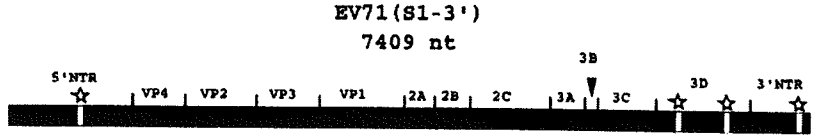


| | PV1 (Mahoney) | PV1 (Sabin) | EV71 (BrCr-TR) | EV71 (S1-3') |
|---|---|---|---|---|
| 5'NTR | A(480) | G(480) | A(485) | G(485) |
| $3D^{pol}$ | Tyr(73) | His(73) | Tyr(73) | HIS(73) |
| $3D^{pol}$ | Thr(362) | Ile(362) | Cys(363) | Ile(363) |
| 3'NTR | A(7441) | G(7441) | A(7409) | G(7409) |

FIG. 1. Schematic diagram of the EV71(S1-3') genome. The sequences derived from the parental EV71(BrCr-TR) genome are represented by the black regions, and the mutations derived from PV1(Sabin) are represented by the white regions with stars above them. Corresponding mutations of PV1(Mahoney), PV1(Sabin), and EV71(BrCr-TR) are also shown. The numbers in parentheses are the nucleotide positions in the genome or the amino acid positions in the $3D^{pol}$ protein. The figure was adapted from a previously published figure with the permission of the Society for General Microbiology (5).

We constructed an infectious clone of EV71(Nagoya-HIS), which is a mutant of EV71(Nagoya) that contains a histidine tag in the BC loop of VP1 protein between amino acid residues 100 and 101 on the VP1 protein. The clone was constructed from pEV71(Nagoya) by SDM using the primers NagoyaVP1-HIS+ and NagoyaVP1-HIS-. This infectious clone of EV71(Nagoya-HIS) was designated as pEV71(Nagoya-HIS).

**Detection of anti-EV71 IgM and IgG in monkey serum.** A virus solution of EV71(Nagoya-HIS) ($1.0 \times 10^8$ $CCID_{50}$/ml) was diluted with PBS(-) (1:10 dilution) and then added to HisGrab nickel-coated plates (Pierce) (100 μl per well). The plates were incubated at 4°C overnight for the adsorption of EV71(Nagoya-HIS) virions to the plates. After adsorption, 1% skim milk–PBS(-) was added to the plates (100 μl per well), which were then incubated at room temperature for 3 h. The plates were washed three times with 0.05% Tween 20–PBS(-) [T-PBS(-)], followed by addition of 100 μl of diluted monkey serum [1:10,000 or 1:20,000 dilution with 0.5% skim milk–0.5% Tween 20–PBS(-), for detection of IgM or IgG, respectively] and incubation at room temperature for 1 h. The plates were washed three times with T-PBS(-), followed by addition of 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-monkey IgM or IgG antibody per well [1:10,000 dilution with 0.5% skim milk–0.5% Tween 20–PBS(-); Nordic Immunology] for the detection of IgM or IgG, respectively. The plates were incubated at room temperature for 1 h and were then washed three times with T-PBS(-). Finally, 100 μl of substrate solution (0.01% 3,3',5,5'-tetramethylbenzidine) per well was added to the plates. After sufficient incubation at room temperature, the reaction was stopped by adding 100 μl of 2 N $H_2SO_4$ per well. Then, the optical density at 450 nm was measured for each well using a Benchmark Plus microplate spectrophotometer (Bio-Rad).

**Monkey neurovirulence test.** Five female cynomolgus monkeys (*Macaca fascicularis*; age, 7 to 23 years) were inoculated with EV71(S1-3'), followed by lethal challenge with EV71(BrCr-TR). All animal procedures were approved by the Committee for Biosafety and Animal Handling and the Committee for Ethical Regulation of the National Institute of Infectious Diseases, Japan. Animal care, breeding, virus inoculation, and observation were performed in accordance with the guidelines of these committees.

Under light anesthesia with ketaral and xylazine, 1 ml of EV71(S1-3') virus solution (containing $10^7$ $CCID_{50}$ of virus) was intravenously inoculated into the right tibial vein. Monkeys were examined daily for neurological manifestations for the first 10 days and were examined weekly from day 10 p.i. to day 45 p.i. On day 45 p.i., three monkeys were challenged with a lethal dose ($10^7$ $CCID_{50}$) of EV71(BrCr-TR) (5) by intravenous inoculation as described above. Those three monkeys were examined daily for neurological manifestations for the first 10 days after the inoculation of EV71(BrCr-TR) and were examined weekly from 10 to 21 days after the inoculation of EV71(BrCr-TR) (55 to 66 days p.i.) (see Fig. 2. below). Autopsy was performed on two monkeys 4 days after the inoculation of EV71(S1-3'). At autopsy, various parts of the CNS, nonneural tissues, and blood were collected for histopathological and virological analysis. Histological changes in the CNS (lesion score) were evaluated using the method recommended by the WHO (40). Lesions were scored as follows: 0, no lesion; 1, cellular infiltration; 2, cellular infiltration with minimal neuronal damage; 3, cellular infiltration with extensive neuronal damage; 4, massive neuronal damage with or without cellular infiltration. For virus isolation, a portion of each excised tissue was stored at −80°C. We also isolated viruses from nonneural tissues of monkeys previously inoculated with EV71(BrCr-TR) and autopsied on day 6 p.i. (5). After freezing and thawing, tissue homogenates (10% [wt/vol]) in minimal essential medium containing 2% FCS were centrifuged at 10,000 × *g* for 10 min to remove cell debris. Supernatants were used for virus isolation in Vero cells. The cells were observed for CPE for 1 week, and then blind passage was performed for CPE-negative samples after freezing and thawing of the first-round passage. If no CPE was observed in the first- or second-round cultures, the result of the virus isolation was recorded as negative.

## RESULTS

**Clinical symptoms of cynomolgus monkeys inoculated with EV71(S1-3').** To characterize the antigenicity and tissue specificity of EV71(S1-3'), we inoculated five cynomolgus monkeys with $10^7$ $CCID_{50}$ of EV71(S1-3') via an intravenous route (Fig. 1). Three monkeys were used to examine antigenicity (Fig. 2A, Exp. 1), and two monkeys were used to examine tissue specificity in the early phase of infection as indicated by clinical symptoms (day 4 p.i.) (Fig. 2A, Exp. 2).

EV71(S1-3') infection caused tremor in three monkeys (age, 20 to 23 years), but no clinical symptoms were observed in two other monkeys (age, 7 and 12 years) (Fig. 2B). Tremor appeared as early as day 3 p.i., disappeared on day 8 p.i., reappeared on day 10 p.i., and then disappeared again before day 21 p.i. The legs of the three monkeys that exhibited tremor remained weak throughout the period of observation. The virus was isolated from both throat and rectal swabs until day 7 p.i., predominantly from rectal swabs (Fig. 2C), suggesting transient infection of EV71(S1-3') in the inoculated monkeys.

**Humoral immune response of cynomolgus monkeys inoculated with EV71(S1-3').** To examine the humoral immune response of monkeys inoculated with EV71(S1-3'), we measured the neutralizing and binding activities of anti-EV71 IgG and IgM antibodies in sera from the five monkeys inoculated with EV71(S1-3') via the intravenous route. Induction of anti-EV71 antibodies was evaluated using EV71(Nagoya) (genotype B1), which is a prime strain of EV71 (16). We also used a genetically engineered EV71(Nagoya) mutant carrying a histidine tag in the BC loop of the VP1 protein [EV71(Nagoya-His)] (Fig. 3A and C). The neutralizing activity of the monkey sera was comparable to that of a monkey (number 4507) inoculated

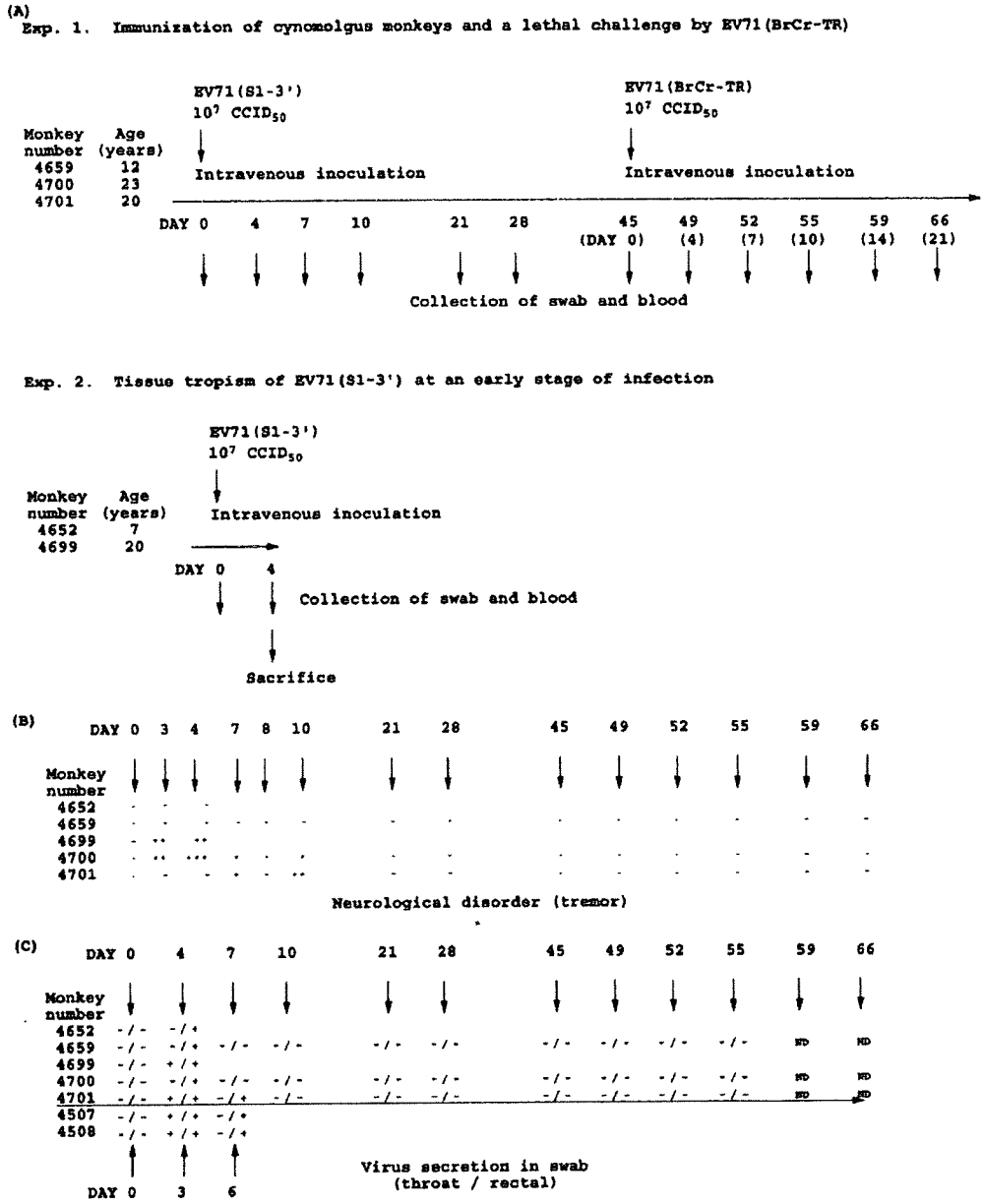


FIG. 2. Experimental schedule, clinical symptoms, and virus excretion of cynomolgus monkeys inoculated with EV71(S1-3'). (A) Experimental schedule. In experiment 1 (upper panel), the antigenicity of EV71(S1-3') in cynomolgus monkeys was examined. Three monkeys were intravenously inoculated with EV71(S1-3') on day 0 and were then challenged by lethal inoculation with EV71(BrCr-TR) on day 45 p.i. In experiment 2 (lower panel), the tissue specificity of EV71(S1-3') in the early phase of infection was examined. Two monkeys were sacrificed on day 4 p.i., and virus distribution and histopathology in those two monkeys were examined. The assigned numbers and ages of individual monkeys are shown. Numbers in parentheses represent days after the lethal challenge with EV71(BrCr-TR). The swabs and blood were collected at the times indicated. (B) Clinical symptoms of the monkeys. Severity of tremor is represented by +, ++, and +++. (C) Virus excretion in the throat and from rectal swabs of the monkeys inoculated with EV71(S1-3'). The swabs from which EV71(S1-3') or EV71(BrCr-TR) was isolated are shown as positive, and swabs from which EV71(S1-3') or EV71(BrCr-TR) was not isolated are shown as negative. Swab samples from monkeys inoculated with EV71(BrCr-TR) (numbers 4507 and 4508) were collected in our previous study (5). ND, not determined.

with virulent parental EV71(BrCr-TR) in a previous study (Fig. 3A, 0 to 6 days p.i.) (5) and peaked (neutralization titer, 1,024 to 2,048) at 14 to 21 days p.i., followed by a gradual decline in activity (primary immune response). After the lethal challenge with EV71(BrCr-TR) on day 45 p.i., the neutralizing activity of the monkey sera markedly increased as a consequence of the secondary immune response. In the secondary immune response, the neutralizing activity peaked (neutralization titer, 16,384 to 32,768) at 7 to 14 days after the lethal challenge (52 to 59 days p.i.) (Fig. 3A).

