

Rapid Detection of H5N1 Subtype Influenza Viruses by Antigen Capture Enzyme-Linked Immunosorbent Assay Using H5- and N1-Specific Monoclonal Antibodies[▽]

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Highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype has caused devastating damage to poultry flocks and sporadic human H5N1 infections. There is concern that this virus subtype may gain transmissibility and become pandemic. Rapid diagnosis and surveillance for H5N1 subtype viruses are critical for the control of H5N1 infection. In this study, we report a robust antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) based on H5- and N1-specific monoclonal antibodies (MAbs) for the rapid detection of H5N1 subtype viruses. The H5 hemagglutinin (HA)-specific MAbs (2D9) targets a conformational epitope which recognized multiple clades of H5N1 viruses, including clades 0, 1, 2.1, 2.2, 2.3, 4, 7, and 8. The N1 neuraminidase (NA)-specific MAbs (8H12) recognized a linear epitope comprising the sequence AELPF. This epitope was 99% conserved in the NA of 708 analyzed H5N1 viruses, while the epitope was absent in NAs of subtypes N2 through N9. The specificity of the AC-ELISA was examined by using 41 H5N1 HPAI strains from multiple clades, 36 non-H5N1 viruses, and 4 influenza B viruses. No cross-reactivity was observed for any of the non-H5N1 viruses tested. The estimated detection limit was 1 to 2 HA titers. It is concluded that this H5N1 AC-ELISA can simultaneously detect H5 and N1 subtype antigens, eliminating the need for secondary testing for the NA subtype. Implementation of this assay in ELISA-like formats suitable for field use, such as dot ELISA, immunofiltration, or electrochemical biosensor technologies, would provide dual on-site detection of H5 and N1 in clinical or environmental specimens.

Influenza A virus is classified into subtypes H1 to H16 and N1 to N9 based on the antigenic specificity of hemagglutinin (HA) and neuraminidase (NA) (8). Only two influenza A subtypes (H1N1 and H3N2) are currently circulating in the human population (23). However, the emergence of the H5N1 highly pathogenic avian influenza (HPAI) virus in poultry, causing devastating outbreaks and sporadic infection in humans, has raised the concern that the H5N1 subtype virus may lead to the next pandemic. As of September 2008, there have been a total of 387 cases of confirmed H5N1 infection in humans, resulting in 245 fatalities (25).

Rapid and sensitive laboratory and field tests for the diagnosis of H5N1 HPAI infection are essential for disease control. Conventional laboratory methods for H5N1 virus detection include virus isolation in embryonated eggs or Madin-Darby canine kidney (MDCK) cells, followed by subsequent HA and NA subtype identification using serological methods. Molecular detection methods such as reverse transcriptase PCR (RT-PCR) have been widely applied for the laboratory diagnosis of influenza infections and HA subtype identification (11, 21, 22). In addition, several studies have reported the use of real-time PCR assays and DNA microarray analysis for detection of

influenza virus in the laboratory (7, 11, 13, 17). However, both conventional and laboratory methods are technically demanding and are not suitable for on-site use in field investigations. The development of rapid H5 subtype influenza virus detection tests in dot ELISA (enzyme-linked immunosorbent assay), AC-ELISA (antigen-capture ELISA), and chromatographic strip formats (5, 6, 10) using H5 monoclonal antibodies (MAbs) have been reported. However, these assays do not directly identify the N1 NA in the H5N1 viruses, and many can yield positive results with H5 avian influenza viruses with other NA subtypes (e.g., H5N2) that have never been reported to cause infection in humans due to the lack of the ability to identify the NA subtype. The identification of the NA subtype is largely done by RT-PCR (3, 20). NA inhibition assay is the conventional method for NA subtype identification (18). However, it is rarely used because the procedure is very cumbersome and NA subtype-specific antisera are not commercially available.

Here we describe an AC-ELISA for the rapid diagnosis of HPAI H5N1 virus infection, based on H5- and N1-specific MAbs that mediate positive identification of H5 HA and N1 NA in a single assay. The selection of MAbs for the development of this H5N1 AC-ELISA was based on detailed characterizations of their binding properties. The sensitivity and specificity of this assay were evaluated using multiple HPAI H5N1 strains and other subtypes of influenza A viruses as well as influenza B viruses. The H5N1 AC-ELISA described here is a proof of concept for the future development of a field test. Our

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TABLE 1. Influenza viruses tested by H5N1 AC-ELISA^a

