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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Characterization of Neutralizing Antibodies in Adults After Intranasal Vaccination With an Inactivated Influenza Vaccine

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The levels and properties of neutralizing antibodies in nasal wash and serum collected from five healthy adults were examined after intranasal administration of an A/Uruguay/716/2007 (H3N2) split vaccine (45 μg hemagglutinin (HA) per dose; five doses, with an interval of 3 weeks between each dose). Prior to the assays, nasal wash samples were concentrated so that the total amount of antibodies was equivalent to about 1/10 of that found in the natural nasal mucus. Vaccination induced virus-specific neutralizing antibody responses, which increased with the number of vaccine doses given. Neutralizing antibodies were produced more efficiently in the nasal passages than in the serum: A ≥4-fold increase in nasal neutralization titres was observed after the second vaccination in four out of five subjects, whereas a rise in serum neutralization titres was observed only after the fifth vaccination. Nasal and serum neutralizing antibodies were mainly found in the polymeric IgA and monomeric IgG fractions, respectively, after gel filtration. Taken together, these results suggest that intranasal administration of an inactivated split vaccine induces high levels of nasal neutralizing antibodies (primarily polymeric IgA) and low levels of serum neutralizing antibodies (primarily monomeric IgG). ***J. Med. Virol. 84:336–344, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: influenza; vaccine; neutralizing antibody

INTRODUCTION

To prevent influenza, protective immunity must be induced in advance by administration of a vaccine. Currently available inactivated vaccines, detergent disrupted split-viruses, or purified glycoproteins (surface antigen vaccines) are given via parenteral injection [Murphy and Webster, 1996]. Parenteral vaccination, that is, vaccination via the non-mucosal route, induces serum IgG antibodies, which are highly protective against homologous virus infection, but less effective against heterologous virus infection. Thus, intramuscular vaccination of seasonal influenza vaccine would be less effective in protecting against a heterologous virus epidemic.

A large number of studies show that the protective immunity induced by influenza virus infection is mainly mediated by secretory IgA (S-IgA) and IgG antibodies within the respiratory tract. S-IgA is carried to the mucus by transepithelial transport, while serum IgG is transported from the serum to the mucus by diffusion [Murphy and Clements, 1989; Brandtzag et al., 1994; Murphy, 1994; Asahi et al., 2002; Asahi-Ozaki et al., 2004]. S-IgA in the upper respiratory tract prevents viral infection, while IgG supports S-IgA-mediated protection by neutralizing newly-generated viruses [Ito et al., 2003; Renegar et al., 2004]. IgG is the main antibody involved in anti-viral protection in the lungs [Ramphal et al., 1979; Palladino et al., 1995; Renegar et al., 1998; Ito et al., 2003]. Also, polymeric S-IgA neutralizes viruses more effectively than monomeric IgA or IgG [Taylor

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and Dimmock, 1985; Renegar et al., 1998]. The polymeric nature of S-IgA also explains why S-IgA cross-reacts with variant influenza viruses to a greater extent than serum IgG [Tamura et al., 1990, 1991, 1992; Asahi-Ozaki et al., 2004]. Thus, intranasal administration of an inactivated influenza vaccine is advocated to elicit S-IgA and IgG responses and improve the protective efficacy of current vaccination procedures [Tamura and Kurata, 2004; Tamura et al., 2005, 2010].

Several clinical trials have examined the induction of both S-IgA and IgG following intranasal administration of inactivated influenza vaccines, either with or without adjuvant [Kuno-Sakai et al., 1994; Hashigucci et al., 1996; Muszkat et al., 2000; Greenbaum et al., 2002; Durrer et al., 2003; Treanor et al., 2006; Atmar et al., 2007]. The antibody responses after intranasal administration of inactivated influenza vaccines were assessed by measuring hemagglutination inhibition (HI) titres in the serum, and anti-hemagglutinin (HA) IgA and IgG titres in nasal wash samples. They did not measure the titre of neutralizing antibodies, which is considered to be a better criterion for functional protective antibodies. Neutralization titres can directly inhibit the complex process involved in virus replication, which include virus attachment and entry to the host cells, and release of newly-synthesized virus from the infected cells in tissue culture. In addition, a previous study found that HI titres were lower, or higher, than the corresponding neutralization titres, depending on a strain of influenza A or B virus used for the assay [Okuno et al., 1990], whereas other studies show that anti-H5 HI antibodies fail to detect H5N1 viruses [Lu et al., 1982; Rowe et al., 1999]. Thus, neutralizing antibody responses following intranasal administration of an inactivated influenza vaccine remain to be fully characterized.

Therefore, the aim of the present study was to examine the levels and properties of neutralizing-antibodies in nasal wash and serum samples from healthy adults after intranasal administration of an inactivated vaccine (five doses, with an interval of 3 weeks between each dose). The inactivated vaccine used in this study was a concentrated split-virus vaccine (containing 45 μg HA per dose), prepared from the A/Uruguay/716/2007 (H3N2) strain. A concentrated split-virus vaccine was chosen because the vaccine has already been shown to induce mucosal antibody responses after intranasal vaccination [Kuno-Sakai et al., 1994]. To ensure that neutralization titres specific for the A/Uruguay/716/2007 virus were assayed at equivalent levels in both serum and nasal wash samples, the neutralization titres were measured using concentrated nasal wash samples (1 mg/ml total protein) that contained approximately 1/10 of the IgA found in undiluted mucus [Kurono and Mogi, 1987]. The properties of the neutralizing IgA and IgG antibodies induced by intranasal vaccination were then examined, and their relative levels and molecular size were determined.

MATERIALS AND METHODS

Subjects

Five healthy male subjects (P1, P2, P3, P4, and P5) were enrolled in the study (aged 22, 32, 42, 42, and 68 years, respectively, at the time of the first vaccination). All participants had already acquired some degree of immunity to H1N1 and H3N2 influenza A virus subtypes after previous exposure to these viruses and/or as a result of previous vaccinations. Each subject provided informed consent and the study protocol and other relevant documentation were reviewed and approved by the Ethics Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Virus and Vaccine

The A/Uruguay/716/2007 (A/Uruguay; H3N2) influenza virus strain was propagated in the allantoic cavity of 10-day-old embryonated hen's eggs and purified from the allantoic fluid. The $TCID_{50}$ (50% infectious dose in tissue culture) of the virus was estimated as described previously [Tobita et al., 1975; Kadowaki et al., 2000]. In brief, 10-fold serial dilutions of the allantoic fluid containing the virus were inoculated into Madin-Darby canine kidney (MDCK) cells (ATCC No. CCL-34) cells in 96-well culture plates and incubated for 4 days at 37°C in a 5% CO_2 humidified atmosphere. The cytopathic effects in the virus-containing wells were monitored under a microscope and the $TCID_{50}$ was calculated using the Reed–Muench method. The split product virus vaccine was supplied by the Research Foundation for Microbial Disease of Osaka University (BIKEN, Kanonji, Japan). The vaccine was prepared from purified viruses, which were sedimented through a linear sucrose gradient according to the manufacturer's protocol. The viruses were then treated with ether and formalin according to the manufacturer's protocol, which was based on the method of Davenport et al. [1964]. The concentrated split vaccine containing 45 μg HA was the product of a process used to prepare a trivalent vaccine comprising A/H1N1, A/H3N2, and B type vaccines, each containing 15 μg HA.

Vaccinations

All participants were immunized intranasally with a threefold concentrated split H3N2 virus vaccine (A/Uruguay, containing 45 μg HA). Each received five doses, with an interval of 3 weeks between each dose. Intranasal vaccination was performed by spraying 0.25 ml of the split vaccine into each nostril (0.5 ml total) using an atomizer (Keytron, Ichikawa, Japan). The mean droplet diameter was 56.5 μm, ranging in size between 10 μm and 90 μm.