The binding activity and/or the amounts of anti-EV71 IgG and anti-EV71 IgM antibodies were measured by ELISA. Pre-

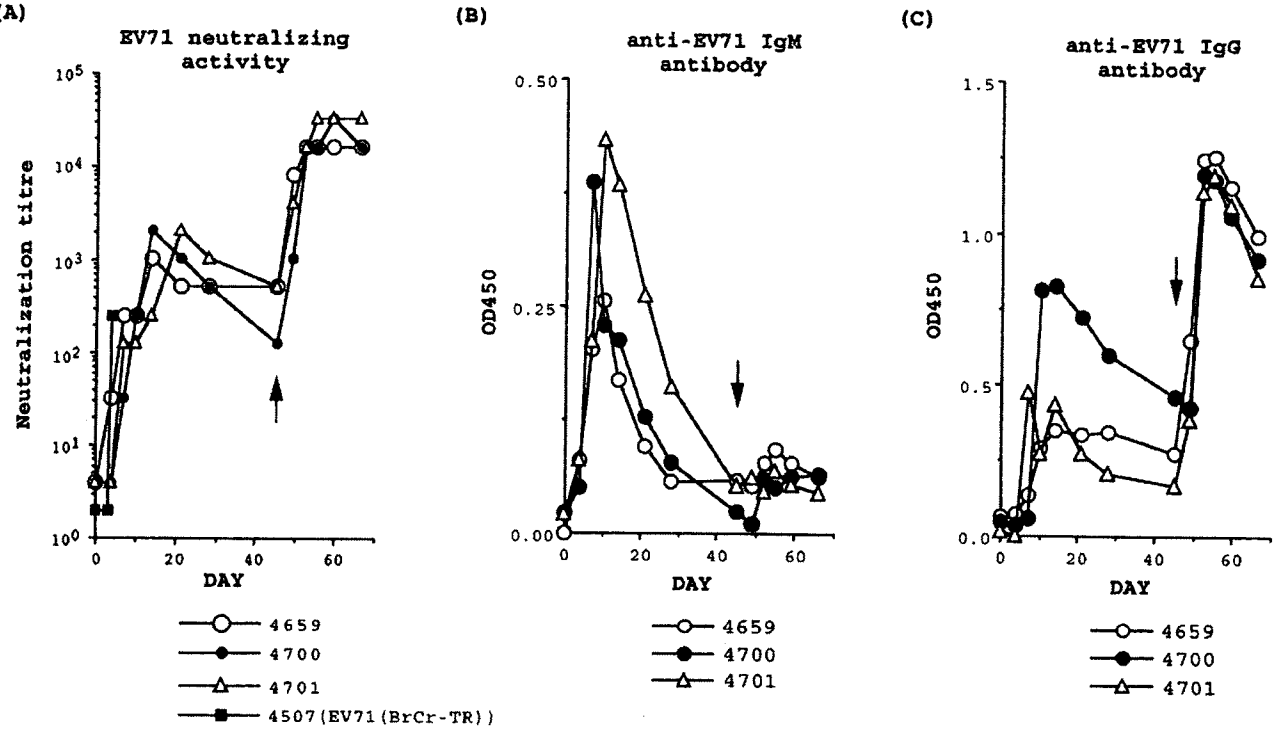


FIG. 3. Anti-EV71 antibody titers of monkeys inoculated with EV71(S1-3'). The time point of lethal challenge with EV71(BrCr-TR) is indicated by an arrow. (A) Anti-EV71 neutralization activity of monkey serum. The neutralization titer of 50 μl of the serum, determined by the CPE method, is shown over the course of the infection. The serum of a monkey (number 4507) inoculated with virulent EV71(BrCr-TR) was collected in a previous study (5). (B and C) Measurement of anti-EV71 IgM (B) or anti-EV71 IgG (C) in monkey serum. Histidine-tagged EV71 virions were adsorbed on a nickel-coated plate and then monkey serum was added to the plate. Monkey anti-EV71 antibodies bound to the histidine-tagged virions were detected by HRP-conjugated goat anti-monkey IgM or anti-monkey IgG antibodies. OD450, optical density at 450 nm.

viously, we developed an IgM capture ELISA for the detection of anti-EV71 IgM in human serum (52). However, that system is not suitable for monkey serum because of the strong cross-reactivity between the antibodies, which results in high background levels (data not shown). To minimize cross-reactivity between the antibodies, we developed a new ELISA system using histidine-tagged EV71(Nagoya-His) virus as the antigen (Fig. 3B and C). In this ELISA system, histidine-tagged EV71 virions are adsorbed on a nickel-coated plate and then monkey serum is added to the plate. The anti-EV71 IgG or IgM antibodies bound to the histidine-tagged virions are detected using goat anti-monkey IgG or anti-monkey IgM antibodies conjugated with HRP (see Materials and Methods). Using this ELISA system, we detected anti-EV71 IgM and IgG antibodies in the monkey sera (Fig. 3B and C). IgM was the predominant type of anti-EV71 antibody in the primary immune response (peak at 7 to 10 days p.i.), but apparently no anti-EV71 IgM antibody was induced in the secondary immune response after the lethal challenge with EV71(BrCr-TR). In contrast, the amount or the binding capacity of anti-EV71 IgG antibody was low in the primary immune response (peak at 10 to 14 days p.i.) and markedly increased in the secondary immune response (peak at 7 days after the lethal challenge [52 days p.i.]). These results indicate that the infection with EV71(S1-3') induced an efficient humoral immune response in cynomolgus monkeys.

**Antigenicity of EV71(S1-3') in cynomolgus monkeys.** Next, we examined the genotype specificity of the neutralizing activity of the monkey sera. We examined neutralizing activity against EV71 strains belonging to genotypes A, B1, B4, C2, and C4 (Tables 2 and 3). First, we determined the neutralization titer by observing the CPE caused by virus that escaped neutralization (CPE method; see Materials and Methods). The highest neutralization titer was against homotypic strain EV71(BrCr-TR) [the parental strain of EV71(S1-3')]. The order of decreasing neutralization activity in the CPE assay was as follows: BrCr-TR (genotype A) > Nagoya (genotype B1) > 75-Yamagata-2003 (genotype C4) and 2399-Yamagata-2003 (genotype C4) > C7-Osaka (genotype B4) and 1095 (genotype C2). However, we were unable to determine the neutralization titer against strain 1530-Yamagata-2003 (genotype C4) using this method because of the late appearance of CPE, attributable to the nonneutralizable aggregated form of virus (54). Therefore, we determined the neutralization titer against strain 1530-Yamagata-2003 by indirect immunofluorescence with rabbit anti-EV71 antiserum (IF method; see Materials and Methods). The IF method is useful for strains of enterovirus that contain a small amount of nonneutralizable aggregated form of the virus, which can account for 0.005% to 30% of total virions (54). The nonneutralizable aggregated form of virus makes it difficult to determine the end point, due to

TABLE 2. Neutralization titers of monkey sera against EV71 strains as determined by CPE assay

| Monkey | Day p.i.[a] | Neutralization titer (% relative to BrCr-TR)[b] with EV71 strain (genotype)[c]: | | | | | |
|---|---|---|---|---|---|---|---|
| | | BrCr-TR (A) | Nagoya (B1) | C7-Osaka (B4) | 1095 (C2) | 75-Yamagata-2003 (C4) | 2399-Yamagata-2003 (C4) |
| 4659 | 14 | 3.6 (100) | 3.3 (50) | 2.1 (3.1) | 2.4 (5.8) | 3.6 (100) | 3.3 (50) |
| | 59 (14) | 4.5 (100) | 3.9 (25) | 2.7 (1.6) | 2.7 (1.6) | 3.6 (13) | 3.6 (13) |
| 4700 | 14 | 3.3 (100) | 3.0 (50) | 2.4 (13) | 2.4 (13) | 3.0 (50) | 2.1 (6.3) |
| | 59 (14) | 4.8 (100) | 3.9 (13) | 3.3 (3.1) | 3.3 (3.1) | 3.9 (13) | 3.9 (13) |
| 4701 | 21 | 3.0 (100) | 2.7 (50) | 2.1 (13) | 2.1 (13) | 2.4 (25) | 1.8 (6.3) |
| | 59 (14) | 4.8 (100) | 4.2 (25) | 3.3 (3.1) | 3.3 (3.1) | 3.9 (13) | 3.9 (13) |

[a] Numbers in parentheses represent the day postinfection at which a lethal challenge with EV71(BrCr-TR) was administered to the monkeys.
[b] The $\log_{10}$ of the neutralization titer in 50 μl of monkey serum. Numbers in parentheses represent the percentage of the neutralization titer relative to the titer against the BrCr-TR strain, which was taken as 100%.
[c] The genotype of each EV71 strain is shown in parentheses. For strain 1530-Yamagata-2003, the neutralization titer could not be determined in this assay.

incomplete neutralization by the antibody. The monkey sera had substantial neutralizing activity against strain 1530-Yamagata-2003 (Table 3). The order of decreasing neutralization activity in the IF assay was as follows: BrCr-TR (genotype A) > Nagoya (genotype B1) and 75-Yamagata-2003 (genotype C4) > 1530-Yamagata-2003 (genotype C4) > C7-Osaka (genotype B4) > 1095 (genotype C2) and 2399-Yamagata-2003 (genotype C4).

Thus, the monkey sera exhibited a broad spectrum of neutralization activity against EV71 strains belonging to different genotypes, with the highest activity against the homotypic genotype A and the lowest activity against genotype C2 (1.6% of neutralization titer against homotypic strain).

**Protection of cynomolgus monkeys from lethal challenge by virulent EV71(BrCr-TR).** Three monkeys inoculated with EV71(S1-3′) were challenged with a lethal dose ($10^7$ $CCID_{50}$) of EV71(BrCr-TR) via an intravenous route (Fig. 2) (5, 39). After the challenge, no clinical symptoms were observed in any of the three monkeys. Virus was not isolated from swab samples obtained after the challenge (Fig. 2B). These results indicate that EV71(S1-3′) acted as an effective antigen that protected the monkeys from lethal infection with the homotypic virulent strain.

**Tissue specificity of EV71(S1-3′) in cynomolgus monkeys.** We examined the tissue specificity of EV71(S1-3′) in the cynomolgus monkeys on day 4 p.i., in the early phase of the infection as indicated by clinical symptoms (Tables 4 and 5). In our previous study (5), in the late phase of the infection (day 10 p.i.), virus was isolated only from the CNS of the monkeys inoculated with EV71(S1-3′), although inflammation was observed in a broad area of the CNS.