Virus	Subtype
A/Indonesia/CDC7/06.....	H5N1
A/Indonesia/CDC326/06.....	H5N1
A/Indonesia/CDC329/06.....	H5N1
A/Indonesia/CDC370/06.....	H5N1
A/Indonesia/CDC390/06.....	H5N1
A/Indonesia/CDC523/06.....	H5N1
A/Indonesia/CDC594/06.....	H5N1
A/Indonesia/CDC595/06.....	H5N1
A/Indonesia/CDC597/06.....	H5N1
A/Indonesia/CDC610/06.....	H5N1
A/Indonesia/CDC623/06.....	H5N1
A/Indonesia/CDC644/06.....	H5N1
A/Indonesia/CDC669/06.....	H5N1
A/Indonesia/TLL01/06.....	H5N1
A/Indonesia/TLL02/06.....	H5N1
A/Indonesia/TLL60/06.....	H5N1
A/Indonesia/TLL177/06.....	H5N1
A/Indonesia/TLL298/06.....	H5N1
A/Indonesia/TLL485/06.....	H5N1
A/Indonesia/TLL530/06.....	H5N1
A/Indonesia/TLL535/06.....	H5N1
A/Indonesia/TLL540/06.....	H5N1
A/Indonesia/TLL561/06.....	H5N1
A/Indonesia/TLL565/06.....	H5N1
A/Chicken/Indonesia/TLL101/06.....	H5N1
A/Duck/Indonesia/TLL102/06.....	H5N1
A/Common Iora/Indonesia/F89/11/95.....	H5N2
A/Chicken/Singapore/Singapore/92.....	H4N1
A/Chicken/Singapore/Singapore/02.....	H3N2
A/Chicken/Singapore/94.....	H7N1
A/Chicken/Singapore/Singapore/98.....	H9N2
A/Mandarin Duck/Singapore/Singapore/93.....	H10N5

^a All H5N1 virus strains isolated from Indonesia belong to clade 2.1.

results indicate that this antibody pair could be particularly useful for on-site use in field investigations of H5N1 infection, when incorporated into a rapid field test format based on dot ELISA, immunofiltration, or electrochemical biosensor technologies.

MATERIALS AND METHODS

Virus cultivation. Twenty-four human and two avian H5N1 influenza strains (clade 2.1) isolated from Indonesia were obtained from the Ministry of Health, Indonesia. Five non-H5 subtype strains (Table 1) were obtained from the Agri-Food and Veterinary Authority of Singapore. Fourteen non-H5N1 virus strains (multiple subtypes; see Tables 1 and 2), sixteen H1N1, six H3N2, and four influenza B virus strains were isolated from human clinical samples by the Department of Pathology, Singapore General Hospital. Viruses were inoculated into the allantoic cavities of 11-day-old embryonated chicken eggs and harvested following 48 h of incubation at 37°C. Virus titers were determined using hemagglutination assays according to standard methods (2). H5N1 subtype viruses were inactivated with formaldehyde as described previously (12). All experiments with live H5N1 and H7N7 subtype viruses were performed in a biosafety level 3 containment laboratory in compliance with CDC/NIH and WHO recommendations (4, 24) and also were approved by the Agri-Food and Veterinary Authority and the Ministry of Health of Singapore.

Reverse genetics. The HA and NA genes of sixteen H5N1 viruses from clade 1, clade 2, clade 4, clade 7, and clade 8 and seven non-H5 subtype viruses (H2N9, H6N8, H7N7, H8N4, H12N5, H13N6, and H16N3) (Table 2) were synthesized by GenScript based on the sequences from the NCBI influenza database. The synthesized HA and NA genes were cloned into a dual-promoter plasmid for influenza A reverse genetics (26). The reassortant viruses were rescued by transfecting plasmids containing HA and NA together with the remaining six gene plasmids derived from A/Puerto Rico/8/34 (H1N1) into a coculture of 293T and MDCK cells by using Lipofectamine 2000 (Invitrogen Corp.). A/Puerto Rico/8/34 virus was also generated by plasmid-based reverse genetics. At 72 h post-

TABLE 2. Reassortant influenza viruses generated from reverse genetics

Virus	Clade	Subtype
A/Hong Kong/213/03	1	H5N1
A/Vietnam/1203/04	1	H5N1
A/muscovy duck/Vietnam/33/07	1	H5N1
A/Indonesia/CDC1031/07	2.1	H5N1
A/turkey/Turkey1/05	2.2	H5N1
A/barheaded goose/Qinghai/12/05	2.2	H5N1
A/Nigeria/6e/07	2.2	H5N1
A/muscovy duck/Rostovon Don/51/07	2.2	H5N1
A/Anhui/1/05	2.3	H5N1
A/Jiangsu/2/07	2.3	H5N1
A/Vietnam/HN31242/07	2.3	H5N1
A/Hong Kong/156/97	0	H5N1
A/chicken/Shanxi/2/06	7	H5N1
A/goose/Guizhou/337/06	4	H5N1
A/chicken/Henan/12/04	8	H5N1
A/Puerto Rico/8/34		H1N1
A/duck/Nanchang/4-184/2000		H2N9
A/shorebird/DE/12/04		H6N8
A/Netherlands/219/03		H7N7
A/duck/Yangzhou/02/05		H8N4
A/pintail/Alberta/49/03		H12N5
A/gull/Maryland/704/1977		H13N6
A/herring gull/Delaware/712/1988		H16N3

