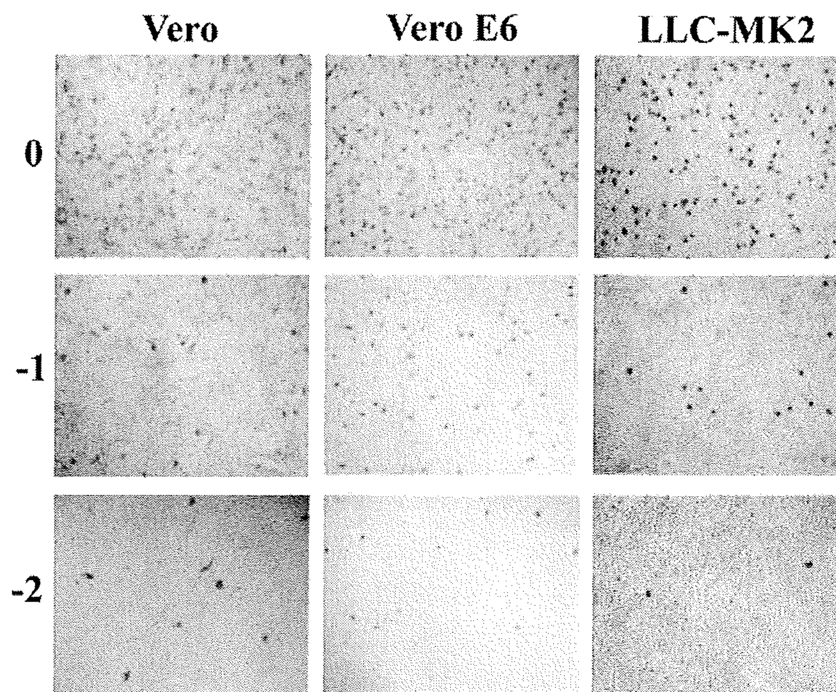

FIG. 2. Microscopic views of Vero, Vero E6, and LLC-MK2 cells infected with hMPV strain Sendai-1311-04, which was originally isolated using LLC-MK2 cells and had been passaged 10 times in the same cell line. We inoculated the cultures in parallel with 10^0, 10^{-1}, and 10^{-2} dilutions of the virus stock, fixed the culture at 24 h postinfection, and immunostained them for the viral antigen.

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MK2-passaged viruses infected 5 to 7.5 times more cells in Vero and Vero E6 cells than in LLC-MK2 cells. Examples of stained images of the three cell lines inoculated with the strain Sendai-1311-04 are shown in Fig. 2. The mean numbers of infected cells for four wells at the dilution of 10^{-2} were 121 in Vero cells, 132 in Vero E6 cells, and 26.5 in LLC-MK2 cells.

Sequence and phylogenetic analysis. As shown in Fig. 3, phylogenetic analysis revealed that 17 out of the 18 strains isolated in 2004 clustered in the B2 subgenogroup and only 1 strain in the B1 subgenogroup. In 2005, there was a mixture of three subgenogroups: 27 subgenogroup A2 strains (44.3%), 18 subgenogroup B1 strains (29.5%), and 16 subgenogroup B2 strains (26.2%). The nucleotide identities among the strains within subgenogroups A2, B1, and B2 from Yamagata were between 97 and 100%, 99 and 100%, and 98 and 100%, respectively. The nucleotide differences between the results for 2004 and 2005 were only 0 to 1% for subgenogroup B1 strains and 0 to 2% for subgenogroup B2 strains.

The monthly distribution of subgenogroups is shown in Fig. 4a. Outbreaks of hMPV subgenogroup B2 strains occurred between February and April 2004, peaking in April, whereas subgenogroup A2 and B1 strains had peaks between April and June 2005, when 21 and 13 isolates, respectively, were recovered. Ten out of 16 subgenogroup B2 viruses were isolated between July and August 2005.

Outbreaks at primary schools and nurseries. During the study period, there were two nurseries and one primary school at which we isolated more than five hMPV strains. Between 2 May and 13 June 2005, we isolated five subgenogroup A2 strains, which had 99 to 100% nucleotide identity with each other, at one primary school (Fig. 4b). At one nursery, we isolated six subgenogroup A2 strains, which had 100% identical nucleotide sequences, between 16 and 28 April 2005 (Fig. 4c). At another nursery, we isolated one subgenogroup B2 strain in April 2004, one subgenogroup A2 strain in May 2005, and eight subgenogroup B2 strains between 19 July and 10 August 2005 (Fig. 4d). The nucleotide identity among the eight subgenogroup B2 isolates in 2005 was 100%, and there was 99% identity between these isolates and the one isolate from 2004. Interestingly, we isolated a subgenogroup A2 strain in May 2005 and a subgenogroup B2 strain in August 2005 from the same 2-year-old girl.

DISCUSSION

First, we report the effectiveness of the Vero E6 cell line for the isolation of hMPV. The appearance of a recognizable hMPV CPE can take 2 or more weeks with the LLC-MK2 cell line (3, 12, 23, 35). In our experience with the Vero E6 cell line, we could identify the CPE for only six strains within 6 to 7 days after specimen inoculation whereas 2 to 4 weeks was needed to detect the CPE for the other strains. For this reason, we could not shorten the time needed to detect CPE using the Vero E6 cell line compared with that for the LLC-MK2 cell line. However, blind passage was often necessary to isolate hMPVs using the LLC-MK2 cell line after approximately 2 weeks of primary incubation (35). The merit of the Vero E6 cell line is that it forms a monolayer that can be maintained in a stable condition for a long period and enable us to observe hMPV CPE for approximately 1 month without passage or medium change. Furthermore, the infectivity assay in this study indicated that the infection efficiency of hMPV in Vero E6 cells was better

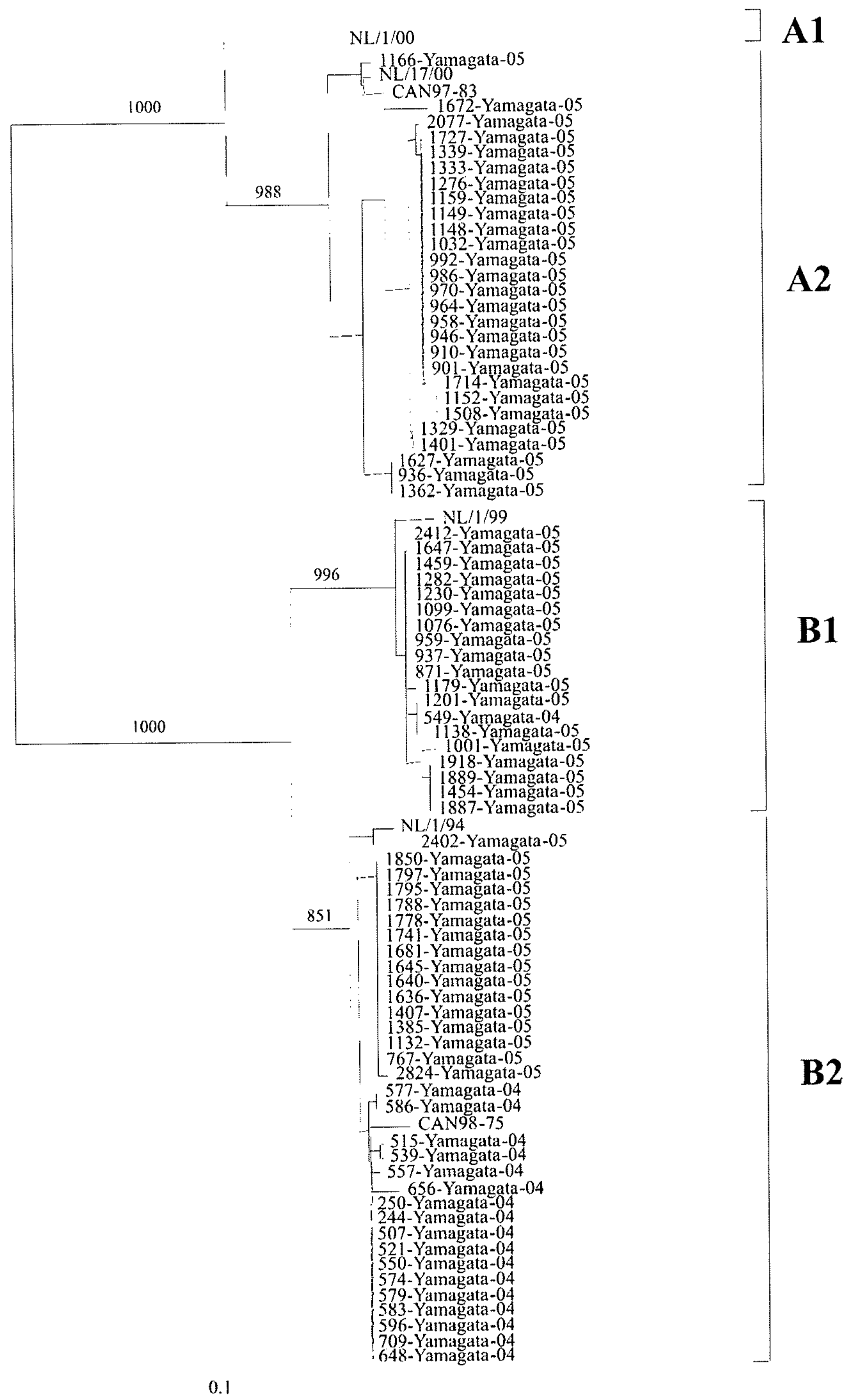

FIG. 3. Phylogenetic tree for the partial (441-bp) sequence of the fusion regions of hMPV strains isolated in Yamagata, Japan, between 2004 and 2005 as well as for the reference strains. The branch lengths are proportional to the numbers of nucleotide differences. The numbers above the branches are the bootstrap probabilities (%). The marker denotes the measurement of relative phylogenetic distance. The reference strains (CAN98-75, CAN97-83, NL/1/00, NL/17/00, NL/1/99, and NL/1/94) were based on references 25 and 32.

than that in LLC-MK2 cells. Thus, the cell line stability and the better infectivity efficiency suggested that the Vero E6 cell line is effective for the isolation of the slow-growing hMPV.

Our infectivity assay suggested that Vero cells are generally sensitive to hMPVs. van den Hoogen et al. found that Vero cell clone 118 permitted infection with viruses from all four lineages and CPEs were easily observed, whereas the CPE of prototype strain NL/1/00, which belongs to the A lineage, was more clearly observed than that of prototype strain NL/1/99, which belongs to the B lineage, on the tMK cell line (33). The findings that the Vero E6 cell line and Vero cell clone 118 possess characteristics that make them advantageous for the

