Development of Two Types of Rapid Diagnostic Test Kits To Detect the Hemagglutinin or Nucleoprotein of the Swine-Origin Pandemic Influenza A Virus H1N1

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Since its emergence in April 2009, pandemic influenza A virus H1N1 (H1N1 pdm), a new type of influenza A virus with a triple-reassortant genome, has spread throughout the world. Initial attempts to diagnose the infection in patients using immunochromatography (IC) relied on test kits developed for seasonal influenza A and B viruses, many of which proved significantly less sensitive to H1N1 pdm. Here, we prepared monoclonal antibodies that react with H1N1 pdm but not seasonal influenza A (H1N1 and H3N2) or B viruses. Using two of these antibodies, one recognizing viral hemagglutinin (HA) and the other recognizing nucleoprotein (NP), we developed kits for the specific detection of H1N1 pdm and tested them using clinical specimens of nasal wash fluid or nasopharyngeal fluid from patients with influenza-like illnesses. The specificities of both IC test kits were very high (93% for the HA kit, 100% for the NP kit). The test sensitivities for detection of H1N1 pdm were 85.5% with the anti-NP antibody, 49.4% with the anti-HA antibody, and 79.5% with a commercially available influenza A virus detection assay. Use of the anti-NP antibody could allow the rapid and accurate diagnosis of H1N1 pdm infections.

In April 2009, a novel influenza A virus of swine origin (H1N1) appeared rapidly and quickly spread. The WHO decided on 12 June 2009 to declare a pandemic (level 6). Although infections appeared to be relatively mild, there had been significant mortality and hospitalization and a gradually increasing number of fatal cases. As of 6 August 2010, more than 214 countries, overseas territories, and communities had reported laboratory-confirmed cases of pandemic influenza A virus H1N1 2009 (H1N1 pdm) infection, including over 18,449 deaths (WHO, http://www.who.int/csr/don/2010_08_06/en/index.html).

Comprehensive phylogenetic analyses revealed H1N1 pdm to have a triple-reassortant genome that comprised genes derived from the avian (PB2 and PA), human H3N2 (PB1), Eurasian avian-like swine (NA and M), and classical swine (HA, NP, and NS) lineages (18, 20). The 2009 CDC guidelines recommended prompt treatment with antiviral drugs and management using specific infection control precautions for high-risk and hospitalized patients (1). A point-of-care strategy with the rapid diagnosis of H1N1 pdm infection is crucial for efficient clinical treatment and the implementation of infection control measures. In the early days of the pandemic, several countries tried to diagnose H1N1 pdm infections using immunochromatography (IC) and subjecting the positive samples to real-time PCR and, if necessary, to viral isolation. The IC test kits, essentially developed for seasonal human influenza A and B viruses, were found to be less sensitive than other tests, such as PCR (4, 6, 8, 11, 16, 25). Several kits were significantly less sensitive to the detection of H1N1 pdm than to seasonal influenza viruses (7, 11, 14), although others showed comparable results (2, 10, 12, 22). Generally, the diagnosis of this infection by real-time PCR is difficult, because relatively few hospitals have the medical expertise and equipment required. Therefore, a new test kit that can easily and rapidly diagnose H1N1 pdm infections without a need for subsequent PCR is needed.

In addition, the test kit for H1N1 pdm may have the utility of influenza A virus subtyping, since antiviral drug resistance of H1N1 pdm is still rare (21). In this study, we prepared murine monoclonal antibodies (MAbs) that react with H1N1 pdm but not with seasonal influenza A (H1N1 and H3N2) or B viruses. Interestingly, we obtained an antibody specific to the nucleoprotein (NP), in addition to an antibody specific to the hemagglutinin (HA), of H1N1 pdm. As expected, the kit developed using the anti-NP antibody showed higher specificity and sensitivity than the test developed using the anti-HA antibody and even existing kits for seasonal influenza viruses.

MATERIALS AND METHODS

Viruses. Several strains of influenza A and B viruses were used: A/Osaka/168/09 and A/Suita/01/09 as H1N1 pdm; A/Sw/Hokkaido/2/81 as swine H1N1; A/Yamagata/32/89, A/Beijing/262/95, A/New Caledonia/20/99, and A/Brisbane/
cells from the mice were used to prepare hybridomas. The spleen cells from immunized mice were fused with PAI myeloma cells, as described previously (27). Specific antibody production was screened on the basis of reactions with A/Osaka/168/09 and A/Suita/01/09 viral particles which had been partially purified from the culture fluid of infected MDCK cells by ultracentrifugation. Three days after the last immunization, the spleen cells from the mice were used to prepare hybridomas.

