Characterization of monoclonal antibodies against H5N1 subtype highly pathogenic avian influenza virus and their use for analysis of viral antigenicity and diagnosis

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H5N1 亜型高病原性鳥インフルエンザウイルス に対する単一抗体の特徴づけとこれらの抗体の 抗原分析と診断への利用

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Abbreviations and unit abbreviations

Abbreviations

A	AF	allantoic fluid
	AGIDT	agar gel immunodiffusion test
	AI	avian influenza
	AIV	avian influenza virus
	APMV	avian paramyxovirus
B	BSL	biosafety level
	BSA	bovine serum albumin
C	CPE	cytopathic effect
	cDNA	complementary deoxyribonucleic acid
E	ELISA	enzyme-linked immunosorbent assay
F	FBS	fetal bovine serum
	FITC	fluorescein isothiocyanate
H	HA	hemagglutinin
	HIT	hemagglutination inhibition test
	HPAI	highly pathogenic avian influenza
	HPAIV	highly pathogenic avian influenza virus
I	IFAT	Indirect fluorescence antibody test
L	LAT	latex agglutination test
	LPAI	low pathogenic avian influenza
	LPAIV	low pathogenic avian influenza virus
M	mAb	monoclonal antibody
	MDCK	Madin-Darby canine kidney
	mRNA	messenger ribonucleic acid
	M1	matrix 1
	M2	matrix 2

N	NA	neuraminidase
	NP	nucleoprotein
	NS1	non-structural 1
	NS2	non-structural 2
P	PA	polymerase acidic
	PBS	phosphate buffered saline
	PB1	polymerase basic 1
	PB2	polymerase basic 2
R	RNA	ribonucleic acid
	RT-PCR	reverse transcription-polymerase chain reaction
	RDE	receptor destroying enzyme
S	SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
	SPF	specific pathogen free
T	TBS	tris buffered saline
V	VNT	virus neutralization test
W	WB	western blotting

Unit abbreviations

bp	base pair	mM	millimolar
°C	Celsius degree	min	minute
EID_{50}	50% egg infective dose	ml	milliliter
g	gravity	µl	microliter
h	hour	µm	micrometer
HAU	hemagglutinin unit	µg	microgram
kDa	kilodalton	%	percentage
M	molar	$TCID_{50}$	50% tissue culture infective dose

General introduction

Overview of AI

AI is a viral disease of birds caused by AIV. It was believed that first outbreak of AI occurred in Italy in 1878. This disease was historically known as fowl plague that caused rapidly severe outbreak with high mortality in chicken flocks (Perroncito, 1878). However, the etiology of the fowl plague could not be identified at that time. By 1955, the causative agent of the fowl plague of chickens was identified as influenza A virus, and this virus was related to influenza A viruses that infected humans and animals (Schäfer, 1955). Subsequently, the name "fowl plague" was replaced by HPAI (Alexander and Brown, 2000**)**. In addition to discovery of HPAIV, another pathotype of AIV that caused mild illness and low mortality in chickens was identified and termed as LPAIV. Currently, AIV can be subtypes, based on antigenicity of HA and NA into H1−H16 and N1−N9 subtypes, respectively (Webster et al., 1992). Most of AIVs isolated in wild birds and poultry are LPAIVs, whereas some strains of H5 and H7 subtypes isolated in poultry are HPAIV (Alexander, 2007). During 1957−1997, more than eighteen outbreaks of HPAI in chickens, turkeys and ducks occurring in America, Europe and Asia have been recorded (Lupiani and Reddy, 2009). The etiology of those outbreaks was related to H5 and H7 subtype viruses. Because of high mortality in poultry, AI also impacted on the poultry industry. Many infected poultry have been culled to control the AI outbreak, leading to economic losses of the poultry industry (Davison et al., 1999).

AIVs infect not only avian species but also humans. In 1997, the first outbreak of the human infection caused by H5N1 HPAIV was reported in Hong Kong. Six cases of 18 infected people died. This human H5N1 infection was associated with the direct and indirect contact with infected live or dead poultry (Chan, 2002). After the outbreak in Hong Kong, the H5N1 HPAIV has rapidly spread to more than 15 countries in Asia, Africa, and the Middle East. Although the number of the H5N1 infected people was relatively few as compared to seasonal human flu, the case fatality rate of the H5N1 virus infection in humans was very high (approximately 62%) (WHO, 2008). It concerns that wild aquatic birds are natural reservoirs of LPAIVs, and occasionally transmit the viruses to poultry. It is believed that some strains of H5 and H7 subtype LPAIVs may rarely mutate into HPAIVs while the viruses repeat infection and transmission among poultry. The mutated HPAIVs have caused deadly outbreaks in poultry, leading to economic losses in poultry industry. Moreover, some strains of H5 and H7 HPAIVs may acquire ability to cause pandemics in human population. Therefore, prompt laboratory diagnosis, and ceaseless monitoring of newly emerging and circulating AIVs are required to prevent or control AI.

Classification of AIV

AIV is an influenza A virus, a member of the genus *influenzavirus A*, belonging to the family *Orthomyxoviridae*. This family contains 7 genera, including *Influenzavirus A, B, C, Thogotovirus, Isavirus* (Bouvier and Palese, 2008)*, Quaranjavirus* (L'Vov D

et al., 2014), and newly *Influenzavirus* D has been identified (Collin et al., 2015). Influenza A viruses can infect many hosts including mammals and avian species, whereas influenza B viruses seem to infect only humans, and influenza C viruses have been found in humans and swine. Thogotoviruses are tick-borne viruses that have been isolated from animals and humans. Quaranjaviruses mainly infect arthropods and birds. Isaviruses can infect fish causing infectious salmon anemia disease. Newly, influenza D viruses that have been identified only in animals including bovine and swine cause mild disease in these animals.

Structure of AIV and its function of viral proteins

AIV is enveloped, segmented-negative sense, single-stranded RNA virus, containing eight segmented RNA molecules that encode at least 10 viral proteins, including structural and non-structural proteins. The structural proteins consist of surface and internal proteins that are found in virus particle. The surface proteins include HA, NA and M2 proteins, and the internal proteins comprise M1, NP, and the polymerase complex including PA, PB1 and PB2 proteins. Moreover, the non-structural proteins, NS1 and NS2, are not found in the viral particle, but these proteins are produced during viral replication process in the infected host cells (Lamb and Krug, 2001).

The HA proteins play a role for binding of the virus to sialic acid receptors on host cell membranes and entry into host cells by receptor-mediated endocytosis. This

protein is the major antigenic determinant targets for neutralizing antibodies (Steinhauer and Wharton, 1998). The function of the NA protein cleaves sialic acid on the host cell membrane to allow new progeny virions to be exited from the host cells (Colman, 1998). The M1 protein plays an essential role in viral assembly of viral replication process, whereas the M2 protein is an ion channel transmembrane protein that supports virus uncoating during initial stages of viral replication (Roberts et al., 1998). The NS1 protein has numerous functions such as a regulator of both viral mRNA splicing and translation, and it also plays an important role to antagonize the interferon production of host cells (Hale et al., 2008). The NS2 protein facilitates the export of newly synthesized ribonucleoproteins from the nucleus to cytoplasm, thus this protein is also called nuclear export protein (Paterson and Fodor, 2012). The PB1, PB2 and PA proteins form the viral polymerase complex, and their function generate viral mRNAs including synthesizing positive-sense complementary RNA templates, and transcribing the complementary RNA templates into the virion RNA segments that are combined into progeny viruses (Maier et al., 2008).

Subtypes and pathotypes of AIV

Influenza A viruses can be classified into subtypes based on antigenic properties of their two surface proteins (HA and NA). Currently, 18 HA (H1–H18) and 11 NA subtypes (N1–N11) of influenza A viruses have been identified. H1–H16, and N1–N9 subtypes of AIV have found in wild birds that are the naturally major reservoir hosts

(Webster et al., 1992), whereas the H16–H18, and N10 subtypes have been identified only in bats (Wu et al., 2014).

AIVs can be pathotyped as LPAIVs and HPAIVs, based on the pathogenicity in chickens. LPAIVs usually cause mild or asymptomatic infections, whereas HPAIVs do severe clinical signs with high morbidity and mortality rate in chickens (Pantin-Jackwood and Swayne, 2009). Now, most of the strains of H1–H16 subtypes of AIV are LPAIV, whereas some strains of H5 and H7 subtypes have been reported as HPAIV (Horimoto and Kawaoka, 1995; Senne et al., 1996).

Natural hosts of AIV

Generally, wild aquatic birds of the orders *Anseriformes* including ducks and geese, and *Charadriiformes* including gulls and shorebirds are natural reservoirs of LPAIV. The replication of the viruses is limited to the epithelial cells of the intestinal tract of the natural reservoirs (Webster et al., 2007). The infected reservoirs remain asymptomatic but can shed the viruses into the environment via feces. Occasionally, LPAIVs from the reservoirs can spread to poultry such as chickens, turkeys and quails, causing unapparent or mild respiratory disease. The clinical signs of those infected poultry persist around 10 days (Pantin-Jackwood and Swayne, 2009). Conversely, HPAIVs have not been maintained in the natural reservoirs, but it is believed that some strains of H5 and H7 subtype LPAIVs circulating in poultry populations could mutate into the HPAIVs (Garcia et al., 1996), and these mutated viruses cause severe disease

and death within 48–96 h, with almost 100% mortality rate in susceptible poultry (Horimoto and Kawaoka, 1995).

Antigenic variation

One of the unique characteristics of influenza A virus is antigenic variations. Antigenic variations compose of antigenic drift or antigenic shift and occurs mainly on two surface glycoproteins of the virus (HA and NA). Antigenic drift is minor or small changes in the genes of influenza A viruses, which occurs continually over time as the virus replicates. The accumulation of the point mutations in the viral genes may result in amino acid changes in the antigenic sites. Although the antigenic sites are altered, the subtypes of these mutated viruses are not changed (same HA and NA). The antigenic drift results in viral escape from the host immune response (Escorcia et al., 2008). Existing antibodies against the wild type viruses cannot recognize the mutated viruses. Thus, the antigenic drift is responsible for influenza epidemics.

