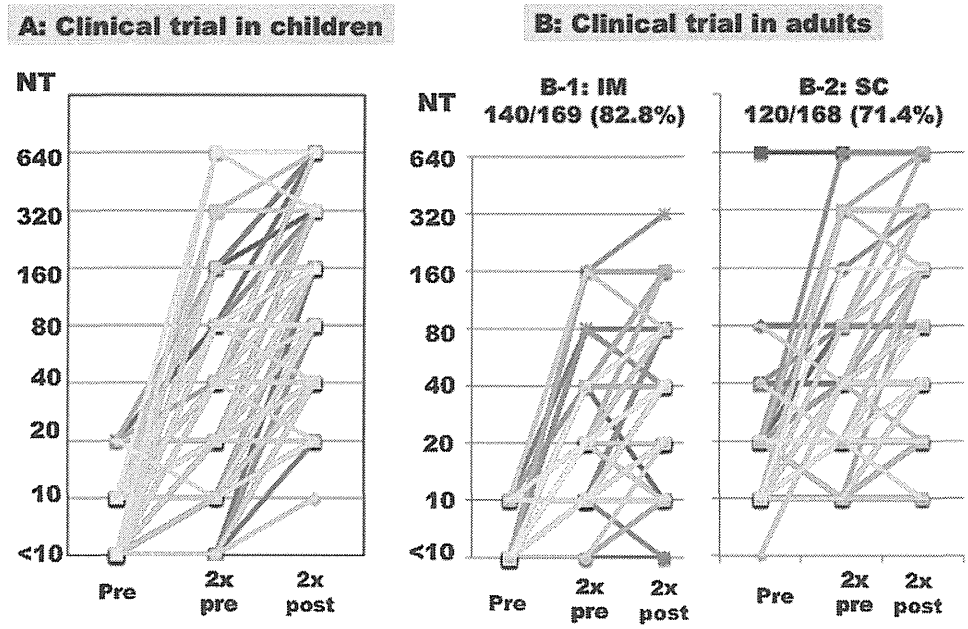

Fig. 1. Development of NT antibodies in clinical trials. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). The development of NT antibodies is shown in a clinical trial in children (A), in adults for intramuscular immunization (B-1), and for subcutaneous immunization (B-2). NT antibody titters are shown in *Y*-axis.

Functionally different IgG subclass antibody responses have been extensively investigated in mouse models, but human IgG subclass antibodies are not always functionally similar to the mouse. In mice, Th1 responses correlate with IgG2a, IgG2b and IgG3, regulated by the production of type I interferon (IFN), but in humans, IgG responses have not been strictly identified [7,8]. IgG1 is most abundant more than 50% of total IgG and IgG4, least abundant [9]. Human IgG1 reflected not only Th1 cytokine response but also Th2 cytokine activation. IgG4 subclass switch depends on IL-4 and IL-13, which are considered part of a Th2 response [10,11]. Primary antibody responses require T-cell help through functionally different Th1 and Th2 cytokines secreted by antigen-presenting macrophages or dendritic cells [12,13]. Therefore, the analysis of IgG subclass antibody responses after vaccination provides supportive evidence of CD4-positive T cell functions for modulating acquired immunity. In this report, IgG subclass responses were investigated in children immunized with alum-adjuvanted H5N1 WIV.

2. Summary of alum-adjuvanted vaccine trials in adults and children

An alum-adjuvanted H5N1 WIV clinical study was conducted, involving 337 subjects aged 20–59 years. Two doses H5N1 vaccine were administered with an approximately 4 weeks interval. Serum samples were obtained just before the first dose, and just before and one month after the second dose. H5N1 vaccines induced poor immunogenicity when assayed by HI tests. The NT assay was carried out by micro-neutralization methods using homologous vaccine strain [14]. NT antibody responses against H5N1 in adults and children are shown in Fig. 1. In adult study, 337 subjects were enrolled and divided into two groups: 169 of intramuscular inoculation and 168 of subcutaneous inoculation. The results of NT response are shown in Fig. 1B. Sero-conversion was observed in 260/337 subjects (77%), demonstrating four-fold or higher responses after the second dose immunization: 140/169 (82.8%) in the intramuscular immunization group (Fig. 1B-1) and 120/168 (71.4%) in the subcutaneous immunization group (Fig. 1B-2). The incidence of a febrile reaction ≥37.5 °C was reported at 3% in an adult vaccination study [15]. Using the same vaccine, a clinical trial was performed involving 374 subjects aged 6 months to 19 years. The results of NT antibodies are shown in Fig. 1A. All recipients became sero-converted in NT antibodies, but, unexpectedly, a high incidence of a febrile reaction ≥38.0 °C was demonstrated in recipients aged less than 7 years. The incidence of a febrile reaction (≥38.0 °C) after vaccination declined with age: 5/5 (100%) in subjects less than one year, 52/92 (57%) in those 1–3 years, 48/90 (53%) in those 4–6 years, 39/134 (29%) in those 7–12 years, and 3/53 (6%) in those 13–19 years. Higher NT antibody titers seemed to be found in those with a higher body temperature after vaccination [15].

3. IgG subclass antibodies against H5N1

A quantitative enzyme immunoassay (EIA) was performed to detect IgG subclass antibodies against the H5N1 vaccine virus in 193 cases where informed consent was re-obtained. H5N1 WIV antigen was adjusted to 333 ng/ml in PBS (−) and wells of a 96-well plate were coated with 33 ng. Serial dilutions of serum samples were incubated, starting at 1:200 for IgG1, IgG2, and IgG4, and 1:20 for IgG3. HRP-conjugated monoclonal antibody against each human IgG1, G2, G3, and G4 was added and stained with o-Phenylenediamine enzyme substrate. The EIA titer was expressed as the reciprocal dilution of 100×2^n that gave two-fold OD in the negative control wells by linear regression assay. The results of IgG1 antibody responses in 193 children are shown in Fig. 2A, where informed consent was re-obtained for EIA assay. Many subjects possessed high levels of IgG1 antibodies ($\geq 100 \times 2^4$) before vaccination and did not demonstrate a significant immune response after vaccination. A significant IgG1 antibody response was observed in 67 (34.7%). The IgG1 antibody response was examined in 20 randomly chosen adults and high levels of IgG1 titer were noted just before immunization without any significant increase after the vaccination (Fig. 2B).

A significant increase in IgG2 antibodies was observed in 12 subjects (6.2%) and that in IgG3 antibodies in four (2.1%). The IgG4

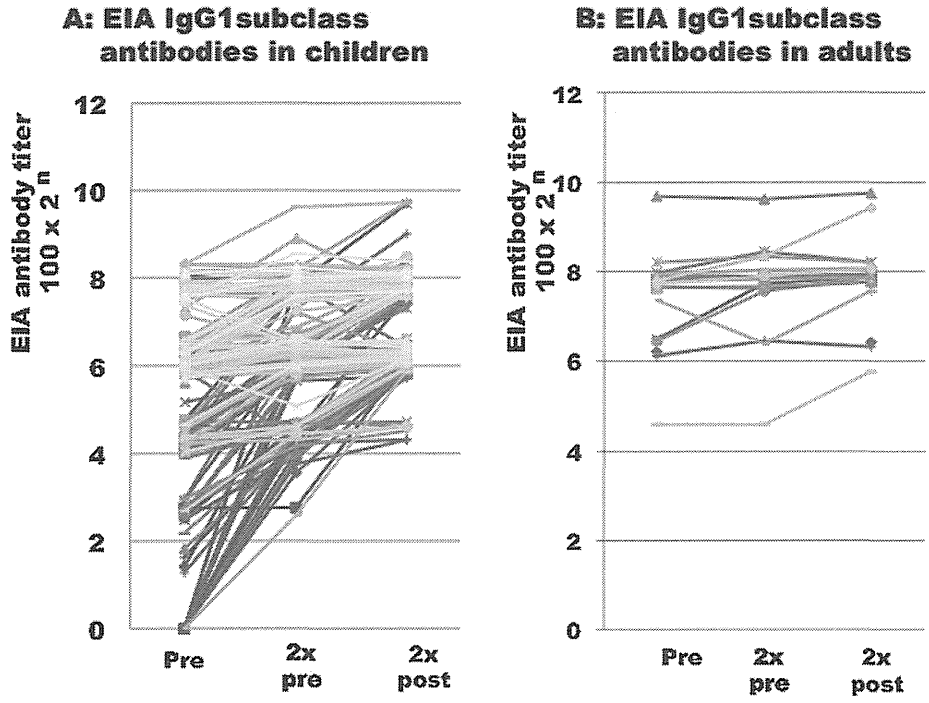

Fig. 2. IgG1 antibody responses in children and adults. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). EIA antibody titers are shown in *Y*-axis for 100×2^n. EIA IgG1 subclass response in children is shown in panel A and that in adults in panel B.

response is shown in Fig. 3A. In 134 recipients, IgG4 antibody was negative before vaccination without a significant response after two dose vaccinations and 42 recipients (21.8%) showed positive responses. Competition assay was performed and the results of three sera are shown in Fig. 3B. Serial dilutions of antigen were mixed with serum samples and binding activity was examined. All serum samples became negative after competition and thus the IgG subclass assay was specific against H5N1 antigens.