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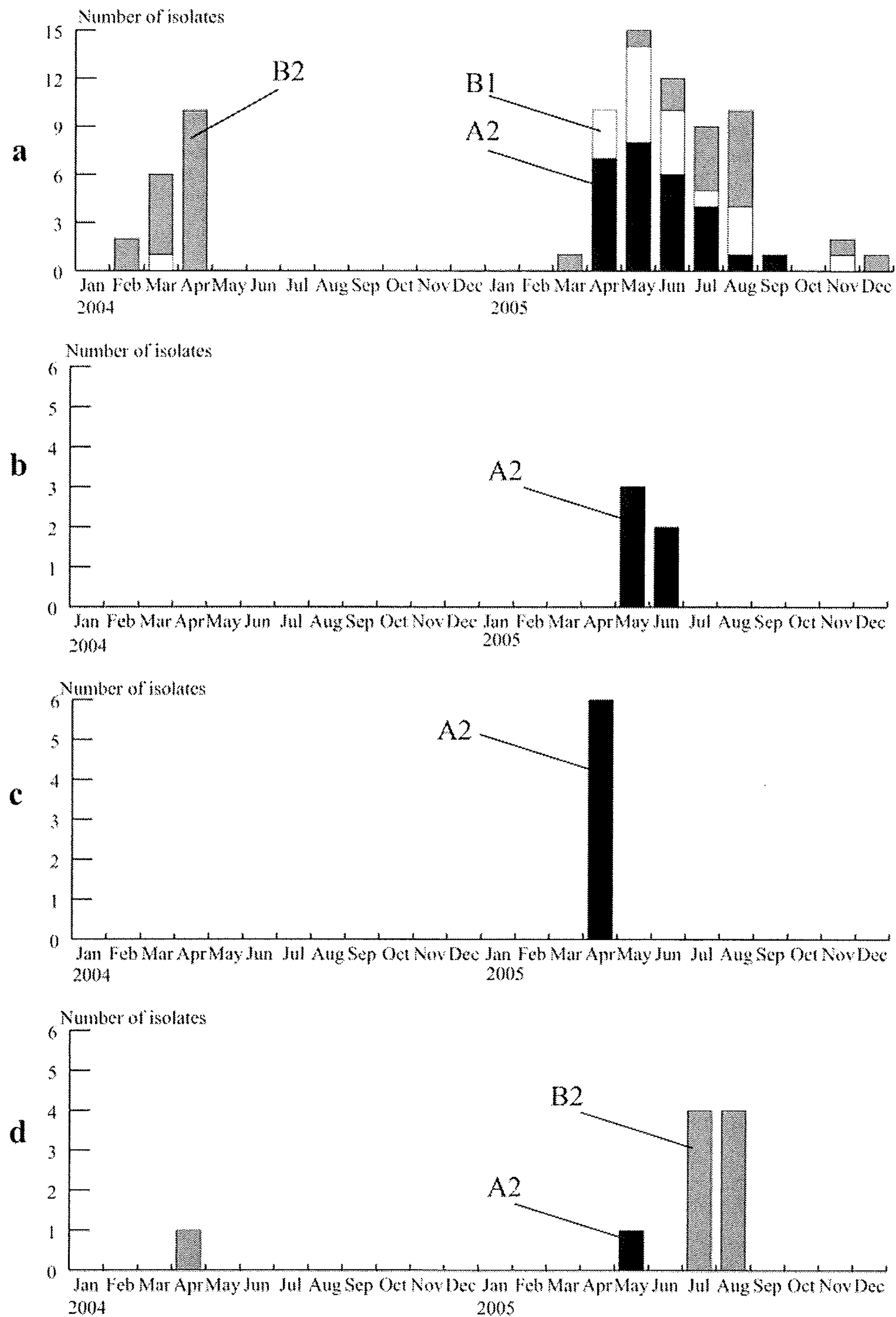

FIG. 4. Monthly distribution and subgenogroups of hMPV strains isolated in Yamagata, Japan, in 2004 and 2005. (a) Subgenogroups A2, B1, and B2 were grouped according to the phylogenetic analysis whose results are shown in Fig. 3. Monthly distributions are also shown for one primary school (b) and two nurseries (c and d) at which we isolated more than five hMPV strains.

isolation of hMPV and for the observation of its CPE suggest that some clones of the Vero cell line might be especially suitable for the isolation of hMPVs. We have not confirmed that hMPVs belonging to the A1 lineage can be replicated in the Vero E6 cell line. Since all four subgenogroups do not always appear within a 2-year period (1), we should continue to investigate whether we are able to isolate subgenogroup A1 viruses using Vero E6 cells.

hMPV has its main clinical impact in the winter months in countries with moderate climates (2, 3, 11, 30, 31, 36). However, a few reports have mentioned the detection of hMPV in summer months (7, 22, 34, 35). Although our limited results

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showed that hMPV infections occurred with a peak in April 2004 and in May 2005, we also isolated hMPVs over the summer, from June to September, in 2005. In particular, we isolated eight strains at one nursery between July and August in 2005 and five strains at one primary school between May and June 2005 (Fig. 4b and d). Therefore, the results for our virus isolation support the notion that hMPV infections occur throughout the year (35, 36).

Cocirculation of hMPV genogroups and subgenogroups has been previously reported (1, 11, 16, 36). Ludewick et al. reported that a shift in the predominant group from subgenogroup B2 to A1 was observed between 2000 and 2002 (16). Williams et al. reported that the cocirculation of multiple hMPV subgenogroups had continued for 20 years, with subgenogroups substituting from year to year (36). We also found the cocirculation of two or three subgenogroups in the Yamagata area in 2004 and 2005, as shown in Fig. 4a. Interestingly, the hMPV subgenogroup isolated from each of the three clusters at two nurseries and one primary school was specific to that place, though two or three subgenogroups had been cocirculating in Yamagata during the respective periods. These results suggest that close contact among children is an important factor in the transmission of hMPVs. Furthermore, observations from one nursery (Fig. 4a and d) suggested that hMPVs circulating within a community can enter facilities such as primary schools and nurseries.

In 1987, on the 25th anniversary of the isolation of Vero cells from African green monkey kidney tissue, Earley and Johnson suggested that Vero cells had been one of the most powerful basic resources in the entire field of virology and should continue to be a major cell substrate for virologists in years to come (9). In this paper, we report that one clone of Vero, the Vero E6 cell line, is sensitive not only to measles virus, Ebola virus, Cremian-Congo hemorrhagic fever virus, and SARS coronavirus, as has been already reported (20, 26, 27, 29), but also to hMPVs. Since only a few hMPVs have been isolated using the tissue culture method, we hope that the Vero E6 cell line might contribute to further research on hMPVs, especially toward clarification of the epidemiology and etiology of this virus.

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REFERENCES

1. **Agapov, E., K. C. Sumino, M. Gaudreault-Keener, G. A. Storch, and M. J. Holtzman.** 2006. Genetic variability of human metapneumovirus infection: evidence of a shift in viral genotype without a change in illness. J. Infect. Dis. **193:**396–403.
2. **Bastien, N., D. Ward, P. van Caeseele, K. Brandt, S. H. S. Lee, G. McNabb, B. Klisko, E. Chan, and Y. Li.** 2003. Human metapneumovirus infection in the Canadian population. J. Clin. Microbiol. **41:**4642–4646.
3. **Boivin, G., Y. Abed, G. Pelletier, L. Ruel, D. Moisan, S. Côté, T. C. T. Peret, D. D. Erdman, and L. J. Anderson.** 2002. Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory-tract infections in all age groups. J. Infect. Dis. **186:**1330–1334.
4. **Boivin, G., G. De Serres, S. Côté, R. Gilca, Y. Abed, L. Rochette, M. G. Bergeron, and P. Déry.** 2003. Human metapneumovirus infections in hospitalized children. Emerg. Infect. Dis. **9:**634–640.
5. **Chan, P. K. S., J. S. Tam, C. Lam, E. Chan, A. Wu, C. Li, T. A. Buckley, K. Ng, G. M. Joynt, F. W. T. Cheng, K. To, N. Lee, D. S. C. Hui, J. L. K. Cheung, I. Chu, E. Liu, S. S. C. Chung, and J. J. Y. Sung.** 2003. Human metapneumovirus detection in patients with severe acute respiratory syndrome. Emerg. Infect. Dis. **9:**1058–1063.
6. **Chan, P. K. S., K. To, A. Wu, G. M. K. Tse, K. Chan, S. Lui, J. J. Y. Sung, J. S. Tam, and B. Tomlinson.** 2004. Human metapneumovirus-associated atypical pneumonia and SARS. Emerg. Infect. Dis. **10:**497–500.
7. **Chano, F., C. Rousseau, C. Laferrière, M. Couillard, and H. Charest.** 2005. Epidemiological survey of human metapneumovirus infection in a large pediatric tertiary care center. J. Clin. Microbiol. **43:**5520–5525.
8. **Døllner, H., K. Risnes, A. Radtke, and S. A. Nordbø.** 2004. Outbreak of human metapneumovirus infection in Norwegian children. Pediatr. Infect. Dis. J. **23:**436–440.
9. **Earley, E. M., and K. M. Johnson.** 1988. The lineage of the Vero, Vero 76 and its clone C1008 in the United States, p. 26–29. *In* B. Simizu and T. Terasima (ed.), Vero cells—origin, properties, and biomedical applications. Department of Microbiology, School of Medicine, Chiba University, Chiba, Japan.
10. **Ebihara, T., R. Endo, H. Kikuta, N. Ishiguro, H. Ishiko, M. Hara, Y. Takahashi, and K. Kobayashi.** 2004. Human metapneumovirus infection in Japanese children. J. Clin. Microbiol. **42:**126–132.
11. **Esper, F., R. A. Martinello, D. Boucher, C. Weibel, D. Ferguson, M. L. Landry, and J. S. Kahn.** 2004. A 1-year experience with human metapneumovirus in children aged <5 years. J. Infect. Dis. **189:**1388–1396.
12. **Falsey, A. R., D. Erdman, L. J. Anderson, and E. E. Walsh.** 2003. Human metapneumovirus infections in young and elderly adults. J. Infect. Dis. **187:** 785–790.
13. **Hamelin, M., Y. Abed, and G. Boivin.** 2004. Human metapneumovirus: a new player among respiratory viruses. Clin. Infect. Dis. **38:**983–990.
14. **Ijpma, F. F. A., D. Beekhuis, M. F. Cotton, C. H. Pieper, J. L. L. Kimpen, B. G. van den Hoogen, G. J. J. van Doornum, and D. M. E. Osterhaus.** 2004. Human metapneumovirus infection in hospital referred South African children. J. Med. Virol. **73:**486–493.
15. **Landry, M. L., D. Ferguson, S. Cohen, T. C. T. Peret, and D. D. Erdman.** 2005. Detection of human metapneumovirus in clinical samples by immunofluorescence staining of shell vial centrifugation cultures prepared from three different cell lines. J. Clin. Microbiol. **43:**1950–1952.
16. **Ludewick, H. P., Y. Abed, N. van Niekerk, G. Boivin, K. P. Klugman, and S. A. Madhi.** 2005. Human metapneumovirus genetic variability, South Africa. Emerg. Infect. Dis. **11:**1074–1078.
17. **Matsuzaki, Y., K. Sugawara, E. Takashita, Y. Muraki, S. Hongo, N. Katsushima, K. Mizuta, and H. Nishimura.** 2004. Genetic diversity of influenza B virus: the frequent reassortment and cocirculation of the genetically distinct reassortant viruses in a community. J. Med. Virol. **74:**132–140.
18. **Mizuta, K., C. Abiko, H. Goto, T. Murata, and S. Murayama.** 2003. Enterovirus isolation from children with acute respiratory infections and presumptive identification by a modified microplate method. Int. J. Infect. Dis. **7:**138–142.
19. **Mizuta, K., N. Katsushima, S. Ito, K. Sanjoh, T. Murata, C. Abiko, and S. Murayama.** 2003. A rare appearance of influenza A(H1N2) as a reassortant in a community such as Yamagata where A(H1N1) and A(H3N2) co-circulate. Microbiol. Immunol. **47:**359–361.
20. **Ng, M., S. Tan, E. See, E. Ooi, and A. Ling.** 2003. Proliferative growth of SARS coronavirus in Vero E6 cells. J. Gen. Virol. **84:**3291–3303.
21. **Numazaki, Y., T. Oshima, A. Ohmi, A. Tanaka, Y. Oizumi, S. Komatsu, T. Takagi, M. Karahashi, and N. Ishida.** 1987. A microplate method for isolation of viruses from infants and children with acute respiratory infections. Microbiol. Immunol. **31:**1085–1095.
22. **Osterhaus, A., and R. Fouchier.** 2003. Human metapneumovirus in the community. Lancet **361:**890–891.
23. **Pelletier, G., P. Déry, Y. Abed, and G. Boivin.** 2002. Respiratory tract reinfections by the new human metapneumovirus in an immunocompromised child. Emerg. Infect. Dis. **8:**976–978.
24. **Percivalle, E., A. Sarasini, L. Visai, M. G. Revello, and G. Gerna.** 2005. Rapid detection of human metapneumovirus strains in nasopharyngeal aspirates and shell vial cultures by monoclonal antibodies. J. Clin. Microbiol. **43:**3443–3446.
25. **Peret, T. C. T., G. Boivin, Y. Li, M. Couillard, C. Humphrey, A. D. M. E. Osterhaus, D. D. Erdman, and L. J. Anderson.** 2002. Characterization of human metapneumoviruses isolated from patients in North America. J. Infect. Dis. **185:**2660–2663.
26. **Rota, J. S., K. B. Hummel, P. A. Rota, and W. J. Bellini.** 1992. Genetic variability of the glycoprotein genes of current wild-type measles isolates. Virology **188:**135–142.
27. **Saijo, M., T. Qing, M. Niikura, A. Maeda, T. Ikegami, C. Prehaud, I. Kurane, and S. Morikawa.** 2002. Recombinant nucleoprotein-based enzyme-linked immunosorbent assay for detection of immunoglobulin G antibodies to Crimean-Congo hemorrhagic fever virus. J. Clin. Microbiol. **40:**1587–1591.
28. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
29. **Sanchez, A., and M. P. Kiley.** 1987. Identification and analysis of Ebola virus messenger RNA. Virology **157:**414–420.
30. **Stockton, J., I. Stephenson, D. Fleming, and M. Zambon.** 2002. Human