In the CNS of the inoculated monkeys, inflammation (perivascular cuffing with mononuclear cells) was observed in a limited area, mainly in gray matter, on day 4 p.i. (Table 4). In one monkey (number 4652), inflammation was observed in the pons, the medulla, and the cervical and lumbar spinal cord. In another monkey (number 4699), inflammation was observed only in the lumbar spinal cord. Virus was isolated from the lumbar spinal cords of both monkeys. In one monkey (number 4513) on day 10 p.i., no virus was isolated and no inflammation was observed. In another monkey (number 4514), virus was isolated only from the lumbar spinal cord, but inflammation was observed in all CNS tissues. These results suggest that EV71(S1-3′) infection can cause inflammation in a broad range of CNS tissues with inefficient replication. Efficient replication of EV71(S1-3′) was limited to the spinal cord, predominantly the lumbar spinal cord.

Next, we isolated virus from extraneural tissues of the monkeys inoculated with EV71(S1-3′) or with the virulent parental EV71(BrCr-TR) (Table 5). Virus was isolated from the spleen, kidney, deep cervical lymph node, and dorsal root ganglion. There was no clear difference in extraneural tissue specificity between EV71(S1-3′) and EV71(BrCr-TR). In the kidney and dorsal root ganglion, inflammation was observed, suggesting

TABLE 3. Neutralization titers of monkey sera against EV71 strains as determined by IF assay

| Monkey | Day p.i.[a] | Neutralization titer (% relative to BrCr-TR)[b] with EV71 strain (genotype): | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | BrCr-TR (A)[c] | Nagoya (B1) | C7-Osaka (B4) | 1095 (C2) | 75-Yamagata-2003 (C4) | 2399-Yamagata-2003 (C4) | 1530-Yamagata-2003 (C4) |
| 4659 | 14 | 4.1 (100) | 3.9 (57) | 3.6 (32) | 3.1 (8.3) | 3.9 (57) | 3.6 (35) | 4.0 (87) |
| | 59 (14) | 5.5 (100) | 5.3 (69) | 4.7 (15) | 4.1 (4.0) | 5.4 (73) | 4.4 (7.1) | 5.2 (50) |
| 4700 | 14 | 4.6 (100) | 4.2 (43) | 4.2 (40) | 3.9 (25) | 4.4 (60) | 3.7 (13) | 4.6 (92) |
| | 59 (14) | 5.5 (100) | 5.0 (37) | 4.6 (16) | 4.4 (8.1) | 5.1 (43) | 5.1 (38) | 5.0 (33) |
| 4701 | 21 | 3.9 (100) | 4.3 (230) | 3.5 (36) | 3.6 (41) | 3.7 (50) | 3.5 (36) | 3.9 (88) |
| | 59 (14) | 5.8 (100) | 5.6 (63) | 5.1 (18) | 5.2 (21) | 5.1 (20) | 4.7 (7.6) | 4.9 (12) |

[a] Numbers in parentheses represent the day postinfection at which a lethal challenge with EV71(BrCr-TR) was administered to the monkeys.
[b] The neutralization titer was determined by indirect immunofluorescence and is the $\log_{10}$ of the $NU_{50}$ in 50 μl of monkey serum (see Materials and Methods). Numbers in parentheses represent the percentage of the neutralization titer relative to the titer against the BrCr-TR strain, which was taken as 100%.

TABLE 4. Tissue specificity of EV71(S1-3') in the CNS of cynomolgus monkeys

| Tissue | Virus isolation (inflammation) for monkey no. [treatment][a]: | | | | | |
|---|---|---|---|---|---|---|
| | 4652 [EV71(S1-3'), day 4 p.i.] | 4699 [EV71(S1-3'), day 4 p.i.] | 4513[b] [EV71(S1-3'), day 10 p.i.] | 4514[b] [EV71(S1-3'), day 10 p.i.] | 4507[c] [EV71(BrCr-TR), day 6 p.i.] | 4508[c] [EV71(BrCr-TR), day 6 p.i.] |
| Cerebrum | − (−) | − (−) | − (−) | − (+) | + (+) | + (+) |
| Midbrain | − (−) | − (−) | − (−) | − (+) | + (+) | + (+) |
| Pons | − (+) | − (−) | − (−) | − (+) | + (+) | + (+) |
| Cerebellum | − (−) | − (−) | − (−) | − (+) | + (+) | + (+) |
| Medulla | − (+) | − (−) | − (−) | − (+) | + (+) | + (+) |
| Cervical cord | + (+) ($10^{1.75}$) | − (−) | − (−) | − (+) | + (+) | + (+) |
| Lumbar cord [lesion score][d] | + (+) ($10^{3.5}$) [0.9] | + (+) ($10^{3.0}$) [0.3] | − (−) [0.0] | + (+) [1.3] | + (+) [2.9] | + (+) [3.2] |

[a] The virus isolation results are shown as positive or negative. The presence of inflammation in the tissues is shown in parentheses as positive or negative. The inoculated viruses and the day sacrificed are also indicated. The virus titer ($CCID_{50}$) in 100 μl of the homogenate is shown, in parentheses following the isolation and inflammation results, for the samples collected from two monkeys, numbers 4652 and 4699.

[b] Samples from monkeys numbers of 4513 and 4514 were collected in a previous study (5).

[c] Data on monkeys inoculated with virulent EV71(BrCr-TR) (numbers 4507 and 4508) were adapted from a previous publication with the permission of the Society for General Microbiology (5).

[d] The lesion score of the lumbar cord was determined for each monkey and is shown in brackets.

that viral replication occurred in these tissues. However, on day 10 p.i. after inoculation of EV71(S1-3'), virus was not isolated from extraneural tissues (data not shown). Thus, EV71(S1-3') infected extraneural tissues of cynomolgus monkeys in the early phase of the infection, but the infection of extraneural tissues was transient.

## DISCUSSION

To evaluate the antigenicity of EV71(S1-3') in cynomolgus monkeys, we used an intravenous inoculation route instead of the oral route. This is because in a previous study EV71 infection via the oral route did not efficiently cause neurological disorders in the inoculated monkeys (1 out of 10 inoculated monkeys) (18).

In the present study, the monkeys inoculated with EV71(S1-3') via the intravenous route exhibited tremor followed by a slight weakness of the legs (Fig. 2C and data not shown). One monkey (number 4699) clearly exhibited tremor on day 3 and day 4 p.i. However, in the CNS of that monkey, the only lesions that were observed were in the lumbar spinal cord, which had a low lesion score (0.3, perivascular cuffing with mononuclear cells) (Table 4). Also in that monkey, no extrapyramidal lesions were detected in the CNS (40). Extrapyramidal lesions directly cause tremor, suggesting that extrapyramidal lesions present at undetectable levels can cause tremor in EV71-infected monkeys and that detectable pyramidal lesions would exacerbate such tremors. This suggests that the tremors observed in the present monkeys inoculated with EV71(S1-3') have a different origin than those observed in monkeys inoculated with the virulent parental strain EV71(BrCr-TR) in previous studies, in which extrapyramidal lesions were detected (39).

The spinal cord is a niche of enterovirus infection (4, 13). Actually, a PV replicon caused severe poliomyelitis-like paralysis in inoculated mice with limited lesions of the lumbar spinal cord (<1.4% of motor neurons) (3). However, EV71 replication in the spinal cord of cynomolgus monkeys was not sufficient to cause tremor. In the young monkeys (ages, 7 and 12 years), EV71 replication was detected in the lumbar spinal cord, but the animals did not exhibit tremor, in contrast to the older monkeys (ages, 20 to 23 years). Interestingly, the lumbar spinal cord of monkey 4699 had a lower lesion score (0.3) than

TABLE 5. Tissue specificity of EV71(S1-3') and EV71(BrCr-TR) in extraneural tissues of cynomolgus monkeys

| Tissue | Virus isolation (inflammation) for monkey no. [treatment][a]: | | | |
|---|---|---|---|---|
| | 4652 [EV71(S1-3'), day 4 p.i.] | 4699 [EV71(S1-3'), day 4 p.i.] | 4507[b] [EV71(BrCR-TR), day 6 p.i.] | 4508[b] [EV71(BrCR-TR), day 6 p.i.] |
| Tonsil | − (−) | − (−) | − (−) | − (−) |
| Heart | − (−) | − (−) | − (−) | − (−) |
| Lung | − (−) | − (−) | − (−) | − (−) |
| Liver | − (−) | − (−) | − (−) | − (−) |
| Spleen | − (−) | − (−) | + (−) | − (−) |
| Kidney | − (−) | + (+) ($10^{5.75}$) | + (−) | + (+) |
| Muscle | − (−) | − (−) | ND | ND |
| Deep cervical lymph node | + (−) (<$10^{0.5}$) | + (−) ($10^{2.5}$) | + (−) | + (−) |
| Dorsal root ganglion | + (−) ($10^{1.25}$) | − (−) (<$10^{0.5}$) | + (+) | + (+) |

[a] The virus isolation results are shown as positive or negative. The presence of inflammation in the tissues is shown in parentheses as positive or negative. The inoculated viruses and the day sacrificed are also indicated. The virus titer ($CCID_{50}$) in 100 μl of the homogenate is shown, in parentheses following the isolation and inflammation results, for the samples collected from two monkeys, numbers 4652 and 4699.

[b] Samples of monkeys numbers 4507 and 4508 were collected in our previous study (5). ND, not determined.

that of monkey 4652 (lesion score, 0.9), which exhibited no clinical symptoms. In poliomyelitis, adults are much more likely than children to be severely affected by infection in the spinal cord (42). Thus, detectable lesions and viral replication are not the only determinants of the occurrence of tremor in monkeys inoculated with EV71(S1-3′); other factors are critical.

Consistent with the mild neurological symptoms in the present cynomolgus monkeys inoculated with EV71(S1-3′), the distribution of EV71(S1-3′) in the CNS of the monkeys was limited to a small region on day 4 p.i. in the early phase of the infection (Table 4). Inflammation (perivascular cuffing with mononuclear cells) was observed in a broad area of the CNS without a detectable level of viral antigen. The virus was isolated from only the lumbar spinal cord on day 10 p.i. (5). Therefore, the inflammation observed in a broad area on day 10 p.i. was caused by inefficient infection of EV71(S1-3′) and was not caused by the vigorous infection in the early phase. Previously, we isolated an EV71 mutant [EV71(3′)], which contains temperature-sensitive mutations in the $3D^{pol}$ and 3′ NTR, from both the lumbar spinal cord and brain stem of inoculated monkeys (5). Thus, it appears that suppressed infection of EV71(S1-3′) in the brain stem is promoted by mutation in the 5′ NTR or by a combination of mutations in the 5′ NTR, $3D^{pol}$, and 3′ NTR. Interestingly, studies indicate that PV1 and EV71 viruses with a mutation in the 5′ NTR [PV1(Sabin) and EV71(S1-3′)] are fairly stable in the lumbar spinal cord of inoculated monkeys (5, 24). The lesions of the brain stem are critical causes of fatality from EV71 infection (10, 21, 25, 31, 57), suggesting that the introduced attenuation determinant of PV1(Sabin) contributes to EV71-specific pathogenesis.

In the present study, we observed transient infection of EV71(S1-3′) in extraneural tissues, including the kidney, deep cervical lymph node, and dorsal root ganglion (Table 5). The infection in the dorsal root ganglion may simply reflect EV71 infection in sensory neurons (40). The deep cervical lymph node is thought to play a central role in establishment of the viremic phase of PV infection, according to a model proposed by Bodian (6; reviewed in reference 35). The kidney may also play an important role in the establishment of viremia as a transiently susceptible extraneural tissue in EV71 infection, as Sabin has proposed for PV infection, although the identity of the extraneural tissue involved in PV infection remains unknown (46).

