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# Establishment of the cross-clade antigen detection system for H5 subtype influenza viruses using peptide monoclonal antibodies specific for influenza virus H5 hemagglutinin

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ABSTRACT

The H5 subtype of highly pathogenic avian influenza (H5 HPAI) viruses is a threat to both animal and human public health and has the potential to cause a serious future pandemic in humans. Thus, specific and rapid detection of H5 HPAI viruses is required for infection control in humans. To develop a simple and rapid diagnostic system to detect H5 HPAI viruses with high specificity and sensitivity, we attempted to prepare monoclonal antibodies (mAbs) that specifically recognize linear epitopes in hemagglutinin (HA) of H5 subtype viruses. Nine mAb clones were obtained from mice immunized with a synthetic partial peptide of H5 HA molecules conserved among various H5 HPAI viruses. The antigen-capture enzyme-linked immunosorbent assay using the most suitable combination of these mAbs, which bound specifically to lysed H5 HA under an optimized detergent condition, was specific for H5 viruses and could broadly detect H5 viruses in multiple different clades. Taken together, these peptide mAbs, which recognize linear epitopes in a highly conserved region of H5 HA, may be useful for specific and highly sensitive detection of H5 HPAI viruses and can help in the rapid diagnosis of human, avian, and animal H5 virus infections.



## 1. Introduction

The H5 subtype of highly pathogenic avian influenza (H5 HPAI) virus has spread from Southeast Asia to China, Russia, Europe, Middle East, and Africa after 2003 [1,2]. It is still prevalent in wild birds and poultry in many areas including Japan, and these areas have suffered enormous economic damage as a result of H5 HPAI infections [3]. A high mortality rate of H5N1 HPAI virus infection in humans has also been confirmed, resulting in 454 deaths of 860 infected people in 16 countries as of December 7, 2017 [4]. Recently, several H5N1 HPAI viruses have acquired NA genes from unrelated avian influenza viruses via reassortment. H5N2, H5N3, H5N6, and H5N8 reassortant viruses isolated from ducks and poultry in China were reported during or after 2010 [5]. H5N6 viruses have also caused sporadic human infections in China; at least 17 human infections have been reported since 2014 [6]. H5 HPAI virus transmission from human to human is still limited, however, there is concern about the emergence of mutated or reassortant viruses that are easily propagated in humans. Therefore, establishment of a rapid and specific diagnostic test for H5 HPAI virus infection is necessary. A number of commercially available rapid influenza diagnostic tests have been developed, which are immunochromatography assays for influenza A and B virus infections targeting the viral nucleoprotein. However, these tests cannot distinguish HA subtypes of influenza A virus such as H1, H3, H5, and H7; thus, human seasonal influenza A viruses and avian influenza viruses are indistinguishable [7].

The antigen-capture enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (mAbs), which specifically recognize H5 HA antigen, prepared by immunization of virus particles or recombinant HA protein has been used to diagnose H5 virus infection in humans [8–12]. Although the produced mAbs recognized HA antigen of the clade of the immunized virus, it is difficult to obtain mAbs that recognize clades of other H5 viruses. Preparation of mAbs using a synthetic partial peptide as an immunizing antigen has also been reported [13,14]. By using this method, it is possible to obtain specific mAbs that recognize the

*Abbreviations:* HPAI, highly pathogenic avian influenza; mAbs, monoclonal antibodies; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay.

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target amino acid sequence of the synthetic partial peptide.

All recently disseminated H5 viruses are classified into HA clades 1 or 2. These clades have been further diverged into several subclades. H5N1 clade 1 virus was introduced and caused the first wave of major outbreaks in Vietnam in 2003 [2]. Thereafter, the WHO/OIE/FAO H5 Evolution Working Group identified three actively circulating H5 clades (2.1.3.2a, 2.2.1, and 2.3.4) [5]. H5N1 clade 2.1.3.2a viruses circulated in Indonesia from 2009 to 2014. H5N1 clade 2.2.1 viruses continued to evolve through 2013 and 2014 in Egypt. H5N1 clade 2.3.4 viruses have circulated in Asia, Europe, and North America since 2005. Furthermore, recently spreading avian H5N6 viruses, which cause sporadic infection in humans, and H5N8 avian viruses were classified into clade 2.3.4.4, which originated from H5N1 clade 2.3.4 viruses [5]. We hypothesized that it is possible to detect multiple clades of H5 viruses by using mAbs prepared from a synthetic partial peptide that contains a conserved amino acid sequence among the above representative H5 HA molecules.

This report describes the preparation of mAbs by immunization of a synthetic partial peptide designed to target a highly conserved region in H5 HA molecules and the establishment of a specific and highly sensitive antigen-capture ELISA using these cross-reactive mAbs to H5 viruses.