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metapneumovirus as a cause of community-acquired respiratory illness. Emerg. Infect. Dis. **8**:897–901.
31. **van den Hoogen, B. G., J. C. de Jong, J. Groen, T. Kuiken, R. de Groot, R. A. M. Fouchier, and A. D. M. E. Osterhaus.** 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat. Med. **7**:719–724.
32. **van den Hoogen, B. G., S. Herfst, L. Sprong, P. A. Cane, E. Forleo-Neto, R. L. de Swart, A. D. M. E. Osterhaus, and R. A. M. Fouchier.** 2004. Antigenic and genetic variability of human metapneumoviruses. Emerg. Infect. Dis. **10**: 658–666.
33. **van den Hoogen, B. G., A. D. M. E. Osterhaus, and R. A. M. Fouchier.** 2004. Clinical impact and diagnosis of human metapneumovirus infection. Pediatr. Infect. Dis. J. **23**:S25–S32.
34. **van den Hoogen, B. G., G. J. J. van Doornum, J. C. Fockens, J. J. Cornelissen, W. E. P. Beyer, R. de Groot, A. D. M. E. Osterhaus, and R. A. M. Fouchier.** 2003. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. J. Infect. Dis. **188**:1571–1577.
35. **Williams, J. V., P. A. Harris, S. J. Tollefson, L. L. Halburnt-Rush, J. M. Pingsterhaus, K. M. Edwards, P. F. Wright, and J. E. Crowe, Jr.** 2004. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. N. Engl. J. Med. **350**:443–450.
36. **Williams, J. V., C. K. Wang, C. Yang, S. J. Tollefson, F. S. House, J. M. Heck, M. Chu, J. B. Brown, L. D. Lintao, J. D. Quinto, D. Chu, R. R. Spaete, K. M. Edwards, P. F. Wright, and J. E. Crowe, Jr.** 2006. The role of human metapneumovirus in upper respiratory tract infections in children: a 20-year experience. J. Infect. Dis. **193**:387–395.

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Development of a new neutralization test for measles virus

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Abstract

Sero-epidemiological studies are required to identify populations susceptible to measles. The hemagglutination inhibition (HI) test is no longer sensitive enough to confirm immunity to measles, and at present the particle agglutination (PA) test and enzyme-linked immunosorbent assay (EIA) are employed. The most reliable method is the neutralization test (NT), but it is time-consuming and requires experience. To simplify the NT, a recombinant measles AIK-C virus expressing green fluorescence protein (GFP-MVAIK) was constructed and used as a challenge virus. Plaques and cytopathic effects were visualized under ultraviolet light and detected easily, and measuring the intensity of the fluorescence enabled a reduction in the time-consuming steps. Neutralizing antibody titers of a complete inhibition neutralization test were equivalent to those of a 90% plaque reduction neutralization test. Comparison of four methods, HI, PA, EIA and the complete inhibition neutralization test, showed that only the results of EIA correlated well with those of the complete inhibition neutralization test, but sera with borderline levels by EIA were sometimes negative by the complete inhibition neutralization assay.
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Keywords: Recombinant measles virus; Neutralization test (NT); Hemagglutination inhibition (HI); Particle agglutination (PA); Enzyme-linked immunosorbent assay (EIA)

1. Introduction

Measles virus is a *morbillivirus* of the family *Paramyxoviridae*. Acute infection with measles virus starts in the upper respiratory tract and the virus spreads by secondary viremia to other organs and tissues, causing a variety of clinical symptoms. The virus infects immuno-competent cells, lymphocytes, and macrophages, and causes a transient immuno-suppressive state. Although measles infection is debilitating, the majority of patients recover completely without any serious complications (Griffin and Bellini, 2001; Strebel et al., 2004). Attenuated live measles vaccines have been developed and the number of cases of measles has decreased with the widespread acceptance of vaccinations in developed countries. Nevertheless, 0.45 million people are estimated to have died of measles or measles-related complications in 2004 (WHO, 2006). The Expanded Program on Immunization (EPI) has targeted as its highest priority the elimination of measles, but cannot attain the goal of controlling the disease because of the unexpectedly high infectivity of the virus, relatively poor immunogenicity of measles vaccines in infants under 9 months of age, and problems with the distribution of vaccines. The EPI recommends measles vaccination at 9 months of age in developing countries (Whittle et al., 1988).

Sero-epidemiological studies are needed to determine how many individuals are susceptible for the elimination of measles. The hemagglutination inhibition (HI) test is not sufficiently sensitive, and the particle agglutination (PA) test or enzyme-linked immunosorbent assay (EIA) is now employed (Neumann et al., 1985; Miyamura et al., 1997). The PA assay is a simple and sensitive method that does not require specialized equipment. Gelatin particles are coated with purified viral antigens and agglutinated by measles-specific antibodies. Titers of ≥1:16 are considered positive, but neutralizing antibodies are not detected among PA-positive sera with low titers (Miyamura et al., 1997). The most reliable test for confirming susceptibility is the neutralization

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test (NT), which is considered the "gold" standard, but is complicated and requires skill and experience (Neumann et al., 1985; Lee et al., 1999). The most time-consuming and critical part of the test is the final observation of cytopathic effects (CPE) under a microscope or the counting of plaques.

Current global efforts regarding vaccination are aimed at accelerating measles control. A sensitive and simple way to measure accurately levels of protective antibodies is needed urgently. A recombinant measles virus expressing green fluorescence protein (GFP-MVAIK) was developed in this study. The virus grew well in both Vero and B95a cells. Green fluorescence protein (GFP) was expressed simultaneously with measles antigens, and the growth of the virus was detected by fluorescent intensity. Using this virus as a challenge virus in the neutralization test could automate the final step in the test systems.

2. Materials and methods

2.1. Construction of recombinant cDNA of measles virus

A recombinant measles virus was rescued from full-length cDNA by reverse genetics (Schneider et al., 1997; Takeda et al., 2000; Nakayama et al., 2001; Kumada et al., 2004). An *Asc* I site was introduced between the phosphoprotein (P) and membrane (M) protein region, adding the GGCGCG sequence at position 3432 of the AIK-C genome (Mori et al., 1993). The cloned GFP genome was inserted at the Asc I site and the recombinant plasmid was co-transfected with N, P, and L expression helper plasmids under the control of T7 RNA polymerase into B95a cells. Infectious virus (GFP-MVAIK) was recovered after two passages in B95a cells. In this study, GFP-MVAIK was used instead of the Edmonston strain, a parental strain of the AIK-C vaccine used in the conventional neutralization test.

2.2. Serum samples

Sera were obtained every year from students in the first and fourth grades of a private primary school in Tokyo for a health assessment. One hundred and thirty-two pupils enter the school each year. The purpose of the study was explained to the parents and written informed consent was obtained from all participants. Each subject's history of measles immunization and past history of natural infection were confirmed through immunization records. The sera were stored at −20 °C.

2.3. Serological tests

The HI antibody titers of test sera against measles virus were examined. Serum samples were treated with 25% kaolin in 1/15 M phosphatebuffered saline to remove nonspecific factors. The supernatant was mixed with 10% green monkey red blood cells to remove nonspecific factors for agglutination and at this final stage serum samples were diluted 1:8. Serum samples were diluted serially two-fold and four units of hemagglutinin antigen was added. After 0.5% green monkey red blood cells were added, the reciprocal of the dilution which completely inhibited hemagglutination was taken as the HI antibody titer. Sera with a HI titer of ≥1:8 were considered HI-positive.

In the PA test, gelatin particles were coated with purified measles virus antigen (Serodia®-Measles, Fuji Rebio, Tokyo, Japan). Sera were diluted serially two-fold starting at a dilution of 1:8, and each diluted serum was mixed with an equal volume of gelatin particles to detect agglutination, according to the recommendation of the manufacturer. The PA antibody titers were expressed as the reciprocal of the serum dilution which induced agglutination. Sera with a PA titer of ≥1:16 were considered positive.

Purified viral particle antigens were coated for EIA on a 96-well plate and 100 μl of serum diluted 1:200 was added to each well (Measles IgG (II)-EIA "Seiken", Denka Seiken, Tokyo, Japan). After incubation with the serum sample for 60 min, anti-human IgG antibody conjugated with peroxidase was added and stained, as recommended in the instruction manual. The EIA antibody titer was expressed as the ratio of absorbance of the serum sample to that of the weak positive control reference serum supplied by the manufacturer. Sera with an EIA titer of ≥1 were considered EIA-positive.

Sera were treated for the neutralization test at 56 °C for 30 min to inactivate the complement. Fifty microliters of diluted serum was mixed with an equal volume of challenge virus containing approximately 50 PFU for the plaque reduction NT. After neutralization at 37 °C for 90 min in a 5% CO_2 incubator, the mixture was placed on a monolayer of Vero cells at 37 °C for 60 min in a 24-well plate, and overlaid with MEM containing 0.5% agarose. The plate was kept at 37 °C for 7 days in 5% CO_2. Neutralization titers were determined by calculating the serum dilution that reduced plaque numbers by 50 or 90%. Sera with a neutralization titer of ≥1:4 were considered NT-positive.

In the complete inhibition NT, sera were diluted serially two-fold, starting at an initial dilution of 1:4. Twenty-five microliter of each dilution was then mixed with an equal volume of the challenge virus approximately 100 TCID 50 and the mixture was kept at 37 °C for 90 min in 5% CO_2. The mixture was placed subsequently on a monolayer of B95a cells in a 96-well plate for complete inhibition of the cytopathic effects. The plates were kept at 37 °C for 7 days in 5% CO_2 and the neutralization titers were taken as the reciprocal of the dilution that induced complete inhibition of the appearance of CPE in B95a cells in a 96-well plate.

In order to calculate the titers automatically, the plates were processed to detect fluorescence intensity (Fluoro-Units: FU) at an emission wavelength of 528 nm and excitation wavelength of 485 nm using a fluorescence reader, FLx800 (BIO-TEK Instruments Inc., Vermont, USA).