transfection, culture medium from the transfected cells was inoculated into 11-day-old embryonated chicken eggs or MDCK cells. The HA and NA genes of reassortant viruses were sequenced to confirm the presence of introduced HA and NA genes.

Production of recombinant neuraminidase protein (rNA). Viral RNA was extracted from allantoic fluid containing A/Indonesia/CDC669/06 (H5N1) using a commercial guanidium-phenol solution (Trizol; Invitrogen). The NA gene was amplified from the viral RNA by RT-PCR using N1-specific primers. The PCR product was cloned into a pGEX-4T-1 glutathione S-transferase fusion (GST) vector (GE Healthcare) and transformed into *Escherichia coli* BL21 competent cells for protein expression. The fusion protein expression was induced by addition of 1 mmol/liter IPTG (isopropyl- β -D-thiogalactopyranoside) to the culture; bacterial cells were harvested after 3 h of incubation and NA protein was subsequently purified using a gel eluter (Bio-Rad).

Production and characterization of MAbs. BALB/c mice were immunized subcutaneously twice, 2 weeks apart, with 25 μ g of rNA protein (for N1 MAb production) in 0.1 ml of phosphate-buffered saline (PBS) or 200 μ l of inactivated A/Indonesia/CDC669/06 (H5N1) with an HA titer of 256 with an equal amount of adjuvant (SEPPIC, France). Mice were boosted with the same dose of rNA or H5N1 virus 3 days before splenocytes were collected and fused with SP2/0 cells (28). The fused cells were seeded in 96-well plates, and supernatants were screened by immunofluorescence assays as described below. The isotypes of antibodies secreted by cloned hybridoma cells were determined using a one-minute isotyping kit (Amersham Bioscience, England).

IFA. HA1-specific MAbs were identified by immunofluorescence assay (IFA) using Sf-9 insect cells infected with a recombinant baculovirus harboring the HA1-encoding region of the HA gene from A/Indonesia/CDC669/06 (H5N1) virus as described previously (16). Sf-9 or MDCK cells cultured in 96-well plates were infected with H5-HA1 recombinant baculovirus or with a panel of H5N1 and non-H5 subtype influenza viruses, respectively (Table 1). At 36 h (for Sf-9 cells) or 24 to 48 h (for MDCK cells) postinfection, the cells were rinsed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature, and washed three times with PBS. The fixed cells were incubated with hybridoma culture supernatant at 37°C for 1 h followed by incubation with a 1:40 dilution of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (Dako, Denmark). Antibody binding was evaluated by wide-field epifluorescence microscopy (Olympus IX71). N1-specific hybridomas were identified by IFA in 293T cells transfected with a plasmid expressing the N1 gene of A/Indonesia/CDC669/06 (H5N1) by using Lipofectamine 2000 (Invitrogen Corp.).

HI assay. The hemagglutination inhibition (HI) assay was performed as described previously (2). Briefly, receptor-destroying enzyme-treated sera were serially diluted (twofold) in V-bottom, 96-well plates and mixed with an equal

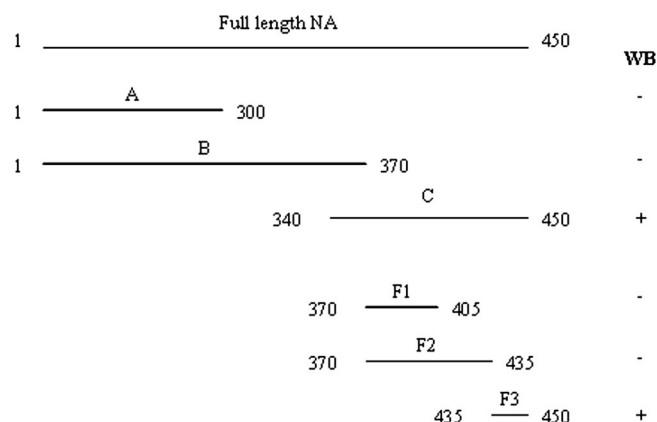


FIG. 1. Epitope mapping of the 8H12 MAb. Three overlapping NA fragments of A/Indonesia/CDC610/06 (H5N1) (A, B, and C) and sub-fragments (F1 to F3) were expressed as GST fusion proteins and analyzed by Western blotting (WB) using 8H12 MAb.

volume of virus. Plates were incubated for 30 min at room temperature, and 1% chicken red blood cell was added to each well. The HI endpoint was the highest serum dilution in which agglutination was not observed.