Nasal Wash and Serum Samples

About 100 ml of nasal wash was collected from each participant in polypropylene tubes by washing the

nasal cavity several times using a nose irrigation device (Hananoa; Kobayashi Pharmaceutical, Osaka, Japan) filled with saline solution according to the manufacturer's instructions. Pieces of dental cotton (Dental Cotton Roll; B.S.A. Sakurai, Nagoya, Japan) were then immersed in the collected nasal washes. Dental cotton pieces (containing a combined absorbed volume of about 25 ml of nasal wash) were then placed into a filter insert (Oxi Fil filter insert; TOHO, Tokyo, Japan) with bottoms drilled to create several pores, and placed in 50 ml polypropylene centrifuge tubes. Clean nasal wash was separated from mucopolysaccharides and other debris by centrifugation at 2,200*g* for 5 min at room temperature. This procedure was repeated for the entire 100 ml nasal wash sample from each participant. The pooled, clean nasal wash was then concentrated to a final volume of approximately 0.5 ml using Vivaspin centrifugal concentrators (Vivaspin 20, MWCO = 30,000; Sartorius Stedim Biotech, Aubagne, France). The concentrated nasal wash was stored at −80°C before use.

Quantitation of IgA, IgG and IgM Antibodies and Other Proteins

The levels of human IgA, IgG, and IgM antibodies in the nasal wash and serum samples were estimated using human IgA, IgG, or IgM ELISA kits (Bethyl Laboratories, Montgomery, USA). The level of human serum albumin in the nasal wash samples was estimated using a Human Albumin ELISA kit (Bethyl Laboratories). The protein concentration in the samples was measured using either a BCA Protein Assay Kit, or a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions.

Neutralization Assays

The level of serum antibodies against the vaccine viruses was examined using micro-neutralization assays as previously described [Belshe et al., 2000; Kadowaki et al., 2000] with minor modifications. In brief, serum samples were treated with a receptor-destroying enzyme (RDE(II); Denka Seiken, Tokyo, Japan) overnight at 37°C and heat-inactivated for 30 min at 56°C before use. The first dilution tested in the assays was 1:10. The concentrated nasal wash samples [1 mg/ml total protein, corresponding to about 1/10 of the total IgA found in nasal mucus (2.20 mg/ml)] [Kurono and Mogi, 1987] were also treated with RDE(II) and heat-inactivated before use. The first dilution tested in the nasal wash assays was 1:20. Twofold serial dilutions of the serum samples were mixed with an equal volume (50 μl) of diluent containing influenza virus equivalent to 100 $TCID_{50}$. Each mixture was added to the wells of a 96-well plate containing a monolayer of MDCK cells. Four control wells were included on each plate and contained either virus or diluent alone. The plates were then incubated for 4 days at 37°C in a 5% CO_2-humidified atmosphere. The monolayer in each well was observed for the presence or absence of cytopathic effects, fixed with 10% formalin for more than 5 min at room temperature, and stained with Naphthol blue black. After the plates were washed and dried, the stained cells were solubilized with 0.1 M NaOH and the absorbance (A) was measured at 630 nm. The average $A_{630\ nm}$ value was determined from quadruplicate virus-infected wells (A_{virus}) and cell culture-only controls (A_{cell}). All values above 50% of the specific signal, calculated using the formula: $X = (1/2) \times (A_{cell} - A_{virus}) + A_{virus}$, were considered positive for neutralization. The titres recorded were the reciprocal of the highest dilution, where A_{630} was >X.

Hemagglutination Inhibition

The antibody responses to the vaccine viruses were examined in serum and nasal washes using HI antibody assays incorporating a microtiter method as described elsewhere [Hierholzer et al., 1969]. All samples were pre-treated with RDE(II) at 37°C for 18 hr, subsequently inactivated at 56°C for 30 min, and mixed with packed red blood cells to remove any nonspecific inhibitors. The starting material for the assays was a 1:10 dilution for the serum samples and a 1:40 dilution for the nasal wash samples.

Fractionation of Nasal and Serum Samples

The concentrated nasal wash samples (100 μl, 6 mg/ml) and diluted serum samples (10-fold dilution, 100 μl, about 6 mg/ml) were fractionated on a Superose 6 10/300 GL gel filtration column using an FPLC-AKTA chromatography system (GE Healthcare, Little Chalfont, UK). The concentrated nasal wash sample was treated with 1 μg/ml of lysozyme (Sigma-Aldrich, St. Louis, MO) for 1 hr at 37°C to decrease the viscosity and then centrifuged using Vivaspin to remove the lysozyme prior to gel filtration. Fractions (each 500 μl) were collected in PBS at a flow rate of 0.1 ml/min; little or no change in the fractionation pattern of the antibodies in the concentrated nasal wash samples was observed following lysozyme treatment. Molecular weight marker proteins (Kit for Molecular Weights 29,000–700,000 Da; Sigma-Aldrich) were eluted under the same conditions to determine the size of each fraction.

RESULTS

Measurement of Neutralization and HI Titres in Concentrated Nasal Wash Samples

The total protein level and the levels of IgA, IgG, and IgM and human serum albumin in 100 ml of unconcentrated nasal wash and in approximately 0.5 ml of concentrated nasal wash are shown in Table I. About 70% of the total nasal wash proteins were lost during the concentration process. Also, a fraction of the higher molecular weight (MW) proteins and lower MW proteins (less than 30 kDa) was lost by

TABLE I. Concentration of IgA, IgG, IgM and HSA in 0.5 ml of Solution Concentrated From 100 ml of Nasal Wash (n = 10)*

Unit	Concentration: Mean ± SD				
	Total protein	IgA	IgG	IgM	HSA
Nasal wash (n = 10)					
Unconcentrated					
mg/100 ml	5.875 ± 1.856	1.132 ± 0.678	0.125 ± 0.057	0.032 ± 0.021	0.531 ± 0.280
Concentrated					
mg/0.43 ± 0.06 ml	1.647 ± 0.549	0.375 ± 0.193	0.093 ± 0.044	0.007 ± 0.006	0.292 ± 0.214
Concentration calculated in terms of total protein (mg/ml)	1.00	0.217	0.057	0.004	0.177

*The concentration was calculated using two nasal wash samples collected from five participants (with a 1 week interval).

adsorption to the cotton and during Vivaspin centrifugation, respectively. However, better recovery was observed for IgA and IgG. When the concentration of the enriched nasal washes was adjusted to 1 mg/ml total protein, the amount of IgA was 0.217 mg/ml. This amount of IgA in the concentrated nasal wash corresponded to about 1/10 of the levels of total IgA recovered from nasal mucus (2.20 mg/ml) by aspiration as reported by Kurono and Mogi [1987] (Table I). In subsequent experiments, neutralization and HI titres in the nasal wash samples were measured using concentrated nasal wash proteins (1 mg/ml of total protein), which contained 1/10 of the IgA found in mucus, to ensure that the nasal and serum neutralization titres were assayed at equivalent levels.

The amount of total IgA and total IgG in the nasal wash samples from each participant varied slightly at each sampling time. Also, the level of total IgA and IgG antibodies did not increase significantly between pre-vaccination and post-vaccination in any of the participants. Thus, the average amount of total IgA or total IgG in the nasal wash samples from the five participants was relatively constant (data not shown).

