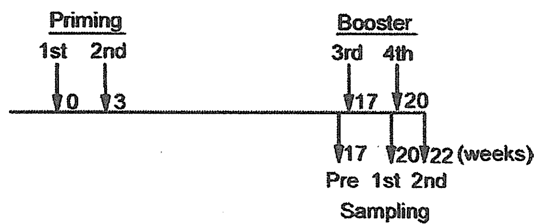

Fig. 1. Vaccination and blood sampling schedule of Table 1.

as Original Antigenic Sin (OAS) [13–15]. It is unclear whether or not an OAS-like phenomenon occurs in a prime–boost regimen other than the combination of clade 1 for priming and clade 2 as the booster. Therefore, we investigated several different prime–boost regimens in a mouse model, particularly combinations other than clade 1 for priming and clade 2 as the booster.

2. Methods

2.1. Vaccine strains

NIBRG-14, Indo/05/2005(H5N1)/PR8-IBCDC-RG2, A/Bar-headed goose/Qinghai Lake/1A/05 SJ163222, and Anhui01/2005(H5N1)-PR8-IBCDC-RG5 were the vaccine strains used. These 4 clades/subclades of H5N1 have been stockpiled in Japan. NIBRG-14 is one of the vaccine reference strains, which was attenuated by reverse genetic engineering from the A/Vietnam/1194/2004(H5N1) virus (clade 1) by the UK National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK). Indo/05/2005(H5N1)/PR8-IBCDC-RG2 (Indo05) and Anhui01/2005(H5N1)-PR8-IBCDC-RG5 (Anhui01) are attenuated vaccine strains derived from the A/Indonesia/5/2005(H5N1) virus (clade 2.1) or the A/Anhui/01/2005 (H5N1) virus (clade 2.3.4), respectively, by the Centers for Disease Control and Prevention (CDC, Atlanta, USA). A/bar-headed goose/Qinghai Lake/1A/05 SJ163222 (Qinghai1A) is an attenuated vaccine strain derived from A/bar-headed goose Qinghai Lake/1A/05 by St. Jude Children's Research Hospital (Tennessee, USA) [2].

The vaccine seed viruses were cultured in embryonated hen eggs, purified from allantoic fluid by zonal centrifugation, and then inactivated with formalin to prepare the inactivated whole virion. The vaccine was formulated by adding aluminum hydroxide (final concentration: 0.3 mg/ml) as the adjuvant. The formulation of the vaccine is the same as the Japanese H5N1 vaccine [16,17]. The HA concentration was determined by SDS-PAGE/densitometry analysis, which method is validated [16].

2.2. Priming effect of the alum adjuvanted whole vaccine to the other clades of vaccine

Specific-pathogen-free female BALB/c mice aged 6–8 weeks (Japan SLC, Inc.) were used in all experiments. The protocol for these animal experiments was approved by the animal experimentation ethical committee of Kaketsuken (Kumamoto, Japan).

An outline of the experiment schedule is shown in Fig. 1. Mice were primed by two intramuscular injections at a 3-week interval, each containing 0.2 μg HA/dose of H5N1 whole-virion antigen (NIBRG-14, Indo05, Anhui01, or Qinghai1A) with 0.03 mg of alum adjuvant (injection volume: 0.1 ml). Control mice were injected with alum-containing PBS. Each group of mice comprised 7 or 8 animals. Four months after priming, the mice were boosted by injecting 0.2 μg HA/dose of H5N1 whole-virion antigen with 0.03 mg of alum adjuvant (injection volume: 0.1 ml). The strain of the booster vaccine was different from that of the priming vaccine to achieve heterologous combinations. Serum samples were obtained before the booster injection (pre), before the 2nd booster injection (1st), and 14 days after the 2nd booster injection (2nd). The antibody responses to both primed and booster strains were measured by hemagglutination-inhibition (HI) assay and micro-neutralization test as described previously [9,16].

2.3. ELISPOT assay

ELISPOT (Enzyme-Linked Immunosorbent SPOT) assays were performed using mouse IFN-γ (InterFeroN-γ) and IL-4 (InterLeukine-4) ELISPOT kits (Mabtech AB, Nacka, Sweden). BALB/c mice were immunized twice intramuscularly at a 3-week interval with 0.2 μg HA/dose of H5N1 whole-virion antigen (NIBRG-14, Indo05, or Anhui01) plus 0.03 mg of alum adjuvant (injection volume: 0.1 ml). Control mice were injected with alum-containing PBS. Each group consisted of eight mice. Single-cell suspensions of spleen cells from all mice in each group were prepared at 14 weeks after the second injection, pooled, and dispensed at 3×10^5 cells/well into polyvinylidene difluoride-coated 96-well plates for incubation with anti-IFN-γ or anti-IL4. The cells were exposed to 0.1 μg HA of each H5N1 whole antigen as the plates were incubated overnight at 37 °C, and then the cells were discarded. After washing with PBS, INF-γ and IL-4 were detected by incubation with biotinylated antibodies for these cytokines, followed by addition of streptavidin-alkaline phosphatase and development with BCIP/NBT substrate solution. The number of spots for INF-γ or IL-4 was counted by an automated ELISPOT reader (AID, Strassberg, Germany).

2.4. Transfer of antiserum to naïve mice

To prepare antisera, BALB/c mice were injected twice at a 3-week interval intramuscularly with 0.2 μg HA/dose of H5N1 whole-virion antigen (NIBRG-14 or Indo05) and 0.03 mg of alum adjuvant. The anti-NIBRG-14 and anti-Indo05 antisera were harvested at 2 weeks after the second immunization and pooled.

For treatment with the antiserum, BALB/c mice were divided into 3 groups. Group 1 received intraperitoneal injection of 0.2 ml of antiserum at one day before the 1st vaccination. (The volume of antiserum was determined by preliminary experiments. We examined 2 different serum volumes (1 ml, 0.2 ml). The results of the 1 ml and 0.2 ml volumes were approximately the same, so we chose 0.2 ml/dose because it decreased the numbers of the mice.) Subsequently, the mice were immunized twice at a 3-week interval by intramuscular injection of 0.2 μg HA/dose of heterologous antigen with 0.03 mg of alum adjuvant (mice given anti-NIBRG-14 serum were immunized with the Indo05 vaccine and vice versa, injection volume: 0.1 ml.). Group 2 mice were actively immunized as in group 1, but the antiserum was injected at one day before the 2nd vaccination. Group 3 mice were treated as in group 1, but were injected with normal BALB/c serum (control). All groups were sacrificed on Day 35 and serum samples were evaluated by the HI assay (against the vaccine strain) described previously [9].

2.5. Statistics

HI titers were transformed into $\log_{10}$ values for calculation of the geometric mean titer (GMT) and 95% CL at every time of assessment. All data manipulations and statistical computations were done with Microsoft Excel software (version 2002).

3. Results

3.1. Priming effect of alum-adjuvanted whole-virion vaccines on the booster response to other clades/subclades with different antigenicity

We investigated the priming effect of alum-adjuvanted whole-virion vaccines on the recall response evoked by boosting with vaccines from different clades/subclades. Four months after priming, most of the mice had high levels of HI antibody for the homologous strain, but had lower levels of cross-reactive antibodies for the heterologous clades/subclade strains (Table 1A).

When mice were primed with the NIBRG-14 strain and boosted with the Indo05 or Anhui01 strains, HI antibodies to the booster strain as well as the priming strain were elevated after one or two booster vaccinations, compared with the HI titer of unprimed animals (shown as PBS). When Indo05 and Anhui01 were used for the priming and booster injections, respectively, as well as with the reverse combination, the HI antibody response to each strain also increased after booster immunization, although it was more specific for Indo05 and Anhui01. Unexpectedly, the combination of priming with Indo05 or Anhui01 and boosting with NIBRG-14 resulted in low titers of HI antibody to NIBRG-14 even after two booster injections. Compared with the response of unprimed mice, priming with Indo05 or Anhui01 actually suppressed the booster response to NIBRG-14. Similar results were obtained with the neutralizing antibody test (data not shown).

The prime–boost effect of the Qinghai1A vaccine was also investigated (Table 1B). Four months after priming and before booster immunization, mice retained a high titer of HI antibodies for the homologous Qinghai1A but had lower levels of antibodies cross-reacting with the heterologous booster strain, NIBRG-14 and Anhui01. Cross-reactivity between the Indo05 and Qinghai1A strains was relatively higher than with the other combinations. After one or two booster doses, HI antibody response to both the priming and booster strains increased.

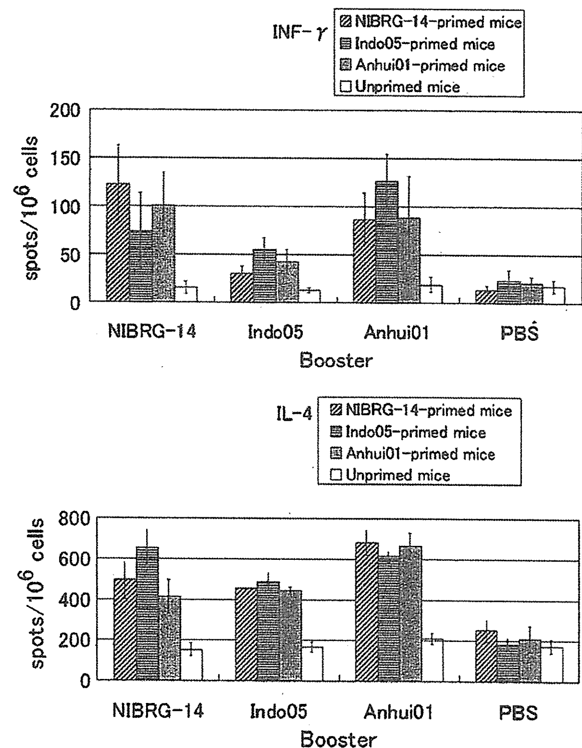

Fig. 2. BALB/c mice were injected twice intramuscularly with 0.2 μg HA/dose of an H5N1 strain plus 0.03 mg of alum adjuvant at a 3-week interval. As a control, mice were injected with alum-containing PBS alone. Spleen cells were harvested at 14 weeks after the second injection, and dispensed into polyvinylidene difluoride-coated 96-well plates with anti-IFN-γ or anti-IL4 at 3×10^5 cells/well. Then cells were stimulated with 0.1 μg HA of each H5N1 whole-virion vaccine. The plates were incubated overnight at 37 °C and the number of spots was counted (spots/10^6 cells, 95% CL).

3.2. Cellular immune responses of mice primed with different H5N1 strains

To investigate the mechanisms that enhanced (or suppressed) the booster response, the helper T cell activity of mice immunized with different clade/subclade strains was compared by investigating cytokine secretion from lymphocytes (Fig. 2). Lymphocytes from mice primed with any of the vaccine strains produced a similar amount of IFN-γ and IL-4 after stimulation with a combination of homologous and heterologous antigens. On the other hand, unprimed mice did not produce these cytokines. Cytokine secretion was not impaired by priming with Indo05 or Anhui01 followed by the booster with NIBRG-14 which led to failure of the booster response.

3.3. Effect of pre-existing antibody on active immunization with antigenically different strains

As a possible mechanism of OAS, Lambert et al. proposed antibody-mediated inhibition of the naïve B cell response to specific epitopes of the new strain [18]. Therefore, we investigated whether pre-existing antibodies could affect the immune response by injection of anti-H5N1 serum before active immunization with different clades/subclades (Table 2).

The HI antibody response of mice injected with the antiserum before the 1st vaccination (Group 1) was suppressed compared with the control mice injected with normal mouse serum (Group 3). There was no difference in the suppressive effect of anti-NIBRG antiserum and anti-Indo05 antiserum. On the other hand, the HI response of mice injected with the antiserum before the 2nd vaccination (Group 2) was not impaired and was comparable to that of the control mice (Group 3).

4. Discussion

We evaluated the priming effect of four H5N1 vaccines belonging to different clades/subclades on booster vaccination with heterologous combinations. NIBRG-14 (clade 1)-primed BALB/c mice developed an enhanced cross-clade booster response after injection of any of the vaccines tested. Qinghai1A (clade 2.2)-vaccination also induced a similar priming effect. In contrast, mice primed with Indo05 (clade 2.1) or Anhui01 (clade 2.3.4) did not respond to booster vaccination with NIBRG-14 and no enhancement of secondary antibody response to both the homologous and heterologous vaccines was observed. The successful prime–boost regimen employing NIBRG-14 (priming) and Indo05 (booster) confirmed the results of our previous study [9]. In addition, prime–boost responses were confirmed with some combinations of different clade 2 vaccines, but we did not expect to find failure of the response with other combinations.