Selections of 2D9 MAb escape mutants. Serial 10-fold dilutions of A/Indonesia/CDC669/06 (H5N1) virus were mixed with an equal volume of 2D9 MAb (1 µg/µl) and incubated at room temperature for 30 min. The mixtures were inoculated onto MDCK monolayers and cultured for 7 days at 35°C in Dulbecco's minimal essential medium containing 1 µg/ml TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone)-treated trypsin (Sigma) and 0.001% DEAE-dextran (Sigma). The cell culture supernatant was subsequently collected for virus isolation and sequence analysis. Viral RNA was isolated using LS Trizol reagent (Invitrogen) as specified by the manufacturer. Reverse transcription and PCR were performed with specific primers for the HA gene of H5 subtypes. The PCR amplicon sequences were compared to those of the wild-type virus to establish the location of coding mutations.

Western blotting. Cell lysates of IPTG-induced *E. coli* cultures or 20 µl of viruses with a HA titer of 256 in allantoic fluid were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins in the gel were then transferred onto a nitrocellulose membrane and blocked with 5% nonfat milk in PBST (1× PBS and 0.1% Tween 20) for 1 h at room temperature. The membrane was incubated with MAb hybridoma supernatant, rinsed with PBST, and incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) (Dako, Denmark) for 1 h at room temperature. Following washing with PBST, the membrane was developed by incubation with ECL reagents (Amersham Biosciences) as described previously (9).

Epitope mapping of 8H12 MAb. Three overlapping fragments encoding the open reading frame of the NA of A/Indonesia/CDC610/06 (H5N1) (Fig. 1, "A" to "C") were amplified by PCR and cloned into the pGEX-4T-1 GST fusion vector (GE Healthcare). The fusion peptides were expressed in *E. coli* as described above and analyzed by Western blotting. Three additional truncated peptides (Fig. 1, "F1" to "F3") were expressed and analyzed. A panel of eleven mutants of fragment F3 was generated by substitution of amino acids 438 to 448 (P438A, D439A, G440D, A441D, E442K, L443F, P444A, F445A, T446A, I447A, and D448A). The mutations were generated by site-directed mutagenesis with a commercial kit (QuikChange; Stratagene), and the introduced mutations were confirmed by sequence analysis.

8H12 epitope conservation analysis. To analyze the variability of the 8H12 epitope, 3,439 NA genes of influenza A viruses (N1 to N9), isolated from avian and human sources, with full-length NA sequences available from NCBI Influenza Virus Resource internet database (<http://www.ncbi.nlm.nih.gov/>), were analyzed. Identical sequences were removed. Alignment was performed using the MUSCLE alignment tool installed locally in the database website.

Development of H5N1 AC-ELISA. Ninety-six-well, round-bottom microtiter plates (Nunc, Roskilde, Denmark) were coated with 1 µg/well of 8H12 MAb in 100 µl of carbonate buffer (73 mM sodium bicarbonate and 30 mM sodium carbonate, pH 9.7) overnight at 4°C or 37°C for 2 h. The plates were washed twice with PBST, followed by two washes with PBS after each incubation with antibody or antigen. The antibody-coated plates were blocked by incubation with 100 µl of blocking buffer (PBS containing 5% skim milk) for 1 h at room

temperature and then incubated at 37°C for 1 h with 100 µl of virus-containing samples diluted in PBST. Virus binding was detected by incubation for 1 h at 37°C with 100 µl of horseradish peroxidase-conjugated 2D9 MAb (800 ng) (in-house labeling; Roche). Chromogen development was mediated by the addition of 100 µl of freshly prepared substrate solution (*o*-phenylenediamine dihydrochloride; Sigma). The reaction was stopped by adding 0.1 N sulfuric acid, and the optical density at 490 nm was recorded. The sensitivity of the H5N1 AC-ELISA was determined based on the HA titer using selected H5N1 virus strains. Viruses were twofold serially diluted in PBST and subjected to AC-ELISA. The detection limit was determined by the optical density value that gave a signal-to-noise ratio of 3. The sensitivity of the H5N1 AC-ELISA was compared with that of a commercial kit specific for the nucleoprotein of influenza A virus (Rockeby avian influenza virus antigen test; Rockeby Biomed, Singapore).

