and buffer, and was incubated at 42°C for 2 h. PCR was performed using the COG1F/Tx30SXN or the COG2F/Tx30SXN primer pair with 5 µL cDNA and 45 µL of a mixture containing 350 µM dNTPs, 0.3 µM PCR primers, and 3.5 units Taq polymerase, and incubated at 94°C for 10 s, at 60°C for 30 s, and at 68°C for 5 min for 35 cycles (31). Semi-nested PCR, using primer pairs G1SKF/Tx30SXN for GI and G2SKF/Tx30SXN for GII, was conducted for samples that showed no PCR amplification product using the COG1F/Tx30SXN or the COG2F/Tx30SXN primer pairs.

**Sequencing analysis:** PCR products were purified using the NucleoSpin Extract II kit (MACHEREY-NAGEL, Duren, Germany), and were directly sequenced with the BigDye Terminator v1.1 Cycle Sequencing kit (Thermo Fisher Scientific). NoV strains were classified using the sequence typing tool in NoroNet (http://www.rivm.nl/mpf/norovirus/typingtool). Nucleotide sequence analysis and phylogenetic tree construction were carried out using the GENETYX software (GENETYX, Tokyo, Japan).

NoV GIL.4 VP1 sequences were analyzed and compared to previously reported reference strains using GENETYX software to determine amino acid (aa) substitutions in the capsid P2 domain (10,32). PCR products from other enteric viruses were directly sequenced using the method described above and were classified as previously reported (24,26,27,33,34). In addition, the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov) was used to identify homologous genes (35).

**RESULTS**

**Detection of enteric viruses in fecal specimens:** NoV was the most commonly detected virus among the 70 foodborne gastroenteritis outbreaks (48 outbreaks, 68.6%). SaV, RVC, and AstV were each detected in one outbreak (1.4%), RVA and AdV were not detected, and no outbreaks were found to be associated with 2 or more virus types (Table 1). NoV was detected in 128 of the 307 fecal specimens examined (41.7%, Table 1), RVC and AstV were each detected in 3 specimens (0.98%), and SaV was detected in one specimen (0.33%). NoV was detected in all outbreaks that involved the consumption of raw oysters (n = 21; Table 1).

**Genetic characterization of detected NoV, RV, AstV, and SaV:** Conventional PCR was performed on all samples determined NoV-positive by real-time PCR. The resultant 125 PCR products (44 of 56 GI and 81 of 94 GII) were directly sequenced. When identical sequences were detected from multiple patients in an outbreak, one sample, selected as the unique sequence, was genotyped. Based on partial sequence of the capsid N/S region, 72 unique NoV sequences were identified and classified using the typing tool in NoroNet. These unique sequences included 9 GI (GI.1, GI.2, GI.3, GI.4, GI.5, GI.6, GI.8, GI.9, and GI.NA), 13 GII (GI.1, GI.2, GI.3, GI.4, GI.5, GI.6, GI.7, GI.8, GI.9, GI.13, GI.16, GI.17, and GI.22), and one GIV sequence (Table 2, Fig. 1). One of the GI.NA strains was subsequently reclassified as GI.8 based on the complete VP1 sequence (Fig. 1). No predominant genotype was observed among the 22 detected genotypes. Frequently detected genotypes were GI.8 (8.3%), GI.5 (8.3%), GI.5 (6.9%), GI.4 (6.9%), and GI.8 (6.9%) (Table 2). The detected SaV strain was classified as GI.2 and shared 98.5% nucleotide sequence similarity with the Parkville strain (U73124). The AstV strain was classified as type 5 and shared 97.3% nucleotide sequence similarity with the Goiania/GO/12 strain.
Table 1. Enteric viruses and norovirus genotypes detected in archived fecal specimens collected between 1966 and 1983