The mechanism leading to such failure is uncertain, but two possibilities can be suggested: (1) failure of Th cell responses or (2) failure of B cell responses. ELISPOT assay showed that spleen cells from primed BALB/c mice produced similar amounts of cytokines irrespective of the combination of priming and boosting antigens, including combinations that led to inhibition of the booster response (Fig. 2). These data showed that helper T cell function

Table 1
Priming effect of alum-adjuvanted whole-virion vaccines for heterologous booster vaccines.

Priming	Booster	HI titer against NIBRG-14			HI titer against Indo05			HI titer against Anhui01		
		Pre	1st	2nd	Pre	1st	2nd	Pre	1st	2nd
(A) Prime-boost responses among the NIBRG-14, Indo05, and Anhui01 strains										
Indo05	NIBRG-14	29.7 (16.5–53.6)	23.8 (14.4–39.2)	32.8 (18.5–58.3)	144.9 (76.0–276.4)	226.3 (91.4–560.0)	226.3 (108.0–474.2)	33.6 (13.8–82.2)	80.0 (36.0–177.9)	59.4 (22.4–158.0)
Anhui01	NIBRG-14	28.3 (16.8–47.7)	13.0 (6.2–27.1)	25.9 (10.5–64.0)	25.9 (11.1–60.7)	28.3 (9.5–84.1)	36.7 (13.2–101.9)	95.1 (46.1–196.3)	174.5 (86.3–352.9)	160.0 (68.1–376.0)
PBS	NIBRG-14	5.0 (5.0–5.0)	10.9 (7.6–15.7)	95.1 (32.4–279.8)	5.0 (5.0–5.0)	5.0 (5.0–5.0)	10.9 (5.1–23.5)	5.0 (5.0–5.0)	5.9 (4.0–8.9)	21.8 (9.1–52.4)
NIBRG-14	Indo05	207.5 (113.9–377.9)	1660.0 (584.6–4713.6)	1810.2 (635.7–5154.3)	25.9 (13.3–50.7)	246.8 (68.7–886.9)	349.0 (138.0–882.7)	33.6 (13.1–86.4)	349.0 (110.7–1099.6)	415.0 (160.1–1075.4)
Anhui01	Indo05	30.8 (18.4–51.8)	11.9 (6.6–21.3)	8.2 (4.2–16.1)	16.8 (11.3–25.1)	123.4 (55.7–273.4)	160.0 (72.0–355.8)	95.1 (63.8–141.9)	380.5 (212.0–683.0)	452.5 (247.3–828.0)
PBS	Indo05	5.0 (5.0–5.0)	6.5 (4.3–9.9)	11.2 (5.2–24.1)	5.0 (5.0–5.0)	28.3 (16.8–47.7)	269.1 (180.4–401.3)	5.0 (5.0–5.0)	10.9 (6.2–19.1)	103.7 (46.8–229.9)
NIBRG-14	Anhui01	190.3 (127.6–283.7)	1076.3 (419.1–2764.3)	1280.0 (575.6–2846.4)	28.3 (15.5–51.8)	146.7 (48.5–444.0)	246.8 (105.4–577.9)	21.8 (10.8–44.1)	207.5 (70.0–615.0)	415.0 (198.8–866.4)
Indo05	Anhui01	23.8 (13.3–42.7)	18.3 (10.5–32.1)	10.9 (6.2–19.1)	146.7 (61.0–352.8)	452.5 (203.5–1006.4)	640.0 (305.4–1341.3)	43.6 (21.6–88.2)	246.8 (111.4–546.8)	293.4 (145.1–593.5)
PBS	Anhui01	5.0 (5.0–5.0)	7.1 (4.2–12.8)	10.0 (2.5–39.9)	5.0 (5.0–5.0)	5.0 (5.0–5.0)	8.4 (5.1–13.9)	5.0 (5.0–5.0)	13.0 (7.7–21.8)	80.0 (47.4–135.0)

Priming	Booster	HI titer against booster strain			HI titer against priming strain		
		Pre	1st	2nd	Pre	1st	2nd
(B) Prime-boost responses between Qinghai1A and the other three strains as boosters							
Qinhai1A	NIBRG-14	21.8 (7.9–60.6)	174.5 (92.3–329.7)	160.0 (79.6–321.4)	640.0 (379.3–1079.9)	2347.5 (1634.3–3372.1)	1413.2 (820.6–2433.8)
PBS	NIBRG-14	5.0 (5.0–5.0)	21.8 (13.6–35.0)	226.3 (123.7–414.0)	–[a] –[a]	–[a] –[a]	–[a] –[a]
Qinhai1A	Indo05	113.1 (57.6–222.3)	293.4 (204.3–421.5)	246.8 (147.0–414.1)	586.9 (408.6–843.0)	1280.0 (903.1–1814.2)	1076.3 (721.8–1605.1)
PBS	Indo05	5.0 (5.0–5.0)	36.7 (16.1–83.6)	246.8 (111.4–546.8)	–[a] –[a]	–[a] –[a]	–[a] –[a]
Qinhai1A	Anhui01	30.8 (13.2–72.2)	452.5 (295.2–693.7)	349.0 (217.8–559.2)	538.2 (326.1–888.1)	1660.0 (1090.2–2527.6)	1660.0 (911.5–3022.8)
PBS	Anhui01	5.0 (5.0–5.0)	26.9 (13.6–53.4)	80.0 (48.8–131.0)	–[a] –[a]	–[a] –[a]	–[a] –[a]
NIBRG-14	Qinhai1A	67.3 (29.0–156.0)	452.5 (268.2–763.6)	452.5 (295.2–693.7)	415.0 (227.9–755.7)	905.1 (536.4–1527.3)	905.1 (536.4–1527.3)
Indo05	Qinhai1A	146.7 (68.2–315.7)	697.9 (369.4–1318.7)	538.2 (260.8–1110.4)	452.5 (268.2–763.6)	697.9 (369.4–1318.7)	452.5 (203.5–1006.4)
Anhui01	Qinhai1A	14.1 (5.4–36.8)	103.7 (33.6–320.4)	269.1 (99.9–724.5)	207.5 (106.0–406.0)	987.0 (588.1–1656.4)	761.1 (328.3–1764.6)
PBS	Qinhai1A	5.0 (5.0–5.0)	73.4 (41.9–128.4)	190.3 (106.0–341.5)	–[a] –[a]	–[a] –[a]	–[a] –[a]

Priming vaccines were injected into BALB/c mice twice at a three-week interval. After 4 months, booster vaccines were injected twice at a three-week interval (A: Prime-boost responses among the NIBRG-14, Indo05, and Anhui01 strains, B: Priming with Qinghai1A and use of the other 3 strains as boosters). HI antibody titers for booster and priming strains (GMT, 95% CL) were measured before the first booster vaccination (Pre), two weeks after the first booster vaccination (1st), and two weeks after the second booster vaccination (2nd). All vaccines were given at are 0.2 μg HA/dose. The grey indicates the HI titer against booster strain, which is the most important results.

[a] Not tested because animals were primed with PBS (control).

Table 2
Effect of pre-existing antibodies on active immunization for antigenically different strains.

Vaccine	Antiserum	Group 1 (antiserum: before the 1st vaccine)	Group 2 (antiserum: before the 2nd vaccine)	Group 3 (normal serum: before the 1st vaccine)
NIBRG-14	Indo05	13.2 (6.5–26.9)	121.3 (48.9–300.8)	69.6 (24.6–196.8)
Indo05	NIBRG-14	8.7 (2.1–36.2)	242.5 (57.0–1031.3)	320.0 (120.6–849.2)

Antiserum was obtained from mice after 2 intramuscular injections of 0.2 mg HA/dose of antigen with 0.03 mg of alum adjuvant at a 3-week interval. All group mice were immunized twice with at a 3-week interval by intramuscular injection of 0.2 mg HA/dose of heterologous antigen with 0.03 mg of alum adjuvant. Group 1 mice were injected intraperitoneally with 0.2 ml of antiserum (anti-NIBRG-14 or anti-Indo05) at one day before the 1st vaccination. Group 2 mice were injected with antiserum before the 2nd vaccination. Group 3 mice were treated as for group 1, but received normal serum (control). All groups were sacrificed on Day 35 and serum was harvested, then HI antibody titers for vaccine strains (GMT, 95%CL) were measured.

was normal even when there was failure of the prime–boost response. Therefore, impairment of B cell immunity was suggested.

As a possible B cell-related mechanism of OAS, Kim et al. proposed that OAS may occur due to competition for common epitopes between Ag-specific memory and naïve B cells [19]. Alternatively, Lambert et al. proposed that pre-existing cross-reactive antibodies may inhibit the activation of naïve B cells. Our experiments are in accordance with the proposal of Lambert et al. because anti-H5N1 antiserum inhibited the antibody response when injected before primary vaccination suggesting that antibody-mediated inhibition of naïve B cells is one of the mechanisms leading to failure of the prime–boost effect (Table 2). However, the reason why the prime–boost regimen was successful in other combinations is unclear. As shown in Table 1, antibodies for Indo05, Anhui01, and Qinghai1A had little or modest cross-reactivity with NIBRG-14. Therefore, the antibodies present after booster injection of Indo05, Anhui01, or Qinghai1A into NIBRG-14-primed mice should have been specific to NIBRG-14. However, in fact antibodies specific to strains other than NIBRG-14 were also present, so there might be a mechanism that overcomes antibody-mediated inhibition of naïve B cell activation, e.g., naïve B cells could be activated by direct binding of influenza virus to cell surface sialic acid irrespective of their B cell receptor's specificity [20], or very small populations of cross-reactive memory B cells might be generated by primary vaccination.

Although we found an OAS-like phenomenon after H5N1 vaccination in mice, other groups have reported that cell culture (Vero)-derived whole virus (H5N1) vaccines based on wild-type strains could elicit heterologous prime–boost reactions in CD1 mice (priming: clade 2.1, booster: clade 1) [21]. Therefore, the prime–boost response may be dependent on the vaccine formulation or the genetic background of vaccinated hosts. In a human study, it was shown that an AS03-adjuvanted split vaccine (clade 1) induced an increased HI antibody response to a clade 2 booster vaccine while split vaccines without AS03 did not [11]. There has also been a similar report about MF59-adjuvanted vaccines [22]. One of the reasons for successful priming would be that adjuvanted H5N1 vaccines could stimulate a wide range of antibody repertoire and expand cross-reactivity to different clades/subclades in primary immunization [23,24]. Our results show that the antibody response was strongly suppressed only in the naïve mice and not in the primed mice by transferring cross-reactive antiserum. Therefore, the priming with such adjuvants to induce a broader cross-reactivity might overcome the OAS-like suppression. For the similar reasons, universal vaccines might contribute towards overcoming such OAS-like suppression [25].

Although we have recently experienced an influenza pandemic with H1N1 2009 virus, there are still many remaining issues regarding the vaccination program. As the next candidate for causing a pandemic, H5N1 virus still has great potential. The optimum prime–boost regimen, as well as other remaining issues, should be decided before any pandemic due to H5N1 occurs. In particular, prime–boost regimens with clades 2.1 or 2.3.4 for priming and clade 1 as the booster have not yet been investigated in humans. Therefore, it is urgent to confirm whether an OAS-like phenomenon occurs with human vaccination.

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References

[1] WHO. Cumulative number of confirmed human cases of avian influenza A (H5N1) reported to WHO, http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_07_01/en/index.html (accessed 1/07/09).
[2] WHO. Status of development and availability of A(H5N1) candidate vaccine viruses, http://www.who.int/csr/disease/avian_influenza/guidelines/201009_H5_H9_VaccineVirusUpdate.pdf (accessed 27/12/10).
[3] Osterhaus AD. Pre- or post-pandemic influenza vaccine? Vaccine 2007;25(June (27)):4983–4.
[4] Stohr K. Vaccinate before the next pandemic? Nature 2010;465(May (7295)):161.
[5] Ninomiya A, Imai M, Tashiro M, Odagiri T. Inactivated influenza H5N1 whole-virus vaccine with aluminum adjuvant induces homologous and heterologous protective immunities against lethal challenge with highly pathogenic H5N1 avian influenza viruses in a mouse model. Vaccine 2007;18(May):3554–60.
[6] Govorkova EA, Webby RJ, Humberd J, Seiler JP, Webster RG. Immunization with reverse-genetics-produced H5N1 influenza vaccine protects ferrets against homologous and heterologous challenge. J Infect Dis 2006;194(July (2)):159–67.
[7] Lipatov AS, Hoffmann E, Salomon R, Yen H-L, Webster RG. Cross-protectiveness and immunogenicity of influenza A/Duck/Singapore/3/97(H5) vaccines against infection with A/Vietnam/1203/04(H5N1) virus in ferrets. J Infect Dis 2006;194(October (8)):1040–3.
[8] Middleton D, Rockman S, Pearse M, Barr I, Lowther S, Klippel J, et al. Evaluation of vaccines for H5N1 influenza virus in ferrets reveals the potential for protective single-shot immunization. J Virol 2009;83(August (15)):7770–8.
[9] Ikeno D, Kimachi K, Kudo Y, Goto S, Itamura S, Odagiri T, et al. A prime–boost vaccination of mice with heterologous H5N1 strains. Vaccine 2009;27(May (23)):3121–5.
[10] Ehrlich HJ, Muller M, Fritsch S, Zeitlinger M, Berezuk G, Low-Baselli A, et al. A Cell culture (Vero)-derived H5N1 whole-virus vaccine induces cross-reactive memory responses. J Infect Dis 2009;200(October (7)):1113–8.
[11] Leroux-Roels I, Roman F, Forgus S, Maes C, De Boever F, Drame M, et al. Priming with AS03 A-adjuvanted H5N1 influenza vaccine improves the kinetics, magnitude and durability of the immune response after a heterologous booster vaccination: an open non-randomised extension of a double-blind randomised primary study. Vaccine 2010;28(January (3)):849–57.
[12] Schwarz TF, Horacek T, Knuf M, Damman HG, Roman F, Drame M, et al. Single dose vaccination with AS03-adjuvanted H5N1 vaccines in a randomized trial induces strong and broad immune responsiveness to booster vaccination in adults. Vaccine 2009;27(October (45)):6284–90.
[13] Francis Jr T. Influenza: the new acquayantance. Ann Intern Med 1953;39(August (2)):203–21.