Neutralizing Antibody Responses in Nasal Wash and Serum Samples

Next, antibody responses in the nasal wash and serum samples were examined in all five study participants. The responses are presented as neutralization titres against the A/Uruguay (H3N2) virus in Table II. The responses recorded in the four young adults (between 18- and 50-years-old) are also shown as geometric neutralization titres (Fig. 1). The nasal wash and serum neutralization titres increased in all participants as the number of vaccinations increased, although the degree of increase differed between participants. In addition, nasal wash neutralization titres increased more rapidly than serum titres. The nasal wash titres showed at least a fourfold increase after the second vaccination in the four young participants (all of whom had a nasal wash neutralization titre of 1:20 or 1:40 before vaccination). By contrast, a fourfold increase in the serum titre was observed only after the fifth vaccination in three of the participants (all of whom had serum titres of <1:10, 1:20, or 1:40 before vaccination). Participant P5, who was 67 years old, showed at least a fourfold increase in nasal wash titre after the fourth vaccination, but no significant

TABLE II. Neutralizing Antibody Responses in Subjects who Received the Threefold Concentrated A/Uruguay/716/2007 (H3N2) Split Vaccine

Weeks (vaccination)	Neutralization titre against A/Uruguay virus (H3N2)[a]									
	P1		P2		P3		P4		P5	
	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum
0 (1st)	20 (1)	40 (2)	20 (1)	<10 (<0)	40 (2)	20 (1)	20 (1)	<10 (<0)	20 (1)	<10 (<0)
3 (2nd)	80 (3)	160 (4)	20 (1)	<10 (<0)	80 (3)	20 (1)	20 (1)	<10 (<0)	40 (2)	<10 (<0)
6 (3rd)	160 (4)	160 (4)	80 (3)	10 (0)	320 (5)	20 (1)	80 (3)	<10 (<0)	40 (2)	<10 (<0)
9 (4th)	320 (5)	160 (4)	160 (4)	20 (1)	1280 (7)	40 (2)	160 (4)	10 (0)	40 (2)	<10 (<0)
12 (5th)	320 (5)	160 (4)	320 (5)	40 (2)	2560 (8)	80 (3)	80 (3)	10 (0)	80 (3)	<10 (<0)
15	1280 (7)	160 (4)	320 (5)	40 (2)	2560 (8)	80 (3)	160 (4)	20 (1)	160 (4)	10 (0)
28	640 (6)	160 (4)	160 (4)	40 (2)	1280 (7)	80 (3)	N.D.	N.D.	80 (3)	10 (0)

N.D., not done.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.

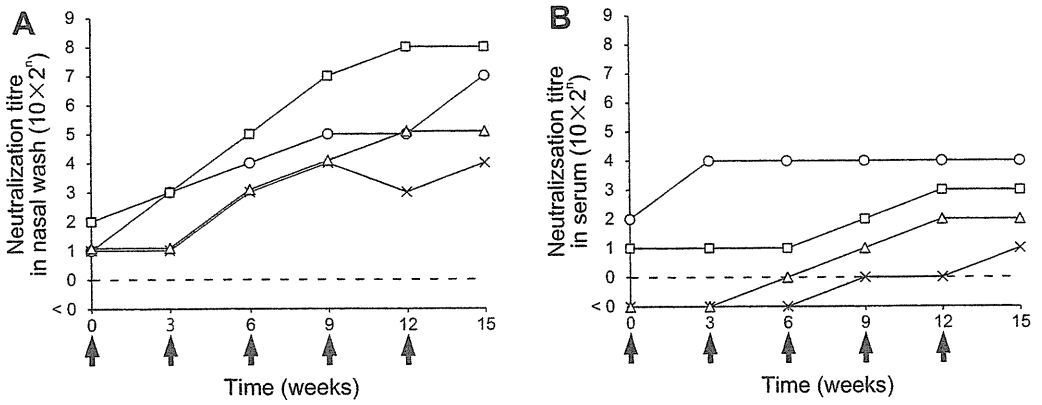

Fig. 1. Nasal wash and serum sample neutralization titres against A/Uruguay (H3N2) (pre- and post-intranasal immunization). Neutralization titres against the A/Uruguay virus in nasal washes (A) and serum (B) were determined in samples collected from four participants (18–60 years old; P1, open circle; P2, open triangle; P3, open square; and P4, cross). The participants were given five doses of the threefold concentrated A/Uruguay split influenza vaccine intranasally with an interval of 3 weeks between doses (each arrow indicates a point of vaccination). The neutralization titre shown is the geometric titre.

increase was observed in serum titre after five vaccinations. In all participants, the nasal wash and serum titres were largely maintained, even at 16 weeks after the fifth vaccination, at which point the nasal wash titre decreased only slightly, while no decrease was observed in the serum titre (Table II).

HI Antibody Responses in Nasal Wash and Serum Samples

Antibody responses were also examined by measuring the HI titre against the A/Uruguay (H3N2) virus. Table III shows the pre-vaccination HI titres of the nasal wash and serum samples from two participants, and the HI titres 3 weeks after each of the five vaccinations. For each participant, the HI titres were lower than the neutralization titres shown in Table II. The HI titres were approximately 1/4–1/8 the level of the neutralization titres. Statistical correlation analysis of the data presented in Tables II and III showed a strong correlation between the HI titres and the neutralization titres ($r = 0.8699$). Thus, the HI titre correlated with the neutralization titre, although it was less sensitive than the neutralization titre.

TABLE III. Hemagglutinin Inhibition (HI) Antibody Responses in Subjects who Received the Threefold Concentrated A/Uruguay/716/2007 (H3N2) Split Vaccine

	HI titre against A/Uruguay virus (H3N2)[a]			
	P1		P2	
Weeks (vaccination)	Nasal wash	Serum	Nasal wash	Serum
0 (1st)	N.D.	10 (0)	<40 (<2)	<10 (<0)
3 (2nd)	<40 (<2)	20 (1)	<40 (<2)	<10 (<0)
6 (3rd)	<40 (<2)	20 (1)	<40 (<2)	<10 (<0)
9 (4th)	40 (2)	20 (1)	<40 (<2)	10 (0)
12 (5th)	40 (2)	20 (1)	80 (3)	20 (1)
15	160 (4)	40 (2)	80 (3)	20 (1)

N.D., not done.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.

Fractionation of The Nasal Wash and Serum Samples

The types of antibody present in the nasal wash and serum samples were examined after fractionation on a gel filtration column. The concentrated nasal wash samples (100 μl, about 6 mg/ml) and diluted serum samples (10-fold diluted sera, 100 μl, about 6 mg/ml) were fractionated on a Superose 6 column in PBS. The antibody concentration in each fraction was then measured by ELISA. Figure 2 shows the profiles for IgM, IgA, and IgG antibodies, together with the absorbance values for the total protein in each fraction. The nasal wash samples contained IgM, which comprised less than 1% of the total protein and showed a peak MW of 970 kD; IgA, which comprised about 20% of the total protein and showed a peak MW of about 660 kD; and IgG, which comprised about 6% of the total protein and showed a peak MW of 150 kD. The MW of the nasal IgA (150 kD–900 kD, with a peak MW of 660 kD) appeared to correspond to that of tetrameric IgA (the MW of dimeric IgA is estimated to be about 360 kD). The maximum absorbance value observed in the protein profile (at around 66 kD) was due to the presence of human serum albumin (Fig. 2A).

The serum samples contained IgM, which comprised about 3% of the total protein and showed a peak MW of 970 kD; IgA, which comprised about 6% of the total protein and showed a peak MW of about 150 kD; and IgG, which comprised about 23% of the total protein and showed a peak MW of 150 kD

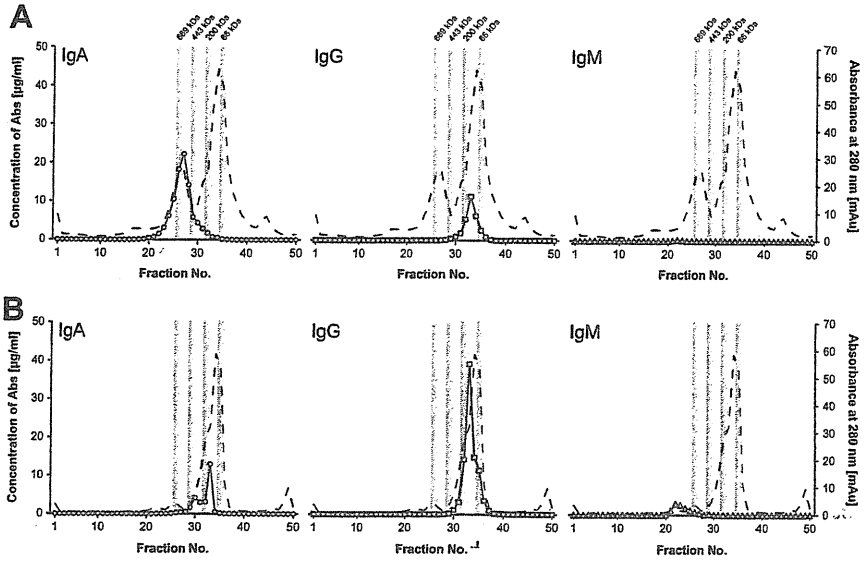

Fig. 2. Fractionation of nasal wash (A) and serum (B) samples from representative participants on Superose 6 columns. Quantification (µg/ml) of IgA (open circles), IgG (open squares), or IgM (open triangles) antibody levels and the absorbance at 280 nm (mAu, broken line) are shown. The grey zones in the upper part of the curves indicate the positions of the molecular weight markers [thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), and bovine serum albumin (66 kD)].