RESULTS

Characterization of MAbs. Five independent hybridomas secreting antibody to A/Indonesia/CDC669/06 (H5N1) revealed a pattern of cytoplasmic staining by IFA in recombinant H5 HA1 baculovirus-infected insect cells and H5N1 virus-infected MDCK cells. The subtype specificity of the MAbs was tested by applying this IFA method to cell monolayers infected with non-H5 subtype viruses. Hybridoma 2D9 MAb proved to be strictly H5 subtype specific and was therefore selected for development of the H5N1 AC-ELISA (Fig. 2). Isotype analysis revealed that the 2D9 hybridoma secretes IgG1. In addition, MAb 2D9 demonstrated neutralizing activity to all of the 41 H5N1 strains with HI titers ranging from 4 to 128 but not to other subtype viruses listed in Tables 1 and 2. The approximate location of the 2D9 epitope was analyzed by selection of neutralization escape mutant viruses. Escape H5N1 mutant viruses were found to have single amino acid substitutions at Arg193 or Ser227 of A/Indonesia/CDC669/06 HA.

Three cloned hybridoma cultures secreted MAb that recognized H1N1-, H5N1-, and H7N1-infected MDCK cells as well as N1 protein expressed in 293T cells (data not shown). One hybridoma, 8H12, was selected for further development of H5N1 AC-ELISA due to its high sensitivity and specificity to N1 (Fig. 1). The specificity of 8H12 MAb against N1 subtype NA was also confirmed by Western blot analysis of influenza viruses of the N1 to N9 subtypes (Fig. 3). The 8H12 MAb was identified as the IgG1 class. The epitope targeted by the 8H12 MAb was subsequently mapped by scanning with a set of overlapping open reading frame expression clones.

Epitope mapping of 8H12. The MAb 8H12 reacted with the NA protein in Western blot assays, suggesting that the epitope is either linear or readily refolded after the removal of denaturing agents from its environment. To further identify the epitope, overlapping NA fragments expressed in *E. coli* were probed by Western blot analysis (Fig. 3). The 8H12 MAb reacted with NA fragment C but not A or B, indicating that the epitope was within amino acids 370 to 450 (Fig. 2). NA fragments F1 to F3 were further generated to refine the epitope and revealed that 8H12 MAb reacted with fragment F3, indicating that the epitope comprised amino acids 435 to 449. A panel of mutant NA F3 fragments with single amino acid substitutions from amino acids 438 to 448 was subsequently constructed, expressed, and analyzed by Western blotting. The Western blot results indicated that the 8H12 MAb failed to react with NA F3 fragments carrying single amino acid substitutions at A441, E442, L443, P444, and F445, suggesting that

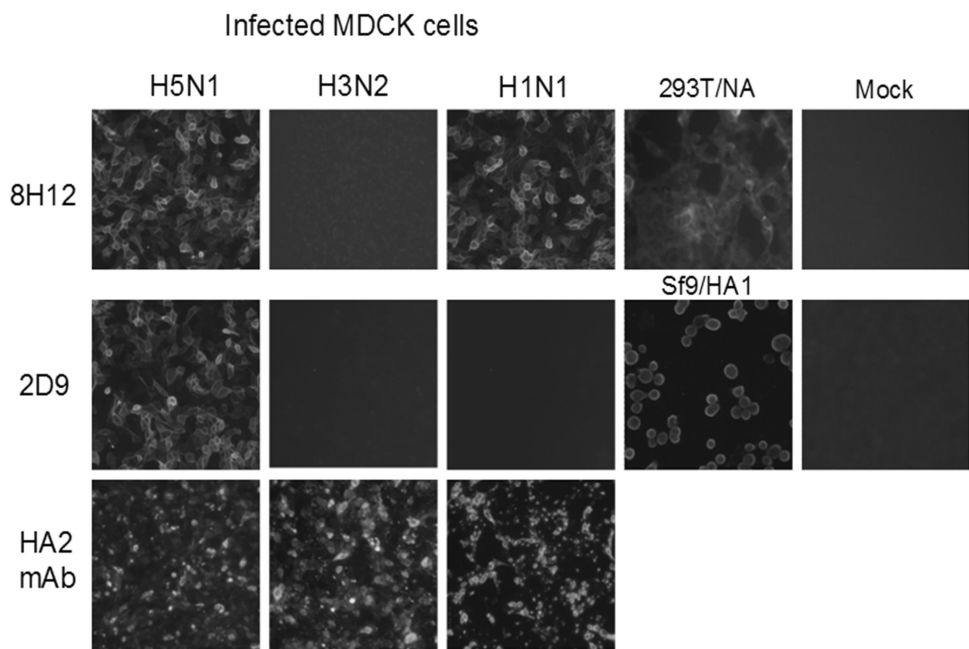


FIG. 2. Immunofluorescent analysis of 8H12 (N1-specific) and 2D9 (H5-specific) MAb. A MAb that recognizes the HA2 region of HA protein was included to confirm the presence of viruses in infected MDCK cells. NA protein expressions in 293T cells further confirm the specificity of 8H12 MAb, whereas Sf-9 cells infected with HA1 recombinant baculoviruses further confirm the specificity of 2D9 MAb.

the epitope recognized by the 8H12 MAb requires at least the following peptide sequence: AELPF (single-letter code).