| Outbreak No. | Date      | Number of samples tested | Number of detected** | Analyzed genotype | Outbreak No. | Date      | Number of samples tested | Number of detected** | Analyzed genotype |
|--------------|-----------|--------------------------|----------------------|-------------------|--------------|-----------|--------------------------|----------------------|-------------------|
|              |           |                          |                      |                   |              |           |                          |                      |                   |
| 1*           | Dec. 1966 | 5                        | 3                    | 1                 | GI. 13       | 36        | Sep. 1976               | 5                    | 1                 | GI. 6             |
| 2            | Dec. 1966 | 5                        | 4                    | GI. 22            |              | 37        | Oct. 1976               | 5                    | (SaV)             |
| 3            | Jan. 1967 | 5                        | 4                    | GI. 8             |              | 38        | Oct. 1976               | 5                    |                   |
| 4            | Jan. 1967 | 5                        |                      |                   |              | 39        | Nov. 1976               | 5                    | 2                 | GI. 5             |
| 5            | Feb. 1967 | 5                        | 5                    | GI. 8             |              | 40*       | Dec. 1976               | 1                    | 1                 | GII. 8            |
| 6*           | Dec. 1967 | 5                        | 3                    | GI. 13            | 41*         | Dec. 1976 | 1                        | 1                    | GI. 1             |
| 7            | Jun. 1968 | 5                        |                      |                   |              | 42*       | Dec. 1976               | 2                    | 2                 | GI. 5, 8, GII. 5  |
| 8            | Jun. 1968 | 5                        | 2                    | GI. 3             |              | 43*       | Dec. 1976               | 1                    | 1                 | GI. 3             |
| 9            | Jun. 1968 | 5                        | 5                    | GI. 6             |              | 44        | Dec. 1976               | 5                    | 4                 | GI. 6             |
| 10           | Nov. 1968 | 5                        | 3                    | GI. 1             |              | 45*       | Jan. 1977               | 5                    | 2                 | GI. 4, NA         |
| 11           | Jan. 1969 | 5                        | 2                    | GI. 3, 8          |              | 46*       | Feb. 1977               | 3                    | 2                 | GI. 5, 8****      |
| 12           | Feb. 1969 | 5                        | 1                    | GI. 7             |              | 47        | Feb. 1977               | 4                    | 1                 | GI. 8             |
| 13           | Jun. 1969 | 5                        |                      |                   |              | 48*       | Dec. 1977               | 2                    | 2                 | GI. 13            |
| 14           | Jun. 1969 | 5                        |                      |                   |              | 49        | Dec. 1977               | 4                    | 1                 | GI. 9             |
| 15           | Feb. 1970 | 5                        | 3                    |                   |              | 50        | Dec. 1978               | 3                    |                   |
| 16           | Mar. 1970 | 5                        | 3                    | GII. 17           | 51          | Jan. 1978 | 5                        | 4                    | GI. 1             |
| 17           | Feb. 1970 | 5                        |                      |                   |              | 52        | Nov. 1980               | 5                    | 3                 | GI. 4             |
| 18           | Jun. 1970 | 3                        | (RVC)                | 53                | Nov. 1980   | 5          | 2                        | GI. 4                |                   |
| 19*          | Dec. 1972 | 3                        | 1                    |                   |              | 54        | Nov. 1980               | 5                    |                   |
| 20*          | Jan. 1975 | 5                        | 4                    | GI. 1, 3, 8, 9    | 55*         | Dec. 1981 | 5                        | 2                    | 1                 | GI. 1, 2, 5       |
| 21           | Mar. 1975 | 5                        | 1                    | GI. 3, GI. 4      | 56          | Dec. 1981 | 5                        | 2                    | GI. 8             |
| 22           | Mar. 1975 | 5                        | 1                    | GI. 16            | 57*         | Dec. 1981 | 2                        | 1                    |                   |
| 23           | Mar. 1975 | 3                        | 1                    | GI. 4             | 58*         | Dec. 1981 | 5                        | 1                    | 2                 | GI. 6, GI. 5      |
| 24           | Jul. 1975 | 5                        | 1                    | GI. 2             | 59          | Jan. 1982 | 5                        | 4                    | GI. 2, 6, 8       |
| 25           | Feb. 1976 | 5                        | 1                    | GI. 2             | 60*         | Jan. 1982 | 5                        | 5                    | GI. 5, 6, 17      |
| 26           | Mar. 1976 | 5                        | 1***                 | GIV                | 61          | May. 1982 | 5                        | 6                    |                   |
| 27           | Mar. 1976 | 5                        | 1                    | GI. 8, GI. 13, 17 | 62          | May. 1982 | 5                        | 6                    |                   |
| 28           | Jun. 1976 | 5                        | 2                    | GI.4,8,GI.13,17   | 63          | Oct. 1982 | 5                        | 6                    |                   |
| 29           | Jun. 1976 | 2                        | 1                    | GI. 3             | 64          | Feb. 1983 | 5                        | 2                    | 3                 | GI. 2, 5, GI. 4   |
| 30           | Jun. 1976 | 5                        |                      |                   | 65*         | Mar. 1983 | 5                        | 2                    | GI. 6, 9          |
| 31           | Jun. 1976 | 1                        |                      |                   | 66*         | Unknown   | 5                        | 4                    | 1                 | GI. 5             |
| 32           | Jul. 1976 | 5                        |                      |                   | 67*         | unknown   | 2                        | 2                    | GI. 2             |
| 33           | Jul. 1976 | 5                        |                      |                   | 68*         | Unknown   | 5                        | 2                    | GI. 8             |
| 34           | Jul. 1976 | 5                        |                      |                   | 69          | unknown   | 5                        | 6                    |                   |
| 35           | Sep. 1976 | 5                        |                      |                   | 70*         | unknown   | 5                        | 2                    | GI. 3             |

