Fig. 3 Polio surveillance in Japan since 1950. A peak number of patients with polio was observed in 1960, and the live polio vaccine was introduced in 1961 (*upper panel*). After 1962, the number of patients with polio decreased, and no wild strain has been isolated since 1980
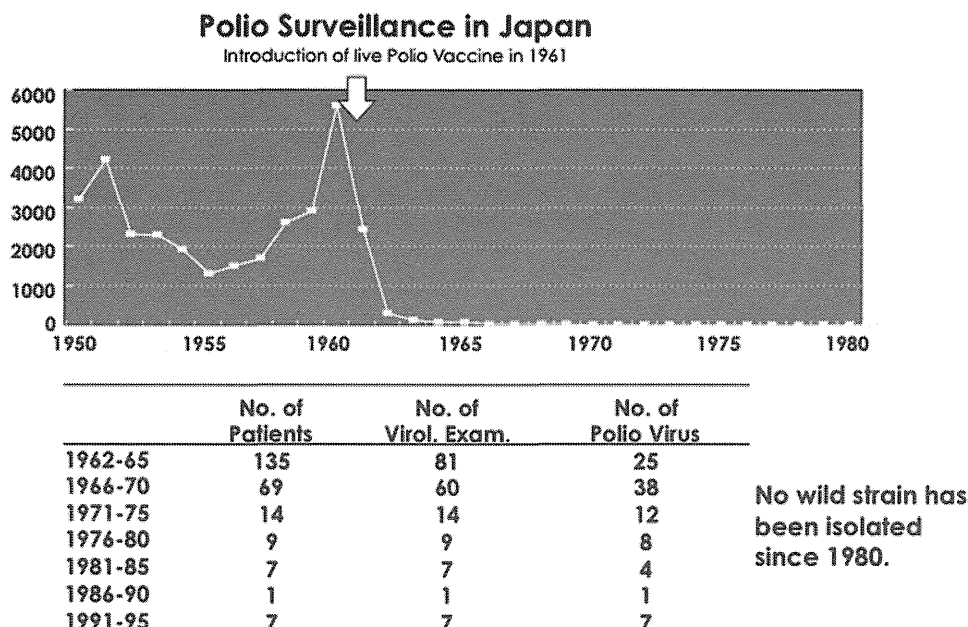
Polio Surveillance in Japan
Introduction of live Polio Vaccine in 1961

	No. of Patients	No. of Virol. Exam.	No. of Polio Virus
1962-65	135	81	25
1966-70	69	60	38
1971-75	14	14	12
1976-80	9	9	8
1981-85	7	7	4
1986-90	1	1	1
1991-95	7	7	7

No wild strain has been isolated since 1980.

Fig. 4 History of the pertussis vaccine and surveillance of the number of reported cases of pertussis and pertussis deaths. The DPT vaccine was recommend in 1968. *P* Pertussis vaccine, *DP* Diphtheria toxoid combined with pertussis vaccine, *DPT* Diphtheria and tetanus toxoids combined with pertussis vaccine, *DTaP* acellular pertussis vaccine combined with diphtheria and tetanus toxoids

History of pertussis vaccine and surveillance of pertussis
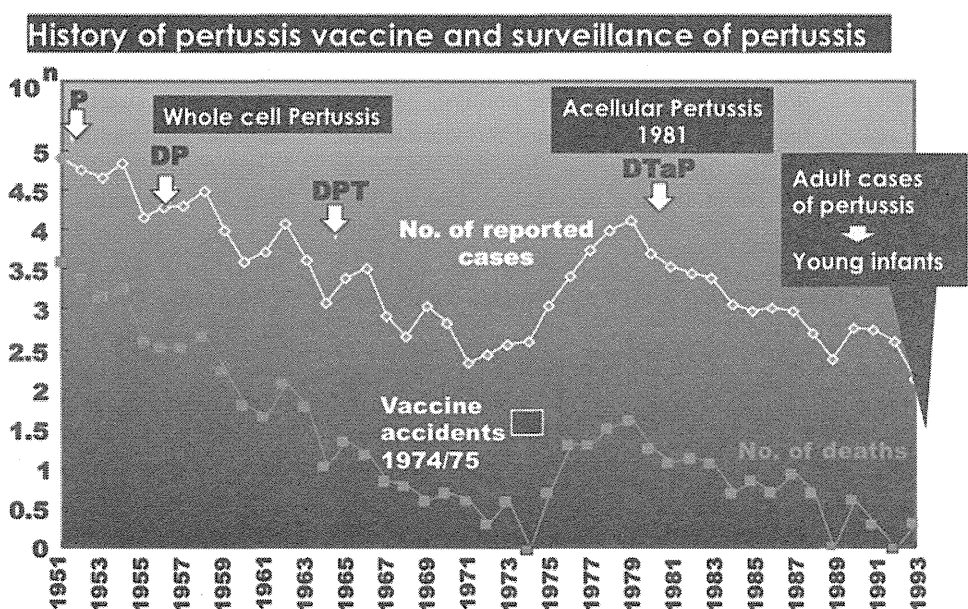
DTaP/IPV vaccines [33]. The wild poliovirus was imported in several situations from countries where wild polio has been circulating, and the high levels of vaccine coverage have been maintained. In addition to disease surveillance, environment surveillance of the vaccine for polio virus should focus on sewage monitoring [34].

Is the DTaP vaccine effective in controlling pertussis?

Pertussis is still a serious illness in young infants, and causes whooping cough, apnea, cyanosis, choking, and encephalopathy [35]. In Japan, the whole-cell pertussis vaccine was developed in 1949 and was combined with diphtheria and tetanus toxoids (DTwP). The results of pertussis surveillance and changes in vaccine strategy are shown in Fig. 4. Although febrile adverse illness was observed in 10 % of the recipients of DTwP, with local reactions of redness at 50–60 % and induration at 20 %, this vaccine was accepted. A routine immunization schedule was implemented with DTwP in 1968, resulting in a reduction in the reported cases of pertussis and pertussis deaths. In 1974–1975, two accidental deaths were reported after the administration of DTwP and, thereafter, DTwP

was temporarily discontinued. It was reintroduced for children aged 2 years old and older, or the DT vaccine was used instead of DTwP. The number of pertussis patients and pertussis deaths increased because of the low vaccine coverage [36, 37].

In 1981, a new type of acellular pertussis was developed, and a combined vaccine (DTaP) was introduced into recommended immunization practice. Principally, two types of DTaP vaccine (Biken-type, B-type; Takeda-type, T-type) were developed: the B-type consisted of two major antigens, pertussis toxin (PT) and filamentous hemagglutinin (FHA), and the T-type contained pertactin and fimbriae in addition to PT and FHA [38, 39]. Nationwide monitoring of clinical adverse events demonstrated low reactogenicity and sufficient antibody responses similar to natural infection. Since 1981, the number of pertussis patients has decreased after the acceptance of DTaP. However, the incidence of pertussis has recently been increasing in adults since 2002 in Japan, and several outbreaks on university campuses and in high schools and offices have been reported [40, 41]. Adult patients of pertussis are difficult to diagnose because of nontypical clinical features, including a prolonged cough. Also, the isolation of *Bordetella* or detection of the pertussis genome is not always successful because of the short duration of excretion of *Bordetella* influenced by the empirical administration of antibiotics or vaccination history [41, 42]. A surveillance system is currently under construction in Japan, based on a genetic diagnosis by loop-mediated isothermal amplification (LAMP) for detection of the pertussis genome [43].

DTaP was adopted by global vaccine makers because of the lower incidence of adverse events than that with DTwP, and multivalent combined vaccines, such as DTaP/Hib/IPV/HBV hexavalent vaccines, were developed based on DTaP. Pertussis is principally an infectious children's illness causing whooping and prolonged cough, and the Advisory Committee on Immunization Practices (ACIP) recommended a five-dose DTaP schedule, at ages 2, 4, 6, 15–18 months, and 4–6 years, instead of the previous DTwP in the US in 1997 [44]. In the 1990s, the incidence of pertussis at an older age increased in many countries because of waning immunity after primary childhood immunization and antigenic changes in pertussis, and adolescent pertussis was identified as the source of the transmission of pertussis to young infants through enhanced surveillance studies [45]. In Japan, the number of newborn pertussis cases increased in household contact [46]. In 2005, the tetanus toxoid, combined with a reduced concentration of diphtheria toxoid and acellular pertussis components (Tdap) vaccine, was licensed in the US, and the ACIP recommended that adolescents aged 11–18 years old should receive a single dose of Tdap for a booster

immunization [47]. It is now recommended for all generations from 19 to more than 64 years of age who have not been vaccinated in the past 10 years [48]. In Japan, DT was recommended at the age of 11–12 years, and the lack of pertussis booster immunization is one of the reasons why the number of patients with pertussis has increased in adults. The booster effect of a reduced volume of DTaP was investigated instead of the DT vaccine at 11–12 years of age, and 0.2 ml DTaP induced sufficient antibodies against PT and FHA without serious adverse events [49]. Even with high vaccine coverage, the number of pertussis patients increased globally because of the short duration of vaccine immunity. Several DTaP candidates containing additional protective antigen(s) are now under investigation [50].

Does the influenza split vaccine prevent infection?