The monkeys inoculated with EV71(S1-3′) were protected from a lethal challenge with EV71(BrCr-TR) (Fig. 2). We immunized monkeys with EV71(S1-3′) via an intravenous route with a high dose ($10^7$ $CCID_{50}$) intended to cause experimentally transient high-level viremia ($2.9 \times 10^4$ to $5.1 \times 10^4$ $CCID_{50}$ per ml). Even the viremogenic PV1(Mahoney) has been shown to cause viremia on the order of $10^5$ $CCID_{50}$ per ml in cynomolgus monkeys (6). Therefore, if EV71(S1-3′) were inoculated via the oral route (i.e., the natural route of EV71 infection), a much weaker humoral immune reaction would be expected in terms of levels of anti-EV71 IgG and IgM in the serum. The effectiveness of EV71(S1-3′) inoculation via the oral route, which depends on the induction of anti-EV71 secretory IgA for protection, remains to be further evaluated.

The neutralizing titers of the sera of monkeys immunized with EV71(S1-3′) were highest against the homotypic strain EV71 (genotype A) and were lowest against strains belonging to genotype C2, with a maximum difference of 60-fold between neutralization titers for different strains (Tables 2 and 3). It should be noted that genotype specificity was not eliminated after a single booster with the homotypic strain. Antigenic heterogeneity within a single serotype is a typical feature of enterovirus; for example, for echovirus 30, differences in the neutralization titer of >100-fold of the sera of rhesus monkeys have been observed (58). There have also been reports of antigenic heterogeneity among EV71 isolates, including a maximum difference in neutralization titers of 32-fold between different rabbit sera after EV71 inoculation (19). Frequent changes in the genotype of circulating EV71 may in part reflect antigenic variation in cross-neutralization between genotypes that may be critical for the perpetuation of EV71 (27, 37, 41, 55).

EV71(S1-3′) is a promising vaccine candidate, although it is still neurovirulent, at least when inoculated via the intravenous route. Several strategies have been proposed for attenuation and stabilization of the phenotype of PV (4, 9, 12, 15, 22, 32, 38). However, in general, attenuated PV strains exhibit impaired virus growth, even in cells cultured in vitro. Therefore, evaluation of the fitness of EV71(S1-3′) required for effective antigenicity is necessary to ensure that further attenuation produces effective vaccine strains.

In summary, we found that the attenuated strain EV71(S1-3′), which belongs to genotype A, acts as an effective antigen against several genotypes of EV71 and has attenuated neurovirulence in cynomolgus monkeys.

## ACKNOWLEDGMENTS

We are grateful to Ayako Harashima, Yuko Sato, and Junko Wada for their excellent assistance.

This work was supported by grants-in-aid from the Japan Society for Promotion of Science and for Research on Emerging and Reemerging Infectious Diseases from the Ministry of Health, Labor and Welfare.

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# Antigenic characterization of a formalin-inactivated poliovirus vaccine derived from live-attenuated Sabin strains

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Received 17 April 2007; received in revised form 7 July 2007; accepted 30 July 2007
Available online 17 August 2007

**Abstract**

A candidate inactivated poliovirus vaccine derived from live-attenuated Sabin strains (sIPV), which are used in the oral poliovirus vaccine (OPV), was prepared in a large-production scale. The modification of viral antigenic epitopes during the formalin inactivation process was investigated by capture ELISA assays using type-specific and antigenic site-specific monoclonal antibodies (MoAbs). The major antigenic site 1 was modified during the formalin inactivation of Sabin 1. Antigenic sites 1–3 were slightly modified during the formalin inactivation of Sabin 2 strain. Sites 1 and 3 were altered on inactivated Sabin 3 virus. These alterations were different to those shown by wild-type Saukett strain, used in conventional IPV (cIPV). It has been previously reported that type 1 sIPV showed higher immunogenicity to type 1 cIPV whereas types 2 and 3 sIPV induced lower level of immunogenicity than their cIPV counterparts. Our results suggest that the differences in epitope structure after formalin inactivation may account, at least in part, for the observed differences in immunogenicity between Sabin and wild-type inactivated poliovaccines.


*Keywords:* Poliovirus; Inactivated poliovirus vaccine; Sabin strains; Antigenicity

## 1. Introduction

Polioviruses (family *Picornaviridae*, genus *Enterovirus*) are non-enveloped positive strand RNA viruses, and are the causative agents of paralytic poliomyelitis. As well as the qualified acute flaccid surveillance and laboratory diagnosis activities, the exhaustive and global use of poliovirus vaccines is one of the key components of the WHO Global Polio Eradication Initiative [1]. Since the programme was launched in 1988, the incidence of polio cases has been drastically reduced and the circulation of indigenous wild polioviruses was interrupted in all but four countries of the world by the end of 2006 [2].

A live-attenuated oral polio vaccine (OPV) is highly effective against all three serotypes of polioviruses and has been used to interrupt wild poliovirus transmission for the Global Polio Eradication Initiative. OPV strains can effectively induce both humoral and mucosal immunities against poliovirus; however, they have some inherent disadvantages due to their genetic instability and rapid generation of revertants during viral replication in vaccine recipients [3]. The incidence of vaccine-associated paralytic poliomyelitis (VAPP), which mainly occurs among OPV recipients or their close contacts, has been considered to be acceptably low (one case per 2.5 million OPV doses and about one case per 6 million among contact cases) [4] during the polio endemic era. However, continuous use of OPV in polio-free areas will maintain the risk of VAPP as the only source of paralytic disease. During the final stages of global polio eradication, the risk of paralytic polio out-

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0264-410X/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2007.07.060

breaks due to vaccine-derived polioviruses (VDPVs) cannot be ruled out as long as the use of OPV continues. For programmatic reasons, VDPVs showing at least 1.0% VP1 nucleotide sequence diversity from the parental Sabin strains have been further classified into two different categories from the epidemiological point of view. Long-term carriers (i.e., >1 year) of VDPVs among immunodeficient patients (iVDPV) appear to be rare, but more than 23 of such cases have been identified in the past 40 years of widespread use of OPV [5–7]. Recently, a number of independent episodes of paralytic polio outbreaks associated with circulating VDPVs (cVDPVs) have been reported [7]. cVDPV isolates seemed to have regained neurovirulent and highly transmissible phenotypes typical of wild-type polioviruses. To minimize the potential risk of polio outbreaks associated with either cVDPV and iVDPV strains, and to reduce the disease burden related to the use of OPV, the introduction of inactivated poliovirus vaccine (IPV) use in middle- and/or low-income countries, already implemented in most developed countries, is urgently needed particularly for the post-eradication era.

Currently, trivalent IPVs are mainly produced using the virulent wild-type poliovirus strains Mahoney, MEF-1, and Saukett, for serotypes 1–3, respectively. The probability of wild poliovirus escape from IPV production sites to the community would be very small but the possible risk of inadvertent release of wild polioviruses into increasingly unimmunized populations should be carefully considered, particularly after global polio eradication is achieved. In this regard, the use of live-attenuated poliovirus strains for IPV production instead of the wild-type strains used at present has been proposed to minimize the risk of poliomyelitis outbreaks due to virus release to the community during the post-eradication era [8].

As we have previously reported, a novel formalin-inactivated poliovirus vaccine derived from the live-attenuated Sabin strains has been developed under good manufacturing practice conditions in a large production-scale [9,10]. It is well known that polioviruses have two different kinds of antigens, namely D- (N) and C- (H) antigens. D-antigen is expressed in native virus particles and can induce type-specific protective neutralization antibodies in individuals after poliovirus infection or vaccination with OPV or IPV. The D-antigen can be converted to non-protective C antigen by mild heating, therefore, the measurement of D-antigen content has been used for the potency test and quality assurance methods to assess the protective immunogenicity of IPV products against polioviruses.

In this study, we have investigated the modification of some antigenic determinants during the formalin inactivation process of all three serotypes of Sabin strains using D-antigen ELISA assays and type- and site-specific monoclonal antibodies in an effort to understand the differences observed in immunogenic properties with respect to the wild-type poliovirus strains used in conventional IPV (cIPV).

## 2. Materials and methods

### 2.1. Large-scale preparation of sIPV

For the production of sIPV, the seed viruses of OPV product, Sabin type 1 (LSc, 2ab), type 2 (P712, Ch, 2ab) and type 3 (Leon, $12a_1b$), were used. Each serotype of the Sabin strains was propagated in Vero cells at 34 °C and the viruses were purified from the virus culture (40 l) by centrifugation and filtration as described previously [9,10]. Before formalin inactivation, the purified virus preparations were diluted ten times with M-199 (medium 199 Earle's base solution without Ca, Mg, $PO_4$ and phenol red, Dainihon Seiyaku Co., Osaka, Japan) and filtered through a 0.22 μm pore size filter (Fuji filter Mfg. Co. Ltd., Tokyo, Japan) in the presence of 0.5% (w/v) of glycine. Each filtered virus fluid was inactivated with 0.025% formalin for 12 days at 37 °C. To assess the time course of antigenic modification, small aliquots of the sample preparations were collected 0, 24, 48, 72 h and 12 days after the formalin treatment for the Sabin 3 strain. For type 3 poliovirus, the virus culture fluid of the Saukett strain was purified and inactivated in the same manner as the Sabin strains. Inactivated antigens were diluted with M-199 to 1:70 for types 1 and 3 and 1:290 for type 2 in order to measure the D-antigen units (DU/ml) by ELISA assays using type-specific monoclonal antibodies (MoAbs) (see below).

### 2.2. Monoclonal antibodies and D-antigen titration

D-antigen-specific monoclonal antibodies (for each poliovirus serotype, MA107-8W against type 1 Mahoney, MA201-159 against Sabin 2, and MA303-182L against type 3 Suwa, were prepared at the Japan Poliomyelitis Research Institute (JPRI) and used for an in-house capture ELISA assay to determine the D-antigen contents in the IPV preparations [9,10]. Other poliovirus type- and site-specific MoAbs used in this study were prepared at the National Institute for Biological Standards and Control (NIBSC), and some of them were well characterized and previously used for the analysis of antigenic properties of inactivated poliovirus vaccines [11–13].

### 2.3. ELISA

One hundred microliters of MoAb per well (mouse ascitic fluid diluted 1:1000 for site-specific MoAb from NIBSC, and 1:20,000 for D-antigen specific MoAb from JPRI in 0.05 M sodium carbonate buffer) were used to coat 96-well microimmunoplate (Nunc-Immuno Plate Maxisorp, Nunc A/S Kamstrupvej, Roskilde, Denmark) and the plate was incubated for 3 h at 36 °C. After coating, plates were washed three times with 250 μl per well of washing buffer (0.01 M PBS with 0.05% Tween 20). Fifty microliters of dilution buffer (0.01 M PBS with 0.05% Tween 20 and 1% BSA) were added to each well and then the virus antigen was added to three wells at the bottom of the plate. Virus anti-

gen was added in twofold dilutions. The plate was incubated overnight at 4 °C and washed three times with washing buffer. In-house anti-poliovirus type-specific rabbit serum (1:1000 for types 1 and 3, 1:2000 for type 2) was added (50 μl per well) and was incubated for 1 h at 36 °C. After washing the plate, horseradish peroxidase conjugated anti-rabbit IgG (0.21 μg protein/ml, ICN/Cappel, Aurora, OH, USA) was added to each well (50 μl per well) and the plates were incubated for 1 h at 36 °C. *o*-Phenylenediamine substrate with hydrogen peroxide was added and the plates were incubated for 20 min at 36 °C. The reaction was stopped by addition of 2 M $H_2SO_4$ and absorbance at 492 nm was measured. Assay data were evaluated by a parallel line analysis of log dose versus log absorbance. D-antigen units were calculated from the mean OD values based on those of the in-house referential standards adjusted to D-antigen units of the control sample of a WHO collaborative study (F 91/672) [14].

### 2.4. Neutralizing assay

Neutralizing titers of the MoAbs against polioviruses were determined by a standard microneutralization assay [15]. Serial twofold dilution of each MoAb was prepared in quartet, and 50 μl per well of the dilutions were incubated with 50 μl per well of poliovirus strain (56–180 $CCID_{50}$). After incubation, HEp-2C cells in suspension then added to each well. The plates were incubated for 5–7 days and the cytopathic effect was observed by microscope. Neutralizing titer was calculated by the Kärber method.

