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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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 human H5N1 infections, 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 effectively identify the H5 subtype 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
A/Puerto Rico/8/34 virus was also generated by plasmid-based reverse genetics. At 72 h post-infection, MDCK cells by using Lipofectamine 2000 (Invitrogen Corp.). A/Puerto Rico/8/34 - D-thiogalactopyranoside) to the culture; A/chicken/Henan/12/04 - H5N1 A/goose/Guiyang/337/06 - H5N1 A/Hong Kong/156/97 - H5N1 A/Anhui/1/05 - H5N1 A/Puerto Rico/8/34 H1N1 A/chicken/Henan/12/04 H5N1 A/goose/Guiyang/337/06 H5N1 A/chicken/Henan/12/04 H5N1 A/Indonesia/CDC597/06 - H5N1 A/Indonesia/CDC390/06 - H5N1 A/Indonesia/CDC370/06 - H5N1 A/Indonesia/CDC329/06 - H5N1 VOL. 16, 2009 H5N1 DETECTION USING H5 AND N1 727 TABLE 1. Influenza viruses tested by H5N1 AC-ELISA a

| Virus                                      | Subtype |
|--------------------------------------------|---------|
| A/Indonesia/CDC7/06                        | H5N1    |
| A/Indonesia/CDC32/06                       | H5N1    |
| A/Indonesia/CDC39/06                       | H5N1    |
| A/Indonesia/CDC37/06                       | H5N1    |
| A/Indonesia/CDC52/06                       | H5N1    |
| A/Indonesia/CDC59/06                       | H5N1    |
| A/Indonesia/CDC59/05                       | H5N1    |
| A/Indonesia/CDC59/07                       | H5N1    |
| A/Indonesia/CDC61/06                       | H5N1    |
| A/Indonesia/CDC62/06                       | H5N1    |
| A/Indonesia/CDC64/06                       | H5N1    |
| A/Indonesia/CDC69/06                       | H5N1    |
| A/Indonesia/TLL01/06                       | H5N1    |
| A/Indonesia/TLL02/06                       | H5N1    |
| A/Indonesia/TLL04/06                       | H5N1    |
| A/Indonesia/TLL06/06                       | H5N1    |
| A/Indonesia/TLL07/06                       | H5N1    |
| A/Indonesia/TLL29/06                       | H5N1    |
| A/Indonesia/TLL48/05                       | H5N1    |
| A/Indonesia/TLL53/06                       | H5N1    |
| A/Indonesia/TLL53/05                       | H5N1    |
| A/Indonesia/TLL54/06                       | H5N1    |
| A/Indonesia/TLL56/06                       | H5N1    |
| A/Indonesia/TLL56/06                       | 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           | H4N2    |
| A/Chicken/Singapore/94                     | H7N1    |
| A/Chicken/Singapore/98                     | H7N2    |
| A/Mandarin Duck/Singapore/Singapore/95     | 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/CNH 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-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 the reassortant influenza 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/CDC69/06 (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-thiogalactoside) 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 μg of rNA protein (for N1 MAb production) in 0.1 ml of phosphate-buffered saline (PBS) or 200 μl of inactivated A/Indonesia/CDC69/06 (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 spleenocytes 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. HAI-specific MAbs were identified by immunofluorescence assay (IFA) using SF-9 insect cells infected with a recombinant baculovirus harboring the HAI-encoding region of the HA gene from A/Indonesia/CDC69/06 (H5N1) 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 SF-9 cells transfected with a plasmid expressing the N1 gene of A/Indonesia/CDC69/06 (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
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 (Rockebay avian influenza virus antigen test; Rockeby Biomed, Singapore).

RESULTS

Characterization of MAb. 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 435 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
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). Therefore, 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
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 (Table 4). No cross-reactivity was observed for any of the non-H5N1 subtype viruses tested, and the average absorbance of the 41 H5N1 viruses 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 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

| Virus | Clade | OD<sub>490</sub> |
|-------|-------|----------------|
| 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/Guanyi/337/06 | 4 | 0.851 |
| A/chicken/Henan/12/04 | 8 | 1.250 |
| A/Indonesia/CDC7/06 | 2.1.1 | 1.368 |
| A/Indonesia/CDC26/06 | 2.1.3 | 1.517 |
| A/Indonesia/CDC37/06 | 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 |

* Optical density at 490 nm. Values represent the mean absorbances from triplicate wells.
A virus detection (Rockeby) was determined to be 200 µl 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 MAbs.

Selection of the H5 HA- and N1 NA-specific MAbs 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 is believed to be extremely low, as comprehensive analyses have demonstrated the high specificity of the selected H5- and N1-specific MAbs. 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 µl of HA titers of 1 to 2 versus 200 µl 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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