4. IgG1 responses in different age groups

193 children were classified into four groups: aged <4 years (47 subjects), 4–6 years (42 subjects), 7–12 years (72 subjects), and ≥13 years (32 subjects). IgG1 EIA antibody responses are shown in Fig. 4 and Table 1. Among 47 subjects aged <4 years, 22 showed positive for IgG1 antibody prior to vaccination and 15 showed positive response after vaccination. Among the 25 infants in which no IgG1

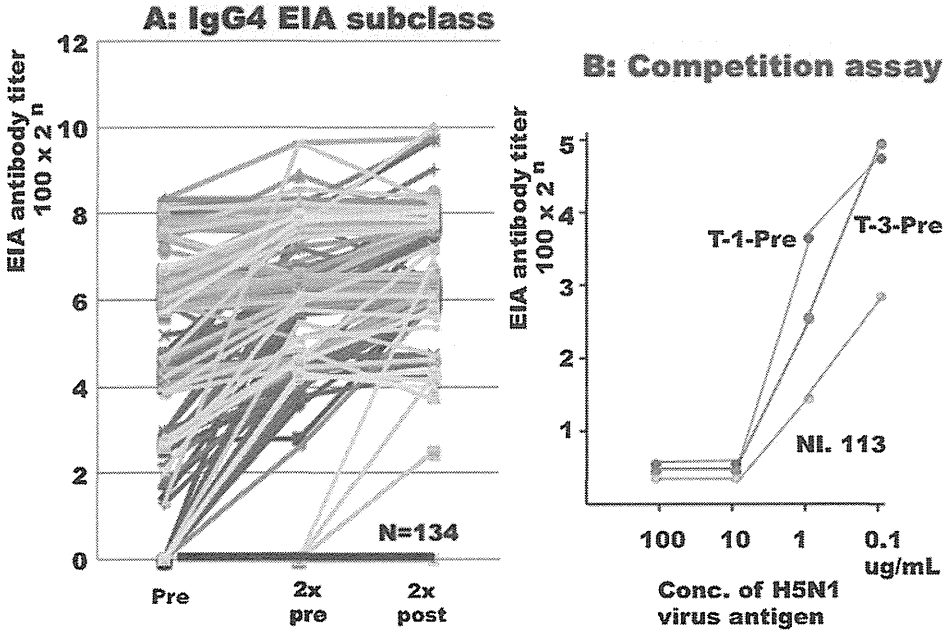

Fig. 3. IgG4 responses in children and the results of competition assay. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). EIA IgG4 antibody titers are shown in *Y*-axis for 100×2^n in panel A. Among 193 subjects, 134 had no EIA responses in IgG4. The results of competition assay are shown in panel B. Serial 10-fold dilutions of H5N1 WIV antigen were mixed with serum samples. The EIA activity was examined.

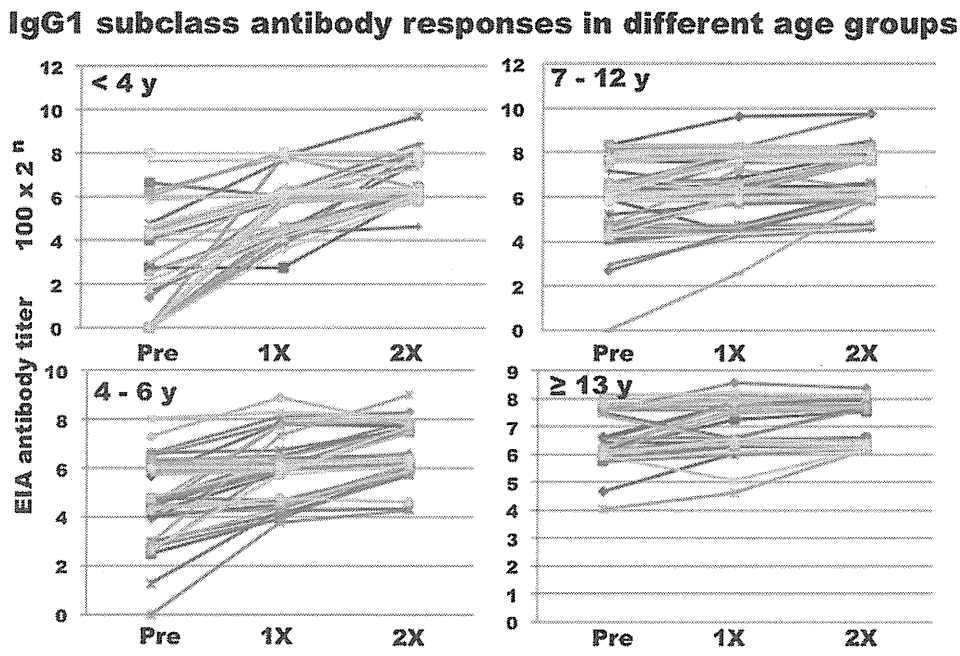

Fig. 4. Different IgG1 responses in different age groups: ≤4 years, 4–6 years, 7–12 years, and ≥13 years. Serum samples were obtained before the first dose (Pre) and before (1X) and four weeks after the second immunization (2X). IgG1 antibody titers are shown in *Y*-axis for 100×2^n.

EIA antibodies were detected before immunization, 17 showed positive after the first dose and the remaining 8 after the second dose. Of 42 subjects aged 4–6 years, 41 showed positive for IgG1 antibody before vaccination and 18 showed a significant response. One was negative pre-vaccination and sero- converted after the first dose. Of the 72 subjects aged 7–12 years, 71 were positive before vaccination and 7 showed a significant response. Among the 32 subjects aged ≥13 years, all tested positive before vaccination and only one showed a significant response. Most subject aged ≤4 years sero-converted, demonstrating four-fold or higher responses after the second immunization. Whereas, in subjects ≥7 years, IgG1 EIA antibody was detected before vaccination with a lower sero-conversion rate, similar to that observed in children ≥13 years and adults.

5. Discussion

Recent investigation on innate immunity has suggested that the development of acquired immunity against a specific antigen is modulated by the production of cytokines through functionally different Th1 and Th2 antigen-specific CD4-positive T lymphocytes [13]. The innate immune system consists of Toll-like receptors (TLRs), retinoic acid inducible gene-based (RIG)-like receptors, and nucleotide oligomerization domain (NOD)-like receptors (NLRs), known as inflammasome [16–18]. Investigation on cytokine production is essential for the better understanding of immune responses. In Japan, alum-adjuvanted H5N1 WIV has been licensed for adults but not for children. In a clinical trial of alum-adjuvanted H5N1 WIV in a pediatric group, a higher incidence of febrile reactions (≥38.0 °C) was observed with sufficient immune responses after vaccination. Cytokine productions were investigated in PBMCs obtained from non-vaccinated donors to know the reason behind these phenomena. H5N1WIV induced the higher levels of IFN-α, IL-6, IL-17, TNF-α, and MCP-1 than the control culture. With alum-adjuvanted H5 WIV, enhanced production of IL-1ß was demonstrated and IL-6 and TNF-α were produced similar to the levels obtained with H5N1 WIV [15]. WIV has genomic RNA that is recognized by TLR-7, inducing the production of IFN-α, which was essential for the antibody response in mice [19]. Inflammasome consists of NLRP3 and IL-1ß, IL-6, TNF-α, and IL-18 are induced in response to alum adjuvants through NLRP3 or other mechanisms. Alum-adjuvanted H5N1 WIV generated high titers of NT antibodies in young children, and, in this report, IgG subclass antibodies were investigated after immunization with alum adjuvanted H5N1 WIV.

IgG1 antibodies against H5N1 WIV antigens were detected in children ≥4 years of age and adult recipients before vaccination. The H5N1 influenza virus is not spread from human to human and has no history of large-scale outbreaks. The H5N1 WIV was a reassortant strain, whose envelop proteins, HA and NA were from H5N1/Vietnam/1194/2004 and remaining inner protein genes were from H1N1/PR8. Therefore, most subjects had IgG1 antibodies before vaccination. Approximately half of the recipients <4 years

Table 1
IgG1 EIA antibody responses in different age groups.

IgG1	<4 years (n = 47)	4–6 years (n = 42)	7–12 years (n = 72)	≥13 years (n = 32)
+++	22 (15)[a]	41 (18)[a]	71 (7)[a]	32 (1)[a]
– ++	17	1	1	0
– – +	8	0	0	0
– – –	0	0	0	0

+++: IgG1 antibody was positive before vaccination, one month after immunization of the first and second dose.
– ++: IgG1 antibody was negative before vaccination, and became positive after the first dose.
– – +: IgG1 antibody was negative before vaccination, and became positive after the second dose.
– – – IgG1 antibody was negative before vaccination, without antibody response after vaccination.
[a] Number of recipients with a significant responses.

had no detectable IgG1 antibodies before vaccination with a significant immune response, who did not experience H1N1 and H3N2 infections.

As for influenza infections, Garcon et al. [20] first reported high levels of IgG1 antibodies with lower amounts of IgG2 and IgG3 after immunization with different vaccine formulations; cold-adapted live recombinant, trivalent inactivated, and purified HA-conjugated vaccines to diphtheria toxoids. Hocart et al. [21] compared the subclass responses in natural infection with H3N2, and IgG1 levels in natural infection showed an 18-fold increase after infection and the other IgG subclasses, a 5- to 8-fold increase. The levels of IgG1 and IgG3 increased after immunization with live cold-adapted vaccines, and inactivated vaccines produced IgG1, IgG2, and IgG3 subclasses. IgG subclass responses were different from the vaccine formulations and also the increased levels of IgG1 differed with the serological status before vaccination. Stepanova et al. [22] reported different responses according to vaccine formulation and age. IgG1 and IgG4 responses were observed only in young adults immunized with the live influenza vaccine, the inactivated vaccine generating IgG1 and IgG3 in young adults, and IgG1 alone in the elderly. Human IgG1 reflected not only a Th1 cytokine response but also Th2 cytokine activation. IgG4 subclass switch depends on IL-4 and IL-13, which are considered part of a Th2 response [10,11].

In this report, a significant IgG1 and IgG4 antibody responses were observed after immunization with alum-adjuvanted H5 WIV especially in young infants <4 years. It provided efficient immune response in young naïve infants. Considering the previous report that alum-adjuvanted vaccine induced inflammatory cytokines, including IFN-α, IL-1ß, IL-6, and TNF-α, they would modulate the expression of co-stimulatory molecules recognized by naïve CD4 helper T cells. Therefore, the IgG4 antibody response seems to be T cell-dependent, induced by innate immune impacts of WIV with alum adjuvant. But, it caused high incidence of febrile reactions, and efficient influenza vaccine formulation for priming in young infants is expected with low incidence of febrile reactions.