Epitope analysis. Sequence alignments of the available N1 to N9 subtype NA genes of influenza A viruses isolated from human and avian sources revealed that the AELPF epitope is highly conserved in N1 subtype viruses but absent in other subtypes (Table 3). The conservation rate of the 8H12 MAb epitope was calculated for 708 N1 fragments of H5N1 and 579 N1 fragments of H1N1 influenza strains. Seven of 708 and 20 of 579 of these viruses have a single amino acid substitution in the epitope AELPF. Thus, the AELPF epitope is conserved in 99% of H5N1 NA and 96.5% of H1N1 NA samples sequenced to date.

Development of H5N1 AC-ELISA. The H5- and N1-specific MABs were used reversibly as capture and detector antibodies to determine their performance characteristics in H5N1 AC-ELISA. H5 MAB (2D9)-coated microtiter plates resulted in lower specific absorbance readings than those from microtiter plates coated with N1 MAB (8H12) (data not shown). There-

fore, the 8H12 MAB was selected as the capture antibody, while 2D9 served as the detector antibody for further analysis. Optimal concentrations of 8H12 and 2D9 MABs for capture and detection were determined by two-way titration of MAB concentrations. The combination that gave the highest signal-to-noise ratio was determined to be 1 µg/well of capture 8H12 MAB and 800 ng/well of 2D9 MAB for detection.

Specificity of H5N1 AC-ELISA. The specificity of the H5N1 AC-ELISA was tested with a total of 41 HPAI H5N1 strains isolated from humans and avian species and 40 non-H5N1 subtype influenza virus strains, including 26 seasonal influenza virus strains (H1N1, H3N2, and B subtypes) circulating in humans. Viruses of H5N1 or HA subtypes not available in our laboratory were rescued by reverse genetics with the six internal genes from A/Puerto Rico/8/34. The reactivity and specificity of the H5N1 AC-ELISA were examined with 100 µl of allantoic fluid or MDCK cell culture supernatant containing the H5N1 strains adjusted to an HA titer of 16. Non-H5N1 viruses with HA titers of ≥16 were used in order to eliminate

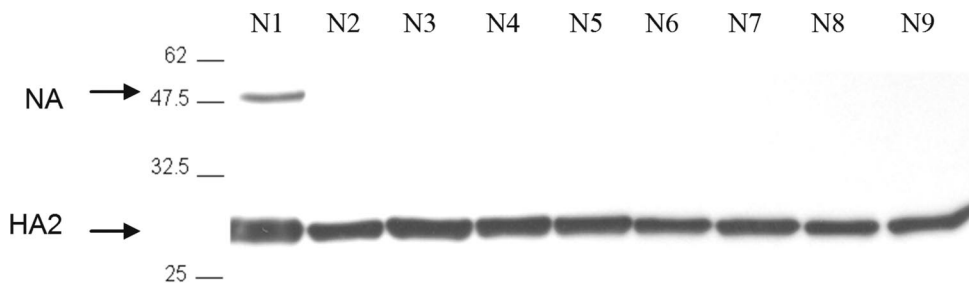


FIG. 3. Analysis of 8H12 MAb specificity by Western blotting. Viruses (20 µl of virus with an HA titer of 256) with NA subtypes N1 to N9 were fractionated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and analyzed with a mixture of 8H12 (N1-specific) MAb and MAB targets of the conserved region of HA2 protein.

TABLE 3. Epitope analysis of the N1 epitope targeted by 8H12 MAb among N1 to N9 subtypes of NA

NA subtype	Consensus epitope ^a	No. of strains in alignment	No. of strains without 8H12 epitope
N1 of H5N1	AELPF	708	7
N1 of H1N1	AELPF	579	20
N1 of HA subtype viruses other than H5 and H1	AELPF	192	40
N2	ADINL	1,337	1,337
N3	SNIGF	142	142
N4	ALLPF	47	47
N5	AILPF	46	46
N6	AEIYY	118	118
N7	AQIQY	61	61
N8	AILPF	147	147
N9	AKIEY	62	62
Total		3,439	

^a The consensus epitope shown in the alignment. The AELPF epitope was not observed in N2 to N9 sequences.

false-positive results. Table 4 shows the absorbance readings of H5N1 virus strains from different clades, covering clades 1, 2.2, 2.3, 0, 7, 4, and 8, and representative H5N1 Indonesia isolates, which belong to clade 2.1. Absorbances of all the 41 H5N1 strains tested were at least 10 times higher than that of the non-H5N1 subtype viruses, with an average absorbance of 1.2 (Table 4). No cross-reactivity was observed for any of the non-H5N1 subtype viruses tested, and the average absorbance reading was as low as 0.06 (Table 5).