## 3. Results and discussion

In the present study, we evaluated the antigenic profile of sIPV during the process of formalin inactivation using a conventional capture ELISA assay with type- and site-specific panels of MoAbs. The measurement of overall D-antigen content by ELISA assays has been widely used for the potency test and quality assurance of cIPV derived from virulent wild-type poliovirus strains. In order to compare the antigenicity and immunogenicity of the newly developed sIPV with those of cIPVs and OPV and to evaluate possible antigenic changes during the inactivation process in more detail, the antigenic properties of native and inactivated Sabin strains of each serotype were analyzed using panels of MoAbs with different epitope specificities. Furthermore, differences in antigenic properties between cIPV and sIPV preparations have been recently revealed [16,17] and therefore, comparative analysis of antigenic profiles is important for further standardization of the in vitro potency test and quality control of sIPV to ensure effective immunity against wild and vaccine-derived polioviruses among sIPV vaccinees.

The ELISA reactivity to native and inactivated Sabin 1 antigens was compared by measuring the antigen content using 10 MoAbs (Table 1). A lower ELISA reactivity to the inactivated antigen than the native one was found for site 1-specific MoAbs (955 and 956) but not for other MoAbs specific to antigenic site 2 (237, 425, 429, 430), site 3 (423, 958) or site 4 (234). The antigenic modification on site 1 epitope of sIPV during formalin-inactivation is consistent with a recent report by Rezapkin et al. [13] using a block-ELISA method with the MoAbs 955 and 956. Although it would be of interest that ELISA reactivity of the inactivated Sabin 1 antigen to MoAbs to antigenic sites 2 and 3 (237, 429, 430, 423, 958) was generally higher than that to the native one (Table 1), the increased ELISA reactivity of sIPV was not apparent by the block-ELISA method using the same MoAbs [13].

For the Sabin 2 antigen, the antigen content measured with site 1-specific (269, 969), site 2-specific (1251 and 1268), and site 3-specific (1050) MoAbs were generally lower for the inactivated antigen than the native one (Table 2), and the results are consistent with those by the block-ELISA assay for site 1-specific MoAbs 269 and 969 [13]. Although significant antigenic difference between native and inactivated type 2 MEF-1 antigens has been observed particularly for antigenic site 1 of cIPV [11], the changes in antigenic contents for Sabin 2 were rather moderate. For instance, several site 1-specific MoAbs (269 and 437) failed to react with inactivated MEF-1

Table 1
D-antigen contents of native or inactivated poliovirus samples (Sabin 1)

| Antigenic site | Monoclonal antibody | D-antigen content (DU/ml)[a] | | Ratio (inactivated/native) | Neutralization reactivity[b] | |
|---|---|---|---|---|---|---|
| | | Native | Inactivated | | Sabin 1 | Mahoney |
| Unknown | MA107-8W | 26.4 ± 8.48 | 21.0 ± 2.20 | 0.80 | + | + |
| 1 | 955 | 14.3 ± 0.53 | 0.00 | 0.00 | + | – |
| 1 | 956 | 23.8 ± 6.88 | 10.1 ± 0.64 | 0.42 | + | – |
| 2 | 237 | 18.7 ± 4.50 | 23.2 ± 3.03 | 1.24 | + | + |
| 2 | 425 | 12.5 ± 0.86 | 22.7 ± 1.11 | 1.82 | + | + |
| 2 | 429 | 11.4 ± 7.86 | 16.5 ± 1.15 | 1.45 | + | + |
| 2 | 430 | 18.2 ± 4.12 | 22.4 ± 1.12 | 1.23 | + | + |
| 3 | 423 | 12.5 ± 2.03 | 18.4 ± 0.35 | 1.47 | + | – |
| 3 | 958 | 11.9 ± 2.23 | 30.2 ± 6.89 | 2.54 | + | – |
| 4 | 234 | 20.1 ± 1.29 | 23.4 ± 0.55 | 1.16 | + | + |

[a] Data presents mean D-antigen contents of three independent assays ± S.D.
[b] +, neutralization; –, no neutralization.

Table 2
D-antigen contents of native or inactivated poliovirus samples (Sabin 2)

| Antigenic site | Monoclonal antibody | D-antigen content (DU/ml)[a] | | Ratio (inactivated/native) | Neutralization reactivity[b] | |
|---|---|---|---|---|---|---|
| | | Native | Inactivated | | Sabin 2 | MEF-1 |
| Unknown | MA201-159 | 28.1 ± 2.44 | 23.2 ± 4.99 | 0.83 | + | + |
| 1 | 269 | 18.8 ± 0.95 | 11.2 ± 1.90 | 0.60 | + | − |
| 1 | 435 | 12.8 ± 4.56 | 17.4 ± 1.01 | 1.36 | + | + |
| 1 | 437 | 14.2 ± 3.65 | 20.4 ± 2.40 | 1.44 | + | + |
| 1 | 969 | 21.0 ± 2.81 | 14.8 ± 2.38 | 0.70 | + | + |
| 2 | 1251 | 17.7 ± 1.87 | 10.7 ± 0.47 | 0.60 | + | + |
| 2 | 1268 | 14.2 ± 0.99 | 11.0 ± 0.21 | 0.77 | + | + |
| 3 | 1050 | 17.7 ± 4.22 | 13.0 ± 0.10 | 0.73 | + | + |
| 3 | 1103 | 12.0 ± 1.67 | 13.3 ± 0.53 | 1.11 | + | + |

[a] Data presents mean D-antigen contents of three independent assays ± S.D.
[b] +, neutralization; −, no neutralization.

Table 3
D-antigen contents of native or inactivated poliovirus samples (Sabin 3)

| Antigenic site | Monoclonal antibody | D-antigen content (DU/ml)[a] | | Ratio (inactivated/native) |
|---|---|---|---|---|
| | | Native | Inactivated | |
| Unknown | MA303-182L | 43.1 ± 10.4 | 40.2 ± 3.42 | 0.93 |
| 1 | 132 | 31.7 ± 3.32 | 10.7 ± 3.15 | 0.34 |
| 1 | 134 | 35.9 ± 0.44 | 14.9 ± 0.81 | 0.42 |
| 1 | 175 | 38.7 ± 6.65 | 22.4 ± 1.40 | 0.58 |
| 1 | 208 | 32.4 ± 8.77 | 18.6 ± 0.40 | 0.57 |
| 1 | 439 | 3.03 ± 1.68 | 4.39 ± 4.45 | 1.45 |
| 1 | 495 | 41.2 ± 2.33 | 32.0 ± 0.85 | 0.78 |
| 1 | 520 | 30.4 ± 7.15 | 22.0 ± 0.51 | 0.72 |
| 2 | 877 | 35.7 ± 3.30 | 40.7 ± 2.13 | 1.14 |
| 2 | 882 | 49.4 ± 4.02 | 32.8 ± 0.92 | 0.66 |
| 3 | 138 | 56.3 ± 7.12 | 8.28 ± 0.66 | 0.15 |
| 3 | 140 | 39.4 ± 11.5 | 9.96 ± 3.86 | 0.25 |
| 3 | 888 | 47.1 ± 1.63 | 39.9 ± 1.53 | 0.85 |
| 3 | 889 | 35.2 ± 2.07 | 36.3 ± 6.47 | 1.03 |

[a] Data presents mean D-antigen contents of three independent assays ± S.D.

antigen in a previous study [11] but retained reactivity with inactivated Sabin 2 antigen.

When the reactivities against native and inactivated Sabin 3 antigens were compared by ELISA, the antigenic content of the inactivated antigen was generally lower than that of the native virus when site 1-specific MoAbs were used with the exception of MoAb 439 (Table 3). On the other hand, the ELISA reactivity against MoAb 439 was severely impaired

Table 4
D-antigen contents of native or inactivated poliovirus samples (Saukett)

| Antigenic site | Monoclonal antibody | D-antigen content (DU/ml)[a] | | Ratio (inactivated/native) |
|---|---|---|---|---|
| | | Native | Inactivated | |
| Unknown | MA303-182L | 50.2 ± 0.91 | 44.3 ± 1.01 | 0.88 |
| 1 | 132 | 66.2 ± 8.41 | 45.1 ± 1.22 | 0.68 |
| 1 | 134 | 86.6 ± 6.65 | 66.6 ± 15.7 | 0.77 |
| 1 | 175 | 49.7 ± 3.78 | 39.9 ± 1.29 | 0.80 |
| 1 | 208 | 79.9 ± 3.65 | 54.8 ± 4.20 | 0.69 |
| 1 | 439 | 118 ± 68.9 | 43.5 ± 13.3 | 0.37 |
| 1 | 495 | 61.3 ± 6.41 | 38.9 ± 2.01 | 0.63 |
| 1 | 520 | 39.9 ± 13.6 | 37.4 ± 7.05 | 0.94 |
| 2 | 877 | 51.4 ± 5.18 | 48.3 ± 3.40 | 0.94 |
| 2 | 882 | 53.1 ± 5.70 | 42.0 ± 2.05 | 0.79 |
| 3 | 138 | 0.00 | 0.00 | − |
| 3 | 140 | 33.0 ± 13.3 | 43.0 ± 19.1 | 1.30 |
| 3 | 888 | 0 | 0 | − |
| 3 | 889 | 0 | 0 | − |

[a] Data presents mean D-antigen contents of three independent assays ± S.D.

Table 5
Predicted amino acid sequences of Sabin 3 and Saukett strains at the antigenic sites

| Virus | Site 1 VP1 89-100 | Site 2a VP1 217-223 | Site 2b VP1 164-172 | Site 3a VP1 286-290 |
|---|---|---|---|---|
| Sabin 3 | EVDNEQPTTRAQ | TDANDQI | TPKSWDDYT | RNNLD |
| Saukett | ---------- | ------- | --------- | KD--N |

during cIPV inactivation [11]. The antigenic modification on site 1 was observed in the conventional and block- ELISA assays with some of the site 1-specific MoAbs (132, 134) for cIPV and sIPV preparations [11,13]. The antigenic content of the inactivated Sabin 3 antigen measured with site 2-specific MoAb 882 was lower than that of the native one, while antigenic content with 877 was similar. Meanwhile, the antigenicity against two out of four site 3-specific MoAbs (138, 140) changed following formalin inactivation, while antigenic content with 889 was similar as previously reported using the block-ELISA assay [13].

The inactivated Sabin 3 antigen was less immunogenic than Sabin 1 and 2 antigens in the previous phase I clinical trial among healthy adult volunteers, at least after the first immunization with sIPV [9], and differences in antigenicity between inactivated Sabin 3 and Saukett (wild-type 3 strain used in cIPV) antigens have also reported previously [17]. Therefore, we compared the antigenic profiles of inactivated Sabin 3 and Saukett antigens (Table 4). Differences in antigenic reactivities against type 3-specific MoAbs were found between Sabin 3 and Saukett strains. Three out of four site 3-specific MoAbs failed to react with both native and inactivated Saukett antigens. However, these three MoAbs recognized native and inactivated Sabin 3 antigens. Furthermore, the ELISA reactivity of Saukett against MoAb 140 was not impaired whereas that of Sabin 3 was reduced after formalin inactivation (Table 4). Sequencing analysis of the VP1 region of the Saukett strain used in this study identified it as a variant of Saukett COP or Saukett A (data not shown). Therefore, the different antigenic characteristics between Saukett and Sabin 3 strains was due to differences in amino acid residues found in antigenic site 3a (Table 5). During the formalin inactivation process, changes in the antigenicity of the site 3 epitope recognized by MoAb 140 were evident in a time-dependent manner (Fig. 1). MoAb 140 did not possess apparent neutralization activity against Sabin 3 and Saukett strains, thus it has not been used by the ELISA assays in previous studies. MoAb 495 neutralized Sabin 3 virus more effciciently whereas the neutralization titer of MoAb 882 was higher against the Saukett strain. Similar neutralization titers against both strains were found for some of the MoAbs that showed neutralization activities (MA303-182L, 175, 520) (Table 6). Therefore the antigenic changes of Sabin 3 antigen detected by the ELISA assay do not necessarily correlate with neutralization or immunogenic epitopes of type 3 polioviruses. For type 1 and 2 strains, all of the MoAbs tested in the ELISA assay possessed neutralizing activities against the corresponding Sabin 1 and 2 strains (Tables 1 and 2). Changes in reactivity between Sabin 1 and 2, and wild-type 1 and 2 strains, Mahoney and MEF-1, respectively, correlated with changes in amino acid sequences on the corresponding antigenic sites.