Acknowledgements

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ORIGINAL ARTICLE

Antibodies against mumps virus component proteins

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Abstract The neutralization (NT) test is regarded as the most reliable method for detection of protective antibodies, but is labor-intensive and time consuming. Enzyme-linked immunosorbent assay (EIA) is frequently used in sero-epidemiological studies because of its simplicity and ease of use. In this study, immunofluorescent (IF) antibodies against nucleocapsid (N), fusion (F), and hemagglutinin–neuraminidase (HN) proteins were investigated in comparison with NT and EIA antibodies. The antibody against N protein was dominant in serum samples obtained from patients with a previous history of mumps infection. Titers of antibodies against F and HN proteins were very low. Many serum samples were positive for EIA but negative for NT, and no significant correlation was noted between NT and EIA antibodies. Among the three component proteins, correlation of EIA and IF antibodies with N protein was relatively good. After vaccination with mumps vaccine, EIA positivity was closely related to the IF antibodies against N protein, and after vaccination NT-positive sera became positive for IF antibodies against F and HN proteins. IF antibodies against F and HN proteins were considered to have a strong association with NT antibodies, and those against N protein were considered to have a strong association with EIA antibodies.

Keywords Neutralization (NT) test · Enzyme-linked immunosorbent assay (EIA) · Fusion (F) protein · Hemagglutinin–neuraminidase (HN) protein · Nucleocapsid (N) protein

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Introduction

Mumps virus is a member of the genus *Ruburavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, and is a major source of epidemic parotitis in childhood [1]. Symptoms of infection include swelling and tenderness in one or both salivary glands, and fever. Although the prognosis is benign, complications include aseptic meningitis, encephalitis, epididymo-orchitis, and oophoritis. Mumps virus is transmitted via droplet infection or direct contact with saliva. The incubation period is usually 16–18 days, and infectivity lasts from several days before the onset of illness until the parotid swelling disappears [2]. Individuals with asymptomatic infection also excrete infectious virus, and are sources of further transmission.

Among patients with typical parotid swelling, mumps infection is easily diagnosed, and virological examination is rarely needed. However, when aseptic meningitis occurs without other symptoms, it is necessary to distinguish the mumps virus from other pathogens, for example enteroviruses. Laboratory-based diagnosis of mumps infection depends on serological examination, isolation of the virus, and detection of the viral genome by polymerase chain reaction (PCR) assay. Isolation of the virus is regarded as the best method for diagnosis of mumps,

and reverse transcription (RT)-PCR is highly sensitive and specific [3, 4], but few laboratories in Japan can perform both examinations. Therefore serological testing is commonly used, by use of the complement fixation (CF) test, the hemagglutination inhibition (HI) test, the virus neutralization (NT) test, or enzyme-linked immunosorbent assay (EIA). The CF and HI tests are less sensitive and specific than the NT test and EIA, and another disadvantage of the HI test is cross-reaction of the antibodies with other paramyxoviruses including parainfluenza type 3 virus [5, 6].

The NT test is regarded as the most reliable indicator of immunity against virus infection, but it is very laborious, difficult to perform, and time-consuming [7, 8]. EIA is simple, rapid, and appropriate for automation, and so ideally applied for large-scale serological surveillance [7]. Diagnosis involves detection of the immunoglobulin-M (IgM) antibody in the acute stage, or a more than fourfold increase in the immunoglobulin-G (IgG) antibody in paired serum samples. Many studies on EIA of mumps virus have been published, and some have reported that the assay is more sensitive than the NT test [9–13]. However, the NT test can detect functionally protective antibodies, whereas EIA can detect IgG or IgM-binding antibodies only [8]. EIA may not be suitable for protection against infection. It is important to evaluate NT antibodies and to investigate antibodies against the component proteins of mumps virus. The mumps virus genome encodes seven proteins; nucleocapsid (N), phospho (P), membrane (M), fusion (F), small hydrophobic (SH), hemagglutinin–neuraminidase (HN), and large (L) proteins. F and HN proteins are enveloped proteins located in the outer membrane. Antibodies directed against these two proteins have been shown to neutralize the virus [14–19]. Therefore, it is necessary to evaluate the association of the NT test and EIA with antibodies against these component proteins.

Materials and methods

Serum samples

Serum samples were obtained every year from new students of the nursing school in Ashikaga city, Tochigi prefecture, for health assessment. The purpose of the study was explained and written informed consent was obtained from 299 participants. Thirty-one individuals had no history of immunization with mumps vaccine or clinically apparent mumps infection, so EIA antibody titers were negative. They received mumps vaccine after informed consent was obtained. Serum samples were obtained 4–6 weeks later and stocked at −20°C.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (EIA) was performed with the mumps IgG-EIA Kit (Denka Seiken, Tokyo, Japan). Briefly, purified viral particle antigens were coated on a 96-well plate and 100 μl serum diluted 1:200 was added to each well. After incubation with the serum sample for 60 min, anti-human IgG antibody labeled with peroxidase was added and stained, as recommended in the instruction manual. The kit contained reference standards relevant to NT titers, negative, $2^{1.0}$, $2^{2.0}$, $2^{3.0}$, $2^{5.0}$, $2^{6.0}$, and $2^{7.0}$. The EIA antibody titer was determined by referring to a linear regression curve obtained from the references. Sera with an EIA titer of $\geq 2^{2.0}$ were considered EIA-positive.

NT test

Serum samples were treated at 56°C for 30 min to inactivate the complement. Wild-type Mp/Tokyo.JPN/40.02 (genotype G) was used as the challenge virus. Then, 100 μl serial twofold dilutions in Eagle's medium containing 2% fetal calf serum (FCS) was mixed with an equal volume of virus (100 $TCID_{50}$/100 μl). After neutralization at 37°C for 90 min in a 5% CO_2 incubator, the mixtures were placed on a monolayer of Vero cells in a 96-well plate, in duplicate, and kept at 37°C in a 5% CO_2 incubator. The plate was observed for cytopathic effects (CPE) under a light microscope until day 7 of culture. NT antibody titer was expressed as the reciprocal of the dilution with no sign of CPE.

Immunofluorescent (IF) antibodies against N, F, and HN proteins

N, F, and HN genes were cloned from the KO.3 mumps Hoshino vaccine strain into pBluescript SK II(−) (Agilent Technologies, Santa Clara, CA, USA) using multi-cloning sites downstream of the T7 RNA promoter. 293T cells were infected with recombinant vaccinia virus expressing T7 RNA polymerase, and plasmids were transfected with Mirus Superfect III (Invitrogen Life Technologies, Carlsbad, CA, USA) [20]. Transfected cells were fixed in preparation glasses and stored at −80°C. More than 80% of the cells were found to express N, F, and HN proteins after staining with anti-mumps polyclonal antibodies raised in rabbits. Serum samples were diluted at 1:10 and twofold serially diluted from 1:10 to 1:640. The serial dilutions were added to the wells on the glass slide and incubated at 37°C for 60 min.

Each well was then incubated with anti-human IgG labeled with fluorescein isothiocyanate (FITC) (FITC conjugated-goat anti-human polyvalent immunoglobulin IgG fraction of antiserum; Sigma–Aldrich, St Louis, MO,

USA) at 37°C for 40 min. FITC-positive cells were identified under a fluorescence microscope. A field with one or more FITC-positive cells, observed at a magnification of 1:40, was judged as positive. In addition, all these judgments were made by the same person.

Statistical analysis

Statistical analysis with t and χ^2 tests was performed using StatMate IV (ATMS, Tokyo, Japan).

Results

Relationship between NT and EIA antibody titers

Titers of NT and EIA antibodies against mumps virus were measured for 299 serum samples. The correlation between the two titers was very low ($R^2 = 0.30$) (Fig. 1), mainly because 67 of 255 EIA-positive samples (26.3%) were negative for NT. Only one of 188 samples (0.5%) was negative for EIA but positive for NT.

Comparison of EIA antibody with IF antibodies against F, HN, and N proteins

More than 80% of transfected cells were positive for the respective proteins in assays using hyperimmune serum. The correlations between EIA and IF antibody titers are shown in Fig. 2. The correlation coefficient between EIA and IF antibodies against F protein was 0.17 (Fig. 2a). That between EIA and IF antibodies against HN protein was 0.33 (Fig. 2b). Among 252 EIA-positive samples, 89 were negative for IF antibody against F protein, and 43 were also negative for the antibody against HN protein. In contrast, the correlation coefficient was 0.54 between EIA and IF

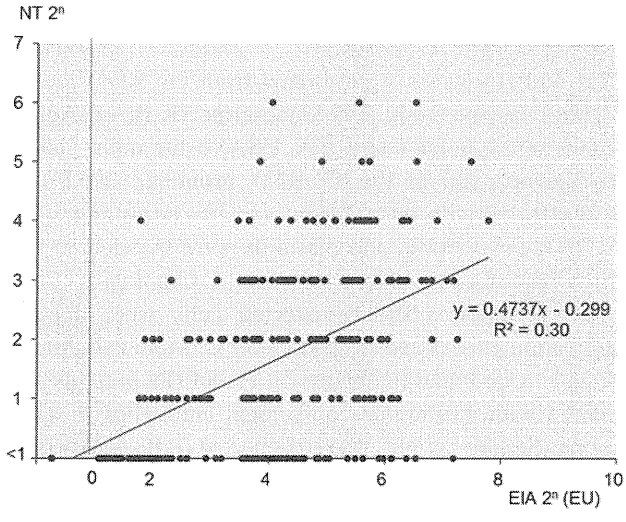

Fig. 1 Correlation between NT and EIA antibodies against mumps virus. NT antibody titers were assayed by 100% inhibition of CPE. EIA antibody titers were calculated by referring to standard positive sera supplied by the manufacturer. Both titers were expressed as 2^n. NT titers $\geq 2^{1.0}$ were regarded as positive. EIA $\geq 2^{2.0}$ were regarded as positive. EIA $<2^{0.0}$ was negative or uncertain

