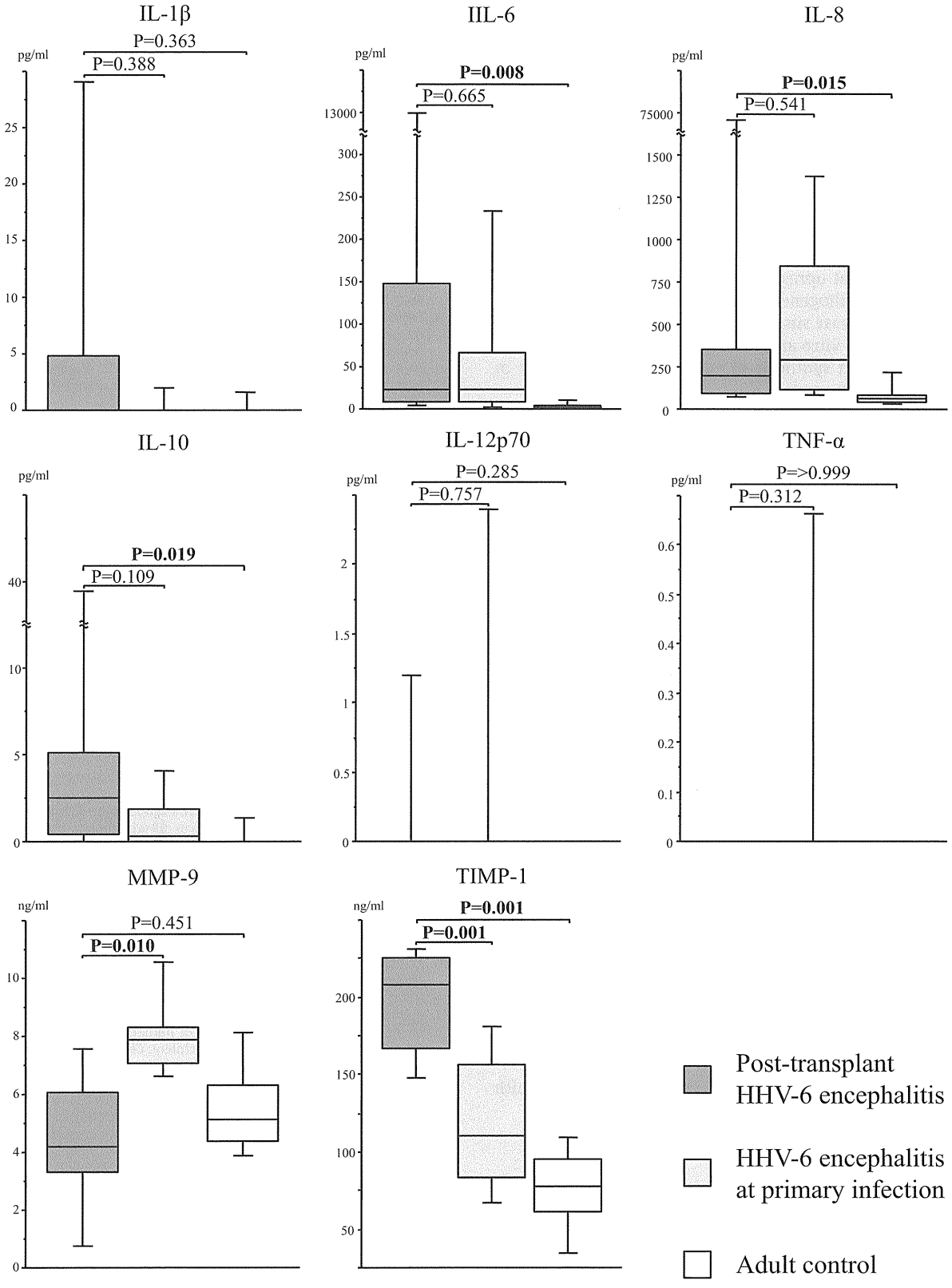

Fig. 3. Comparison of the eight cerebrospinal fluid (CSF) biomarkers (IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, MMP-9, and TIMP-1) levels among patients with post-transplant HHV-6 encephalitis ($n = 7$), HHV-6 encephalitis in primary infection ($n = 22$), and adult controls ($n = 8$). Data are shown as box plots, where the boxes represent the first through third quartiles, the lines within the boxes represent the median, and the lines outside the boxes represent the minimum and maximum values (excluding outliers).

Although transient CSF IL-8 elevation has been reported in patients with aseptic meningitis or bacterial meningitis,[25,26] the role of elevated CSF IL-8 in the HHV-6 encephalitis in primary infection patients remains undefined, because most of the CSF collected from the patients did not show pleocytosis. The primary role of IL-8 is the recruitment and activation of neutrophils, but it also serves as a chemotactic for T cells[27] and monocytes.[28] Therefore, it is possible that elevated CSF IL-8 may accelerate invasion of HHV-6 resident

cells into CNS, which establishes a suitable condition for HHV-6 latency in brain tissue.

In CNS infections, MMPs are thought to play an important role in promoting destructive inflammatory processes including disruption of the blood brain barrier, edema, and disintegration of the neurovascular unit.[29,30] As in the case with other viral encephalitis,[31] MMP-9 and TIMP-1 were significantly higher in patients with HHV-6 encephalitis in primary infection than those patients with non-HHV-6 FC. The expression and activity of MMP-9 can be upregulated by cytokines and chemokines.[32] Therefore, we speculated that elevated IL-8 or IL-6 would induce MMP-9 elevation in HHV-6 encephalitis in primary infection patients. It has been suggested that upregulation of MMP-9 and TIMP-1 was associated with the pathogenesis of multiple sclerosis, which is a major neurological disease suspected to be associated with HHV-6 infection.[33–35] Thus, in vitro analysis to determine whether HHV-6 might be involved in synthesis of either MMP-9 or TIMP-1 in neurological cell lines is needed.

Although adult control subjects were not perfect in this study because of differences in host conditions and age from post-transplant HHV-6 encephalitis patients, similar cytokines elevation was observed in post-transplant HHV-6 encephalitis patients as HHV-6 encephalitis at primary infection. Furthermore, CSF IL-10 concentrations were also significantly higher in post-transplant HHV-6 encephalitis patients than controls ($P = 0.019$). IL-10 is the prototypical anti-inflammatory cytokine, and elevation of CSF IL-10 concentration might reflect strong protective reaction against CNS inflammation caused by direct HHV-6 replication. Additionally, as the ratio of MMP-9 and TIMP-1 was different between HHV-6 encephalitis in primary infection and post-transplant HHV-6 encephalitis, this discrepancy may be associated with the distinct pathological mechanisms between HHV-6 encephalitis in primary infection and viral reactivation in transplant recipient. Thus, in addition to different concentrations of direct HHV-6 replication in brain tissue, a complex network of cytokines and inflammatory mediators is likely to be associated with the pathological mechanisms that distinguish HHV-6 encephalitis in primary infection from viral reactivation in transplant recipient.

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Ethical approval

This study was approved by the review boards of Fujita Health University (No. 08-183).

Conflict of interest

The authors do not have any commercial or other associations that might pose a conflict of interest.

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Evaluation of Reverse Transcription Loop-Mediated Isothermal Amplification Assays for Rapid Diagnosis of Pandemic Influenza A/H1N1 2009 Virus

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Two genetic diagnosis systems using reverse transcription-loop-mediated isothermal amplification (RT-LAMP) technology were evaluated: one for detecting the HA gene of the pandemic influenza A/H1N1 2009 virus (H1pdm RT-LAMP) and the other for detecting the matrix gene of the influenza A virus (TypeA RT-LAMP). The competence of these two RT-LAMP assay kits for the diagnosis of the pandemic influenza A/H1N1 2009 virus was compared using real-time RT-PCR assays developed recently on viruses isolated and clinical specimens collected from patients with suspected infection. TypeA RT-LAMP and H1pdm RT-LAMP showed almost the same sensitivity as real-time RT-PCR for viruses isolated. The sensitivity and specificity of TypeA RT-LAMP and H1pdm RT-LAMP were 96.3% and 88.9%, respectively, for clinical specimens. Considering that the ability of the two RT-LAMP assay kits for detection of the pandemic influenza A/H1N1 2009 virus was comparable to that of the real-time RT-PCR assays, and that the assays were completed within 1 hr and did not require any expensive equipment, these two RT-LAMP assays are promising rapid diagnostic tests for the pandemic influenza A/H1N1 2009 virus at the hospital bedside. ***J. Med. Virol. 83:10–15, 2011.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: rapid diagnosis; RT-LAMP; pandemic influenza A/H1N1 2009 virus

INTRODUCTION

The novel H1N1 subtype influenza A virus (pandemic influenza A/H1N1 2009 virus) has spread worldwide since it was identified in Mexico and the United States in March and April 2009 [WHO, 2009a,b]. As of November 6, 2009, the mortality rates of pandemic influenza A/H1N1 2009 infection (deaths per million population) ranged from 1.8 to 14.6 in temperate zone countries, while it was only 0.2 in Japan [WHO, 2009c]. In Japan, only 197 deaths from pandemic influenza A/H1N1 2009 infection were confirmed as of March 9, 2010, although the estimated number of cases was about 20.6 million according to the Japanese Ministry of Health, Labor, and Welfare. The low case to fatality rate in Japan may have resulted from the aggressive early treatment strategy adopted by hospitals; thus, it is important to develop highly specific and sensitive early diagnostic tests for the pandemic influenza A/H1N1 2009 virus that can be performed easily in the clinic.

A novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), was described by Notomi et al. [2000]. Amplification is conducted under isothermal conditions ranging from 60 to 65°C with DNA polymerase and usually with 4 primers recognizing 6 distinct target regions (4-primer-based LAMP), making this assay highly specific. If two additional "loop primers" are included in the LAMP assay (6-primer-based LAMP), the reaction time can be reduced [Nagamine et al., 2002]. Nucleic acid amplification-based diagnostic assays have become the gold standard for the rapid diagnosis of viral infections. Several PCR assays, such as conventional RT-PCR and real-time RT-PCR (rRT-PCR), have been reported for

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the pandemic influenza A/H1N1 2009 virus [Bose et al., 2009; Carr et al., 2009; Hall et al., 2009; He et al., 2009; Lam et al., 2009; Lau et al., 2009; LeBlanc et al., 2009; Pabbaraju et al., 2009; Wang et al., 2009; Yang et al., 2009; Nakauchi et al., 2010; Chidlow et al., 2010]; however, these methods require high-precision instruments, such as the LightCycler Real-Time PCR System (Roche Diagnostics Ltd., Mannheim, Germany). On the other hand, the LAMP assay can be carried out without using such instruments; furthermore, viral genomes can be detected within a shorter time and in a real-time manner. Recently, LAMP-based assays for several virus infections [Hong et al., 2004; Mori et al., 2006; Shirato et al., 2007; Yoneyama et al., 2007; Iizuka et al., 2009] and influenza virus infections [Poon et al., 2005; Imai et al., 2006, 2007; Ito et al., 2006; Kubo et al., 2010] have been reported.

In this study, two genetic diagnosis kits using RT-LAMP technology were evaluated: one for detecting the pandemic influenza A/H1N1 2009 virus and the other for detecting the influenza A virus. These two RT-LAMP assay kits (Eiken Chemical, Tokyo, Japan) contain the Loopamp Extraction Reagent that does not require an RNA purification step, RNA Amplification Reagent (Dried Form) that eliminates the need to dispense the enzyme and reaction buffer, and Primer Set for FluA (for Dried Form) or Primer Set for H1 pdm 2009 (for Dried Form); as a result, RT-LAMP is simpler and easier to perform using these kits than conventional RT-LAMP assays. The competence of the two RT-LAMP assay kits for the diagnosis of the pandemic influenza A/H1N1 2009 virus was compared with rRT-PCR assays using isolated viruses and clinical specimens collected from patients with suspected infection.

MATERIALS AND METHODS

Viruses and Cells

All influenza virus isolates included in this study were grown in Madin–Darby Canine Kidney (MDCK) cells. Pandemic influenza A/H1N1 2009 virus isolates are listed in Table III.

The following influenza viruses were used to evaluate the specificity of the RT-LAMP assays: A/New Caledonia/20/1999 (H1N1); A/Moscow/13/1998 (H1N1); A/Panama/2007/1999 (H3N2); A/Wyoming/03/2003 (H3N2); A/New York/55/2004 (H3N2); A/Sydney/05/1997 (H3N2); A/Hong Kong/156/1997 (H5N1); A/Hong Kong/213/2003 (H5N1); A/Vietnam/HN30259/2004 (H5N1); A/Vietnam/HN30262/2004 (H5N1); A/Netherlands/219/2003 (H7N7); A/Netherlands/33/2003 (H7N7); A/duck/Hokkaido/55/96 (H1N1); A/duck/Hong Kong/278/78 (H2N9); A/duck/Ukraine/1/63 (H3N8); A/duck/Hong Kong/365/78 (H4N6); A/chicken/Yamaguchi/7/04 (H5N1); A/duck/Hong Kong/716/79 (H6N1); A/duck/Hong Kong/293/78 (H7N2); A/turkey/Ontario/6118/68 (H8N4); A/duck/Hong Kong/702/79 (H9N5); A/duck/Hong Kong/560/79 (H10N8); A/duck/England/56 (H11N6); A/duck/Alberta/60/76 (H12N6); A/gull/Maryland/704/77 (H13N6); A/mallard/Gurjev/263/82 (H14N5); A/duck/Australia/341/83 (H15N8); B/Shangdong/07/2002; B/Shanghai/361/2002; and B/Brisbane/32/2002.

Clinical Specimens

Nasal swabs were taken in duplicate from patients with suspected infection at the same time. One of the two swabs, designated as swab 1, was suspended in 4 ml of Loopamp Extraction Reagent for Influenza virus (Eiken Chemical). The other swab, designated as swab 2, was suspended in 4 ml of viral transport medium (VTM) (Becton-Dickinson and Company, Franklin Lakes, NJ), and was used for RNA extraction and virus isolation. The study protocol was approved by the Ethics Committee at NIID and Fujita Health University School of Medicine, and the study was performed in compliance with the Declaration of Helsinki. Informed consent was obtained from all patients.

RNA Extraction

Supernatants of the cultured MDCK cells were cleared by centrifugation at 10,000*g* for 10 min. Viral RNA was prepared using the MagMAX 96 Viral Isolation Kit (Ambion, Austin, TX) from 50 μl of the supernatant with a KingFisher Flex (Thermo Fisher Scientific, Waltham, MA) according to the manufacturers' instructions. Total RNA from the clinical specimens was also prepared using the MagMAX 96 Viral Isolation Kit (Ambion) from 50 μl of sample. Total RNA was eluted in 30 μl of elution buffer (Ambion) and stored at −70°C until used.

RT-LAMP

RT-LAMP was carried out using the RNA Amplification Reagent (Dried Form) (Eiken Chemical) that consists of 200 μl tubes with dried enzyme and reaction buffer for RT-LAMP fixed on the inside of the cap. Ten microliters of purified RNA or a nasal swab suspended in Loopamp Extraction Reagent for Influenza virus (Eiken Chemical) was added to the bottom of a tube containing 15 μl of Primer Set for FluA (for Dried Form) or Primer Set for H1 pdm 2009 (for Dried Form) (Eiken Chemical), and the tube was then inverted to resuspend the enzyme and buffer. The reaction mixture was collected at the bottom of the tube by a quick spin down. The mixture was incubated using a Loopamp real-time turbidimeter (LA-320C; Eiken Chemical) for 35 min at 62.5°C and then for 5 min at 80°C to terminate the reaction. The locations, names, and sequences of the RT-LAMP primers specific for the pandemic influenza A/H1N1 2009 virus HA gene (Primer Set for H1 pdm 2009 (for Dried Form)) and the influenza A virus matrix gene (Primer Set for FluA (for Dried Form)) are given in Tables I and II, respectively (information provided by Eiken Chemical).

Real-Time RT-PCR

Real-time RT-PCR assays for detecting the Type A influenza virus and specifically the pandemic influenza

TABLE I. Primer Set for H1 pdm 2009 (for Dried Form) (Eiken Chemical)

Primer name	Sequence (5'–3')	Genome position	Length (bp)
H1F3-1	AGCTAAGAGAGCAATT	350–365	16
H1B3-1	TTTCCCTTTATCATTAATGTAGGATTTG	537–564	28
H1FIP-1[a] (F1c-1 + (T) + F2-1)	ACCTTTGTTCGAGTCATGATTGG-(T)CTCAGTGTCATCATTTGAAAGGTTT	422–444 (F1c-1) 369–393 (F2-1)	49
H1BIP-1[b] (B1c-1 + B2-1)	TAACGGCAGCATGTCCTCA-GTATGAATTTCCTTTTTTAACTAGCCA	446–464 (B1c-1) 499–525 (B2-1)	46
H1FL-1	CCATGAACTTGTCTTGGGGAATA	398–420	23
H1BL-1	TGCTGGAGCAAAAAGCTTCTAC	465–486	22
H1F3-2	ACCTTCTAGAAGACAAGCATAA	143–164	22
H1B3-2	TCCTCATAATCGAT	337–350	14
H1FIP-2[c] (F1c-2 + F2-2)	TGGATTTCCCAGGATCCAGC-GGAAACTATGCAAACTAAGAGG	227–246 (F1c-2) 167–188 (F2-2)	42
H1BIP-2[d] (B1c-2 + B2-2)	TCCACAGCAAGCTCATGGTC-TCCTGGGTAACACGTTCC	262–281 (B1c-2) 313–330 (B2-2)	38
H1FL-2	CCAAATGCAATGGGGCTAC	190–208	19
H1BL-2	CTACATTGTGGAAACATCTAGTTCAG	282–307	26

[a]H1FIP-1 primer consisted of F1c-1, a T linker, and F2-1.
[b]H1BIP-1 primer consisted of B1c-1 and B2-1.
[c]H1FIP-2 primer consisted of F1c-2 and F2-2.
[d]H1BIP-2 primer consisted of B1c-2 and B2-2.

A/H1N1 2009 virus were performed as described previously [Nakauchi et al., 2010].

Virus Isolation

One hundred microliters of VTM containing a nasal swab was diluted with an equal volume of Opti-MEM (Invitrogen, Carlsbad, CA). This was then added to MDCK cells in a 12.5 cm^2 flask and incubated at 34°C for 1 hr. The cells were washed twice with Opti-MEM, and then cultured in 2 ml of Opti-MEM containing 5 μg/ml acetylated trypsin (Sigma-Aldrich Corp., St. Louis, MO), 200 μg/ml penicillin/streptomycin (Invitrogen), 100 μg/ml gentamicin (Invitrogen), and 0.5 μg/ml fungizone (Invitrogen) until a cytopathic effect was observed.