| total        | 307       | 34                      | 72                   | 22                |                   |

NA: classification not assigned by the sequence typing tool in NoroNet (http://www.rivm.nl/mpf/norovirus/typingtool).<ref>
*: outbreaks associated with raw oyster consumption.
**: detected by real-time PCR.
***: amplified and detected using primer pairs and a probe for NoV GII by real-time and conventional PCR.
****: classified as NA according to the partial N/S sequence, and classified as GI.8 based on the complete VP1 sequence.
</ref>

(DQ028623). The VP7 of the RVC strain was classified as G4 and shared 97.2% nucleotide sequence similarity with the ad1015 strain (U20994).

Identification of recombinant NoV strains: To identify recombinant NoV strains, the region between the partial RdRp and N/S region was amplified in a single PCR. Of the 72 unique strains, 28 strains produced an amplicon, and 17 (60.7%) were classified as different genotypes according to their ORF1 and ORF2 sequences (Table 3). This included one strain each of GI.NA_GI.NA, GII.P5_GII.1, GII.P4_GII.2, GII.Pk_GII.6, and GII.Pk_GII.7, 2 strains each of GII.P22_GII.5, 3 strains of GII.NA_GII.17, and 6 strains of GII.P22_GII.5.

Comparison of the GII.17 strains from archived specimens with reported sequences: Partial sequences of the RdRp and the capsid N/S regions of NoV GII.17 strains detected were compared to deposited sequences. The NoV GII.17 strain detected in samples from 1970 to 1982 clustered independently from previously reported NoV GII.17 strains (Fig. 2A and 2B). Based on the sequences of the partial RdRp region, 2 strains (Tokyo/27-3/1976 and Tokyo/28-3/1976) were classified as GI.NA.

Sequence analysis of the VP1 of the GII.4 strains: The complete VP1 aa sequences of 4 out of 5 strains, Tokyo/21-5/1975 (AB684675), Tokyo/52-2/1980 (AB684704), Tokyo/53-1/1980 (AB684705), and Tokyo/64-3/1983 (AB684720), were analyzed and compared with previously reported NoV GII.4 strains.
Table 2. Genotypes of noroviruses detected in samples from 70 outbreaks between 1966 and 1983 in Tokyo, Japan