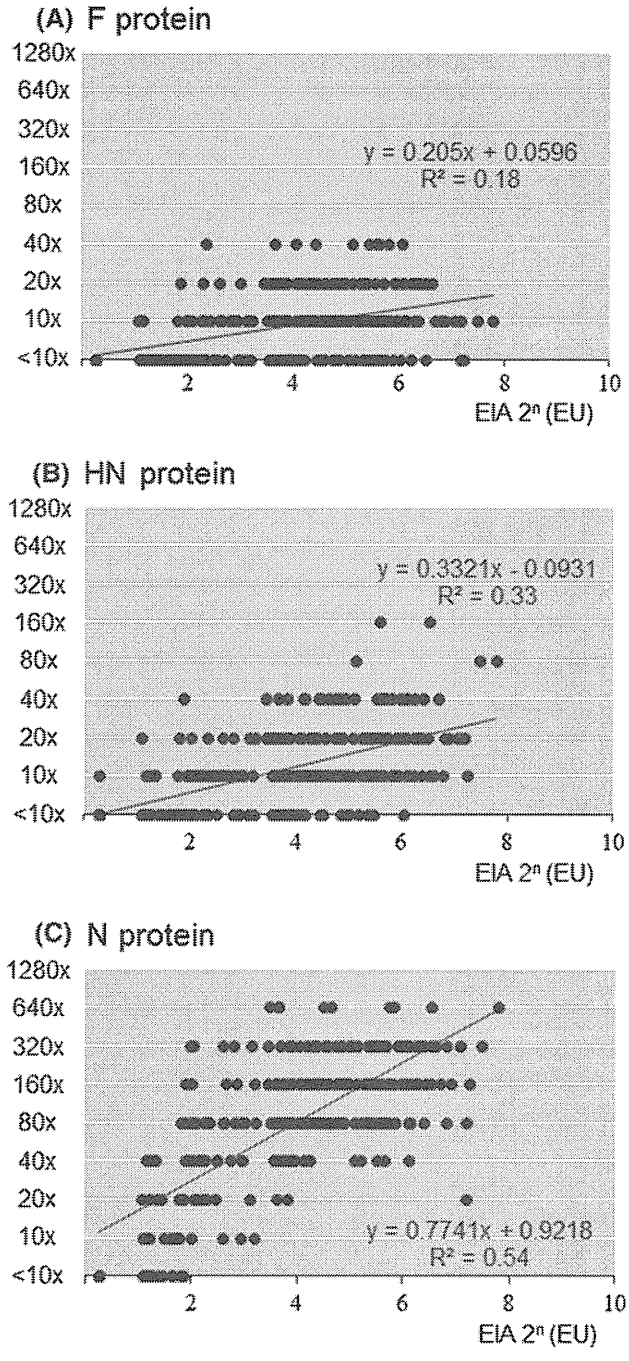

Fig. 2 Correlations between EIA and IF antibodies against three proteins. **a** Correlation between the EIA antibody and that against F protein. **b** Correlation between the EIA antibody and that against HN protein. **c** Correlation between the EIA antibody and that against N protein

Table 1 Serologic positive conversion of antibody titers against mumps before and after vaccination

	EIA	EIA titer (EU)	NT	NT titer ($\times 2^n$)	IF		
		GMT (95% CI)		GMT (95% CI)	Against F	Against HN	Against N
Before vaccination	0/31[a]	1.74 (1.15–2.65)	0/31[a]	0 (0–0)	1/31[a]	2/31[a]	11/31[a]
After vaccination	30/31[a]	12.12 (4.59–27.86)	14/31[a]	1.15 (0–2.34)	15/31[a]	19/31[a]	31/31[a]
NT-positive		13.92 (5.66–34.30)			14/14[a]	14/14[a]	14/14[a]
NT-negative		10.56 (4.29–26.00)			1/17[a]	5/17[a]	16/17[b]

Statistical evaluation was by use of the χ^2 test. Thirty-one subjects negative for EIA were immunized with mumps vaccine. Paired sera were obtained before and 4–6 weeks after vaccination

GMT geometric mean titer

[a] $P < 0.01$

[b] NS

antibodies against N protein (Fig. 2c). Among samples positive for EIA antibody, only one was negative for IF antibody against N protein. Therefore, there were statistically significant differences among the three groups of IF antibody against each protein ($P < 0.001$).

Comparison of NT antibody titers with those for F, HN, and N proteins

The correlation coefficient was 0.39 between NT and IF antibodies against F protein, 0.45 between NT and IF antibodies against HN protein, and 0.38 between NT and IF antibodies against N protein. Among 188 NT-positive samples, 39 and 19 were negative for IF antibody against F and HN proteins, but there was no negative result for IF antibodies against N protein. In contrast, among 111 NT-negative samples, 28, 48, and 84 were positive for IF antibodies against F, HN, and N proteins, respectively (data not shown).

Comparison of antibody titers before and after vaccination

The results are shown in Table 1. Thirty-one subjects were immunized with mumps vaccine and EIA, NT, and IF antibodies against N, F, and HN proteins were investigated before and after vaccination. Thirty out of 31 (96.8%) became EIA-positive and 14 (45.2%) became NT-positive. IF antibodies against F protein were positive in one subject before vaccination and in 15 after vaccination. Those against HN protein were detected in two before vaccination and in 19 after vaccination. Those against N protein were found in 11 before vaccination and in all after vaccination. The mean EIA titer was 13.92 EU for 14 subjects of the NT-positive group, and 10.56 EU for the NT-negative group. IF antibodies increased in one subject (5.9%) for F protein and five subjects (29.4%) for HN protein in the NT-negative group. Those against F and HN protein increased in all subjects with the positive response to NT ($P < 0.01$). All subjects in the NT-positive group and 16 subjects (94.1%) in the negative group had antibodies for N protein.

Discussion

In Japan, mumps, although an important infectious disease, is thought of a minor disease in comparison with measles and rubella. In most countries including the United States and European countries, implementation of a two-dose MMR vaccination scheme has dramatically reduced the number of mumps infections [21–23]. In Japan, MMR vaccine was administered from 1989 to 1993, but was discontinued because of an unexpectedly high incidence of aseptic meningitis [24, 25]. Since 1993, a monovalent mumps vaccine has been used but vaccine coverage is estimated to be less than 30% [26]. Therefore, outbreaks of mumps infection have often been found in schoolchildren. It is necessary to introduce routine vaccination of young children to suppress these outbreaks.

The immunity induced by a natural infection is considered life-long, and vaccine-acquired immunity is also believed to last for a long period [27], but some patients with clinically apparent mumps infection have had a clinical history of vaccination or even previous infection [28, 29]. Mumps outbreaks have recently been reported in several countries in which routine MMR vaccination was adopted [28–31]. In a few cases, individuals who had one or two doses of the MMR vaccine caught mumps. There was no significant difference in clinical symptoms between vaccinated and unvaccinated individuals in the case of a mumps outbreak in a summer camp in New York in 2005 [30]. In a mumps outbreak in Canada from 2009 to 2010 the effectiveness of one dose of the MMR vaccine ranged from 49.2 to 81.6% whereas for two doses it was estimated to be 66.3 to 88.0%; similar results have been reported in several studies [28]. Multistate outbreaks of mumps in

2006 showed two doses of the vaccine to be 76–88% effective, with attack rates not significantly different between one and two doses, and that immunity for mumps might decrease from 10 years after vaccination [31]. Also, the effectiveness of two doses of mumps vaccine declined from 98.8% in children 5–6 years of age to 86.4% in children 11–12 years of age [32]. The efficacy of live attenuated mumps vaccine was reported to be approximately 95% in phase III prelicensure serological responses [27]. Mumps virus is classified into 12 different genotypes (from A to L) on the basis of the sequence diversity of the SH gene, but it is thought to be serologically monotypic. Although antigenic differences might lead to reduced effectiveness, mumps vaccines have had high protective efficacy in outbreaks [31, 33]. With the exception of the Rubini strain vaccine, all available mumps vaccines worldwide, for example Jeryl-Lynn and Urabe Am9, have similar seroconversion rates and clinical efficacy and are regarded as acceptable for use in immunization programs [23]. Therefore, the age-related decrease in the vaccine's effectiveness seems to result from a waning of vaccine-acquired immunity. IgG-EIA and IgM-EIA antibody titers are used for diagnosis of mumps and to judge antibody prevalence in sero-epidemiological studies. EIA is superior in sensitivity, specificity, and simplicity, and suitable for large-scale surveillance. There is a problem with whether EIA antibody titers reflect protection against mumps infections.

In this study, a correlation between EIA and specific antibodies against mumps N protein was demonstrated, although there was a negative correlation of NT and IF antibodies against F and HN proteins. Many sera were negative for IF antibodies against F and HN proteins with high EIA titers, and the correlations between the EIA antibody and the antibodies against F and HN proteins were lower. From the results obtained for the serological responses for each antibody before and after vaccination, the EIA antibody became seropositive in 96.8% (30/31) of samples, but the seroconversion rate for the NT antibody was 45.2% (14/31). The IF antibody against N protein was found in all cases where the EIA antibody converted to positive. However, there were few cases where IF antibodies against F and HN proteins increased without any response in NT. Conversely, all sera with a positive response in NT were positive for IF antibodies against F and HN proteins. Thus, EIA antibody of mumps reflected the presence of antibodies against N protein. This shows that the development of EIA antibodies did not reflect protective immunity, because few antibodies against F and HN proteins were included in EIA antibodies. Several authors have reported that secondary vaccine failures were frequently observed in cases of natural mumps infections; IgM-EIA antibodies were detected, reflecting poor immunological memory of mumps infections [29, 34]. After measles virus infection, large amounts of antibodies were produced against N protein [35, 36]. The target of cell-mediated immunity is believed to be N protein. The presence of EIA reflects the immunological memory of N protein.

NT is the most reliable serological test for protective immunity, but has practical disadvantages. EIA has been widely used for serological diagnosis and immunological surveillance. Both serological methods have advantages and disadvantages, and each should be used taking into consideration its limitations.