Sensitivity of H5N1 AC-ELISA. The tested virus was considered to be positive in the H5N1 AC-ELISA when the absorbance was three times higher than that of the non-H5N1 viruses. Four H5N1 viruses from different clades that had absorbance readings ranging from 0.6 to 1.6 were selected for determination of detection limit based on virus HA titer. With

TABLE 4. Absorbance readings of H5N1 viruses tested by H5N1 AC-ELISA

Virus	Clade	OD ₄₉₀ ^a
A/Hong Kong/213/03	1	1.657
A/Vietnam/1203/04	1	0.981
A/muscovy duck/Vietnam/33/07	1	0.921
A/turkey/Turkey1/05	2.2	1.661
A/barheaded goose/Qinghai/12/05	2.2	1.629
A/Nigeria/6e/07	2.2	1.804
A/muscovy duck/Rostovon Don/51/07	2.2	2.069
A/Anhui/1/05	2.3	0.617
A/Jiangsu/2/07	2.3	1.234
A/Vietnam/HN31242/07	2.3	1.221
A/Hong Kong/156/97	0	1.102
A/chicken/Shanxi/2/06	7	0.994
A/goose/Guiyang/337/06	4	0.851
A/chicken/Henan/12/04	8	1.250
A/Indonesia/CDC7/06	2.1.1	1.368
A/Indonesia/CDC326/06	2.1.3	1.517
A/Indonesia/CDC370/06	2.1.3	1.339
A/Indonesia/CDC523/06	2.1.3	1.197
A/Indonesia/CDC594/06	2.1.2	0.878
A/Indonesia/CDC669/06	2.1.3	1.021

^a Optical density at 490 nm. Values represent the mean absorbances from triplicate wells.

TABLE 5. Absorbance readings of non-H5N1 viruses tested by H5N1 AC-ELISA

Virus	OD ₄₉₀ ^a
H1N1	0.058
H2N9	0.057
H3N2	0.059
H4N1	0.069
H5N2	0.058
H6N8	0.057
H7N7	0.069
H7N1	0.055
H8N4	0.056
H9N2	0.055
H10N5	0.067
H12N5	0.059
H13N6	0.055
H16N3	0.056
H1N1 ^b	0.059
H3N2 ^b	0.059
Influenza B ^b	0.061

^a Optical density at 490 nm. Values represent the mean absorbances from triplicate wells.

^b Mean absorbance values of 16 H1N1, 6 H3N2, and 4 influenza B viruses isolated from human clinical samples in Singapore General Hospital.

a cutoff value of 0.18, the detection limit was determined to be 100 µl of sample containing 1 HA titer of virus for viruses that had average and higher-than-average absorbance, while it was 2 HA titers for viruses that had lower-than-average absorbance (Fig. 4). The detection limit of the commercial kit for influenza

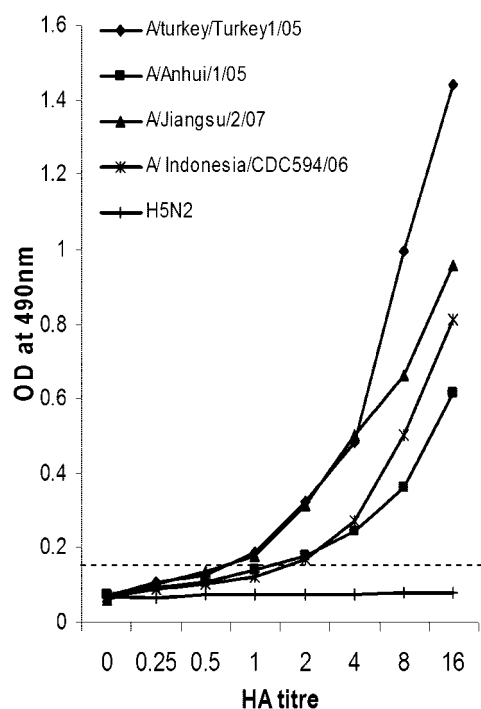


FIG. 4. Sensitivity of H5N1 AC-ELISA. Tested viruses with an HA titer of 16 were twofold diluted and subjected to H5N1 AC-ELISA assay. Values represent the means of absorbances of duplicate wells from two independent tests. OD, optical density; dotted line, cutoff values.