Two types of influenza virus vaccines are now globally available, inactivated and cold-adapted live attenuated vaccines. There are three types of inactivated vaccines: whole virion, split, and subunit inactivated vaccines. The whole virion inactivated vaccine induced febrile reactions after the vaccination, and thereafter the split vaccine was licensed in 1972 in Japan, which has been used for more than 40 years with a lower incidence of febrile reactions. The split vaccine is made by destroying the structure of virus using detergents and ether to remove their lipid components from the formalin-inactivated whole virion. The HA subunit vaccine is purified from the HA fragments zone [51]. Changes in immunization policies, vaccine production, and the population aged less than 15 and more than 65 years are shown in Fig. 5. The transmission of influenza was believed to be associated with contact with schoolchildren, and, thereafter, the influenza vaccine has been recommended every year as school immunization in primary schools since 1962 [52]. In the 1960s, the pediatric population (<15 years of age) was more than 20 million, and more than 25 million doses of influenza vaccine were produced. The effects of school immunization on decreasing the social impact of influenza were questionable, and a comparative study was performed. There was no difference in the number of reported cases, number of hospital visits, and cost of healthcare insurance among several cities with or without school immunization in Gunma Prefecture in the early 1980s. This study provided evidence that school immunization had no effect on reducing the impact of influenza in the community, but had a limited effect on an individual basis [53, 54]. The influenza vaccine strategy was shifted from an obligatory routine vaccine to a voluntary vaccine in 1994. School immunization was interrupted in 1995, and the total

Fig. 5 Changes in the immunization strategy of the influenza vaccine, population more than 65 years and less than 15 years of age, and vaccine production in million doses
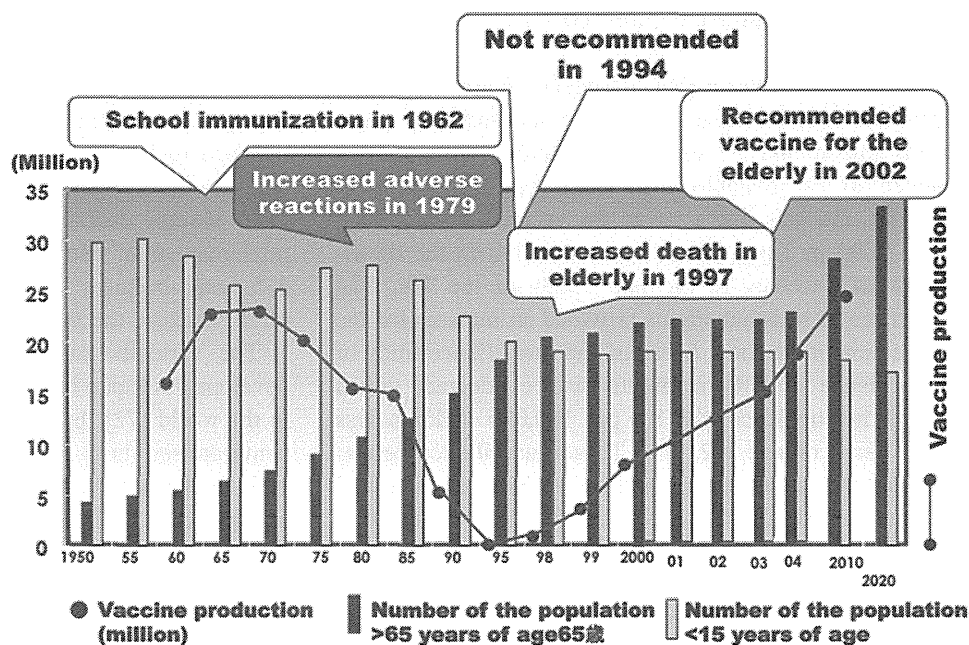
amount of vaccine produced was at its lowest, 0.35 million doses. A large outbreak of H3N2 was observed in 1997, and several deaths were reported in many nursing homes for the elderly as social topics. It has been recommended as a routine recommended vaccine for the elderly more than 65 years of age since 2002 for the benefits of vaccine recipients [55].

Three pandemics of influenza occurred in the 20th century. The most devastating pandemic dated back to 1918 and was known as Spanish flu. It was caused by a highly pathogenic H1N1 influenza virus transmitted through some animals from an avian pathogenic virus and is estimated to have killed 40–50 million people [56]. In 1957, Asian influenza A/H2N2 caused the second pandemic, and Hong Kong influenza A/H3N2 appeared as the third pandemic in 1968. Seasonal influenza outbreaks or epidemics are caused by an antigenic drift of A/H1N1 or A/H3N2, whereas these pandemics appeared as an antigenic shift, leading to a new strain, which is thought to be a re-assortment with the non-preexisting features of hemagglutinin (HA) or neuraminidase (NA) in human influenza viruses. After the 1968 pandemic of A/H3N2, several cases and small local outbreaks were reported, caused by new strains, H5, H7, or H9, and were considered to be from poultry, with H5 being very close to humans as a target for vaccine development [57]. A regional outbreak of H5 was reported in Hong Kong in 1997, and 6 of 18 patients died, causing an H5 pandemic threat [58]. Sporadic H5 transmission on poultry farms and in migratory birds has spread across Asia to the EU and Africa, and approximately 610 cases of human H5 infection have been reported at present

in 2013 since 2003, showing a high mortality rate of approximately 60 %. Most cases have involved close and direct contact with poultry, with no definite evidence of human-to-human transmission. There are several barriers to human-to-human transmission: receptor usage of the HA protein, cleavage efficiency by cellular protease, and host factors. H5N1 is considered to be a target for the pandemic vaccine, and the WHO addressed sharing viruses and sequence information for a future pandemic vaccine development [57, 59]. The development of an effective and safe vaccine is expected to mitigate the threat of a pandemic.

Several types of H5 vaccines have been developed, basically based upon the HA split, subunit vaccine, or whole virion inactivated with adjuvant. In Japan, alum-adjuvanted H5N1 whole virion inactivated vaccine (WIV) (alum concentration, 300 μg/ml) was developed using a genetically engineered reassortant, the NIBRG-14 strain, originating from H5N1/A/Vietnam/1194/2004. In a clinical phase II/III trial in healthy adults, alum-adjuvanted WIV (HA protein, 15 μg) led to favorable immunogenicity, >70 % sero-conversion rate in neutralization tests (NT) antibodies, without causing any serious systemic illnesses [60]. However, when it was administered to young infants and children at a reduced dose, 7.5 or 3 μg, a high body temperature (≥ 38.0 °C) was observed in approximately 60 % of recipients less than 7 years of age, and, unexpectedly, NT antibody titers were higher in children than in the clinical trial in adults. These phenomena were associated with the enhanced production of inflammatory cytokines [61].

Introduction of Hib, PCV7, and HPV

Hib and PCV are the major pathogens of bacterial meningitis and invasive systemic bacteremia, and they cause serious pneumonia. In the past, bacterial infection was believed to be treatable with antibiotics through early diagnosis and was not a target for vaccine development before 2000 in Japan. However, a shift led to the development of vaccines in the late 1980s in the US. The appearance of resistant strains provided an impetus for the introduction of vaccines. In Japan, the surveillance study of the incidence of Hib meningitis was conducted, which showed the incidence was 8.3 per 100,000 children less than 5 years of age [62, 63]. These surveillance results estimated 600 cases of serious invasive Hib infection, and then, the Hib vaccine was introduced. In the postmarketing study, the practical usage of Hib simultaneously administered with DPT was confirmed to be safe and effective, similar to separate administration [64]. It was allowed in 2008, and Hib was the first vaccine imported from a foreign country. Thereafter, PCV7, HPV, and Rota vaccines were licensed. Hib, PCV7, and HPV vaccines were temporarily adopted as routine recommended vaccines in 2010 with tentative financial support and were engaged to be covered as routine recommended vaccines in 2013 [10]. After the introduction of Hib and PCV7, the incidence of serious invasive infection decreased whereas the *Streptococcus pneumoniae* 6B and 19A serotypes uncovered by PCV7 are increasing, with a higher number of penicillin-resistant strains [65, 66]. Hib infection became controlled but *S. pneumoniae* has approximately 100 serotypes, using serotype replacement after the introduction of PCV7 and PCV13 to be licensed.

Action for the routine immunization of mumps, zoster, and hepatitis B vaccines

Five live mumps vaccine strains were developed in the 1970s from domestic wild strains isolated in the 1960s and 1970s [67, 68]. MMR vaccines containing four domestic vaccine strains were used, but were discontinued because of the unexpected high incidence of aseptic meningitis. Thereafter, monovalent mumps vaccines were used and the incidence of aseptic meningitis was evaluated. We reported that the incidence of aseptic meningitis was 13/1,051 (1.24 %) in patients with symptomatic natural mumps infection and was estimated to be 0.7–1.1 % of overall infections considering asymptomatic infections, and 10/21,465 (0.05 %) in vaccine recipients [69]. Although aseptic meningitis is considered to be an apparent adverse event of the mumps vaccine, its incidence is considerably lower than among those with symptomatic natural infections. It provides informative findings for consideration of resuming the mumps vaccine as a part of a routine immunization schedule for Japanese children. Regarding mumps deafness, the incidence of deafness was previously believed to be 1 in 15,000 [70], but irreversible mumps deafness occurred at a higher incidence, in 1 case per 1,000 [71]. Mumps deafness is one of the targets for vaccine implementation. Mumps outbreaks were observed every 3–5 years because of low vaccine coverage, less than 40 %, because the vaccine was voluntary.

The varicella zoster virus vaccine OKA strain was developed in Japan in 1974, and is the only strain available in the world [72]. Initially, it was developed for immunocompromised hosts who develop serious complications with chickenpox [73]. It causes no serious adverse reaction and protects against the onset of illness by immediate inoculation within 3 days of contact with patients in pediatric wards [74]. It was allowed for use in healthy infants, but the yearly epidemiological pattern did not change because of the low vaccine coverage, less than 40 % [75].

Mumps and zoster vaccines were universal vaccines in the US and EU but were voluntary in Japan [10, 12, 75]. The hepatitis B vaccine (HBV) is still a voluntary vaccine, as HBV was developed to interrupt the carrier through vertical transmission from carrier mothers positive for the HBe antigen [76]. HBV was given at 2, 3, and 5 months of age, and the number of carriers became markedly reduced. Recently, cases of nosocomial infections or horizontal transmission cannot be neglected, and HBV should be adopted as a universal vaccination [77]. Mumps, zoster, and HBV are still voluntary vaccines in Japan although they are globally recommended as universal vaccines. These vaccines are anticipated to be routine recommended vaccines.

Requirement for future immunization

The disease surveillance system in Japan is based on 3,000 sentinel clinics or hospitals for pediatric infectious diseases and reflects the tendency of infectious diseases, not population-based incidences. The immunization strategy is decided based upon disease surveillance, and monitoring of vaccine-associated adverse events is important to assess the safety. It is now based on postmarketing surveillance in Japan and should be developed in a systematic administrative form, together with laboratory investigations. It is difficult to identify the relationship of vaccination to serious adverse events occurring within a few weeks after immunization, and, in most cases, a direct relationship could not be identified. In 2005, a serious case of acute disseminated encephalomyelitis (ADEM) was reported

after vaccination with the Japanese encephalitis vaccine (JEV). At that time, JEV was produced from purified virus particles from mouse brains infected with Japanese encephalitis virus. Therefore, JEV has the potential to cause allergic encephalomyelitis. JEV was suddenly discontinued in a shortsighted political decision, without considering the effects of blank periods without JEV. At that time, tissue-culture JEV was ready to be marketed. Comprehensive decisions are required and should be made after scientific discussion.