[14] Haaheim LR. Original antigenic sin. A confounding issue? Dev Biol (Basel) 2003;115:49–53.
[15] Davenport FM, Hennessy AV, Francis Jr T. Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. J Exp Med 1953;98(December (6)):641–56.
[16] Ikeno D, Kimachi K, Kino Y, Harada S, Yoshida K, Tochihara S, et al. Immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1 NIBRG-14) vaccine administered by intramuscular or subcutaneous injection. Microbiol Immunol 2010;54(February (2)):81–8.
[17] Tada Y. Characterization of a whole, inactivated influenza (H5N1) vaccine. Influenza Other Respir Viruses 2008;2(November (6):261–6.
[18] Lambert PH, Liu M, Siegrist CA. Can successful vaccines teach us how to induce efficient protective immune responses? Nat Med 2005;11(April (4 Suppl.)):S54–62.
[19] Kim JH, Skountzou I, Compans R, Jacob J. Original antigenic sin responses to influenza viruses. J Immunol 2009;183(September (5)):3294–301.
[20] Doucett VP, Gerhard W, Owler K, Curry D, Brown L, Baumgarth N. Enumeration and characterization of virus-specific B cells by multicolor flow cytometry. J Immunol Methods 2005;303(August (1–2)):40–52.
[21] Sabarth N, Howard MK, Savidis-Dacho H, van Maurik A, Barrett PN, Kistner O. Comparison of single, homologous prime–boost and heterologous prime–boost immunization strategies against H5N1 influenza virus in a mouse challenge model. Vaccine 2010;28(January (3)):650–6.
[22] Galli G, Hancock K, Hoschler K, DeVos J, Praus M, Bardelli M, et al. Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine. Proc Natl Acad Sci USA 2009;106(May (19)):7962–7.
[23] Leroux-Roels I, Bernhard R, Gerard P, Drame M, Hanon E, Leroux-Roels G. Broad Clade 2 cross-reactive immunity induced by an adjuvanted clade 1 rH5N1 pandemic influenza vaccine. PLoS ONE 2008;3(2): e1665.
[24] Khurana S, Chearwae W, Castellino F, Manischewitz J, King LR, Honorkiewicz A, et al. Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. Sci Transl Med 2010;2(January (15)):ra5.
[25] Nabel GJ, Fauci AS. Induction of unnatural immunity: prospects for a broadly protective universal influenza vaccine. Nat Med 2011;16(December (12)):1389–91.

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Rapid Discrimination of Oseltamivir-Resistant 275Y and -Susceptible 275H Substitutions in the Neuraminidase Gene of Pandemic Influenza A/H1N1 2009 Virus by Duplex One-Step RT-PCR Assay

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Pandemic influenza A/H1N1 2009 (A/H1N1pdm) virus caused significant outbreaks worldwide last year (2009). A number of oseltamivir-resistant A/H1N1pdm viruses possessing an H275Y substitution in the neuraminidase (NA) protein were reported sporadically in several countries, including Japan, but they were sensitive to zanamivir and did not spread in the community. In this study, to monitor rapidly and simply oseltamivir-resistant A/H1N1pdm viruses possessing H275Y, a duplex one-step RT-PCR assay (H275Y RT-PCR assay) was developed based on an endpoint genotyping analysis method. H275Y RT-PCR assay evaluated using several subtypes/types of influenza A and B viruses and other respiratory pathogenic viruses and shown to have high sensitivity and high specificity. Forty-four clinical specimens were tested after RNA purification using the H275Y RT-PCR assay, resulting in one clinical specimen being found to contain a virus possessing the H275Y mutation. Seventy-three clinical isolates were then tested with the H275Y assay by using clinical isolates in the cultured supernatants of cells directly, without RNA purification, and the results were consistent with the NA sequencing. Since the H275Y RT-PCR assay could detect the H275Y mutation in clinical isolates without RNA purification, as well as a H275Y mutated virus in clinical specimens after RNA purification, the assay was considered a powerful tool for surveillance screening of oseltamivir-resistant A/H1N1pdm virus activity. ***J. Med. Virol. 83:1121–1127, 2011.*** © 2011 Wiley-Liss, Inc.

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KEY WORDS: oseltamivir resistant; pandemic influenza A/H1N1 2009 virus; rapid discrimination

INTRODUCTION

The novel H1N1 subtype influenza A virus (pandemic influenza A/H1N1 2009 virus) has been spreading worldwide since it was identified in Mexico and the United States in March and April 2009 [Garten et al., 2009; Smith et al., 2009; WHO, 2009a,b]. The initial characterization of pandemic influenza A/H1N1 2009 (A/H1N1pdm) virus isolates showed that they were susceptible to the neuraminidase inhibitors (NAIs) zanamivir and oseltamivir, but resistant to the ion channel M2 blocker adamantane [Smith et al., 2009]. Therefore, NAIs, especially oseltamivir, are pharmaceutical options for the prophylactic drugs and

Additional Supporting Information may be found in the online version of this article.

The authors declare no potential conflict of interest relevant to this article.

[†]See supplemental material in the online version of this article for complete list of investigators involved in the influenza virus surveillance group of Japan.

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therapeutic drugs administered to A/H1N1pdm virus-infected individuals.

After the introduction of two NAIs into clinical use, a low rate of resistant viruses for any subtypes and strains was observed until the 2006/2007 influenza season [Monto et al., 2006; Kramarz et al., 2009; Tashiro et al., 2009]. However, in the 2007/2008 influenza season, from November 2007, oseltamivir-resistant seasonal influenza A/H1N1 viruses possessing a substitution in the neuraminidase (NA) protein from the histidine to tyrosine residue at position 275 (H275Y) were isolated predominantly in EU countries. In the 2008/2009 influenza season, oseltamivir-resistant viruses spread dramatically worldwide, including Japan [Lackenby et al., 2008; Meijer et al., 2009; Ujike et al., 2010] (http://www.who.int/csr/disease/influenza/h1n1_table/en/index.html).

In the 2009/2010 influenza season, A/H1N1pdm viruses spread worldwide, replacing seasonal influenza A/H1N1 viruses, but only 285 oseltamivir-resistant A/H1N1pdm viruses possessing H275Y substitution in the NA protein were reported sporadically in several countries, including Japan, as of April 14, 2010 (http://www.who.int/csr/disease/swineflu/laboratory07_05_2010/en/index.html). In the NAI surveillance in Japan, 5422 A/H1N1pdm clinical isolates were sequenced for identification of the H275Y substitution marker in the NA gene, and only 67 oseltamivir-resistant viruses possessing H275Y substitution were found, as of April 5, 2010 (http://idsc.nih.go.jp/iasr/graph/tamiful09-10.gif). Those oseltamivir-resistant viruses did not cause localized epidemics in Japan. Most oseltamivir-resistant viruses were isolated from cases administered prophylactics or therapeutics, and at this point oseltamivir use was associated with the emergence of oseltamivir-resistant A/H1N1pdm viruses [Baz et al., 2009; WHO, 2009c].

Currently, the detection of oseltamivir-resistant A/H1N1pdm viruses in Japan is performed by partial sequencing of the NA gene of the clinical isolates to identify the H275Y substitution marker, followed by NA inhibition assay using a chemiluminescence system. Those methods, however, require skill and involve laborious procedures.

In this study, to monitor rapidly and simply oseltamivir-resistant A/H1N1pdm viruses possessing H275Y, a duplex RT-PCR assay (H275Y RT-PCR assay) was developed using an endpoint genotyping analysis method. This assay could discriminate an oseltamivir-resistant A/H1N1pdm virus possessing the H275Y mutation in clinical specimens. RNA purification is required for using the assay to detect the H275Y mutation in clinical specimens, since specimens from nasal or nasopharyngeal swabs may contain some components that inhibit the RT-PCR reaction. The assay also could detect the H275Y mutation of the clinical isolates in cultured supernatants directly without RNA purification. Since the RNA purification procedure for each sample increased the risk of cross-contamination and was time-consuming, the ability to perform the assay without RNA purification is advantageous in situations where a large number of clinical isolates are handled. The assay developed in this study is a useful tool for monitoring oseltamivir-resistant viruses.

MATERIALS AND METHODS

Primer and Probe Design

The nucleotide sequences of the NA gene from A/H1N1pdm viruses posted on the Global Initiative on Sharing Avian Influenza Data (GISAID) database between August and October 2009 were aligned using the Clustal W software [Larkin et al., 2007]. Based on these sequences, two probes for detecting oseltamivir-susceptible H275 and oseltamivir-resistant Y275 were designed to discriminate C and T nucleotides at position 823 of the NA gene, respectively. The primer and probe sequences are listed in Table I.

Viruses

Influenza viruses were isolated from clinical specimens using Madin–Darby Canine Kidney (MDCK) cells or Caco 2 cells (human colonic carcinoma cells), and typing and subtyping of the clinical isolates were determined by a hemagglutination inhibition (HI) test. All clinical isolates of influenza viruses

TABLE I. Primers and Probes

Names	Sequences (5′–3′)	Orientation	Position[a]
Primers for RT-PCR			
H1N1NA-F690-719	ATGTGCATGTGTAAATGGTTCTTGCTTTAC	+	690–719
H1N1NA-R847-872	ACACATGTGATTTCACTAGAATCAGG	−	847–872
Probes for RT-PCR			
FAM-274Ya-swH1N1-F823-835 (for detecting resistant virus)	(FAM)TACTATGAGGAAT(MGB)	+	823–835
VIC-H274a-swH1N1-F823-835 (for detecting susceptible virus)	(VIC)CACTATGAGGAAT(MGB)	+	823–835
Primers for sequencing			
N1-676-694F	ACACAAGAGTCTGAATGTG	+	676–694
N1-1130-1111R	GGATCCCAAATCATCTCAAA	−	1130–1111

[a]The nucleotide positions of NA genes are based on cRNA sequences obtained from GISAID database. Isolate ID numbers for NA genes of A/Denmark/524/2009 and A/Denmark/528/2009 are EPI_ISL_33836 and EPI_ISL_33837.

included in this study were cultured by passage through MDCK cells. The MDCK cells were cultured in Opti-MEM (Invitrogen, Carlsbad, CA) containing 5 μg/ml acetylated trypsin (Sigma–Aldrich Corp., St. Louis, MO) and 200 μg/ml penicillin/streptomycin (Invitrogen) until a cytopathic effect was observed, and hemagglutination (HA) assays were performed using 0.5% turkey red blood cells so that the HA titer was confirmed to be higher than 8. As a control for the assay, A/Denmark/524/2009 (susceptible to NAI, possessing H275) and A/Denmark/528/2009 (resistant to NAI, possessing Y275) were obtained from the European Centre for Disease Prevention and Control (ECDC). A/Denmark/524/2009 and A/Denmark/528/2009 were isolated from the same patient before and after treatment with oseltamivir, respectively. These two isolates were plaque-purified and used as controls for the H275Y RT-PCR assay.

All A/H1N1pdm viruses used in this study are listed in Supplemental Table. The several subtypes/types of seasonal influenza viruses used for validating the specificity of the assay were as follows: A/Yamaguchi/26/2009 (H1N1), A/Oita/64/2009 (H1N1), A/Wakayama/198/2009 (H1N1), A/Sendai/85/2008 (H1N1), A/Toyama/104/2008 (H1N1), A/Kobe/41/2008 (H1N1), A/Uruguay/716/2007 (H3N2), A/Toyama/123/2008 (H3N2), A/Yamanashi/135/2008 (H3N2), A/Hiroshima-C/41/2008 (H3N2), B/Sakai/41/2008, and B/Mie/1/2009.