Preparation of hybridomas. The spleen cells from immunized mice were fused with PAI myeloma cells, as described previously (27). Specific antibody production was screened on the basis of reactions with A/Osaka/168/09 and A/Suita/01/09 but no reaction with A/New Caledonia/20/09 by indirect immunofluorescence assay (IFA). The cells in the wells producing the specific antibody were cloned by limiting dilution and then subjected to secondary screening as described above.

IFA. IFA was performed as detailed previously (24). Briefly, 2.5 × 10^5 MDCK cells per well in a 96-well plate were infected with various viruses. After 6 to 12 h, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100 in PBS. The bound antibody was visualized by a further reaction with an Alexa Fluor 488-conjugated secondary antibody (1:1,000; Invitrogen). For the staining of infected MDCK cells with positive-control MAbs, C179 and C43 were used as anti-influenza A virus HA, and NP, respectively (19).

Isotyping. The MAbs obtained were isolated using an Isoclip kit for mouse monoclonal isotyping (Sigma-Aldrich Corporation).

Western blotting. The MDCK cells infected with A/Suita/01/09 or A/New Caledonia/20/09 were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subjected to SDS-PAGE with a 10% polyacrylamide gel. Horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) was used for the secondary antibody. The peroxidase reaction was visualized using ECL plus (GE Healthcare UK Ltd., Buckinghamshire, United Kingdom).

Influenza virus HA or NP expression. The coding regions for the HA and NP derived from A/Suita/01/09 were amplified by reverse transcriptase PCR (RT-PCR) and cloned into a pCAGGS II expression plasmid, as described previously (5). 293T cells transfected with the plasmids were used as viral antigens for the reaction with the MAbs in ascitic fluid by IFA.

Preparation of ascitic fluid. BALB/c mice (6 weeks old, female) were intraperitoneally treated with pristane (Sigma-Aldrich Corporation). After 1 week, the mice were injected with hybridoma cells (about 4 × 10⁶ cells per mouse). Ascitic fluid samples obtained at 1 to 2 weeks postinjection were used for the characterization of individual MAbs.

Assembly for rapid diagnostic testing using the IC method. The IgG fraction purified from murine ascitic fluid was used to develop the IC test kit with a system from Alfresa Pharma Corporation, Osaka, Japan. The anti-H1N1 pdm-specific MAbs (MAbs N-SW2-6 and N-SW4-6) prepared in this study were immobilized onto a nitrocellulose membrane (0.6 and 0.4 μg/test, respectively) for the test line to capture H1N1 pdm protein. To prepare the control line, an anti-mouse IgG antibody (Nippon Biotest Laboratories, Tokyo, Japan) was immobilized onto a nitrocellulose membrane (1.8 μg/test) to capture mouse IgG. A conjugated pad containing the same N-SW2-6 or N-SW4-6 MAb used for the test line was labeled with colloidal gold, impregnated onto glass fibers, dried, and placed between the test line and the sample-dropping region. The nitrocellulose membrane and glass fiber pad were assembled with a glass fiber sample pad on a plastic sheet with a plastic cover. This assembled kit was stored in a bag with desiccant at room temperature until use.

Evaluation of test kits. The IC test kits were evaluated with samples of nasal wash from patients with influenza-like symptoms (n = 126) collected in 2009 and 2010 at Baba Pediatric Clinic, Osaka, Japan. As control samples of seasonal influenza virus, used to confirm the specificity of the primers for PCR, specimens of nasopharyngeal fluid from influenza patients (n = 42) collected before the H1N1 pdm pandemic by Baba Pediatric Clinic and Alfresa Pharma Corporation (Osaka, Japan) were used. As a control test, an existing IC kit for rapid detection of the NPs of seasonal influenza A and B viruses (Capilia Flu A+B; Alfresa Pharma Corporation) was used.