No organization for decision making on immunization is currently systematized in Japan, such as the Advisory Committee on Immunization Practices (ACIP) of the US [12]. An investigational Committee on Immunization was organized to propose immunization strategies to decision makers and to discuss problematic issues based on the scientific evidence. However, this committee has been organized in the administrative agency, the Ministry of Health, Labor, and Welfare. Although issues on immunization should be discussed based on scientific evidence as a third party, it belongs to the political side at present. It may be hard to listen to the clinical needs of general physicians for the improvement of immunization practice. It should be organized for the purpose of promoting public health with a longitudinal vision for immunization policies and prompt responses to the critical issues, without the influence by political changes.

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Sensitive detection of measles virus infection in the blood and tissues of humanized mouse by one-step quantitative RT-PCR

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Live attenuated measles virus (MV) has long been recognized as a safe and effective vaccine, and it has served as the basis for development of various MV-based vaccines. However, because MV is a human-tropic virus, the evaluation of MV-based vaccines has been hampered by the lack of a small-animal model. The humanized mouse, a recently developed system in which an immunodeficient mouse is transplanted with human fetal tissues or hematopoietic stem cells, may represent a suitable model. Here, we developed a sensitive one-step quantitative reverse transcription (qRT)-PCR that simultaneously measures nucleocapsid (N) and human RNase P mRNA levels. The results can be used to monitor MV infection in a humanized mouse model. Using this method, we elucidated the replication kinetics of MV expressing enhanced green fluorescent protein both *in vitro* and in humanized mice in parallel with flow-cytometric analysis. Because our qRT-PCR system was sensitive enough to detect MV expression using RNA extracted from a small number of cells, it can be used to monitor MV infection in humanized mice by sequential blood sampling.

Keywords: measles virus infection, humanized mouse, quantitative RT-PCR, EGFP expression, flow cytometry

INTRODUCTION

Measles, a highly contagious childhood disease caused by the measles virus (MV), affects more than 20 million people each year. MV infection is characterized by a high fever with typical Koplik's spots followed by the appearance of a generalized maculopapular rash, and is often associated with respiratory and neuronal complications (Griffin, 2007). Since the implementation of vaccination programs using an effective live attenuated MV vaccine, global measles deaths have decreased dramatically. Nevertheless, measles is still one of the leading causes of death among young children under the age of 5 years, especially in countries with weak health infrastructures, and approximately 158,000 measles death occurred in 2011 (http://www.who.int/mediacentre/factsheets/fs286/en/). The ongoing global vaccination strategy aims to protect small children at high risk.

The MV vaccine is safe, effective, and inexpensive. Based on its long and successful vaccination history, several groups have taken advantage of reverse-genetics technology to utilize the live attenuated MV vaccine strain as a viral vector to elicit immune responses against foreign antigens from various pathogens, such as Env or Gag of human immunodeficiency virus (HIV; Lorin et al., 2004; Stebbings et al., 2012), hepatitis B surface (S) antigen (Singh et al., 1999; Reyes-del Valle et al., 2009), fusion protein of respiratory syncytial virus (Sawada et al., 2011), and envelope glycoprotein of West Nile virus (Despres et al., 2005; Brandler et al., 2012). MV is a human-tropic virus that uses CD46, signaling of lymphocyte activation molecule (SLAM, CD150), and the recently identified epithelial-cell receptor nectin-4 (PVRL4, see review in Kato et al., 2012) as receptors. To test the immune response against MV-based recombinant vaccines, both MV receptor-transgenic mice (Singh et al., 1999; Lorin et al., 2004; Despres et al., 2005) and non-human primates have been used as animal models (Reyes-del Valle et al., 2009; Brandler et al., 2012; Stebbings et al., 2012).

Although non-human primates are susceptible to MV, and they develop pathologies similar to those that occur in humans, the expense of using monkeys in research limits the number of animals that can be used for studies. To overcome such practical problems, various types of human MV receptor-transgenic mice expressing CD46 or CD150 have been developed (review in

Sellin and Horvat, 2009). Unfortunately, MV infection of all of these human MV receptor-expressing mouse models is severely restricted by the presence of murine type I IFN; to establish MV infection, it is necessary to introduce the IFNα receptor knockout into the MV receptor-transgenic mice, even in strains expressing CD150 driven by a native human promoter (Ohno et al., 2007). The IFNα receptor knockout/CD150 knock-in mouse is highly susceptible to MV infection and reproduces some aspects of MV infection in humans, including immunosuppression (Koga et al., 2010). This makes it a useful mouse model for study purposes. However, one problem is the lack of an initial innate immune response, which may modify the outcome of MV infection. Thus, the model may not truly reflect the outcome in humans.

In the early 2000s, a series of immunodeficient mice were developed that allow efficient transplantation of human cells or tissues; these systems are collectively termed "humanized mice." A large number of studies have described the development of human hematopoietic cells and their immunological functions in humanized mice, and technical modifications have been made for the study of various human diseases (Ito et al., 2012). Currently, humanized mouse systems are widely used as alternatives to non-human primate models, especially for the study of human-tropic infectious diseases such as HIV, human T cell leukemia virus (HTLV), dengue virus, HCV, and EB virus (Akkina, 2013). Of the different humanized mice models, the BM/Liver/Thymus transplanted (BLT) mouse, which is transplanted with human fetal liver and thymus tissue in addition to hematopoietic stem cells (HSCs), is recognized as the model that most closely mimics the human immune response (Wege et al., 2008). However, the use of this model is limited, mainly because of the ethical issues surrounding human fetal organs/tissues.

We have recently established an HIV infection model in NOD/SCID/Jak3null (NOJ) mouse transplanted with human cord blood HSCs (Terahara et al., 2013). To study MV infection in humanized NOJ (hNOJ), we infected an MV vaccine strain (AIK-C) expressing enhanced green fluorescent protein (EGFP) into hNOJ and analyzed the MV-infected cells by flow cytometry. The hNOJ mouse is highly susceptible to MV infection; in that study, we observed that GFP$^+$ cells were present in systemic lymphoid tissues and bone marrow (BM). Because it is important to assess MV infection kinetics in an animal without sacrificing the infected mouse, we developed a highly sensitive one-step quantitative reverse transcription-PCR (qRT-PCR) system to monitor MV infection in human peripheral blood mononuclear cells (PBMCs) circulating in the blood of humanized mice. In this study, we describe how this monitoring system works and demonstrate that the results obtained reflect the actual frequency of MV-infected cells, as determined by flow cytometry.

MATERIALS AND METHODS
CELL FRACTIONATION OF PBMCs
Peripheral blood mononuclear cells were obtained from human blood samples of healthy volunteers. Samples were collected after obtaining the approval of the institutional ethical committee of the National Institute of Infectious Diseases (NIID; No.

350) and written informed consent from each subject. PBMCs were separated by Ficoll–Hypaque density-gradient centrifugation (Lymphosepal; IBL, Gunma, Japan).

To obtain monocyte-derived dendritic cells (MDDCs), monocytes were enriched from PBMCs using CD14 microbeads (Miltenyi Biotec) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics in the presence of interleukin-4 (IL-4) and granulocyte–macrophage colony-stimulating factor (GM-CSF; both 10 ng/ml, from Pepro-Tech Inc., London, UK) for 1 week. T cells were isolated from CD14-negative PBMCs using the Total T Cell Enrichment Kit (STEMCELL technologies, Vancouver, BC, Canada).

PREPARATION OF RNA
Total RNA was extracted from mouse blood, BM, and spleen of humanized mice, human PBMCs, and Jurkat cells expressing human SLAM (Jurkat/hSLAM) using the RNeasy Mini Kit (QIA-GEN, Valencia, CA, USA) or the Total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, USA).

To prepare a standard of MV RNA, the cDNA encoding measles virus nucleocapsid (N) (MV-N: AB052821) was subcloned into the pBluescript II vector, and then MV-N RNA was produced by in vitro RNA transcription using the T7 RiboMAX^{TM} Express Large Scale RNA Production System (Promega, Madison, WI, USA). The RNA product was purified by DNase treatment, followed by phenol–chloroform extraction and ethanol precipitation, according to the protocol supplied by the manufacturer. The final concentration of RNA was measured using an ND-1000 spectrophotometer (Thermo, Waltham, MA, USA).

PREPARATION OF STANDARD TEMPLATE DNA
To prepare a standard template DNA, cDNAs of human CD45 (hCD45: NG_007730) and RNase P (NM_006413) were synthesized from total RNA of CEM cells by reverse transcription (RT)-PCR using SuperScript III RT/Platinum Taq Mix (Invitrogen, Carlsbad, CA, USA). The products were further amplified by PCR using TaKaRa Ex Taq Hot Start Version (TAKARA, Otsu, Shiga, Japan) for hCD45, or AmpliTaq Gold 360 (Applied Biosystems, Carlsbad, CA, USA) for RNase P. These PCR products of hCD45 and RNase P were subcloned into plasmids using the pGeneBLAzer TOPO TA Expression kit (Invitrogen) and pGEM-T (Easy) Vector Systems (Promega), respectively.

REAL-TIME RT-PCR ASSAY
To perform real-time qRT-PCR, SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) was used according to the manufacturer's instructions. Briefly, each reaction contained 1× reaction mix, ROX reference dye, SuperScript III RT/Platinum TaqMix, 0.2 μM specific primers, and 0.1 μM TaqMan probe. Reactions were performed on an Mx3000P qPCR system (Agilent Technologies). Thermocycling parameters included a RT step at 50°C for 20 min, followed by a DNA polymerase activation step at 95°C for 2 min and 50 PCR cycles (95°C for 20 s, 60°C for 30 s). Threshold cycle (C_t) values were calculated for each reaction; C_t represents the cycle at which a statistically significant increase in the emission intensity of the reporter relative to the passive reference dye is first detected.