(Fig. 2B). Serum IgA (which showed a lower peak at about 360 kD in addition to a peak at about 150 kD) appeared to comprise both monomeric and dimeric IgA.

Taken together, the results of the fractionation analysis suggests that highly polymeric IgA is the predominant nasal antibody, and can be separated from nasal IgG and IgM. By contrast, the monomeric forms of IgG are the major component of total serum antibodies.

Neutralization Activity of the IgA and IgG Antibodies in The Nasal Wash and Serum Samples

To determine the isotype of the antibodies responsible for the neutralization activity induced by intranasal administration of the inactivated vaccine, nasal wash and serum samples from participant P1, who showed relatively high neutralization titres after the fifth vaccination, were separated on a Superose 6 column and the neutralization titre of the resulting antibody fractions assayed. The nasal polymeric IgA fraction (No. 27) showed a neutralization titre of 1:10, whereas the nasal monomeric IgG fraction (No. 33) showed a reciprocal neutralization titre of <1:10. However, the serum dimeric IgA fraction (No. 30) showed a neutralization titre of <1:10, whereas the serum peak monomeric IgG fraction (No. 33) showed a neutralization titre of 1:10 (Table IV). The respective peak fractions in the nasal wash were then concentrated to 100 µg/ml, and the neutralization activity of the nasal IgA antibodies (a mixture of fractions 26 and 27) was compared with that of the nasal IgG antibodies (a mixture of fractions 33 and 34). The nasal IgA fractions showed a neutralization titre of 1:40, whereas the nasal monomeric IgG fractions showed a neutralization titre of 1:10. Similarly, the neutralization activity of the serum IgA antibodies (100 µg/ml; a mixture of fractions 30 and 31) was compared with that of serum IgG antibodies (a mixture of fractions 33 and 34). The serum IgA fractions showed a neutralization titre of <1:10, whereas the serum

TABLE IV. Neutralization Titre of the IgA and IgG Fractions From the Nasal Wash and Serum Samples Following Separation on Superose Columns

	Neutralization titre[a]			
	Nasal wash		Serum	
	Polymeric IgA	IgG	Dimeric IgA	IgG
A/Uruguay (A/H3N2)				
Peak fraction: Separated on Superose column	10 (0)	<10 (<0)	<10 (<0)	10 (0)
Concentrated fraction (100 µg/ml)	40 (2)	10 (0)	<10 (<0)	10 (0)

The samples were collected from a representative subject vaccinated five times with an interval of 3 weeks between vaccinations.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.

IgG fractions showed a neutralization titre of 1:10 (Table IV).

The peak polymeric IgA fraction (about 600 kD) from the nasal wash samples, as measured using an IgA ELISA, contained no IgG antibodies when measured using an IgG ELISA; however, the peak monomeric IgG fractions (about 150 kD) from the nasal wash comprised about 1/4 of IgA (data not shown). By contrast, about 1/10 of the peak dimeric IgA (about 380 kD) from the serum samples comprised IgG antibodies, whereas about 1/10 of the peak monomeric IgG fractions from the serum comprised IgA (data not shown). This suggests that nasal polymeric IgA is responsible for the neutralization activity observed in the peak polymeric IgA fractions (about 600 kD) from the nasal wash samples. Serum monomeric IgG appears to be responsible for the neutralization activity observed in the peak monomeric IgG fractions (about 150 kD) from the serum, because the IgA content of the IgG fractions was very small. In those nasal monomeric IgG fractions that contained a relatively high amount of IgA, both IgG and IgA may be responsible for the neutralization activity. Taken together, these results show that the main neutralizing antibody in the nasal mucus is highly polymeric IgA, while the main neutralizing antibody in the serum is monomeric IgG.

DISCUSSION

In the present study, neutralizing antibody responses and their properties were examined in nasal and serum samples from healthy adults after intranasal administration of a concentrated, inactivated split A/Uruguay (H3N2) vaccine (containing 45 μg HA per dose). The first intranasal administration of a concentrated split vaccine in young adults was conducted by Kuno-Sakai et al. [1994] and showed that both serum HI- and nasal HA-specific IgA antibodies were induced after two aerosol vaccinations, which protected against a challenge infection with a cold-adapted live virus vaccine. In the present trial, neutralizing antibody responses were examined in both serum and nasal wash samples obtained from adults given five doses of vaccine, with an interval of 3 weeks between doses. The nasal wash samples were concentrated to ensure that nasal and serum neutralization titres were assayed at equivalent levels (Table I).

To measure the concentration of IgA and IgG antibodies in the concentrated nasal wash samples, the standardized nasal wash samples were adjusted to 1 mg/ml of total protein, and contained about 1/10 amount of IgA and IgG found in natural nasal mucus [Kurono and Mogi, 1987]. Previous studies show that the total amounts of IgA and IgG increase between pre-vaccination and post-vaccination in BALB/c mice [Tamura et al., 1990, 2010]; however, the results of the present study show that the amount of total IgA (and other antibodies) recovered from the nasal mucus showed small variations at each sampling time, although this was not related to vaccination status (data not shown). Even allowing for small variations in the recovery of total IgA and IgG from the nasal mucus of each subject, the neutralization titres in the standardized nasal wash samples after vaccination appeared to be a reasonable reflection of the absolute antibody titre in the nasal mucus.

A ≥4-fold increase in the nasal neutralization titre was observed after the second vaccination in the four younger subjects, whereas a rise in the serum neutralization titre was observed only after the fifth vaccination in the three younger subjects (Table II and Fig. 1). Intranasal administration of a vaccine tends to induce inferior serum antibody responses, but superior nasal IgA responses, compared with intramuscular injection [Atmar et al., 2007]. The present study also showed that neutralization titres correlated well with HI titres, although the HI titres were lower than the corresponding neutralization titres (Table III). This result confirms the work of Okuno et al. [1990], who showed that HI titres are sometimes lower than the corresponding neutralization titres, depending on the strain of influenza A or B virus used in the HI assay.

Healthy adults who had already acquired immunity to influenza viruses due to previous natural infections or vaccinations (seropositive adults) showed both nasal and serum antibody responses induced by the nasal vaccine (Tables II and III, and Fig. 1). Clinical trials show that intranasal administration of inactivated vaccines induces both mucosal and systemic antibody responses in seropositive adults [Kuno-Sakai et al., 1994; Hashigucci et al., 1996; Muszkat et al., 2000; Greenbaum et al., 2002; Durrer et al., 2003; Treanor et al., 2006; Atmar et al., 2007]. The induction of antibody responses in seropositive people by the nasal vaccine can be explained by the notion that the seropositive people have immunological memory for influenza viruses. Previous reports show that administration of an intranasal split vaccine plus adjuvant induces both local and systemic antibody responses in naive mice, and that the adjuvant is not required for a booster dose to induce an enhanced anamnestic immune response 4 weeks later [Tamura et al., 1989, 1992]. Administration of an adjuvant together with the vaccine stimulates innate immunity via several classes of pattern-recognition receptors (such as Toll-like receptors), which leads to the acquisition of specific immune responses, including immunological memory [Tamura et al., 1991, 2005; Tamura and Kurata, 2004].