An RT-PCR-based analysis for the highly sensitive detection of viral RNA as a “gold standard” was also performed. Primer sets that were newly designed on the basis of the sequence of the NP region using swine-derived H1N1 isolates (Table 1), together with those recommended by WHO (http://www.who.int/entity/ce/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf) for the specific detection of the HA region of the H1N1 pdm genome, were used. The primer sets for individual seasonal A (H1N1 and H3N2) and B viruses (Table 1) were prepared as described by Yamada et al. (26) and used for multiplex PCR. Briefly, viral RNA was extracted from the clinical samples with a QIAamp viral RNA minikit (Qiagen, Tokyo, Japan). RT-PCR was performed with a one-step RT-PCR kit (Qiagen) for H1N1 pdm and a SuperScript III one-step RT-PCR system with Platinum Taq High Fidelity (Invitrogen) for seasonal influenza viruses. The RT-PCR conditions were as follows: 50°C for 30 min; 94°C for 3 min; then 40 cycles consisting of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s; and then 72°C for 7 min.

Ethics. The research protocols for human samples as well as the mouse experiments for the preparation of MAbs and ascitic fluids were approved by the Ethics Committee of the Research Institute for Microbial Diseases at Osaka University.

RESULTS
Preparation of MAbs and development of IC test kits for rapid detection of HA and NP of H1N1 pdm. From BALB/c mice immunized with an H1N1 pdm particle fraction, three hybridoma clones (N-SW2-6, N-SW4-5, and N-SW4-6) were obtained as producers of antibodies specifically reactive to several strains of H1N1 pdm but not seasonal H1N1 by screening using IFA. The isotypes of these MAbs were determined using kits: IgG1 (kappa chain) in N-SW2-6 and N-SW4-6 and IgA (kappa chain) in N-SW4-5. The three MAbs were confirmed to react only to H1N1 pdm and swine H1N1 and not to seasonal influenza A and B viruses, using several strains of H1N1 pdm and seasonal A (H1N1 and H3N2) and B viruses, as described in Materials and Methods. The staining pattern for N-SW2-6 indicated a reaction to HA, with membrane/cytoplasmic staining being similar to that for seasonal influenza A and B viruses.

| Subtype | Primer | Sequence | Positions | Reference strain | GenBank accession no. |
|---------|--------|----------|-----------|-----------------|----------------------|
| H1N1 pdm NP | N-SW2-6 | CGACGCCAGTACGTGG | 1206–1223 | A/California/04/09 | FJ966083 |
|         | N-SW4-6 | CGGTGTCGCCCTCCTTAT | 1315–1296 |                   |                      |
| Seasonal H1N1 | HAI-1N | TGAGGGAGCAATTAGGTCA | 320–339 | A/New Caledonia/20/09 | EF566076.1 |
|         | HAI-1C | TGCCCTAAATTTATTTGTT | 747–728 |                   |                      |
| Seasonal H3N2 | HA3-5al | GAGCTGTTAGCAGGCTCCT | 197–216 | A/Saita/2/68 | M55095.1 |
|         | HA3-3al | GTGACCTAAAGGAGGGA | 407–386 |                   |                      |
| Seasonal influenza B virus | N-Sbl | GAAGTAGGTGAGACGAGGAGG | 1387–1407 | B/Malaysia/2506/04 | CY038290.1 |
|         | N-Sbl | GTAAACCACACTTCCAACG | 1777–1756 |                   |                      |

TABLE 1. Primer sets used to determine the subtype of influenza virus in the patients’ specimens by RT-PCR