For detection of hCD45 mRNA, the following sequences were used: forward primer, 5'-GGA AGT GCT GCA ATG TGT CAT T-3'; reverse primer; 5'-CTT GAC ATG CAT ACT ATT ATC TGA TGT CA-3'; TaqMan probe; 5'-FAM-ACA ACT AAA AGT GCT CCT CCA AGC CAG GTC T-BHQ1-3' (Hamaia et al., 2001). For detection of RNase P mRNA: forward primer, 5'-AGA TTT GGA CCT GCG AGC G-3'; reverse primer, 5'-GAG CGG CTG TCT CCA CAA GT-3'; TaqMan probe, 5'-FAM-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1-3' (Kimberly et al., 2005). For detection of MV-N RNA: forward primer, 5'-CGA TGA CCC TGA CGT TAG CA-3'; reverse primer, 5'-GCG AAG GTA AGG CCA GAT TG-3'; TaqMan probe, 5'-FAM-AGG CTG TTA GAG GTT GTC AG AGT GAC CAG-BHQ1-3' (Hummel et al., 2006).

GENERATION OF HUMANIZED MICE

Humanized NOD/SCID/JAK3null mice were established as described previously (Terahara et al., 2013). In brief, NOJ mice were transplanted with human HSCs ($0.5–1 \times 10^5$ cells) enriched from human umbilical cord blood cells into the livers of irradiated (1 Gy) newborn mice within 2 days after birth. All mice were maintained under specific pathogen-free conditions in the animal facility at NIID and were treated in accordance with the guidelines issued by the Institutional Animal Care and Committee of NIID.

Human umbilical cord blood was donated by the Tokyo Cord Blood Bank (Tokyo, Japan) after obtaining informed consent. The use of human umbilical cord blood cells was approved by the Institutional Ethical Committees of NIID and the Tokyo Cord Blood Bank. Human HSCs were isolated using the CD133 MicroBeads Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity was approximately 90% as assessed by flow cytometry.

PREPARATION AND INFECTION OF MV

Recombinant wild-type MV (IC323: AB016162) expressing EGFP (IC323-EGFP; Hashimoto et al., 2002) and a recombinant vaccine strain of MV (AIK-C: S58435) expressing EGFP (AIK-C-EGFP; Fujino et al., 2007) were grown in Vero/hSLAM cells. Virus titers were determined by plaque assay using Vero/hSLAM cells.

Jurkat/hSLAM cells were infected with various doses of MV [multiplicity of infection (MOI) = 0.25, 0.05, and 0.01] by incubation at 37°C for 1 h, washed twice with phosphate buffered saline (PBS), and seeded on 24-well plates. Cells were harvested immediately after washing (time 0) or 6, 12, 18, or 24 h later. The harvested cells were either lysed for RNA extraction or analyzed by flow cytometry.

Humanized NOD/SCID/JAK3null mice were challenged intravenously (i.v.) with different doses [200, 2,000, 10,000, or 20,000 plaque-forming units (pfu)] of AIK-C-EGFP. Peripheral blood was obtained from MV-infected hNOJ mice at 3, 5, 7, 10, 14, and 21 days post-infection (p.i.). In some experiments, MV-infected hNOJ mice were sacrificed at day 7 p.i. At the time of sacrifice, peripheral blood, BM, spleen, and mesenteric lymph nodes (MLNs) were harvested, and red blood cells were lysed in ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA-2Na; pH 7.2–7.4).

FLOW-CYTOMETRIC ANALYSIS OF MV-INFECTED CELLS

PE-conjugated anti-human CD150 (A12) and Pacific Blue-conjugated anti-hCD45 (HI30) monoclonal antibodies (mAbs) were purchased from BioLegend Inc. (San Diego, CA, USA). Cells were stained with these mAbs, fixed with 2% formalin/PBS for 15 min at room temperature, washed, and kept at 4°C prior to flow-cytometric analysis. Dead cells were stained with a LIVE/DEAD Fixable Dead Cell Stain Kit (L34957; Invitrogen). Data were collected using a FACScanto (BD Biosciences, San Jose, CA, USA) and analyzed using the FACSDiva (BD Biosciences) or FlowJo (Tree Star, San Carlos, CA, USA) software.

STATISTICAL ANALYSIS

Non-parametric one-way ANOVA was performed to compare cell type-specific differences in hCD45 and RNase P mRNA expression. Spearman's rank correlation coefficient test was also performed to compare the level of MV-N expression and frequency of MV-infected cells. Prism ver.5 software (GraphPad Software, San Diego, CA, USA) was used for all analyses. $P < 0.05$ was considered statistically significant.

RESULTS

HUMAN-SPECIFIC qRT-PCR SYSTEM FOR THE DETECTION OF MV INFECTION

For the detection of MV infection in clinical specimens, Hummel et al. (2006) established a sensitive qRT-PCR system that used primer and probe sets targeting the MV-N gene. In our humanized mouse model, it is necessary to analyze endogenous mRNA expression in human PBMCs to determine the level of human cell-associated MV infection in mouse blood. We initially assumed that hCD45 expression would be suitable to discriminate human hematopoietic cells from co-existing mouse hematopoietic cells *in vivo*. On that basis, we designed human-specific primer and TaqMan probe sets for hCD45 and compared their usefulness with a primer/probe set for a widely used housekeeping gene, RNase P. RNA was extracted from humanized (hu-mouse) or non-humanized (non-hu-mouse) murine splenocytes, and the level of mRNA was measured by one-step qRT-PCR. Both hCD45 and RNase P primer/probe sets detected mRNA expression of target genes from human PBMCs present in hu-mouse spleen, at similar sensitivities, but neither set detected expression in non-hu-mouse (**Figure 1A**). Thus, both primer/probe sets are human-specific. Next, we enriched CD14$^+$ monocytes and T cells from PBMCs by positive and negative magnetic-bead selection, respectively, and then determined the copy numbers of hCD45 and RNase P in these cell fractions from each of five donors. In **Figure 1B**, the expression levels of hCD45 (left panel) and RNase P (right panel) in monocytes and T cells are depicted relative to the level in each donor's PBMCs. Because RNase P expression was less affected by cell type than CD45 expression (*$P < 0.05$), in subsequent experiments we exclusively used RNase P primer/probe sets as an endogenous control for mRNA expression.

PARALLEL INCREASE IN THE TIME COURSE OF MV-INFECTED CELL FREQUENCY AND MV-N RNA LEVEL *IN VITRO*

Because wild-type MV mainly utilizes SLAM as the receptor for entry into lymphoid cells (Tatsuo et al., 2000), the kinetics of MV
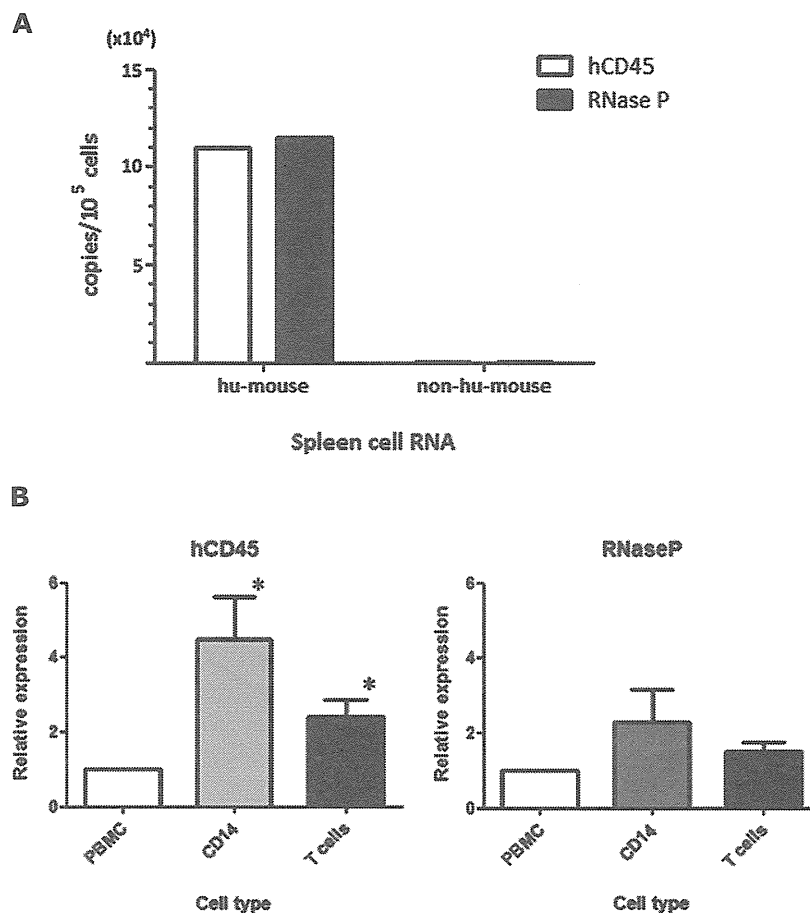
FIGURE 1 | Selection of an endogenous control for the analysis of MV-infected human PBMCs. (A) RNA was extracted from spleen cells of hNOJ and non-humanized NOJ, and one-step qRT-PCR was performed using primer and probe sets designed against the human-specific hCD45 and RNaseP mRNAs. To calculate copy numbers of these genes, the PCR products of human CD45 and RNase P were subcloned into plasmids and used as standard DNAs. (B) Human PBMCs from five donors were fractionated into CD14$^+$ monocytes and T cells. RNA from these cell populations was extracted, and the expression levels of hCD45 and RNase P were analyzed by qRT-PCR. The graph depicts the expression levels in these fractionated cells relative to the levels in PBMCs (defined as 1). Statistical differences in hCD45 and RNase P expression among these cell populations were evaluated by non-parametric one-way ANOVA test ($^*P<0.05$).

infection in Jurkat/hSLAM cells can be clearly visualized by flow cytometry. We infected Jurkat/hSLAM cells with a wild-type MV encoding EGFP (IC323-EGFP) at MOI of 0.01, 0.05, and 0.25. Cells were washed and harvested at 6, 12, 18, or 24 h after MV infection. A subset of the cells in each sample was analyzed by flow cytometry, and the remainder of the sample was used for RNA extraction. The mRNA levels of MV-N and RNase P were determined by qRT-PCR, and the level of MV-N mRNA relative to RNase P RNA was calculated. Representative results of three experiments are shown in Figure 2A (flow cytometry) and Figure 2B (qRT-PCR). Because of the rapid and strong cytopathic effect by MV at the highest MOI (0.25), we omitted the flow cytometry data corresponding to that condition. At MOI 0.01, a similar frequency of GFP$^+$ cells was detectable at 12 and 18 h p.i., whereas at MOI 0.05, the GFP$^+$ cell frequency was already high at 12 h p.i. Note that the level of hSLAM was not down-modulated by MV infection. Over the time course, relative MV-N expression level at all three MOIs increased in parallel

over two orders of magnitude, indicating that these two methods yield comparable results (as shown in Figure 2C) and are useful for monitoring the replication kinetics of MV infection *in vitro*.