Analysis of nasal wash and serum samples after passage through Superose 6 columns showed that the major component of nasal mucus antibodies was highly polymeric IgA, while that of serum antibodies was IgG (Fig. 2). In those subjects that received five doses of the intranasal A/Uruguay (H3N2) vaccine, the highly polymeric nasal IgA fractions were responsible for the majority of the neutralizing activity, whereas

the serum IgG fractions were responsible for the majority of the neutralizing activity in the serum (Table IV). These data are in agreement with those obtained in a previous mouse model experiment, in which IgA antibodies with neutralizing activity purified from the respiratory tract of mice immunized intranasally with HA molecules from the A/Puerto Rico/8/34 (H1N1) virus were polymeric, whereas the purified IgG antibodies with neutralizing activity were monomeric [Tamura et al., 1990]. Further study of the detailed structure of IgA, which has higher MW than expected for dimeric IgA [Song et al., 1995] remains to be performed.

Previous studies show that IgA in the respiratory tract is more cross-reactive with variant influenza viruses than IgG [Tamura et al., 1990, 1991]. This cross-reactivity seems to depend on the polymeric nature of IgA [Taylor and Dimmock, 1985; Palladino et al., 1995]. Taken together, these data suggest the potential for intranasally administered inactivated vaccines to induce cross-protection against antigenic variants of viruses in pre-immunized adults.

Both serum and mucosal HA-specific ELISA antibody responses after nasal vaccination need to be examined and compared with the corresponding neutralization and HI titres. In addition, neutralizing antibody responses to other influenza vaccines (from different strains, different subtypes or types of viruses, and from different forms of vaccines such as subvirion and whole virus vaccines) after nasal vaccination remain to be examined to compare the efficacy of nasal vaccines with that of the parenteral vaccine. Some of these studies are ongoing.

In conclusion, intranasal administration of an A/Uruguay split vaccine containing 45 μg HA resulted in induced nasal and serum neutralizing antibody responses in four out of five healthy adult subjects, with a neutralization titre of >1:40 after the second and the fifth administrations, respectively. These neutralizing antibody responses were largely due to the induction of nasal polymeric IgA and serum monomeric IgG.

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Monitoring and Characterization of Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus, Japan, 2009–2010

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To monitor and characterize oseltamivir-resistant (OR) pandemic (H1N1) 2009 virus with the H275Y mutation, we analyzed 4,307 clinical specimens from Japan by neuraminidase (NA) sequencing or inhibition assay; 61 OR pandemic (H1N1) 2009 viruses were detected. NA inhibition assay and M2 sequencing indicated that OR pandemic (H1N1) 2009 virus was resistant to M2 inhibitors, but sensitive to zanamivir. Full-genome sequencing showed OR and oseltamivir-sensitive (OS) viruses had high sequence similarity, indicating that domestic OR virus was derived from OS pandemic (H1N1) 2009 virus. Hemagglutination inhibition test demonstrated that OR and OS pandemic (H1N1) 2009 viruses were antigenically similar to the A/California/7/2009 vaccine strain. Of 61 case-patients with OR viruses, 45 received oseltamivir as treatment and 10 received it as prophylaxis, which suggests that most cases emerged sporadically from OS pandemic (H1N1) 2009, due to selective pressure. No evidence of sustained spread of OR pandemic (H1N1) 2009 was found in Japan; however, 2 suspected incidents of human-to-human transmission were reported.

In March and early April of 2009, a new swine-origin A/H1N1 influenza virus, now called pandemic (H1N1) 2009, emerged in Mexico and the United States and spread rapidly (*1–3*). On June 11, 2009, the World Health Organization (WHO) declared a phase-6 pandemic alert, indicating a global pandemic. The earliest virus isolates were sensitive to the neuraminidase inhibitors (NAIs) zanamivir and oseltamivir, but resistant to M2 inhibitors, such as amantadine and rimantadine (*1,3–5*). Thus, the NAIs have been used globally for treatment and prophylaxis of pandemic (H1N1) 2009 virus inflection.

Oseltamivir-resistant (OR) pandemic (H1N1) 2009 was first detected in Japan, Denmark, and Hong Kong during May–June 2009 and has since been sporadically identified around the world (*6–8*). The OR pandemic (H1N1) 2009 viruses have a specific NA mutation, a histidine-to-tyrosine substitution at amino acid position 275 (N1 numbering, H275Y), that confers resistance to oseltamivir. In a report of 39 OR pandemic (H1N1) 2009 cases (as of October 22, 2009), 16 were associated with treatment, 13 were associated with postexposure prophylaxis, 3 were in NAI-untreated patients, and 7 were of unknown association (*8*). Preliminary global NAI surveillance showed 190 OR pandemic (H1N1) 2009 infections among >15,000 clinical specimens; thus, the global frequency of OR pandemic (H1N1) 2009 was <1.5% (as of January 8, 2010) (*9*). These reports indicated that human-to-human transmission of OR pandemic (H1N1) 2009 was limited but that oseltamivir treatment and prophylaxis could lead to emergence of OR pandemic (H1N1) 2009 virus.

A report for 1997–2007 showed that Japan accounted for ≈70% of the world's oseltamivir consumption (*10*). From August 2009 to March 2010, 9.76 million doses of

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oseltamivir were supplied in Japan, ≈2.3× that of the 2008–09 seasons (data from Chugai Co. Ltd, Tokyo, Japan). Thus, Japan is a high-risk environment for the development of OR pandemic (H1N1) 2009 virus because of drug use pressure. The emergence of such resistance is alarming, because OR seasonal influenza A (H1N1) viruses can rapidly spread worldwide once they acquire the capacity for human-to-human transmission (*11–15*). Additionally, in the 2009–10 season in Japan, almost all cases of influenza were caused by pandemic (H1N1) 2009 viruses (Figure 1). Thus, close surveillance must be maintained to detect pandemic (H1N1) 2009 and changes in its transmissibility and genetic and antigenic characteristics.

We monitored and characterized 4,307 clinical specimens collected in Japan during May 2009–February 2010 from patients with OR pandemic (H1N1) 2009 by NA sequencing, NAI assay, or both. Of them, we found 61 OR pandemic (H1N1) 2009 viruses with the H275Y mutation.

Materials and Methods

Virus Testing

Influenza sentinel clinics and nonsentinel institutes send original samples to local public health laboratories for detection and virus isolation. In total, 4,307 clinical specimens, comprising both original samples (n = 440) and clinical isolates (n = 3,867), underwent either full or partial (nt 695–1110) NA sequencing to detect the H275Y mutation. Samples from 1,088 cases were collected before oseltamivir exposure, 516 were associated with oseltamivir use, 103 were associated with zanamivir use, and for 2,600, antivirual treatment status was unknown. We collected all OR pandemic (H1N1) 2009 isolates and randomly selected OS isolates (≈10%) from local public health laboratories. These representative OS and OR pandemic (H1N1) 2009 isolates underwent NA inhibition assay (421 OS and 61 OR viruses tested), full NA and hemagglutination (HA) sequencing (190 OS and 61 OR), internal gene (PB2/PB1/PA/NP/M/NS) sequencing (138 OS and 20 OR), and hemagglutination inhibition (HI) test (583 OS and 59 OR).

Sequence Analysis

Phylogenetic trees of NA and HA genes were constructed by neighbor-joining method. A phylogenetic tree was constructed by using representative OR and OS pandemic (H1N1) 2009 isolates from several prefectures of Japan. Sequence information of pandemic (H1N1) 2009 from other countries was downloaded from the Global Initiative on Sharing Avian Influenza Data (GISAID) and GenBank. All amino acid positions in the phylogenetic tree were described by N1 numbering.