RESULTS

Sensitivity of RT-LAMP Assays

The sensitivity of the RT-LAMP assay using primer set Influenza A (TypeA RT-LAMP) and primer set AH1pdm (H1pdm RT-LAMP) was evaluated and compared with that of the TypeA rRT-PCR or H1pdm rRT-PCR assays [Nakauchi et al., 2010] using RNA samples diluted serially that were prepared from the pandemic influenza A/H1N1 2009 virus, as indicated in Table III. The assays were carried out twice independently. As shown in Table III, the 100% detectable concentration of each strain of the pandemic influenza A/H1N1 2009 virus by TypeA RT-LAMP or H1pdm RT-LAMP was almost identical to the 100% detectable concentration of each strain by TypeA rRT-PCR or H1pdm rRT-PCR, respectively.

The specificity of the LAMP assays was evaluated using 27 strains of human and avian influenza A viruses (subtypes H1–H15) and 3 strains of influenza B viruses. TypeA RT-LAMP detected all influenza A viruses with no cross-reactivity against influenza B viruses. H1pdm RT-LAMP reacted specifically to pandemic influenza A/H1N1 2009 viruses with no cross-reactivity against other subtype influenza A viruses, except for pandemic influenza A/H1N1 2009 viruses and influenza B viruses.

Evaluation of RT-LAMP Using Clinical Specimens

The TypeA RT-LAMP and H1pdm RT-LAMP assays were evaluated using 45 nasal swabs from patients with

TABLE II. Primer Set for FluA (for Dried Form) (Eiken Chemical)

Primer name	Sequence (5'–3')	Genome position	Length (bp)
FluAF3-1	GACTTGAAGATGTCTTTGC	80–98	19
FluAF3-2	GACTGGAAAGTGTCTTTGC	80–98	19
FluAB3-1	TGTTATTTGGATCCCCATT	259–277	19
FluAB3-2	TGTTGTTCGGGTCCCCATT	259–277	19
FluAFIP[a] (F1c + F2)	TTAGTCAGAGGTGACAGGATTG-CAGATCTTGAGGCTCTC	149–170(F1c) 110–126(F2)	39
FluABIP[b] (B1c + B2)	TTGTGTTCACGCTCACCGTG-TTTGGACAAAGCGTCTACG	185–204(B1c) 226–244(B2)	39
FluAFL	GTCTTGTCTTTAGCCA	133–148	16
FluABL	CAGTGAGCGAGGACTG	207–222	16

[a]FluAFIP primer consisted of F1c and F2.
[b]FluABIP primer consisted of B1c and B2.

TABLE III. The Sensitivity of RT-LAMP Was Directly Compared With Real-Time RT-PCR Assay Using Series Dilutions of Viral RNA

	Dilution rate of virus			
	10^7		10^8	
Virus (TCID50/ml)	TypeA	H1pdm	TypeA	H1pdm
A/Aichi/198/2009 ($10^{6.5}$)				
RT-LAMP	2/2	2/2	1/2	1/2
rRT-PCR	2/2	2/2	2/2	1/2
A/Saitama/85/2009 ($10^{7.5}$)				
RT-LAMP	2/2	2/2	2/2	0/2
rRT-PCR	2/2	2/2	2/2	1/2
A/Shiga/44/2009 ($10^{8.0}$)				
RT-LAMP	2/2	2/2	2/2	2/2
rRT-PCR	2/2	2/2	1/2	1/2
A/Kagoshima/56/2009 ($10^{6.3}$)				
RT-LAMP	2/2	2/2	1/2	0/2
rRT-PCR	2/2	2/2	2/2	0/2
A/Kobe/1/2009 ($10^{8.0}$)				
RT-LAMP	2/2	2/2	2/2	1/2
rRT-PCR	2/2	2/2	2/2	2/2
A/Shiga/2/2009 ($10^{6.7}$)				
RT-LAMP	2/2	2/2	0/2	1/2
rRT-PCR	2/2	2/2	2/2	1/2
A/Kanagawa/140/2009 ($10^{7.3}$)				
RT-LAMP	2/2	2/2	0/2	2/2
rRT-PCR	2/2	2/2	0/2	1/2
A/Hiroshima/310/2009 ($10^{7.5}$)				
RT-LAMP	2/2	2/2	2/2	2/2
rRT-PCR	2/2	2/2	2/2	1/2

suspected pandemic influenza A/H1N1 2009 infection, and the results were compared with those obtained using rRT-PCR (Table IV). Compared with TypeA rRT-PCR, the sensitivity and specificity of TypeA RT-LAMP were 96.3% and 88.9%, respectively. Similarly, compared with H1pdm rRT-PCR, the sensitivity and specificity of H1pdm RT-LAMP were 96.3% and 88.9%, respectively. Among the 45 samples, 2 samples (Samples 27 and 46) were positive for TypeA and H1pdm when tested by RT-LAMP; however, they were negative for TypeA and H1pdm when tested by rRT-PCR (Table V). Two samples (samples 23 and 40) were positive for TypeA and H1pdm when tested by rRT-PCR; however, they were negative for TypeA or H1pdm when tested by RT-LAMP (Table V).

In addition to comparing them with rRT-PCR, the TypeA RT-LAMP and H1pdm RT-LAMP assays were also compared with viral isolation from 45 nasal swabs. As shown in Table IV, 53.3% (24/45) were positive by viral isolation, 60% (27/45) by TypeA rRT-PCR and H1pdm rRT-PCR, and 62.2% (27/45) by TypeA RT-LAMP and H1pdm RT-LAMP.

DISCUSSION

Two RT-LAMP assays were evaluated, namely, TypeA RT-LAMP and H1pdm RT-LAMP and demonstrated that these assays have approximately the same sensitivity as the TypeA and H1pdm rRT-PCR assays, respectively (Table III). The results of assays using

TABLE IV. Comparison of the Results of RT-LAMP and Those of rRT-PCR and Virus Isolation

	RT-LAMP: H1pdm		
	+		−
TypeA rRT-PCR (+) (n = 27)	26 (23)		1 (1)
TypeA rRT-PCR (−) (n = 18)	2 (0)		16 (0)
Sensitivity		96.3%	
Specificity		88.9%	
	RT-LAMP: H1pdm		
	+		−
H1pdm rRT-PCR (+) (n = 27)	26 (23)		1 (1)
H1pdm rRT-PCR (−) (n = 18)	2 (0)		16 (0)
Sensitivity		96.3%	
Specificity		88.9%	

The number of the samples which were virus isolation positive were shown in parentheses.

TABLE V. Comparison of the Results of Assays Performed on Four Clinical Specimens With RT-LAMP, rRT-PCR, and Virus Isolation

	RT-LAMP		rRT-PCR		
Sample ID	TypeA	H1pdm	TypeA	H1pdm	Virus isolation
23	+	−	+	+	+
27	+	+	−	−	−
40	−	+	+	+	+
46	+	+	−	−	−

clinical specimens (Table IV) also suggested that the RT-LAMP assays have the same sensitivity as the rRT-PCR assays. However, four samples showed different results when tested with RT-LAMP and rRT-PCR (Table V). It was presumed that these discrepancies were due to the fact that swab 1 (suspended directly in Loopamp Extraction Reagent for Influenza virus), which was used for the LAMP assay, and swab 2 (suspended in VTM), which was used for the real-time RT-PCR assay and virus isolation, did not necessarily contain the same quantity of virus even though they were collected from the same patient. For samples 23 and 40, swab 2 was positive for TypeA and H1pdm by rRT-PCR and also positive for virus isolation, whereas swab 1 was negative for Type A or H1pdm by RT-LAMP (Table V). The remaining RNA extracted from swab 2 of samples 23 and 40 was further tested using the RT-LAMP assays. As a result, it was demonstrated that swab 2 from samples 23 and 40 was positive for TypeA and H1pdm using the RT-LAMP assays (data not shown). Swab 1 from samples 27 and 46 was positive for TypeA and H1pdm by the RT-LAMP assays, whereas both swab 2 samples were negative for TypeA and H1pdm by rRT-PCR and also negative for virus isolation. The rRT-PCR assays were carried out after RNA extraction from swab 1 of samples 27 and 46 using the Loopamp Extraction Reagent for Influenza virus. As a result, it was demonstrated that both swab 1 samples were positive for TypeA and H1pdm by rRT-PCR (data not shown). Unfortunately, virus isolation could not be performed using swab 1 because the viral particles were disrupted by some component of the Loopamp Extraction Reagent for Influenza virus. These facts suggests that swab 1 from samples 23 and 40 contained insufficient amounts or quality of virus for the RT-LAMP assays, and swab 2 from samples 27 and 46 contained insufficient amounts or quality of virus for the rRT-PCR assays and virus isolation. Considering these results, the ability of the TypeA and H1pdm RT-LAMP assays to detect the pandemic influenza A/H1N1 2009 virus was comparable to the ability of the TypeA and H1pdm rRT-PCR assays, respectively.

A diagnostic method was developed recently to detect specifically the pandemic influenza A/H1N1 2009 virus using RT-LAMP [Kubo et al., 2010]; however, as this assay targeted only one region of the pandemic influenza A/H1N1 2009 virus HA gene, the RT-LAMP assay may fail to detect viruses with mutations in the target region that are difficult to amplify. Although the H1pdm RT-LAMP assay is more tolerant to mutations of the HA gene than the assay of Kubo et al. because it includes two primer sets targeted to different regions of the HA gene (Table I), it is important to check the nucleotide sequence of recently circulating viruses and to modify the primers when viruses emerge with mutations in the target regions. When only the TypeA RT-LAMP assay is positive, a mutant of the pandemic influenza A/H1N1 2009 virus or a different subtype influenza virus circulating in the community is considered because of the failure of the H1pdm assay and if necessary, further analysis, such as sequencing, should be performed. Furthermore, to reduce the risk of detection failure of the influenza A virus, it is important to perform simultaneously the subtype-specific H1pdm RT-LAMP assay and the TypeA RT-LAMP assay on the same samples.