PARALLEL INCREASE OF MV-INFECTED CELL FREQUENCY AND MV-N RNA LEVELS *IN VIVO*

We then applied these detection systems *in vivo* in MV-infected hNOJ mice. hNOJ mice were infected with an MV vaccine strain expressing EGFP (AIK-C-EGFP) at 2000 pfu, and the animals were sacrificed 7 days later. Blood PBMCs and BM cells were washed with PBS, and a subset of the cells in each sample were stained with anti-hCD45 mAb. Representative results of flow-cytometric analysis of BM cells from three mice are shown in Figure 3A. The percentages of GFP$^+$ cells in mice 127-1, 127-4, and 127-5 mice were low (0.002%), high (0.35%), and intermediate (0.028%), respectively. The number of human PBMCs obtained from mouse blood was not sufficient to determine GFP$^+$
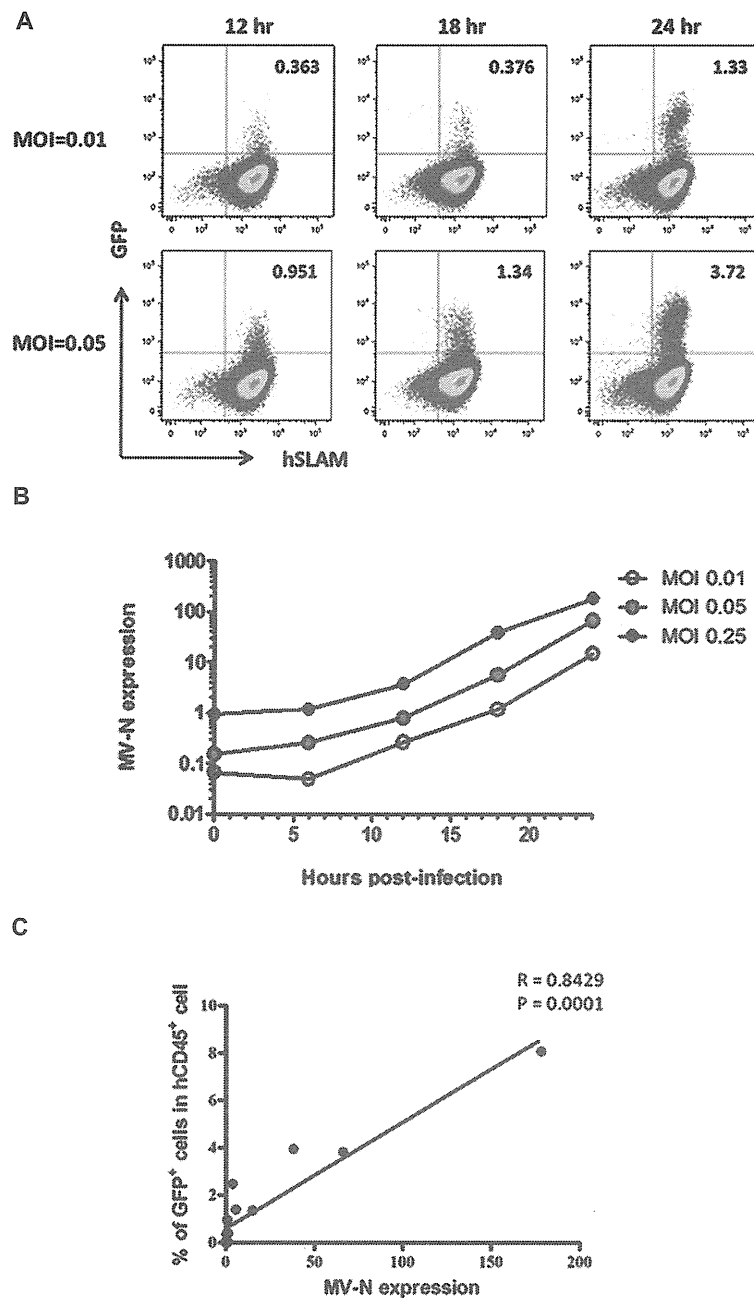
FIGURE 2 | Time course of MV infection in vitro. Jurkat/hSLAM cells were infected with wild-type MV IC323-EGFP at MOI of 0.01, 0.05, and 0.25, washed, and harvested at the indicated time points. **(A)** Cells were stained with PE-conjugated anti-hSLAM mAb, fixed with 2% formalin/PBS, and GFP expression was analyzed. **(B)** RNA was extracted from cells, and expression levels of MV-N and RNase P were analyzed by one-step qRT-PCR. The copy numbers of MV-N and RNase P were determined, and the ratio of MV-N copies to RNase P copies is depicted on the vertical axis. **(C)** Correlation between the percentage of GFP$^+$ Jurkat/SLAM cells and the time course of MV-N expression. Spearman's rank correlation coefficient was used for statistical analysis.

cell frequencies by flow cytometry. Next, we extracted RNA from PBMCs and BM cells and analyzed MV-N expression by qRT-PCR, as described in the previous section. MV-N expression paralleled the GFP$^+$ frequencies in BM (**Figure 3B**). Notably, a high level of MV-N expression was also detected in PBMCs of mouse 127-4, suggesting that the level of MV-N expression per single

hematopoietic cell is similar between blood and BM. We plotted the GFP$^+$ frequency and MV-N expression level in BM cells of eight mice. As shown in **Figure 3C**, these values were well correlated ($R = 0.9286$). Taken together, these data indicate that MV infection in vivo is detectable in BM by both flow cytometry and MV-N RNA qRT-PCR analysis, but only MV-N RNA qRT-PCR is
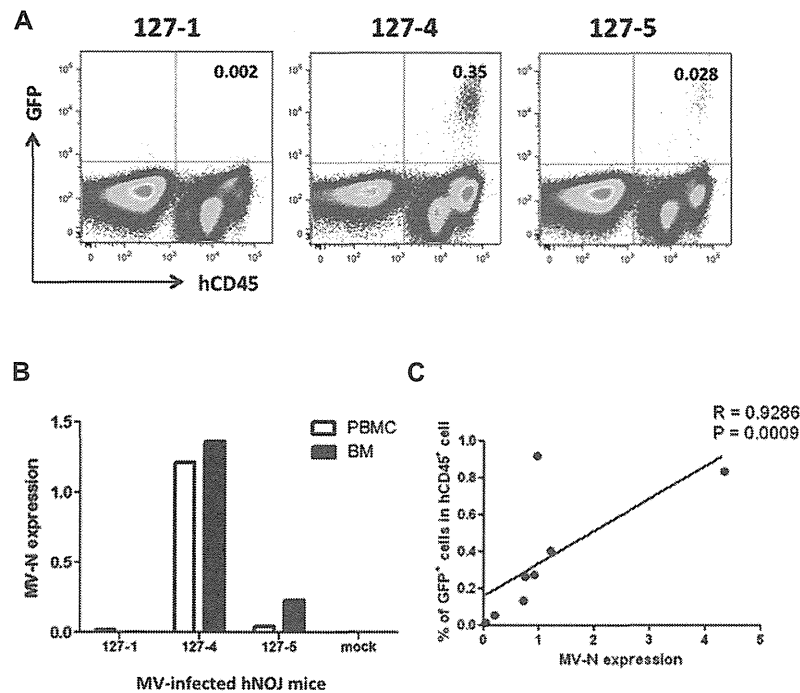
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FIGURE 3 | Analysis of MV infection *in vivo*. Three hNOJ mice (127-1, -4, and -5) were infected intravenously with 2,000 pfu of the MV vaccine strain, AIK-C-EGFP. Mice were sacrificed at day 7 post-infection, and blood and bone marrow cells (BM) were obtained. **(A)** BM cells were stained with PB-anti-human CD45 mAb, fixed with 2% formalin/PBS, and GFP expression was analyzed. **(B)** PBMCs from blood and BM cells were lysed, and RNA was prepared. The expression MV-N and RNase P was analyzed as described in the legend for **Figure 2B**. **(C)** Correlation between the percentage of GFP$^+$ cells among hCD45$^+$ cells in BM and the level of MV-N expression in MV-infected hNOJ mice, at day 7 ($n = 4$) or day 10 ($n = 4$) p.i. Spearman's rank correlation coefficient was used for statistical analysis.

sensitive enough to detect PBMC-associated MV infection in the blood.

