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ORIGINAL ARTICLE

Simultaneous discrimination and detection of influenza A(H1N1)pdm09 and seasonal influenza A viruses using a rapid immunogold biosensor

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Abstract A rapid immunogold biosensor for the simultaneous discrimination of influenza A(H1N1)pdm09 and seasonal influenza A viruses was developed successfully. Monoclonal antibodies (mAbs) that were specific for the hemagglutinin protein of the A(H1N1)pdm09 virus were produced, and the best mAb pairs were selected. Using an mAb that was specific for the influenza A nucleoprotein, a rapid immunogold biosensor for the discrimination and detection of A(H1N1)pdm09/seasonal influenza viruses was developed. When tested with 72 virus isolates, the system achieved 100 % detection of the A(H1N1)pdm09 virus without cross-reactivity against seasonal influenza A (H1, H3 subtypes) and B viruses, parainfluenza viruses, respiratory syncytial viruses, and adenoviruses. The detection limits for A(H1N1)pdm09 and seasonal strains were 5×10^2-7.5×10^3 and 1×10^3-7.5×10^5 $TCID_{50}$/mL, respectively. When tested with 49 clinical specimens, the specificity was high (100 %). The sensitivity for the detection of A(H1N1)pdm09 and seasonal strains was 90 % and 100 %, respectively, which correlated with the results of real-time reverse transcription polymerase chain reaction as a reference method. The ability of the system to detect and discriminate the A(H1N1)pdm09 strain from the seasonal strains suggests that this method may be beneficial for investigation of outbreaks and diagnostic applications. Furthermore, this method might be a useful platform for developing a rapid diagnostic system for the simultaneous discrimination of other influenza virus subtypes during future outbreaks.

Introduction

Influenza A virus causes recurrent epidemics and occasionally causes severe pandemics. Although recurrent epidemics occur on a regular basis, the severity of epidemics varies greatly. The outbreak of the influenza A(H1N1)pdm09 virus in April 2009, which resulted in millions of people in more than 214 countries becoming infected by the virus, is a prime example of a pandemic-level outbreak [1]. However, the impact of this outbreak was not limited to the A(H1N1)pdm09 outbreak [2]. Human infections with a new variant of avian influenza A (H7N9) virus also continue to be reported [3, 4]. When such an outbreak arises, the requirement for an efficient diagnostic test that can detect and distinguish the pandemic strain from the circulating seasonal strains becomes urgent for a prompt treatment regimen and for control of infection.

An emerging A(H1N1)pdm09 outbreak represents one influenza A outbreak that has had a significant impact on

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the human population and has spread rapidly across the world [5]. Although the World Health Organization (WHO) has announced that the A(H1N1)pdm09 virus is in its post-pandemic period, localized outbreaks with different magnitudes are likely to continue to be reported in many countries, particularly in high-risk patients and in younger age groups [2]. In addition, there are concerns that reassortment of this virus with other circulating strains could generate a new variant with higher pathogenicity [6, 7]. Therefore, identification of and discrimination between pandemic and seasonal strains may continue to be important for diagnosis, surveillance, and infection control [8].

Phylogenetic analysis of the A(H1N1)pdm09 virus revealed that this virus was derived from a mixed ancestry of a Eurasian avian-like swine virus and triple-reassortment viruses [9]. Because the A(H1N1)pdm09 virus resulted from a change in several genes, including the gene encoding the hemagglutinin (HA) protein, this method allows the detection and discrimination between the A(H1N1)pdm09 virus and seasonal viruses to be possible [10]. Several detection systems have been developed for diagnostic and screening purposes and are available commercially. Examples of these systems include molecular-based assays and rapid antigen detection tests, including a new-generation point-of-care (POC) test with automated reading [11–13]. Although rapid antigen detection tests allow direct visualization and rapid screening, the performance of these tests in the detection of and distinction between the A(H1N1)pdm09 virus and seasonal viruses varies greatly [14, 15]. Novel assays that employ nucleic acid amplification techniques offer broader virus detection with increased sensitivity [13, 16]. However, routine diagnosis using these techniques may be difficult, because these techniques require technical expertise and expensive instruments.

In this study, the development of a rapid immunogold biosensor for the detection and discrimination of A(H1N1)pdm09 and seasonal influenza viruses is reported. Monoclonal antibodies were produced against the HA protein of the A(H1N1)pdm09 virus (HA mAbs) to detect antigenic differences between the A(H1N1)pdm09 virus and seasonal influenza A viruses, and the best mAb pair was selected for diagnostic purposes. A clinical evaluation of the rapid immunogold biosensor for detecting A(H1N1)pdm09 virus and differentiating it from seasonal influenza A viruses in comparison with the molecular approach was also performed and is discussed. This study provides a basis for the further development of a rapid diagnostic system for the simultaneous discrimination of pandemic and seasonal influenza A strains, which may be useful for diagnostic applications and investigation of outbreaks.