Antigenic shift is major changes in the genes of influenza viruses. This antigenic variation only occurs with influenza A virus because it has a wide host range and has segmented genomes. The genetic reassortment causes new HA and NA subtype viruses when two influenza A strains from different species can simultaneously infect the same target cells during viral replication (Scholtissek, 1995). Because hosts have no neutralizing antibodies to protect the infection, these new viruses are able to spread very quickly. The antigenic shift is responsible for influenza pandemics.

Evolution of H5N1 HPAIV

Since outbreaks of the H5N1 HPAIV in Hong Kong in 1997, the H5N1 HPAIVs have been identified in more than 60 countries including Asia, Africa and Europe. The H5N1 HPAIV initially categorized as genotypes were then identified into several lineages. However, the genotyping system was not suitable, because the H5N1 viruses have undergone evolution rapidly and continuously. A unified nomenclature of the H5N1 HPAIVs based on the phylogenetic tree of HA genes was used instead, and then phylogenetically distinct clades (0–9) have been identified so far. Currently, some clades of the H5N1 HPAIVs have not spread (clades 3–6, and 8–9), whereas other clades are continuously evolving (clades 1, 2 and 7) that are further classified into new second-order (clades 1.1, 2.2 and 7.1), third-order (clades 1.1.1 and 2.2.1), fourth-order (clades 2.2.2.1, 2.3.2.1 and 2.3.4.1) and fifth-order clade groups (clades 2.3.2.1a, 2.3.2.1b, 2.3.2.1c) (WHO/OIE/FAO, 2014). H5N1 clade 1 viruses have been detected mainly in Asia including Cambodia, Thailand, and Vietnam, whereas the clade 2 viruses had been spread into many regions including Asia, Europe, Middle East and Africa (Nguyen et al., 2012). Moreover, the clade 2 viruses have the highest diversity and had potential for human infection. Several subclades of the H5N1 viruses such as clade 2.1.3.2a have a geographically restricted distribution in Indonesia (Smith et al., 2015). The clade 2.2.1 viruses are still circulating in Egypt, and the newly emerged clade 2.2.1.2 viruses were found in this country (Arafa et al., 2016). Recently, the clades 2.3.2.1a, 2.3.2.1c and 2.3.4.4 viruses were detected in Asia (Smith et al., 2015),

and the clade 2.3.2.1c viruses were first detected in Africa (Tassoni et al., 2016). Additionally, the clade 7 viruses were found in China and Vietnam (Le and Nguyen, 2014).

Laboratory diagnosis of H5N1 HPAIV

Generally, the methods of laboratory diagnosis for detecting H5N1 infection in chickens can be categorized into those that directly detect the viral antigens using specific antibodies and viral RNA by molecular techniques such as RT-PCR, those that isolate viruses using cell cultures or embryonated chicken eggs, and those that detect specific antibodies in infected animals, generally using HIT and AGIDT (Beard, 1970; Charlton et al., 2009; Marche and van den Berg, 2010; Spackman et al., 2008; Spackman and Killian, 2014; Suarez et al., 2007).

Currently, the isolation of H5N1 virus using cell cultures or embryonated chicken eggs is a highly sensitive method (gold standard) for detecting viruses from clinical specimens (Spackman and Killian, 2014). MDCK cells and 9−11 days old embryonated chicken eggs are usually selected for viral isolation. After inoculation of clinical samples into the MDCK cells or the embryonated chicken eggs, the H5N1 virus can replicate within 7-day post inoculation. Supernatant fluids from the cell cultures or AFs of the infected embryonated chicken eggs were collected and were further subtyped with other methods such as conventional RT-PCR, real-time RT-PCR and HITs. In addition, infected MDCK cells can be tested to detect viral antigens in the

infected cells using specific antibodies. However, there are several disadvantages of the isolation of HPAIV in these methods (Gangadharan et al., 2013). BSL-3 facilities with special equipment and high maintenance costs are required to isolate HPAIV. In addition, it may take time (around 5–7 days) to obtain the results of the isolation and subtyping of the viruses.

Although there are many molecular methods to detect influenza A virus, conventional RT-PCR and real-time RT-PCR have been developed for detecting H5N1 infection. Both type RT-PCRs may have more sensitivity comparable to the virus isolation (Ng et al., 2006; Chen et al., 2007a). Both molecular methods can directly detect the viral RNA in clinical samples. Although both methods can detect the H5N1 viruses from tracheal and oropharyngeal swabs of turkeys and chickens, the viruses in feces of those animals may not be detected. Some PCR inhibitors may be contained in the fecal sample, resulting in false-negative of RT-PCR result (Das et al., 2006).

Direct detection of viral antigens in clinical specimens is the best way to diagnose H5N1 infection. There are several rapid diagnostic tests that can directly detect influenza A viruses in clinical samples (Woolcock and Cardona, 2005). Most of the tests use mAbs to NP of the influenza A virus to detect the viral antigens in the clinical samples. The tests have the advantage of rapidly detecting the viral viruses as short as around 15 min. However, those tests are able to detect only influenza A viruses, while they cannot be used to differentiate or identify subtypes of the viruses (Wang and Taubenberger, 2010). Detection of H5 HA antigens using specific mAbs may be more

useful than the currently available tests as mentioned above to detect the NP of the influenza A virus. Recently, rapid diagnostic tests using mAbs against H5N1 HA proteins to immunologically detect H5N1 viruses have been reported (Du et al., 2009; Wada et al., 2011; Ohnishi et al., 2012). However, there have been no reports as to whether the mAbs used in those tests could detect currently circulating H5N1 clade viruses. Therefore, production of new anti-H5 HA mAbs and development of diagnostic tests using these mAbs may be required.

AGIDT and HIT are serological assays that have been widely and routinely used to detect specific antibodies to AIV in chicken flocks (Wibawa et al., 2012). The AGIDT detects specific IgM or IgG against AIVs, based on formation of the antigen-antibody complex and precipitation line that can be observed through an agar gel. The AGIDT is an inexpensive test with moderate sensitivity. This test may detect the specific antibody around 5 days post infection (Spackman et al., 2008). However, if HPAIVs infect poultry and cause sudden death within 1-2 days post infection, false negative may occur. In addition, the AGIDT can identify influenza A virus infection, but not AIV subtypes.

HITs detect a specific IgM and IgG that inhibits the binding of the virus to red blood cells via the HA protein, causing non-agglutination. For the detection of H5N1 infection, paired sera are required to compare the specific antibody titers between convalescent and acute sera, which demonstrates more than 4-fold rising of the titers (WHO, 2005). In addition, the HIT can identify the HA subtype of the AIVs. This assay is also a cheap test and has highly sensitivity and specificity. However, in case HPAIVs

kill the infected chickens before collecting the convalescent serum samples, it may lead to misdiagnosis as false-negative. Moreover, newly emerging viruses may not be detected by the HIT with reference antisera, due to mutation of the viruses leading to change in binding properties of the HA protein to antibodies.

Objectives of the present study

It is known that the HA protein is the principal surface antigen on the influenza viruses. The surface antigens play an important role to induce neutralizing antibody, and therefore a protective immune response (Tamura et al., 2005; Kaverin et al., 2007; Wu et al., 2008). However, phylogenetic analysis of the HA genes showed that the H5N1 HPAIVs have undergone considerable genetic changes resulting in 10 viral clades (WHO/OIE/FAO, 2008). These genetic changes may associate with antigenic evolution of the influenza A viruses as the viruses escape from neutralizing antibodies and involve transition from one antigenic cluster to another (Smith et al., 2004; Ndifon et al., 2009). Therefore, genetic and antigenic characterization of H5 viruses is important for control the disease.

Antigenic characterization of AIVs using antibodies has been widely applied to taxonomic classification of the virus and subtyping. This has contributed much toward a better understanding of the structural constituents of AIV. Moreover, highly specific mAbs are especially advantageous for analysis of the antigen determinants present on particular molecules, or localization of antigens in infected materials. MAbs against the

H5N1 viruses could be also very useful and powerful tools to characterize antigenic variant isolates. Obtained data can provide useful information to better understand the isolated strains and improve our knowledge on epidemiology of AIVs. However, H5 viruses with complex biological characteristics have undergone continued evolution in poultry and wild bird populations, and post concern about the challenge in diagnosis, surveillance and vaccine design (Smith et al., 2006; Chen, 2009). Thus, antigenic characterization of H5 viruses using mAbs may help to improve diagnosis, surveillance and vaccine design.

In the present study, the objectives were as follows:

1. To produce mAbs against H5N1 viruses isolated in Japan and chracterize the produced mAbs
2. To characterize various H5 subtype viruses isolated in Japan and Vietnam, and analyze a broad cross-clade epitope(s) on the HA protein of the H5N1 viruses using the produced mAbs
3. To develop LAT (latex agglutination test) for detecting the H5 subtype viruses

Chapter I

Production of monoclonal antibodies against H5N1 highly pathogenic avian influenza viruses

1.1 Introduction

In 1997, the first outbreak of H5N1 HPAIV was recorded in chicken populations in Hong Kong. In this outbreak, unexpectedly, the 18 people were infected and the 6 dead cases were reported. More than 1.5 million chickens were culled to control the outbreak in Hong Kong (Chan, 2002). However, after the recurrence of the outbreak in Hong Kong and the main land of China in 2003, the HPAIVs have caused deadly outbreaks of poultry in many regions including Asia, Africa and Europe (Brown, 2010), and occasionally they have also caused serious diseases highly associated with mortality in humans, leading to an increased awareness of a pandemic outbreak (Amendola et al., 2011).