| Genotype   | 1966 | 1967 | 1968 | 1969 | 1970 | 1971 | 1972 | 1973 | 1974 | 1975 | 1976 | 1977 | 1978 | 1979 | 1980 | 1981 | 1982 | 1983 | Unknown | Total | (%)
|------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|---------|-------|
| GI. 1      | 1    | 1    |      |      | 1    | 1    | 2    | 2    | 1    | 3    | (4.2)|      |      |      |      |      |      |      |      |         | 114   |
| GI. 2      | 1    | 1    | 1    | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 8     |
| GI. 3      | 1    | 2    |      | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 4     |
| GI. 4      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 2     |
| GI. 5      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 5     |
| GI. 6      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 2     |
| GI. 7      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 6     |
| GI. 8      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 6     |
| GI. 9      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 2     |
| GI. NA     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 2     |
| GI. 1      | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 3     |
| GI. 2      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 3     |
| GI. 3      | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 3     |
| GI. 4      | 2    | 2    | 2    | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 6     |
| GI. 5      | 1    | 1    | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 6     |
| GI. 6      | 1    | 1    | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 5     |
| GI. 7      | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 1     |
| GI. 8      | 2    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 5     |
| GI. 9      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 1     |
| GI. 13     | 1    | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 4     |
| GI. 16     | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 1     |
| GI. 17     | 1    | 2    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 4     |
| GI. 22     | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 2     |
| GIV        | 1    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |         | 1     |

NoVs were classified according to the partial sequences of the capsid N/S region using the sequence typing tool in NoroNet (http://www.rivm.nl/mpf/norovirus/typingtool).
NA, classification not assigned by the sequence typing tool in NoroNet; unknown, collected between 1966 and 1983.
*: one of these strains was classified as GI.8 based on the complete VP1 sequence.

Phylogenetic analysis of the complete VP1 region and P2 domain of NoV GII.4 strains Tokyo/21-5/1975, Tokyo/52-2/1980, and Tokyo/53-1/1980 clustered with CHDC2094/1974, CHDC5191/1974, and CHDC4871/1977 collected in the mid-1970s in the USA (Fig. 3). In contrast, a NoV GII.4 strain collected in 1983 formed a new branch independent from reference strains detected from the mid-1970s to 2012. Tokyo/21-5/1975, Tokyo/52-2/1980, and Tokyo/53-1/1980 had RVG(T/I) sequences at aa position 292–295, whereas Tokyo/64-3/1983 had HIRG at this same location (Table 4). The Tokyo/64-3/1983 strain had an alanine (A) at aa position 410 of the VP1 region, whereas reference strains detected after 1987 had a conserved arginine (R) in this position (Table 4).

Deposition of sequences: All sequences were deposited in the DDBJ database under accession numbers AB684658–AB684729 for NoV, AB769837 for SaV, AB769838 for AstV, and AB769839 for RVC.

DISCUSSION

This is the first report detailing the distribution of NoV detected in foodborne outbreaks of nonbacterial gastroenteritis between 1966 and 1983 in Tokyo, Japan. Enteric viruses were detected in 51 of 70 outbreaks (72.9%). The results showed that viral gastroenteritis played a major role in outbreaks of nonbacterial gastroenteritis. At the time of sample collection, detection using molecular biological techniques and genetic characterization were not possible because techniques such as RT-PCR and sequencing had not been developed. In our study, we show that the oldest NoV outbreaks (outbreak number 1 and 2) occurred in 1966 (Table 1). This result showed that NoV was responsible for outbreaks of gastroenteritis in Tokyo, Japan prior to the first official report in a primary school in Ohio, USA in 1968 (4). One of the oldest NoV-related gastroenteritis outbreaks (outbreak number 1) occurred following consumption of raw oysters (Table 1). SaV, RVC, and AstV were detected in gastroenteritis outbreaks that occurred in 1976 (outbreak number 37), 1970 (outbreak number 18), and 1976 (outbreak number 33), respectively (Table 1). SaV and RVC were also detected in samples collected prior to the first reports describing these viruses in 1977 and 1986, respectively (5,36). In our study, NoV GI.1, GI.3, GI.6, GI.8, GI.13, and GI.22 were detected in outbreaks that occurred between 1966 and 1968 (Table 2). Based on this result, the prevalence of various genotypes of NoV caused gastroenteritis outbreaks even before the prototype virus was reported (37). In total, 22 NoV genotypes (8 GI, 13 GII, and one GIV) were detected. Two strains, Tokyo/45-5/1977 (AB684696) and Tokyo/46-2/1977 (AB684698), with identical N/S region sequences, could not be classified using the sequence typing tool in NoroNet. These sequences showed 92% similarity to the Gyeonggi/S4/2002 strain (EU43786) over 289 bp as determined by BLAST search. However, based on the complete VP1
Fig. 1. Phylogenetic tree of NoV strains based on partial nucleotide sequences of the capsid gene, specifically, the N-terminus and Shell (N/S) region (GI: 298 bp, GII and GIV: 282 bp) with bootstrap analysis of 1,000 replicates generated by the neighbor joining method. Bootstrap values are shown at nodes of the tree. The scale bar indicates nucleotide substitutions per site. Strain names are shown as host/genogroup/outbreak number/sample number/Tokyo/year collected/Japan: accession number. Reference strains are shown in bold. The N/S region sequences of NoV strain detected were classified as Genogroup I (9 genotypes), Genogroup II (13 genotypes), and Genogroup IV. *: based on its complete VP1 sequence, Tokyo/46-2/1977 was classified as GI.8 using the sequence typing tool in NoroNet. unknown, collected between 1966 and 1983.
Table 3. Genotyping of 28 selected NoV strains based on the ORF1 and ORF2 sequences