NAI Assay

A chemiluminescent NAI assay was performed with the NA-star kit (Applied Biosystems, Tokyo, Japan) (*13*). Briefly, final drug concentration was 0.03–6,500

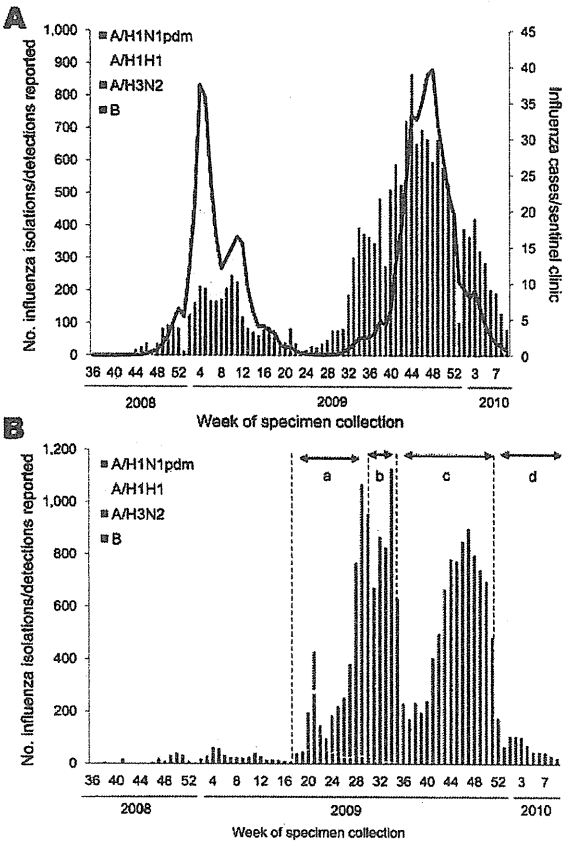

Figure 1. Weekly cases of influenza and isolation or detection of influenza viruses by influenza sentinel clinics (A) and nonsentinel clinics (B) from week 36 of 2008 to week 9 of 2010 in Japan (as of March 9, 2010). Pandemic (H1N1) 2009 (A/H1N1pdm) surveillance in Japan was divided into 4 stages depending on the prevalence situation, as shown in panel B: a) case-based surveillance (April 28–July 23), b) outbreak and hospitalization surveillance (July 24–August 24), c) hospitalization surveillance (August 25–December 20), and d) severe/fatal case surveillance (December 21 onwards). The sentinel clinics, consisting of 3,000 pediatric clinics and 2,000 internal medical clinics, collected samples randomly, while the nonsentinel clinics collected samples depending on the surveillance stage. Local public health laboratories randomly selected these samples for neuraminidase (NA) surveillance from both sentinel and nonsentinel clinics. In this study, 4,307 clinical specimens, comprising both original samples (n = 440) and isolates (n = 3,867), were subjected to full or partial NA sequencing for detection of the H275Y mutation. All oseltamivir-resistant (n = 61) and ≈10% of oseltamivir-susceptible pandemic (H1N1) 2009 (n = 421) isolates were then subjected to NA assay. The treatment history of the 4,307 cases consists of NA inhibitor–untreated (n = 1,088), oseltamivir use (n = 516), zanamivir use (n = 103), and unknown history (n = 2,600). Black line in panel A indicates weekly cases of influenza-like illness per influenza sentinel clinic.

nM for oseltamivir and 0.03–12,500 nM for zanamivir. Chemiluminescence was assayed with an LB940 plate reader (Berthhold Technologies, Bad Wilbad, Germany). Drug concentrations required for 50% inhibitory concentration of NA activity (IC_{50}) were calculated with MikroWin 2000 software (ver. 4; Mikrotek Laborsysteme GmbH, Overath, Germany). To validate the NAI assay, we used already characterized drug-resistant viruses and sensitive counterparts as controls: A/Hokkaido/15/2002 (155H) and A/Hokkaido/9/2002 (155Y), zanamivir (*16*); A/Denmark/528/2009pdm (275Y), A/Denmark/524/2009pdm (275H), seasonal-H1N1 A/Yamagata/68/2008 (275Y), A/Yamagata/41/2008 (275H), oseltamivir.

Statistical Analyses

Box-and-whisker plots were used to determine the cutoff value between NAI-resistant (outlier) and -sensitive viruses. The box contains 50% of the results, representing the middle 2 quartiles (25%–75%). The length of the box shows the interquartile range (IQR). The cutoff value was defined as the upper quartile + 3.0 × interquartile range from the 25th to 75th percentile. For statistical analyses, OR pandemic (H1N1) 2009 viruses with the H275Y mutation were excluded from the overall population.

HI Test

An HI test was performed to evaluate the reactivity of ferret antiserum against the 2009/10 vaccine strain A/California/7/2009, as described in the WHO Manual (*17*). The efficacy of ferret postinfection antiserum against egg-grown A/California/7/2009 was used as a reference. Antiserum was treated with receptor-destroying enzyme II (Denka Seiken, Tokyo, Japan) and adsorbed with turkey erythrocytes before testing, to prevent nonspecific reactions. A 0.5% suspension of turkey erythrocytes was used for the HI test.

Results

Geographic Distribution of OR Pandemic (H1N1) 2009

The 4,307 clinical specimens isolated during May 2009–February 2010 were collected from 41 of 47 prefectures in Japan, and the H275Y mutation was detected by NA sequencing. In total, 61 (1.4%) OR pandemic (H1N1) 2009 viruses possessed the H275Y (n = 48) or 275H/Y mixed (n = 13) mutations (Figure 2). OR pandemic (H1N1) 2009 emerged sporadically in several prefectures and was detected over a period of several months (Figures 2, 3).

Patient Treatment History and Epidemiologic Background

Of 4,307 case-patients, 516 had oseltamivir treatment, 103 had zanamivir treatment, 1,088 were NAI-untreated, and the treatment history of 2,600 was unknown. Of the 61 cases of OR pandemic (H1N1) 2009, 45 were associated with oseltamivir treatment, 10 with postexposure prophylaxis, and 6 occurred in NAI-untreated patients; thus, oseltamivir treatment and prophylaxis likely accelerated emergence of OR pandemic (H1N1) 2009. The relationship between time of sampling (days after oseltamivir treatment) and OR pandemic (H1N1) 2009 detection showed that OR pandemic (H1N1) 2009 was generally detected at least 4 days after oseltamivir treatment (Table 1).

Of the 61 case-patients, 36 (59%) were male; 19 (31%) were 0–4 years, 25 (41%) were 5–18 years, 12 (20%) were 19–50 years, and 5 (8%) were >50 years. Underlying medical conditions were known for 41; 24 had chronic underlying medical conditions (pulmonary [13], neurologic [4], blood [3], diabetes [1], kidney disease [1], immunocompromised [5], other conditions [2]).

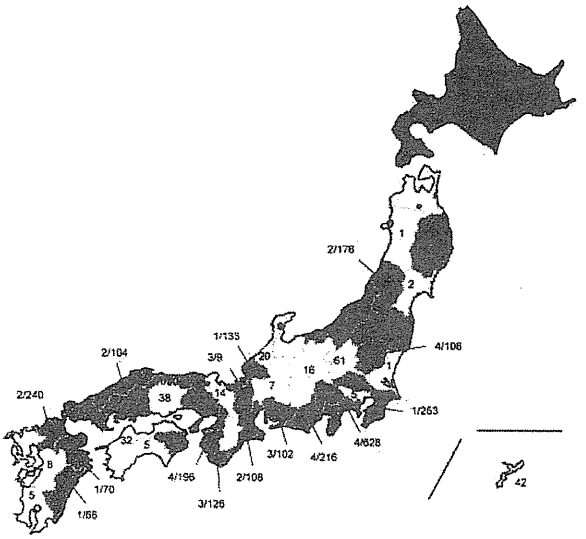

Figure 2. Geographic distribution of H275Y-harboring oseltamivir-resistant pandemic (H1N1) 2009 viruses in Japan, May 2009–February 2010. Values are no. oseltamivir-resistant isolates/total no. tested. Overall prevalence in Japan was 1.4% (61/4,307).