The viral respiratory pathogens used for validating the specificity of the assay were as follows: Respiratory syncytial viruses A and B, human parainfluenza viruses 1–4, human rhinoviruses 1B, 2, 14, 36, and 89, human metapneumovirus, and human coronaviruses OC43, 229E, and NL63. Human metapneumovirus and human parainfluenza viruses 2 and 4 were obtained from Sendai Medical Center. Human rhinoviruses 1B, 2, 14, 36, and 89 were obtained from Nagasaki Prefectural Institute for Environmental Research and Public Health. Respiratory syncytial viruses A and B, human parainfluenza viruses 1 and 3, and human coronaviruses were stored in the National Institute of Infectious Diseases (NIID).

Clinical Specimens

Nasal or pharyngeal swabs collected from suspected and contact cases of influenza suspended in virus transport medium were obtained from hospitals and clinics in Japan. All specimens were collected between September 2009 and February 2010. The study protocol was approved by the Ethics Committee at NIID, and the study was performed in compliance with the declaration of Helsinki. Informed consent was obtained from all patients.

RNA Preparation

Supernatants of the cultured MDCK cells inoculated with influenza viruses were clarified by centrifugation at 10,000*g* for 10 min. Viral RNA was prepared from 140 μl of the supernatant using the QIAamp® Viral RNA kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions, except that the viral RNA was eluted in 70 μl of AVE (Qiagen).

Total RNA was prepared from clinical specimens using the QIAamp® Viral RNA kit (Qiagen) (using 140 μl of clinical specimen) or MagMAX™ 96 Viral Isolation Kit (Ambion, Austin, TX) (using 50 μl of clinical specimen) with KingFisher Flex (Thermo Fisher Scientific, Waltham, MA) according to each manufacturer's instructions. Total RNA from the 140 μl clinical specimen was eluted with 60 μl of AVE (Qiagen), whereas total RNA from the 50 μl clinical specimen was eluted with 30 μl of elution buffer (Ambion).

One-Step Duplex RT-PCR Assay

The reaction was performed using a QuantiTect® Virus + ROX Vial Kit (Qiagen) according to the manufacturer's instructions. Briefly, for testing clinical isolates using cultured medium, the 20 μl assay contained 4 μl of 5× QuantiTect Virus NR Master Mix, 0.2 μl of QuantiTect Virus RT Mix, 1.2 μl of 10 μM forward primer, 1.2 μl of 10 μM reverse primer, 0.4 μl for each of two 5 μM probes for H275Y RT-PCR or 0.4 μl of 5 μM probe for Type A rRT-PCR, 7.6 μl or 8 μl of water, and 2 μl of culture medium. For testing clinical specimens using extracted RNA, 5 μl of RNA template was used. Cycling was performed as follows: 20 min at 50°C to activate RT, followed by initial denaturation for 5 min at 95°C, with a subsequent 45 cycles of amplification (denaturation at 95°C for 15 sec and annealing as well as extension at 56°C for 45 sec) using LightCycler® 480 (Roche, Basel, Switzerland). Fluorescent signals were collected during annealing and extension steps, and amplification data and endpoint data were analyzed using the Light Cycler® 480 SW1.5 software according to the manufacturer's instructions.

To evaluate the sensitivity of the H275Y RT-PCR assay, the influenza A virus detection assay (simplex real-time RT-PCR assay designated as Type A rRT-PCR assay) was performed using previously designed primers and probe to correspond to a conserved region of the matrix gene segment as a control [Nakauchi et al., 2010].

To confirm the subtype of influenza virus, all clinical specimens were tested by simplex real-time RT-PCR assays for detecting specifically A/H1N1pdm virus (H1pdm rRT-PCR assay) as described previously [Nakauchi et al., 2010].

Sequence

To amplify the partial NA gene between 676 and 1111 (from the start codon) using extracted RNA from the isolated virus, RT-PCR was carried out with the following paired primers: swine N1-676-694F and swine N1-1130-1111R (Table I) using the One-Step RNA PCR Kit (AMV) (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. The RT-PCR

conditions were as follows: 30 min at 50°C to activate RT, followed by initial denaturation for 2 min at 94°C, with a subsequent 30 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, and extension at 72°C for 2 min). The PCR products were purified, and then sequenced using swine N1-676-694F primer with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) by Applied Biosystems DNA Analyzer. Total RNA was prepared as described in the RNA Preparation Section.

RESULTS

Development of Duplex Real-Time RT-PCR Assay for Detecting Oseltamivir-Resistant Pandemic Influenza A/H1N1 2009 Virus

The specificity of the duplex endpoint genotyping RT-PCR assay for detecting oseltamivir-resistant A/H1N1pdm virus (H275Y RT-PCR) was evaluated using human seasonal influenza A/H1N1 and A/H3N2 viruses, influenza B viruses, and other viral respiratory pathogens. The H275Y RT-PCR assay reacted specifically to A/H1N1pdm viruses but showed no cross-reactivity against other subtypes/types of influenza A and B viruses or other viral respiratory pathogens (data not shown).

The sensitivity of the H275Y RT-PCR assay was compared with that of the Type A rRT-PCR assay using serial dilutions of A/Denmark/524/2009 (H1N1)pdm and A/Denmark/528/2009 (H1N1)pdm viruses in six replicates for each assay. As shown in Table II, the minimum viral titer for 100% detection of A/Denmark/524/2009 (H1N1)pdm and A/Denmark/528/2009 (H1N1)pdm using the Type A rRT-PCR and H275Y RT-PCR assays was 3.16×10^{-2} TCID50/reaction, respectively.

Endpoint fluorescence plots of the H275Y RT-PCR (Fig. 1A) and the standard curve of the Type A rRT-PCR assay were also generated (Fig. 1B,C). The standard curve showed a linear relationship between the log of viral titer and the crossing point (Cp) value (Fig. 1B for A/Denmark/524/2009 and Fig. 1C for A/Denmark/528/2009). The correlation coefficient of the standard curve was 0.99, indicating a precise log-linear relationship between the viral titer and Cp value (Fig. 1B,C).

Validation of H275Y RT-PCR Assay Performed Using Clinical Specimens

From October 2009 to February 2010, clinical specimens (nasal or pharyngeal swabs, or nasal aspirate) from suspected cases of A/H1N1pdm virus infection (n = 44) were obtained from hospitals and clinics in Japan. The clinical specimens were tested by Type A and H1pdm rRT-PCR assays after RNA purification. Of 44 clinical specimens, 39 clinical specimens were confirmed to be positive for Type A and H1pdm, and 5 clinical specimens were confirmed to be negative for Type A and H1pdm (Table III). Of 39 Type A- and H1pdm-positive clinical specimens tested by H275Y RT-PCR assay, 38 specimens were determined as H275 (oseltamivir-susceptible) and 1 specimen was determined as Y275 (oseltamivir-resistant) (Table III). All five Type A- and H1pdm-negative clinical specimens were confirmed as negative by H275Y RT-PCR assay (Table III). Some of these results are also shown as endpoint fluorescence plots (Fig. 2).

Validation of H275Y RT-PCR Assay Performed Using Clinical Isolates Without RNA Purification

To facilitate the assay scheme, the H275Y RT-PCR assay was performed by using the cultured supernatants of infected MDCK cells directly, without RNA purification, as the template of the assay for screening H275Y mutation from a large number of clinical isolates. Twenty-four A/H1N1pdm clinical isolates possessing H275, 45 A/H1N1pdm clinical isolates possessing Y275, and 4 A/H1N1pdm clinical isolates having a combination of viruses possessing H275 and Y275 were used directly for the validation of the H275Y RT-PCR assay without RNA purification. Three isolated seasonal influenza A/H1N1 viruses possessing H275 and three isolated seasonal influenza A/H1N1 viruses possessing Y275 were also used directly as negative controls for the H275Y RT-PCR assay without RNA purification. All the isolated seasonal influenza A/H1N1 viruses showed no cross-reactivity and were negative in the assay (Fig. 3). In the assay,

TABLE II. Detection Limits of RT-PCR Assays Using Serial Dilutions of Viral RNA

Viral titer (TCID50/reaction)	A/Denmark/524/2009			A/Denmark/528/2009		
	No. of positive replicates/ No. of tests for each assay (positive %)			No. of positive replicates/ No. of tests for each assay (positive %)		
		H275Y RT-PCR			H275Y RT-PCR	
	Type A rRT-PCR	H275	Y275	Type A rRT-PCR	H275	Y275
3.16×10^{-1}	6/6 (100)	6/6 (100)	0/6 (0)	6/6 (100)	0/6 (0)	6/6 (100)
3.16×10^{-2}	6/6 (100)	6/6 (100)	0/6 (0)	6/6 (100)	0/6 (0)	6/6 (100)
3.16×10^{-3}	2/6 (33.3)	2/6 (33.3)	0/6 (0)	3/6 (50)	0/6 (0)	3/6 (50)
3.16×10^{-4}	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)

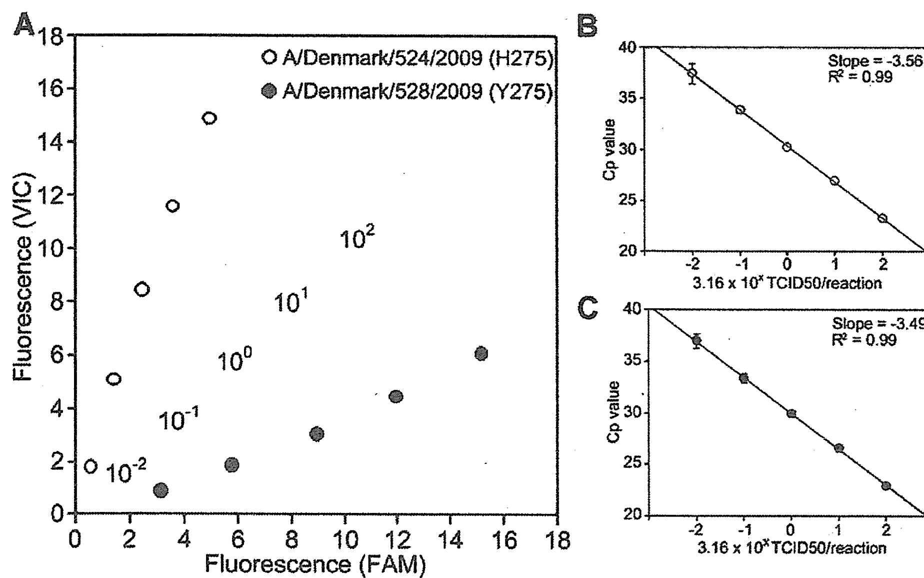

Fig. 1. **A**: Endpoint fluorescence plot. Serial dilutions of A/Denmark/524/2009 (open circle) and A/Denmark/528/2009 (filled circle) were analyzed by H275Y RT-PCR assay. Viral titers of these two isolates were 3.16×10^2, 3.16×10^1, 3.16×10^0, 3.16×10^{-1}, and 3.16×10^{-2} TCID50/reaction and are represented in the centerline of the plot as 10^2, 10^1, 10^0, 10^{-1}, and 10^{-2}, respectively. The plot was generated using the average of the results obtained from the assay performed in six replicates. Relative H275 (VIC) and Y275 (FAM) fluorescence are plotted on the y-axis and x-axis, respectively. **B**,**C**: Serial dilutions of A/Denmark/524/2009 (B) and A/Denmark/528/2009 (C) were analyzed by Type A rRT-PCR assay. The standard curves were generated using average crossing point (Cp) values obtained from the assay performed in six replicates. The correlation coefficient and slope of the standard curve are represented in the graphs.

all 24 and 45 isolated A/H1N1pdm viruses possessing H275 and Y275 were confirmed as H275 (Fig. 3) and Y275 (the results of 5 clinical isolates are shown in Fig. 3), respectively. The four clinical isolates having a combination of viruses were confirmed as "mix" (Fig. 3).

DISCUSSION

In this study, a rapid and simple duplex one-step endpoint genotyping RT-PCR assay for discriminating A/H1N1pdm viruses possessing the H275Y mutation (H275Y RT-PCR assay) was developed. The sensitivity of the H275Y RT-PCR assay was comparable to that of the Type A rRT-PCR assay (Table II), with a detection limit of 7.5 copies/reaction [Nakauchi et al., 2010]. No cross-reactivity or nonspecific reactions were observed in the assay performed using other subtypes/type of influenza viruses (Fig. 3) or clinical specimens (Table III and Fig. 2). These results demonstrated that the H275Y RT-PCR assay was highly specific and sensitive at detecting the H275Y mutation.