Materials and methods

Preparation of a recombinant HA protein

Recombinant HA protein (rHA) of A(H1N1)pdm09 virus (A/Thailand/104/2009 (H1N1)) was expressed in a baculovirus system [17]. Briefly, the purified HA cDNA was cloned into the pFastBacHtb (Invitrogen, USA) donor plasmid. The recombinant plasmid was isolated and used to transform DH10BAC^{TM} *E. coli* competent cells (Invitrogen). The recombinant bacmid was isolated and used to transfect *Spodoptera frugiperda* insect cells for the production of recombinant baculovirus. The expressed protein was analyzed using SDS-PAGE, western blot, and hemadsorption [17, 18].

Production of monoclonal antibodies

A mAb that was specific for the influenza A virus NP (NP mAb) was supplied by Innova Biotechnology, Thailand. HA mAbs were produced using the purified inactivated A(H1N1)pdm09 virus (isolate: A/Thailand/104/2009) as the antigen for immunization [17, 19]. Briefly, 6- to 8-week-old BALB/c mice (National Laboratory Animal Center, Mahidol University, Thailand) were immunized with 5 μg of the antigen in complete Freund's adjuvant. After three subsequent immunizations, a final boost with 2 μg of the antigen was performed 3 days before harvesting the spleen. Hybridomas were produced by fusion of the spleen cells to P3-X63-Ag8.653 myeloma cells and were screened using ELISA and western blot [17]. Purification and isotyping of HA mAb clones were performed using a HiTrap Protein G column (GE Healthcare, UK) with fast performance liquid chromatography (AKTA purifier, GE Healthcare) and an mAb isotyping kit (GE Healthcare).

Hemagglutination inhibition assay

HA mAbs were characterized using a hemagglutination inhibition (HAI) assay [20]. Briefly, 25 μL of a serial twofold dilution of an HA mAb in phosphate-buffered saline (PBS) was incubated with an equal volume of the A(H1N1)pdm09 virus (4 HA units) at room temperature (RT) for 1 h, followed by addition of 50 μL of erythrocytes. The highest reciprocal dilution of an HA mAb that showed no hemagglutination was determined to be the HAI titer.

Competitive ELISA

A competitive ELISA was performed to analyze the epitope that was recognized by the mAbs. The wells were coated with 50 μL of the purified A(H1N1)pdm09 virus (8

HA units) and incubated overnight at 4 °C. After washing and blocking with 3 % bovine serum albumin (BSA) in PBS that contained 0.05 % Tween-20 (PBST), 50 µL of purified mAb (1 µg/mL) was added to the wells and incubated for 30 min at 37 °C. For the competitive binding to the antigen of each mAb, 50 µL of a biotinylated mAb (1 µg/mL) (EZ-link NHS-PEG4 Biotinylation Kit, Thermo Scientific, USA) was added to the wells and incubated for 30 min at 37 °C. Bound mAbs were detected using a goat anti-mouse IgG horseradish peroxidase (HRP) conjugate antibody (1:10,000). Color development was performed using SureBlue™ TMB Microwell Peroxidase Substrate (KPL, USA). The absorbance of the HRP products was measured at 450 nm (A_{450}) using a PowerWave™ XS/XS2 Microplate Spectrophotometer (BioTek Instrument, USA). The percent inhibition (PI) of each mAb was calculated as described below. Two mAbs that corresponded to the unlabeled and biotinylated mAbs were considered to share an epitope when the PI value was 50 % or higher.

$$PI = [1 - (A_{450} \text{ of the tested well}/A_{450} \text{ of the negative well})] \times 100.$$

Sandwich ELISA

A sandwich ELISA was performed using each of the HA mAbs as either the capture or detector antibodies. The detector antibody was biotinylated to prevent the detection of the capture antibody. Briefly, the wells were coated with 50 µL of purified HA mAb (1 µg/mL in 50 mM carbonate buffer, pH 9.6) and incubated overnight at 4 °C. After washing and blocking with 2 % tryptone in PBST, 50 µL of the purified A(H1N1)pdm09 virus (8 HA units) was added into each well and incubated for 30 min. A volume of 50 µL of the detector antibody was added to the wells and incubated for 30 min at 37 °C. After washing, the immunocomplexes were detected using a streptavidin-HRP conjugate (1:2,000, KPL). The color development and the measurement of the A_{450} signal were performed as described previously.