In Japan, there were six outbreaks caused by HPAIVs in birds during 2004 to 2016. The first to fourth outbreaks were caused by some strains of clades 2.5 (Mase et al., 2005), 2.2 (Sugiura et al., 2009), 2.3.2.1 (Uchida et al., 2008) and 2.3.2.1c (Sakoda et al., 2012; Bui et al., 2013) H5N1 HPAIV, respectively. The fifth outbreak was caused by the H5N8 HPAIV (clade 2.3.4.4) in 2014 (Kanehira et al., 2015). Recently, the

newly emerging H5N6 HPAIV related to clade 2.3.4.4 caused outbreaks in 2016 (Okamatsu et al., 2017).

Rapid diagnosis of H5N1 infection is important to prevent the outbreaks caused by the H5N1 HPAIV. Although there are several diagnostic techniques for identifying H5N1 viruses, including viral isolation followed by serological tests to determine HA and NA subtypes or RT-PCR, those techniques have some disadvantages in terms of high costs, rapidness, necessary expertise, and the need for a BSL-3 laboratory (Suarez et al., 2007).

Antigen detections with specific mAbs are suitable for a rapid diagnosis of H5N1 infection. MAbs against NP of influenza A virus are available for diagnosis and widely used in field investigations as rapid diagnostic tests for H5N1 HPAIV (Chan et al. 2002). However, since these mAbs recognize the NP which antigenicity is common among influenza A viruses, they can not differentiate HA or NA subtypes of influenza A virus (Abdel-Ghafar et al., 2008). Therefore, mAbs against HA proteins of H5 subtype AIV (anti-H5 mAbs) are more valuable than the mAbs against NP in rapid diagnosis of H5N1 infections.

Rapid diagnostic tests using anti-H5 mAbs to immunologically detect H5N1 viruses have been reported (Chen et al., 2007; Miyagawa et al., 2011; Sakurai et al., 2013; Kobayashi-Ishihara et al., 2014; Wada et al., 2011). However, there have been no reports as to whether the anti-H5 mAbs used in these tests could detect currently circulating H5N1 clade viruses. Although Uchid et al. (2008) produced two mAbs against the Ck/Yamaguchi/7/04 strain (clade 2.5) that was also used as immunogen in

this study, their mAbs could not recognize two of five strains belonging to clade 1. Therefore, it is important to continuously make efforts to search for anti-H5 mAbs with broad reactivity with the epitopes on HA proteins in the development of diagnostic systems that are highly sensitive and can rapidly detect H5N1 HPAIVs, especially the H5N1 viruses that are currently circulating and the newly emerging H5N1 clades.

In this chapter, the production of anti-H5 mAbs against Ck/Yamaguchi/7/04 (clades 2.5) and Ck/Miyazaki/K11/07 (clade 2.2) of H5N1 HPAIV isolated in Japan were described, and the produced anti-H5 mAbs were further characterized.

1.2. Materials and methods

Viruses

Ck/VN-HT/33/03 (clade 1), Ck/VN-HT/30/10 (clade 2.3.2.1a), Mdk /VN-HN/77/07 (clade 2.3.4) H5N1 HPAIVs isolated in Vietnam were kindly afforded by the National Institute of Veterinary Research (NIVR), Vietnam. Ck/Yamaguchi/7/04 (clade 2.5), Ck/Miyazaki/K11/07 (clade 2.2) H5N1 HPAIVs isolated in Japan were kindly afforded by the National Institute of Animal Health, Japan. All the viruses were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs. The infective AFs were collected and kept at -80 °C until use. All experiments with the viruses were conducted in a BSL-3 laboratory approved by the relevant committee at the institution.

Cell culture

Mouse myeloma cells (SP2/0) that were kindly provided by Dr. X. Xuan, Obihiro University of Agriculture and Veterinary Medicine were cultured in the growth medium RPMI 1640 (Nissui Pharmaceutical Co., Ltd.), supplemented with 10% FBS, 10% Daigo's GF 21 growth factor (Wako Junyaku, Tokyo, Japan), and OPI Media Supplement (Sigma-Aldrich Japan, Tokyo, Japan) at 37°C in the presence of 5% CO_2.

Preparation of H5N1 viruses for immunization to mice

Ck/Yamaguchi/7/04 and Ck/Miyazaki/K11/07 viruses were inactivated with neutral buffered formalin at a final concentration of 0.1%. The inactivated H5N1 viruses were purified through a 30% and 60% discontinuous sucrose density gradient as described previously (Imai et al., 2012). In some experiments, the purified inactivated H5N1 viruses were lysed with 0.4% Triton-X100 and 0.2M KCl, followed by incubating at 4°C for 30 min before the use for immunization to mice.

Production of mAbs

Two female BALB/c mice (8-week-old) were immunized with 0.2 ml of the inactivated purified H5N1 viruses or the inactivated and lysed ones, resepectively, emulsified in a squalene-based adjuvant (AddaVAX™, InvivoGen, San Diego, CA) of the inoculum at a HA titer of 1,280 by intraperitoneal injection three times at intervals of 2–3 weeks. Final immunization was performed with the same viruses in 0.1 ml of

PBS (pH 7.4) intravenously without the adjuvant three days before cell fusion. Fusion of the mouse spleen cells with the SP2/0 cells was performed, and the fused cells were cultivated as previously described (Trinh et al., 2015), except that an OPI Media Supplement and an Endothelial Cell Growth Supplement (Sigma-Aldrich Japan) were applied. Antibody-producing hybridomas were first screened using an ELISA and then anti-HA antibody-producing hybridomas were selected using a HIT, as described below. The anti-HA antibody-producing hybridomas were cloned by a single cell pick up method, and these hybridomas were injected into the peritoneal cavity of mice to produce ascitic fluids containing mAbs, as described previously (Harlow and Lane, 1988). The IgG fraction of the ascitic fluids were precipitated with a 50% saturated ammonium sulphate solution, followed by dialyzing against the PBS. All mouse studies were conducted in compliance with the institutional rules for the care and use of laboratory animals using protocols approved by the relevant committee at the institution.

ELISA

ELISA procedures were modified from those described by Nishikawa et al. (1983). Briefly, the purified, formalin-inactivated Ck/Yamaguchi/7/04 or Ck/Miyazaki/K11/07 was lysed with 0.4% Triton-X100 and 0.2M KCl, followed by incubating at 4°C for 30 min. The lysed viruses were diluted in carbonate-bicarbonate buffer (pH 9.6) to a concentration of 20 μg/ml and coated at 50 μl/well of 96-well ELISA microplates

(Thermo Fisher Scientific, K.K., Yokohama, Japan). The coated plates were blocked with 3% BSA in PBS at 37°C for 2 h, and washed with PBS (pH 7.4) containing 0.05% Tween-20 (PBS-T). Culture supernatants of the hybridomas or ascitic fluids were added to each well (50 µl/well) followed by incubation at 37°C for 1 h. After washing with PBS-T, peroxidase-conjugated goat anti-mouse polyvalent Igs (IgA, IgG and IgM) (Sigma-Aldrich Japan) were added to each well at 50 µl/well followed by incubating at 37°C for 1 h. After washing, TMB Substrate Reagents (BD Bioscience Pharmigen, San Diego, CA) were added to each well. The optical density of the samples were measured at 450 nm by an ELISA reader (TECAN, Kawasaki, Japan).

WB analysis

The viral antigens (Ck/Yamaguchi/7/04 or Ck/Miyazaki/K11/07) were separated by 12% SDS-PAGE with reducing condition, and transferred to polyvinylidene difluoride membranes using a Trans-Blot® SD Semi-dry Electrophoretic Transfer Cell (BIO-RAD, Hercules, CA). The membranes were blocked with 3% BSA in TBS (pH 7.5) at room temperature for 1.5 h and washed once with 0.05% Tween-20 in TBS (TBS-T). The membranes were incubated with ascitic fluids containing mAbs at 37°C for 2 h and washed three times with TBS-T. Anti-H5 mAb to A/Vietnam/1203/04 (H5N1), (Rockland Immunochemicals, Limerick, PA) was used as positive control. Peroxidase-conjugated goat anti-mouse polyvalent Igs (IgA, IgG and IgM) were used as secondary antibodies and incubated on the membranes at 37°C for 1 h. The

conjugated mouse antibodies were visualized with ECL Plus Western Blotting Detecion system s (GE Healthcare UK Ltd., Buckinghamshire, UK) and captured using a LAS-3000 Image analyzer (Fujifilm, Tokyo, Japan).

IFAT

IFATs were used to determine the mAbs isotypes. Briefly, 95% confluent MDCK cells on the 8-well chamber slides (Thermos Fisher Scientific, K.K.) were inoculated with the Ck/Yamaguchi/7/04 or Ck/Miyazaki/K11/07 and incubated for 18 h. The infected cells were fixed with cold acetone for 10 min, and incubated with mAbs (1:100) for 1h. The infected cells were then incubated with FITC or Rhodamine-conjugated anti-mouse IgG subclasses (IgG1, IgG2a, or IgG2b), (Rockland Immunochemicals). The fluorescent signal was observed under a fluorescence microscope (Biorvo BZ-9000, Keyence, Japan).

HIT

HITs were performed according to Manual on Animal Influenza Diagnosis and Surveillance (WHO, 2005) using 0.5% chicken red blood cells. Briefly, serial 2-fold dilutions of the culture supernatant of hybridomas or the RDE-treated-ascitic fluids containing mAbs were mixed with 4 HAU/25 μl of the viruses, and incubated at room temperature for 30 min. Chicken red blood cells were then added to the mixtures, and

the reciprocal of the highest dilution of mAb that completely inhibited hemagglutination was taken as the HI titer.