3. Results

3.1. Expression of GFP and measles antigens

The recombinant AIK-C virus expressing GFP (GFP-MVAIK) grew well in both B95a and Vero cells. GFP was visualized in the cells infected with GFP-MVAIK with a fluorescence microscope. Measles antigens were stained by polyclonal

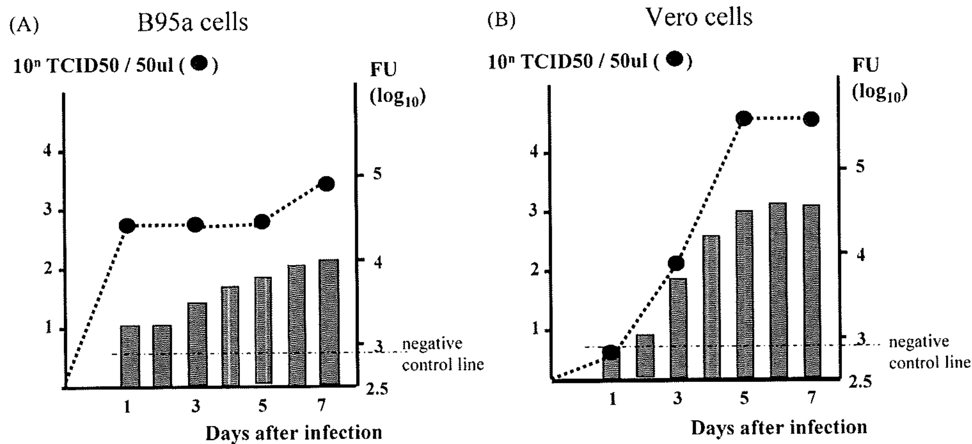
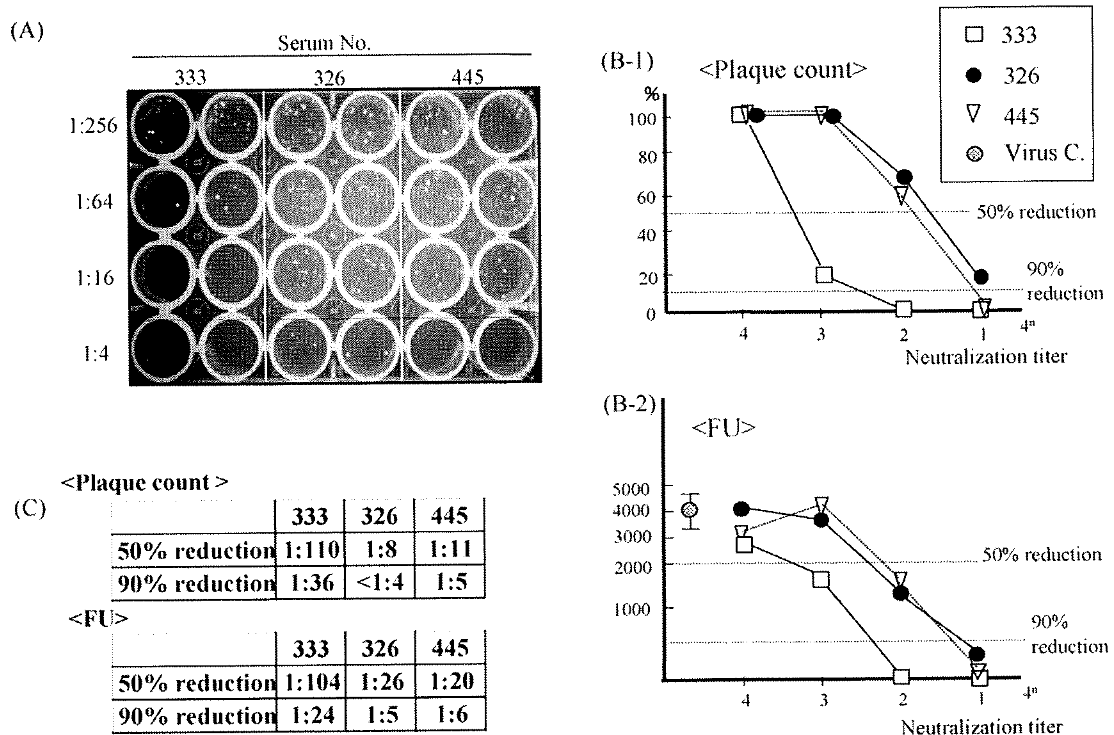


Fig. 1. Viral growth and the expression of GFP in B95a cells and Vero cells infected with GFP-MVAIK. B95a (A) or Vero cells (B) were infected with GFP-MVAIK at a moi of 0.01. Culture fluid was obtained on days 1, 3, 5, and 7 of infection, and the infectivity was examined in B95a cells. The infectivity is shown as 10^n TCID50/50 μl (closed circles). The dotted column shows the intensity in FU. The value in the control well was approximately 800 FU ($10^{2.9}$ FU) as a negative control.

antibodies against measles virus at the same locations GFP was observed under the microscope (data not shown). The correlation between the intensity of GFP and growth of the virus is shown in Fig. 1. GFP-MVAIK grew better in B95a cells than in Vero cells up to day 3 of the infection, but on day 5 or 7, growth was greater in the Vero cells. The kinetics of the GFP expression were similar to the viral growth both in B95a and in Vero cells.

3.2. Comparison of the neutralization titers assayed by conventional and new methods

The results of the conventional plaque NT are shown in Fig. 2. GFP-MVAIK was used as a challenge virus and neutralization titers of several sera were examined. The results for three samples (Nos. 326, 333, and 445) are shown. The assay plate was examined under ultraviolet light, and plaques were visualized as white spots. After the plaques were enumerated in virus control wells and at each dilution, a reduction curve was drawn, from which the theoretical titer of a 50% reduction in the plaque count was calculated (Fig. 2, B-1). The titers of sera Nos. 333, 326, and 445 were calculated as 1:110, 1:8, and 1:11, respectively. Based on a 90% reduction in the plaque count, the results were 1:36, negative, and 1:5, respectively (Fig. 2C). The intensity of the fluorescence in each well was also examined. The mean value for cell control wells was subtracted from the value for virus control wells, and in this experiment, the difference in intensity



<Plaque count >

	333	326	445
50% reduction	1:110	1:8	1:11
90% reduction	1:36	<1:4	1:5

<FU>

	333	326	445
50% reduction	1:104	1:26	1:20
90% reduction	1:24	1:5	1:6

Fig. 2. The plaque reduction NT using Vero cells and GFP-MVAIK. Three sera were used. (A) Plaques expressing GFP were visualized using ultraviolet light. (B-1) Reduction in terms of the number of plaques. (B-2) Reduction in terms of Fluoro-Units (FU). (C) Neutralizing antibody titers based on 50 and 90% reductions.

was 3897 ± 544 Fluoro-Units. From this difference, 50% and 90% reduction levels were calculated to be approximately 1950 and 400 FU, respectively. A reduction line was drawn based on the results for serial dilutions and the neutralization titer showing 1950 FU (50% reduction) and 400 FU (90% reduction) was taken as the value at the point where the lines were crossed (Fig. 2B-2). Neutralization titers were 1:104, 1:26, and 1:20 based on a 50% reduction in FU, and 1:24, 1:5, and 1:6 for a 90% reduction in FU (Fig. 2C).

In another NT for measles virus, neutralization titers were calculated as 100% inhibition of CPE in B95a cells. The results are shown in Fig. 3. CPE was visualized as a patchy region. Neutralization titers were expressed as the reciprocal of the serum dilutions that completely prevented the appearance of CPE. The mean values for the cell control wells were 658, 674, and 680 FU. CPE was not observed at a 1:8 dilution of Serum No. 445 in duplicate wells, and a 1:16 dilution of serum did not neutralize GFP-MVAIK, the mean value being 1528 FU. Thus, the neutralization titers for Nos. 445, 333, and 326 were 1:8, 1:32, and 1:4, respectively. The antibody titers determined by the complete inhibition of CPE were similar to those obtained based on a 90% reduction in plaque numbers or in FU of GFP expression. The NT for complete inhibition of CPE is simple and the neutralization titers are calculated automatically by a fluorescence microplate reader.

In most laboratories, the wild-type Edmonston strain is used for NT. The AIK-C vaccine strain was attenuated from the wild-type Edmonston strain (genotype A). The difference in neutralization titers using the Edmonston and GFP-MVAIK strains as the challenge virus was compared in 43 sera. There was no significant difference in titers in 38 sera. Five sera had four-fold lower titers by the NT using Edmonston than that using GFP-MVAIK. The correlation coefficient of neutralization titers against these two viruses was 0.936 ($p < 0.001$).

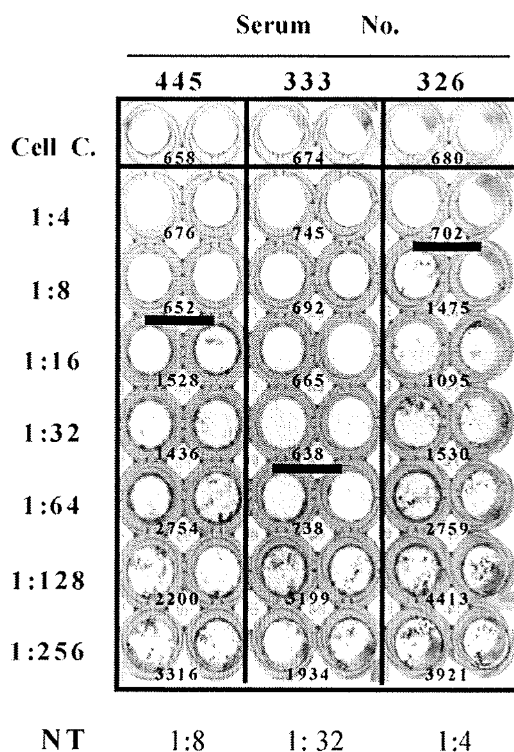


Fig. 3. The complete inhibition NT using B95a cells and GFP-MVAIK. CPE were visualized through GFP expression. Numbers between two wells demonstrate the mean value in Fluoro-Units (FU) for duplicated wells. Neutralizing antibody titers for Nos. 445, 333, and 326 were determined as 1:8, 1:32, and 1:4 based on the complete inhibition of CPE.

3.3. *Comparison of HI, PA, EIA, and NT tests*

Two hundred and thirty-seven serum samples were obtained from pupils in the first and fourth grades in 2002. About 98% of the students had been immunized against measles. With these

(A) HI

HI	<2	2	3	4	5	6	7	≥ 8
512x								1
256x								
128x								3
64x								
32x								5
16x						1	7	14
8x						15	12	6
<8x	8	2	22	24	55	46	15	1

NT 2^n

(B) PA

PA	<2	2	3	4	5	6	7	≥ 8
8192x							1	11
4096x						1	8	5
2048x			1		8	18	6	11
1024x			1	3	18	22	14	2
512x			5	10	24	17	5	1
256x		1	8	7	4	4		
128x	1		6	1	1			
64x	1			3				
32x	1	1						
16x								
<16x	5		1					

NT 2^n

(C) EIA

EIA	<2	2	3	4	5	6	7	≥ 8
≧ 6					4	8	12	24
5					7	11	9	4
4				2	12	17	9	
3			2	6	22	11	4	1
2	1	1	8	10	9	14		1
1.5-2			5	2	1			
1-1.5			6	4		1		
±	1	1	1					
—	6							

NT 2^n

Fig. 4. Comparison of antibody titers assayed by different methods. (A) Comparison of antibody titers between NT and HI. (B) Comparison of antibody titers between NT and PA. (C) Comparison of antibody titers between NT and EIA.

Table 1
Distribution of neutralizing antibody titers of sera with PA antibodies less than or equal to 1:128

PA titers	Neutralization titers (2^n)					
	<2	2	3	4	5	6
≤16 (n = 20)	19		1			
32 (n = 2)	1	1				
64 (n = 15)	7	3	1	3		1
128 (n = 32)	6	8	11	4	3	
Total	33	12	13	7	3	1

sera, serological tests were carried out using HI, PA, the EIA, and the NT. The results are shown in Fig. 4. The correlation between HI and the NT is shown in Fig. 4(A). Only 64 samples were HI-positive (27.0%), whereas 229 samples were NT-positive (96.6%). The correlation between PA and the NT is shown in Fig. 4(B). The PA test was started at a dilution of 1:16 and sera with a PA titer of ≥1:16 were considered positive. All 216 sera with PA titers equal to or higher than 1:256 were NT-positive but some sera with PA titers less than 1:256 were NT-negative. For a more thorough analysis, 48 sera with PA titers of less than 1:256 obtained from junior and senior high school students in 2002 were added. The results for the 69 sera are shown in Table 1. Six (18.8%) of 32 sera with a PA titer of 1:128 were NT-negative, and 7 (46.7%) of 15 sera with a PA titer of 1:64 were NT-negative. The correlation between the EIA and NT is shown in Fig. 4(C). A good correlation was observed between EIA and NT, and most EIA-positive sera were NT-positive. Three sera with borderline levels of EIA (±) showed a low neutralization titer (1:4 and 1:8) and one of the three was NT-negative.