KINETICS OF MV GROWTH CAN BE MONITORED IN THE BLOOD OF hNOJ MOUSE

Finally, we measured MV growth kinetics *in vivo* by qRT-PCR analysis using sequential blood samples obtained from MV-infected hNOJ mice; it was not feasible to perform these measurements by flow cytometry because of the paucity of human PBMCs in the blood. Two or three hNOJ mice in each group were infected intravenously with 200, 2000, or 20,000 pfu AIK-C-EGFP and followed up to 21 days p.i. The level of PBMC-associated MV RNA in individual mice is shown in **Figure 4A**. We noticed two peaks of MV replication, the first at around day 3 p.i., and the second at day 10 p.i., irrespective of the initial inoculum. Two mice infected with 20,000 pfu MV exhibited a high level of MV replication that peaked at day 10 p.i. One mouse infected with 2,000 pfu exhibited a high level of MV replication at day 3 p.i., followed by a small peak at day 10 p.i. For some mice, we counted the number of human cells per 50 μl of blood used for RNA extraction. The data are shown in **Figure 4B**. We were able to detect high levels of MV in samples containing less than 2,000 cells, indicating that the qRT-PCR system is sensitive enough to detect low numbers of MV-infected human cells.
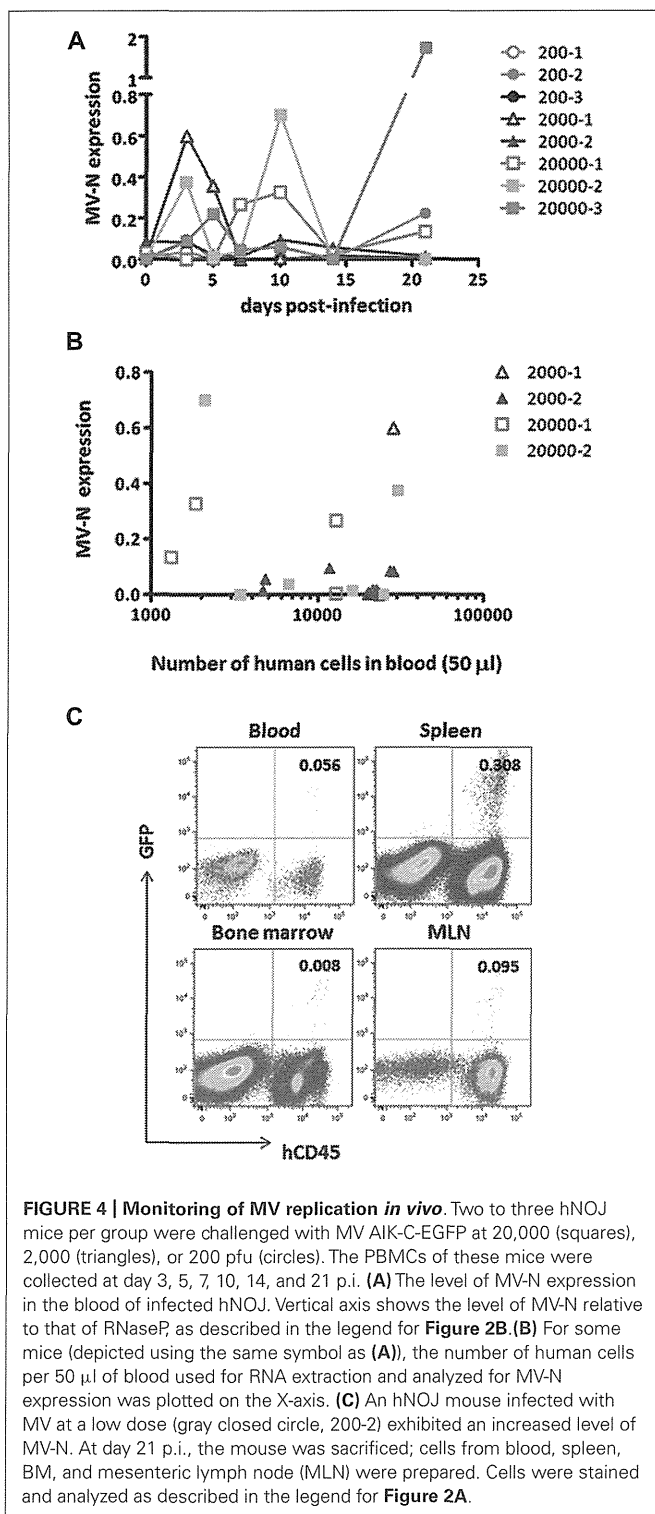
Although MV replication was not obvious in three mice infected with the smallest dose (200 pfu), one of these animals exhibited an increase in MV RNA expression at day 21 p.i. (gray circle). We sacrificed this particular mouse and used flow cytometry to analyze GFP expression in its blood, spleen, MLN, and BM. As shown in **Figure 4C**, GFP$^+$ cells were present in spleen (0.308%) and all the other tissues, albeit at a lower frequency, indicating that MV infection can occur even at a low dose (200 pfu) and spread slowly in the systemic lymphoid tissues of hNOJ.

It may be necessary to acquire at least 30,000 events to be sure of having >10,000 cells for flow cytometry analysis. This is because of the substantial amount of sample loss that occurs in this system. The flow cytometry data presented in **Figure 4C** were obtained by analyzing ~0.4 ml blood from a sacrificed mouse. However, even under these conditions, the proportion of MV-infected cells detected was only 0.056%; indeed, the cells are barely visible on the plot. Therefore, it appears that flow cytometry is not a suitable method for the sequential monitoring of infected (GFP$^+$) cells. Thus, the qRT-PCR system we have developed here allowed us to monitor systemic MV replication using a small volume of blood from humanized mice.

DISCUSSION

Based on a highly sensitive MV-N RNA detection method previously developed by Hummel et al. (2006), which could detect one copy of synthetic MV RNA/reaction, we developed a novel one-step real-time qRT-PCR system for the purpose of monitoring MV replication in the blood of MV-infected humanized mice.

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FIGURE 4 | Monitoring of MV replication *in vivo*. Two to three hNOJ mice per group were challenged with MV AIK-C-EGFP at 20,000 (squares), 2,000 (triangles), or 200 pfu (circles). The PBMCs of these mice were collected at day 3, 5, 7, 10, 14, and 21 p.i. **(A)** The level of MV-N expression in the blood of infected hNOJ. Vertical axis shows the level of MV-N relative to that of RNaseP, as described in the legend for **Figure 2B**. **(B)** For some mice (depicted using the same symbol as **(A)**), the number of human cells per 50 μl of blood used for RNA extraction and analyzed for MV-N expression was plotted on the X-axis. **(C)** An hNOJ mouse infected with MV at a low dose (gray closed circle, 200-2) exhibited an increased level of MV-N. At day 21 p.i., the mouse was sacrificed; cells from blood, spleen, BM, and mesenteric lymph node (MLN) were prepared. Cells were stained and analyzed as described in the legend for **Figure 2A**.

types present in human PBMCs, we found that RNase P expression was less dependent than CD45 expression on cell type. Using this qRT-PCR system with RNase P as an internal control, we can reliably detect MV replication with high sensitivity in humanized mice *in vivo*. When MV expressing GFP was used for infections *in vitro* or *in vivo*, the level of MV-N RNA was closely correlated with the frequencies of GFP$^+$ MV-infected cells determined by flow cytometry.

Our qRT-PCR system allowed us to follow MV replication *in vivo* using a small amount of blood, with no need to sacrifice mice at each time point. Although flow-cytometric analysis provides valuable information, such as the proportions of various cell types and the surface phenotypes of MV-infected cells, the small number of human cells circulating in the mouse blood may not be sufficient for precise estimation of MV-infected cells by flow cytometry. By contrast, our qRT-PCR system was able to detect MV-N RNA in fewer than 2,000 human PBMCs (**Figure 4B**). This is an important technological advantage considering that individual humanized mice exhibit variable levels of human cell engraftment, i.e., chimerism (Terahara et al., 2013); moreover, there may exist donor-to-donor variation in susceptibility to MV infection. Thus, it should be possible to select humanized mice with a degree of MV infection appropriate for the purpose of a given experiment.

In this study, MV was inoculated through the tail vein, and infected cells were distributed to systemic lymphoid tissues as well as BMs, where human hematopoietic cells localize in humanized mice (Traggiai et al., 2004). MV may also be distributed to other organs, such as lung and intestinal tissue, as demonstrated in the case of HIV infection using the BLT mouse (Sun et al., 2007). To our surprise, by monitoring MV replication in PBMCs of humanized mice, we noticed two peaks of MV replication, at around 3 and 10 days p.i., in some mice. This pattern of MV replication did not depend on the initial dose of MV inoculum. We do not know why MV replication showed two peaks in many animals. However, it was recently reported in a monkey model that MV RNA persists in PBMCs for more than 1 month after primary infection, and declined in three phases (Lin et al., 2012). The authors of that study hypothesized that both T cells, including regulatory T cells (Treg), and antibody responses contributed to the dynamics of MV replication *in vivo*. Although hNOJ mice are reported to show poor immune responses, the role of regulatory T cells should be considered. This is because these cells regulate HIV-1 infection in humanized mice (Jiang et al., 2008). Alternatively, it may be that the intravenous injection of MV rapidly kills the target cells (probably those showing an activated phenotype) within 3 days. The low number of MV-infected cells then gradually transmits the virus to the human cells that are replenished from the BM stem cell pool. Further investigations are required to clarify this issue.

The humanized mouse model is expected to be a useful tool for studying virus infection (Akkina, 2013). Although the human immune system is not fully reconstructed by the transplantation of human HSCs alone, we believe that further improvements are possible, which will allow us to utilize this mouse model to not only evaluate vaccine and drug efficacy but also to increase our understanding of the pathogenesis of MV infection. The described novel method of monitoring MV-infected human cells in the blood will

Because MV replication usually occurs in association with cells (Griffin, 2007), it is necessary to evaluate the endogenous RNA expression level of human PBMCs that co-exist with mouse blood cells. To this end, we designed human-specific primer/probe sets for the CD45 and RNase P mRNAs. When we analyzed the detection efficiencies of these two primer/probe sets using distinct cell

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be useful for studying MV-based vaccines in humanized mouse models without the need to sacrifice the mice.