Preparation of gold nanoparticles and a mAb-gold nanoparticle conjugate

Gold nanoparticles (GNP, 40 nm) were prepared by the citrate reduction method [21]. Briefly, 100 mL of 0.01 % (w/v) tetrachloroauric acid was heated to boiling and stirred continuously. After 1 mL of 1 % (w/v) sodium citrate was added rapidly, the solution was boiled with stirring until the color of the solution changed from bright yellow to deep red. The mixture was cooled to RT and stored at 4 °C until used. The mAb-GNP conjugates were prepared as described by Roth [22]. Briefly, 30 µL of 0.1 mg/mL mAb in 2 mM borate buffer, pH 9 (buffer A), was mixed with 900 µL of GNP solution. After mixing gently, 100 µL of buffer A was added to the mixture and incubated for 15 min at RT. The residual surfaces of the GNP were coated with 100 µL of 10 % (w/v) BSA. After incubation for 15 min, the conjugates were collected by centrifugation at 6,000 × *g* for 30 min at 4 °C and resuspended in 20 µL of Tris/HCl saline buffer (pH 8.2) that contained 1 % (w/v) BSA and 0.1 % (w/v) sodium azide.

Assembly of the rapid immunogold biosensor system

A rapid immunogold biosensor test strip consisted of a sample pad, a conjugate pad, a Hi-Flow Plus 180 membrane (Merck Millipore, USA), and an absorbent pad (Fig. 1). Free HA mAb (clone H109.1.1) and free NP mAb (1 mg/mL) were dispensed at 1 µL/cm onto the NC membrane using a BioDot dispenser (USA) to generate the A(H1N1)pdm09 test line 1 (A(H1N1)pdm09 T1) and the Flu A test line 2 (Flu A T2), respectively. A goat anti-mouse IgG secondary antibody (1 mg/mL) was immobilized onto the NC membrane, at 0.5 cm downstream of the Flu A T2 line, to generate the control (C) line. The conjugate pad was soaked with the desired volume of the conjugates and dried at RT. The test strip was then packaged in a plastic bag with desiccant and stored at 4 °C until used.

Evaluation of the performance of the rapid immunogold biosensor system

Madin-Darby canine kidney (MDCK) cell cultures that were infected with influenza A virus were obtained using

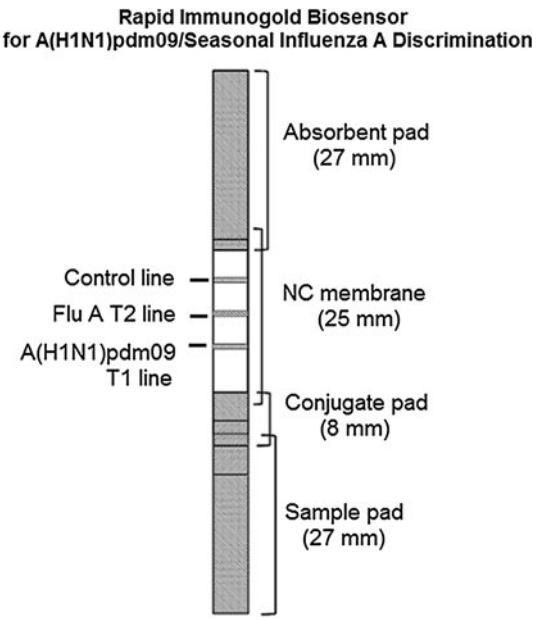


Fig. 1 Schematic diagram of the rapid immunogold biosensor system components

the following clinical human isolates: two isolates of the seasonal influenza A H1N1 virus (H1N1 14/2004 and H1N1 20/2007), two isolates of the seasonal influenza A H3N2 virus (H3N2 04/2004 and H3N2 01/2007), and two isolates of the A(H1N1)pdm09 virus (H1N1 104/09/01 and H1N1 104/09/02) [17]. In addition to MDCK cell cultures, a purified inactivated avian influenza A virus (A/Nakorn-Pathom/CU-K2/2004 (H5N1)), which was provided by Prof. Yong Poovoravan, Chulalongkorn University, Thailand, and a purified inactivated influenza A H7N1 virus (A/mallard/Alberta/34/2001), which was provided by Prof. Robert Webster, St. Jude's Children's Hospital, USA, were also included [17]. The virus isolates were diluted sequentially in viral transport medium, and 100 μL of the viral suspension was mixed with 375 μL of extraction buffer (PBS buffer containing BSA, sodium azide, detergents, and stabilizers). The test strip was dipped into the sample suspension and allowed to develop for 15 min before the signal was read. All viral manipulations were performed under biosafety level 2 plus laboratory conditions.