Two Possible Cases of Human-to-Human Virus Transmission

Almost all OR pandemic (H1N1) 2009 cases emerged sporadically and were not epidemiologically linked. However, 2 cases of human-to-human transmission were suspected. One was observed in Niigata Prefecture where 2 children hospitalized in the same room were infected with OR pandemic (H1N1) 2009 virus within a few days. Symptoms developed first in a 4-year-old girl on October 10,

2009, and she received oseltamivir. OR pandemic (H1N1) 2009 virus was isolated from this patient on October 14. A 6-year-old boy in the same room received prophylaxis (by treatment dosage) with oseltamivir beginning October 10. However, he experienced symptoms on October 13, and OR pandemic (H1N1) 2009 virus was isolated on October 14. The patients were in a double room, and patients with pandemic (H1N1) 2009 were not around them. Genetic analyses of the 2 viruses (i.e., A/Niigata/1233/2009 and A/Niigata/1234/2009) showed only 1 aa difference (D to G), at position 256 in PB2, and they also shared unique changes in NS1 and in PB1 (Table 2). Transmission possibilities were as follows: 1) OR pandemic (H1N1) 2009 was directly transmitted from the female patient or 2) an OS pandemic (H1N1) 2009 was transmitted from the female patient and an OR pandemic (H1N1) 2009 virus emerged in the male patient.

The other suspected instance of human-to-human transmission occurred in Tottori Prefecture. In a 9-year-old boy, symptoms developed on December 18, 2009, and OR pandemic (H1N1) 2009 virus was isolated from a sample collected on the same day, before oseltamivir use. However, the patient's 2 brothers were both infected with pandemic (H1N1) 2009 virus and had received oseltamivir since December 15. Although samples from these persons were not available, OR pandemic (H1N1) 2009 likely emerged in 1 patient and was transmitted to the other.

Case Unrelated to Oseltamivir Use

Detailed epidemiologic information was available for 2 of 6 persons with OR pandemic (H1N1) 2009 infections untreated by NAIs. Besides the case in Tottori Prefecture, another occurred in Oita Prefecture. The index patient had a mild cough beginning on July 12, and typical

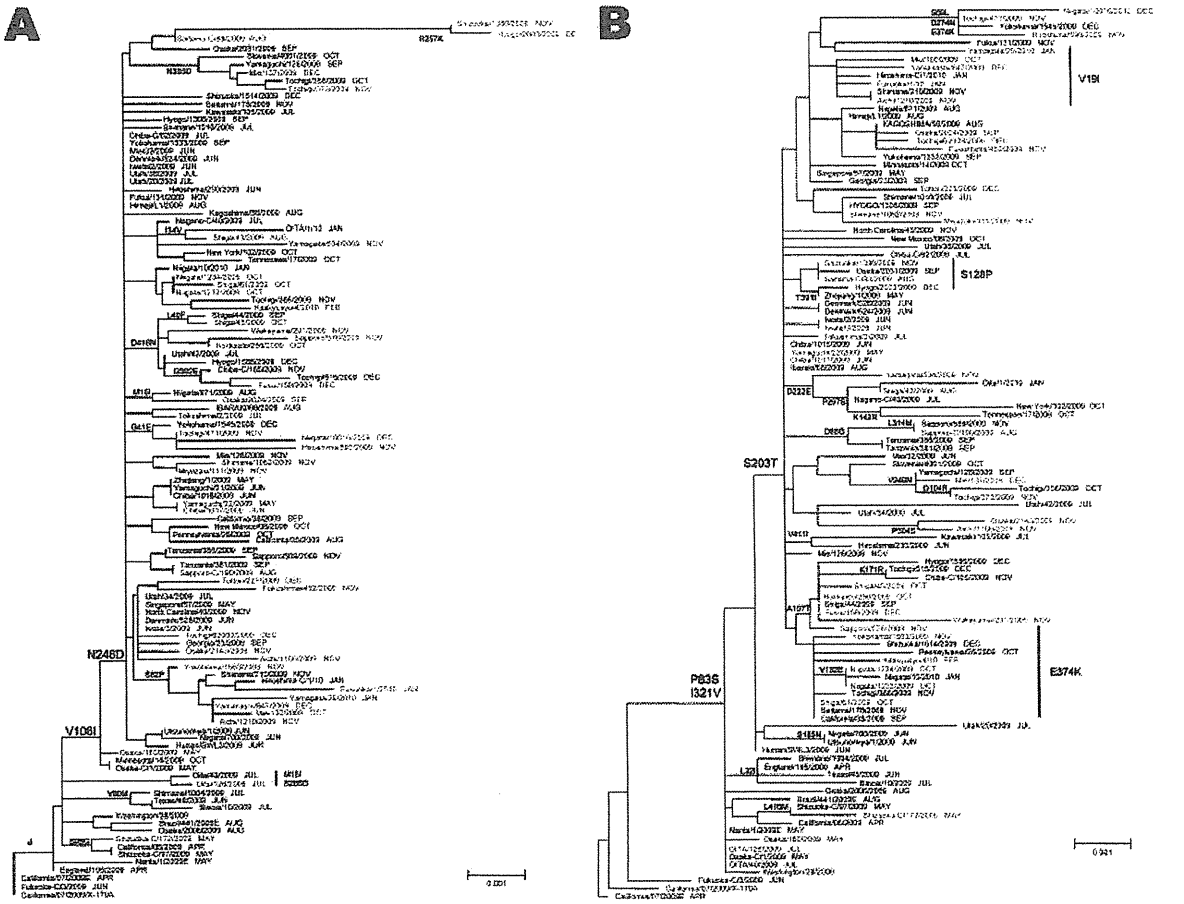

Figure 3. Phylogenetic analysis of influenza pandemic (H1N1) 2009 viruses neuraminidase (NA) (A) and hemagglutinin (HA) genes (B). Most pandemic (H1N1) 2009 viruses possessed the amino acid substitutions S203T in HA and V106I and N248D in NA. Red, oseltamivir-resistant pandemic (H1N1) 2009 from Japan; green, oseltamivir-resistant pandemic (H1N1) 2009 from outside Japan; black, oseltamivir-susceptible (OS) pandemic (H1N1) 2009; purple, 2009–10 current vaccine strains. The sampling month of each isolate is listed following the strain name. The phylogenetic tree of NA and HA genes was constructed by using the neighbor-joining method. Scale bars indicate nucleotide substitutions per site.

Table1. Relationship between detection of oseltamivir-resistant pandemic (H1N1) 2009 and interval from oseltamivir treatment to sample collection, Japan, 2009–2010*

	Days after oseltamivir treatment, N = 516†											
No. samples	Unknown	0‡	1	2	3	4	5	6	7	8	9	>10
Total no.	169	54	116	54	37	31	36	7	6	1	3	2
No. oseltamivir-resistant pandemic (H1N1) 2009	3	0	4	2	4 (1)	13 (1)	19 (1)	3 (2)	2	1 (1)	2 (2)	2 (2)

*Of total 4,307 specimens tested, neuraminidase inhibitor treatment history was available for 1,707; of these specimens, 516 were from patients who had received oseltamivir treatment.
†Parentheses indicate prophylactic use, e.g., 4 (1) = 1 of 4 total uses was for prophylaxis.
‡Day 0 represents the samples collected within 24 h after oseltamivir use.

influenza symptoms developed on July 15. OR pandemic (H1N1) 2009 virus was detected in a sample taken on July 16, before oseltamivir use. However, symptoms had developed in the index patient's son on July 11; the boy received zanamivir on July 12 (OR pandemic (H1N1) 2009 virus was not detected from a sample taken that day). No reports have indicated that zanamivir can induce OR virus with the H275Y mutation. The OR pandemic (H1N1) 2009 virus may have thus emerged naturally, with no selective pressure. However, the index patient may have been exposed to an oseltamivir-treated person outside of her household who harbored OR pandemic (H1N1) 2009 virus.

