



## ORIGINAL ARTICLE

# Development of a Specific and Rapid Diagnostic Method for Detecting Influenza A (H1N1) pdm09 Virus Infection Using Immunochromatographic Assay

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**Abstract**

**Objectives:** The aim of this study was to develop an immunochromatographic assay (ICA) for the detection of influenza A (H1N1) pdm09 virus infection.

**Materials and methods:** Several monoclonal antibodies against influenza A (H1N1) pdm09 virus were generated and an ICA (pdm09-ICA) was developed for the rapid and specific detection of influenza A (H1N1) pdm09 virus infection. The specificity and sensitivity of the developed assay were compared with that of hemagglutination assay and real-time reverse-transcription polymerase chain reaction (rRT-PCR).

**Results:** The detection limit was estimated to be 1/2 (8) hemagglutinating unit; the sensitivity and specificity rates of pdm09-ICA were 75.86% (110/145) and 100% (43/43), respectively, compared with rRT-PCR. The cross-reactivity for 20 influenza viruses, including seasonal H1N1 viruses, was found to be negative except for the H1N1 virus (A/Swine/Korea/GC0503/2005).

**Conclusion:** These results indicate that the proposed method can be easily used for rapid and specific detection of the pdm09 infection. The assay developed in this study would be a useful tool for distinguishing the pdm09 infection from seasonal influenza A and B infections.

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## 1. Introduction

The influenza A (H1N1) pdm09 virus (pdm09) was first reported in the spring of 2009 in Mexico and in the United States. The virus spreads rapidly and its infection has become a world pandemic [1,2]. In August 2010, the World Health Organization (WHO) announced the postpandemic period. However, WHO recommended that the pdm09 virus is expected to continue as a seasonal virus. According to the report, younger age groups and pregnant women will continue to be affected disproportionately from the pdm09 infection [3].

The Centers for Disease Control and Prevention recommends using the real-time reverse-transcription polymerase chain reaction (rRT-PCR) analysis for the confirmation of pdm09 infection [4]. Although rRT-PCR is a standard method and is highly sensitive and specific for the detection of influenza viruses [5–7], it requires specialized equipment, trained technicians, and takes more than 5–24 hours to generate results from clinical specimens [1,7,8]; in addition, rRT-PCR is available only in limited diagnostic laboratories [9,10]. Even though commercial influenza antigen tests can distinguish between influenza A and B viruses, they are unable to differentiate between the pdm09 and seasonal influenza A viruses [4].

Detection of pdm09-specific hemagglutinin (HA) gene can be used for the confirmation of pdm09 virus and for differentiating it from other seasonal influenza viruses [7]. Thus, antibodies against this HA can be useful agents to develop methods that can distinguish between the pdm09 and the seasonal influenza A and B viruses.

In this study, we have developed monoclonal antibodies against the HA of pdm09 virus and a rapid antigen test specifically to detect pdm09 based on the principle of immunochromatographic assay (pdm09-ICA).

## 2. Materials and methods

### 2.1. Patient and clinical specimens

During the period from October 2009 to November 2009, two nasopharyngeal swab specimens were collected simultaneously using flocked swabs (Copan Diagnostics, Murrieta, CA, USA) from each patient ( $n = 188$ ) at the Cheonan Hospital (Soonchunhyang University, Korea). One specimen was placed in a tube containing 0.3 mL of assay buffer for pdm09-ICA, and the other was placed in a tube containing 1 mL of viral transport medium for rRT-PCR.

### 2.2. Preparation of pdm09

The pdm09 virus strain (A/Korea/01/2009) was obtained from the Korea Centers for Disease Control and Prevention. A total of  $2^5$  hemagglutinating units (HAU) of the binary ethylenimine-inactivated pdm09 were centrifuged at  $4000 \times g$  for 30 minutes at  $4^\circ\text{C}$ ;

their supernatants were harvested and centrifuged at  $70,000 \times g$  for 3 hours at  $4^\circ\text{C}$  (L8-70M; Beckman, USA). The pellets were resuspended with 1% sucrose solution, followed by ultracentrifugation on a discontinuous sucrose density gradient, as described previously [11]. Moreover, fractions that displayed hemagglutination with 1% chicken red blood cell were collected to be used as antigen in the monoclonal antibody preparation process.