The specificity of the system was evaluated using 72 clinical human isolates that were collected from 2002-2009 (Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand). These included 15 isolates of seasonal influenza A H1N1 virus, 13 isolates of seasonal influenza A H3N2 virus, 5 isolates of A(H1N1)pdm09 virus, 9 isolates of influenza B virus, 12 isolates of parainfluenza virus, 8 isolates of respiratory syncytial virus (RSV), and 10 isolates of adenovirus. Influenza virus isolates were characterized using a WHO influenza reagent kit for the identification of influenza isolates and indirect immunofluorescent methods, whereas other virus isolates were characterized using commercial virus-specific antibodies (Merck Millipore). The sample application was performed as described previously.

Clinical evaluation of the rapid immunogold biosensor system

Fifty nasopharyngeal wash samples (Table S1, electronic supplementary material; ESM), obtained with written permission from the Virology Laboratory, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand, were included in a preliminary clinical evaluation of the developed system. These samples were leftover samples from specimens that had been collected for clinical purposes. The samples were screened for A(H1N1)pdm09 and seasonal influenza A viruses using a real-time reverse transcription polymerase chain reaction assay (rRT-PCR) with a panel of specific primer pairs and probes for the *in vitro* detection and characterization of swine influenza A virus in respiratory specimens [23].

The clinical evaluation of the rapid immunogold biosensor system was performed by mixing 100 μL of the samples with 400 μL of the extraction buffer. The test strip was dipped into the treated sample suspension and processed as described previously. The presence of a signal at the A(H1N1)pdm09 T1, Flu A T2, and C lines indicated a valid positive result for the A(H1N1)pdm09 virus. The presence of the signal at the Flu A T2 and C lines only indicated a valid result for the seasonal influenza A virus. The diagnostic sensitivity and specificity of the system were determined [24].

Results

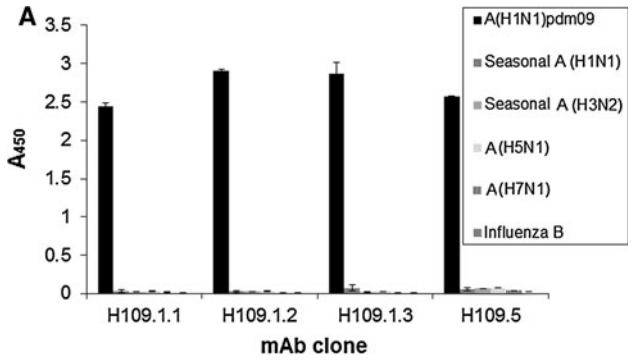
Monoclonal antibodies recognizing the HA of A(H1N1)pdm09 virus

In total, 34 stable hybridoma clones with reactivity against A(H1N1)pdm09 virus were obtained from an initial screening of the hybridoma clones. The 12 clones with the highest activity were selected and subjected to an extensive selection process. As shown in Fig. 2A, there were only four mAb clones (H109.1.1, H109.1.2, H109.1.3, and H109.5) that recognized the purified A(H1N1)pdm09 virus antigens specifically with no cross-reactivity to purified seasonal influenza A (H1, H3 subtypes), other influenza A (H5, H7 subtypes) and influenza B virus antigens. The HAI assay showed that all four clones exhibited HAI activity against the A(H1N1)pdm09 virus. Western blot analysis demonstrated the high reactivity of these clones against the rHA proteins of the A(H1N1)pdm09 virus and of the purified A(H1N1)pdm09 virus (data not shown). Characteristics of these mAb clones are shown in Fig. 2B.

The epitopes that were recognized by the aforementioned mAbs were analyzed using a competitive ELISA (Fig. 2C). The PI value of the selected clones showed that the H109.1.1, H109.1.2, and H109.1.3 clones may recognize either identical or overlapping conformational epitopes of the HA protein of the A(H1N1)pdm09 virus, whereas the H109.5 clone recognized a different epitope from the other three clones.

Selection of mAb pairs to the HA proteins

The mAbs were selected to achieve highly reactive mAb pairs. These mAb pairs were selected by alternating each mAb as capture or detector antibodies and monitoring the reactivity of each mAb pair using a sandwich ELISA. As the detector, the H109.1.1 clone was superior to all other clones, as demonstrated by the highest A_{450} signal, whereas all four clones were considered equal as capture antibodies (Fig. 3).