| strain          | ORF1 | ORF2 |
|-----------------|------|------|
| Tokyo/2-3/1966  | GII. P22 | GII. 22 |
| Tokyo/8-2/1968  | GII. Pg | GII. 3 |
| Tokyo/9-3/1968  | GII. Pk | GII. 6 |
| Tokyo/12-3/1969 | GII. Pk | GII. 7 |
| Tokyo/20-3/1975 | GII. P1  | GII. 1 |
| Tokyo/22-2/1976 | GII. P16 | GII. 16 |
| Tokyo/27-3/1976 | GII. NA  | GII. 17 |
| Tokyo/28-3/1976 | GII. NA  | GII. 17 |
| Tokyo/36-3/1976 | GII. NA  | GII. 6 |
| Tokyo/39-5/1976 | GII. P22 | GII. 5 |
| Tokyo/42-2/1976 | GII. P22 | GII. 5 |
| Tokyo/43/1976   | GII. Pg  | GII. 3 |
| Tokyo/45-4/1977 | GII. P4  | GII. 4 |
| Tokyo/45-5/1977 | GII. NA  | GII. NA |
| Tokyo/46-3/1977 | GII. P22 | GII. 5 |
| Tokyo/51-1/1979 | GII. P5  | GII. 1 |
| Tokyo/52-2/1980 | GII. P4  | GII. 4 |
| Tokyo/55-3/1981 | GII. P22 | GII. 5 |
| Tokyo/55-4/1981 | GII. P4  | GII. 2 |
| Tokyo/56-4/1981 | GII. P8  | GII. 8 |
| Tokyo/58-4/1981 | GII. P22 | GII. 5 |
| Tokyo/59-2/1982 | GII. P8  | GII. 8 |
| Tokyo/60-1/1982 | GII. P22 | GII. 5 |
| Tokyo/60-2/1982 | GII. NA  | GII. 17 |
| Tokyo/60-4/1982 | GII. P6  | GII. 6 |
| Tokyo/64-3/1983 | GII. P4  | GII. 4 |
| Tokyo/65-3/1983 | GII. NA  | GII. 6 |
| Tokyo/68-4/unknown | GII. P22 | GII. 22 |

NA, classification not assigned by the sequence typing tool in NoroNet (http://www.rivm.nl/mpf/norovirus/typingtool); unknown, collected between 1966 and 1983.

As a conclusion, four enteric viruses, NoV, SaV, RVC, and AstV, were detected in fecal samples from outbreaks of nonbacterial foodborne gastroenteritis collected between 1966 and 1983. NoV was the most commonly detected virus in this study, and 22 NoV genotypes and 9 recombinant sequences (between ORF1 and ORF2) were observed.

Conflict of interest None to declare.