4. Discussion

In Japan, the cumulative measles vaccine coverage was 80% by 90 months of age, but the actual coverage during the most susceptible period, around 1 year of age, was less than 60% (Nakayama et al., 2003). Regional pediatric associations have launched a campaign promoting measles vaccination. At present, vaccine coverage has increased to 90% by 90 months of age. The two-dose strategy was introduced in 2006.

Most measles patients are diagnosed clinically and WHO has recommended the detection of measles-specific IgM antibodies in acute phase serum samples for the diagnosis of measles (WHO, 2003). It is important to carry out serological studies that can measure levels of protective antibody accurately to determine how many people are susceptible to measles, especially for promoting vaccination. Most studies employed the HI test which was believed to be sensitive enough to detect immunity. In the present study, 165 (95.4%) of 173 HI-negative sera were NT-positive, thus the HI test is not suitable for epidemiological studies. The PA test is highly sensitive and useful for epidemiological studies. The cut-off titer was thought to be 1:16, but some sera with a PA titer of ≤1:64 were NT-negative and all sera with a PA titer of ≥1:128 were NT-positive (Miyamura et al., 1997). In this study, 6 (18.8%) of 32 sera at 1:128 were NT-negative. At ≥1:256 in PA, all sera were NT-positive. In contrast, EIA was related more closely to the NT. The EIA test has been used in sero-epidemiological studies (Ratnam et al., 1995; Lee et al., 1999; van den Hof et al., 2003). But sera with borderline levels in the EIA were thought to be negative or have low neutralization titers. PA or EIA antibodies do not always correlate with NT activity. The antibodies detected in the NT are considered mainly anti-H and -F antibodies, which are the most important factors for preventing a measles infection. In PA or EIA, purified viral particle antigens are used which contain some components other than H and F antigens. To determine which populations are susceptible to measles, the neutralizing antibody titer should be checked, especially in the sera with a PA titer of ≤1:128 or borderline levels in the EIA. Recently, de Swart et al. (2005) reported that measles virus neutralizing antibodies were directed mainly against the H protein. Bouche et al. (1998) reported that EIA titers against an H protein antigen prepared from crude membrane preparations of H protein-transfected BHK-21 cells correlated more closely with results of the NT and HI than those of whole-virus-based EIA.

Two different forms of the NT were used in this study, a conventional plaque reduction assay using Vero cells and a complete inhibition assay using B95a cells. The complete inhibition assay is a simpler procedure than the plaque reduction assay. Ward et al. (1999) reported that the 50% plaque reduction assay was the "gold standard" and correlated well with the complete inhibition assay when neutralization titers were moderate or high, but there were discrepancies in sera with low levels of neutralizing antibodies. NT-negative sera by the complete inhibition assay showed various titers in the 50% plaque reduction assay. In the present study, results obtained by the complete inhibition assay were equivalent to a 90% reduction in plaques by the conventional assay. The complete inhibition assay is more reliable when considering levels of protection from measles in vivo, especially in the controllable stage of infection. However, the current plaque reduction NT is not suitable for large-scale serological studies because it is cumbersome and requires experience. The new method described in this study overcomes these problems by measuring the intensity of fluorescence automatically and makes it possible to test with many samples.

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References

Bouche, F., Ammerlaan, W., Berthet, F., Houard, S., Schneider, F., Muller, C.P., 1998. Immunosorbent assay based on recombinant hemagglutinin protein produced in a high-efficiency mammalian expression system for surveillance of measles immunity. J. Clin. Microbiol. 36, 721–726.

de Swart, R.L., Yuksel, S., Osterhaus, A.D., 2005. Relative contributions of measles virus hemagglutinin- and fusion protein-specific serum antibodies to virus neutralization. J. Virol. 79, 11547–11551.

Griffin, D.E., Bellini, W.J., 2001. Measles virus. In: Knipe, D.M., Howley, P.M. (Eds.), Fields virology, fourth ed. Williams & Wilkins, Lippincott, Philadelphia, pp. 1267–1312.

Kumada, A., Komase, K., Nakayama, T., 2004. Recombinant measles AIK-C strain expressing current wild-type hemagglutinin protein. Vaccine 22, 309–316.

Lee, M.S., Cohen, B., Hand, J., Nokes, D.J., 1999. A simplified and standardized neutralization enzyme immunoassay for the quantification of measles neutralizing antibody. J. Virol. Methods 78, 209–217.

Miyamura, K., Sato, T.A., Sakae, K., Kato, N., Ogino, T., Yashima, T., Sasagawa, A., Chikahira, M., Itagaki, A., Katsuki, K., Matsunaga, Y., Utagawa, E., Takeda, N., Inouye, S., Yamazaki, S., 1997. Comparison of gelatin particle agglutination and hemagglutination inhibition tests for measles seroepidemiology studies. Arch. Virol. 142, 1963–1970.

Mori, T., Sasaki, K., Hashimoto, H., Makino, S., 1993. Molecular cloning and complete nucleotide sequence of genomic RNA of the AIK-C strain of attenuated measles virus. Virus Genes 7, 67–81.

Nakayama, T., Zhou, J., Fujino, M., 2003. Current status of measles in Japan. J. Infect. Chemother. 9, 1–7.

Nakayama, T., Komase, K., Uzuka, R., Hoshi, A., Okafuji, T., 2001. Leucine at position 278 of the AIK-C measles virus vaccine strain fusion protein is responsible for reduced syncytium formation. J. Gen. Virol. 82, 2143–2150.

Neumann, P.W., Weber, J.M., Jessamine, A.G., O'Shaughnessy, M.V., 1985. Comparison of measles antihemolysin test, enzyme-linked immunosorbent assay, and hemagglutination inhibition test with neutralization test for determination of immune status. J. Clin. Microbiol. 22, 296–298.

Ratnam, S., Gadag, V., West, R., Burris, J., Oates, E., Stead, F., Bouilianne, N., 1995. Comparison of commercial enzyme immunoassay kits with plaque reduction test for detection of measles virus antibody. J. Clin. Microbiol. 33, 811–815.

Schneider, H., Spielhofer, P., Kaelin, K., Dotsch, C., Radecke, F., Sutter, G., Billeter, M.A., 1997. Rescue of measles virus using a replication-deficient vaccinia-T7 vector. J. Virol. Methods 64, 57–64.

Strebel, P.M., Papania, M.J., Halsey, N.A., 2004. Measles vaccine. In: Plotkin, S.A., Orenstein, W.A. (Eds.), Vaccine, fourth ed. Saunders, Philadelphia, pp. 389–440.

Takeda, M., Takeuchi, K., Miyajima, N., Kobune, F., Nagata, N., Suzuki, Y., Tashiro, M., 2000. Recovery of pathogenic measles virus from cloned cDNA. J. Virol. 74, 6643–6647.

van den Hof, S., van Gageldonk-Lafeber, A.B., van Binnendijk, R.S., van Gageldonk, P.G., Berbers, G.A., 2003. Comparison of measles virus-specific antibody titers as measured by enzyme-linked immunosorbent assay and virus neutralization assay. Vaccine 21, 4210–4214.

Ward, B.J., Aouchiche, S., Martel, N., Bertley, F.M.N., Bautista-Lopez, N., Serhir, B., Ratna, m.S., 1999. Measurement of measles virus-specific neutralization of the syncytium inhibition assay in comparison with the plaque reduction neutralization test. Diagn. Microbial. Infect. Dis. 33, 147–152.

Whittle, H.C., Mann, G., Eccles, M., O'Neill, K., Jupp, L., Hanlon, P., Hanlon, L., Marsh, V., 1988. Effects of dose and strain of vaccine on success of measles vaccination of infants aged 4–5 months. Lancet 30, 963–966.

WHO, 2003. WHO-recommended surveillance standard of measles. WHO-recommended standards for surveillance of vaccine-preventable diseases, 13–17.

WHO, 2006. Progress in reducing global measles deaths: 1999-2004. Wkly. Epidemiol. Rec. 81, 90–94.

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Identification and Characterization of Two Strains of Human Parechovirus 4 Isolated from Two Clinical Cases in Fukuoka City, Japan[▽]

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Reverse transcription-PCR targeting the VP0 gene of human parechoviruses (HPeVs) was used to identify two isolates from two Japanese children's stool specimens. Molecular analysis revealed that these isolates belonged to HPeV type 4, and their nucleotide identity in the P1 region was 85.0%.

Human parechoviruses (HPeVs), members of the *Parechovirus* genus of the *Picornaviridae* family, are genetically classified into six types. HPeVs have been associated with gastrointestinal and respiratory symptoms and also with severe symptoms such as transient paralysis and neonatal sepsis in young children (3, 5). HPeV type 1 (HPeV1) and HPeV3 infections are common worldwide (5, 6); however, other HPeV infections, especially HPeV4 infections, are less common. HPeV4 strains have been isolated from young children with fever, TORCH syndrome, and lymphadenitis in a few instances (2, 10, 11).

This paper describes the identification and characterization of HPeV4 strains using molecular and immunological techniques. In 2001 and 2005, we isolated viruses from stool specimens from a 1-year-old girl with herpangina and from a 3-year-old boy with acute gastroenteritis, respectively, in Fukuoka City, Japan. The stool specimens were cultured weekly at 37°C for three passages on Caco-2, RD-18S, VeroE6, and HEp-2 cells. *Picornaviridae*-like cytopathic effects were observed in the Caco-2, RD-18S, and VeroE6 cell lines. To identify these isolates, a molecular typing method based on reverse transcription-PCR (RT-PCR) and direct sequencing was carried out. Viral RNAs were extracted from cell culture supernatants by using a QIAamp viral RNA mini kit (Qiagen, Germany). RT-PCRs were performed with specific primers (187/011, 188/011, and 189/011 for the enterovirus VP1 gene and E23P1/HPV-N1 for the HPeV VP0 gene) (5, 9).

Table 1 shows the molecular typing results from the cell culture samples. In case 1, the Caco-2 and RD-18S cell culture samples were positive by RT-PCR for enterovirus, and human coxsackievirus group A type 2 was identified by direct sequencing of the VP1 amplicon. In both case 1 and 2, all of the Caco-2, RD-18S, and VeroE6 culture samples were positive for HPeV by RT-PCR. The VeroE6 culture sample from case 1 and the Caco-2 culture sample from case 2 were designated Fuk2001-282 and Fuk2005-123, respectively. These amplicon sequences were compared with sequences from HPeV reference strains HPeV1 Harris, HPeV2 Williamson, HPeV3 A308-99, HPeV4 K251176-02, HPeV4 T75-4077, HPeV5 T92-15, and HPeV6 NII561-2000.

Both Fuk2001-282 and Fuk2005-123 showed high similarities to the VP0 gene of the HPeV4 reference strains (positions 710 to 1,339 on HPeV1 Harris); the nucleotide identities of Fuk2001-282 to HPeV4 K251176-02 and HPeV4 T75-4077 were 84.9% and 89.5%, respectively, and those of Fuk2005-123 to K251176-02 and T75-4077 were 85.4% and 85.2%, respec-

TABLE 1. Results of molecular typing based on RT-PCR and direct sequencing[a]

Case	Age (yr)	Sex	Date of onset	Clinical diagnosis	Specimen	Cell culture	Molecular typing result		Strain
							Enterovirus	HPeV	
1	1	F	September 2001	Herpangina	Stool	Caco-2	CA2	HPeV4	Fuk2001-282
						RD-18S	CA2	HPeV4	
						VeroE6		HPeV4	
2	3	M	December 2005	Acute gastroenteritis	Stool	Caco-2		HPeV4	Fuk2005-123
						RD-18S		HPeV4	
						VeroE6		HPeV4	

[a] F, female; M, male; CA2, human coxsackievirus group A type 2.