VNT

VNTs with a constant mAb and diluted virus condition (α-neutralizing procedure) were performed. Briefly, serial 10-fold stepwise dilutions of the virus were mixed with ascetic fluids containing mAb (1:10) or viral growth medium (VGM) (Imai et al., 2012), and the mixtures were incubated for 2 h at 37°C. Then, the mixtures were added to the MDCK cells grown in 96-well microplates and incubated for 2 h. After washing with VGM, the mixtures were replaced with VGM, and the cells were cultured for 4 days. The virus titer (log_{10} $TCID_{50}$) of the mixture was determined by the Behrens-Kärber method (Behrens and Kärber, 1934). The neutralizing index was calculated by subtracting the log_{10} $TCID_{50}$ of the mAb-virus mixture from that of the virus control. When the neutralizing index was higher than 0.7, the mAb was regarded to have neutralizing activity.

1.3. Results

Establishment of hybridomas producing mAbs against H5N1 HPAIVs

At first, inactivated purified H5N1 viruses (Ck/Yamaguchi/7/04 and Ck/Miyazaki/K11/07 strains) were lysed with lysis buffer (0.4% Triton-X100 and 0.2M KCl) and used for immunization to mice. However, hybridomas secreting anti-

H5 mAbs against the Ck/Yamaguchi/7/04 strain were not obtained, except that only one anti-H5 mAb against the Ck/Miyazaki/K11/07 strain was established (data not shown). Therefore, the whole inactivated purified H5N1 virus was used as immunogen instead of the lysed virus. Consequently, after screening by ELISA and HITs, two hybridomas secreting anti-H5 mAbs against the Ck/Yamaguchi/7/04 strain and one hybridoma secreting the anti-H5 mAb against the Ck/Miyazaki/K11/07 strain were generated.

Three hybridomas producing mAbs against the Ck/Yamaguchi/7/04 (clade 2.5) and Ck/Miyazaki/K11/07 (clade 2.2) strains were designated as 3B5.1 and 3B5.2, and 1G5, respectively (Table 1.1). Hemagglutination inhibition of the mAbs (3B5.1, 3B5.2 and 1G5) were examined for their homologous reactivity against the Ck/Yamaguchi/7/04 (clade 2.5) or Ck/Miyazaki/K11/07 (clade 2.2) strains of H5N1 HPAIV using HITs. The 3B5.1 (HI titers 2,560) and 3B5.2 mAbs (HI titers 640) to the clade 2.5 strain showed higher HI titers than the 1G5 mAb (HI titers 320) to the clade 2.2 strain of H5N1 HPAIV (Table 1.1).

Additionally, the neutralizing reactivity against their homologous strains of the mAbs was determined. The results demonstrated that the 3B5.1 and 3B5.2 mAbs to Ck/Yamaguchi/7/04 (clade 2.5) had neutralizing indexes higher than the 1G5 mAb to Ck/Miyazaki/K11/07 (clade 2.2) (Table 1.1).

Isotyping of mAbs and WB analysis

The isotypes of the mAbs were characterized by IFAT using FITC- or Rhodamine-conjugated anti-mouse IgG subclasses (IgG1, IgG2a, or IgG2b). All three mAbs (3B5.1, 3B5.2 and 1G5) were isotyped as IgG1 (Table 1.1).

WB analysis of mAbs was performed with their homologous strains. The results of WB showed that the 3B5.2 and 1G5 mAbs reacted only to a 75 kD protein band of the HA0 under non-reducing conditions. These results indicated that these mAbs recognized the discontinuous (conformational) epitope of the HA proteins. However, the 3B5.1 mAb recognized both the 75 and 50 kD proteins of the HA (HA0 and HA1) under non-reducing and reducing conditions, respectively (Fig. 1.1). This suggested that the 3B5.1 mAb specifically recognized linear and conformational epitopes of the HA proteins.

Cross-reactivity of the mAbs with heterologous strains of H5N1 HPAIV

Cross-reactivity of the mAbs (3B5.1, 3B5.2 and 1G5) with heterologous strains of H5N1 HPAIV isolated in Japan and Vietnam was examined using HITs. Interestingly, the 3B5.1 and 3B5.2 mAbs recognized all the strains of six heterologous clades (1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1c, 2.3.4). The HI titers of the 3B5.1 mAb to those heterologous H5N1 viruses ranged from 160–640, and those of the 3B5.2 mAb ranged from 20–640 (Table 1.2). In contrast, the 1G5 mAb recognized only clade 1 and 2.5 viruses with the HI titers ranging from 20–80.

1.4. Discussion

Although the inactivated purified H5N1 viruses (Ck/Yamaguchi/7/04 and Ck/Miyazaki/K11/07 strains) lysed with the lysis buffer (0.4% Triton-X100 and 0.2M KCl) for immunization of mice were first used, no hybridomas secreting anti-H5 mAbs were obtained except one hybridoma against the Ck/Miyazaki/K11/07 strain. In contrast, Nishikawa et al. (1983, 1987) successfully produced many mAbs using Newcastle disease viruses treated with the similar lysis buffer as immunogen. Although the reason of the discrepancy in mAb production seen between the two viruses is unclear, it was suggested that the lysis buffer might destroy immunogenicity of the HA proteins of the H5N1 virus, leading to reducing the number of anti-H5 mAb-hybridomas produced.

On the other hand, two neutralizing anti-H5 mAbs (3B5.1 and 3B5.2) against the clade 2.5 virus (Ck/Yamaguchi/7/04), and one (1G5 mAb) against the clade 2.2 virus (Ck/Miyazaki/K11/07) were successfully established when the whole inactivated purified viruses were used (Table 1.1). Interestingly, the 3B5.1 and 3B5.2 mAbs reacted to all the 6 H5N1 viruses tested of clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1c, and 2.3.4 isolated in Japan and Vietnam, suggesting the possibility that the mAbs possess broad cross-reactivity to various clades of H5 subtype HPAIV, whereas the 1G5 mAb did not react to all clades examined (Table 1.2).

Although anti-H5 mAbs against clade 2.5 strains of H5N1 HPAIV (Ck/Yamaguchi/7/04, Crow/Kyoto/53/04) isolated in Japan were established (Uchida et al., 2008; Du et al., 2009), a clade 2.3.2.1 H5N1 HPAIV strain (Who.sw./Akita/1/08) was not recognized by the mAbs (Uchida et al., 2008), and the information on the reactivity of the anti-H5 mAbs to the newly emerging clade H5N1 HPAIVs (ex. 2.3.2.1a and 2.3.2.1c) was unknown (Uchida et al., 2008; Du et al., 2009). In contrast to the mAbs reported by Uchida et al. (2008), the mAbs of this study (3B5.1 and 3B5.2) demonstrated the reactivity to the Who.sw./Hokkaido/1/08 strain (clade 2.3.2.1) that was isolated during the same outbreak in which the Who.sw./Akita/1/08 strain (clade 2.3.2.1) was isolated (Table 1.2). Thus, since epitopes on the HA proteins recognized by mAbs are various even among the H5N1 strains belonging to the same clade, exploring of the mAbs recognizing the conserved epitopes on the HA proteins could be important for development of a mAb-based diagnosis.

The outbreaks caused by H5N1 HPAIVs are still serious problems that have an impact on both animal and public health. Therefore, rapid diagnostic tests are essential for the detection and monitoring of circulating H5N1 viruses to prevent an outbreak. This study indicated that the 3B5.1 and 3B5.2 mAbs have potential for this application. However, broad cross-reactivity to other clades of the H5N1 viruses should be further evaluated. Additionally, the epitopes of H5 proteins recognized by the 3B5.1 3B5.2 mAbs should be determined as well. If the recognized H5 epitopes are found in other

clade H5N1 viruses, the 3B5.1 and 3B5.2 mAbs may be applied to detection of all clade H5N1 viruses.

1.5. Summary

H5N1 HPAIVs are a threat to both animal and public health; therefore, the specific and rapid detection of the virus is required for prompt disease control. In this chapter, two neutralizing anti-H5 mAbs (3B5.1, 3B5.2) using the clade 2.5 H5N1 HPAIV (Ck/Yamaguchi/7/04) and one neutralizing anti-H5 mAb using the clade 2.2 (1G5) H5N1 HPAIV (Ck/Miyazaki/K11/07), which viruses were isolated in Japan, were successfully established. The anti-H5 mAbs were IgG1 subclass and were able to recognize the HA proteins of the H5N1 viruses examined. The 3B5.2 and 1G5 mAbs recognized the conformational epitope on the HA proteins (HA0), whereas the 3B5.1 recognized the linear and conformational epitope on the HA proteins (HA1 and HA0). The 3B5.1 and 3B5.2 mAbs against the clade 2.5 virus showed cross-reactivity to each strain examined from the 6 clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1c and 2.3.4 isolated in Japan and Vietnam, suggesting the mAbs may have broad cross-reactivity against other H5N1 clades. Conversely, the 1G5 mAb against the clade 2.2 virus showed reactivity only to clades 1 and 2.5 strains. Therefore, the 3B5.1 and 3B5.2 mAbs may be useful for the specific detection of H5N1 HPAIVs currently circulating in the field.

Table 1.1 Characterization of anti-H5 mAbs to H5N1 HPAIVs

Immunogen			HIT*	VNT*
Strain (Clade)	mAbs	Isotypes	HI titers	Neutralizing index
Ck/Yamaguchi/7/04 (Clade 2.5)	3B5.1	IgG1	2,560	>7
	3B5.2	IgG1	640	6
Ck/Miyazaki/K11/07 (Clade 2.2)	1G5	IgG1	320	3.75

*The HIT and VNT were performed using the homologous viral strain.