A virus detection (Rockeby) was determined to be 200 μ l of sample containing at least 1.5 HA titer of virus.

DISCUSSION

Although avian H5 subtype viruses have been identified in combination with various NA subtypes, the major threat to animal and human health is currently posed by H5N1 viruses. Most importantly, H5N1 viruses have infected hundreds of people, killing approximately 60% of those infected (25). On the other hand, most of the Eurasian H5 avian influenza viruses of NA subtypes other than N1 (H5N2-N9) do not pose an immediate threat to human health. Conventional methods for H5N1 virus detection are time-consuming and technically demanding, and most importantly, these methods are not practical for field investigation. Several rapid diagnostic kits for the detection of H5 subtype viruses have been reported (5, 6). However, since not all H5 viruses are highly pathogenic, the inability of these kits to indicate the NA subtype of H5 viruses might cause unnecessary concerns over low-pathogenicity H5 virus infection. In this study, we described a robust AC-ELISA for the rapid and specific detection of H5N1 subtype viruses based on concurrent use of H5- and N1-specific MAb.

Selection of the H5 HA- and N1 NA-specific MAb for the development of the H5N1 AC-ELISA was based on detailed analyses of their binding properties. The selected H5-specific MAb 2D9 demonstrated high specificity to H5 HA in the HI assay and IFA. Neutralization escape mutants with MAb 2D9 showed substitutions at amino acid positions Arg193 and Ser227. These amino acids are two of the key residues in the H5 receptor-binding site of the globular head of the HA molecule (19). H5N1 HPAI viruses are classified into distinct phylogenetic clades based on their phylogenetic divergence (27). The 2D9 MAb recognizes multiple clades of H5N1 viruses, including clades 0, 1, 2.1, 2.2, 2.3, 4, 7, and 8 in the H5N1 AC-ELISA. This result could suggest that the epitope-binding site of the 2D9 MAb is highly conserved in H5N1 viruses. Further investigation, however, is needed to determine the exact residues that constitute the 2D9 epitope.

The N1 NA-specific MAb (8H12) used in this study was found to recognize an epitope comprising the amino acid sequence AELPF in N1 NA. This epitope is located at positions 441 to 445, which is near the C terminus of the NA protein. In contrast to HA, which is anchored to the membrane by a hydrophobic amino acid near the C terminus, NA is anchored by a series of hydrophobic amino acids near the N terminus (1). Analysis of the AELPF epitope in 3,439 NA sequences of influenza A viruses revealed that the epitope is highly conserved in H5N1 viruses. The conservation rate of the epitope was found to be 99% in 708 analyzed H5N1 viruses isolated from avian or human species, whereas it was not found in the N2 to N9 subtypes of NA. Western blot analysis of 8H12 MAb against N1 to N9 NA subtypes further demonstrated the specificity of this MAb in recognizing only N1 NA.

The specificity of the H5N1 AC-ELISA was evaluated using 41 HPAI H5N1 strains and 40 non-H5N1 subtype influenza virus strains isolated from human and avian species. No cross-reactivity was observed for the 40 non-H5N1 viruses tested. It is noted that, although the 40 non-H5N1 viruses tested constitute only a small subset of the possible viruses and only limited

HA and NA combinations were studied, the possibility of cross-reactivity of the H5N1 AC-ELISA with other subtype viruses is believed to be extremely low, as comprehensive analyses have demonstrated the high specificity of the selected H5- and N1-specific MAb. Further evaluation of the H5N1 AC-ELISA on field samples, however, is needed to determine the specificity of the assay in a more quantitative way.

The sensitivity of the H5N1 AC-ELISA was determined to be higher than that of the Rockeby avian influenza virus antigen test (100 μ l of HA titers of 1 to 2 versus 200 μ l of an HA titer of 1.5). Furthermore, the sensitivity of the H5N1 AC-ELISA is believed to improve upon refinement of horseradish peroxidase-labeled detection of MAb (2D9) efficiency and removal of unlabeled MABs, which were not performed in this experiment.

In conclusion, the H5N1 AC-ELISA developed in this study provides dual, positive identification of HA and NA subtypes for rapid detection of H5N1 HPAI viruses, thus eliminating the need for subsequent NA subtyping. This H5N1 AC-ELISA shows enough specificity and sensitivity for detection of multiple clades of H5N1 HPAI virus strains. This study suggests that the H5N1 AC-ELISA based on concurrent use of H5- and N1-specific MABs described here could be formatted as a rapid field test based on dot ELISA, immunofiltration, or electrochemical biosensor technologies (14), which would greatly facilitate field investigations.

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We declare no competing interests.

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