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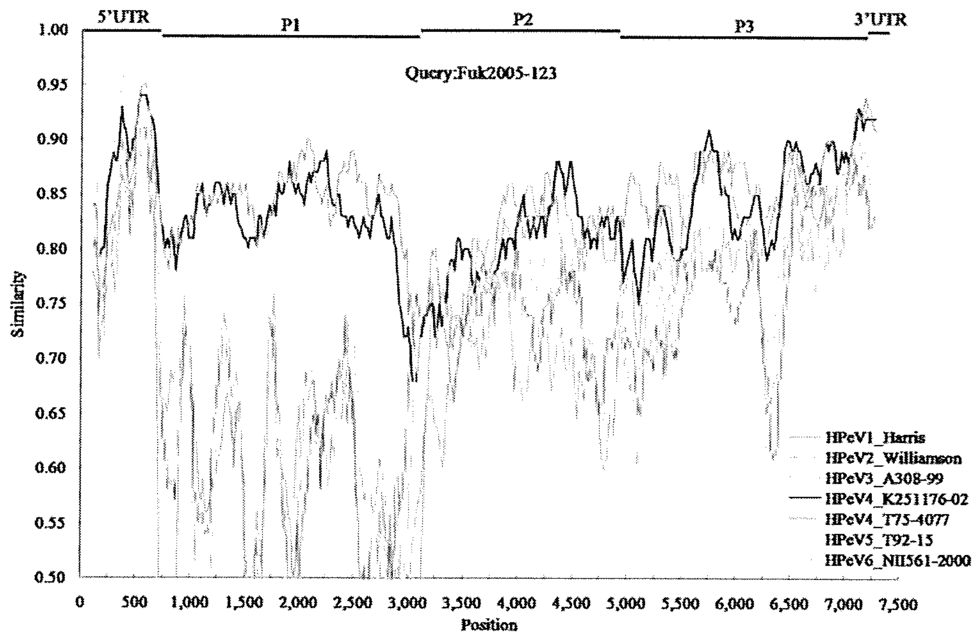

FIG. 1. SimPlot analysis of HPeVs against Fuk2005-123. The nucleotide sequences of the reference strains were obtained from the GenBank database (GenBank accession numbers are in parentheses): HPeV1 Harris (L02971), HPeV2 Williamson (AJ005695), HPeV3 A308-99 (AB084913), HPeV4 K251176-02 (DQ315670), HPeV4 T75-4077 (AM235750), HPeV5 T92-15 (AM235749), and HPeV6 NII561-2000 (AB252582).

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tively. The identity between Fuk2001-282 and Fuk2005-123 was 85.4%; therefore, these strains were not genetically identical (data not shown).

The full-length nucleotide sequence of the Fuk2005-123 strain was determined by using a primer-walking strategy. The complete genome of Fuk2005-123 was 7,349 nucleotides long, containing an open reading frame of 6,549 nucleotides. HPeV1, -2, -4, -5, and -6 contain RGD (arginine-glycine-aspartic acid) motifs, which are known to associate with cell attachment (4, 5). Fuk2005-123 also contained an RGD motif at the C terminus of the VP1 gene.

A SimPlot analysis of complete sequences of the reference HPeV strains against the sequence of the Fuk2005-123 strain was performed to analyze genetic relationships and recombination events between HPeVs (Fig. 1) (8). Nucleotide sequence alignments were generated with MEGA software, version 3.1 (7), and the plots were created with SimPlot software, version 3.5.1. The plots for the P1 region show that Fuk2005-123 is relatively closer to the HPeV4 reference strains than to the HPeV1, -2, -3, -5, and -6 reference strains, whereas in the P2, P3, and untranslated regions, the identities between Fuk2005-123 and the reference strains vary. Therefore, the P1 region is suitable for comparing nucleotide identities among HPeVs. Recent studies reveal that recombination plays a role in the evolution of HPeVs (1); however, the recombination breakpoint of Fuk2005-123 was not detected.

A phylogenetic tree based on the P1 region nucleotide sequences of Fuk2001-282, Fuk2005-123, and the HPeV reference strains was constructed by the neighbor-joining method using MEGA (Fig. 2). These strains cluster into six groups; Fuk2001-282 and Fuk2005-123 belong to the HPeV4 group. Fuk2001-282 is closely related to the HPeV4 NII370-93 refer-

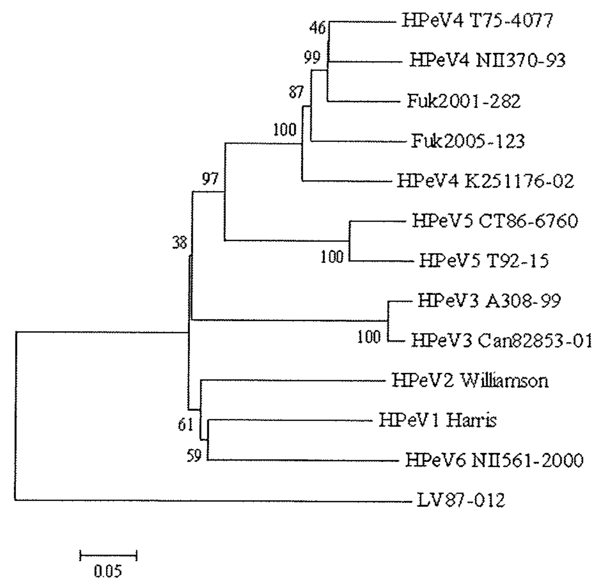

FIG. 2. Phylogenetic tree based on nucleotide sequences of the P1 region showing the relationship between Fuk2001-282, Fuk2005-123, HPeV reference strains (HPeV1 Harris [L02971], HPeV2 Williamson [AJ005695], HPeV3 A308-99 [AB084913], HPeV3 Can82853-01 [AJ889918], HPeV4 K251176-02 [DQ315670], HPeV4 T75-4077 [AM235750], HPeV4 NII370-93 [AB434673], HPeV5 CT86-6760 [AF055846], HPeV5 T92-15 [AM235749], and HPeV6 NII561-2000 [AB252582]), and Ljungan virus (LV87-012 [AF020541]) (GenBank accession numbers are in square brackets). The bootstrap values are shown for the major nodes as a percentage of the data with 1,000 bootstrap replicates. The scale bar represents the number of nucleotide substitutions per site.

TABLE 2. Comparisons between the P1 region of the Fuk2001-282 strain, the Fuk2005-123 strain, and the HPeV4 reference strains

Strain	% Nucleotide identity (% amino acid identity)[a]				
	Fuk2001-282	Fuk2005-123	HPeV4 K251176-02	HPeV4 T75-4077	HPeV4 NII370-93
Fuk2001-282	100 (100)				
Fuk2005-123	85.0 (97.7)	100 (100)			
HPeV4 K251176-02	84.2 (97.9)	84.6 (97.8)	100 (100)		
HPeV4 T75-4077	88.0 (97.8)	85.8 (97.6)	85.4 (97.6)	100 (100)	
HPeV4 NII370-93	87.6 (97.9)	84.4 (97.4)	84.5 (97.6)	88.3 (97.6)	100 (100)

[a] GenBank accession numbers (in parentheses) are as follows: HPeV4 K251176-02 (DQ315670), HPeV4 T75-4077 (AM235750), and HPeV4 NII370-93 (AB434673).

ence strain, which was isolated from a 5-year-old Japanese boy with lymphadenitis in 1993 (11). The P1 region nucleotide and amino acid identities between the Fuk2001-282 strain, the Fuk2005-123 strain, and the HPeV4 reference strains were 84.2 to 88.3% and 97.4 to 97.9%, respectively (Table 2); these results indicate that Fuk2001-282 and Fuk2005-123 are HPeV4 strains. The nucleotide identity between Fuk2001-282 and Fuk2005-123 was only 85.0%, although these strains were isolated in the same region within only four years of each other.

To identify HPeV4 by immunological assay, neutralization tests were performed using a specific antiserum against the Fuk2005-123 strain (12). The antiserum was obtained from a rabbit that had been injected subcutaneously with purified strain Fuk2005-123 at the Nippon Biotest Laboratories (Japan) (11). The antiserum against Fuk2005-123 did not cross-react with HPeV1, -2, -3, and -6 strains and neutralized both the Fuk2001-282 and Fuk2005-123 strains (data not shown).

In our infectious disease surveillance, two HPeV4 strains, Fuk2001-282 and Fuk2005-123, were isolated in Fukuoka City. HPeV4 strains have rarely been isolated in Japan; the first isolation was described by Watanabe et al. (11), and this report is the second. The results of our molecular study demonstrate that the Fuk2001-282 and Fuk2005-123 strains are genetically distinct; however, it is uncertain whether these strains are indigenous or nonindigenous to Fukuoka City. In the future, a seroepidemiological study should be undertaken to understand the epidemiology of HPeV4 infection in Japan.

Nucleotide sequence accession numbers. The full-length sequence of Fuk2005-123 and the partial sequence of the P1 region of Fuk2001-282 have been deposited in GenBank under accession numbers AB433629 and AB433630, respectively.

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REFERENCES

1. **Al-Sunaidi, M., Ç. H. Williams, P. J. Hughes, D. P. Schnurr, and G. Stanway.** 2007. Analysis of a new human parechovirus allows the definition of parechovirus types and the identification of RNA structural domains. J. Virol. **81:**1013–1021.
2. **Benschop, K. S. M., J. Schinkel, M. E. Luken, P. J. M. van den Broek, M. F. C. Beersma, N. Menelik, H. W. M. van Eijk, H. L. Zaaijer, C. M. J. E. VandenBroucke-Grauls, M. G. H. M. Beld, and K. C. Wolthers.** 2006. Fourth human parechovirus serotype. Emerg. Infect. Dis. **12:**1572–1575.
3. **Benschop, K. S. M., J. Schinkel, R. P. Minnaar, D. Pajkrt, L. Spanjerberg, H. C. Kraakman, B. Berkhout, H. L. Zaaijer, M. G. H. M. Beld, and K. C. Wolthers.** 2006. Human parechovirus infections in Dutch children and the association between serotype and disease severity. Clin. Infect. Dis. **42:**204–210.
4. **Boonyakiat, Y., P. J. Hughes, F. Ghazi, and G. Stanway.** 2001. Arginine-glycine-aspartic acid motif is critical for human parechovirus 1 entry. J. Virol. **75:**10000–10004.
5. **Ito, M., T. Yamashita, H. Tsuzuki, N. Takeda, and K. Sakae.** 2004. Isolation and identification of a novel human parechovirus. J. Gen. Virol. **85:**391–398.
6. **Joki-Korpela, P., and T. Hyypiä.** 1998. Diagnosis and epidemiology of echovirus 22 infections. Clin. Infect. Dis. **26:**129–136.
7. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5:**150–163.
8. **Lole, K. S., R. C. Bollinger, R. S. Paranjape, D. Gadkari, S. S. Kulkarni, N. G. Novak, R. Ingersoll, H. W. Sheppard, and S. C. Ray.** 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J. Virol. **73:**152–160.
9. **Oberste, M. S., K. Maher, M. R. Flemister, G. Marchetti, D. R. Kilpatrick, and M. A. Pallansch.** 2000. Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. J. Clin. Microbiol. **38:**1170–1174.
10. **Schnurr, D., M. Dondero, D. Holland, and J. Connor.** 1996. Characterization of echovirus 22 variants. Arch. Virol. **141:**1749–1758.
11. **Watanabe, K., M. Oie, M. Higuchi, M. Nishikawa, and M. Fujii.** 2007. Isolation and characterization of novel human parechovirus from clinical samples. Emerg. Infect. Dis. **13:**889–895.
12. **World Health Organization.** 2004. Polio laboratory manual. World Health Organization, Geneva, Switzerland.

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ORIGINAL ARTICLE

Evaluation of a two-dose administration of live oral poliovirus vaccine for wild and virulent vaccine-derived poliovirus type 1, 2, 3 strains in Japan

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Abstract

We evaluated the efficacy of Japan's vaccination policy, a 2-dose administration of live oral poliovirus vaccine (OPV) against wild and virulent vaccine-derived poliovirus (VDPV) type 1, 2, 3 strains, by investigating the neutralizing antibody titers of residents in Toyama Prefecture, Japan. Seropositivities against the virulent type 1 and 2 strains were more than 90%, but the values against the virulent type 3 strains were approximately 60%. Also, while geometric mean antibody titers against virulent type 1 and 2 strains were more than 180, those against the virulent type 3 strains were 58–59, and 9–12, in particular, at 10 to 19 y of age. A booster dose of the vaccine for the type 3 virus is recommended for adolescents. However, high herd immunity against type 1, 2 and 3 viruses has been maintained for these 22 y, although the seropositivity against type 3 virus was always lower than other types. Our results suggest that Japan's vaccination policy might be enough to prevent an epidemic of poliomyelitis caused by wild and virulent VDPV type 1, 2, 3 strains, even though the titers against type 3 viruses were the lowest.