The positive rate of pandemic influenza A/H1N1 2009 virus detection in the clinical specimens using TypeA or H1pdm RT-LAMP was higher than that obtained with virus isolation and it was almost identical to the rates of the TypeA and H1pdm rRT-PCR assays. The virus isolation method is only able to detect an infectious virus, and the quantity and amount of infectious viral particles depend on the preparation and storage conditions of the sample. Thus, the detection rate of the pandemic influenza A/H1N1 2009 virus using the virus isolation method is lower than that using genetic diagnosis methods such as RT-LAMP and rRT-PCR. Three out of 27 samples were positive for TypeA and H1pdm by rRT-PCR, but were negative for virus isolation (Table IV); however, the crossing point (C_p) values for the TypeA and H1pdm rRT-PCR assays for these three samples were high (data not shown), suggesting that there was only a low amount of virus in these three samples.

The two RT-LAMP assay kits did not require the enzyme or reaction buffer to be aliquoted. In addition, by using the Loopamp Extraction Reagent for Influenza virus (Eiken Chemical), the assays did not require RNA purification. Thus, the RT-LAMP assay kits are easy to use compared with the RT-PCR assays that are employed routinely for the diagnosis of pandemic influenza A/H1N1 2009 infection. The RT-LAMP assays can be completed within 1 hr without the need for any expensive equipment, such as a thermal cycler. The two RT-LAMP assay kits are considered valuable for the diagnosis of the pandemic influenza A/H1N1 2009 virus in clinics and show promise for bedside diagnosis.

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Analysis of rotavirus antigenemia in hematopoietic stem cell transplant recipients

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Abstract : Systemic rotavirus infection, such as rotavirus antigenemia, has been found in immunocompetent rotavirus gastroenteritis patients. However, the pathogenesis of rotavirus infection in immunocompromised transplant recipients remains unclear. Enzyme-linked immunosorbent assay was used to measure rotavirus antigen levels in serially collected serum samples obtained from 62 pediatric patients receiving allogeneic hematopoietic stem cell transplants (HSCT). Rotavirus antigen was detected in 43 (6.8%) of 633 serum samples (8 of 62 patients). The duration of rotavirus antigenemia ranged between 1 and 10 weeks, and diarrhea was concurrent with rotavirus antigenemia in Cases 3, 6, 7, and 8. The level of viral antigen in the transplant recipients (0.19 ± 0.20) was significantly lower than that observed in serum samples collected from immunocompetent patients on either day 1 (0.49 ± 0.18, $P = 0.0011$) or day 3 (0.63 ± 0.09, $P = 0.0005$). A patient who received a graft from a human leukocyte antigen (HLA)-mismatched donor was at significant risk for rotavirus antigenemia ($P = 0.024$; odds ratio = 9.44) in comparison to patients who received grafts from HLA-matched donors. Although the duration of antigenemia was clearly longer in HSCT patients than in immunocompetent rotavirus gastroenteritis patients, the levels of viral antigen were not as high. Therefore, mismatched HLA may be a risk factor for rotavirus antigenemia after HSCT.

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Key words: rotavirus; antigenemia; hematopoietic stem cell transplantation; pediatric

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Rotavirus is the major cause of gastroenteritis in young children worldwide. Severe dehydration caused by rotavirus-induced diarrhea and vomiting can be fatal in developing countries. In addition, the gastroenteritis induced by rotavirus infection causes a large economic burden in developed countries. Rotaviral infection is generally benign and self-limited in immunocompetent children. In contrast, it has been reported that rotavirus can cause severe diarrhea resulting in fatal outcomes for immunocompromised transplant recipients (1–5). Thus, the pathogenesis of rotaviral infection may differ between immunocompetent and immunocompromised individuals.

Initially, rotavirus replication was thought to be limited to the gastrointestinal tract in patients with rotavirus gastroenteritis. However, recently, rotavirus antigen and RNA were detected in the sera of rotavirus-infected children (6–9). In addition, rotavirus antigen was detected not only in the serum but also in several organs, including the stomach, intestine, liver, lung, spleen, kidney, pancreas, thymus, and bladder in rotavirus-infected animals (10). These findings suggest that rotavirus spreads beyond the intestine in children with rotavirus gastroenteritis, resulting in systemic viral infection. Recently, we found that rotavirus antigenemia was frequently observed during the acute phase of rotavirus

gastroenteritis (11). Rotavirus antigen peaked on day 2 of the illness, with the amount of viral antigen gradually decreasing to nearly undetectable levels by day 6. We also found that cytokines were involved in controlling antigenemia levels. The results of this study, together with those from previous studies (7, 8, 12, 13), suggested that host immune responses have important roles in regulating viral replication. Therefore, the kinetics of rotavirus antigenemia in transplant recipients may be different from those in immunocompetent rotavirus gastroenteritis patients. In this study, we sought to elucidate the kinetics of rotavirus antigenemia in hematopoietic stem cell transplant (HSCT) recipients. We measured rotavirus antigen levels in serum samples serially collected from pediatric HSCT recipients and analyzed the associations between antigenemia and clinical features.

Patients and methods

Patient characteristics

Between September 2004 and February 2007, 62 patients received allogeneic HSCT (17 with allogeneic bone marrow transplant [BMT] from human leukocyte antigen [HLA]-matched siblings, 9 with allogeneic BMT from HLA-mismatched siblings, 22 with allogeneic BMT from unrelated donors, 2 with peripheral blood stem cell transplants, and 12 with umbilical cord blood transplants) at the Division of Hematology-Oncology at the Children's Medical Center, the Japanese Red Cross Nagoya First Hospital, or the Department of Pediatrics at the Nagoya University Graduate School of Medicine. The patients' guardians provided written consent for their participation in this study. This study was approved by the review boards of the 3 institutes. Patient characteristics are summarized in Table 1 and include age, gender, underlying diseases, type of graft, HLA matching, having received total body irradiation (TBI) or anti-thymocyte globulin (ATG) in conditioning regimen, and occurrence of acute graft-versus-host disease (GVHD).

Patient management

Details of the conditioning regimen and GVHD prophylaxis have been previously described (14, 15). In brief, patients with hematologic malignancies were conditioned with high-dose chemotherapy consisting of melphalan (180 mg/m^2) plus busulfan (16 mg/kg) or TBI (12 Gy). Patients with severe aplastic anemia were conditioned with 200 mg/kg cyclophosphamide and 10 mg/kg rabbit ATG for transplantation from a matched sibling donor. For patients transplanted with an unrelated bone marrow donor, TBI (5–10 Gy) was added (16). GVHD prophylaxis consisted of cyclosporine or tacrolimus with short-term methotrexate. All patients received trimethoprim–sulfamethoxazole orally or inhaled pentamidine as prophylaxis against *Pneumocystis jirovecii*. The standard doses of oral amphotericin B and acyclovir were administered as prophylaxis for fungal and herpes simplex virus infections. Intravenous γ-globulin preparations were administered weekly during the first 3 months as prophylaxis for cytomegalovirus (CMV) infection. In addition, ganciclovir was given as preemptive therapy against CMV infection following a positive result from a CMV antigenemia assay. Acute and chronic GVHD was diagnosed and graded according to established criteria.

Experimental design

Serum samples were collected from 62 recipients at the time of HSCT, weekly for 3 or 4 months post transplant. Ultimately, 633 serum samples were analyzed in this study. In addition to these samples, 15 serum samples were collected from rotavirus gastroenteritis patients on days 1, 3, and 5 of illness and used as controls. Clinical data were collected retrospectively and assessed to determine associations with rotavirus antigenemia.

Rotavirus antigen detection

Rotavirus antigen was measured using a previously described enzyme-linked immunosorbent assay (ELISA) for the detection of VP6 antigen of the virus (11). Diluted serum (1:16, 50 μL) was used to detect rotavirus antigen. Ninety-six–well well plates (Nalgen Nunc, Rochester, New York, USA) coated with a monoclonal antibody against the VP6 antigen of rotavirus (YO-156) (17) were used for the ELISA (18). The plates were incubated with 50 μL of diluted serum at 4°C overnight. Then, 50 μL of anti-human rotavirus hyperimmune rabbit serum (diluted 1:5000 with phosphate buffered saline [PBS] containing 0.05% Tween-20 [PBST] and 2.5% skim milk) was added to each well. Peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) (diluted

Patient characteristics and risk factors for rotavirus antigenemia after hematopoietic stem cell transplantation

	Rotavirus antigenemia			
Categories	Yes (n = 8)	No (n = 54)	Odds ratio (95% CI)	P
Age (years)	7.4 ± 5.6	8.3 ± 5.1	...	0.652
Sex				
Male	4	37	0.46 (0.10–2.06)	0.312
Female	4	17		
Underlying disease*				
Malignancy	4	36	2.0 (0.44–8.93)	0.368
Non-malignancy	4	18		
Total body irradiation				
Yes	6	41	1.05 (0.18–5.85)	0.955
No	2	13		
Anti-thymocyte globulin				
Yes	4	11	0.25 (0.05–1.19)	0.080
No	4	43		
Acute graft-versus-host disease (grade 2–4)				
Yes	3	17	0.77 (0.16–3.58)	0.705
No	5	37		
Source of the graft				
Related donor	3	23	1.0	
Unrelated donor	3	19	1.21	0.827
CBT	1	11	0.70	0.760
PBSCT	1	1	7.67	0.206
HLA matching				
Match	1	31	9.44 (1.09–82.11)	0.024
Mismatch	7	23		

*Acute lymphoblastic leukemia, 23; aplastic anemia, 12; acute myeloid leukemia 11; myelodysplastic syndrome, 4; rhabdomyosarcoma, 1; malignant lymphoma, 2; neuroblastoma, 1; others, 8.
CI, confidence interval; CBT, cord blood transplant; PBSCT, peripheral blood stem cell transplant; HLA, human leukocyte antigen.