Table 1.2 Cross-reactivity of anti-H5 mAbs to different clade strains of the H5N1 HPAIV

Clades	Strains	Country	HI titers of mAb		
			3B5.1	3B5.2	1G5
1	Ck/VN-HT/33/03	Vietnam	320	20	40
2.2	Ck/Miyazaki/K11/07	Japan	640	640	320*
2.3.2.1	Who.sw./Hokkaido/1/08	Japan	320	20	<10
2.3.2.1a	Ck/VN-HT/30/10	Vietnam	160	20	<10
2.3.2.1c	Who.sw./Hamanaka/11	Japan	320	20	<10
2.3.4	Mdk /VN-HN/77/07	Vietnam	160	80	<10
2.5	Ck/Yamaguchi/7/04	Japan	2,560*	640*	80

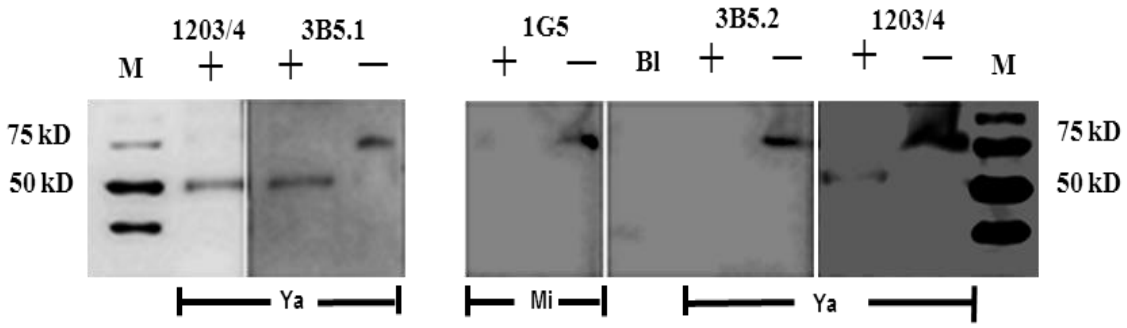

Fig. 1.1 Identification of anti-H5 mAbs by WB analysis using the inactivated purified Ck/Yamaguchi/7/04 (clade 2.5) and Ck/Miyazaki/K11/07 (clade 2.2) strains as the antigens under reducing and non-reducing conditions. One anti-H5 mAb (3B5.1) had reactivity to the HA1 proteins (50 kDa) under a reducing condition and to the HA proteins (75 kDa) under a non-reducing condition of Ck/Yamaguchi/7/04. Two anti-H5 mAbs (3B5.2 and 1G5) had reactivity to the HA proteins (75 kDa) of Ck/Yamaguchi/7/04 and Ck/Miyazaki/K11/07. 1203/4: anti-H5 mAb to A/Vietnam/1203/4; M: molecular marker; +: reducing condition; -: non-reducing condition; Bl: Blank; Ya: inactivated purified Ck/Yamaguchi/7/04 antigen; Mi: inactivated purified Ck/Miyazaki/K11/07 antigen.

Chapter II

Characterization of cross-clade mAbs against H5N1 HPAIV and their application to the antigenic analysis of diverse H5 subtype viruses

2.1 Introduction

The HA protein is an antigenic glycoprotein which is found on the surface of influenza A viruses. Function of this glycoprotein is responsible for binding the viruses to the host cells (Wilson et al., 1981). Neutralizing antibodies to the HA protein can bind this glycoprotein and prevent entry of the viruses to the host cells (Han and Marasco, 2011).

To evade host immune response, influenza A viruses have to evolve overtime as the virus replicates. This phenomenon is called "antigenic drift" (Suarez and Schultz-Cherry, 2000). A mutation of the HA gene commonly occurs, resulting in antigenic modification of the HA proteins. The mutated HA proteins could not be recognized by the existing neutralizing antibodies.

HPAIVs have been classified as clades 0–9 based on the phylogenetic analysis of their HA genes (WHO/OIE/FAO, 2008). However, clades 0, 3, 4, 5, 6, 8, and 9 have

not been recently detected. Only the clades 1, 2 and 7 have continued evolution of their HA genes, leading to the emergence of new phylogenetic groups (WHO/OIE/FAO, 2012, 2014; Smith et al., 2015). Especially, the clade 2 viruses have more diversity than the other clades, and have been phylogenetically classified into second-order (clades 2.1–2.5), third-order (2.1.1–2.1.3, 2.2.1, 2.2.2, 2.3.1–2.3.4), fourth-order (2.1.3.1–2.1.3.3, 2.2.1.1, 2.2.1.2, 2.2.2.1, 2.3.2.1 and 2.3.4.1–2.3.4.4), and fifth-order groups (2.1.3.2a, b, 2.2.1.1a, and 2.3.2.1a, b, c). Some new fifth-order clade group strains (2.3.2.1a, 2.3.2.1c) have caused outbreaks in Asia. Recently, the clade 2.3.2.1a and 2.3.2.c viruses have been detected in the outbreaks of Africa and Europe (WHO, 2014, 2015, 2016). However, not all strains of H5 subtype are highly pathogenic: only a small number of H5 LPAIV has mutated to the HPAIV (Swayne, 2007). LPAIVs can be classified into Eurasian and American clades (EA-nonGsGD and Am-nonGsGD) that were originated in Asia and America, respectively (Spackman et al., 2007). Therefore, an accurate, rapid, and specific diagnostic system for HPAIV and LPAIV infection is essential for the establishment of prompt disease control measures. For this purpose, it is important to continuously search for anti-H5 mAbs with broad reactivity with the epitopes on HA proteins in the development of diagnostic systems that are highly sensitive and can rapidly detect H5 subtype viruses, especially the H5N1 viruses that are currently circulating and newly emerging H5N1 clades.

Recently, anti-H5 mouse and human mAbs recognized broad cross-reactive epitopes of several clade viruses: 1, 2.1.3.2, 2.2, and 2.3.4 (Kobayashi-Ishihara et al., 2014), 0, 1, 2.2, 2.3.2.1, and 2.3.4 (Wu et al., 2014), and 0–9, except for clade 7.2 (Hu

et al., 2012). Unfortunately, however, the newly circulating H5N1 clades 2.3.2.1a and 2.3.2.1c were not evaluated using those mAbs (Hu et al., 2012; Kobayashi-Ishihara et al., 2014; Wu et al., 2014). Recently, Xiong et al. (2015) reported that the broad cross-reactivity anti-H5 human mAbs reacted to clade 0, 1 2.1.3, 2.2, 2.2.1, 2.3.2, 2.3.2.1a, 2.3.4, and 2.5 viruses; however, some strains of clade 2.3.2.1a were not recognized by the mAbs.

In this chapter, three neutralizing anti-H5 mAbs (3B5.1, 3B5.2 and 1G5) described in Chapter I were evaluated for cross-clade reactivity using various H5N1 HPAIV strains isolated in Japan and Vietnam in addition to Eurasian-H5 (clade EA-nonGsGD) and American-H5 LPAIV strains (clade Am-nonGsGD). Additionally, possible antigenic sites on the HA proteins recognized by the anti-H5 mAbs were analyzed.

2.2 Materials and methods

Viruses and cells

A total of 39 strains of influenza A viruses, including different clades of the H5N1 HPAIVs isolated in Japan and Vietnam, escape mutant H5N1 viruses, LPAIVs, and human influenza A virus were used in this study (Table 2.1). All viruses were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs. All experiments with the viruses were conducted in a BSL-3 laboratory approved by the relevant committee at the institution.

MDCK cells were cultured in 10% FBS in Dulbecco's modified Eagle's minimum essential medium supplemented with 2 mM L-glutamine, in a humidified incubator with 5% CO_2 at 37°C.

HIT

HITs were performed according to the Manual on Animal Influenza Diagnosis and Surveillance (WHO 2005) using 0.5% chicken red blood cells. Briefly, serial 2-fold dilutions of the culture supernatant of hybridomas or the RDE-treated-ascitic fluids containing mAbs were mixed with 4 HAU/25 μl of the virus, and incubated at room temperature for 30 min. Chicken red blood cells were then added to the mixtures, and the reciprocal of the highest dilution of mAb that completely inhibited hemagglutination was taken as the HI titer.

Blocking IFAT

Blocking IFATs were used to determine whether three anti-H5 mAbs (3B5.1, 3B5.2 and 1G5) recognized similar or different binding sites on HA proteins. Initially, 95% confluent MDCK cells on the 8-well chamber slides (Thermo Fisher Scientific, K.K., Waltham, MA) were inoculated with the Ck/Yamaguchi/7/04 and Ck/Miyazaki/K11/07 strains and incubated for 18 h. The infected cells were fixed with cold acetone for 10 min and reacted with anti-H5 (3B5.1, 3B5.2, or 1G5) mAbs for 30 min at 37°C. Then, the 3B5.1 mAb labeled with Alexa Fluor® 488 using a Fluorescein

Labeling kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan) or the 3B5.2 and 1G5 mAbs labeled with Alexa Fluor® 594 using a Zenon® Mouse IgG Labeling kit (Thermo Fisher Scientific, K.K.) were reacted for 30 min. The fluorescent signal was observed under a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

Selection of escape mutants

Serial dilutions of the virus were incubated with an excess of mAb. After incubate for 1 h at 37°C, the mixtures were inoculated into MDCK cells and incubated for 3 days at 37°C. The mutant viruses that were not neutralized, indicated by HA positive in culture supernatants and CPE in MDCK cells, were cloned 2 times by limiting-dilution in embryonated chicken eggs.

Sequencing of escape mutants

Viral RNAs were extracted from the mutant-infected AFs using ISOGEN II (NIPPON GENE, Tokyo, Japan). The construction and purification of cDNA libraries which were used in next-generation sequencing were conducted according to a previous report (Masuda et al. 2014). The sequencing was carried out on a MiSeq bench-top sequencer (Illumina, San Diego, CA) to generate 51-bp single-end reads. The FASTQ-formatted sequence data was generated using MiSeq Reporter program (Illumina). The contiguous sequences were assembled from the short sequence reads

using CLC Genomics Workbench version 6.5.1 (CLC bio, Aarhus, Denmark). The consensus sequence was determined using BLAST.