Introduction

The trivalent live oral poliovirus vaccine (OPV) is constituted of attenuated Sabin strains of each of the 3 serotypes. It is regarded as one of the most effective and safest vaccines in current use, and it has been used as a major tool for the global poliomyelitis eradication program of the World Health Organization (WHO). Two additional merits of the OPV are extreme simplicity of administration and low cost of manufacture. In Japan, following the introduction of the 2-dose administration of OPV, which was imported from Canada and the Soviet Union, to children of 3 months to 12 y of age in 1961–1963, the number of patients decreased markedly, while 1000 to 5000 paralytic cases of poliomyelitis were reported annually before the introduction of the OPV [1,2]. A 2-dose administration of domestic OPV to infants 3–48 months of age (between 1964–1994) and 3–90 months (since 1995 until now) of age at intervals longer than 6 weeks has been performed routinely since 1964. A wild poliovirus was isolated from 1 patient with poliomyelitis in 1980, and from 2 patients with non-acute flaccid paralysis in 1984 and 1993, respectively, but since then no wild poliovirus has been isolated from patients with poliomyelitis in Japan [3].

OPV is usually given to an individual 3 times or more, which confers a high seropositivity against 3 types of polioviruses [4–7]. Moreover, it is in Japan that the epidemic of poliomyelitis has been prevented with only a 2-dose administration of OPV. A large-scale investigation was carried out to evaluate antibody titers that neutralize virulent wild polioviruses by the vaccination of imported monovalent OPV

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(prepared by British Pfizer Co.) in 1961 before the introduction of mass vaccination [8,9]. However, both the virus titer of each type of poliovirus and the administration method that inoculated each type of monovalent vaccine virus, respectively, in the investigation were different from Japan's current trivalent OPV policy. Furthermore, it was suggested that natural infections of poliovirus influenced the results because of continually occurring epidemics of poliomyelitis caused by poor public health conditions at that time in Japan. Therefore, a regular measurement program of the neutralizing antibody titers against Sabin attenuated strains has been performed by the Ministry of Health and Welfare in Japan since 1973, but few investigations on antibody titers against virulent wild polioviruses have been carried out.

The global poliomyelitis eradication program of the WHO is close to the final stage, at which the virulent wild-type poliovirus is replaced with the vaccine strains from OPV [10]. Recently, epidemics of poliomyelitis by vaccine-derived polioviruses (VDPVs) [11,12] and imported wild polioviruses [13] have been reported worldwide. We have surveyed polioviruses regularly since 1979 in Toyama Prefecture. Although the wild poliovirus was not isolated, a total of 78 VDPV strains (type 1: 16 strains, type 2: 31 strains, type 3: 31 strains) were isolated from the river water and wastewater from sewage disposal plants between October 1993 and September 1995 [14]. Furthermore, using the method of 'mutant analysis by PCR and restriction enzyme cleavage (MAPREC)' to estimate the ratio of polioviruses containing genomes of a virulent nature in a vaccine-virus population [15,16], we have found that some strains of type 1 [17,18], type 2 [19] and type 3 [20] VDPV possess virulent genotype.

We have been considering why an epidemic of poliomyelitis caused by virulent polioviruses like virulent VDPV has not occurred in Japan, and have studied this using virulent poliovirus type 1 strains [21]. In this study, we attempt to assess retrospectively the efficacy of Japan's current OPV vaccination policy in preventing epidemics of poliomyelitis caused by wild and virulent VDPV type 1, 2, 3 strains.

Material and methods

Serum samples of residents used for this study were obtained with the agreement of the residents or their guardians in October, 1998.

Viruses

Type 1, 2 and 3 strains of Sabin and wild-type Mahoney, Lansing and Saukett were obtained from the National Institute of Infectious Diseases, Tokyo, Japan. Type 1, 2 and 3 VDPV G4-12, G18-2 and G5-1 strains were originally isolated from a sewage disposal plant located downstream of the Oyabe River, Toyama Prefecture, Japan [14]. They were estimated to be strong neurovirulent strains by the molecular analysis method (MAPREC method) that quantifies genomic changes [17,19,20]. Differences in both nucleotide and amino acid sequences in the VP1 capsid protein region and 5' non-coding region between the genomes of Sabin strains and the isolated VDPVs are described in Table I. The VDPVs that had strong neurovirulence and the most abundant nucleotide mutations in the VP1 region were used in this research. According to WHO criteria, a VDPV is defined as a strain provided there is 1–15% drift in the VP1 region at the nucleotide level [12]. Although the G4-12 and G5-1 strains do not belong to the category of VDPV since their nucleotide divergences

Table I. Differences in nucleotide sequences between the genomes of Sabin strains and VDPVs.

		Change from Sabin to VDPV [a]			
		VP1 region		5′ non-coding region	
	VDPV	Nucleotide	Amino acid	Nucleotide	Ratio of reversion [b]
Type 1	G4-12	C→U (2743)		G→A (480)	
		A→G (2795)	T→A (106)	U→C (525)	92.1%
Type 2	G18-2	T→C (2537)	V→A (19)	A→G (481)	83.2%
		G→A (2566)	D→N (29)		
		C→T (2568)			
Type 3	G5-1	G→A (2536)	A→T (54)	U→C (472)	88.6%
		C→T (2713)			
		T→C (2790)	M→T (105)		
		A→G (2815)			

[a] Numbers in parentheses are positions.
[b] Contents of 480-A + 525-C for type 1, 481-G for type 2 and 472-C for type 3 by using the MAPREC method.

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were less than 1% in the VP1 region, we included them as VDPVs in this research.

Sera

In this research, the serum that remained in the regular measurement program between 1984 and 2005 was used. The selection of serum was decided based on the following criteria: 1) serum which had been collected at the time in 1993 to 1995 close to when the virulent VDPVs were isolated; 2) sufficient amount of serum was residual for measurement of neutralizing antibody titer. Consequently, a total of 191 individual serum samples from residents of Toyama Prefecture, Japan, of 0–76 y of age, collected in 1998, were used for this study. Of these serum samples, 85 that received the standard 2 doses of OPV were measured for neutralizing antibody titers against wild and virulent VDPV strains. The vaccination history of individual groups of residents is shown in Table II. In addition, 67–295 serum samples every y between 1984 and 2005 (total 4050 samples) which contain non-vaccinated, 1- or 2-dose OPV-vaccinated individuals from residents of Toyama Prefecture were measured for neutralizing antibody titers against Sabin type 1, 2 and 3 strains.

Measurement of neutralizing antibody titers

The neutralizing test was performed in 96-well microtiter plates using Vero cells according to the standard method recommended by the WHO [22]. The original virus titers and the challenge virus titers used for the neutralization test were as follows: Sabin 1: $10^{6.75}$, $10^{2.08}$, Sabin 2: $10^{6.75}$, $10^{2.06}$, Sabin 3: $10^{6.75}$, $10^{2.36}$, Mahoney: $10^{6.38}$, $10^{2.32}$, Lansing: $10^{6.50}$, $10^{2.11}$, Saukett: $10^{6.25}$, $10^{2.25}$, G4-12: $10^{6.75}$, $10^{2.25}$, G18-2: $10^{5.50}$, $10^{2.06}$ and G5-1: $10^{6.25}$, $10^{2.17}$ $TCID_{50}$/0.05 ml, respectively. The cytopathic effect was scored 7 d after infection. A serum sample with a neutralizing antibody titer equal to or more than 8 was regarded as seropositive.

Results

Seropositivity and neutralizing antibody titers against Sabin type 1, 2, 3 vaccine strains

The serum samples were measured for neutralizing antibody titers against each type of Sabin vaccine strain using a microneutralization assay. In individuals who were born after the enforcement of domestic OPV vaccination and received the standard 2 doses of OPV, the seropositivities against the Sabin type 1, 2 and 3 strains were 98.8%, 100% and 68.2%, respectively (Table II). The rates were higher than those of either non-vaccinated individuals (corresponding rates were 40.0%, 80.0% and 20.0%, respectively), or 1-dose OPV-vaccinated individuals (75.0%, 87.5% and 37.5%, respectively). Similarly, the geometric means of neutralizing antibody titers of the 2-dose vaccinated individuals against each type of Sabin strain was obviously higher (569, 426 and 72.1, respectively) compared to those of the non-vaccinated individuals (corresponding titers were 79.2, 180 and 6.4, respectively). Although both the seropositivity and antibody titers against the Sabin type 3 strain were the lowest among 3 types of Sabin strains, the efficacy of 2-dose administration of OPV against all types

Table II. Vaccine history, seropositivity and neutralizing antibody titers against Sabin type 1, 2 and 3 strains of residents in Toyama Prefecture.

	Vaccine history [a] (no. of OPV doses)	No. of serum samples [b]	Seropositivity (%) [c]/ neutralizing antibody titer		
			Sabin 1	Sabin 2	Sabin 3
Residents born after 1964 [d] (after introduction of domestic OPV)	Non-vaccinated	5	40.0/79.2	80.0/180	20.0/6.4
	Vaccinated (1)	8	75.0/649	87.5/489	37.5/69.5
	Vaccinated (2)	85	98.8/569	100/426	68.2/72.1
	Unknown	24	87.5/323	95.8/365	66.7/41.3
Residents born before 1964 [e] (before introduction of domestic OPV)	0	69	92.8/158	94.2/212	85.5/90.9

[a] The OPV, prepared by the Japan Poliomyelitis Research Institute, Tokyo, Japan, contained $10^{6.0\pm0.5}$, $10^{5.0\pm0.5}$, $10^{5.5\pm0.5}$ $TCID_{50}$ of Sabin type 1, 2 and 3 strains, respectively, in 1 dose.
[b] Serum specimens were collected in October, 1998.
[c] A titer equal to or more than 8 was regarded as seropositive.
[d] <35 y of age in 1998.
[e] Equal to or more than 35 y of age in 1998.

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of Sabin strains seems to be valid compared to those of non-vaccinated individuals.

High seropositivity (92.8%, 94.2% and 85.5%, respectively) and high geometric mean antibody titers (158, 212 and 90.9, respectively) were observed in individuals who were born before the enforcement of domestic OPV vaccination and, as a rule, were not vaccinated (Table II). This may be explained by natural infection, as there were continually occurring epidemics of poliomyelitis because of poor public health conditions at that time in Japan.

Seropositivity and neutralizing antibody titers against virulent type 1, 2, 3 strains

Efficacy of the 2-dose administration of OPV against virulent strains was analysed. Seropositivity and geometric mean antibody titers against type 1, 2 and 3 virulent wild strains of Mahoney, Lansing and Saukett, and virulent VDPVs G4-12, G18-2 and G5-1 are shown, in Figure 1, in the residents who received the standard 2-dose administration of domestic OPV. The seropositivities against virulent type 1 and 2 strains were more than 90%, but the values against virulent type 3 strains were 62.4% (Figure 1A). Differences in seropositivity between the Sabin vaccine strains and virulent strains in all types were hardly observed.

The geometric mean antibody titers against type 1 strains of Sabin, Mahoney and G4-12 were 569, 186 and 190, respectively (Figure 1B). Similarly, the titers against type 2 strains of Sabin, Lansing and G18-2 were 426, 260 and 325, respectively. Although high antibody titers were observed against virulent type 1 and 2 strains, those were obviously lower than those of the Sabin attenuated strains. In contrast, the titers against type 3 strains of Sabin, Saukett and G5-1 showed lower values of 72, 58 and 59, respectively, compared with those of the other 2 types.