B

mAb clone	Isotype	HAI	Western blot	
		A(H1N1) pdm09	rHA	A(H1N1) pdm09
H109.1.1	G1, Kappa	+	+	+
H109.1.2	G1, Kappa	+	+	+
H109.1.3	G1, Kappa	+	+	+
H109.5	G1, Kappa	+	+	+

C

Unlabeled purified mAb	Percent Inhibition (%) Competing biotinylated mAb		
	H109.1.1	H109.1.2	H109.5
H109.1.1	96	95	33
H109.1.2	92	94	29
H109.1.3	89	84	0
H109.5	24	0	82

Fig. 2 (A) Reactivity of the mAb clones H109.1.1, H109.1.2, H109.1.3, and H109.5 against the A(H1N1)pdm09 virus compared with other influenza A virus subtypes and influenza B virus. (B) Characteristics of the four selected mAb clones that were generated against the A(H1N1)pdm09 virus and the rHA protein. (C) Percent inhibition of each mAb, which corresponded to the extent to which each mAb clone may share an overlapping epitope

Development of the rapid immunogold biosensor system

To assemble the test system, the two detector mAbs (HA mAb and NP mAb) were conjugated to GNP to produce HA mAb-GNP and NP mAb-GNP conjugates. For HA mAb, it was shown that only the H109.1.1 and H109.5 clones were able to retain their activity after GNP conjugation. Thus, these clones were selected as the detector antibodies. In the case of the capture antibody, the H109.1.1 and H109.1.3 clones were selected because these clones were able to produce a strong positive T line signal. The reactivity of these HA mAb pairs in a rapid immunogold biosensor system was evaluated. Three mAb pairs (H109.1.1/H109.1.1-GNP, H109.1.3/H109.1.1-GNP, and H109.1.3/H109.5-GNP) were shown to produce a strong (+++) T line signal intensity (Table S2A, ESM). These three selected mAb pairs were evaluated for their performance using fourfold serial dilutions of the virus with known HA titers. It was shown that prototypes 1 and 2 provided good sensitivity with a lower limit of detection of

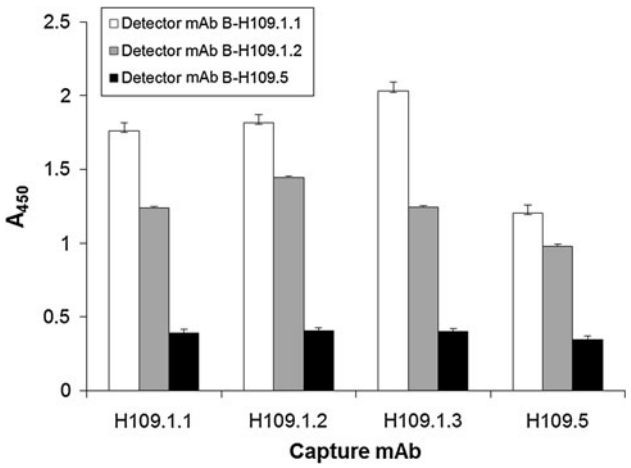


Fig. 3 The reactivity of HA mAb pairs in a sandwich ELISA for the detection of the A(H1N1)pdm09 virus

1 HA unit compared with prototype 3 (Table S2B, ESM). Prototype 1 was selected as a final candidate for developing the system.

The selection of the NP mAb pair to target the NP protein of influenza A virus was based on our previous study, which demonstrated that this NP mAb pair demonstrated a high reactivity in a sandwich ELISA against the recombinant NP protein and the NP protein in MDCK cells that were infected with either A(H1N1)pdm09 or seasonal influenza A viruses [17]. The high reactivity of NP mAb against A(H1N1)pdm09 and seasonal influenza viruses in a rapid immunogold biosensor system was demonstrated by a strong T line signal intensity (data not shown). Based on these results, a rapid immunogold biosensor system to discriminate between A(H1N1)pdm09 and seasonal influenza A viruses was produced using the selected HA mAb and NP mAb pairs.

Performance of the rapid immunogold biosensor

The performance of the test system to discriminate between A(H1N1)pdm09 and seasonal influenza A viruses was evaluated and is shown in Table 1A. A strong A(H1N1)pdm09 T1 line signal intensity was observed, with a lower limit of detection between 5.0×10^2 and 7.5×10^3 $TCID_{50}$/mL when tested with the A(H1N1)pdm09 virus. The lower detection limit of the Flu A T2 line of the system when using seasonal influenza A viruses was in a range between 1.0×10^3 and 7.5×10^5 $TCID_{50}$/mL. All cases that were positive for influenza A virus, either the A(H1N1)pdm09 virus or other influenza A virus subtypes, were confirmed by the presence of the Flu A T2 line signal. Invariably, the system showed the presence of the Flu A T2 line signal and C line signal, which indicated a valid positive result.