Analysis of epitopes recognized by mAbs

The HA1 amino acids sequences (positions 1-322, corresponding to H5 numbering) of the parental strains (Ck/Yamaguchi/7/04 and Ck/Miyazaki/K11/07) and their escape mutants (3B5.1, 3B5.2, and 1G5 mts) were aligned and the mutation sites were further analyzed in the HA1 protein. The 3D-structural model of the HA1 protein of the Ck/Yamaguchi/7/04 strain was constructed by using the Phyre2 web server (Kelley et al. 2015), based on homology modeling. The HA1 crystal structure of the A/Vietnam/1194/04 strain was downloaded from the protein data bank (PDB) (PDB ID: 2IBXA) and used as the template for this construction.

The sixteen HA1 amino acid sequences of the different clades of H5N1 HPAIVs, escape mutants, and H5 subtype LPAIVs used in this study were aligned to predict a cross-clade epitope recognized by the anti-H5 mAbs. Subsequently, the predicted cross-clade epitope was analyzed and mapped to the HA1 structural model of the Ck/Yamaguchi/7/04 strain using PyMOL (Rigsby and Parker 2016). A predicted cross-clade epitope was further analyzed to find the conserved amino acid residues in the HA1 protein among the 5,366 HA amino acid sequences of clades 0–9 of the H5N1 viruses, obtained from the GenBank and Influenza Research Database.

2.3 Results

Reactivity of the mAbs with heterologous H5 subtype influenza viruses

The 3B5.1 and 3B5.2 mAbs against the Ck/Yamaguchi/7/04 strain (clade 2.5) had reactivity to all the 26 heterologous strains examined of clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1b, 2.3.2.1c, and 2.3.4 of the H5N1 HPAIV, isolated in Japan and Vietnam. The HI titers of the 3B5.1 mAb to these heterologous H5N1 viruses ranged from 160–640, and those of the 3B5.2 mAb ranged from 10–640 (Table 2.2). Conversely, the 1G5 mAb against the Ck/Miyazaki/K11/07 strain (clade 2.2) recognized only clades 1, 2.3.4 and 2.5 viruses with the HI titers ranging from 20–320. However, only one of the five strains examined in clade 2.3.4 reacted with this mAb (titer of 1:20) (Table 2.2).

The 3B5.1, 3B5.2, and 1G5 mAbs also recognized the H5N1, H5N2, and H5N3 LPAIV strains belonging to the EA-nonGsGD clade with the HI titers ranging from 80 to 1,280, except the Ck/Ibaraki/1/05 (H5N2) and Whis.sw./Shimane/499/83 (H5N3) strains belonging to the Am-nonGsGD clade, and the H9N2 and H1N1 strains (Table 2.3).

Blocking IFAT

Blocking IFAT was performed to verify whether the three anti-H5 mAbs could recognize different antigenic sites in the HA1 protein or not. Binding of all the fluorescein-conjugated 3B5.1, 2B5.2, and 1G5 mAbs were not blocked by any competitor mAbs, except the homologous mAbs (Fig. 2.1), indicating that each of the mAbs recognized different antigenic sites.

Analysis of the escape mutants and epitopes recognized by the mAbs

Three escape mutants to the anti-H5 mAbs (3B5.1 mt, 3B5.2 mt, and 1G5 mt) were generated and sequenced to identify the mutated amino acid positions in H5 HA1 associated with the mAbs binding sites. The 3B5.1 mt carried point mutations at amino acid positions 56 (S56N), and 162 (R162I) (according to mature H5 numbering), whereas the 3B5.2 mt had two mutation sites at the positions 162 (R162G) and 184 (A184G) in the HA1 protein. The 1G5 mt had only a single point mutation at position 139 (G139R) (Table 2.4).

Subsequently, these mutated amino acid positions (56, 139, 162 and 184) were mapped to the HA1 crystal structure (2IBXA). The result showed that the three mutated amino acid residues (positions 139, 162 and 184) were located on the HA1 protein (Figs. 2.2 and 2.3). The mutated amino acid at position 139 was located in the antigenic site A in H3 (Rudneva et al. 2010) and Ca in H1 HA (Shore et al., 2013), whereas the mutated amino acid at position 184 was located in the antigenic site B in H3 (Rudneva et al., 2010) and Sb in H1 HA (Shore et al., 2013). The mutated amino acid at position 162 was located in the antigenic site Sa in H1 HA (Shore et al., 2013). However, the mutated amino acid at position 56 was not located on any antigenic sites of H1 and H3 HA (Fig. 2.2). The HA amino acid residue at position 184 (E184) in clades 2.3.2.1, 2.3.2.1a, and 2.3.2.1c used in this study (data not shown) was different from that of the parental strain (A184), but these clade strains were recognized by the 3B5.1 and 3B5.2 mAbs (Table 2.2). Altogether, these results suggested that the HA amino acid residues

at position 162 is a major epitope recognized by the 3B5.1 and 3B.2 mAb. The 1G5 mAb recognizes the amino acid residues at position 139 in the HA protein.

In this chapter, the 3D-structural model of the Ck/Yamaguchi/7/04 strain was analyzed to identify the binding site of 3B5.1 and 3B5.2 in HA1 protein. Two different amino acid residue portions were found to be located adjacent to or surrounding the HA1 amino acid residue at position 162. These portions consisted of the amino acid residues at positions 115–128 (115QIIPKSSWSDHEAS128) that is located in the antigenic site A in H3 and at positions 158–170 (158PTIKRSYNNTNQE170) that is located in the antigenic site Sa in H1 HA (Yang et al., 2016) (Fig. 2.4). These two portions may affect the binding of the 3B5.1 and 3B5.2 mAbs.

However, because no evidence was found in this study to support that 3B5.1 and 3B5.2 mAbs recognized the HA1 amino acids at positions 115–128, the amino acid residue positions 158–170 containing the mutated sites at position 162 seemed to be an epitope recognized by these mAbs. Thus, the HA1 amino acids at positions 158–170 were analyzed to identify the antigenic sites of the 3B5.1 and 3B5.2 mAbs. The presence of 158PTIKRSYNNTNQE170 residues in the HA1 protein of the Ck/Yamaguchi/7/04 strain was examined to verify whether the residues were conserved among the H5 subtype strains, including seven H5N1 HPAIV strains and seven LPAIV (clades EA-nonGsGD and Am-nonGsGD) strains used in this study (Table 2.5) The amino acid sequence of six HPAIV strains was the same as that of the Ck/Yamaguchi/7/04 strain, except at position 162 of clades 2.3.2.1, 2.3.2.1a, and 2.3.2.1c (K162), and at position 163 of clades 2.3.2.1a and 2.3.2.1c (G163). However,

the lysine residues at position 162 (K162) did not affect the binding ability of the 3B5.1 and 3B5.2 mAbs, although the mAbs could not react to the mutants with R162I or R162G (Table 2.4).

In addition, five EA-nonGsGD clade strains recognized by the 3B5.1 and 3B5.2 mAbs had the same amino acid sequence (158PTIKRSYNNTNQE170) as the Ck/Yamaguchi/7/04 strain, except K169 residue of the Dk/Hong Kong/820/83 (and D170 of the Md/Hokkaido/24/09 (Table 2.5). However, these mutated amino acid residues (Q169K and E170D) did not affect the binding ability of the 3B5.1 and 3B5.2 mAbs. Unexpectedly, in two Am-nonGsGD strains, the Ck/Ibaraki/1/05 strain which possessed P158K, I160L, S163N, N165T, and Q169V substitutions and the Whis.sw./Shimane/499/83 strain which possessed K161E, S163T, and Q169V substitutions, the R162 residues in their HA1 protein were not recognized by the 3B5.1 and 3B5.2 mAbs (Table 2.5).

Conservation of the HA1 epitope (158–170) among divergent H5N1 HPAIVs recognized by the mAbs

The 158PTIKRSYNNTNQE170 epitope was aligned and compared among 5,366 strains of clades 0–9 of the H5N1 viruses to analyze the conservation of this epitope. The result showed that the HA1 amino acid at positions 158, 160, 164, and 166 (P158, I160, Y164, and N166) were highly conserved (>90%) among all clades 0–9 of the H5N1 virus (Table 2.6). The R162 and K162 in the HA1 amino acid residues were also

highly conserved among all clades of the H5N1 virus, except clades 2.2.1.1a (E162), 2.2.2 (I162), 2.2.2.1 (I162), 2.3.1 (I162), 2.3.4.4 (I162), 7.1 (V162), and 7.2 (V162). The amino acid positions 159, 163, 165, and 167–170 (T159, S163, N165, T167, N168, Q169, and E170) were completely mutated and highly conserved in clades 2.3.2.1b (I159 and K169), 7.1 (P159, N163, and T165), 7.2 (P159, N163, T165, and A167), 2.1.3.2a (T163), 2.1.3.2b (T163), 2.3.2.1c (G163), 2.2.1.1 (H165), 2.2.1.1a (H165), 2.1.3 (E168), and 2.3.4.4 (R169) and mutated and moderately conserved (50–90%) in clades 1.1.1 (D168), 2.3.4.1 (S168), 2.3.2.1c (R169), and 7.2 (K170).

2.4 Discussion

In this chapter, the 3B5.1 and 3B5.2 mAbs reacted to all HPAIV strains of clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1b, 2.3.2.1c, and 2.3.4 isolated in Japan and Vietnam during 2003–2012, indicating that the mAbs recognized the cross-clade epitope; whereas the 1G5 mAb did not react to all clades examined (Table 2.2).