Using the H275Y RT-PCR assay, purified RNA from 44 clinical specimens was tested (Table III and Fig. 2). Of 44 clinical specimens tested, 39 were positive for Type A and H1pdm (Table III and Fig. 2). Among 39 specimens, the H275Y RT-PCR assay detected 38 oseltamivir-susceptible viruses with H275 and 1 oseltamivir-resistant virus with Y275. These results corresponded to those of NA sequencing. There was no discrepancy between the H275Y RT-PCR assay and NA sequencing, suggesting that the H275Y RT-PCR assay could discriminate A/H1N1pdm viruses possessing H275 and Y275 in clinical specimens.

The clinical isolates in the cultured supernatants of MDCK cells (Fig. 3) were used directly for the H275Y RT-PCR assay, without RNA purification. The assay results were compared with the results of NA sequencing, and no discrepancies were found between

TABLE III. Detection of Pandemic Influenza A/H1N1 2009 Virus Having H275Y in Clinical Specimens by H257Y RT-PCR Assay

	Results of H275Y RT-PCR assay		
Results of Type A and H1pdm rRT-PCR assays	H275	Y275	Negative
Type A- and H1pdm-positive (n = 39)	38	1	0
Type A- and H1pdm-negative (n = 5)	0	0	5

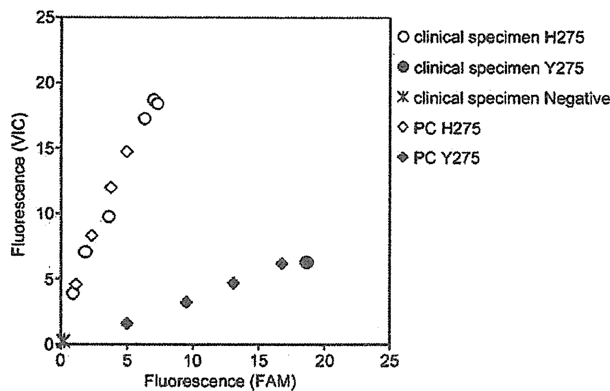

Fig. 2. Endpoint fluorescence plot of H275Y RT-PCR assay using clinical specimens. Relative H275 (VIC) and Y275 (FAM) fluorescence are plotted on the y-axis and x-axis, respectively. Clinical specimens discriminated as H275 or Y275 are represented as open circles or filled circles, respectively. Viral titers of 3.16×10^1, 3.16×10^0, 3.16×10^{-1}, and 3.16×10^{-2} TCID50/reaction of A/Denmark/524/2009 (PC H275, open diamond) and A/Denmark/528/2009 (PC Y275, filled diamond) were used as positive controls for the assay.

the assays (data not shown, all sequence data of the NA gene of the clinical isolates used in this study have been deposited in the GISAID database). Four clinical isolates, A/Aichi/1210/2009, A/Mie/137/2009, A/Tochigi/373/2009, and A/Niigata/1459/2009 (Fig. 3a–d) were found to be a mixed population of the viruses possessing H275 and Y275 by sequencing of the NA gene (data not shown), and were detected as "mix" in the H275Y RT-PCR assay (Fig. 3). These results suggested that the H275Y RT-PCR assay could discriminate specifically A/H1N1pdm viruses possessing H275, Y275, or a mix of H275 and Y275.

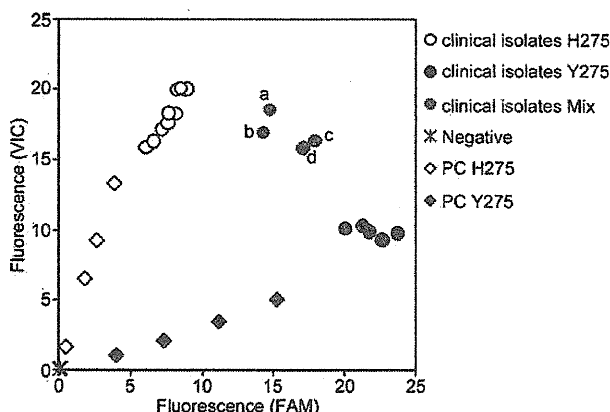

Fig. 3. Endpoint fluorescence plot of H275Y RT-PCR assay using clinical isolates. Relative H275 (VIC) and Y275 (FAM) fluorescence are plotted on the y-axis and x-axis, respectively. Clinical isolates discriminated as H275, Y275, or mix are represented as open circles, filled circles, or gray circles, respectively. Viral titers of 3.16×10^1, 3.16×10^0, 3.16×10^{-1}, and 3.16×10^{-2} TCID50/reaction of A/Denmark/524/2009 (PC H275, open diamond) and A/Denmark/528/2009 (PC Y275, filled diamond) were used as positive controls for the assay.

It is noteworthy that the H275Y RT-PCR assay could detect the H275Y mutation in clinical isolates without RNA purification, since a sufficient amount of viral RNA may be released from the virion under the RT conditions (50°C, 20 min). When considering the handling of a large number of clinical isolates, the H275Y RT-PCR assay can be performed rapidly and simply with a lower risk of cross-contamination, because RNA purification is unnecessary.

Recently, an assay was developed using probes containing LNA nucleotides for discriminating A/H1N1pdm possessing H275Y [van der Vries et al., 2010]. Their assay was highly specific, and the results were interpreted easily; however, the oseltamivir-resistant A/H1N1pdm virus has not been evaluated with their assay. Two real-time RT-PCR assays were also developed: one for detecting A/H1N1pdm viruses possessing H275, and the other for detecting those possessing Y275 [Hindiyeh et al., 2010]. However, in the two independent assays described above for discriminating H275Y, it is not simple to interpret the results, and doing so is costly.

The H275 RT-PCR assay developed in this study could be performed rapidly and simply, since the cultured supernatants of infected cells were used directly without RNA purification (Fig. 3), and the endpoint genotyping analysis program of the Light Cycler® 480 SW1.5 software interpreted the results of the assay automatically by using positive controls, like A/Denmark/524/2009 (H1N1)pdm and A/Denmark/528/2009 (H1N1)pdm, suggesting that this assay is useful for the screening of the H275Y mutation in NAI surveillance activity. Additionally, the H275Y RT-PCR assay could detect whether patients were infected with oseltamivir-susceptible or -resistant viruses by testing clinical specimens, suggesting that this assay is a powerful diagnostic method for determining how to manage patients.

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REFERENCES

Baz M, Abed Y, Papenburg J, Bouhy X, Hamelin ME, Boivin G. 2009. Emergence of oseltamivir-resistant pandemic H1N1 virus during prophylaxis. N Engl J Med 361:2296–2297.

Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ,

Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD Jr, Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI, Cox NJ. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325:197–201.

Hindiyeh M, Ram D, Mandelboim M, Meningher T, Hirsh S, Robinov J, Levy V, Orzitzer S, Azar R, Grossman Z, Mendelson E. 2010. Rapid detection of influenza A pandemic (H1N1) 2009 virus neuraminidase resistance mutation H275Y by real-time RT-PCR. J Clin Microbiol 48:1884–1887.

Kramarz P, Monnet D, Nicoll A, Yilmaz C, Ciancio B. 2009. Use of oseltamivir in 12 European countries between 2002 and 2007—Lack of association with the appearance of oseltamivir-resistant influenza A(H1N1) viruses. Euro Surveill 14: pii19112.

Lackenby A, Thompson CI, Democratis J. 2008. The potential impact of neuraminidase inhibitor resistant influenza. Curr Opin Infect Dis 21:626–638.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948.

Meijer A, Lackenby A, Hungnes O, Lina B, van-der-Werf S, Schweiger B, Opp M, Paget J, van-de-Kassteele J, Hay A, Zambon M. 2009. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007–08 season. Emerg Infect Dis 15:552–560.

Monto AS, McKimm-Breschkin JL, Macken C, Hampson AW, Hay A, Klimov A, Tashiro M, Webster RG, Aymard M, Hayden FG, Zambon M. 2006. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob Agents Chemother 50:2395–2402.

Nakauchi M, Yasui Y, Miyoshi T, Minagawa H, Tanaka T, Tashiro M, Kageyama T. 2010. Improving one-step real-time reverse transcription-PCR assays for detecting and subtyping pandemic (H1N1) 2009, seasonal H1N1, and seasonal H3N2 influenza viruses. J Virol Methods 171:156–162.

Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JS, Guan Y, Rambaut A. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122–1125.

Tashiro M, McKimm-Breschkin JL, Saito T, Klimov A, Macken C, Zambon M, Hayden FG. 2009. Surveillance for neuraminidase-inhibitor-resistant influenza viruses in Japan, 1996–2007. Antivir Ther 14:751–761.

Ujike M, Shimabukuro K, Mochizuki K, Obuchi M, Kageyama T, Shirakura M, Kishida N, Yamashita K, Horikawa H, Kato Y, Fujita N, Tashiro M, Odagiri T, Working Group for Influenza Virus Surveillance in Japan. 2010. Oseltamivir-resistant influenza viruses A (H1N1) during 2007–2009 in fluenza seasons, Japan. Emerg Infect Dis 16:926–935.

van der Vries E, Jonges M, Herfst S, Maaskant J, Van der Linden A, Guldemeester J, Aron GI, Bestebroer TM, Koopmans M, Meijer A, Fouchier RA, Osterhaus AD, Boucher CA, Schutten M. 2010. Evaluation of a rapid molecular algorithm for detection of pandemic influenza A (H1N1) 2009 virus and screening for a key oseltamivir resistance (H275Y) substitution in neuraminidase. J Clin Virol 47:34–37.

WHO. 2009a. Global influenza surveillance network: Laboratory surveillance and response to pandemic H1N1 2009. Wkly Epidemiol Rec 84:361–365.

WHO. 2009b. New influenza A(H1N1) virus infections: Global surveillance summary, May 2009. Wkly Epidemiol Rec 84:173–179.

WHO. 2009c. Oseltamivir-resistant pandemic (H1N1) 2009 influenza virus, October. Wkly Epidemiol Rec 84:453–468.

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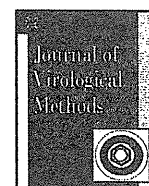

One-step real-time reverse transcription-PCR assays for detecting and subtyping pandemic influenza A/H1N1 2009, seasonal influenza A/H1N1, and seasonal influenza A/H3N2 viruses

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Pandemic influenza A/H1N1 2009 (A/H1N1pdm) virus has caused significant outbreaks worldwide. A previous one-step real-time reverse transcription-PCR (rRT-PCR) assay for detecting A/H1N1pdm virus (H1pdm rRT-PCR assay) was improved since the former probe had a low melting temperature and low tolerance to viral mutation. To help with the screening of the A/H1N1pdm virus, rRT-PCR assays were also developed for detecting human seasonal A/H1N1 (H1 rRT-PCR assay) and A/H3N2 influenza viruses (H3 rRT-PCR assay). H1pdm, H1, and H3 rRT-PCR assays were evaluated using *in vitro*-transcribed control RNA, isolated viruses, and other respiratory pathogenic viruses, and were shown to have high sensitivity, good linearity ($R^2 = 0.99$), and high specificity. In addition, the improved H1pdm rRT-PCR assay could detect two viral strains of A/H1N1pdm, namely, A/Aichi/472/2009 (H1N1)pdm and A/Sakai/89/2009 (H1N1)pdm, which have mutation(s) in the probe-binding region of the hemagglutinin gene, without loss of sensitivity. Using the three rRT-PCR assays developed, 90 clinical specimens collected between May and October 2009 were then tested. Of these, 26, 20, and 2 samples were identified as positive for A/H1pdm, A/H3, and A/H1, respectively, while 42 samples were negative for influenza A viruses. The present results suggest that these highly sensitive and specific H1pdm, H1, and H3 rRT-PCR assays are useful not only for diagnosing influenza viruses, but also for the surveillance of influenza viruses.

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1. Introduction

Since the identification of the novel H1N1 subtype of influenza A virus (pandemic influenza A/H1N1 2009 virus) in Mexico and the United States in March and April 2009, respectively, (WHO, 2009a,b), the virus has spread worldwide. According to WHO, as of February 7, 2010, more than 212 countries and overseas territories or communities have reported laboratory-confirmed cases of pandemic influenza, including at least 15,292 deaths (http://www.who.int/csr/don/2010_02_12/en/index.html).