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\text{Subtype} & \text{Primer} & \text{Sequence} & \text{Positions} & \text{Reference strain} & \text{GenBank accession no.} \\
\hline
\text{H1N1 pdm NP} & \text{N-SW2-6} & \text{CGACGCCAGTACGTGG} & 1206–1223 & \text{A/California/04/09} & \text{FJ966083} \\
& \text{N-SW4-6} & \text{CGGTGTCGCCCTCCTTAT} & 1315–1296 & \text{A/New Caledonia/20/09} & \text{EF566076.1} \\
\text{Seasonal H1N1} & \text{HAI-1N} & \text{TGAGGGAGCAATTAGGTCA} & 320–339 & \text{A/New Caledonia/20/09} & \text{EF566076.1} \\
& \text{HAI-1C} & \text{TGCCCTAAATTTATTTGTT} & 747–728 & \text{A/Saita/2/68} & \text{M55095.1} \\
\text{Seasonal H3N2} & \text{HA3-5al} & \text{GAGCTGTTAGCAGGCTCCT} & 197–216 & \text{A/Saita/2/68} & \text{M55095.1} \\
& \text{HA3-3al} & \text{GTGACCTAAAGGAGGGA} & 407–386 & \text{A/Saita/2/68} & \text{M55095.1} \\
\text{Seasonal influenza B virus} & \text{N-Sbl} & \text{GAAGTAGGTGAGACGAGGAGG} & 1387–1407 & \text{B/Malaysia/2506/04} & \text{CY038290.1} \\
& \text{N-Sbl} & \text{GTAAACCACACTTCCAACG} & 1777–1756 & \text{B/Malaysia/2506/04} & \text{CY038290.1} \\
\end{array}
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the control anti-seasonal H1N1 HA (C179), whereas the staining patterns for N-SW4-5 and N-SW4-6 indicated reactions to NP, with nucleus staining being similar to that for the control anti-influenza virus NP (C43) (Fig. 1). However, Western blotting using viral proteins did not show any band reactive with the antibodies (data not shown), indicating that all three may recognize conformational epitopes. Therefore, we next examined 293T cells transfected with the plasmid expressing HA or NP of H1N1 pdm using IFA (Fig. 2). The results clearly showed the reactivities of N-SW2-6 to the HA protein and N-SW4-5 and N-SW4-6 to NP in transfected 293T cells. Therefore, we used N-SW2-6 and N-SW4-6 to develop the IC test kit: N-SW2-6 for the H1N1 pdm anti-HA kit, to rapidly detect HA of H1N1 pdm and not seasonal influenza A (H1N1 and H3N2) or B viruses, and N-SW4-6 for the H1N1 pdm anti-NP kit, to rapidly detect NP of H1N1 pdm but not seasonal influenza A (H1N1 and H3N2) or B viruses. N-SW4-5 was not used because it shows the IgA isotype and slightly lower activity than N-SW4-6.

Evaluation of IC test kits using influenza viruses produced in cultured cells. First, we produced H1N1 pdm (A/Suita/01/09) and seasonal influenza A (H1N1, A/New Caledonia/20/99; H3N2, A/Wyoming/2/03) and B (B/Malaysia/2506/04) viruses in MDCK cells. Compared with the primer sets recommended by the WHO, those newly designed in this study (Table 1) were about 10-fold more sensitive in the real-time PCR (data not shown). Therefore, we preferred our primers for the specific amplification of H1N1 pdm and used primer sets for individual seasonal influenza A and B viruses (Table 1) in a multiplex PCR, according to a previous paper (26).

Next, serial 10-fold dilutions of the H1N1 pdm (A/Suita/01/09) solution, corresponding to $1.0 \times 10^2$ to $1.0 \times 10^{-2}$ focus-forming units (FFU)/ml, were subjected to RT-PCR with the same newly designed primer set used for H1N1 pdm. The virus titer (FFU) assay was performed by the peroxidase-antiperoxidase staining method as described previously (23). The primers could amplify $1 \times 10^4$ FFU/ml or even $1 \times 10^5$ FFU/ml, a trace amount of the viral genome (Fig. 3A). Then, the same samples were tested with the newly developed kits (Fig. 3B). The H1N1 pdm anti-NP kit ($1.0 \times 10^3$) was significantly more sensitive than the H1N1 pdm anti-HA kit ($1.0 \times 10^1$). As a control for the detection of both seasonal A and B viruses and H1N1 pdm, we used the Capilia Flu A+B kit. This kit showed moderate sensitivity ($1.0 \times 10^2$) (Fig. 3B).

Evaluation of IC test kits using clinical specimens from patients with influenza-like syndromes. A total of 126 specimens from the Baba Pediatric Clinic sampled in 2009 and 2010 after the appearance of H1N1 pdm and 42 specimens collected by the Baba Pediatric Clinic and Alfresa Pharma Corporation before the epidemic of H1N1 pdm were used. The results of subtyping by RT-PCR using the primer sets listed in Table 1 were as follows: 83 of the 126 (nasal wash) samples from the Baba Pediatric Clinic were H1N1 pdm positive, while the other 43 samples tested negative for all influenza A and B viruses, including H1N1 pdm, and all 42 samples (nasopharyngeal fluid) collected by the Baba Pediatric Clinic and Alfresa Pharma Corporation were positive for seasonal influenza virus (5, 27, and 10 samples for influenza A H1N1 and H3N2 viruses and B viruses, respectively).