Table 1

1:5000, Jackson ImmunoResearch Laboratory Inc., West Grove, Pennsylvania, USA) was used as a secondary antibody. Finally, the amount of rotavirus VP6 antigen bound to specific monoclonal antibody was assessed by adding substrate. The optical density (OD) was read using spectrophotometry at 492 nm, and an appropriate cut-off value was established based on data from 20 serum samples collected from control subjects. As the mean OD of the control samples was 0.084 ± 0.014, we defined 0.13 (mean + 3 standard deviations [SD]) as the baseline value in this study.

Measurement of rotavirus-specific IgG

Serum anti-rotavirus IgG antibody titer was determined using sandwich ELISA. Briefly, 96-well plates were coated with rabbit anti-rotavirus serum diluted 1:10,000 in 10 mM PBS overnight at 4°C. After the plates were washed twice with 10 mM PBS containing 0.05% PBST, then 1% bovine serum albumin in PBST was added, and the plates were incubated for 4 h at 4°C. The plates were washed twice with PBST. SA-11-infected culture fluid was then added to the plates and incubated for 1 h at 37°C. Serum samples diluted with 2.5% skim milk in

PBST were allowed to react for 1 h at 37°C. After washing 4 times with PBST, donkey anti-human IgG (H+L) conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratory Inc.) diluted 1:1000 in 2.5% skim milk in PBST was added. The plates were incubated for 1 h at 37°C, then washed 4 times with PBST, and o-phenylenediamine-2HCl substrate was added. The OD was read by spectrophotometry at 492 nm. As the mean OD of the control samples was 0.047 ± 0.026, we defined 0.124 (mean + 3 SD) as the baseline value in this study.

Statistical analyses

Statistical analyses were performed using JMP7 (SAS Institute, Cary, North Carolina, USA). A Mann–Whitney *U*-test was used to compare the levels of rotavirus antigenemia between sera collected from transplant recipients and immunocompetent rotavirus gastroenteritis patients (days 1, 3, and 5). The antigen levels in 16-fold and 4-fold diluted serum samples were compared using a Wilcoxon's signed-ranks test. The anti-rotavirus IgG antibody levels with and without rotavirus antigenemia were compared using a Student's *t*-test.

To elucidate risk factors for rotavirus antigenemia in transplant recipients, pre-transplant variables and transplant variables were compared between recipients with and without antigenemia. Pre-transplant variables included age, gender, and underlying diseases. Transplant variables included TBI, ATG, HLA matching, type of graft source, and acute GVHD. The ages of the recipients with and without rotavirus antigenemia were compared using a Student's *t*-test. Odds ratios (and 95% confidence intervals) were based on 2 × 2 contingency tables and were calculated to assess the association between rotavirus antigenemia and demographics. The significance of measurement was determined by chi-square and Fisher's exact tests.

Results

Rotavirus antigen was detected in 43 (6.8%) of 633 serum samples (8 of 62 patients). The kinetics and season of rotavirus antigenemia are shown in Figure 1. Rotavirus antigenemia lasted between 1 and 10 weeks. Rotavirus antigenemia started within 4 weeks of the transplant in all 8 recipients. Although the endemic seasons for rotavirus gastroenteritis are generally in the winter and spring in Japan, rotavirus antigenemia was observed in non-endemic periods in Cases 1, 2, 3, 4, and 7. Figure 1 also shows a temporal relationship between rotavirus antigenemia and diarrhea. Diarrhea was concurrent with rotavirus antigenemia in Cases 3, 6, 7, and 8. Meanwhile, rotavirus antigenemia persisted after the cessation of diarrhea in Cases 6 and 7. Moreover, diarrhea was not observed during rotavirus antigenemia in Cases 1, 2, and 5.

To determine whether the amount of serum rotavirus antigen was higher in HSCT recipients than in immunocompetent rotavirus gastroenteritis patients, the antigen levels were compared between the 2 groups (Fig. 2). As expected, rotavirus antigen peaked on day 3 after illness onset in the serum samples collected from immunocompetent rotavirus gastroenteritis patients. The levels of rotavirus antigenemia in the transplant recipients (0.22 ± 0.19) and day 5 serum samples collected from immunocompetent rotavirus gastroenteritis patients (0.19 ± 0.20) were similar ($P = 0.9060$). The level of viral antigen in the transplant recipients was significantly lower than that observed in either day 1 (0.49 ± 0.18, $P = 0.0011$) or day 3 (0.63 ± 0.09, $P = 0.0005$) of serum samples collected from immunocompetent rotavirus gastroenteritis patients. Although a remarkable peak in rotavirus antigen levels was observed in immunocompetent rotavirus gastroenteritis patients (11), no such peak was seen in the kinetics of rotavirus antigenemia in HSCT recipients (data not shown).

As rotavirus antigenemia levels were low in HSCT recipients, antigen level was measured using less dilute

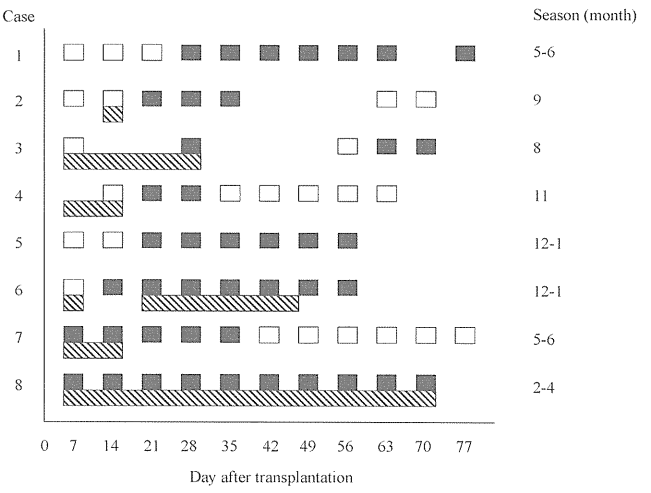

Fig. 1. Associations between rotavirus antigenemia (black boxes) and diarrhea (shaded bars) are shown. White boxes indicate antigenemia-negative serum samples.

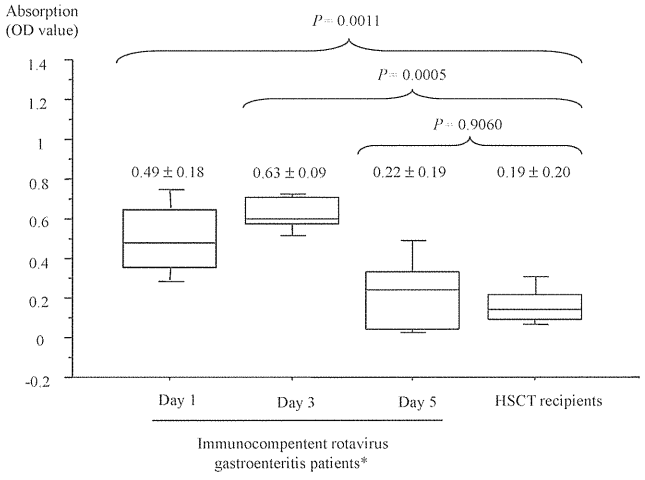

Fig. 2. Comparison of rotavirus antigen levels between immunocompetent rotavirus gastroenteritis patients and hematopoietic stem cell transplant (HSCT) recipients. *Days after onset of the illness. OD, optical density.

sera (1:4) to determine whether these positive samples contained low levels of rotavirus antigen. Forty-three antigen-positive serum samples and 40 randomly selected antigen-negative samples were used in this experiment (Fig. 3). The lower dilutions of antigen-positive serum samples (1:4) demonstrated significantly higher rotavirus antigen levels than the 1:16 diluted sera ($P < 0.0001$). However, no statistical difference was observed between 1:4 and 1:16 dilutions of antigen-negative samples ($P = 0.2733$). In addition, the immune response against rotavirus was also examined to confirm rotavirus infection; only one recipient with rotavirus antigenemia demonstrated a marked increase in rotavirus IgG antibody titers (Fig. 4).

Table 1 summarizes the results of statistical analyses identifying risk factors for rotavirus antigenemia in HSCT recipients. No statistical difference was seen between the ages of recipients with (7.4 ± 5.6 years) and without (8.3 ± 5.1 years) rotavirus antigenemia ($P = 0.652$). Moreover, neither gender ($P = 0.312$) nor underlying disease ($P = 0.368$) correlated with occurrence of rotavirus antigenemia. Of the 4 transplant-related variables, neither having received TBI ($P = 0.955$), having received ATG ($P = 0.080$), complications from acute GVHD ($P = 0.705$), nor type of graft source (related vs. unrelated; $P = 0.827$, related vs. cord blood; $P = 0.760$, related vs. peripheral blood stem cell transplant; $P = 0.206$) were associated with occurrence of rotavirus antigenemia. However, a patient who received a graft from an HLA-mismatched donor was at significant risk for rotavirus antigenemia ($P = 0.024$; odds ratio = 9.44) in comparison to patients who received a graft from an HLA-matched donor.