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Novel Clinical Features of Recurrent Human Respiratory Syncytial Virus Infections

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Children and elderly individuals are often infected easily and repeatedly with human respiratory syncytial virus (HRSV); however, the features of recurrent infection in the same individual are defined poorly. To clarify the clinical significance of repeated HRSV infections in relation to subgroup epidemiology, this study performed prospective and longitudinal analyses in children with lower respiratory tract infections over 20 consecutive epidemics between 1985 and 2005 at a pediatric outpatient clinic in Kawasaki, Japan. HRSV infections were confirmed by 2 types of reverse-transcription PCR. Samples obtained from patients with repeated infections were subjected to sequence analysis and cloning analysis. A total of 1,312 lower respiratory tract infections observed in 1,010 patients were diagnosed as HRSV infections. Repeated HRSV infections occurred in 208 of the 1,010 patients. Analysis of the patients with repeated infections revealed that children were often infected multiple times even within a single short epidemic. Some patients were re-infected with strains having the same or virtually identical N gene sequences. In patients infected more than 4 times, cloning analysis revealed more frequent dual infections with both subgroups (23.8%). The HRSV-A subgroup caused subsequent homologous infections more frequently than did HRSV-B; furthermore, HRSV-A infections provided no protection from a second homologous infection. In contrast, HRSV-B infections offered significant protection against a second homologous infection. Statistical analysis revealed alleviation of symptoms with a reduced rate of dyspnoeic attacks only in the group re-infected with homologous HRSV-A strains. Thus, this study elucidates new clinical features of recurrent HRSV infection. *J. Med. Virol.*

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INTRODUCTION

Human respiratory syncytial virus (HRSV), of the family *Paramyxoviridae*, subfamily *Pneumovirinae*, genus *Pneumovirus*, is the leading cause of lower respiratory tract infections in infants and children [Parrott et al., 1973]. All infants experience at least 1 HRSV infection by 2 years of age [Glezen et al., 1986]. Despite the presence of circulatory antibodies against HRSV, recurrent infections in older children and adults occur throughout life, and protective immunity against re-infections is incomplete and brief [Hall et al., 1991]. There are 2 major antigenic subgroups, A (HRSV-A) and B (HRSV-B), and viruses from these subgroups are considered genetically distinct on the basis of sequencing data [Matheson et al., 2006]. Several findings have raised the possibility that antigenic differences between HRSV subgroups may contribute to re-infection, although the results were not conclusive [Mufson et al., 1987]. Both subgroups are subdivided into

several strains or genotypes according to the attachment glycoprotein (G) gene. More than 3 genotypes in each dominant subgroup usually co-circulate in epidemics, with some strains being replaced each year [Hall et al., 1990; Sullender, 2000; Galiano et al., 2005; Matheson et al., 2006]. The difference between genotypes might be considered to play a role in the establishment of re-infections; however, this requires further investigation [Sullender et al., 1998]. A birth cohort study in Kenya observed that 4 of 12 repeated infections recurred with an identical virus within the same short epidemic [Scott et al., 2005]. Furthermore, in a study with adult volunteers, clinical re-infections occurred repeatedly, even when the subjects were infected with the same strain of HRSV-A within a few months after a natural HRSV-A infection [Hall et al., 1991]. From a study of hospitalized children in Finland, no direct evidence of protection against re-infection with a homologous subgroup was found even within a single season [Waris, 1991]. To clarify the clinical characteristics and significance of HRSV re-infection, a prospective and longitudinal analysis of HRSV infections during 20 consecutive epidemics at the same outpatient clinic was conducted.

MATERIALS AND METHODS

Patients and Clinical Specimens

This study was performed at a private pediatric outpatient clinic in Kawasaki, Japan. A pediatrician in the clinic monitored children with symptoms of lower respiratory tract infection prospectively during the period from December 1985 to August 2005. The diagnosis of lower respiratory tract infection was based on major clinical manifestations such as expiratory wheezing, shortness of breath, hoarseness, barking cough with or without inspiratory stridor, deep or wet chest cough, rhonchi, and rales. Duration of fever $\geq$38°C and the existence of respiratory difficulty (retraction, expiratory wheezing, tachypnoea $\geq$50 breath/min, and/or orthopnoea) on the day of visit or during the illnesses were recorded as clinical features by the pediatrician. Nasopharyngeal secretions or nasal swabs were collected from all patients with lower respiratory tract infections, as described previously [Yui et al., 2003]. If an HRSV infection occurred $\geq$14 days after a previous infection or if the 2 infections were determined to involve different subgroups, the event was defined as a separate infection [Hall et al., 1991]. When a patient was diagnosed with HRSV infection for the first time during a visit to the outpatient clinic, this was determined as the first HRSV infection.

Informed consent for participation in this study was obtained from the parents of all the children. The study protocol was approved by the ethics committee of the Kitasato Institute for Life Sciences, Kitasato University.

Detection of HRSV Antigen

From a total of 1,735 clinical specimens, 1,690 were examined immediately for the presence of HRSV antigens with an enzyme-linked immunosorbent assay (ELISA) kit (Ortho Diagnostics, Raritan, NJ), a TestPack RSV enzyme immunoassay (EIA) kit (Abbott Laboratories, North Chicago, IL) or immunochromatography (IC) using ImmunoCard STAT RSV (Meridian Bioscience, Cincinnati, OH). From 1985 to 1986, an ELISA kit was used for direct antigen detection. Then, EIA was performed until December in 2004, when the manufacturer stopped providing the test kit. The IC test was used subsequently (Fig. 1).

RNA Extraction and RT-PCR

Total RNA was extracted directly from respiratory specimens (nasopharyngeal secretions or nasal swabs) using acid guanidinium thiocyanate phenol–chloroform with minor modifications [Yui et al., 2003], as described previously or using the High Pure Viral RNA Kit (Roche Applied Science, Manheim, Germany) according to the manufacturer's instructions. To amplify HRSV-A and HRSV-B simultaneously, an N gene region conserved between the 2 subgroups was selected (Table I). Early in the study, reverse-transcription PCR (RT-PCR) was performed using primer set 1 (Prs1), as described previously [Yui et al., 2003]. Later in the study, an increased number of samples tested negative by PCR using Prs1, although they were positive by EIA and IC. Because HRSV sequence mutation(s) at the position of Prs1 may cause a failure of PCR amplification, another primer set (Prs2) was synthesized (Table I). First-strand cDNA synthesis was carried out using the CN3 primer (for viral RNA) and CCN6 primer (for mRNA). cDNA was amplified by the first PCR using n-F1(+) and n-B1(−), followed by nested PCR using EcoF3′ (+) and NotB3 (−). When an HRSV infection was suspected clinically or epidemiologically, RT-PCR was conducted irrespective of the rapid antigen detection assay (Fig. 1). All RT-PCR procedures were performed according to the protocol described by Kwok and Higuchi [1989]. Every assay was performed with a negative control.

Restriction Fragment-Length Polymorphism

HRSV subgroups were distinguished using restriction fragment-length polymorphisms (RFLP) of the RT-PCR products, as reported previously [Yui et al., 2003]. In brief, the RT-PCR product of HRSV-A was digested with BglII and that of HRSV-B was digested with HaeIII. All PCR products were subjected to RFLP, except the clones obtained from the samples selected for cloning.

Nucleotide Sequencing

All the samples obtained from patients with repeated infections were subjected to sequence analysis.
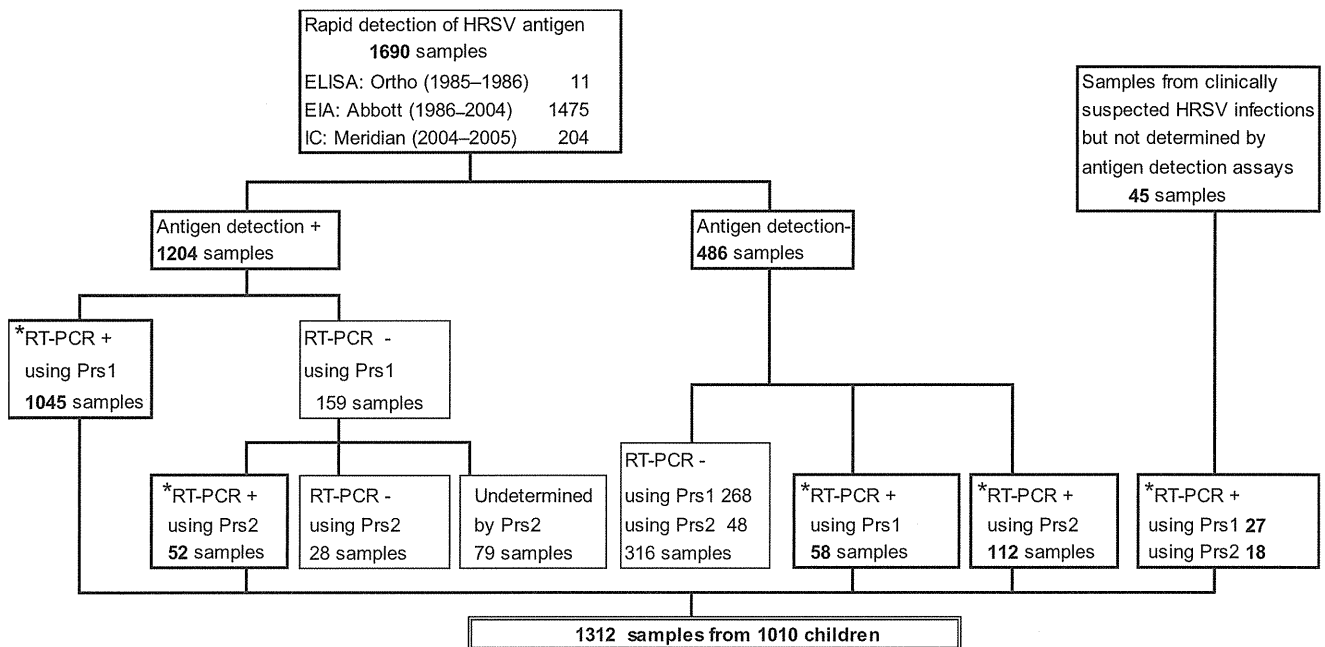
Fig. 1. Study flow diagram. Among the 1,735 clinical specimens, 1,690 were subjected to rapid antigen detection assay and 45 samples from clinically suspected HRSV infections were subjected to RT-PCR. A total of 1,312 specimens obtained from 1,010 children with symptoms of lower respiratory tract infections were confirmed to be infected with HRSV using 2 types of RT-PCR using the primers Prs1 and Prs2. The asterisk represents confirmed HRSV infections. ELISA, enzyme-linked immunosorbent assay; EIA, enzyme immunoassay; IC, immunochromatography; Prs1, RT-PCR primer set 1 shown in Table I; Prs2, RT-PCR primer set 2 shown in Table I.