Table 1 Evaluation of the performance of the rapid immunogold biosensor system

Virus (isolate number/year of isolation)	Stock virus ($TCID_{50}$/mL)	LOD ($TCID_{50}$/mL)	
		A(H1N1)pdm09 T1 line	Flu A T2 line
(A)			
A(H1N1)pdm09 104/2009/1	3.0×10^5	7.5×10^3	7.5×10^3
A(H1N1)pdm09 104/2009/2	2.0×10^4	5.0×10^2	5.0×10^2
Seasonal influenza A H1N1 14/2004	2.0×10^7	Negative	5.0×10^5
Seasonal influenza A H1N1 20/2007	3.0×10^7	Negative	7.5×10^5
Seasonal influenza A H3N2 04/2004	1.0×10^5	Negative	1.0×10^3
Seasonal influenza A H3N2 01/2007	6.0×10^6	Negative	6.0×10^4

Virus	Number of positive result/ total isolates tested	
	A(H1N1)pdm09 T1 line	Flu A T2 line
(B)		
A(H1N1)pdm09	5/5	5/5
Seasonal influenza A H1N1	0/15	15/15
Seasonal influenza A H3N2	0/13	13/13
Influenza B	0/9	0/9
Parainfluenza	0/12	0/12
Respiratory syncytial virus	0/8	0/8
Adenovirus	0/10	0/10

(A) The lower detection limit and (B) the specificity of the system were determined using known titers of A(H1N1)pdm09 and seasonal influenza A viruses and other viruses

$TCID_{50}$ 50 % tissue culture infectious dose

The specificity of the test system is shown in Table 1B. In total, 33 influenza A virus isolates, including five A(H1N1)pdm09 isolates, 15 seasonal influenza H1N1 isolates, and 13 seasonal influenza H3N2 isolates, were included in this study. Other viruses that were recognized as being respiratory viruses, including nine influenza B virus isolates, 12 parainfluenza (subtype 1, 2, 3, and 4) virus isolates, 8 RSV isolates, and 10 adenovirus isolates, were included. There was 100 % specificity for the detection of the A(H1N1)pdm09 virus and 100% specificity for influenza A virus, with no cross-reactivity to other respiratory viruses. Invariably, a strong C line signal was observed. This result indicated that the system provided valid positive and negative results. The results demonstrate that the test system can discriminate between A(H1N1)pdm09 and seasonal influenza A viruses effectively. Figure 4 shows examples of positive and negative

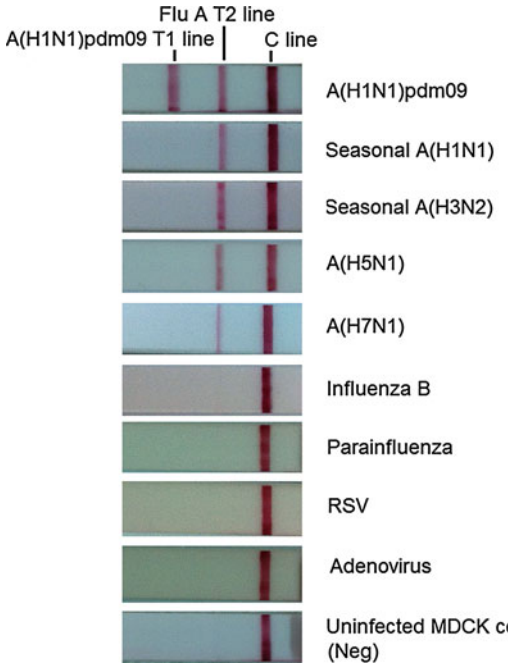


Fig. 4 Examples of rapid immunogold biosensor test results. The test showed positive A(H1N1)pdm09 T1 and Flu A T2 line signals for the A(H1N1)pdm09 virus but showed a negative A(H1N1)pdm09 T1 result for seasonal influenza A viruses (H1, H3 subtypes) and other influenza A subtypes (H5, H7 subtypes). The test showed negative A(H1N1)pdm09 T1 and Flu A T2 results for other viruses, including influenza B virus, parainfluenza virus, respiratory syncytial virus, and adenovirus. Uninfected MDCK cells were used as a negative control

results from the test system when tested with A(H1N1)pdm09, other influenza A subtypes, and other viruses.