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ORIGINAL ARTICLE

Mumps Hoshino and Torii vaccine strains were distinguished from circulating wild strains

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Abstract Aseptic meningitis and acute parotitis have been observed after mumps vaccination. Mumps outbreaks have been reported in Japan because of low vaccine coverage, and molecular differentiation is required to determine whether these cases are vaccine associated. RT-nested PCR was performed in the small hydrophobic gene region, and viruses were differentiated by restriction fragment length polymorphism assay. A total of 584 nucleotides were amplified. The PCR product of the Hoshino strain was cut into two fragments (313 and 271 nucleotides) by *Mfe*I; that of the Torii strain was digested with *Eco*T22I, resulting in 332- and 252-nucleotide fragments. Both strains were genotype B and had an *Xba*I site, resulting in two fragments: 299 and 285 nucleotides. Current circulating wild types were cut only by *Xba*I or *Mfe*I. However, the *Mfe*I site of the wild types was different from that of the Hoshino strain, resulting in 451- and 133-nucleotide fragments. Using three restriction enzymes, two mumps vaccine strains were distinguished from wild types, and this separation was applied to the identification of vaccine-related adverse events.

Keywords Mumps Hoshino strain · Mumps Torii strain · Molecular differentiation · Wild circulating genotypes

Introduction

In Japan, the MMR vaccine was introduced in 1989 but discontinued in 1993 because of an unexpectedly high incidence of aseptic meningitis caused by components of the mumps vaccine [1, 2]. The mechanisms responsible for the high incidence of aseptic meningitis with the MMR vaccine have not been elucidated in comparison to monovalent mumps vaccines used since 1993. Nagai et al. [3] investigated the incidence of aseptic meningitis after vaccination and identified 10 cases among 21,465 vaccine recipients. Moreover, 13 patients with aseptic meningitis were reported among 1,051 cases of naturally acquired mumps confirmed by viral isolation together with genome detection. The incidence of aseptic meningitis after vaccination was 1/27 of that observed for natural infections. However, in the post marketing study, the incidence of aseptic meningitis was approximately 0.01 % (1 case in 10,000 recipients) and that of acute parotitis, 2–3 %.

The mumps virus strains were divided into 12 genotypes based upon the sequence diversity of the small hydrophobic (SH) genome region [4, 5]. Parental strains of the Hoshino and Torii vaccine strains, isolated in the 1960s, are genotype B [6, 7]. Circulating wild-type strains were all genotype B in the 1970s and earlier and were genotypes J and B in the 1980s to 1990s. Genotype G appeared in the 2000s. Genotypes D, I, and L have been isolated sporadically [8–10], and recently genotype G was globally the major circulating genotype [11]. Large outbreaks have been observed every 3–5 years because of the low vaccine coverage, 30 % to 40 %. The mumps vaccine is voluntary (its cost is not covered by the government), and a guardian's decision usually depends on information on mumps outbreaks. Some recipients were immunized during the incubation period of natural infection, making it difficult to determine whether the mumps illness was caused by a natural infection or the vaccine.

In previous reports, the Hoshino vaccine strain was distinguished from circulating wild strains using the

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reverse transcription-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) in the hemagglutinin-neuraminidase (HN) gene with *Sca*I and *Afl*II [12]. A simpler method was also reported through digestion with *Sca*I after DNA amplification by reverse transcription loop-mediated isothermal amplification (RT-LAMP) [13]. These methods are applied after immunization with the Hoshino vaccine. Now, two vaccine strains of the Torii and Hoshino are used, but no method of differentiation has been developed for the Torii strain. In this report, 584 nucleotides were amplified in the SH gene, and the two vaccine strains were distinguished from circulating wild types by unique restriction enzyme sites.

Materials and methods

Mumps virus and clinical samples

The Hoshino (Kitasato Institute, Tokyo, Japan) and Torii (Takeda Pharmaceutical, Osaka, Japan) vaccine strains were recovered from marketed vaccines. MuVi/Tokyo.JPN/77 (genotype B), MuVi/Akita.JPN/93-AK (genotype I), MuVi/Tokyo.JPN/94-H (genotype J), MuVi/Tokyo.JPN/94-0K (genotype B), and MuVi/Tokyo.JPN/01-III-10 (genotype L) were used as wild-type representatives for genotypes B, J, and L, which have already been reported [8–10]. A total of 47 clinical samples were examined: 20 cases of aseptic meningitis after immunization with the Torii strain, 25 cases after immunization with the Hoshino strain, and 2 cases of orchitis after immunization with the Hoshino strain. Two wild-type strains (MuVi/Tokyo.JPN/10-K and MuVi/Tokyo.JPN/10-F) were isolated and identified as genotype G. Cerebrospinal fluid (CSF) samples from the patients with aseptic meningitis and two salivary swab samples or nasopharyngeal swab (NPS) from the patients with orchitis were used.

RNA extraction

Total RNA was extracted from 200 μl CSF and salivary swabs or NPS using a magnetic bead RNA purification kit (MagExtractor-viral RNA; Toyobo, Osaka, Japan) and the RNA pellet was suspended in 30 μl distilled water.

RT-PCR and RLFP

RNA was transcribed to cDNA with a random hexamer using a PrimeScript RT reagent Kit (TaKaRa Bio, Japan) and amplified using Ex *Taq* DNA polymerase (TaKaRa Bio). The first PCR was done using MP F 921+ (5′TCTAT AATTCAATTGCCAGA) and MP HN241− (5′TGTCTGC AATTGAAGACAAC) and the nested PCR, using MpF0+ 5′GTCGATGATCTCATCAGGTAC) and Mp HN1− (5′C CAATATTCGGAAGCAGGTTCGGA), amplifying 584 nucleotides including the primer sequences from the genome positions 6139 to 6722 [10]. PCR products underwent electrophoresis after digestion with *Eco*T22I, *Mfe*I, and *Xba*I (New England BioLabs Japan).

Sequence analysis

PCR products were excised from low-melting gel electrophoresis and purified. DNA sequences were determined by the dye terminator method using an Applied Biosystems 3130 (Life Technologies Japan).

Results

Sequence analysis and restriction enzyme sites

The Hoshino and Torii strains were sequenced; alignments at the restriction enzyme sites are depicted in Fig. 1. The *Eco*T22I site (genome position 6386–6391) was unique to

	EcoT22 I	*Xba I*	*Mfe I*	*Mfe I*
	6386 6391	6437 6442	6451 6456	6589 6594
Torii [B]	ATGCAT	TCTAGA	CAGTTG	CAACTG
Hoshino [B]	-----G	------	--A---	T-----
JPN/77 [B]	-----G	------	---C--	----C-
JPN/93AK [I]	-----G	------	-----A	---T--
JPN/94H [J]	-----G	------	--T--A	----C-
JPN/94OK [B]	-----G	------	---C--	----C-
JPN/01-III-10 [L]	-----A	------	-----T	------
JPN/10K [G]	-----G	-----G	-----A	---T--
JPN/10F [G]	-----G	-----G	-----A	---T--

Fig. 1 Sequence alignment of the Torii and Hoshino vaccine strains and representative wild strains. MuVi/Tokyo.JPN/77 (genotype B), MuVi/Akita.JPN/93-AK (genotype I), MuVi/Tokyo.JPN/94-H (genotype J), MuVi/Tokyo.JPN/94-0K (genotype B), and MuVi/Tokyo.JPN/01-III-10 (genotype L) were used. MuVi/Tokyo.JPN/10-K and MuVi/Tokyo.JPN/10-F (genotype G) are isolated in this study. Nucleotide changes are depicted in comparison with the Torii strain, and restriction enzyme sequences are highlighted in *grey*

Table 1 DNA sizes of restriction fragments after treatment with *Eco*T22I, *Mfe*I, and *Xba*I

Mumps strains	*Eco*T22I	*Mfe*I	*Xba*I
Hoshino genotype B	−	+ (313/271)	+ (299/285)
Torii genotype B	+ (332/252)	−	+ (299/285)
Wild genotypes B, J, L	−	−	+ (299/285)
Wild genotype I	−	+ (451/133)	+ (299/285)
Wild genotype G	−	+ (451/133)	−

the Torii strain and *Mfe*I site (6451–6456) to the Hoshino strain. Genotypes B, I, J, and L had an *Xba*I (6437–6442) site, and old genotype I and the currently circulating genotype G had an *Mfe*I site (6589–6594) newly introduced by nucleotide change, not at position 6451–6456 of the Hoshino strain. Based on the results of the sequence analysis, RFLP and predicted fragment lengths are shown in Table 1. The PCR product of the Hoshino strain was cut into two fragments (313 and 271) by *Mfe*I and that of the Torii strain into two fragments (332 and 252) by *Eco*T22I. These two strains were also cut by *Xba*I into two fragments (299 and 285). RFLP of the circulating wild type had mainly two patterns: genotypes B, J, and L were cut by *Xba*I and genotype G by *Mfe*I but differently from the Hoshino strain.

The results of RFLP are shown in Fig. 2. The PCR product of the Hoshino vaccine strain was cut by *Mfe*I and *Xba*I, and that of the Torii strain by *Eco*T22I and *Xba*I. As for the RFLP of wild type, the PCR product of MuVi/Akita.JPN/93-AK (genotype I) was cut by both *Mfe*I and *Xba*I with different fragment sizes from the Hoshino strain. MuVi/Tokyo.JPN/94-OK (genotype B) was cut by *Xba*I, and the same RFLP pattern was noted for MuVi/Tokyo.JPN/94-H (genotype J) and MuVi/Tokyo.JPN/01-III-10 (genotype L). PCR products of MuVi/Tokyo.JPN/10-K and/10-F (genotype G) were cut by *Mfe*I. They showed different patterns from the vaccine strains, as predicted from the sequencing results.