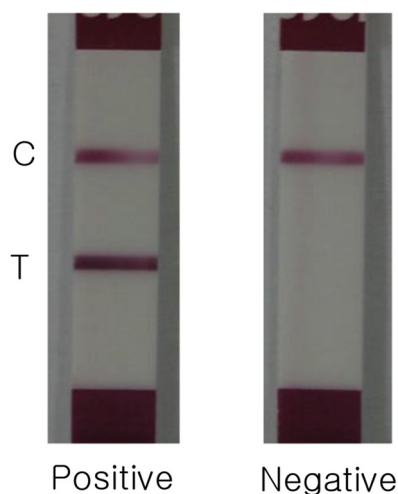
### 2.3. Production of mouse monoclonal antibodies against pdm09

Hybridoma producing mouse monoclonal antibody (MAb) against the pdm09 virus was prepared as follows: female BALB/c mice, 7 weeks of age, were intraperitoneally immunized with 200  $\mu\text{g}$  of concentrated pdm09. They were emulsified in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, USA) for the first immunization and in incomplete Freund's adjuvant for the second and third immunizations at 1-week intervals. Spleen cells of the immunized mice were fused with SP2/0 myeloma cells using polyethylene glycol 1500 (Roche Diagnostics GmbH, Mannheim, Germany). The hybridomas, which secrete specific antibodies against the HA of the pdm09 virus, were screened using the hemagglutination inhibition (HI) assay. Cell culture supernatants of the hybridomas were diluted in phosphate-buffered saline (pH 7.2) at a ratio of 1:1, and  $2^2$  HAU of the pdm09 cells were mixed and incubated for 30 minutes at room temperature (RT). Approximately 1% chicken red blood cell was added and the mixture was incubated for 30 minutes at RT. Finally, hybridomas that completely inhibited the hemagglutination were selected.

In the screening test, hybridomas producing positive monoclonal antibodies were subcloned by limiting the dilution, and were grown as ascites-producing tumors in the peritoneal cavities of Freund's adjuvant-primed BALB/c mice. The monoclonal antibodies were purified from the ascites fluid by chromatography on protein G-Sepharose 4B fast flow medium (GE Healthcare Biosciences, Uppsala, Sweden).

### 2.4. Development of pdm09-ICA

The pdm09-ICA, which was developed for the detection of pdm09 using monoclonal antibodies, is specific to the pdm09 virus. The test strip includes a sample pad, a gold conjugate pad, a nitrocellulose membrane (Millipore Co., USA), and an absorbent pad. The captured antibodies were immobilized to two different lines on a nitrocellulose membrane, namely, the control line (1.0 mg/mL goat antimouse immunoglobulin G) and the test (2.0 mg/mL pdm09 MAb). Detector antibodies were conjugated with colloidal gold particles (Standard Diagnostic, Korea) and the gold-conjugated pdm09 MAb were dried on a glass fiber. Samples were diluted with assay buffer (0.4 M Tricine, 0.1 M NaCl, and 1% TritonX-100) in test tubes, and the test strips were added into the test tube. The pdm09 was combined with the antibody–colloidal gold conjugates,



**Figure 1.** Specific detection of the influenza A (H1N1) pdm09 virus by pdm09-ICA. The result is negative if only one purple band appears, whereas it is positive if two purple bands appear. ICA = immunochromatographic assay.

and complexes were migrated to the immobilized antibodies. The positive samples showed a purple band, whereas the negative samples did not.

### 2.5. rRT-PCR

The viral RNA was extracted using a QIAamp viral RNA minikit (Qiagen, Germany), according to the manufacturer's instructions. Each reaction mixture for rRT-PCR was prepared according to the manufacturer's instructions, and includes 5  $\mu$ L of extracted RNA and PCR reagents (Influenza A/H1N1 Detection set; Roche Applied Science, Germany). Amplification and detection were conducted on LightCycler 480 (Roche Applied Science).

## 3. Results

### 3.1. Production of anti-pdm09 monoclonal antibodies

Mice were immunized with pdm09 emulsified with incomplete Freund's adjuvant. Splens of mice showing

seroreactivity to the pdm09 virus were isolated and fused to myeloma cells. Specific antibodies against the pdm09 virus were selected by the HI assay, which was conducted with the pdm09 and seasonal H1N1 viruses. Ten anti-pdm09 antibodies reacted with pdm09, but did not react with the seasonal H1N1. The selected antibodies were produced as ascites of BALB/c mice and purified from the ascites fluid by protein G-affinity resin. Two clones, i38 and d383, showing high reactivity toward pdm09 were selected for pdm09-ICA.