The new kits, together with the Capilia Flu A+B kit as a control, were then used to test these clinical samples for H1N1 pdm. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the individual test kits are summarized in Table 2. The H1N1 pdm anti-NP
kit showed 85.5% sensitivity, 100% specificity, a PPV of 100%, and an NPV of 87.6%. These values were significantly higher than those for the H1N1 pdm anti-HA kit and slightly higher than those for the Capilia Flu A/H11001 B kit. Six clinical samples that tested negative for all subtypes of influenza virus by RT-PCR showed a positive reaction with the H1N1 pdm anti-HA kit, probably due to a nonspecific reaction with the N-SW2-6 MAb. The sensitivities of the Capilia Flu A+B kit were 84.8% for the detection of both H1N1 pdm and seasonal influenza A virus and 79.5% for the detection of H1N1 pdm. One clinical sample that tested negative for all subtypes by RT-PCR showed bands for both influenza A and B viruses with the Capilia Flu A+B kit, again, probably due to a nonspecific reaction.

FIG. 2. Specific immunostaining of H1N1 pdm HA protein and NP in transfected 293T cells with the newly prepared murine MAb. 293T cells were mock transfected with empty plasmid or transfected with plasmid expressing the HA protein or NP of H1N1 pdm. The ascitic fluid samples for N-SW2-6, N-SW4-5, and N-SW4-6 were diluted and used for staining, as described in the legend to Fig. 1. The control murine MAbs were C43 and C179, also as described in the legend to Fig. 1.

FIG. 3. Typical banding profiles of the rapid diagnostic test kits with the newly prepared murine MAbs. (A) Serial 10-fold dilutions of the H1N1 pdm (A/Suita/01/09) solution, corresponding to $1.0 \times 10^0$ to $1.0 \times 10^{-2}$ FFU/ml, were subjected to RT-PCR with the newly designed primer set for H1N1 pdm; (B) representative profiles of the H1N1 pdm anti-NP and anti-HA test kits using the H1N1 pdm samples with the same dilutions used for panel A. As a control, the results for the Capilia Flu A+B kit obtained with the same samples are shown. C, control line; T, test line (detection of H1N1 pdm); A, influenza A virus detection line; B, influenza B virus detection line.
In this study, we developed two rapid diagnostic test kits using newly prepared murine MAbs that react with the HA protein and NP of H1N1 pdm but not those of seasonal influenza viruses. The H1N1 pdm anti-NP kit showed high sensitivity and specificity, reacting with H1N1 pdm but not seasonal influenza A viruses in clinical specimens. The estimates for this test kit were slightly higher than those for the Capilia Flu A+B kit developed for the detection of seasonal influenza A or B viruses.

Similar approaches to the rapid diagnosis of H1N1 pdm infections using an anti-NP MAb have been reported. Miyoshi-Akiyama et al. (17) developed a test kit that can be used to distinguish H1N1 pdm from seasonal influenza A (H1N1 and H3N2) and B viruses. They used an anti-NP MAb obtained from mice immunized with H5N1 and evaluated it using five clinical specimens. Choi et al. (3) evaluated the newly available SD Bioline Influenza Ag A/B/H1N1 (Pandemic) test kit with a large number of clinical specimens, although they did not give any information on the antibody in the kit.

Several rapid diagnostic test kits, so-called point-of-care or on-the-spot diagnostic test kits, are commercially available in many countries. Kits for the diagnosis of seasonal influenza A or B viral infections have been widely used, especially in Japan, to avoid delayed diagnostic testing, which could affect antiviral treatment. In addition, patients with underlying diseases such as diabetes and renal disease as well as obese individuals and pregnant women are often severely affected by infections of H1N1 pdm (13, 15). Therefore, a point-of-care strategy with the rapid diagnosis of H1N1 pdm infections seems to be crucial for efficient clinical treatment. In addition, infections with this virus can also cause severe illness in the pediatric population (9), although the sensitivities of existing test kits were shown to be significantly higher for children 5 years of age or younger (4, 10).

The H1N1 pdm anti-NP test kit could be useful not only for the confirmation of H1N1 pdm infection in hospitals but also for the surveillance of the antigenic shift and drift of seasonal and zoonotic influenza viruses in laboratories.

### DISCUSSION

In this study, we developed two rapid diagnostic test kits using newly prepared murine MAbs that react with the HA protein and NP of H1N1 pdm but not those of seasonal influenza viruses. The H1N1 pdm anti-NP kit showed high sensitivity and specificity, reacting with H1N1 pdm but not seasonal influenza A viruses in clinical specimens. The estimates for this test kit were slightly higher than those for the Capilia Flu A+B kit developed for the detection of seasonal influenza A or B viruses.

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