Differentiation of vaccine strains from wild types

A total of 47 clinical samples were obtained: 20 cases of aseptic meningitis after immunization with the Torii strain, 25 cases after immunization with the Hoshino strain, and 2 cases of orchitis after immunization with the Hoshino strain. The results of RT-PCR and RFLP are shown in Table 2. RT-PCR was negative for two CSF samples from the recipients of the Torii strain, and among 18 RT-PCR positives, 16 were identified as the Torii vaccine strain. Among 25 CSF samples obtained from the recipients of the Hoshino strain, 3 were negative by RT-PCR, and 20 were considered positive for the vaccine strain. Two from each

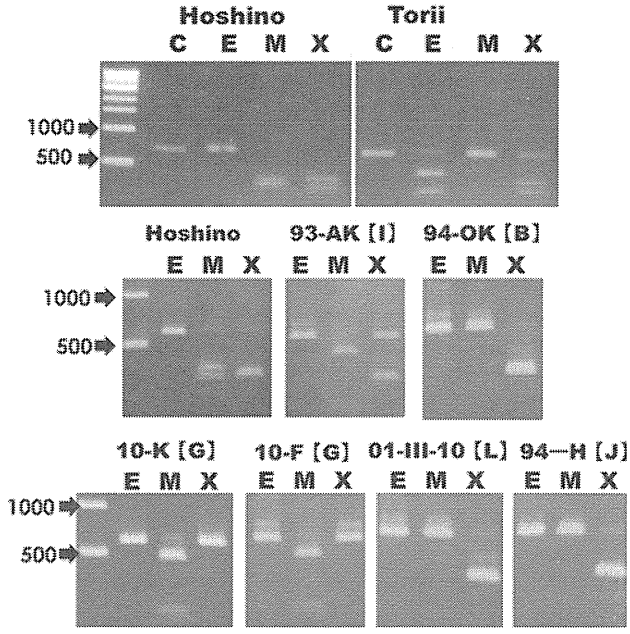

Fig. 2 Restriction fragment length polymorphism (RFLP) of the Hoshino and Torii vaccine strains and circulating wild strains. C, control; E, treatment with *Eco*T22I; M, treatment with *Mfe*I; X, treatment with *Xba*I. 93-AK, MuVi/Akita.JPN/93-AK [genotype I]; 94-H, MuVi/Tokyo.JPN/94-H [genotype J]; 94-OK, MuVi/Tokyo.JPN/94-0K [genotype B]; 01-III-10, MuVi/Tokyo.JPN/01-III-10 [genotype L]; 10-K, MuVi/Tokyo.JPN/10-K [genotype G]; 10-F, MuVi/Tokyo.JPN/10-F [genotype G]

were identified as wild strains. In 2 cases of orchitis after vaccination with the Hoshino strain, RT-PCR was positive in 1 case, identified as the wild type. Five of 45 patients with suspected adverse events were identified as having a concurrent wild-type genotype G.

Some strains identified as causing adverse events were sequenced; the phylogenetic analysis is shown in Fig. 3. Cases 1 and 2 were patients with aseptic meningitis after immunization with the Hoshino strain and cases A and B after that with the Torii strain. The sequencing results showed they were identical to the respective vaccine strains.

Table 2 Results of differentiation of mumps virus genome for clinical samples obtained from patients with aseptic meningitis and orchitis

	PCR negative	PCR positive	
		Vaccine strain	Wild strain
Aseptic meningitis after vaccination with			
Torii ($n = 20$)	2	16	2
Hoshino ($n = 25$)	3	20	2
Orchitis after vaccination with			
Hoshino ($n = 2$)	1		1

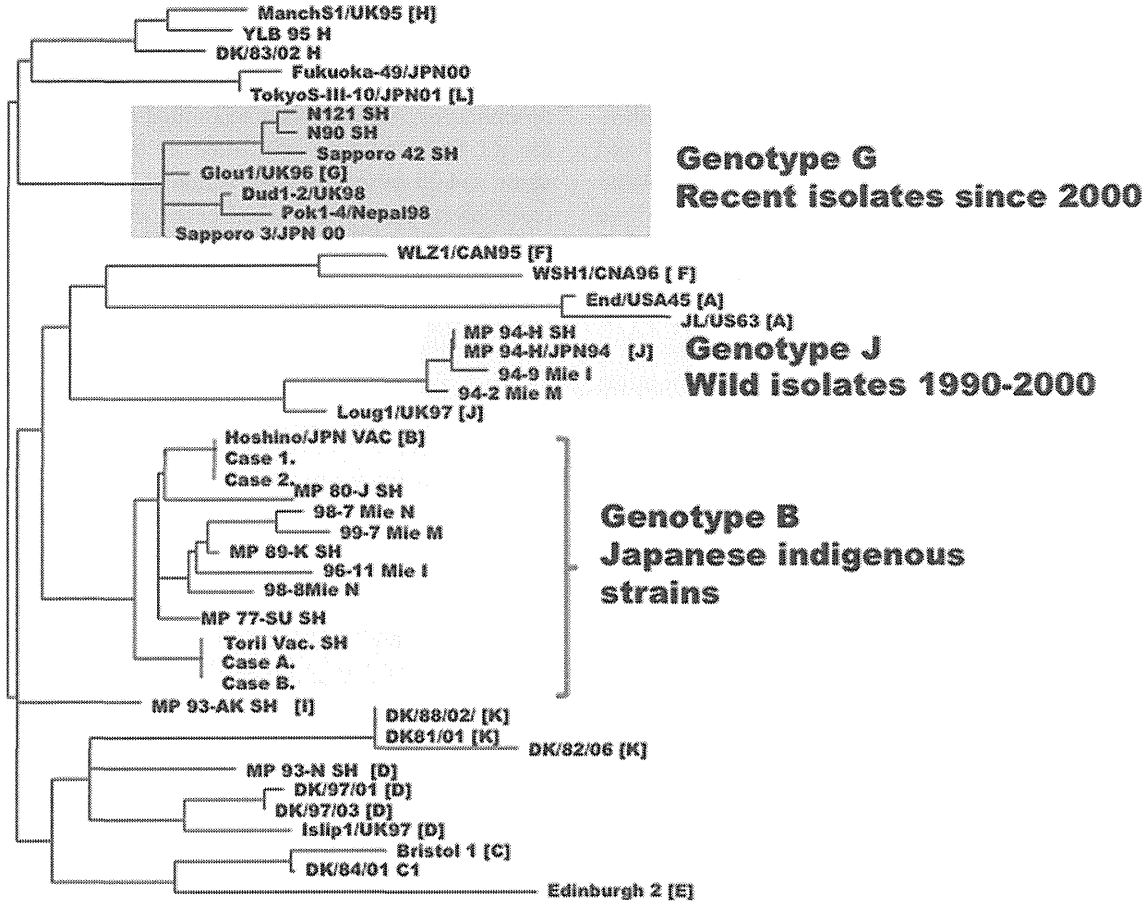

Fig. 3 Phylogenetic analysis of vaccine-associated cases in the small hydrophobic (SH) genome region. Cases 1 and 2 were patients with aseptic meningitis after immunization with the Hoshino strain and cases A and B after immunization with the Torii strain

Discussion

The mumps virus is classified into 12 distinct genotypes, with genotype B indigenous to Japan [4, 5, 8]. Genotype J was a dominant circulating strain with some genotype B strains in 1990–2000, and genotype G appeared in 2000–2012 with sporadic outbreaks of genotype L [8–10]. Mumps has been circulating in Japan because of a low immunization rate, approximately 30–40 %, and mump outbreaks have also been reported in the EU and USA where high immunization coverage was achieved with two doses of MMR [14–16]. The outbreaks were caused by the accumulation of susceptible individuals with an insufficient two-dose MMR vaccination in childhood. Several vaccine strains have been developed, and the Jeryl Lynn strain, belonging to genotype A, has been widely used as a component of MMR. Neutralization test (NT) antibody titers in sera obtained after vaccination with Jeryl Lynn were lower against genotype G than those against the vaccine strain, but they completely neutralized the other genotypes [17, 18]. In contrast, the antigenicity of genotype A of the vaccine strain was quite different from the recent circulating wild types and considered one of the reasons for the recirculation of the mumps virus [19]. There would be some problem with immunogenicity and persistence of immunity after immunization with the Jeryl Lynn strain. In Japan, two vaccine strains, Hoshino and Torii, are used and they belong to genotype B. In our previous report, there was no antigenic difference among circulating wild types [10]. Immunogenicity paralleled the incidence of adverse reactions. The incidence of aseptic meningitis after immunization with the Jeryl Lynn strain was reported to be 1 case in 100,000, and that of Torii or Hoshi was higher. The mump vaccine is still a voluntary one and so the cost is not covered by regional governments. Thus, guardians consider a mumps vaccination only when an outbreak is coming according to surveillance data. Some recipients were vaccinated by chance during the incubation period, and infection with the wild type became mixed into the vaccine-adverse events [12, 13]. In this report, 4 cases were identified as wild types among 45 cases with aseptic meningitis and 1 of the 2 cases of orchitis after immunization. From the results of surveillance reports, mumps outbreaks were observed in moderate grade, and

approximately 10 % of the vaccine-associated cases were infected with the wild type around the immunization day. Most adverse events developed 2–3 weeks after vaccination, but wild-type-related illness developed a few days earlier. There was no difference in clinical symptoms and clinical laboratory findings between vaccine-related adverse events and wild-type-related illness [3]. Five samples in aseptic meningitis and 1 in orchitis showed negative for mumps RT-PCR. Enterovirus RT-PCR for the mump PCR-negative samples showed negative for 5 mumps PCR-negative clinical samples [20]; these were considered to be low virus doses or in inappropriate stocking or transporting conditions.