Although four amino acid mutations (S56N, R162I, R162G, and A184G, H5 numbering) were found in the escape mutants (3B5.1 and 3B5.2 mts) (Table 2.4), the mutations at the positions 56 and 184 did not seem to affect the binding ability of the 3B5.1 and 3B5.2 mAbs since the amino acid at position 56 was buried into the HA1 protein, and clade 2.3.2.1, 2.3.2.1a, and 2.3.2.1c strains containing the E184 (data not shown) were recognized by these mAbs (Table 2.2). In contrast, the positively charged amino acid residue (R162) seemed to highly associate with the binding ability of the

3B5.1 and 3B5.2 mAbs, since the change from positively charged amino acid residue (R162) to the neutral charged residue (I162 and G162) might lead to the inhibition of the binding ability of the 3B5.1 and 3B5.2 mAbs (Table 2.4). However, although the H5N1 clade 2.3.2.1, 2.3.2.1a, and 2.3.2.1c strains have the lysine residue (K162) and not arginine, they were also recognized by these mAbs. Unfortunately, since the HA amino acid sequences of the clade 2.3.2.1b strains used in this study were not available, the 35 HA sequences of clade 2.3.2.1b available in the database were examined instead, and all 35 strains of clade 2.3.2.1b contained the amino acid residue K162 as shown in Table 2.6. Therefore, the 3B5.1 and 3B5.2 mAbs may mainly interact with the positively charged amino acid residues at position 162 in the HA1 protein.

The mutation of amino acids in position 162 of the HA1 protein caused a loss of binding of some anti-H5 mouse mAbs (Kaverin et al., 2007, Rudneva et al., 2010, Masalova et al., 2011). The VN04-2 mAb to the A/Vietnam/1203/04 (clade 1) strain reacted to clade 2.1.1 (R162), but not to clade 2.1.3.1, 2.2, and 2.3.4 strains with the same residue R162 (Kaverin et al., 2007). Four anti-H5 mouse mAbs to Duck/Novosibirsk/56/05 (clade 2.2) (3G9, 5G9, 5F12, and 6E2) seemed to recognize R162, because they failed to react with the escape mutants with the amino acid residue change R162G/K/W; however, these mAbs could not recognize the clade 1 (R162) and clade 2.3.2.1 (K162) and 2.3.2.1c (K162) strains (Rudneva et al., 2010; Masalova et al., 2011). On the other hand, our mAbs (3B5.1 and 3B5.2) could recognize clade 1 (R162) and clades 2.1.3.1 (R162), 2.2 (R162), 2.3.2.1(K162), 2.3.2.1c (K162), and 2.3.4 (R162). Thus, the mAbs of this study also recognized the H5N1 strains with K162

in the HA1 protein (Table 2.5), a result that contradicts with previous studies (Kaverin et al., 2007; Rudneva et al, 2010; Masalova et al., 2011). Although the reason for this contradiction is unclear, the antigenic structures of these regions, including the amino acid residue at position 162 that the mAbs (3B5.1 and 3B5.2) recognized, may be different from those that other mAbs recognized.

Although several broad cross-clade reactivities of anti-H5 HA human and mouse mAbs have been established (Hu et al., 2012; Kobayashi-Ishihara et al., 2014; Wu et al., 2014; Xiong et al., 2015), there is not much information on the reactivity to the newly emerging H5 HPAIVs (clades 2.3.2.1a or 2.3.2.1c). Although a human mAb (FLD194) recognized the A/Hubei/1/10 strain (clade 2.3.2.1a) bearing Q119 in the HA1 protein (H5 numbering) in addition to clade 0, 1, 2.1.3, 2.2, 2.2.1, 2.3.1, 2.3.2, 2.3.4, and 2.5 strains, it could not react to the other two strains of the same clade 2.3.2.1a (A/chicken/Bangladesh/11RS1984-33/11 and A/chicken/Bangladesh/14VIR2665-23/14) bearing R119 and K119, respectively (Xiong et al., 2015). Thus, it may be needed to examine as many H5N1 strains as possible from different origins within clades to define the broad cross-reactivity of established mAbs. On the other hand, the 3B5.1 and 3B5.2 mAbs could react to all six strains of clade 2.3.2.1a strains bearing K119 (Table 2.2) and EA-nonGsGD LPAIV strains bearing R119 (Table 2.3). Although the clade 2.3.2.1a strains used in the previous study (Xiong et al., 2015) were not available in this study, the two strains with the R162 or K162 residues in the HA1 protein seemed to be recognized by the 3B5.1 and 3B5.2 mAbs.

Hu et al. (2012) reported that a human mAb (65C6) binds a conformational epitope, comprised of amino acid residues at positions 118, 121, 161, 164, and 167 (H5 numbering) in the HA1 protein, that is conserved among clade 0, 1, 3–9, 2.1.3.2, 2.2, 2.2.1, 2.3.2.1, 2.3.4, 2.4, and 2.5 strains, except for clade 7.1. However, the amino acid mutation at position 162 did not significantly affect the binding ability of the 65C6 mAb, but did abolish the binding ability of the 3B5.1 and 3B5.2 mAbs used in this study (Table 2.4). These results may suggest that the 3B5.1 and 3B5.2 mAbs recognized different antigenic structures than those recognized by the 65C6 mAb, although each site seemed to be structurally very similar and nearby.

The cross-clade epitope in the HA1 protein at the R162 residue has been reported by Zuo et al. (2015). They suggested that four major vulnerable antigenic sites (VS1–4) exist on the A/Anhui/1/05 HA1 protein based on epitopes recognized by the anti-H5 human and murine mAbs, aligning with other reports. One of the mAbs (65C6) recognized the cross-clade conformational epitope in VS1 (115QIIPKSSWSDH125 and 158PTIKRSYNNTNQE170, H5 numbering) that roughly corresponded to Site A on H3 HA and Sa on H1 HA. However, the 158PTIKRSYNNTNQE170 region only includes amino acid residues at positions 158–163 (H5 numbering) of the antigenic site Sa on H1 HA, but not Site A on H3. In this study, 3B5.1 and 3B5.2 mAbs could not bind to the mutant strains with the mutation R162I or R162G, which may suggest that the epitope recognized by these mAbs overlapped the epitope (158PTIKRSYNNTNQE170).

Therefore, the 158PTIKRSYNNTNQE170 epitope on the HA1 protein was analyzed whether this epitope was conserved among the 5,366 sequences of various clade H5N1 HAs currently available in the database. As shown in Table 5, the R162 or K162 residues in the HA1 protein were highly conserved among 36 clades, including clades 2.3.2.1a and 2.3.2.1c, out of the 43 H5N1 clades reported, except for seven clades (2.2.1.1a, 2.2.2, 2.2.2.1, 2.3.1, 2.3.4.4, 7.1 and 7.2). Out of the 18 amino acid residues of the epitope, in four or five residues (159, 162, 163, 165, and 167) of clades 7.1 and 7.2, a very low level of residue conservation was revealed compared with other clades (Table 2.6). It was expected that the 3B5.1 and 3B5.2 mAbs would fail to react to these clade strains; thus, I believe that clades of the H5N1 HPAIV containing the R162 or K162 residue in the HA1 protein could be recognized by the mAbs, leading to the detection of clade 2.3.2.1a and 2.3.2.1c viruses currently circulating in Asia, Africa, and Europe (WHO 2014, 2015, 2016).

As shown in Table 2.5, the 3B5.1 and 3B5.2 mAbs recognized the EA-nonGsGD clade (Eurasian-lineage) H5 LPAIV strains (H5N1, H5N2, and H5N3) with residue R162 in the HA1 protein. Unexpectedly, these mAbs could not recognize the Am-nonGsGD clade (American-lineage) H5 LPAIV strains (H5N2 and H5N3), even with the residue R162. In the American-lineage strains, the amino acid residue at position 158 in the HA1 protein of the Ck/Ibaraki/1/05 strain (K158) was the positive charge, whereas the residue at position 161 of the Whis.sw./Shimane/499/83 strain (E161) was the negative charge. Those positively and negatively charged amino acid residues were different from the Eurasian-lineage strains of H5 HPAIV and LPAIV (P158 and K161)

(Table 2.5). The shift in the charge from positive to negative that resulted in conformational change of the HA1 surface affecting on the binding of an anti-H5 mAb has been reported (Kobayashi-Ishihara et al., 2014). Herein, the negatively charged amino acid residue (E161) of the Whis.sw./Shimane/499/83 may have affected the binding of the 3B5.1 and 3B5.2 mAbs. However, it was unclear whether the positively charged residue (K159) of the Ck/Ibaraki/1/05 strain similarly affected the binding of these mAbs. We suspected that the HA1 structure of this Am-nonGsGD clade strain was complex and different from that of the HPAIV and EA-nonGsGD clade strains.

Unfortunately, the 3B5.1 and 3B5.2 mAbs may not recognize the clade 2.2.1.1a, 2.2.2, 2.2.2.1, 2.3.1, 2.3.4.4, 7.1, and 7.2 strains, containing the residue E162, I162, or V162 in the HA1 protein (Table 2.6). Therefore, in order to detect clades 2.2.1.1a, 2.2.2, 2.2.2.1, 2.3.1, 2.3.4.4, 7.1, and 7.2, additional mAb should be produced. A combination of our mAbs (3B5.1 and 3B5.2) and the complementary mAbs to the clade 2.2.1.1a, 2.2.2, 2.2.2.1, 2.3.1, 2.3.4.4, 7.1, and 7.2 strains could be useful in the development of a diagnostic test with broad cross-clade reactivity for the detection of all H5N1 clade strains. Previously, a mAb-based dot ELISA was developed for the universal detection of H5N1 viruses including clades 0, 1, 2.1, 2.2, 2.3, 4, 7, and 8 by using two complementary anti-H5 mAbs (He et al., 2010).