In this study, we found antigenic modifications on Sabin 1–3 antigens during the process of formalin inactivation

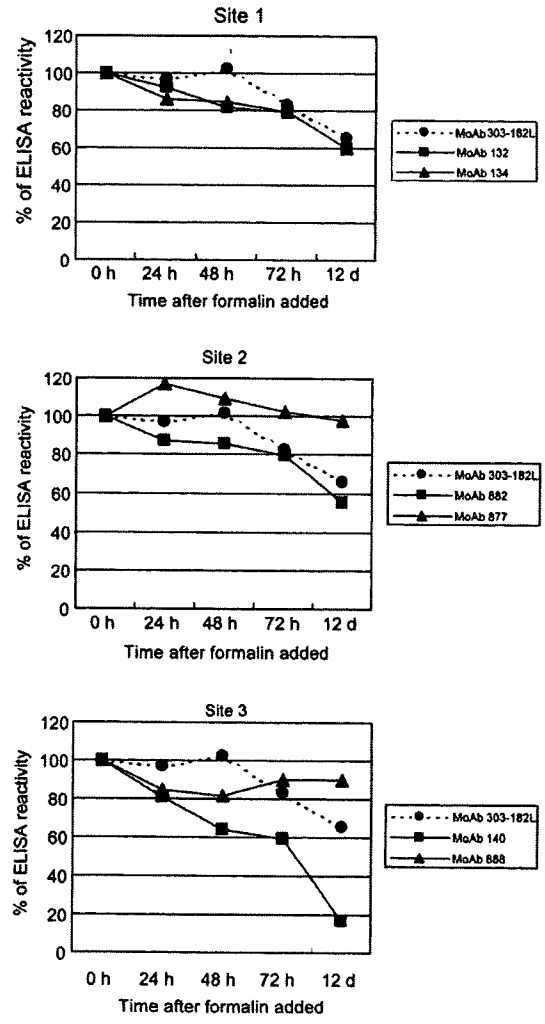


Fig. 1. ELISA reactivity of the Sabin 3 antigen against various site-specific monoclonal antibodies during formalin inactivation. The ELISA OD value for each monoclonal antibody before formalin treatment is represented by 100%.

Table 6
Neutralizing titer of site-specific monoclonal antibodies against type 3 polioviruses

| Recognition site | Monoclonal antibody | Neutralizing titer | |
|---|---|---|---|
| | | Sabin 3 | Saukett |
| Unknown | MA303-182L | >3,200 | >3,200 |
| 1 | 132 | <100 | <100 |
| 1 | 134 | <100 | <100 |
| 1 | 175 | >3,200 | >3,200 |
| 1 | 208 | <100 | <100 |
| 1 | 439 | <100 | <100 |
| 1 | 495 | 3,200 | 673 |
| 1 | 520 | >12,800 | 9,050 |
| 2 | 882 | 1,600 | 5,380 |
| 3 | 140 | <100 | <100 |

in large-scale sIPV production using conventional ELISA assays and type- and site-specific panels of MoAbs. Most of the epitopes on antigenic site 1 were partially modified by the formalin treatment of Sabin 1–3 strains. Although similar antigenic changes during formalin inactivation have been described in commercially available cIPV products [11], the effect of formalin treatment on each epitope was varied, especially for type 3 polioviruses, Sabin 3 and Saukett strains, examined in this study. In a recent study using transgenic mice carrying human poliovirus receptor (TgPVR21), two doses of type 2 sIPV (2–16 DU) induced lower level of antibodies and incomplete protection but cIPV did efficiently protect mice against challenge with type 2 wild-type MEF-1 strain. However, the TgPVR21 mice, which were immunized with 8 DU sIPV, were fully survived from a lethal challenge with a virulent type 2 vaccine derived strain [16]. The relative risk of polio outbreaks due to VDPV, compared with those due to antigenically divergent wild polioviruses, will increase in the future, therefore, sIPV might induce proper population immunity against VDPV strains during the post-eradication era.

In our recent study, a single dose of sIPV exhibited relative immunogenicity, comparable to that of cIPV in rats, for type 1 and 2 strains, but less for type 3 [10]. However, four doses of sIPV (3, 100 and 100 DU, for types 1–3, respectively) induced high-neutralization titers against the attenuated and virulent poliovirus strains for all three serotypes in green monkeys [10]. The results indicate that optimization of the antigen content and different immunization regimens would be still important for further sIPV development. Although more careful and comprehensive antigenic characterizations will be important to standardize and harmonize the potency test and quality assurance methods to assess the protective immunogenicity of sIPV against wild and vaccine-derived polioviruses, sIPV should be an alternative polio vaccine during the post-eradication era.

## Acknowledgements

We thank Shinobu Abe, Yoshihiro Ohta, and Miwako Miyazawa for providing the polio vaccine materials for ELISA. This work was supported, in part, by grants from the Ministry of Health, Labour and Welfare, Japan.

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Jpn. J. Infect. Dis., 60, 402-404, 2007

**Short Communication**

# Sequence and Phylogenetic Analysis of the Nucleoprotein (*N*) Gene in Measles Viruses Prevalent in Gunma, Japan, in 2007

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(Received June 15, 2007. Accepted August 31, 2007)

**SUMMARY**: In 2007, relatively large outbreaks of measles occurred in the Kanto region of Japan, including Gunma Prefecture. We performed sequence and phylogenetic analysis of the nucleoprotein gene (*N* gene) of measles viruses from 3 measles patients in this area in May 2007. The *N* gene sequences of the present strains were identical to each other, and phylogenetic analysis showed these viruses were classified into genotype D5. The results suggest that highly homologous measles viruses may be associated with outbreaks of measles in Gunma, Japan.

The measles virus (MV), genus *Morbillivirus*, family *Paramyxoviridae*, causes acute and highly contagious measles infections in humans (1,2). Measles presents as an unpleasant mild to moderately severe illness, and the most serious complications include blindness, encephalitis (a dangerous infection of the brain causing inflammation), severe diarrhea (possibly leading to dehydration), ear infections, and severe respiratory infections such as pneumonia, the most common cause of death associated with measles. Encephalitis is estimated to occur in 1/1,000 cases, while otitis media (middle ear infection) reportedly occurs in 5-15% of cases and pneumonia in 5-10%. The case fatality rate in developing countries is generally in the range of 1 to 5% but may be as high as 25% in populations with high levels of malnutrition and poor access to health care (http://www.who.int/mediacentre/factsheets/fs286/en/print.html).

The measles vaccine was introduced in the 1978 as a regular vaccination in Japan. According to the World Health Organization's field guidelines for measles elimination (http://www.wpro.who.int/publications/pub_929061126x.htm), over 95% population immunity is needed to interrupt transmission and hence eliminate measles. However, the actual vaccination rate in Japan is thought to be about 70 to 90%, and thus, relatively large outbreaks of measles still occur every 5 to 7 years (3,4).

Between March and June 2007, outbreaks of measles occurred in the Kanto region of Japan, which includes Tokyo, Saitama, Chiba, Kanagawa, Ibaragi, Tochigi, and Gunma Prefectures. Genotyping and phylogenetic analysis are useful tools for understanding the infection routes of various viruses, including the MV (3,5). The MV nucleoprotein gene (*N* gene) is frequently used for genotyping and phylogenetic analysis (3-11). To better understand the molecular epidemiology of the MV prevalent in 2007 in Gunma, Japan, we therefore performed sequence and phylogenetic analysis of the *N* gene. The findings are presented herein.

In Gunma Prefecture, which has a population of approximately 2 million, 131 cases were reported during the period from January to May 2007, compared to only 6 cases in 2006, representing a dramatic increase in the incidence of measles in this area (Fig. 1A). Most patients were teenagers aged 15 to 19 years (22%) and young adults in their twenties (27%) (Fig. 1B). In May 2007, throat swab samples were collected from 3 measles patients showing typical clinical symptoms such as a high fever, cough, conjunctivitis, Koplik's spots inside the mouth, and a rash on the face, trunk, upper neck, back, and, eventually, hands and feet. All patients gave written informed consent prior to participation in this study. One patient (M01) was a senior high school student (17 years old), and the remaining 2 were junior high school students (13 and 15 years, respectively). The 2 junior high school students (M02 and M03) were residents of Tatebayashi City, and the senior high school student lived in Maebashi City; the distance between the two cities is about 50 km. None of the 3 patients had a history of contact with each other. One patient (M03) was immunized in May 1993 (about 13 years before contracting measles), but the remaining 2 were unvaccinated or had an unknown vaccination history, respectively.

Throat swabs were centrifuged at 3,000 × g at 4°C for 30 min, and the supernatants were used for RT-PCR and sequence analysis performed as previously described (12). Briefly, MV RNA was extracted from 140 $\mu$l of the swab supernatants using a QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., USA). The extracted RNA was then suspended in 60 $\mu$l of DNase/RNase-free water. To amplify the *N* gene, we used a one-step RT-PCR kit (Qiagen) with reaction mixture (total volume, 50.0 $\mu$l) consisting of 10.0 $\mu$l of template RNA, 2 $\mu$l of pMvGTf1 and pMvGTr1 primers (20 pmol each), 10.0 $\mu$l of 5 × OneStep RT-PCR Buffer, 2 $\mu$l of dNTP Mix (containing 10 mM of each dNTP), 2 $\mu$l of OneStep RT-PCR enzyme mix, 0.5 $\mu$l of RNase inhibitor (containing 10 units/$\mu$l), and 23.5 $\mu$l of RNase-free water. The samples were incubated for 30 min at 50°C then 15 min at 95°C followed by 40 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min, ending with elongation for an additional 10 min at 72°C (574 bp). Nested PCR (533 bp) was then used

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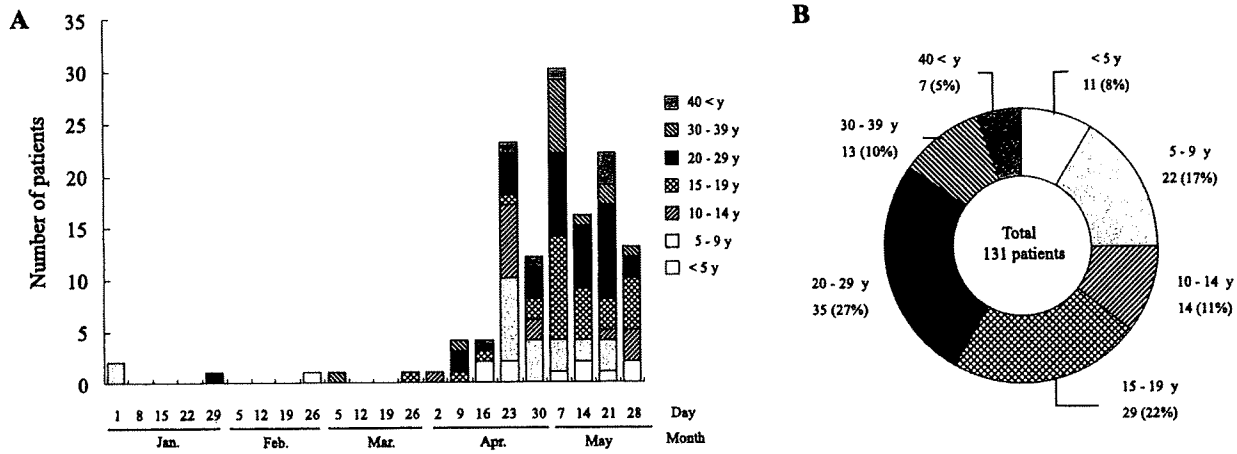


Fig. 1. (A) Epidemic curve showing breakdown by age for patients with measles in Gunma Prefecture, 2007. (B) Breakdown of patients with measles by age and number of cases ($n$ = 131).