Clinical evaluation of the rapid immunogold biosensor

A preliminary clinical evaluation of the test system was performed using the leftover portion of 50 nasopharyngeal wash samples that were collected from the Department of Microbiology, Mahidol University, Thailand. Of the 50 samples, 20 samples were positive for A(H1N1)pdm09 virus, 20 samples were positive for seasonal influenza A H3N2 virus, and 10 samples were negative for both A(H1N1)pdm09 and seasonal influenza A viruses as determined by rRT-PCR assay. It was shown that the system could detect 18 of 20 A(H1N1)pdm09-positive samples correctly, 20 of 20 seasonal-influenza-A-positive samples, and 9 negative samples, respectively (Table 2). One negative sample was excluded from the evaluation. This sample, however, contained a mucous matrix component that prevented the migration of the liquid sample and that could affect the binding of the target antigen and mAbs. However, the A(H1N1)pdm09 T1 and the FluA T2 lines gave concordant results and exhibited a strong C line

Table 2 Clinical evaluation of the rapid immunogold biosensor system was performed using clinical specimens

Rapid immunogold biosensor	rRT-PCR			Total
	A(H1N1)pdm09 positive	Seasonal influenza A positive	Negative	
A(H1N1)pdm09 positive	18	–	–	18
Seasonal influenza A positive	–	20	–	20
Negative	2	–	9	11
Total	20	20	9	49

The performance of the system was compared with that of the rRT-PCR assay, which was used as a reference method

signal, which indicated that the system was invariably functioning correctly. The diagnostic sensitivity of the system was determined to be 90 % and 100 % for A(H1N1)pdm09 and seasonal influenza A viruses, respectively. The diagnostic specificity of the system was high (100 %) for A(H1N1)pdm09 and seasonal influenza A viruses.

Discussion

The emergence of influenza outbreaks often causes a burden on the public healthcare system. The appearance of new variant strains suggests that there is a requirement for comprehensive diagnostic testing to provide critical information for treatment decisions [11]. A diagnosis that is based on the clinical presentation alone may not be sufficient because the symptoms could be very similar to those caused by other respiratory pathogens [25]. Although a viral culture is a reference standard that is used in the laboratory confirmation of influenza A virus infection, this technique is time-consuming and laborious. In addition, these techniques do not discriminate the pandemic strains from the seasonal strains. Although molecular approaches, such as an rRT-PCR assay, are considered to be the method of choice for influenza A diagnosis, these sophisticated techniques may not be readily implemented for rapid diagnosis and screening purposes in simple clinical laboratory and field settings [26]. Therefore, to facilitate the diagnosis, outbreak investigation, and infection control of the pandemic strain, a rapid diagnostic system with the ability to discriminate the pandemic strain from seasonal strains is required.

An outbreak of the A(H1N1)pdm09 infection occurred in 2009 and affected people in more than 214 countries worldwide [1, 27]. Although this outbreak has moved into the post-pandemic period, several reports have shown that the A(H1N1)pdm09 virus continues to circulate worldwide [8]. This continued transmission allows the A(H1N1)pdm09 virus to be an excellent target model for developing a platform for a rapid diagnostic system with the ability to detect and discriminate the pandemic and seasonal strains simultaneously. The rapid immunogold biosensor that was developed in this study was designed to provide an additional benefit compared with most available conventional rapid tests. In this study, the system was not only developed to detect the pandemic strain but also to distinguish it from circulating seasonal strains. Because the HA protein is the dominant surface glycoprotein of the influenza A virion and because there is significant variation in the HA protein between the A(H1N1)pdm09 virus and the seasonal influenza A virus, the HA protein is considered to be an excellent target for the detection of the A(H1N1)pdm09 virus [28]. Although there are major antigenic differences in the HA protein between A(H1N1)pdm09 and seasonal strains, minor antigenic heterogeneity of the NP protein could be observed among different influenza A subtypes. The NP protein is considered a highly conserved protein and is one of the most abundant proteins present in the virion [29]. For these reasons, the NP protein was chosen as the target antigen for seasonal influenza A virus detection.