To prepare for the entry of pandemic influenza A/H1N1 2009 (A/H1N1pdm) virus in Japan, the National Institute of Infectious Diseases (NIID) developed a one-step real-time reverse transcription-PCR (rRT-PCR) assay and conventional RT-PCR method for specifically detecting A/H1N1pdm virus by April 29, 2009 (Kageyama et al., in press). By sharing these laboratory diagnostic systems consisting of primers, probes, reagents, positive controls, and a manual with 75 prefectural and municipal public health institutes and 15 quarantine stations, preparations to detect A/H1N1pdm virus were completed by May 4, 2009 (Kageyama et al., in press). After the first cases of A/H1N1pdm virus infection were identified at Narita Airport Quarantine Station, Japan, on May 9, 2009 (Shimada et al., 2009), the virus spread and was confirmed (using these diagnostic assays) in all prefectures of Japan by July 16, 2009 (report by Infectious Disease Surveillance Center of NIID [http://idsc.nih.go.jp/disease/swine_influenza_e/index.html]).

In August 2009, a previous rRT-PCR assay was improved so as to detect A/H1N1pdm viruses more specifically and sensitively, since the probe that was used previously had a low melting temperature and low tolerance to viral mutation. Furthermore, to help with the screening of the A/H1N1pdm virus, rRT-PCR assays were developed also for detecting human seasonal A/H1N1 influenza (A/H1N1) and A/H3N2 influenza (A/H3N2) viruses. These highly sensitive and specific rRT-PCR assays for detecting and subtyping A/H1N1pdm,

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seasonal A/H1N1, and seasonal A/H3N2 viruses will be useful not only for diagnosing influenza viruses, but also for the surveillance of influenza viruses.

2. Materials and methods

2.1. Primer and probe design

The probe for detecting the A/H1N1pdm virus was redesigned by comparing the former probe sequence and the corresponding sequences of hemagglutinin (HA) genes from A/H1N1pdm viruses posted on the Global Initiative on Sharing Avian Influenza Data (GISAID) database between August and October 2009. The nucleotide sequences were aligned using Clustal W software (Larkin et al., 2007). The sequence of the probe was modified slightly to match the HA gene of the recently circulating virus, and the redesigned probe was elongated (seven nucleotides longer than the former probe) to increase the melting temperature and thus improve the sensitivity and specificity of the assay. Subtyping primers and probes for detecting human seasonal A/H1N1 and A/H3N2 viruses were designed to correspond to each HA gene by comparing sequences posted over the past five years on the GISAID database. The type A typing primers and probe for detecting influenza A virus were designed previously to correspond to conserved regions of the matrix gene segment (Kageyama et al., in press). The rRT-PCR assay for detecting influenza A virus (Type A rRT-PCR assay) was used as a control for evaluating the new and improved rRT-PCR assays. The sequences and position of the primers and probes are listed in Table 1. The sequences of the primers and probes for Type A and H1pdm rRT-PCR assays are disclosed on the Global Influenza Programme website (http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf).

2.2. Viruses

All influenza viruses included in this study were isolated in Madin-Darby Canine Kidney (MDCK) cells. In addition to A/H1N1pdm, several subtypes/types of seasonal influenza viruses used for validating the specificity of the assay were as follows: A/Narita/1/2009 (H1N1)pdm, A/Hiroshima/310/2009 (H1N1)pdm, A/Aichi/472/2009 (H1N1)pdm, A/Sakai/89/2009 (H1N1)pdm, A/Brisbane/59/2007 (H1N1), A/Shiga/8/2008 (H1N1), A/Ehime/38/2008 (H1N1), A/Yokohama/95/2008 (H1N1), A/Kanagawa/69/2008 (H1N1), A/Fukui/91/2008 (H1N1), A/Akita/10/2008 (H1N1), A/Fukui/77/2008 (H1N1), A/Sapporo/16/2008 (H1N1), A/Uruguay/716/2007 (H3N2), A/Toyama/123/2008 (H3N2), A/Yamanashi/135/2008 (H3N2), A/Hiroshima-C/41/2008 (H3N2), B/Sakai/41/2008, and B/Mie/1/2009.

The viral respiratory pathogens used for validating the specificity of the assay were as follows: respiratory syncytial virus A, respiratory syncytial virus B, human parainfluenza viruses types 1–4, Human rhinoviruses types 1B, 2, 14, 36, and 89, human metapneumovirus, and human coronaviruses OC43, 229E, and NL63. Human metapneumovirus and human parainfluenza viruses 2 and 4 were obtained from Sendai Medical Center. Human rhinoviruses types 1B, 2, 14, 36, and 89 were obtained from Nagasaki Prefectural Institute for Environmental Research and Public Health. Respiratory syncytial viruses A and B, human parainfluenza viruses 1 and 3, and human coronaviruses were stored at NIID.

2.3. Preparation of RNA transcript controls

To construct an RNA-positive control for each rRT-PCR assay, each target gene segment was amplified by RT-PCR, and the resulting PCR product containing T7 promoter was then transcribed *in vitro*. The detailed procedure is described below.

The primer Uni12 (5′-AGCAAAAGCAGG-3′) (Hoffmann et al., 2001) was used for RT using a SuperScript® III Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's instructions. The matrix gene of the A/Narita/1/2009 (H1N1)pdm virus was amplified using the following paired primers: Narita_MP_F (5′-ATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTTTCTATCATC-3′) and T7_Narita_MP_R (5′-TAATACGACTCACTATAGGGTTACTCTAGCTCTATGTTGACAAAATGACCATCGTC-3′). The HA gene segments of A/Narita/1/2009 (H1N1)pdm, A/Brisbane/59/2007 (H1N1), and A/Uruguay/716/2007 (H3N2) viruses were amplified using the following paired primers: Narita_HA_F (5′-AGCAAAAGCAGGGGAAAACAAAAGCAACAAAAATGAAGGCAATACTAGTAGTTCTG-3′) and T7_Narita_HA_R (5′-TAATACGACTCACTATAGGGAGTAGAAACAAGGGTGTTTTTCTCATGCTTCTGAAATCCTAATGTTAAATAC-3′), Brisbane_HA_F (5′-AGCAAAAGCAGGGGATAATAAAAACAACCAGAATGAAAGTAAAACTACTGGTCCTGTTAT-3′) and T7_Brisbane_HA_R (5′-TAATACGACTCACTATAGGGTAGTAGAAACAAGGGTGTTTTTCCTTATATTTCTGAAATTCTGGTCTTAGATG-3′), and Uruguay_HA_F (5′-AGCAAAA-

Table 1
Primers and probes.

Primer and probe names	Primer and probe sequences (5′–3′)	Orientation	Target gene	Position[a]
Primers and probe set for the **H1pdm** rRT-PCR assay				
NIID-swH1 TaqMan Primer-F1	AGAAAAGAATGTAACAGTAACACACTCTGT	+	HA	111–140
NIID-swH1 TaqMan Primer-R1	TGTTTCCACAATGTARGACCAT	−	HA	276–297
NIID-swH1 Probe2	(FAM)CAGCCAGCAATRTTRCATTTACC(MGB)[b]	−	HA	208–230
Primers and probe set for the **H1** rRT-PCR assay				
NIID-H1 TaqMan Primer-F1	CCCAGGGYATTTCGCYGACTATGAG	+	HA	324–348
NIID-H1 TaqMan Primer-R1	CATGATGCTGAYACTCCGGTTACG	−	HA	432–455
NIID-H1 Probe1	(FAM)TCTCAAAYGAAGATACTGAACT(MGB)	−	HA	367–387
Primers and probe set for the **H3** rRT-PCR assay				
NIID-H3 TaqMan Primer-F1	CTATTGGACAATAGTAAAACCGGGRGA	+	HA	744–770
NIID-H3 TaqMan Primer-R1	GTCATTGGGRATGCTTCCATTTGG	−	HA	898–921
NIID-H3 Probe1	(FAM)AAGTAACCCCKAGGAGCAATTAG(MGB)	−	HA	799–821
Primers and probe set for the **Type A** rRT-PCR assay				
MP-39-67For	CCMAGGTCGAAACGTAYGTTCTCTCTATC	+	M	14–42
MP-183-153Rev	TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA	−	M	128–159
MP-96-75ProbeAs	(FAM)ATYTCGGCTTTGAGGGGGCCTG(MGB)	−	M	50–71

The NIID-swH1 Probe2 is seven nucleotides longer than the former probe, NIID-swH1 Probe. The seven nucleotides are underlined.

[a] The nucleotide positions of HA and M genes are based on cRNA sequences obtained from GenBank. Accession numbers for HA genes of A/Narita/1/2009 (H1N1)pdm, A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2) viruses, and the M gene of the A/Narita/1/2009 (H1N1)pdm virus are GQ165815, CY030230, EU716428, and GQ169302, respectively.

[b] Probes are labeled with FAM at the 5′ end and MGB at the 3′ end.

GCAGGGGATAATTCTATTAACCATGAAGACTATCATTGCTTTGAGCT-ACATTCTATG-3′) and T7_Uruguay_HA_R (5′-TAATACGACTC-ACTATAGGGCAGTAGAAACAAGGGTGTTTTTAATTAATGCACTCAAA-TGCAAATGTTGC-3′). PCR was performed using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). The PCR products were purified using NucleoSpin® (Macherey-Nagel GmbH & Co. KG, Duren, Germany) and transcribed using the MegaScript® T7 In Vitro Transcription Kit (Ambion) according to each manufacturer's instructions. After DNase digestion to remove residues of RT-PCR products, the transcribed RNA was purified twice using an RNA Isolation Kit (Agilent Technologies, Santa Clara, CA, USA) and quantified by spectrophotometric analysis.

2.4. Sequencing of the partial HA gene of pandemic influenza A/H1N1 2009

To confirm the sequence of target region for primers and probe of H1pdm rRT-PCR assay, partial HA genes of A/Narita/1/2009 (H1N1)pdm, A/Hiroshima/310/2009 (H1N1)pdm, A/Aichi/472/2009 (H1N1)pdm, and A/Sakai/89/2009 (H1N1)pdm were sequenced. To amplify the partial HA gene between −32 and +366 (from the start codon) using extracted RNA, RT-PCR was carried out with the paired primers H1HA1-BEGIN (5′-AGCAAAAGCAGGGGAAAATAA-3′) and swine H1-385-366R (5′-CAAATGATGACACTGAGCTC-3′) using the OneStep RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RT-PCR conditions were as follows: 30 min at 50 °C to activate RT followed by initial denaturation for 15 min at 95 °C, with a subsequent 45 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 40 s). The PCR products were purified and then sequenced using swine H1-56-76F primer (5′-CATTATGTATAGGTTATCATG-3′). Total RNA was prepared as described in Section 2.6.

2.5. Clinical specimens

Nasal and/or pharyngeal swabs collected from suspected and contact cases of influenza and suspended in virus transport medium were obtained from prefectural and municipal public health institutes and quarantine stations in Japan. These specimens were collected between May and October 2009.

2.6. RNA preparation

Supernatants of cultured MDCK cells were centrifuged at 10,000 × g for 10 min. Viral RNA was prepared from 140 μl of the supernatant using the QIAamp® Viral RNA kit (Qiagen) according to the manufacturer's instructions with a slight modification in that the viral RNA was eluted in 70 μl of AVE (Qiagen).

Total RNA was prepared from clinical specimens using the QIAamp® Viral RNA kit (Qiagen) (using 140 μl of clinical specimen) or MagMAX™ 96 Viral Isolation Kit (Ambion, Austin, TX, USA) (using 50 μl of clinical specimen) with KingFisher Flex (Thermo Fisher Scientific, Waltham, MA, USA) according to each manufacturer's instructions. Total RNA from the 140 μl clinical specimen was eluted with 60 μl of AVE (Qiagen), whereas total RNA from the 50 μl clinical specimen was eluted with 30 μl of elution buffer (Ambion).

2.7. One-step real-time RT-PCR assay

The reaction was performed using a QuantiTect® Probe RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Briefly, the 25 μl assay contained 12.5 μl of 2× QuantiTect Probe PCR Master Mix, 0.25 μl of QuantiTect RT Mix, 0.1 μl of RNase Inhibitor (Applied Biosystems, Foster City, CA, USA), 1.5 μl of 10 μM forward primer, 1.5 μl of 10 μM reverse primer, 0.5 μl of 5 μM probe, 3.65 μl of water, and 5 μl of RNA template. Cycling was performed as follows: 30 min at 50 °C to activate RT, followed by initial denaturation for 15 min at 95 °C with a subsequent 45 cycles of amplification (denaturation at 95 °C for 15 s and annealing as well as extension at 56 °C for 75 s) using LightCycler® 480 (Roche Molecular Biochemicals). Fluorescent signals were collected during annealing and extension steps, and amplification data were analyzed using the Light Cycler® 480 SW1.5 software according to the manufacturer's instructions.

3. Results

3.1. Development of real-time RT-PCR assays

Three rRT-PCR assays were developed (Table 1): H1pdm rRT-PCR assay for detecting pandemic influenza A/H1N1 2009 (A/H1N1pdm) virus, H1 rRT-PCR assay for detecting human seasonal A/H1N1 influenza (A/H1N1) virus, and H3 rRT-PCR assay for detecting A/H3N2 influenza (A/H3N2) virus.