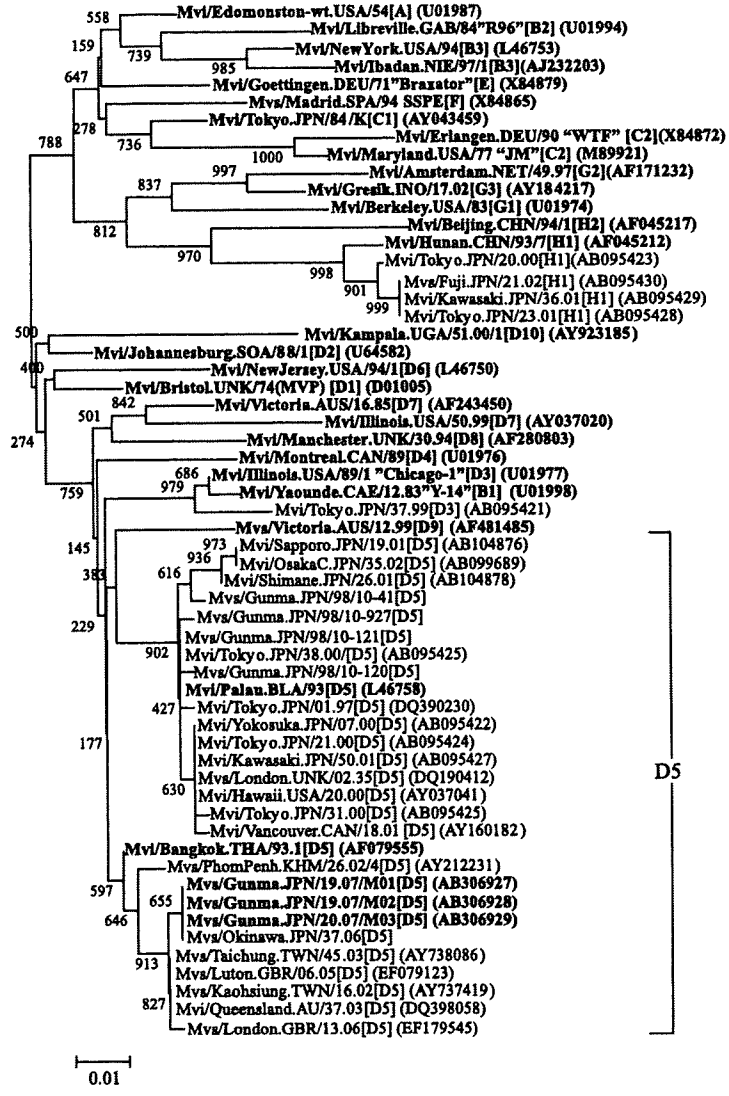


Fig. 2. Phylogenetic tree based on the nucleotide protein ($N$) gene sequences of various strains of the measles virus. The distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. Numbers in parenthesis are GenBank accession numbers. The genotype of reference strains and the present strains are represented in bold type.

to detect the *N* gene. The PCR reaction mixture contained 5 $\mu$l of template DNA, 2 $\mu$l of pMvGTf2 and pMvGTr2 primers (20 pmol each), 25 $\mu$l of PCR Master Mix (Promega, Madison, Wis., USA), and 18 $\mu$l of DNase- and RNase-free distilled water (total volume, 50 $\mu$l). The PCR protocol included incubation for 3 min at 94°C followed by 30 cycles at 94°C for 2 min, 55°C for 3 min, and 72°C for 2.5 min, ending with elongation for an additional 5 min at 72°C. The sizes of the amplified DNA fragments were confirmed by electrophoresis using 3% agarose gel. The DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen), and the nucleotide sequence was determined with an automated DNA sequencer ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA) using a Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems) (12). Nucleotide sequences of the partial *N* gene of the MV (positions 1302 to 1686: 385 bp) were analyzed phylogenetically using the CLUSTAL W program on the DNA database of Japan (DDBJ) homepage (http://clustalw.ddbj.nig.ac.jp/top-e.html) and TreeExplorer (Version 2.12) (http://evolgen.biol.metro-u.ac.jp/TE/). Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenetic trees were constructed using the neighbor-joining (N-J) method (13). Reliability of the trees was estimated using 1,000 bootstrap replications.

The nucleotide sequences of the *N* gene in the obtained MV samples were identical to each other. In addition, these sequences were identical to those of another strain detected in Okinawa in 2006; this case was epidemiologically confirmed during an outbreak in Tokyo. The results suggest that the MVs detected in Gunma are highly homologous to each other as well as strains detected in other areas.

Based on the *N* gene sequences, the genotypes were classified as A to H then were further classified into subgenotypes. A detailed phylogenetic tree based on the *N* gene sequences is shown in Fig. 2. It has been suggested that there is an epidemiological link between certain genotypes/subgenotypes and geographical area (5-11,14,15). The present strains were classified into the same cluster as D5, and they were highly homologous compared to other MV strains detected in areas of Taiwan (Mvs/Taichung.TWN/45.03 [D5]), Australia (Mvi/Queensland.AU/37.03 [D5]), and England (Mvs/Luton.GBR/06.05 [D5]) (Fig. 2). Recently, genotype D5 has mainly been detected in Asian countries including Taiwan, Cambodia, Thailand, and Japan (3,6,10), and recurrent outbreaks have been particularly apparent in Japan in recent years (3). For example, a large measles outbreak that occurred in Gunma in 1998 was due to the D5 virus (16). The results suggested that Japanese outbreaks of measles during the past 2 years were at least partially due to a highly homologous virus(es) belonging to the D5 genotype.

In Japan, during the past 10 years, domestic measles outbreaks occurred in 1998, 2001, 2006, and 2007 (3,4,14-16). In June 2007, outbreaks were still being confirmed in various areas in Japan (http://idsc.nih.go.jp/disease/measles_e/idwr200719.html). Additional information regarding the epidemic status of measles continues to be needed, since there is a high likelihood of the disease spreading to other areas.

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JOURNAL OF CLINICAL MICROBIOLOGY, June 2007, p. 1912–1919 Vol. 45, No. 6
0095-1137/07/$08.00+0 doi:10.1128/JCM.01251-06


# Outbreak of Human Metapneumovirus Detected by Use of the Vero E6 Cell Line in Isolates Collected in Yamagata, Japan, in 2004 and 2005[▽]

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Received 19 June 2006/Returned for modification 24 September 2006/Accepted 9 April 2007

**A number of epidemiological studies have shown human metapneumovirus (hMPV) to be one of the most important viral agents associated with acute respiratory infections in humans. However, due to the difficulty in growing the virus, all epidemiological studies of hMPV infection have been performed on the basis of the molecular method. Thus, the development of a cell line suitable for the isolation of hMPV from clinical specimens is a crucial step for further research. Using the Vero E6 cell line, which could be stably maintained for 1 month without passage or medium change, we succeeded in isolating 79 strains from 4,112 specimens obtained in Yamagata, Japan, in 2004 and 2005. The total isolation rate was 1.9% (79/4,112). The monthly distribution revealed that hMPV infections occurred between February and April in 2004 and throughout most of the year in 2005. Phylogenetic analysis indicated that subgenogroup B2 was predominant in 2004, whereas three subgenogroups, A2, B1, and B2, had cocirculated in 2005. Although multiple subgenogroups cocirculated in 2005, each individual subgenogroup strain was found to predominate at specific sites. An infectivity assay of hMPV strains also indicated that the infection efficiency in Vero E6 cells was better than that in LLC-MK2 cells. Finally, we found that Vero E6 cells are useful for the isolation of hMPVs and that this utility might aid further research into hMPVs beyond the epidemiological data shown in this study.**

In 2001, a new respiratory virus, human metapneumovirus (hMPV), was first isolated from nasopharyngeal specimens from children with acute respiratory infection (ARI) (31). Based on genetic and phylogenetic analyses, hMPV has been categorized as a member of the genus *Metapneumovirus* of the subfamily *Pneumovirinae* of the family *Paramyxoviridae* (31). It has been recognized as a common cause of respiratory infections, ranging from upper respiratory infections to severe bronchiolitis and pneumonia, in both children and adults worldwide (3, 31). The seasonality of hMPV infections resembles that of human respiratory syncytial virus infections, and the epidemic season of hMPV is generally reported to be from winter to early spring (2, 3, 30, 31, 35). hMPVs are separated into two groups, serotypes A and B, with each serotype divided into genetic sublineages 1 and 2 on the basis of genetic differences and neutralization assays (12, 25, 32, 33). However, insufficient epidemiological data for hMPV have been accumulated to date.

With regard to the isolation of hMPV, van den Hoogen et al. first reported that the virus isolates replicated slowly in tertiary monkey kidney (tMK) cells and very poorly in African green monkey kidney (Vero) and human lung adenocarcinoma (A549) cells and could not be propagated in Madin-Darby canine kidney or chicken embryo fibroblast (CEF) cells (31). Since then, hMPV isolation has commonly been performed using tMK and rhesus monkey kidney (LLC-MK2) cell lines, especially LLC-MK2 (3, 4, 5, 6, 10, 12, 13, 14, 15, 23, 24, 35). However, only strains 1 to 38 have been isolated in previous studies using tMK and LLC-MK2 cell lines (3, 4, 10, 14, 24, 25, 35). Furthermore, Døllner et al. detected 50 hMPV-positive cases by reverse transcription-PCR (RT-PCR) but failed to find any cytopathic effect (CPE) using the LLC-MK2 cell line (8). Of course, after 5 to 10 passages, the hMPV titers reach $10^7$ or more tissue culture infective doses per ml, and these viruses show CPEs within 5 days postinfection (24). However, it is still quite difficult to isolate hMPVs directly from clinical specimens, and RT-PCR has since been widely used for the detection and laboratory diagnosis of hMPV. Therefore, if a cell line suitable for the isolation of hMPV can be developed, it will be of great benefit for further research on hMPV.

Historically, the Vero E6 cell line was chosen from seven clones for use in the recovery of the Lassa virus, as it could easily be maintained for a long period (7 to 10 days at that time) without changing of the medium and was found to be superior for use in the replication and plaque production of slow-growing noncytolytic agents infecting humans and rodents (9). This cell line has been further used for the isolation and growth of measles virus, Ebola virus, Crimean-Congo

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▽ Published ahead of print on 25 April 2007.

hemorrhagic fever virus, severe acute respiratory syndrome (SARS) coronavirus, and so on (20, 26, 27, 29), and has potential application for the isolation of other viruses.

After the emergence of SARS coronavirus, we had the opportunity to use the Vero E6 cell line, primarily to isolate SARS coronavirus, in our role as members of a public health laboratory (20). Fortunately, it was not necessary for us to isolate SARS coronavirus. However, by accident, we found that the Vero E6 cell line was sensitive to hMPV when we applied this cell line to the isolation of common respiratory viruses from clinical specimens from children with ARI. Using this Vero E6 cell line, we succeeded in isolating 79 hMPV strains, which could be the highest number of strains yet isolated, after an observation of 4 weeks. In this paper, we describe the effectiveness of this Vero E6 cell line for hMPV isolation as well as our epidemiological findings and further phylogenetic analysis, which we believe to be the first epidemiological data based on virus isolation. On the basis of the results presented herein, it is hoped that this method will be of great use in the further study of hMPV isolation.

## MATERIALS AND METHODS

**Collection of specimens.** Between January 2004 and December 2005, 4,112 nasopharyngeal swab specimens were obtained from patients with ARI at pediatric clinics collaborating with the local health authority of the Yamagata Prefecture for the surveillance of viral diseases in Japan. Among them, 3,958 (96.3%) were from patients ≤15 years old, 108 (2.6%) were from patients of unknown ages, and 46 (1.1%) were from patients >15 years old. The specimens were collected and placed immediately in tubes containing a transport medium and transported to the Department of Microbiology, Yamagata Prefectural Institute of Public Health, for virus isolation.