## 2. Materials and methods

### 2.1. Viruses

A/Vietnam/1194/2004 (H5N1, NIBRG-14, clade 1) virus, which possesses modified HA and neuraminidase (NA) genes derived from the A/Vietnam/1194/2004 virus on the backbone of six internal genes of A/Puerto Rico/8/34 virus, was provided by the National Institute for Biological Standards and Control (Potters Bar, UK). A/Indonesia/05/2005 (H5N1, IBCDC-RG2, clade 2.1.3.2), A/Egypt/N03072/2010 (H5N1, IDCDC-RG29, clade 2.2.1), and A/Anhui/01/2005 (H5N1, IBCDC-RG5, clade 2.3.4) were also developed and provided by the United States Centers for Disease Control and Prevention (Atlanta, GA, USA). A/duck/Hyogo/1/2016 (H5N6, NIIDRG-001, clade 2.3.4.4) was developed in our laboratory. All non-H5 viruses used in this study were obtained from a stockpile in our laboratory (Table 1). These viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs at 34 °C for 48 h and HA titer was determined using 0.5% turkey red blood cells. A/Vietnam/1194/2004 (NIBRG-14) virus was purified through a 20–60% sucrose gradient by ultracentrifugation. The virus was then resuspended in phosphate-buffered saline (PBS) and inactivated by treatment with 0.05% β-propiolactone at 4 °C for 18 h and then at 37 °C for 90 min. Total protein amount of purified NIBRG-14 virus was determined using BCA protein assay kit (Pierce) and then adjusted to a final concentration of 1 mg/mL.

**Table 1**
Influenza A viruses used in this study.

| Subtype | Virus[a] | HA titer |
|---|---|---|
| H5N1 (Clade 1) | A/Vietnam/1194/2004 (NIBRG-14) | 1024 |
| H5N1 (Clade 2.1.3.2) | A/Indonesia/05/2005 (IBCDC-RG2) | 256 |
| H5N1 (Clade 2.2.1) | A/Egypt/N03072/2010 (IDCDC-RG29) | 128 |
| H5N1 (Clade 2.3.4) | A/Anhui/1/2005 (IBCDC-RG5) | 512 |
| H5N6 (Clade 2.3.4.4) | A/duck/Hyogo/1/2016 (NIIDRG-001) | 64 |
| H1N1pdm | A/California/7/2009 (X-179A) | 256 |
| H2N3 | A/duck/Germany/1215/73 | 128 |
| H3N8 | A/duck/Ukraine/1/63 | 256 |
| H4N6 | A/duck/Czechoslovakia/56 | 256 |
| H6N2 | A/turkey/Massachusetts/3740/65 | 128 |
| H7N9 | A/Anhui/1/2013 (NIBRG-268) | 256 |
| H8N4 | A/turkey/Ontario/6118/68 | 128 |
| H9N2 | A/turkey/Wisconsin/1/66 | 256 |
| H10N7 | A/chicken/Germany/N/49 | 1024 |
| H11N6 | A/duck/England/56 | 256 |
| H12N5 | A/duck/Alberta/60/76 | 256 |
| H13N6 | A/gull/Maryland/704/77 | 128 |
| H14N5 | A/mallard/Gurjev/263/82 | 256 |
| H15N8 | A/duck/Australia/341/83 | 128 |

[a] Names in parentheses indicate candidate vaccine viruses.

### 2.2. Preparation and screening of mAbs specific for H5 HA antigen

BALB/c mice were immunized with the synthetic partial peptide, which contains a region conserved among various H5 HA molecules, to obtain mAbs specific for H5 HA antigen. To conjugate the synthetic partial peptide with keyhole limpet hemocyanin, cysteine was added at the C-terminal of the peptide. The mAb-producing hybridomas, in which mouse myeloma cells (P3U1) were fused with fibula lymphocytes harvested from immunized mice, were cloned by BEX Co., Ltd. mAb screening was conducted by ELISA using A/Vietnam/1194/2004 (NIBRG-14) virus antigen, which was lysed with 1% Triton X-100. The lysate was diluted with ELISA-coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6) and coated onto an ELISA plate (MaxiSorp; Thermo Fisher Scientific) at 4 °C overnight. After washing with PBS-Tween, the microplate was blocked with BlockAce (DS Pharma Biomedical Co., Ltd.) for 1 h at room temperature. The microplate was then washed and hybridoma culture supernatants were added and incubated for 1 h at room temperature. After another wash, horseradish peroxidase (HRP)-conjugated anti-mouse IgG mAb (1:8000; Invitrogen) was added to the wells and incubated for 1 h at room temperature. The plate was then washed and TMB substrate (ScyTek Laboratories, Inc.) was added. The reaction was stopped by adding TMB Stop Buffer (ScyTek Laboratories, Inc.), and the optical density at 450 nm ($OD_{450}$) was measured using a multi-well plate reader (SpectraMax Plus 384; Molecular Devices). After screening, mouse ascites that contained mAb were prepared and purified by BEX Co., Ltd. according to the conventional method.

### 2.3. Optimization of detergent condition for detection of H5 viruses

Sodium dodecyl sulfate (SDS) and Empigen BB [n-dodecyl-N,N-dimethylglycine] (Sigma-Aldrich) detergent were selected to determine the optimum detergent condition for the ELISA. A/Vietnam/1194/2004 (NIBRG-14) virus antigen was lysed with these detergents at several concentrations (0.1–0.0025%), diluted with ELISA-coating buffer, and incubated on coated ELISA plates at 4 °C overnight. The ELISA protocol was the same as that described above.

### 2.4. Antigen-capture ELISA

Antigen-capturing mAb (10 μg/mL) was coated onto an ELISA plate and incubated with ELISA-coating buffer at 4 °C overnight. The ELISA plate was then blocked with BlockAce, washed with PBS-Tween, and viral antigen was added to the wells with 0.025% SDS for 1 h at room temperature. After washing, HRP-conjugated mAb (10 μg/mL), for detection of viral antigen, was added to the wells with 0.025% SDS for 1 h at room temperature. After washing, TMB substrate was added, the reaction was stopped by adding TMB Stop Buffer, and the $OD_{450}$ was measured using a multi-well plate reader.