Discussion

Although it has been reported that rotavirus can cause severe clinical manifestations in immunocompromised transplant recipients (1–4), few studies have been conducted to elucidate the full spectrum of

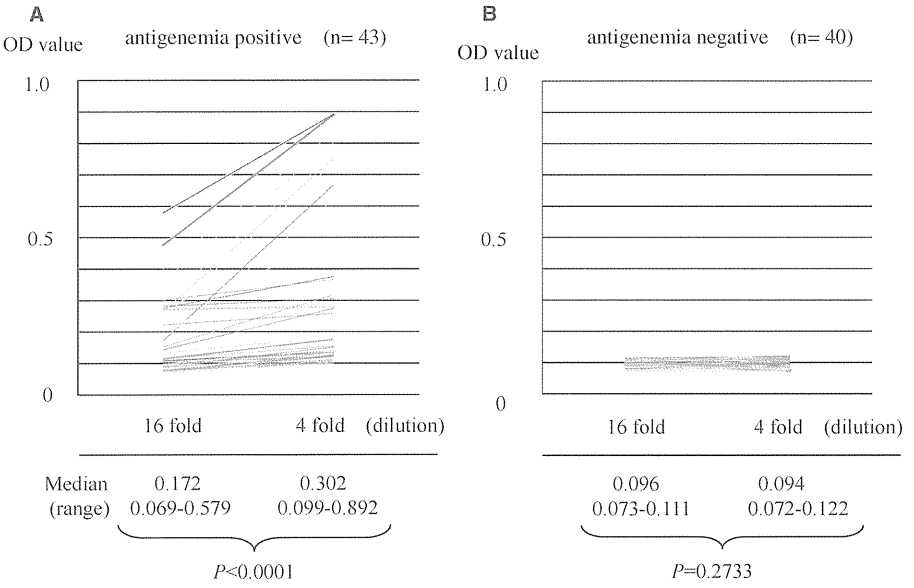

Fig. 3. Comparison of rotavirus antigen levels between 16-fold and 4-fold diluted serum samples to determine rotavirus antigenemia. OD, optical density. (A) Antigenemia positive (n = 43). (B) Antigenemia negative (n = 40).

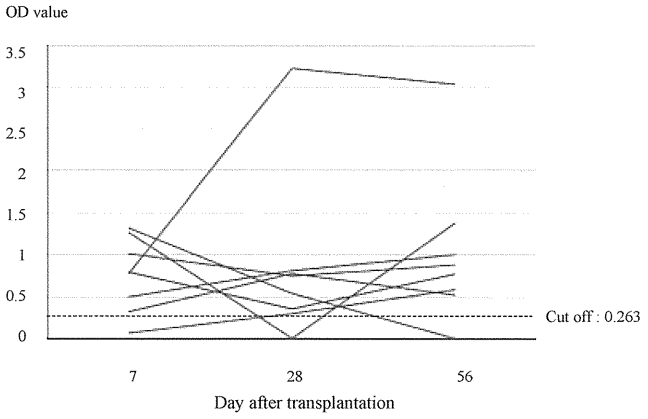

Fig. 4. Kinetics of anti-rotavirus immunoglobulin G (IgG) antibody titers in rotavirus antigenemia-positive patients (n = 8). IgG antibody titers were determined using enzyme-linked immunosorbent assay. OD, optical density.

rotavirus infection in transplant recipients (4, 19–21). In this study, rotavirus antigen was detected in 43/633 (6.8%) serum samples, and rotavirus antigenemia was observed in 8/62 (12.9%) transplant recipients. To the best of our knowledge, this is the first study that demonstrates rotavirus antigenemia in transplant recipients. Stelzmueller et al. (4) demonstrated that rotavirus infection was observed in 1.5% of solid organ transplant (SOT) recipients, and the highest frequency of rotavirus infections was observed in pediatric liver transplant recipients (52%) based on conventional rotavirus antigen detection analysis of stool samples. In addition, previous reports have identified rotavirus infection in 10–12% of pediatric BMT recipients (1, 20). Thus, although the clinical specimens used to detect rotavirus antigen were different, the frequency of antigenemia in our pediatric HSCT recipients was similar to previous studies (1, 20).

Both rotavirus-specific neutralizing antibodies and $CD8^+$ cytotoxic T cells have been shown to play important roles in terminating rotavirus infection (22–25). In addition to adaptive immunity, it has been suggested that the innate immune response is also important for protecting the host from rotaviral infection (13, 26). Thus, it is likely that rotaviral infection would be more severe in transplant recipients with severe immunosuppression than in immunocompetent children. Persistent rotavirus excretion in the stool has been documented in children with a congenital T-cell deficiency (27) and BMT recipients (5). We previously reported that the duration of rotavirus antigenemia in immunocompetent rotavirus gastroenteritis patients was short (11). Our present study suggests that rotavirus antigenemia persists for a longer period in transplant recipients, and this corresponds to rotavirus excretion in the stool (5).

To confirm specificity of the low levels of antigenemia in transplant recipients, antigen levels were measured in less diluted serum samples (Fig. 3). As less dilute (1:4) sera demonstrated statistically higher levels of antigen than that of a higher dilution (1:16), we considered that serum demonstrating low level of antigen really contained low levels of rotavirus antigen. Ray et al. (8) and Blutt et al. (9) reported that serum rotavirus antigen levels were negatively associated with rotavirus antibody levels in children with rotavirus antigenemia. Moreover, serum rotavirus antigen levels were significantly lower in patients who had a subsequent infection than in those with primary infection (7). The ages of the patients in this study ranged between 8 months and 23 years old. Thus, most of the recipients in this study could have previously had a primary rotavirus infection, which may have caused their lower levels of rotavirus antigen. Further detailed immunologic analysis of rotavirus infection is needed to clarify the pathogenesis of the characteristic kinetics of rotavirus antigenemia (low levels and long duration) observed in HSCT recipients.

Seven of 8 recipients with rotavirus antigenemia failed to demonstrate an antibody response against rotavirus. There are 2 possible explanations for such a low immune response rate. One is low immunogenicity of the low levels of antigenemia, and another is severe immunosuppression in transplant recipients. Although it is not clear whether positive antigenemia indicates active rotaviral infection, a serological assay is insufficient for monitoring rotavirus antigenemia in HSCT recipients.

It has been suggested that rotavirus can cause severe diarrhea (1, 3–5) and toxic megacolon (28), and may be confused with enteric GVHD (29), which results in significant morbidity in transplant recipients. As shown in Figure 1, persistent diarrhea and rotavirus antigenemia were concurrent in Cases 3, 6, 7, and 8. Meanwhile, rotavirus antigenemia persisted for a few weeks after the resolution of diarrhea in Cases 3, 6, and 7. Diarrhea was not observed in the 2 cases with persistent rotavirus antigenemia. Although it would be difficult to prove an association between rotavirus antigenemia and persistent diarrhea, because no complete examinations were carried out to exclude all other pathogens that would cause diarrhea, the current findings suggest that rotavirus antigenemia may be involved in the persistent diarrhea in HSCT recipients in some recipients. It was difficult to determine how many patients without rotavirus antigenemia had

diarrhea during the observation period following transplantation, because this study was a retrospective study using stored serum samples. Therefore, future prospective study is needed to elucidate the precise association between rotavirus antigenemia and diarrhea.

Moreover, asymptomatic rotavirus antigenemia was demonstrated in these patients. Asymptomatic rotavirus excretion in stool has also been reported in HSCT recipients, which could potentially make them index cases for nosocomial rotavirus infections (19). Thus, it is important to elucidate the ability of virus to be transmitted from asymptomatic rotavirus antigenemia patients. A prospective study that concurrently monitors rotavirus antigenemia and viral excretion in the stool is currently underway.

Notably, the timing after transplantation and occurrence of rotavirus antigenemia in non-endemic seasons in HSCT recipients were quite different from expected. According to a previous study based on detecting rotavirus antigen in stool, the median duration of rotavirus infection is 20 days after SOT (4). In our study, rotavirus antigenemia started within 4 weeks after transplant in all 6 cases except for Cases 7 and 8. Six of the 8 recipients were cared for in laminar airflow rooms at the beginning of rotavirus antigenemia. Although medical personnel may have caused nosocomial transmission, the likelihood of this possibility is very low, because standard precautions were thoroughly followed. Kang et al. (20) detected rotavirus antigen in the stool of an HSCT recipient at the time of pre-transplant screening. From a clinical standpoint, a large-scale molecular epidemiological study is needed to elucidate the route of viral transmission in HSCT recipients. In addition to the timing of rotavirus antigenemia, the seasons in which the rotavirus antigenemia occurred are another remarkable finding in this study. Although the endemic seasons in Japan for rotavirus infection are the winter and spring, rotavirus antigenemia was observed in the summer and fall in several cases (Fig. 1). Recently, a similar finding was demonstrated in SOT recipients (4). One possible mechanism for rotavirus antigenemia outside of its endemic season is the persistence of rotavirus infections in immunocompromised patients. Further human or animal studies are necessary to determine whether rotavirus can persistently infect a host.