Vaccine safety is a major concern and depends on postmarketing surveillance. Postmarketing surveillance from 1994 to 2010 is summarized, adding new data to the previous report [21], compared with the incidence of natural infections, in Table 3. The incidence of aseptic meningitis was <1–15 % among mumps infections with different incidences [22], and enhanced surveillance data showed 2.9 % of mumps patients were hospitalized, 6.1 % had orchitis, 0.3 % had meningitis, and 0.25 % had pancreatitis in England in 2002–2006 [23]. For the other complications, permanent deafness was considered to occur in approximately 1 per 20,000 cases, but it would actually be higher, 1 per 1,000 cases [24]. The results of postmarketing studies are shown from 1994 to 2010. A total of 3.5 million doses of the Hoshino vaccine were shipped, and acute parotitis was observed in 2–3 % of recipients. Among them, 117 nasopharyngeal swabs were examined and 89 were positive for RT-PCR: 64 were identified as the Hoshino vaccine strain and 25 were wild type. Among CNS complications, 5 cases of encephalopathy, 223 cases of aseptic meningitis, and 3 cases of acute disseminated encephalomyelitis (ADEM) were reported. Two cases were identified as enterovirus infections by RT-PCR [20]. When 85 CSF samples were examined in 223 cases of aseptic meningitis, 58 were considered vaccine-associated illnesses among 66 PCR positives. In this study period from 2008 to 2012, approximately 10 % of the patients suspected of having a vaccine-associated illness were identified as having wild-type infections during the mump outbreaks. Therefore, a simple differentiation method would contribute to further understanding of the safety of mumps vaccines.

Table 3 Complications of mumps and vaccine adverse events after vaccination with the Hoshino strain reported from 1994 to 2010

Complications	Natural infection	Vaccination (3.5 million)
Acute parotitis	70 %	2–3 %[a]
CNS complications		
Encephalopathy	1/5,000–6,000	5 (1: enterovirus)
Aseptic meningitis	1–2 %	223[b]
ADEM		3 (1: enterovirus)
Deafness	1/15,000 (1/1,000)	4
Orchitis	25 % in adolescents	15[c]
Oophoritis	5 %	
Pancreatitis	4 %	2
Other		1: ITP
		1: allergic purpura

Incidence of complications during natural infection refers to Ref. [22]

CNS, central nervous system; ADEM, acute disseminated encephalomyelitis; ITP, idiopathic thrombocytopenic purpura

[a] Of 117 nasopharyngeal swab (NPS) samples examined from patients with acute parotitis after vaccination with the Hoshino strain, PCR was positive in 89; 64 were identified as the vaccine strain and 25 as the wild type

[b] Of 85 CSF samples examined, 66 were PCR positive; 58 were identified as the vaccine strain and 8 as the wild type

[c] Three NPS samples were examined; one was the vaccine strain and two were the wild type

Conflict of interest The author has a conflict of interest. T.N. has received a research fund for the development of a new concept of live recombinant vaccines (20 million yen a year) from Daiichi-Sankyo Pharmaceutical.

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Rhinovirus Load and Disease Severity in Children With Lower Respiratory Tract Infections

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It has not been clarified if there is a correlation between rhinovirus (RV) load and disease severity in the lower respiratory tract infections of hospitalized children. This study was undertaken to elucidate the contribution of the viral load to the development of disease severity in 412 children ≤3 years of age who were hospitalized with lower respiratory tract infections. The RV load in nasopharyngeal aspirates obtained from the patients at the time of admission was measured by real-time quantitative reverse-transcription polymerase chain reaction (PCR), and the clinical symptoms of the patients were assessed using a severity scoring system. Of the 412 patients, 43 (10.4%) were diagnosed with RV infections only, and 15 were determined to have high severity scores. When all patients infected with RV were assessed, there was no correlation between the viral load and the disease severity. However, there was a significant negative correlation between the disease severity and age among children <11 months of age ($n = 15$, $\rho = -0.677$, $P = 0.006$) and a significant positive correlation between the viral load and the disease severity among children ≥11 months of age ($n = 28$, $\rho = 0.407$, $P = 0.032$). Among the patients infected with RV <11 months of age, the disease severity may be associated with an immature immune response and the small diameter of their airways rather than viral load. By contrast, in the patients ≥11 months of age, viral load may contribute to the development of disease severity. ***J. Med. Virol. 84:1135–1142, 2012.***

KEY WORDS: rhinovirus; PCR; disease severity; lower respiratory tract infection; children

INTRODUCTION

Rhinoviruses (RVs) are one of the major causes of the common cold in both children and adults. RVs are members of the family *Picornaviridae*, genus *Enterovirus*, with three species, *RV-A*, *RV-B*, and *RV-C* [Palmenberg et al., 2010]. Since the development of polymerase chain reaction (PCR) technology, RVs not previously identified by conventional methods have been detected in lower respiratory tract infections, and RVs have been shown to be second to respiratory syncytial virus (RSV) as a cause of lower respiratory tract infections [Papadopoulos et al., 2002; Singh et al., 2007; Aramburo et al., 2011; Iwane et al., 2011; Venter et al., 2011]. RV and RSV infections have similar clinical symptoms, and it has been suggested that RV may have an important role in the development of recurrent wheezing and asthma [Lemanske et al., 2005; Jackson et al., 2008].

Many studies have demonstrated a close correlation between the RSV load and the disease severity in children with lower respiratory tract infections [DeVincenzo, 2004; Fodha et al., 2007; Kaplan et al., 2008]. However, there have been no reports on the correlation of the RV viral load with the disease severity. This study assessed the contribution of the viral load and age to the development of severe disease, using real-time quantitative reverse-transcription PCR (qRT-PCR) to measure the RV load in nasopharyngeal aspirates collected from patients. In addition, the degree of disease severity was

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estimated using a severity scoring system for clinical symptoms.

MATERIALS AND METHODS

Patients

Nasopharyngeal aspirates were collected between February 2008 and August 2009 from 412 children ≤3 years of age who were hospitalized for lower respiratory tract infections at Soma General Hospital, located in Soma City in the northeastern part of Fukushima Prefecture, Japan. There is only one hospital in this area that admits children. The study was approved by the Ethical Committee of Soma General Hospital, and informed consent for participation in the study was obtained from the parents of each child enrolled.

Lower respiratory tract infections were considered to include bronchitis, bronchiolitis, and pneumonia. Bronchitis was defined as a disease presenting with cough and the chest radiographic findings within the normal range or with enhanced pulmonary markings [Goodman, 2007]. Bronchiolitis was defined as a disease presenting with tachypnea, expiratory wheezing, dyspnea, and the chest radiographic findings of pulmonary emphysema with or without diffuse atelectasis [Watts and Goodman, 2007]. Pneumonia was defined as a disease presenting with rhinorrhea, cough, tachypnea, moist rales or wheezing on chest auscultation, and the chest radiographic findings of hyperinflation with bilateral interstitial infiltrates and peribronchial cuffing [Sectish and Prober, 2007]. The degree of disease severity of each RV infection was estimated and scored according to a severity scoring system as described previously (Table I) [Gern et al., 2002; Lemanske et al., 2005]. Patients suspected of having a bacterial infection because of a high white blood cell count and/or a high level of C-reactive protein were not eligible for the study.

TABLE I. Severity Scoring System

Symptom	Points
Fever (≥38°C)	1
Cough	
Mild	1
Moderate	2
Severe	3
Rhinorrhea	
Mild[a]	1
Moderate to severe[b]	2
Hoarseness	1
Duration of illness >4 days	1
Apnea	3
Wheezing	5
Cyanosis	5
Retractions	5
Tachypnea	5

[a]Suction 0–4 times/day or wipe every 2 hr or less.
[b]Suction ≥5 times/day or ≥1 wipe/hr.

Measurement of Viral Load Using Real Time qRT-PCR

Nasopharyngeal aspirates were treated as described by Drosten et al. [2003]. Briefly, the nasopharyngeal samples were weighed, gently shaken for 30 min with an equal volume of acetylcysteine (10 g/L) in 0.9% sodium chloride, and centrifuged for 10 min. The supernatants were stored at −80°C until analysis.

Viral RNA was extracted from 200 μl of specimen supernatant using a QIAamp MinElute Virus Spin Kit (Quiagen, Hilden, Germany), and the cDNA was synthesized using a Prime-Script RT reagent Kit (TaKaRa Bio Inc., Shiga, Japan) and an ABI-7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturers' instructions.

The PCR primer pair and probe were designed to detect all RVs as described previously [Scheltinga et al., 2005], and do not differentiate between species. The PCR mixture consisted of final concentrations of 1× Premix Ex Taq (TaKaRa), 400 nM each primer, 340 nM of probe, and 5 μl of target cDNA, and was made up to a volume of 20 μl with nuclease-free water. The cDNA was amplified by 40 two-step cycles (5 sec at 95°C for denaturation, 31 sec at 60°C for annealing and extension) using the ABI-7300.

The virus copy number was determined by comparison with a serially diluted plasmid standard of known concentration. Copy numbers of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were measured in each sample for normalization of samples. Real-time qRT-PCR for RSV [Hu et al., 2003] and parainfluenza virus 3 (PIV-3) [Watzinger et al., 2004], conventional PCR for human metapneumovirus (hMPV) [Ebihara et al., 2004], and the rapid influenza antigen test (Capillia Flu A + B: Alfresa Pharma Corporation, Tokyo, Japan) were also performed. Patients with positive PCR results for RSV, PIV-3, or hMPV, or with positive rapid influenza antigen tests, were excluded from the study.

Statistical Analysis

Non-parametric tests were used for non-normally distributed data. The Spearman rank correlation was used to assess correlations among viral load, severity score and age of patients. The *t*-test was used to compare baseline characteristics among groups. *P*-values ≤0.05 were considered statistically significant. Analyses were performed using the Statistical Package for Social Sciences, version 17.0 (SPSS, Inc., Chicago, IL).

RESULTS

Study Patients and Baseline Characteristics

During the study period, 412 patients, including 50 (12.1%) preterm or low-birth-weight patients, hospitalized because of lower respiratory tract infections

and ≤3 years of age, were enrolled. Of the 412 patients, 37 patients (9.0%) had bronchitis, 53 patients (12.9%) had bronchiolitis, 53 patients (12.9%) had both bronchiolitis and pneumonia, and 269 patients (65.3%) had pneumonia. RV was detected in 43 patients (10.4%). RSV-A was detected in 59 patients (14.3%), RSV-B in 55 (13.3%), influenza virus in 21 (5.1%), PIV-3 in 16 (3.9%), and hMPV in 8 (1.9%). RV co-infections with RSV, influenza virus, and hMPV were demonstrated in 22 (5.3%), 3 (0.7%), and 1 (0.2%) patient, respectively. There were no RV-PIV3 co-infections.