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Fig. 2. Phylogenetic tree of NoV GII.17 based on partial sequences of the RNA-dependent RNA polymerase (RdRp) region (A: 779 bp) and capsid (N-terminus and Shell [N/S] region; B: 338 bp) with bootstrap analysis of 1,000 replicates generated by the neighbor joining method. Hawaii strain (GII.Pm GII.1) was used as an outgroup in the analysis. Bootstrap values are indicated at nodes of the tree. The scale bar indicates nucleotide substitutions per site. The NoV GII.17 strains detected in this study are indicated in bold. The RdRp sequences of Tokyo/16-5/1970, Tokyo/60/2/1982, RS11125/2005, C17/Bonaberi/2009, CSE1/2002, Katrina-17/2005, INCMNSZ01/2007, Sep11-A2/2011, and Kibera209/2012 were unable to be compared (Fig. 2A) due to their short length or no sequence data available for comparison.
Fig. 3. Phylogenetic tree of NoV GII.4 strains based on the complete amino acid sequences of the VP1 region (A; 539 or 540 aa) and P2 domain (B; 126 or 127 aa) with bootstrap analysis of 1,000 replicates generated by the neighbor joining method. The NoV GII.4 strains detected in archived specimens are shown in bold. Hawaii strain (GII.1) was used as an outgroup in the analysis. Accession numbers of GII.4 reference strains and Hawaii strain are indicated in Table 4 and Fig. 1, respectively. Bootstrap values are shown at nodes of the tree. Scale bar indicates amino acid substitutions per site.

Table 4. Comparison of characteristic amino acids in the P2 domain of VP1 of NoV GII.4 strains

| cluster | strain             | (accession numbers) | position of amino acids |
|---------|--------------------|---------------------|-------------------------|
|         |                    |                     | 292 | 293 | 294 | 295 | 410/411** |
| A       | CHDC2094/1974      | (FJ537135)          | R  | V  | G  | I  | T  |
|         | CHDC5191/1974      | (FJ537134)          | R  | V  | G  | I  | T  |
|         | Tokyo/21-5/1975    | (AB684675)          | R  | V  | G  | T  | T  |
|         | Tokyo/52-2/1980    | (AB684704)          | R  | V  | G  | T  | S  |
|         | Tokyo/53-1/1980    | (AB684705)          | R  | V  | G  | I  | S  |
|         | Tokyo/64-3/1983    | (AB684720)          | H  | I  | R  | G  | A  |
|         | CHDC4108/1987      | (FJ537137)          | H  | I  | V  | G  | R  |
|         | Bristol/1993       | (X76716)            | H  | I  | A  | G  | R  |
|         | Camberwell/1994    | (AF145896)          | R  | I  | V  | G  | R  |
|         | US95_96            | (AJ004864)          | H  | I  | A  | G  | R  |
|         | Kaiso 2003         | (AB294779)          | H  | I  | A  | G  | R  |
|         | FarmingtonHills 2002 | (AY902023)     | H  | I  | A  | G  | R  |
|         | Lanzhou 2002       | (DQ364459)          | H  | I  | A  | G  | R  |
|         | Asia 2003          | (AB220922)          | H  | I  | P  | G  | R  |
|         | Hunter 2004        | (DQ078794)          | H  | I  | A  | G  | R  |
|         | Yerseke 2006       | (EF126963)          | H  | I  | A  | G  | R  |
|         | DenHaag 2006b      | (EF126965)          | H  | I  | A  | G  | R  |
|         | Osaka 2007         | (AB434770)          | H  | I  | A  | G  | R  |
|         | Apeldoorn 2007     | (AB445295)          | H  | I  | T  | G  | R  |
|         | New Orleans 2009   | (GU455325)          | H  | I  | P  | G  | R  |
|         | Sydney 2012        | (JX459908)          | H  | I  | T  | G  | R  |

Strains detected in this study are shown in bold.

*: cluster name based on the sequence typing tool in NoroNet (http://www.rivm.nl/mpf/norovirus/typingtool).

**: position of this amino acid was 411, after the Farmington Hills strain.
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