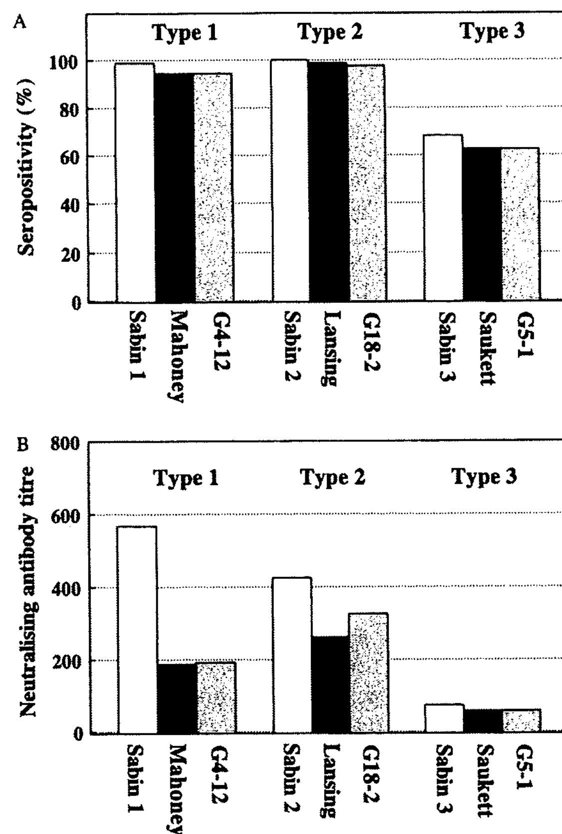

Figure 1. Seropositivity (A) and geometric mean neutralizing antibody titers (B) of residents who were vaccinated with the standard 2-dose administration of OPV against strains of type 1 Sabin, Mahoney and G4-12, type 2 Sabin, Lansing and G18-2, and type 3 Sabin, Saukett and G5-1.

Comparison of neutralizing antibody titers among age group

The geometric mean neutralizing antibody titers among age group of the residents who received the standard 2-dose administration of OPV were investigated, although the number of serum samples used in the investigation was unsatisfactory (85 samples) (Figure 2). Sufficient titers against virulent type 1 and 2 strains were observed in all generations (Figure 2A, B), while the titers against the virulent strains of Mahoney and G4-12 were 2 to 4 times lower than those against the Sabin strain in the case of type 1 viruses. Such large differences were not observed in type 2 and 3 viruses (Figure 2A, B, C). These data are consistent with the results shown in Figure 1B. The geometric mean antibody titers against type 3 strains of Sabin, Saukett and G5-1 were extremely low at the ages of 10 to 13 y (corresponding titers were 19.1, 12.0 and 11.4, respectively) and 14 to 19 y (10.0, 9.0 and 9.0, respectively) compared with other age groups. The corresponding seropositivities in both generations also showed low values of 38.5%, 30.8%, 30.8% and 50.0%, 25.0%, 25.0%, respectively (data not shown).

Furthermore, the seropositivity of total 4050 serum samples from residents of Toyama Prefecture against Sabin type 1, 2 and 3 strains for these 22 y is shown in Figure 3. The vaccine coverage was 90–97% in each y except for the individuals with unknown vaccination history (data not shown). Large difference of the vaccine coverage was not observed compared with the general situation in Japan (90–94%) [23]. Since the seropositivity against type 3 strain was always lower than other

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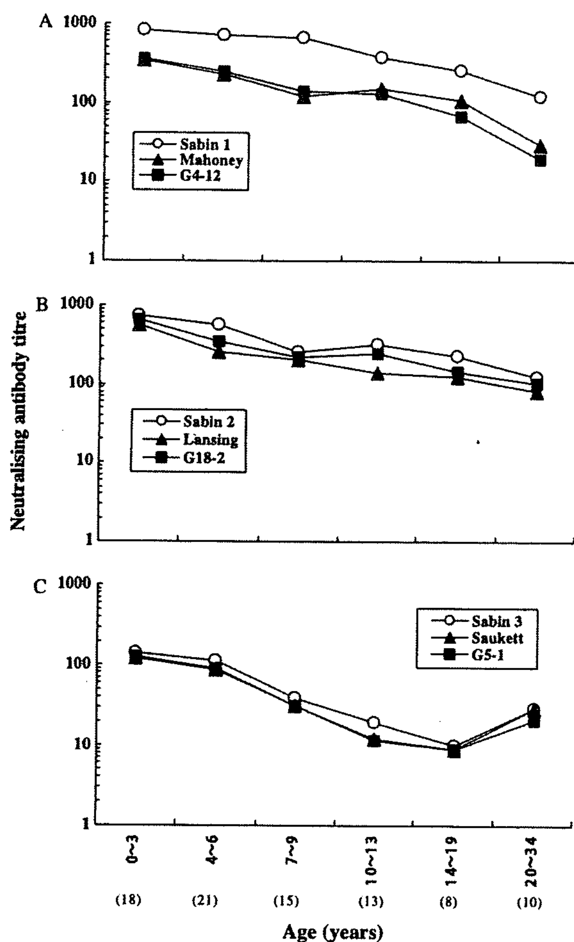

Figure 2. Geometric mean neutralizing antibody titers according to age group of the residents who were vaccinated with the standard 2-dose administration of OPV against strains of type 1 Sabin, Mahoney and G4-12 (A), type 2 Sabin, Lansing and G18-2 (B), and type 3 Sabin, Saukett and G5-1 (C) in each generation. The number of serum samples used in the investigation is shown in parentheses.

types, the antibody titer against type 3 virus would be postulated to be lower than the other types as well. However, high herd immunity against type 1, 2 and 3 viruses has been maintained long term.

Discussion

A 2-dose administration, in spring and autumn, respectively, of OPV is routinely carried out in infants in Japan. The effect of this vaccination protocol has been a concern, because it is only in Japan (of the whole world) that the vaccination is performed just twice. Although there are some reports of a high seropositivity using the 2-dose vaccination of OPV against all types of polioviruses [24], the continuation of the effect is unclear. Therefore, a large-scale surveillance of neutralizing antibody titers against virulent wild polioviruses administrated by imported monovalent OPV was performed before the introduction of mass vaccination [8,9]. However, the virus titers and inoculation method were different from Japan's current trivalent OPV vaccination policy. Furthermore, there is also the possibility that natural infections of poliovirus influenced the results because of continually occurring epidemics of poliomyelitis caused by poor public health conditions at that time in Japan. Nishio et al. [25] reported that the neutralizing antibody titers, particularly against the type 3 virus, decreased gradually for a period of 5 y after 2-dose vaccination of OPV, and thereafter some of them increased due to natural infection.

To assess the effect of the vaccination under current public health conditions with few natural infections, we assayed here the neutralizing antibody titers against type 1, 2 and 3 strains of Sabin, wild, and virulent VDPV of residents in an area of Toyama

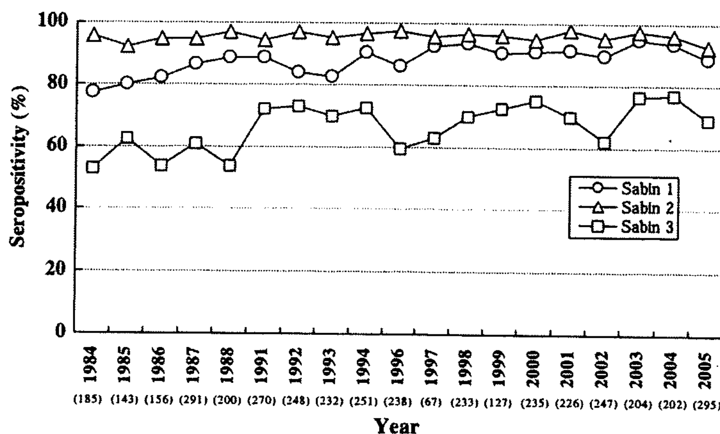

Figure 3. Seropositivity against type 1, 2 and 3 strains of Sabin (1984–2005). The number of serum samples used in the investigation is shown in parentheses.

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Prefecture where the virulent VDPVs were isolated. We have already reported that the current vaccination policy gave a sufficient effect on virulent type 1 viruses [21]. In this study, >90% of the individuals were seropositive, and high neutralizing antibody titers of >180 were observed in virulent type 1 and 2 polioviruses in residents who were vaccinated with the standard 2-dose administration of OPV (Figure 1A, B). Geometric neutralizing antibody titers against isolated VDPV strains were lower than those against Sabin strains, especially in type 1 strains. Challenge virus titers of Sabin 1 and G4-12 strains for neutralizing test were almost the same (see Material and methods). It is therefore considered that the amino acid divergence in the VP1 region takes part in the difference of neutralizing antibody titre. In contrast, remarkably low seropositivity and neutralizing antibody titers were revealed in type 3 viruses. Large differences in antibody titers were also observed among the age groups of type 3 viruses especially at 10 to 19 y of age (Figure 2C). It is unclear why the antibody titers were low in these age groups, although a 2-dose administration of OPV had been received as well as other age groups. This phenomenon was similarly observed not only in 1998, but also in other y (Table III), i.e. a tendency that the antibody titer and seropositivity of 10–19 y age group were lower than those of other age groups was shown between 1996 and 2005. A booster dose of the vaccine in adolescents to ensure personal and herd immunity is recommended.

Lago et al. [4] reported similar results, showing that while the seropositivities of poliovirus type 1 and 2 strains were >90%, that of the type 3 virus was 45.9%, after a 2-dose administration of OPV in Cuba. It has been also reported that the seropositivity of the type 3 virus after a 2-dose vaccination of OPV was approximately 40% in Brazil, which value increased after the booster vaccination [6]. Thus, it should be considered that solid immunity will be acquired by performing the booster vaccination for adolescents in Japan, even though Japan's current OPV vaccination policy has been effective in preventing poliomyelitis caused not only by the wild poliovirus, but also by virulent VDPV in types 1, 2 and 3. Although the objective was an increase in type 1 antibody titer, the notification of the booster vaccination of OPV was initiated by the Ministry of Health and Welfare in Japan in 1996 for individuals who were born in 1975 to 1977, and subsequently the vaccination was performed. The efficacy of the booster vaccination in Japan will be clarified by analysing the effects of this.

Recently, epidemics of poliomyelitis caused by VDPVs have been reported worldwide [11,12]. Also,

Table III. Seropositivity and geometric mean neutralizing antibody titers in each age group of residents who were vaccinated with the standard 2-dose administration of OPV against Sabin type 3 strain in recent y.

Age group	Seropositivity (%)/neutralizing antibody titer against Sabin type 3 strain.								
	1996	1997	1998	1999	2001	2002	2003	2004	2005
0~3	76.9/172 (26) [a]	83.3/106 (6)	61.1/142 (18)	79.2/88.2 (24)	83.3/49.1 (18)	73.3/30.9 (15)	73.3/131 (15)	71.4/182 (14)	83.3/176 (24)
4~6	85.0/63.8 (20)	71.4/102 (7)	76.2/114 (21)	84.0/26.2 (25)	75.0/39.0 (8)	58.8/22.8 (17)	53.8/23.4 (13)	85.7/159 (14)	94.1/93.4(17)
7~9	87.5/28.0 (16)	75.0/45.0 (8)	86.7/38.4 (15)	93.8/87.8 (16)	87.5/76.5 (16)	69.2/21.5 (13)	75.0/28.3 (12)	66.7/52.7 (12)	71.4/30.9 (7)
10~13	53.8/9.2 (13)	– (0)	38.5/19.1 (13)	71.4/32.0 (14)	44.8/16.3 (29)	45.5/9.8 (22)	50.0/21.2 (10)	69.2/45.8 (26)	66.7/28.5 (33)
14~19	42.4/16.4 (33)	66.7/14.7 (3)	50.0/10.0 (8)	42.9/20.6 (7)	63.6/39.3 (11)	46.2/14.5 (13)	68.0/24.0 (25)	71.4/48.0 (7)	57.1/19.4 (14)
20~34	0/4.0 (5)	88.9/61.8 (9)	90.0/28.4 (10)	63.6/23.3 (11)	60.0/17.6 (5)	50.0/20.0 (8)	75.0/9.0 (4)	–(0)	40.0/11.2 (10)

[a] The number of serum samples used in the investigation is shown in parentheses.

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