**Virus isolation and identification.** Virus isolation was carried out using a modified microplate method. Briefly, human embryonic lung fibroblast, human laryngeal carcinoma (HEp-2), Vero, Madin-Darby canine kidney, rhabdomyosarcoma (RD-18S), and green monkey kidney cell lines were prepared on the wells of a 96-well microplate (18, 21). In January 2004, we substituted the Vero E6 cell line, which was provided by the National Institute of Infectious Diseases, Tokyo, Japan, for the Vero cell line. The growth medium for the Vero E6 cell line consisted of Eagle's minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and antibiotics (streptomycin [0.1 μg/ml] and penicillin G [100 units/ml]). The maintenance medium for the Vero E6 cell line consisted of MEM with crystallized trypsin (T-8003; Sigma, St. Louis, MO) (2 μg/ml), 5% MEM vitamin solution (100× concentrate; Sanko Junyaku, Tokyo, Japan), 0.2% glucose, and antibiotics. After the plates were washed with phosphate-buffered saline without calcium and magnesium (PBS), 100 μl of the maintenance medium was added to each well of the plates. Then, each specimen was centrifuged at 3,000 rpm for 15 min and 75 μl of the supernatant was inoculated onto two wells of each cell line. The inoculated plates were incubated at 33°C in a $CO_2$ incubator. We observed the plates two or three times per week for CPEs for 14 days for all cell lines except the Vero E6 cell line, which was observed for approximately 1 month, without passage or medium change. When a suspected hMPV CPE was observed, viral identification was carried out by RT-PCR and sequence analysis.

**Infectivity assay of hMPVs.** We prepared confluent monolayers of Vero, Vero E6, and LLC-MK2 cell lines in 96-well microplates, using MEM containing 10% FBS. Cells were washed twice with PBS and inoculated with 50 μl of serial 10-fold dilutions, ranging from $10^0$ to $10^{-2}$, of hMPV strains 1508-Yamagata-05 and 1918-Yamagata-05, which were isolated in this study and had been passaged twice in Vero E6 cells, and Sendai-1311-04 and Sendai-155-D06, which were isolated at the Virus Research Center, Sendai Medical Center, Sendai, Japan, and had been passaged 10 times in LLC-MK2 cells. We used four replicate wells per dilution of each strain. After centrifugation for 30 min at 2,000 rpm, the virus inocula were incubated for 30 min at 33°C in a $CO_2$ incubator and aspirated, and then 0.1 ml of MEM without trypsin was added as the maintenance medium to each well. At 24 h postinoculation, the medium was removed and the cells were rinsed with PBS twice and then fixed with 70% acetone in PBS for 10 min at room temperature. Fixed cultures were immunostained for the expression of hMPV antigen by incubation for 45 min at 37°C with guinea pig anti-hMPV polyclonal antibodies, which were prepared at the Department of Infectious Diseases, Yamagata University School of Medicine, followed by incubation for 45 min with peroxidase-labeled anti-guinea pig immunoglobulin G antibodies (AP108P; CHEMICON International, Inc.) at room temperature. After each reaction, the cells were washed several times in PBS containing 5% skim milk. In the final step, the peroxidase reaction was allowed to proceed for 20 min using 0.03% $H_2O_2$ and 0.2 mg/ml of 3-3′-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan). The cells were then rinsed with distilled water, and the stained cells were counted microscopically. We determined the numbers of hMPV-infected cells per well for four replicate wells for each virus dilution and calculated the mean number.

**RT-PCR, sequence analysis, and phylogenetic analysis.** RNA extraction, RT-PCR, and sequence analysis were carried out as described previously (17, 19). Briefly, viral RNA was extracted from 100 μl of the viral culture fluid by using ISOGEN-LS (Nippon Gene, Tokyo, Japan). The viral RNA was then transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Nippon Gene, Tokyo, Japan) and a random primer (Takara Bio Inc., Otsu, Japan). Using cDNA, a part of the fusion region was amplified by PCR with 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and then sequenced using a BigDye Terminator V1.1 cycle sequencing kit on an ABI Prism 310 (Applied Biosystems, Foster City, CA) automatic sequencer. For PCR and sequencing analysis, primers MPVF1f, MPVF1r, BF101, and BF104 were used (25, 32). Sequence data were analyzed with CLUSTAL W version 1.83, and a phylogenetic tree was constructed via the neighbor-joining method (28), using the same software.

**Nucleotide sequence accession numbers.** Sequence data were added under accession numbers AB251496 through AB251574 at GenBank.

## RESULTS

**Isolation and CPEs for hMPV in the Vero E6 cell line in Yamagata.** All hMPVs in this study were isolated using the Vero E6 cell line. We succeeded in isolating a total of 79 hMPV strains from 77 children and 2 adults, with a total isolation rate of 1.9% (79/4,112).

The CPEs for hMPV in the primary cultures were accompanied by granular, small, roundup, and refringent cells, but there was no clear syncytium formation (Fig. 1a to d). Most early CPEs were recognized as focal or scattered granular patterns, as shown in Fig. 1a and b, whose images were taken on day 17 after specimen inoculation. Thereafter, the areas of granular, refringent cells spread slowly, as shown in Fig. 1c and d, whose images were taken 29 days after specimen inoculation, with eventual destruction of the cells in some cases. We found the CPE for hMPV in a primary culture within 6 to 7 days after specimen inoculation for 6 strains, within 8 to 14 days for 46 strains, within 15 to 21 days for 23 strains, and after more than 21 days for 4 strains. On the other hand, the CPEs observed after a passage of recovered viral fluid showed large and/or small syncytia within 4 days after inoculation (Fig. 1e and f). Findings of initial CPEs without syncytial formation in the primary culture and with syncytial formation after the passage indicated that the CPEs for hMPVs in the Vero E6 cell line were quite variable. The most remarkable characteristic of the Vero E6 cell line was the stability of the monolayer, which enabled us to observe the hMPV CPE several weeks or even 1 month after cell preparation and specimen inoculation (Fig. 1c, d, and g), whereas regular Vero cells, which had been used until December 2003, degenerated within 14 days. Adenoviruses, enteroviruses, and herpes simplex viruses could also be replicated using the Vero E6 cell line. Since these viruses grow faster than hMPV, we could not isolate hMPVs in cases where these viruses were recovered.

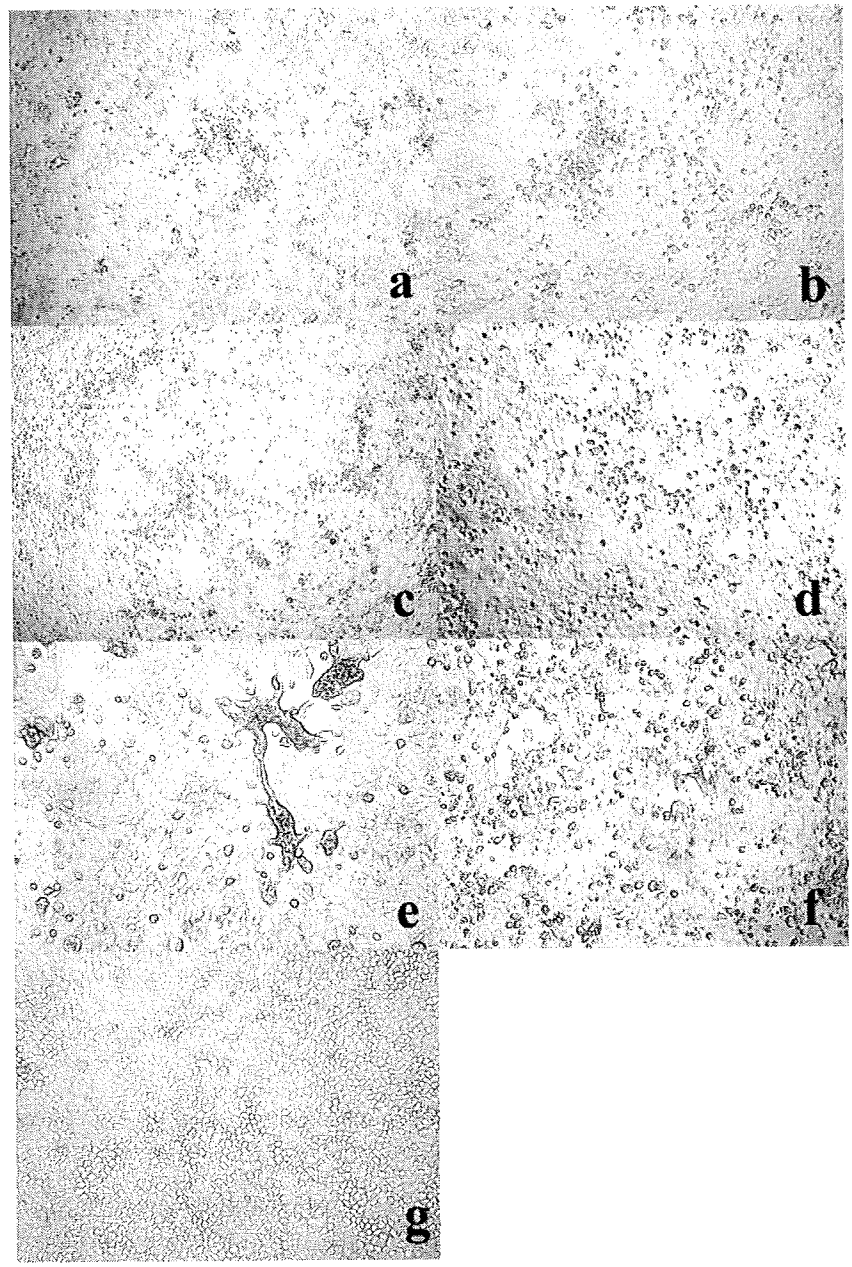


FIG. 1. Microscopic studies of hMPV-infected (a to f) and uninfected (g) Vero E6 cell lines. The various CPEs were induced by the hMPV strains. (a and b) Vero E6 cell primary culture 17 days after inoculation with 1159-Yamagata-05 and 1645-Yamagata-05, respectively. Early CPEs for hMPV were recognized as granular and roundup cell formation, and there was no clear syncytium formation. (c and d) Vero E6 cell primary culture 29 days after inoculation with 970-Yamagata-05 and 1159-Yamagata-05, respectively. The granular formation has spread and progressed, though the Vero E6 monolayer is still stable. (e) Vero E6 cell culture 4 days after inoculation with viral fluid from 1159-Yamagata-05 after two passages. Infected cells showed marked syncytium formation progressing to detachment from the cell monolayer. (f) Vero E6 cell culture 4 days after inoculation with viral fluid from 871-Yamagata-05 after two passages. The CPE consisted of roundup cells and small rather than large syncytia. (g) Uninfected Vero E6 cells 22 days after preparation. Original magnifications, ×100 (a and c) and ×200 (b and d to g).

**Infectivity assay of hMPVs in Vero, Vero E6, and LLC-MK2 cells.** We compared the sensitivities of Vero E6 cells to hMPVs with those of Vero and LLC-MK2 cells. We inoculated the hMPV strains, fixed the cells 24 h after infection, and counted the number of immunostained cells. The two hMPV strains previously passaged twice in Vero E6 cells (1508-Ymagata-05 and 1918-Yamagata-05) infected more than 20 times more cells in Vero E6 cells than in LLC-MK2 cells; however, there were no significant differences in infectivity between Vero E6 and Vero cells. For example, at a dilution of $10^{-2}$, the mean numbers of infected cells for four replicate wells infected with the 1918-Yamagata -05 strain were 63.25 in Vero cells, 74.25 in Vero E6 cells, and 2.5 in LLC-MK2 cells. We also performed the infectivity assay for two strains (Sendai-1311-04 and Sendai-155-D06) previously passaged 10 times in LLC-MK2 cells. In replicate experiments performed on different days, LLC-