We believe that this is the first report to demonstrate HLA mismatches as a significant risk factor for rotavirus antigenemia after HSCT, which is similar to Epstein–Barr virus (EBV) infection (30). ATG administration, which is another well-known risk factor for EBV infection after HSCT (30), tended to be a risk for rotavirus antigenemia, though it did not reach the level of statistical significance. Several characteristic factors have been suggested to pose significant risks for each viral infection in HSCT recipients. The present study suggests that the risk factors for rotavirus antigenemia are similar to those for EBV infection after HSCT. If the clinical significance of rotavirus antigenemia in HSCT recipients is confirmed, predictions about patients at high risk for rotavirus antigenemia would be important for improving their prognosis. Further clinical analysis of rotavirus antigenemia should be continued to determine the significance of rotavirus antigenemia on the morbidity or mortality of HSCT recipients.

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Prevalence and Genetic Characterization of Pertactin-Deficient *Bordetella pertussis* in Japan

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Abstract

The adhesin pertactin (Prn) is one of the major virulence factors of *Bordetella pertussis*, the etiological agent of whooping cough. However, a significant prevalence of Prn-deficient (Prn$^-$) *B. pertussis* was observed in Japan. The Prn$^-$ isolate was first discovered in 1997, and 33 (27%) Prn$^-$ isolates were identified among 121 *B. pertussis* isolates collected from 1990 to 2009. Sequence analysis revealed that all the Prn$^-$ isolates harbor exclusively the vaccine-type *prn1* allele and that loss of Prn expression is caused by 2 different mutations: an 84-bp deletion of the *prn* signal sequence (*prn1*ΔSS, $n=24$) and an IS*481* insertion in *prn1* (*prn1*::IS*481*, $n=9$). The frequency of Prn$^-$ isolates, notably those harboring *prn1*ΔSS, significantly increased since the early 2000s, and Prn$^-$ isolates were subsequently found nationwide. Multilocus variable-number tandem repeat analysis (MLVA) revealed that 24 (73%) of 33 Prn$^-$ isolates belong to MLVA-186, and 6 and 3 Prn$^-$ isolates belong to MLVA-194 and MLVA-226, respectively. The 3 MLVA types are phylogenetically closely related, suggesting that the 2 Prn$^-$ clinical strains (harboring *prn1*ΔSS and *prn1*::IS*481*) have clonally expanded in Japan. Growth competition assays in vitro also demonstrated that Prn$^-$ isolates have a higher growth potential than the Prn$^+$ back-mutants from which they were derived. Our observations suggested that human host factors (genetic factors and immune status) that select for Prn$^-$ strains have arisen and that Prn expression is not essential for fitness under these conditions.

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Introduction

Bordetella pertussis is the causative agent of pertussis or whooping cough, a highly contagious disease of the human upper respiratory tract. Adolescents and adults are its primary reservoir and play a crucial role in the transmission of the microbe to infants and unvaccinated children [1,2]. Immunization is the most effective method for the prevention and control of pertussis. In Japan, acellular pertussis (aP) vaccines were introduced in 1981 and pertussis has been controlled by means of a schedule of three primary doses and a single booster dose at ages 3, 4, 5, and 18–23 months. The vaccine coverage with three primary does has been ≥90%.

B. pertussis produces several virulence factors that contribute to its adherence to the respiratory ciliate epithelium. The virulence factors pertussis toxin (PT) and filamentous haemagglutinin (FHA) are critical antigens responsible for inducing immunity to *B. pertussis* and are included as major antigens in aP vaccines. Some aP vaccines include either the virulence factor pertactin (Prn) and/or fimbriae (Fim) as additional antigen(s). Among four currently used Japanese aP vaccines, two vaccines contain Prn (5–7.5 μg per 0.5 ml dose) and Fim2 (1 μg/dose), and others do not contain both Prn and Fim2 [3]. In contrast, aP vaccines widely used in Europe and the USA contain from 3 to 8 μg per dose of Prn: Infanrix, 8 μg; DAPTACEL, 3 μg. The three-component aP vaccine containing PT, FHA, and Prn is more effective than the two-component aP vaccine consisting of only PT and FHA [4,5]. In vaccine efficacy trials, the anti-Prn antibody level correlates with clinical protection, suggesting an important role for Prn in immunity [6]. In vitro studies also show that anti-Prn antibody is crucial for opsonophagocytosis [7].

Prn belongs to the type V autotransporter family whose members undergo autoproteolytic processing; mature Prn is a 69-kDa protein that is attached to the bacterial cell surface [8,9,10]. This protein contains an RGD (Arg-Gly-Asp) motif, which is implicated in ligand-receptor interactions in eukaryotes [11]. Prn is considered to function as an adhesin that can bind human epithelial cells; however, the host receptor for Prn has not been identified. Besides its potential role as an adhesin, *Bordetella bronchiseptica* Prn has been shown to function as a phage receptor [12,13]. During the last decade, Prn polymorphism has been described among *B. pertussis* strains circulating worldwide. Prn variation is mainly limited to 2 regions, designated as region 1 (R1) and region 2 (R2), which are composed of the repeat motifs (GGXXP)n and (PQP)n, respectively [14]. Most variations are found in R1, which is located adjacent to an RGD motif. Thirteen Prn variants have been identified so far [15,16]. In Japan, Prn1 and Prn2 variants currently predominate; however, the vaccine-

type Prn1 has been gradually replaced with the nonvaccine-type Prn2 since the mid-1990s [17]. A recent study shows that the ability of *B. pertussis* strains to colonize mouse lung decreases in the order Prn1>Prn2 and Prn3 [18].

B. pertussis Prn$^-$ isolates are present in Europe [19,20]. The Prn$^-$ isolates were collected in Italy ($n = 1$) and France ($n = 4$), and this phenotype is due to the deletion of *prn* or insertion of the IS*481* element. The IS*481* is present in multiple copies in the *B. pertussis* chromosome, causing frequent chromosomal rearrangements and deletions [21,22]. The emerging Prn$^-$ strains raise the possibility that the prevalence of Prn$^-$ strains reduces the efficacy of aP vaccines containing Prn. Here, we identified the significant prevalence of Prn$^-$ strains recently circulating in Japan. To obtain detailed insights into these strains with respect to their genetic, temporal, and geographical characteristics, we performed sequence analysis and multilocus variable-number tandem repeat analysis (MLVA). Using an in vitro growth competition assay, we attempted to gain insights into the biological mechanisms responsible for the prevalence of Prn$^-$ strains.

Results

Identification of Prn$^-$ isolates

B. pertussis Prn expression was analyzed by immunoblotting with anti-Prn1 antiserum. Figure 1 shows a representative blot of 6 Prn-positive and 4 negative isolates. Total 33 Prn$^-$ isolates were identified among 121 *B. pertussis* isolates collected in 1990–2009 in Japan, which we acquired from the National Institute of Infectious Diseases (NIID), Japan. Interestingly, all Prn$^-$ isolates harbor vaccine-type *prn1* and *ptxA2* alleles. The expression of other virulence factors PT, FHA, and Fim3 was detected in the recent Prn$^-$ isolates (collected in 2005–2009) by immunoblotting and serotyping. Detailed information on these 121 isolates is listed in Table S1.

Sequence analysis of Prn$^-$ isolates

To investigate the molecular basis for the loss of Prn expression in Prn$^-$ isolates, we sequenced the Prn gene of all 33 Prn$^-$ isolates. Two independent mutations were detected, which had caused the loss of Prn1: a deletion of the *prn1* signal sequence (*prn1*ΔSS) and an IS*481* insertion, *prn1*::IS*481* (Figure 2). The *prn1* signal sequence, which plays an important role in localizing Prn to the *B. pertussis* outer cell membrane, was deleted in 24 (73%) out of 33 Prn$^-$ isolates. All 24 isolates harboring *prn1*ΔSS had the same 84-bp deletion, resulting in the deletion of 28 amino acid residues (Val^9–Trp^{36}) (Figure 2A). Secondary structure analysis also showed that the deleted DNA sequence is predicted to form a hairpin-loop structure (Figure S1). In contrast, 9 (27%) of 33 Prn$^-$ isolates were shown to contain the IS*481* insertion in *prn1*. Eight IS*481* sequences were specifically inserted in the 5′–3′ orientation between a 6-bp direct repeat (ACTAGG, 1593–1598 bp), and 1 was oriented in the opposite direction (Figure 2B).

Temporal and geographical characterization in Prn$^-$ isolates

Figure 3 shows the temporal trend of the frequency of Prn$^-$ strains among 121 *B. pertussis* isolates according to the year of collection. The frequencies of Prn$^-$ isolate harboring *prn1*ΔSS were 0, 0, 27 and 25% in the periods 1990–1994, 1995–1999, 2000–2004 and 2005–2009, respectively. In contrast, the frequencies of Prn$^-$ isolates harboring *prn1*::IS*481* were 0, 5, 11 and 7% in 1990–1994, 1995–1999, 2000–2004 and 2005–2009, respectively. Notably, the total percentage of the Prn$^-$ isolates significantly increased from the 2000s, i.e., 0% in 1990–1994, 5% in 1995–1999, 38% in 2000–2004 and 32% in 2005–2009.