### 3.2. Development of pdm09-ICA

These 10 antibodies were paired by negative and positive reference specimens (data not shown). The pair of i38 antibody (as capture) and d383 antibody (as detector) had different signals in the negative and positive specimens. The i38 antibody was coated on a nitrocellulose membrane as the test line, and the d383 antibody was conjugated with colloidal gold particles.

**Table 2.** Specificity of pdm09-ICA for 20 different influenza viruses

Type	Strain	HAU	Pdm09-ICA results
H1N1	A/PR/8/34	2 <sup>7</sup>	–
	A/Brisbane/59/2007	2 <sup>7</sup>	–
	A/Solomon Islands/3/2006	2 <sup>8</sup>	–
	A/New Caledonia/20/99	2 <sup>7</sup>	–
	A/Swine/Korea/GC0503/2005	2 <sup>6</sup>	±
H2N2	A/Singapore/1/57	2 <sup>7</sup>	–
H3N2	A/Hong Kong/8/68	2 <sup>6</sup>	–
H3N5	A/Swine/Korea/GC0407/2005	2 <sup>7</sup>	–
	A/Duck/Ukraine/1/63	2 <sup>5</sup>	–
H4N6	A/Duck/Czechoslovakia/56	2 <sup>7</sup>	–
	A/Duck/Hong Kong/820/80	2 <sup>6</sup>	–
H6N5	A/Shearwater/Australia/1/72	2 <sup>7</sup>	–
	A/Duck/Hong Kong/301/78	2 <sup>4</sup>	–
H8N4	A/Turkey/Ontario/6118/68	2 <sup>6</sup>	–
	A/Turkey/Wisconsin/1/66	2 <sup>8</sup>	–
H10N7	A/Chicken/Germany/N/49	2 <sup>7</sup>	–
	A/Duck/Hong Kong/562/1979	2 <sup>8</sup>	–
H11N6	A/Duck/England/56	2 <sup>8</sup>	–
	A/Duck/Hong Kong/T37/97	2 <sup>7</sup>	–
B	B/Lee/40	2 <sup>5</sup>	–

HAU = hemagglutinating units; ICA = immunochromatographic assay.

**Table 1.** Analytical sensitivity of pdm09-ICA

Dilution (HAU)	Hemagglutination assay	pdm09-ICA
1:2 (2 <sup>1</sup> )	+	+++
1:4 (2 <sup>2</sup> )	+	++
1:8 (2 <sup>3</sup> )	+	++
1:16 (2 <sup>4</sup> )	+	+
1:32 (2 <sup>5</sup> )	–	+
1:64 (2 <sup>6</sup> )	–	+w
1:128 (2 <sup>7</sup> )	–	±
1:256 (2 <sup>8</sup> )	–	±
1:512 (2 <sup>9</sup> )	–	–

+ = positive; ++ = medium positive; +++ = strong positive; +w = weak positive; ± = trace; – = negative; HAU = hemagglutinating units; ICA = immunochromatographic assay.

**Table 3.** Performance of pdm09-ICA compared with real-time RT-PCR

Real-time PCR results <sup>a</sup> ( <i>n</i> <sup>b</sup> )	No. of specimens with pdm09-ICA		% Clinical sensitivity (95% CI)	% Clinical specificity (95% CI)	PPV (%)	NPV (%)
	Positive	Negative				
Confirmed positive (145)	110	35	75.86 (68.29–82.1)	—	100	—
Confirmed negative (43)	0	43	—	100 (91.8–100)	—	55.1

<sup>a</sup>As determined by Roche real-time PCR; <sup>b</sup>*n*, Number of specimens with result. CI = confidence interval; ICA = immunochromatographic assay; NPV = negative predictive value; PPV = positive predictive value; RT-PCR = reverse-transcription polymerase chain reaction.

Samples were diluted with assay buffer in test tubes. Further, the test strips were added into the test tube and the results were read for 10–15 minutes (Figure 1).

### 3.3. Performance evaluation of pdm09-ICA

The sensitivity of the pdm09-ICA was compared with the hemagglutination assay. In brief, the hemagglutination assay was carried as follows: the pdm09 (A/Korea/01/2009) was diluted twofold in phosphate-buffered saline (pH 7.2), and 1% chicken red blood cell was added into the diluted pdm09, which was then mixed and incubated for 40 minutes at RT. To investigate the sensitivity of the pdm09-ICA, twofold dilutions of the pdm09 were made in assay buffer and applied to the test strip. The detection limit of the pdm09-ICA was estimated at 1/2 (8) HAU, and the detection limit of the hemagglutination assay was 1/2 (4) HAU (Table 1). The result of the pdm09-ICA showed relatively high sensitivity than the hemagglutination assay.