The specificity of each rRT-PCR assay was evaluated using A/H1N1, A/H3N2, and A/H1N1pdm viruses, influenza B viruses, and other viral respiratory pathogens. The improved H1pdm rRT-PCR assay reacted specifically to A/H1N1pdm viruses, but did not cross-react with A/H1N1 and A/H3N2 viruses. The new H1 rRT-PCR assay reacted specifically to A/H1N1 viruses, but did not cross-react with A/H3N2 and A/H1N1pdm viruses, and the H3 rRT-PCR assay reacted specifically with the A/H3N2 viruses, but did not cross-react with A/H1N1 and A/H1N1pdm viruses. The Type A rRT-PCR assay could detect only influenza A viruses. All four assays showed no cross-reactivity against influenza B viruses and other viral respiratory pathogens.

The detection limit of each assay was determined by performing serial dilutions of *in vitro*-transcribed control viral RNA for six replicates in each assay. The detection limit of each assay was determined by calculating the concentration where there is 95% positivity using the results shown in Table 2. The detection limits of Type A, H1pdm, H1, and H3 rRT-PCR were determined to be 7.5, 6.8, 7.3, and 7.1 copies/reaction, respectively.

The sensitivity of improved H1pdm, H1, and H3 rRT-PCR assays was compared with that of the Type A rRT-PCR assay using serial dilutions of A/Narita/1/2009 (H1N1)pdm, A/Aichi/472/2009 (H1N1)pdm, A/Sakai/89/2009 (H1N1)pdm, A/Brisbane/59/2007 (H1N1), or A/Uruguay/716/2007 (H3N2) viruses in six replicates for each assay (Tables 3 and 4). A/Aichi/472/2009 (H1N1)pdm was shown to have an HA gene in which the adenine nucleotide was substituted with a cytosine nucleotide at position 218 from the start codon located in the target region of the former probe (NIID-swH1 Probe1) (Kageyama et al., in press). A/Sakai/89/2009 (H1N1)pdm showed an HA gene in which the guanine nucleotide and the thymine nucleotide were substituted with adenine nucleotides at positions 208 and 210, respectively, located in the target region of the NIID-swH1 Probe 1. Since both viruses had mutation(s) in the probe-binding region of the HA gene, the sensitivity of the former H1pdm rRT-PCR assay decreased for these viruses (Table 4). However, the improved H1pdm rRT-PCR assay could detect these viruses with high sensitivity (Table 4).

As shown in Tables 3 and 4, the minimum viral titers for 100% detection of A/Narita/1/2009 (H1N1)pdm, A/Aichi/472/2009 (H1N1)pdm, and A/Sakai/89/2009 (H1N1)pdm using the Type A and H1pdm rRT-PCR assays were 3.16×10^{-2}, 5.00×10^{-3}, and 1.07×10^{-3} TCID50/reaction, respectively. The minimum viral titer for 100% detection of A/Brisbane/59/2007 (H1N1) using Type A and H1 rRT-PCR assays was 2.19×10^{-3} TCID50/reaction, and that of

Table 2
Detection limits of each assay using serial dilutions of *in vitro*-transcribed control viral RNA.

Template RNA concentrations (copies/reaction)	Number of positive replicates/number of tests for each assay (positive %)			
	Type A[a]	H1pdm[b]	H1[c]	H3[d]
10	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)
5	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)
1	2/6 (33.3)	5/6 (83.3)	3/6 (50)	4/6 (66.7)
0.1	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)

[a] Template RNA for each assay is the matrix gene segment of A/Narita/1/2009 (H1N1)pdm.
[b] Template RNA for each assay is the HA gene segment of A/Narita/1/2009 (H1N1)pdm.
[c] Template RNA for each assay is the HA gene segment of A/Brisbane/59/2007 (H1N1).
[d] Template RNA for each assay is the HA gene segment of A/Uruguay/716/2007 (H3N2).

Table 3
Detection limits of each real-time RT-PCR assay using serial dilutions of viral RNA.

Viral titer ($TCID_{50}$/reaction)	No. of positive replicates/ No. of tests for each assay (positive %)		Viral titer ($TCID_{50}$/reaction)	No. of positive replicates/ No. of tests for each assay (positive %)		Viral titer ($TCID_{50}$/reaction)	No. of positive replicates/ No. of tests for each assay (positive %)	
A/Narita/1/2009 (H1N1)pdm	Type A	H1pdm	A/Brisbane/59/2007 (H1N1)	Type A	H1	A/Uruguay/716/2007 (H3N2)	Type A	H3
3.16×10^{-1}	6/6 (100)	6/6 (100)	2.19×10^{-2}	6/6 (100)	6/6 (100)	4.68×10^{-2}	6/6 (100)	6/6 (100)
3.16×10^{-2}	6/6 (100)	6/6 (100)	2.19×10^{-3}	6/6 (100)	6/6 (100)	4.68×10^{-3}	6/6 (100)	6/6 (100)
3.16×10^{-3}	3/6 (50)	2/6 (33.3)	2.19×10^{-4}	4/6 (66.7)	3/6 (50)	4.68×10^{-4}	1/6 (16.7)	1/6 (16.7)
3.16×10^{-4}	0/6 (0)	0/6 (0)	2.19×10^{-5}	0/6 (0)	0/6 (0)	4.68×10^{-5}	0/6 (0)	0/6 (0)

A/Uruguay/716/2007 (H3N2) using Type A and H3 rRT-PCR assays was 4.68×10^{-3} TCID50/reaction (Table 3). As described above, the minimum viral titer for 100% detection of each virus using H1pdm, H1, or H3 rRT-PCR assays was identical to that for 100% detection of each virus using the Type A rRT-PCR assay (Tables 3 and 4). Detection of further dilutions of each virus was not 100% over six replicates of each assay (Tables 3 and 4).

A standard curve of each assay was also generated (Fig. 1). The standard curve showed a linear relationship between the log of the viral titer and the crossing point (Cp) value for all assays (Fig. 1). The correlation coefficient of the standard curve was 0.99 for all assays, indicating a precise log-linear relationship between the viral titer and Cp value (Fig. 1).

3.2. Validation of real-time RT-PCR assays performed using clinical specimens

From May 2009 to October 2009 clinical specimens (nasal and/or pharyngeal swabs) from suspected cases of pandemic (H1N1) 2009 virus infection ($n = 90$) were obtained from prefectural and municipal public health institutes and quarantine stations in Japan. Type A rRT-PCR, improved H1pdm rRT-PCR, H1 rRT-PCR, and H3 rRT-PCR assays were performed for all clinical specimens. As a result, 48 samples were identified as positive for Type A, whereas 42 samples were negative for Type A. All 48 Type A-positive samples were subtyped, revealing that 26, 20, and 2 samples were positive for H1pdm, H3, and H1, respectively.

4. Discussion

After the worldwide outbreak of pandemic influenza A/H1N1 2009 (A/H1N1pdm), many diagnostic methods for detecting the causative virus, including the rRT-PCR assay, were established by many groups (Beck et al., 2010; Bose et al., 2009; Carr et al., 2009; Chidlow et al., 2010; Dong et al., 2010; Ge et al., 2009; Gunson et al., 2010; Hall et al., 2009; He et al., 2009; Jiang et al., 2010; Kubo et al., 2010; Lau et al., 2009; LeBlanc et al., 2009; Liu et al., 2009; Pabbaraju et al., 2009; Poon et al., 2009; Wang et al., 2009; Wenzel et al., 2009; Whiley et al., 2009; Wu et al., 2010; Yang et al., 2009). Type A rRT-PCR and improved H1pdm rRT-PCR assays, as well as the newly developed H1 rRT-PCR and H3 rRT-PCR assays used for subtyping human seasonal influenza A viruses, were shown to have good linearity ($R^2 = 0.99$) and high sensitivity (Tables 2–4 and Fig. 1). No cross-reactivity or nonspecific reactions were observed in any of the assays performed using isolated viruses and clinical specimens. In the subtyping assay of clinical specimens, all 48 Type A-positive samples were subtyped by H1pdm, H1, and H3 rRT-PCR rRT-PCR assays developed in this study, and there were no unsubtyped Type A-positive samples. These results suggest that H1pdm, H1, H3, and Type A rRT-PCR assays are highly sensitive and specific for each target gene and thus should be of great use for detecting A/H1N1pdm virus and subtyping seasonal influenza A viruses.

The A/Aichi/472/2009 (H1N1)pdm and A/Sakai/89/2009 (H1N1)pdm viruses with mutation(s) in the sequence corresponding to the former probe sequence could not be detected using the

Table 4
Comparison of detection limits of previous and modified H1pdm rRT-PCR assays using serial dilutions of viral RNA.

Viral titer ($TCID_{50}$/reaction)	No. of positive replicates/ No. of tests for each assay (positive %)			Viral titer ($TCID_{50}$/reaction)	No. of positive replicates/ No. of tests for each assay (positive %)		
A/Aichi/472/2009 (H1N1)pdm	Type A	H1pdm	Former H1pdm	A/Sakai/89/2009 (H1N1)pdm	Type A	H1pdm	Former H1pdm
5.00×10^{1}	6/6 (100)	6/6 (100)	0/6 (0)	1.07×10^{0}	6/6 (100)	6/6 (100)	0/6 (0)
5.00×10^{0}	6/6 (100)	6/6 (100)	0/6 (0)	1.07×10^{-1}	6/6 (100)	6/6 (100)	0/6 (0)
5.00×10^{-1}	6/6 (100)	6/6 (100)	0/6 (0)	1.07×10^{-2}	6/6 (100)	6/6 (100)	0/6 (0)
5.00×10^{-2}	6/6 (100)	6/6 (100)	0/6 (0)	1.07×10^{-3}	6/6 (100)	6/6 (100)	0/6 (0)
5.00×10^{-3}	6/6 (100)	6/6 (100)	0/6 (0)	1.07×10^{-4}	5/6 (83.3)	5/6 (83.3)	0/6 (0)
5.00×10^{-4}	5/6 (83.3)	2/6 (33.3)	0/6 (0)	1.07×10^{-5}	3/6 (50)	0/6 (0)	0/6 (0)
5.00×10^{-5}	0/6 (0)	0/6 (0)	0/6 (0)	1.07×10^{-6}	0/6 (0)	0/6 (0)	0/6 (0)