During 1990 to 2000, 5 Prn$^-$ isolates (*prn1*ΔSS, 4 isolates; *prn1*::IS*481*, 1 isolate) were collected only in the Kanto district. Thereafter, Prn$^-$ isolates were collected in several areas during

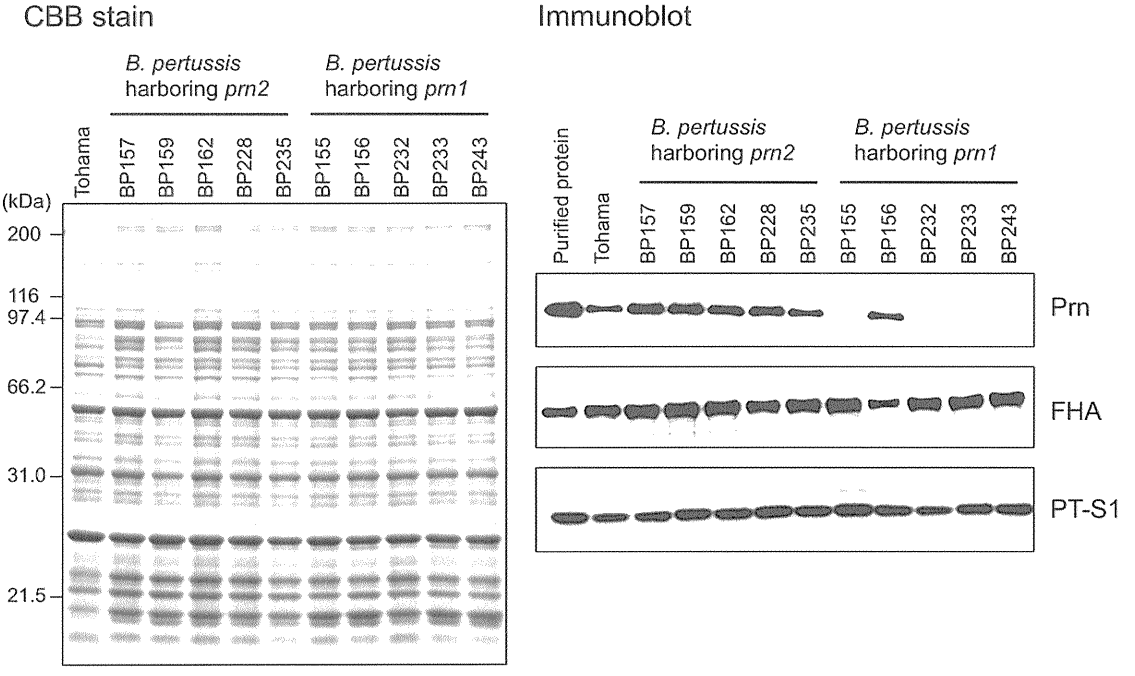

Figure 1. Prn expression in *B. pertussis* clinical isolates. The isolates harboring *prn2* allele (BP157, BP159, BP162, BP228, and BP235) and *prn1* allele (BP155, BP156, BP232, BP233, and BP243) were cultured on CSM plates. Total protein (10 μg) extracted from the bacterial cells was separated by SDS-PAGE followed by CBB R-250 staining (left panel). Immunoblots (1 μg protein/lane) were incubated with anti-Prn1, anti-PT or anti-FHA antiserum (right panel). Ten ng of purified Prn1, PT, or FHA and total protein (1 μg) from *B. pertussis* Tohama were run on the gel as positive controls.
doi:10.1371/journal.pone.0031985.g001

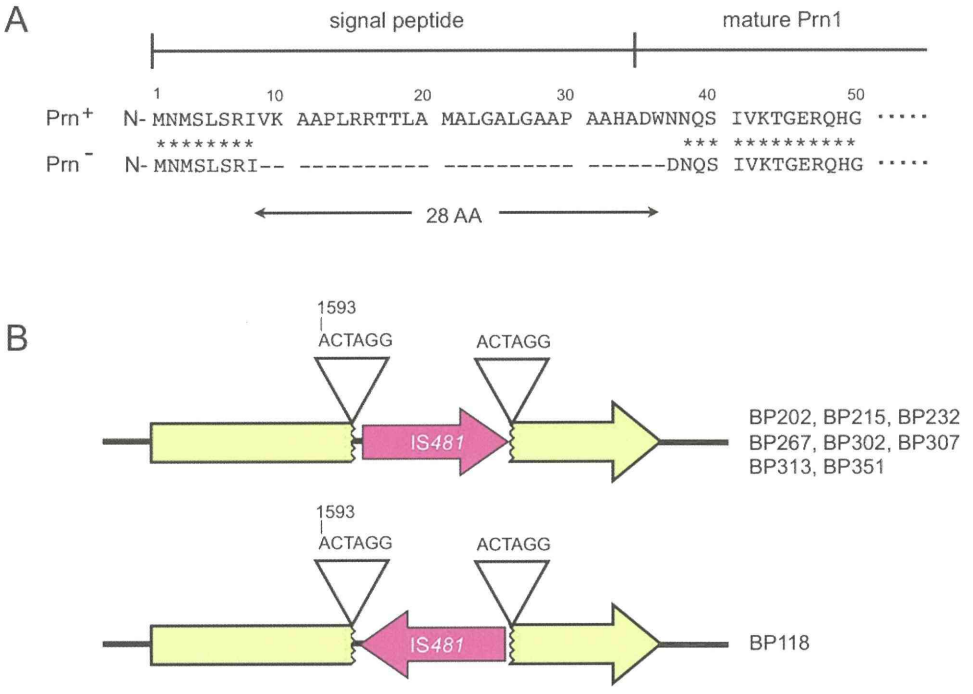

Figure 2. Molecular mechanisms of loss of Prn expression. (A) Deletion of the Prn signal sequence (*prn1*ΔSS). Prn$^-$ isolates ($n = 24$) have an 84-bp deletion, resulting in a 28-amino acid deletion (Val^9 to Trp^{36}) in the N-terminal region. (B) IS*481* insertion mutation in Prn1 gene (*prn1*::IS*481*). Eight Prn$^-$ isolates have an IS*481* insertion in the forward direction at the 6-bp direct repeats (ACTAGG, 1593–1598 bp) of *prn1*, and 1 isolate had the insertion in the reverse.
doi:10.1371/journal.pone.0031985.g002

2001 to 2009 (Figure 4). In the period from 2000 to 2009, 20 Prn$^-$ isolates harboring *prn1*ΔSS were collected from Tohoku, Kanto, Chubu, Kinki, and Kyushu districts, and 8 Prn$^-$ isolates harboring *prn1*::IS*481* were collected from Kanto, Chubu, Kinki, Shikoku, and Kyushu. These findings indicate that Prn$^-$ isolates were present nationwide since 2000.

Molecular epidemiology of Prn$^-$ isolates

Thirty-three Prn$^-$ and 88 Prn$^+$ isolates collected by the NIID between 1990 and 2009 were subjected to MLVA. Among the 121 isolates, 33 different MLVA types were identified, of which 10 were novel (MLVA-223 to -227, -229, -230, -233, -234, and -248) (Figure 5 and Table S1). Twenty-six of these MLVA types were present at low frequencies (each, ≤2% of all isolates). Thirty-three Prn$^-$ isolates belonged to only 3 MLVA types; 24 isolates (73%) were MLVA-186, 6 isolates (18%) were MLVA-194, and 3 isolates (9%) were MLVA-226. MLVA-186 was the predominant type (frequency, 35% of all isolates), whereas MLVA-194 and MLVA-226 were minor (frequency, 5% and 2%, respectively). The 3 MLVA types were closely related phylogenetically. When categorized by their mutations, 24 Prn$^-$ isolates harboring *prn*ΔSS

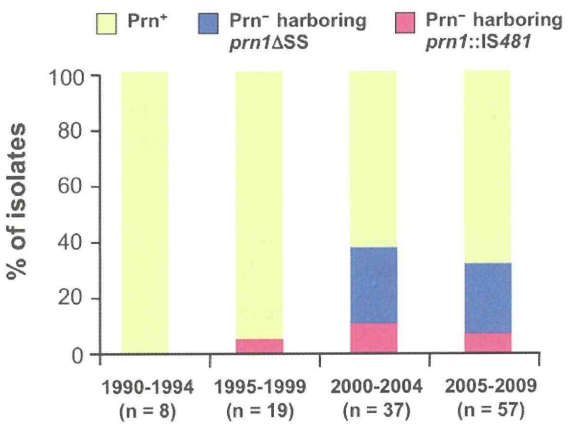

Figure 3. Temporal trend of the occurrence of Prn$^-$ isolates in Japan. The frequencies of Prn$^-$ isolates harboring *prn1*ΔSS and *prn1*::IS*481* were based on 121 *B. pertussis* isolates collected during 1990–2009. Prn$^+$ indicates Prn-expressing isolate.
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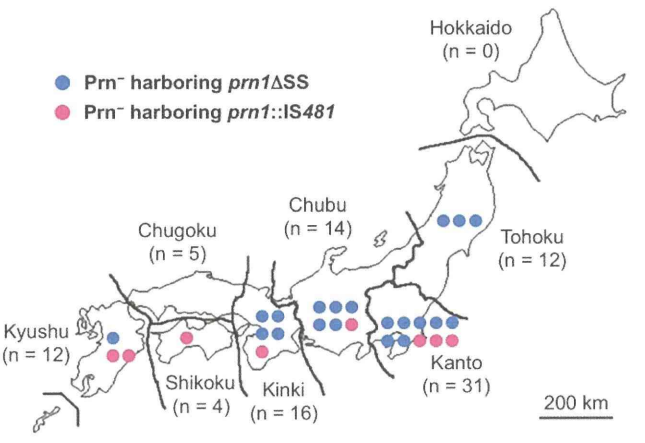

Figure 4. Geographical distribution of Prn$^-$ isolates in Japan during 2001–2009. Blue and red circles indicate Prn$^-$ isolates harboring *prn1*ΔSS and *prn1*::IS*481*, respectively. Numbers of isolates tested are indicated in parentheses.
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