A total of 20 influenza viruses, including the seasonal H1N1 virus, were confirmed with cross-reactivity of the pdm09-ICA for specificity of the pdm09. No positive signal was shown except for the H1N1 (A/Swine/Korea/GC0503/2005), which had a very weak positive signal (Table 2).

We evaluated the sensitivity and specificity of the pdm09-ICA to the pdm09 using clinical specimens consisting of 145 positive and 43 negative specimens, confirmed by rRT-PCR. The sensitivity and specificity of the pdm09-ICA were 75.86% (110/145) and 100% (43/43), respectively (Table 3). The positive rates of the pdm09-ICA, according to the crossing point (CP) values

of rRT-PCR, are presented in Table 4. As the CP values of rRT-PCR increases, the sensitivity of the pdm09-ICA decreases.

## 4. Discussion

Influenza A viruses are made up of eight segments of encoding proteins, namely, polymerase basic proteins 1 and 2 (PB1 and PB2), polymerase acidic protein, HA, nucleoprotein (NP), neuraminidase, matrix proteins 1 and 2 (M1 and M2), and nonstructural proteins 1 and 2 [12,13]. Because HA genes are the most specific genes in the genome of the influenza A virus subtype, the methods based on viral RNA analysis target HA genes to diagnose the pdm09 [7]. However, commercially available influenza antigen tests do not distinguish the pdm09 from seasonal influenza viruses because they use antibodies against the NP of influenza A and B viruses [7,14]. For rapid and specific detection of the pdm09 among seasonal viruses, we have developed antibodies against the HA of the pdm09 and a variety of hybridoma cells, which produced HA-specific monoclonal antibodies screened by the HI assay. We selected one hybridoma producing secreted MAb that showed excellent reactivity in the HI assay.

For the detection of seasonal influenza viruses, several commercial influenza antigen tests have been evaluated for pdm09. Compared with the rRT-PCR analysis, the sensitivity and specificity were 48.7% and 96.5% [6], 47% and 96% [15], 53.4% and 100% [14], respectively. The tests showed relatively low sensitivity for the detection of pdm09. Thus, the development of a

**Table 4.** The positive rates of pdm09-ICA according to the crossing point values of real-time RT-PCR

Real-time PCR CP interval	No. of specimens with pdm09-ICA			Sensitivity(%)
	No. of positive	Positive	Negative	
<20	22	21	1	95.45
20–25	69	61	8	88.4
25–30	34	23	11	67.65
>30	20	5	15	25
Total	145	110	35	75.86

CP = crossing point; ICA = immunochromatographic assay; RT-PCR = reverse-transcription polymerase chain reaction.

more accurate ICA for the screening and diagnosis of pdm09 infection is urgently needed [16]. In this study, we have developed pdm09-ICA that is not only high in sensitivity but also detectable to distinguish between the pdm09 and seasonal influenza A and B viruses using monoclonal antibodies against HA of the pdm09. The pdm09-ICA developed in this study showed 75.86% sensitivity, which is higher than the sensitivity of commercial influenza antigen tests and 100% specificity compared with rRT-PCR results. When the CP value was lower than 20, the sensitivity of the pdm09-ICA was 95.45%; however, when the CP value was higher than 30, the sensitivity of the pdm09-ICA decreased to 25%. Therefore, the sensitivity of pdm09-ICA is closely related with the viral number; thus, it will be useful in the diagnosis of patients who have severe symptoms for influenza [10]. In addition, pdm09-ICA had the specificity for pdm09; 19 of the 20 different influenza viruses chosen did not show reactivity, except for H1N1 (A/Swine/Korea/GC0503/2005), which showed a very weak positive signal because the pdm09 was a result of a reassortment with swine influenza A viruses [12,13].

The pdm 09-ICA can be easily used for rapid and specific detection of the pdm09 virus from other influenza A and B viruses, and positive signals can be visible within 10–15 minutes without using any equipment in the laboratory. This study suggests that the rapid diagnosis of the pdm09 by the pdm09-ICA might alternate with PCR, which requires equipment, cost, and time.

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