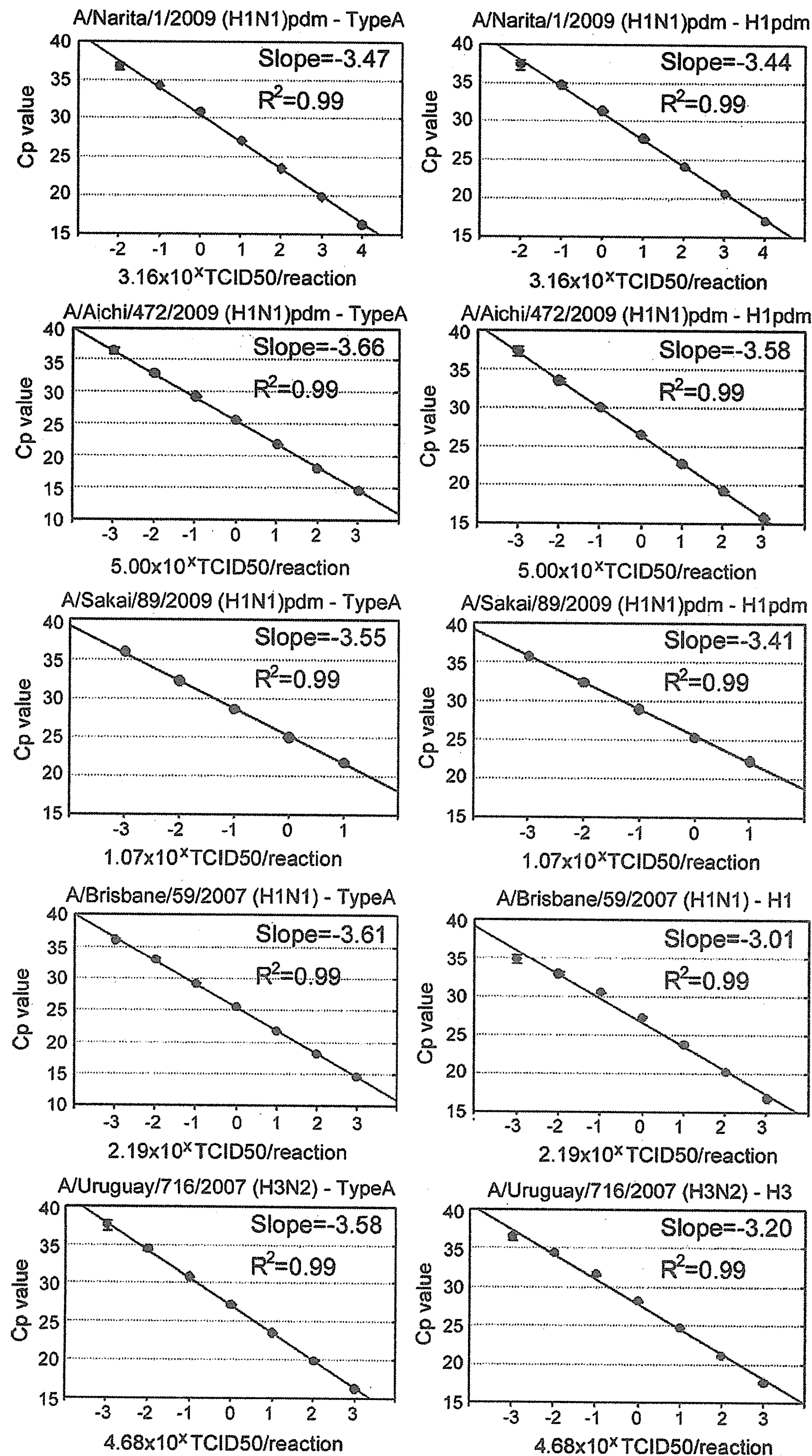

Fig. 1. Standard curves of each assay. Ten-fold serial dilutions of viral RNA were used for each rRT-PCR assay performed in six replicates. The standard curves were generated using average crossing point (Cp) values obtained from the assays performed in six replicates. The correlation coefficient and slope of the standard curve are represented in the graphs. Standard curves were made based on Type A and H1pdm rRT-PCR assays performed using A/Narita/1/2009 (H1N1)pdm, A/Aichi/472/2009 (H1N1)pdm, and A/Sakai/89/2009 (H1N1)pdm (upper 6 graphs), those based on Type A and H1 rRT-PCR assays performed using A/Brisbane/59/2007 (H1N1) (second 2 graphs from the bottom), and those developed based on Type A and H3 rRT-PCR assays performed using A/Uruguay/716/2007 (H3N2) (bottom 2 graphs).

former H1pdm rRT-PCR assay (Table 4). These two viruses were isolated from clinical specimens that failed to be detected by the former H1pdm rRT-PCR assay. The redesigned probe in this study was seven nucleotides longer than the former probe (Table 1). This was done to increase the melting temperature and thus to improve the sensitivity and specificity of the assay. The results of the improved H1pdm rRT-PCR assay performed using serial dilutions of the A/Aichi/472/2009 (H1N1)pdm and A/Sakai/89/2009 (H1N1)pdm viruses demonstrated that the sensitivity of this assay was identical to that of the Type A rRT-PCR assay (Table 4 and Fig. 1), suggesting that the improved H1pdm rRT-PCR assay can detect mutated A/H1N1pdm viruses without losing sensitivity. Thus far, additional mutations of the target sequence of the redesigned probe used in H1pdm rRT-PCR assays have not been observed in recent circulating A/H1N1pdm viruses in Japan.

Using the four rRT-PCR assays, a diagnostic scheme was developed in which H1pdm, H1, and H3 rRT-PCR assays for subtyping and Type A rRT-PCR assay for evaluating the subtyping assays were all performed simultaneously. To prepare for viruses with other possible mutations in the HA gene and emerging influenza viruses of other subtypes, as well as to decrease the risk of false negatives, subtype-specific rRT-PCR assays must always be performed in combination with the Type A rRT-PCR assay.

The highly sensitive and specific H1pdm, H1, and H3 rRT-PCR assays developed in this study and Type A rRT-PCR assay were performed as laboratory diagnostic tests for pandemic influenza A/H1N1 2009 virus at most prefectural and municipal public health institutes and quarantine stations in Japan, and have been confirmed to be very useful not only for laboratory diagnostic tests but also for the surveillance of the spread of influenza viruses.

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The authors declare no potential conflict of interest relevant to this article. None of the primers and probes listed in this article are patented.

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References

Beck, E.T., Jurgens, L.A., Kehl, S.C., Bose, M.E., Patitucci, T., LaGue, E., Darga, P., Wilkinson, K., Witt, L.M., Fan, J., He, J., Kumar, S., Henrickson, K.J., 2010. Development of a rapid automated influenza A, influenza B, and respiratory syncytial virus A/B multiplex real-time RT-PCR assay and its use during the 2009 H1N1 swine-origin influenza virus epidemic in Milwaukee, Wisconsin. J. Mol. Diagn. 12, 74–81.

Bose, M.E., Beck, E.T., Ledeboer, N., Kehl, S.C., Jurgens, L.A., Patitucci, T., Witt, L., LaGue, E., Darga, P., He, J., Fan, J., Kumar, S., Henrickson, K.J., 2009. Rapid semi-automated subtyping of influenza virus species during the 2009 swine origin influenza A H1N1 virus epidemic in Milwaukee, Wisconsin. J. Clin. Microbiol. 47, 2779–2786.

Carr, M.J., Gunson, R., Maclean, A., Coughlan, S., Fitzgerald, M., Scully, M., O'Herlihy, B., Ryan, J., O'Flanagan, D., Connell, J., Carman, W.F., Hall, W.W., 2009. Development of a real-time RT-PCR for the detection of swine-lineage influenza A (H1N1) virus infections. J. Clin. Virol. 45, 196–199.

Chidlow, G., Harnett, G., Williams, S., Levy, A., Speers, D., Smith, W.D., 2010. Duplex real-time RT-PCR assays for the rapid detection and identification of pandemic (H1N1) 2009 and seasonal influenza viruses A/H1, A/H3 and B. J. Clin. Microbiol. 48, 862–866.

Dong, H., Zhang, Y., Xiong, H., Yan, A., Ding, G., Chen, Y., Xie, L., Chen, J., Zhang, G., Hao, P., Cong, L., Lu, Y., Che, X., Wang, X., Li, Y., Yuen, K.Y., Zhao, G., Jin, W., 2010. Detection of human novel influenza A (H1N1) viruses using multi-fluorescent real-time RT-PCR. Virus Res. 147, 85–90.

Ge, Y., Cui, L., Qi, X., Shan, J., Shan, Y., Qi, Y., Wu, B., Wang, H., Shi, Z., 2009. Detection of novel swine origin influenza A virus (H1N1) by real-time nucleic acid sequence-based amplification. J. Virol. Methods.

Gunson, R., Maclean, A., Davies, E., Bennett, S., Miller, R., Carman, W.F., 2010. Development of a multiplex real-time RT-PCR that allows universal detection of influenza A viruses and simultaneous typing of influenza A/H1N1/2009 virus. J. Virol. Methods 163, 258–261.

Hall, R.J., Peacey, M., Huang, Q.S., Carter, P.E., 2009. Rapid method to support diagnosis of swine origin influenza virus infection by sequencing of real-time PCR amplicons from diagnostic assays. J. Clin. Microbiol. 47, 3053–3054.

He, J., Bose, M.E., Beck, E.T., Fan, J., Tiwari, S., Metallo, J., Jurgens, L.A., Kehl, S.C., Ledeboer, N., Kumar, S., Weisburg, W., Henrickson, K.J., 2009. Rapid multiplex reverse transcription-PCR typing of influenza A and B virus, and subtyping of influenza A virus into H1, 2, 3, 5, 7, 9, N1 (human), N1 (animal), N2, and N7, including typing of novel swine origin influenza A (H1N1) virus, during the 2009 outbreak in Milwaukee, Wisconsin. J. Clin. Microbiol. 47, 2772–2778.

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275–2289.

Jiang, T., Kang, X., Deng, Y., Zhao, H., Li, X., Yu, X., Yu, M., Qin, E., Zhu, Q., Yang, Y., Qin, C., 2010. Development of a real-time RT-PCR assay for a novel influenza A (H1N1) virus. J. Virol. Methods 163, 470–473.

Kageyama, T., Shirakura, M., Kishida, N., Nakauchi, M., Obuchi, M., Ujike, M., Itamura, S., Odagiri, T., Tashiro, M., in press. Establishment of pandemic influenza A 2009 (H1N1) virus diagnosis system in Japan using conventional RT-PCR assay and real-time RT-PCR assay. Jpn. J. Infect. Dis.

Kubo, T., Agoh, M., Mai le, Q., Fukushima, K., Nishimura, H., Yamaguchi, A., Hirano, M., Yoshikawa, A., Hasebe, F., Kohno, S., Morita, K., 2010. Development of a reverse transcription-loop-mediated isothermal amplification assay for detection of pandemic (H1N1) 2009 virus as a novel molecular method for diagnosis of pandemic influenza in resource-limited settings. J. Clin. Microbiol. 48, 728–735.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Lau, S.K., Chan, K.H., Yip, C.C., Ng, T.K., Tsang, O.T., Woo, P.C., Yuen, K.Y., 2009. Confirmation of the first Hong Kong case of human infection by novel swine origin influenza A (H1N1) virus diagnosed using ultrarapid, real-time reverse transcriptase PCR. J. Clin. Microbiol. 47, 2344–2346.

LeBlanc, J.J., Li, Y., Bastien, N., Forward, K.R., Davidson, R.J., Hatchette, T.F., 2009. Switching gears for an influenza pandemic: validation of a duplex reverse transcriptase PCR assay for simultaneous detection and confirmatory identification of pandemic (H1N1) 2009 influenza virus. J. Clin. Microbiol. 47, 3805–3813.

Liu, S., Hou, G., Zhuang, Q., Shu, Y., Chen, J., Jiang, W., Li, J., 2009. A SYBR Green I real-time RT-PCR assay for detection and differentiation of influenza A (H1N1) virus in swine populations. J. Virol. Methods 162, 184–187.

Pabbaraju, K., Wong, S., Wong, A.A., Appleyard, G.D., Chui, L., Pang, X.L., Yanow, S.K., Fonseca, K., Lee, B.E., Fox, J.D., Preiksaitis, J.K., 2009. Design and validation of real-time reverse transcription-PCR assays for detection of pandemic (H1N1) 2009 virus. J. Clin. Microbiol. 47, 3454–3460.

Poon, L.L., Chan, K.H., Smith, G.J., Leung, C.S., Guan, Y., Yuen, K.Y., Peiris, J.S., 2009. Molecular detection of a novel human influenza (H1N1) of pandemic potential by conventional and real-time quantitative RT-PCR assays. Clin. Chem. 55, 1555–1558.

Shimada, T., Gu, Y., Kamiya, H., Komiya, N., Odaira, F., Sunagawa, T., Takahashi, H., Toyokawa, T., Tsuchihashi, Y., Yasui, Y., Tada, Y., Okabe, N., 2009. Epidemiology of influenza A (H1N1)v virus infection in Japan, May–June 2009. Euro Surveill., 14.

Wang, R., Sheng, Z.M., Taubenberger, J.K., 2009. Detection of novel (swine origin) H1N1 influenza A virus by quantitative real-time reverse transcription-PCR. J. Clin. Microbiol. 47, 2675–2677.

Wenzel, J.J., Walch, H., Bollwein, M., Niller, H.H., Ankenbauer, W., Mauritz, R., Holtke, H.J., Zepeda, H.M., Wolf, H., Jilg, W., Reischl, U., 2009. Library of prefabricated locked nucleic acid hydrolysis probes facilitates rapid development of reverse-transcription quantitative real-time PCR assays for detection of novel influenza A/H1N1/09 virus. Clin. Chem. 55, 2218–2222.

Whiley, D.M., Bialasiewicz, S., Bletchly, C., Faux, C.E., Harrower, B., Gould, A.R., Lambert, S.B., Nimmo, G.R., Nissen, M.D., Sloots, T.P., 2009. Detection of novel influenza A(H1N1) virus by real-time RT-PCR. J. Clin. Virol. 45, 203–204.

WHO, 2009a. Global influenza surveillance network: laboratory surveillance and response to pandemic H1N1. Wkly. Epidemiol. Rec. 84, 361–365.

WHO, 2009b. New influenza A(H1N1) virus infections: global surveillance summary, May 2009. Wkly. Epidemiol. Rec. 84, 173–179.

Wu, W., Kang, X., Bai, Z., Liu, L., Li, J., Wu, X., Sun, H., Hu, T., Yang, M., Wang, P., Yang, Y., Di, B., Chen, W., 2010. Detection of pandemic influenza A/H1N1/2009 virus by real-time reverse transcription polymerase chain reaction. J. Virol. Methods 165, 294–296.

Yang, J.R., Lo, J., Liu, J.L., Lin, C.H., Ho, Y.L., Chen, C.J., Wu, H.S., Liu, M.T., 2009. Rapid SYBR green I and modified probe real-time reverse transcription-PCR assays identify influenza H1N1 viruses and distinguish between pandemic and seasonal strains. J. Clin. Microbiol. 47, 3714–3716.

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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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