When a subgroup could not be determined by RFLP due to an atypical cutting pattern, the samples were also subjected to direct sequencing. RT-PCR products were extracted from low-melting-temperature 1% agarose gel and used for sequencing. The nucleotide sequence was determined with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) using an automated 3130/3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). RT-PCR products from samples that were suspected of dual infections of HRSV-A and HRSV-B, based on the RFLP pattern and samples from children infected with HRSV more than 4 times, were inserted into pBluescript II SK (−) (Stratagene, La Jolla, CA); then, nucleotide sequences of more than 20 individual clones from each PT-PCR product were determined

TABLE I. Sequences and Positions of the PCR Primers Used

Primer	Sequence (5′–3′)	Positions
RT-PCR primer set 1 (Prs1)		
C-RSN (+)	GGGTCGACAATTCACTGGGTTAATACCTAT	1274–1295*
RSN-F1 (+)	GCCCCGGGGAGATAGAATCTAGAAAATCCT	1477–1498
RSN-B1 (−)	GCGGAGCTCTTTGGGTTGTTCAATATATGG	1998–2018
RSN-F2 (+)	CCGGTACCGAAATGGGAGAGGTAGCTCC	1516–1535
RSN-B2 (−)	CCGCATGCATAAACCTCAACAACTTGTTCC	1938–1959
RT-PCR primer set 2 (Prs2)		
CN3 (+)	GCTCTTAGCAAAGTCAAGTTGAA	1099–1121*
CCN6 (−)	TCTGTACTCTCCCATTATGCCTA	2087–2109
n-F1 (+)	GAGATAGAATCTAGAAAATCCTACAAAA	1477–1504
n-B1 (−)	TGGGTTGTTCAATATATGGTAGA	1994–2016
EcoF3′ (+)	TGGTGAATTCGCTCCAGAATACAGGCA	1531–1547
NotB3 (−)	AGTTGCGGCCGCATAAACCTCAACAACTTGTTCC	1938–1959
Colony direct PCR primers		
M13m4 (+)	GTTTTCCCAGTCACGAC	580–596**
M13RV (−)	CAGGAAACAGCTATGAC	812–828

The number of nucleotides is based on the genomic location of HRSV-A strain A2* (GenBank accession number M11486) and pBluescript II SK (−)** (GenBank accession number X52330). Underlined letters indicate the linker sequences.

(cloning analysis). A pair of primers, M13m4 (+) and M13RV (−) shown in Table I, was used for colony direct sequencing.

Statistical Analysis

Comparisons between mean values, paired but not normally distributed, were assessed with the Wilcoxon rank sum test. Categorical data were compared by the Chi-square test or Fisher's exact test. All comparisons were conducted at the two-tailed 0.05 level of significance using Stat-View 5.0 software (SAS Institute, Tokyo, Japan).

Nucleotide Sequence Accession Numbers

The N-gene sequences determined in this study have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB722450–AB723492.

RESULTS

Laboratory Diagnosis of HRSV Infection

Among the 1,735 clinical specimens, 1,690 were subjected to the rapid antigen detection assay, of which 1,204 (71.2%) tested positive for the HRSV antigen (Fig. 1). Early in the study, RT-PCR using Prs1 were conducted. Of the 1,557 samples subjected to RT-PCR using Prs1, 1,130 (72.6%) were positive for HRSV. On comparing the results of EIA and IC to those of RT-PCR, 159 samples were negative by RT-PCR using Prs1, although the viral antigen was detected by EIA or IC (Fig. 1). A portion (80/159) of these samples was re-examined by RT-PCR using another set of primers, Prs2. Using Prs2, 52 samples (65.0%) tested positive for HRSV RNA. Further, of the 486 samples that were

negative for antigen detection, 58 and 112 samples were detected to be positive by PCR using Prs1 and Prs2, respectively. Consequently, a total of 1,312 lower respiratory tract infections were confirmed as HRSV infections in 1,010 patients, that is, positive by antigen detection and RT-PCR using Prs1 (1,045) or Prs2 (52), negative by antigen detection but positive by PT-PCR using Prs1 (58) or Prs2 (112), and only detected by RT-PCR using Prs1 (27) or Prs2 (18). The patients ranged in age from 5 days to 11 years (median, 18 months), and the duration analyzed was 20 epidemic years. The definition of 1 epidemic year was from September to August of the next year.

Of the 1,312 HRSV infections, 756 (57.6%) and 517 (39.4%) were caused by HRSV-A and HRSV-B, respectively. Through RFLP and cloning analysis, 39 (3.0%) HRSV infections were verified as dual infections with both HRSV-A and HRSV-B. Further, 617 of 756 (81.6%) HRSV-A infections, 487 of 517 (84.7%) HRSV-B infections, and 20 of 39 (51.3%) infections caused by both HRSV-A and HRSV-B were observed in patients aged less than 3 years. These results showed that HRSV-A and HRSV-B co-circulated continuously over 20 epidemic years, and the overall pattern of subgroup prevalence changed every 1 or 2 years (Fig. 2). From 1994 to 2005, 1 year of HRSV-A predominance was followed by 1 or 2 intervening years where the 2 subgroups presented in similar numbers or HRSV-B predominated.

Rate of Repeated HRSV Infection

From the sequencing and cloning analyses, a total of 208 children were noted to have experienced multiple HRSV infections (2–9 times). Consequently,
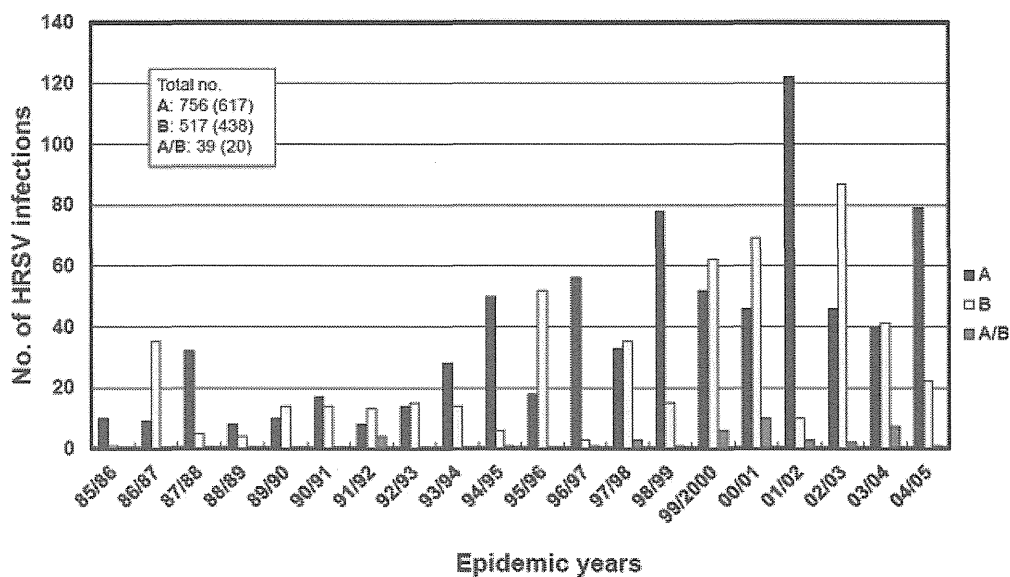

Fig. 2. HRSV subgroup epidemiology. Distribution of HRSV subgroups circulating in Kawasaki, Japan, during 1985–2005. The number in parentheses indicates HRSV infections among patients aged less than 3 years. A, HRSV-A; B, HRSV-B; A/B, dual infection with HRSV-A and HRSV-B.

TABLE II. Patterns of Occurrence Among Children Infected Repeatedly With HRSV

Patterns of occurrence in repeated infections	No. of patients	No. of specimens	HRSV subgroup		
			A	B	A/B
Twice	151	302	186	103	13
Three times	42	126	72	48	6
Four times	6	24	16	4	4
Five times	3	15	8	2	5
Six times	3	18	11	3	4
Eight times	2	16	8	4	4
Nine times	1	9	5	2	2
Total	208	510	306[a]	166[a]	38

[a]Difference between percentages of sample numbers of repeated cases for the total number of HRSV-A and HRSV-B cases is statistically significant ($P = 0.0029$). A/B indicates dual infection with HRSV-A and HRSV-B.

510 infections (510/1,312 = 38.9%) were recorded as multiple infections observed in the 208 children. Among the 510 infections, 306, 166, and 38 infections were caused by HRSV-A, HRSV-B, and both subgroups, respectively (Table II). The rate of repeated HRSV-A infections among the total circulating HRSV-A infections (306/756 = 40.5%) was higher than that of HRSV-B infections (166/517 = 32.1%). The difference in the rate of HRSV-A and HRSV-B infections was statistically significant ($P = 0.0029$). Among the 208 children infected repeatedly with HRSV, 151 and 42 children were re-infected and infected 3 times, respectively. Further, 6, 3, and 3 children were infected 4, 5, and 6 times, respectively. Two children were infected 8 times, and 1 patient was infected 9 times (Table II).

Subgroup Occurrence Among Children Re-Infected With HRSV

During the 20 epidemic years, 151 children experienced HRSV re-infections (302 infections; Table III). The patients had no underlying conditions such as congenital heart disease, immunodeficiencies, neuromuscular disorders, or chronic respiratory disorders, except previously diagnosed asthma (in 10 patients). Five children with low birth weights (<2,500 g) were included in the analysis, as they were not born prematurely, with a gestational age of at least ≥37 weeks. There were 16 (5.3%) cases of hospital admissions (16/302; hospitalization due to the first infection, 12; hospitalization due to a second case of infection, 4) in the group. In the second infection, 60 children were infected with a homologous subgroup, of which 50 and 10 re-infections by a homologous subgroup were caused by HRSV-A and HRSV-B, respectively. Re-infections with a heterologous subgroup were detected in 78 children, of whom 35 and 43 were first infected with HRSV-A and HRSV-B, respectively. Thirteen patients had dual infections with both subgroups in either the first or the second infection. Of the 151 patients, 133 (88.1%) were re-infected after more than 1 season and 18 patients (11.9%) within a single season (Table III). Re-infection with a homologous strain occurred more frequently with HRSV-A than HRSV-B ($P = 0.0065$). During the study period, the overall ratio of circulating HRSV-A/HRSV-B strains in the community was 3:2. Taking circulating HRSV into account, HRSV-A infections provided no protection from a second infection with a homologous strain ($P = 0.91$). However, patients with HRSV-B infections were protected significantly from a second homologous infection

TABLE III. Subgroup Characteristics of HRSV Isolated From Re-Infected Children

Patterns of occurrence	No. of re-infected children	Interval of re-infection		No. of children analyzed for clinical features
		More than 1 separate season	Within a single season	
A–A*	50	43	7	47
A–B	35	32	3	33
B–B	10	9	1	9
B–A	43	39	4	40
A/B	13	10 First (5), Second (5)	3 First (2), Second (1)	13
Total	151	133	18	129

*The first letter designates the subgroup responsible for the first HRSV infection, and the second letter, that for the second infection. A/B indicates that dual infection with both subgroups is included in the first or second infection. The numbers in parentheses indicate dual-infected HRSV samples in the first or second infection. The numbers in italics indicate re-infected children who were enrolled for the analysis of clinical features.