Genetic Analysis

Phylogenetic analyses of the HA and NA genes showed that most shared amino acid changes: S203T in HA and V106I and N248D in NA (Figure 3). In both trees, OR pandemic (H1N1) 2009 isolates were genetically scattered and possessed several sporadic amino acid changes, but each OR pandemic (H1N1) 2009 was genetically close to OS pandemic (H1N1) 2009 (Figure 3). Several OR pandemic (H1N1) 2009 isolates from Japan were also closely related to OR pandemic (H1N1) 2009 isolates from other countries.

Analysis of the genomes of representative OR (n = 20) and OS pandemic (H1N1) 2009 (n = 138) provided further insight into their similarities. First, comparison of the internal amino acid sequences of each OR pandemic (H1N1)

Table 2. Amino acid differences of each internal protein between oseltamivir-resistant (n = 20) and oseltamivir-sensitive (n = 138) pandemic (H1N1) 2009 virus, by strain, Japan, 2009–2010*

Strain	M1	M2	NP	NS1	NS2	PA	PB1†	PB2†
A/Niigata/1459/2009						V122I	**I435V**, N537S	
A/Osaka/2024/2009							**I435V**	R251K
A/Shimane/1062/2009						V127A, T357I	**I435V**	N448S
A/Shimane/188/2009				A102T			**I435V**	R54K
A/Yokohama/1340/2009						S186N	**I435V**	
A/Yokohama/1394/2009							**I435V**, F466Y	
A/Shiga/61/2009			V119I	M93I			**I435V**, A93V, T257A	**K660R**
A/Niigata/1233/2009				M93I, E217K			T257A	**K660R**
A/Niigata/1234/2009				M93I, E217K			T257A	**K660R**, D256G
A/Shiga/45/2009								**K660R**
A/Chiba/1017/2009								
A/Iwate/3/2009	K103R‡							
A/Mie/100/2009		S23N		T94N			V609A	R251K
A/Oita/126/2009			I100V	E55G, V103I		L370I	K480R	
A/Osaka/180/2009	A33T			V103I	E63K		I667T	V649I, E700K
A/Saitama-C/88/2009				E208K	M50I	A70V		V227I
A/Sapporo-C/190/2009		D21G						
A/Shiga/43/2009							A652T	
A/Tokushima/2/2009						M311I		
A/Yamaguchi/22/2009						V379I		

*M, matrix protein; NP, nucleoprotein; NS, nonstructural protein; PA, polymerase A; PB, polymerase B.
†Of the 138 oseltamivir-sensitive pandemic (H1N1) 2009 virus samples, I435V and K660R (**boldface**) were observed from 32 and 12 isolates, respectively. These changes would sporadically occur in both pandemic (H1N1) 2009 isolate types.
‡Lys (K) at position 103 in M1 protein, consensus amino acid among the oseltamivir-resistant pandemic (H1N1) 2009 virus, was replaced with Arg (R).

2009 and OS pandemic (H1N1) 2009 isolate consensus showed that OR viruses possessed several sporadic amino acid changes, but did not exhibit any common amino acid changes unique to OR pandemic (H1N1) 2009 viruses, indicating that the internal genes of OR and OS pandemic (H1N1) 2009 viruses were genetically indistinguishable (Table 2). Second, comparison of a 2 samples from a patient with pandemic (H1N1) 2009 before and after oseltamivir treatment (A/Chiba/1016/2009 and A/Chiba/1017/2009) showed only the H275Y change in NA and no changes in any other proteins. Finally, no evidence of reassortment of pandemic (H1N1) 2009 and seasonal influenza A (H1N1) viruses was detected.

Of 61 pandemic (H1N1) 2009 OR isolates, those from 13 patients were of mixed NA gene populations (H275 and Y275). Because all 13 patients had received oseltamivir, these samples would have been collected during selective pressure–induced generation of OR pandemic (H1N1) 2009 from OS pandemic (H1N1) 2009 (online Appendix Figure, www.cdc.gov/EID/content/17/3/470-appF.htm). Because calculating precise IC_{50} values from a mixed population of NAI-resistant and -sensitive viruses is not possible (*13,18*), the 13 mixed isolates were excluded from the overall population for the purposes of the statistical analysis of OR.

Antiviral Drug Susceptibility

NAI data are summarized in Table 3. The average IC_{50} value of OR pandemic (H1N1) 2009 (n = 48) for oseltamivir was 370-fold higher than that of OS pandemic (H1N1) 2009 (n = 421) viruses. For zanamivir, 3 of 482 viruses were identified as outliers (cutoff >0.60 nM). Compared with the consensus sequence of OS pandemic (H1N1) 2009, one OS pandemic (H1N1) 2009 A/Okayama/17/2009pdm (0.61 nM) had a D151D/N mixture in its NA protein, and 2 OR pandemic (H1N1) 2009, A/Shiga/43/2009pdm (0.64 nM) and A/Yokohama/1538/2009pdm (0.64 nM) possessed I34V and I195V substitutions in the NA protein, respectively (online Appendix Table, www.cdc.gov/EID/content/17/3/470-appT.htm). The IC_{50} values of OS and OR pandemic (H1N1) 2009 viruses were similar to those of their seasonal influenza A (H1N1) counterpart viruses (Table 3).

Susceptibility to M2 inhibitors was determined by M2 sequencing. All tested viruses, including OR (n = 20) and OS pandemic (H1N1) 2009 (n = 138), had an S31N resistance marker in the M2 protein, suggesting that all pandemic (H1N1) 2009 isolates were resistant to M2 inhibitors.

Antigenic Characterization

The HI test was performed to estimate the reactivity of OS (n = 583) and OR pandemic (H1N1) 2009 (n = 59) virus to ferret antiserum against the 2009–10 vaccine strain A/California/7/2009. More than 93% of OS (n = 546) and OR pandemic (H1N1) 2009 (n = 55) isolates were inhibited by anti-A/California/7/2009 ferret antiserum, and 5.8% and 5.1% of OS (n = 34) and OR pandemic (H1N1) 2009 (n = 3), respectively, showed a 4-fold reduced HI titer. Only 0.5% and 1.7% of OS- (n = 3) and OR pandemic (H1N1) 2009 (n = 1), which had either the K153E or G155E changes in deduced antigenic sites in HA protein, showed at least an 8-fold reduction in HI titer. Thus, OS and OR pandemic (H1N1) 2009 are antigenically indistinguishable and similar to the 2009–10 current vaccine strain A/California/7/2009.

Discussion

The data presented here provide no evidence of sustained spread of OR pandemic (H1N1) 2009 in Japan.

Table 3. Summary of neuraminidase inhibition assay of oseltamivir-resistant and oseltamivir-sensitive pandemic (H1N1) 2009 virus to oseltamivir and zanamivir*

	IC_{50}, (nM/L)					
	Oseltamivir			Zanamivir		
Strain	No. isolates	Mean ± SD (range)	Cutoff value	No. isolates	Mean ± SD (range)	Cutoff value
Pandemic (H1N1) 2009						
Oseltamivir-sensitive	421	0.10 ± 0.02 (0.05–0.19)	>0.20	421	0.28 ± 0.06 (0.11–0.61)	>0.60†
Oseltamivir-resistant	48‡	37.28 ± 14.06 (20.69–80.91)	NC	61	0.36 ± 0.11 (0.17–0.64)	
Seasonal influenza (H1N1) (A/Yamagata/41/2008)						
Oseltamivir-sensitive		0.09 ± 0.02§			0.24 ± 0.10	
Oseltamivir-resistant		51.76 ± 9.54			0.37 ± 0.13	

*IC_{50}, 50% inhibitory concentration; NC, not calculated.
†Because both IC_{50} values of OS and OR pandemic (H1N1) 2009 viruses were indistinguishable, the cutoff values for zanamivir were calculated from the overall population (N = 482).
‡IC_{50} values of 13 mixed samples with H275 and Y275 were excluded from overall population in statistical analysis of OR isolates.
§Mean ± SD IC_{50} values of control seasonal influenza A (H1N1) viruses were determined from 10 independent experiments for oseltamivir and 2 for zanamivir.