HA mAbs that were specific for the HA protein of the A(H1N1)pdm09 virus were successfully developed. The specificity of the selected four mAb clones could be attributed to the differences in the sequence and antigenicity of the HA protein between A(H1N1)pdm09 and seasonal influenza A viruses, which were recognized by the HA mAbs. It has been demonstrated that the amino acid sequence of HA of the A(H1N1)pdm09 virus displayed up to approximately 23-28 % difference from seasonal influenza A viruses [30, 31]. An effort to determine the extent to which each HA mAb may share an epitope of the HA protein was made using a competitive ELISA. It was shown that the HA mAbs that were produced in this study may share an identical or overlapping epitope, except for one HA mAb clone (clone H109.5). However, an additional epitope mapping study would provide more detailed information regarding the binding regions in the A(H1N1)pdm09 HA protein that are recognized by each mAb. The further characterization of the HA mAbs to select the best antibody pair revealed that the system that was composed of the HA mAb clone H109.1.1 as the capture and the detector antibody exhibited the lowest limit of detection. Although the capture and the detector antibody were the same mAb clone recognizing an identical epitope, this situation did not affect the total signal that was generated. This result suggested that this specific mAb clone may recognize an immunodominant epitope that is present abundantly on the HA protein.

A preliminary evaluation of the system with a range of influenza A viruses, including H1, H3, H5, and H7 subtypes, during the setup of the assay demonstrated that a rapid immunogold biosensor could distinguish the A(H1N1)pdm09 virus from seasonal viruses. However, a thorough comparison of the system with an alternative POC assay would clarify the performance of the assay. To broaden the diagnostic applications of the test system, the ability to distinguish influenza A virus from influenza B virus would also be more clinically relevant.

A further point that may be worthwhile to discuss is how this system differs from other tests. Several studies also reported that these tests have low analytical sensitivity in detecting the A(H1N1)pdm09 virus [32, 33]. Other studies have been reported in which a rapid diagnostic test was developed for detection of A(H1N1)pdm09 virus. Although these studies evaluated the performance of the kits, it was difficult to make a comparison of the test sensitivity due to differences in the virus quantitation method. Mizuike et al. reported a detection limit of 1×10^1 focus-forming units/mL (FFU/mL), whereas the detection limit of the test that was developed by Miyoshi-Akiyama et al. was 2×10^5 viral copies/kit [26, 34]. Although the previous systems selected the NP protein as a target antigen for the detection of the A(H1N1)pdm09 virus, the HA protein was selected as the antigen of choice in this study to allow the simultaneous detection of and discrimination between A(H1N1)pdm09 and the seasonal influenza A viruses. Rapid detection and differentiation of A(H1N1)pdm09 virus from seasonal influenza viruses using other platforms has also been described by other groups. Examples include the development of a nanoparticle-based genomic nanomicroarray assay and the development of a reverse transcription loop-mediated isothermal amplification assay for the detection and subtyping of A(H1N1)pdm09 [35, 36].

A preliminary clinical evaluation of the test system was performed using clinical specimens (nasopharyngeal wash) and was compared with a molecular approach (rRT-PCR) as a reference method. Notably, the type and quality of specimens are considered important factors in the evaluation of a rapid diagnostic test for influenza A diagnosis. Because nasopharyngeal aspirate/wash and sputum specimens are considered a good source of clinical specimens for influenza diagnosis when compared to swab specimens, nasopharyngeal wash specimens were included in the evaluation [37]. Although molecular approaches are sensitive techniques, these techniques require specific expertise and specialized instruments. A rapid diagnostic kit, however, provides POC testing due to its simplicity and short assay turnaround time for a cost-effective diagnosis of A(H1N1)pdm09 and seasonal influenza A viruses in infected individuals. The ability of the developed system to discriminate the A(H1N1)pdm09 virus from the seasonal viruses, which correlated to the rRT-PCR results, demonstrated that this method could be applied clinically for the detection of A(H1N1)pdm09 virus.

In conclusion, the rapid immunogold biosensor for the simultaneous discrimination of A(H1N1)pdm09 and seasonal influenza A viruses, which is based on HA- and NP-specific mAbs, was developed successfully. The system provides an advantage for not only detecting the A(H1N1)pdm09 virus but also distinguishing the pandemic strain from the seasonal influenza A virus simultaneously in one test. The preliminary evaluation of the system showed that the system appeared to be sufficiently sensitive and reliable to allow an effective analysis of A(H1N1)pdm09 virus with no cross-reaction to other respiratory viral agents. The clinical application of the system demonstrated that this method can be useful as a rapid, on-site, and early screening tool for the detection and discrimination of A(H1N1)pdm09 and seasonal influenza A viruses. This study provides a basis for the design of a rapid diagnostic combination test that may be applicable for diagnostic, subtyping, and surveillance applications during future outbreaks. Furthermore, the system may be useful in several different applications, such as monitoring the emergence of antiviral resistance by differentiating the resistant isolates from the sensitive ones.

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