Among the patients only infected with RV, two (4.7%) had bronchitis, three (7.0%) had bronchiolitis, 12 (27.9%) had both bronchiolitis and pneumonia, and 26 (60.5%) had pneumonia. The characteristics of these patients are shown in Table II.

Viral Load and Severity Score

There were no significant correlations between the viral load and the severity score (Fig. 1), the viral load and age (Fig. 2), or the severity score and age (Fig. 3). However, since the curve in Figure 3 indicated that there was a close correlation between the severity score and age less than 11 months, correlations were also calculated by age based on two age groups, <11 months or ≥11 months. There were no significant differences in the characteristics between patients <11 months and ≥11 months of age, except for the presence of siblings in the family (Table II). The clinical diagnoses and the severity scores of these two groups are shown in Table III. Patients with severity scores <10 and ≥10 were classified with mild-to-moderate and severe disease, respectively.

Although there was a significantly negative correlation between the severity score and age in the patients <11 months of age, no correlation was observed in the patients ≥11 months of age (Fig. 4). There was a significantly positive correlation between the viral load and the severity score in the patients ≥11 months of age, but no correlation was seen in the group <11 months of age (Fig. 5). There was no correlation between the viral load and age of the patients in either age group (Fig. 6).

Clinical Course in the Patients With High Severity Scores

The clinical data of 15 patients with high severity scores (≥10), including three preterm or low-birth-weight infants with RV infection, are shown in Table IV. Of these patients, eight were hospitalized for 7 or more days, and 7 and 10 patients required administration of oxygen and steroids, respectively. Patients with wheezing and dyspnea received intravenous hydrocortisone (5 mg/kg/dose).

DISCUSSION

RV is the major cause of upper respiratory tract infections. Since the optimal temperature range for RV replication in tissue culture is 33–35°C, and the temperature of the lower respiratory tract has been assumed to be higher than 35°C, RV has not been thought to be one of the pathogens causing lower respiratory tract infections. However, direct temperature monitoring has demonstrated that lower airway temperatures can be less than 35°C, which indicates that the lower respiratory tract may be permissive for RV infection and replication [McFadden et al., 1985]. Moreover, an in vitro study has also indicated that some serotypes of RV replicate at core body temperatures and induce the release of pro-inflammatory cytokines and chemokines after infection of human respiratory epithelial cells [Schroth et al., 1999].

Clinical study findings, that RV was detected in specimens collected from the lower respiratory tract of volunteers experimentally infected with RV [Papadopoulos et al., 2000], and that RV was detected by PCR in 14–35% of children hospitalized with lower respiratory tract infections, have proved that RVs can be associated with lower respiratory tract infections [Juvén et al., 2000; Hayden, 2004; Cheuk et al., 2007; Chung et al., 2007; Hamano-Hasegawa et al., 2008]. In this study, 10.4% of the children hospitalized with lower respiratory tract infections were PCR-positive for RV, which also demonstrates that RV is a major cause of lower respiratory tract infections.

Many previous studies have identified risk factors for the development of severe disease in children with RSV infections. In particular, the immature

TABLE II. Characteristics of Patients Infected With Rhinovirus

	All patients	Age: <11 months	Age: ≥11 months	P^a
Patients (no.)	43	15	28	
Age (month)	15.0 ± 9.85[b]	6.0 ± 3.3	19.8 ± 8.8	
Viral load (log_{10} copies/ml)	4.0 ± 1.69	3.6 ± 2.04	4.2 ± 1.47	0.378
Severity score (points)	9.4 ± 5.88	10.0 ± 7.08	9.0 ± 5.23	0.601
GA (week)	38.4 ± 2.71	38.6 ± 1.63	38.2 ± 3.16	0.662
BBW (g)	2934.3 ± 452.9	2909.2 ± 267.16	2947.6 ± 530.64	0.795
Male (%)	60.0	53.0	64.0	0.496
Siblings (%)	58.0	80.0	46.0	0.034[a]
Daycare (%)	42.0	33.0	46.0	0.419

[a]*t*- test.
[b]Means ± standard deviation.
GA, gestational age; BBW, birth body weight.

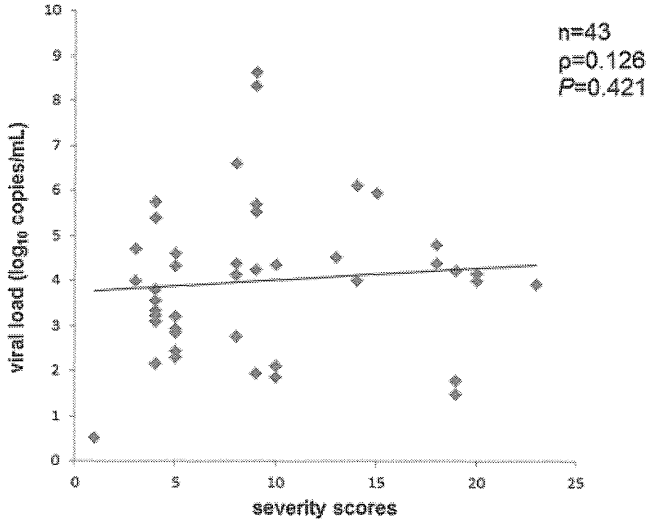

Fig. 1. Correlation of the viral load with the severity score. When all patients infected with rhinovirus (RV) were assessed, there was no significant correlation between the RV load and the severity score.

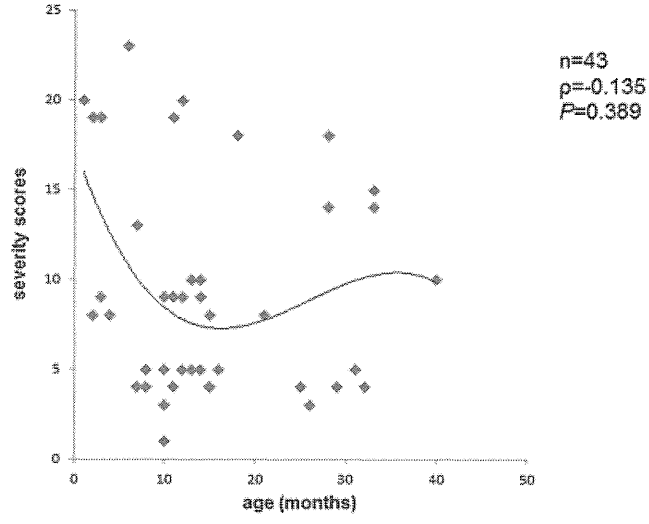

Fig. 3. Correlation of the severity score with age. When all patients infected with rhinovirus (RV) were assessed, there was no significant correlation between the severity score and age. The fitting curve in this figure demonstrates that there was a significant negative correlation between age and severity in the children <11 months of age by the Spearman rank correlation test.

respiratory tracts and the small diameters of the airways of infants contribute to disease severity and infant mortality. RSV-associated bronchiolitis leads to necrosis of the bronchiolar epithelium and destruction of epithelial cells, edema of submucosal and adventitial tissues, and excessive secretion of mucus. Mucus combined with cell debris and inflammatory cells can easily obstruct the small bronchioles of young infants [Mcintosh, 2007]. Moreover, in RSV infection, an immature immune response is responsible for delayed virus elimination and development of serious disease [Aberle et al., 1999; Renzi et al., 1999; van Schaik et al., 1999; Openshaw and Tregoning, 2005]. In addition to host risk factors, some studies have revealed that a high RSV load contributes to increased disease severity [DeVincenzo et al., 2005; Houben et al., 2010].

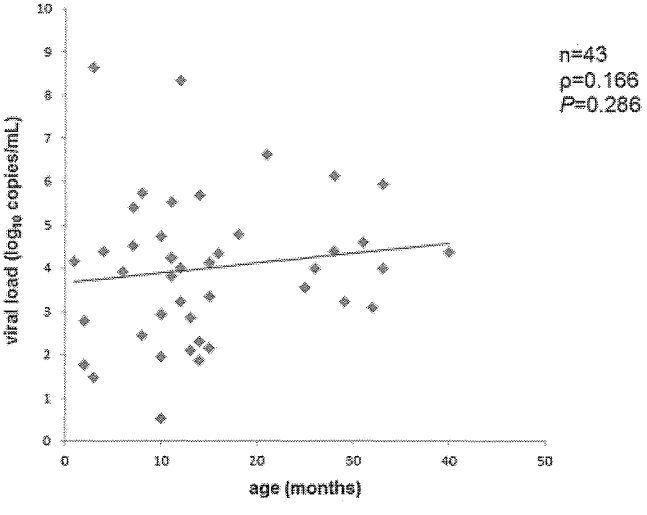

Fig. 2. Correlation of the viral load with age. When all patients infected with rhinovirus (RV) were assessed, there was no significant correlation between the RV load and age.

Although the clinical characteristics of patients infected with RV are similar to the characteristics of patients infected with RSV, maternal atopy is a risk factor for RV-associated bronchiolitis of increased severity [Miller et al., 2011]. Children with RV-associated wheezing are older, and more of these children present with atopic dermatitis and eosinophilia than do children with RSV infection [Korppi et al., 2004; Jartti et al., 2006]. RV infection also exacerbates asthma in adults by inducing inflammatory cytokines and eosinophilic infiltration in the lower respiratory tract [Message et al., 2008].

Correlation of the viral load with the severity of disease has not yet been reported in the patients with RV infections. In this study, 34% of patients who were PCR-positive for RV had high severity scores. This result suggests that RVs cause severe lower respiratory tract infections in infants and children.

Although there was no significant correlation between the viral load in nasal aspirates and the disease severity in children <11 months of age in this study, a significant negative correlation between age and severity was observed in this group. These results may indicate that, as in RSV infections, the immature immune responses and the small diameters of the airways in this age group contribute to the development of increased disease severity in RV infection. By contrast, a significant positive correlation between the viral load and the disease severity was observed among children ≥11 months of age, suggesting that