2.5 Summary

The outbreaks caused by H5N1 HPAIVs are still serious problems that have an impact on both animal and public health. Therefore, rapid diagnostic tests are essential for the detection and monitoring of circulating H5N1 viruses to prevent an outbreak. In this chapter, the 3B5.1 and 3B5.2 mAbs against the clade 2.5 virus showed cross-clade reactivity to all 26 H5N1 strains from clades 1, 2.2, 2.3.2.1, 2.3.2.1a, b, c and 2.3.4, suggesting that the epitope(s) recognized by the 3B5.1 and 3B5.2 mAbs are conserved. Conversely, the 1G5 mAb against the clade 2.2 virus showed reactivity only to clades 1, 2.3.4 and 2.5 strains. Blocking IFATs showed that each mAb recognized different epitopes. An analysis of escape mutants and some clades of the H5N1 viruses recognized by the 3B5.1 and 3B5.2 mAbs suggested that the mAbs bind to an epitope, including amino acid residues at position 162 in the HA1 protein (R162 or K162). Unexpectedly, however, when five Eurasian-origin strains (EA-nonGsGD clade) and two American-origin strains (Am-nonGsGD clade) with R162 in the HA1 protein of H5 LPAIV were examined, the mAbs recognized only EA-nonGsGD clade strains. The R162 and K162 residues in the HA1 protein were highly conserved among 36 of the 43 H5N1 clades reported, including clades 2.3.2.1a and 2.3.2.1c that are currently circulating in Asia, Africa and Europe. The amino acid residues (158PTIKRSYNNTNQE170) in the HA1 protein are probably an epitope responsible for the cross-clade reactivity of the mAbs, considering the epitopes reported elsewhere.

These results indicated that the 3B5.1 and 3B5.2 mAbs with broad cross-reactivity to the H5N1 viruses could be available to develop specific and rapird diagnostic tests for recently circulating H5N1 viruses, especially clade 2.3.2.1a and 2.3.2.1c strains causing lethal infections of poultry in Asia, Africa, and Europe.

Table 2.1 Influenza A viruses used in this study

Influenza viruses	Strains	Clades	Virus source and references
HPAIVs	A/chicken/Yamaguchi/7/04(H5N1),[Ck/Yamaguchi/7/04]	2.5	(Mase et al., 2005)**
	A/chicken/Yamaguchi/7/04(H5N1) [3B5.1-escape mutant]	2.5	*****
	A/chicken/Yamaguchi/7/04(H5N1) [3B5.2-escape mutant]	2.5	*****
	A/chicken/Miyazaki/K11/07(H5N1),[Ck/Miyazaki/K11/07]	2.2	(Suzuki et al., 2010)**
	A/chicken/Miyazaki/K11/07 (H5N1),[1G5-escape mutant]	2.2	*****
	A/whooper swan/Hokkaido/1/08 (H5N1) [Who.sw/Hokkaido/1/08]	2.3.2.1	(Okamatsu et al.,2010)***
	A/whooper swan/Hamanaka/11 (H5N1, [Who.sw/Hamanaka/11]	2.3.2.1c	(Bui et al., 2013)*****
	A/chicken/Vietnam/HN-06/03 (H5N1), [Ck/VN-HN/06/03]	1	*
	A/chicken/Vietnam/HT-33/03 (H5N1), [Ck/VN-HT/33/03]	1	*
	A/chicken/Vietnam/TB-31/04 (H5N1), [Ck/VN-TB/31/04]	1	*
	A/chicken/Vietnam/HN-40/04 (H5N1), [Ck/VN-HN/40/04]	1	*
	A/chicken/Vietnam/HT-30/10 (H5N1), [Ck/VN-HT/30/10]	2.3.2.1a	*
	A/chicken/Vietnam/VP-02/11 (H5N1), [Ck/VN-VP/02/11]	2.3.2.1a	*
	A/chicken/Vietnam/QT-06/11 (H5N1), [Ck/VN-QT/06/11]	2.3.2.1a	*
	A/chicken/Vietnam/HD-04/11 (H5N1), [Ck/VN-HD/04/11]	2.3.2.1a	*
	A/chicken/Vietnam/TB-10/11 (H5N1), [Ck/VN-TB/10/11]	2.3.2.1a	*
	A/chicken/Vietnam/HD-01/12 (H5N1), [Ck/VN-HD/01/12]	2.3.2.1a	*
	A/chicken/Vietnam/NB-05/11 (H5N1), [Ck/VN-NB/05/11]	2.3.2.1b	*
	A/molly duck/Vietnam/BN-02/12 (H5N1), [Mol.dk/VN-BN/02/12]	2.3.2.1b	*
	A/duck/Vietnam/VP-03/12 (H5N1), [Dk/VN-VP/03/12]	2.3.2.1b	*
	A/duck/Vietnam/QB-69/12 (H5N1), [Dk/VN-QB/69/12]	2.3.2.1b	*
	A/duck/Vietnam/BN-06/12 (H5N1), [Dk/VN-BN/06/12]	2.3.2.1b	*
	A/duck/Vietnam/HN-72/12 (H5N1), [Dk/VN-HN/72/12]	2.3.2.1c	*
	A/duck/Vietnam/HN-74/12 (H5N1), [Dk/VN-HN/74/12]	2.3.2.1c	*
	A/duck/Vietnam/HN-75/12 (H5N1), [Dk/VN-HN/75/12]	2.3.2.1c	*
	A/muscovy duck/Vietnam/HN-77/07 (H5N1), [Mdk/VN-HN/77/07]	2.3.4	*
	A/muscovy duck/Vietnam/HN-01/09 (H5N1), [Mdk/VN-HN/01/09]	2.3.4	*
	A/muscovy duck/Vietnam/BN-16/09 (H5N1), [Mdk/VN-BN/16/09]	2.3.4	*
	A/muscovy duck/Vietnam/HN-13/09 (H5N1), [Mdk/VN-HN/13/09]	2.3.4	*
	A/muscovy duck/Vietnam/TB-05/10 (H5N1), [Mdk/VN-TB/05/10]	2.3.4	*

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****Kindly afforded by Dr. T. Ito, Tottori University, Japan.

*****Available in our laboratory, Japan.

Table 2.1 Continued

Influenza viruses	Strains	Clades	Virus source and references
LPAIVs	A/mallard/Hokkaido/24/09 (H5N1), [Md/Hokkaido/24/09]	EA-nonGsGD	(Yamamoto et al., 2009)****
	A/duck/Japan/11UO0023/11 (H5N2), [Dk/Japan/11UO0023/11]	EA-nonGsGD	*****
	A/duck/Japan/11UO0032/11(H5N2), [Dk/Japan/11UO0032/11]	EA-nonGsGD	*****
	A/duck/Japan/11UO0083/11(H5N2), [Dk/Japan/11UO0083/11]	EA-nonGsGD	*****
	A/duck/Hong Kong/820/80 (H5N3), [Dk/Hong Kong/820/80]	EA-nonGsGD	***
	A/chicken/Ibaraki/1/05 (H5N2), [Ck/Ibaraki/1/05]	Am-nonGsGD	(Okamatsu et al., 2007)**
	A/whistling swan/Shimane/499/83 (H5N3)[Whis.sw/Shimane/499/83]	Am-nonGsGD	****
	A/chicken/Yokohama/aq-55/01 (H9N2), [Ck/Yokohama/aq/55/01]		(Eto and Mase, 2003)**
Human influenza A virus	A/Puerto Rico/8/34 (H1N1), [PR/8/34]		*****

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*****Available in our laboratory, Japan.

Table 2.2 Antigenic characterization of different clades of the H5N1 HPAIVs isolated in Japan and Vietnam using the three mAbs produced

Clades	Strains	HI titers		
		3B5.1	3B5.2	1G5
1	Ck/VN-HN/06/03	160	40	160
	Ck/VN-HT/33/03	320	20	40
	Ck/VN-TB/31/04	320	10	160
	Dk/VN-HN/40/04	640	20	320
2.2	Ck/Miyazaki/K11/07	640	640	320*
2.3.2.1	Who.sw/Hokkaido/1/08	320	20	<10
2.3.2.1a	Ck/VN-HT/30/10	160	20	<10
	Ck/VN-VP/02/11	640	40	<10
	Ck/VN-QT/06/11	640	80	<10
	Ck/VN-HD/04/11	640	40	<10
	Ck/VN-HD/01/12	320	20	<10
	Ck/VN-TB/10/12	640	40	<10
2.3.2.1b	Ck/VN-NB/05/11	320	20	<10
	Dk/VN-VP/03/12	640	40	<10
	Dk/VN-QB/69/12	640	80	<10
	Dk/VN-BN/06/12	320	10	<10
	Mol. dk/VN-BN/02/12	640	40	<10
2.3.2.1c	Who.sw/Hamanaka/11	320	20	<10
	Dk/VN-HN/72/12	640	80	<10
	Dk/VN-HN/74/12	640	80	<10
	Dk/VN-HN/75/12	640	80	<10
2.3.4	Mdk /VN-HN/77/07	160	80	<10
	Mdk /VN-HN/01/09	160	320	<10
	Mdk /VN-BN/16/09	320	320	20
	Dk/VN-HN/13/09	320	320	<10
	Mdk /VN-TB/05/10	320	40	<10
2.5	Ck/Yamaguchi/7/04	2,560*	640*	80

Table 2.3 Antigenic characterization of the LPAIVs and other subtypes of influenza A viruses using three mAbs produced

Clades	Subtypes	Strains	HI titers		
			3B5.1	3B5.2	1G5
EA-nonGsGD	H5N2	Dk/Japan/11UO0023/11	1,280	160	80
EA-nonGsGD	H5N2	Dk/Japan/11OG1032/11	1,280	160	80
EA-nonGsGD	H5N2	Dk/Japan/11OG1083/11	1,280	160	80
Am-nonGsGD	H5N2	Ck/Ibaraki/1/05	<10	<10	<10
EA-nonGsGD	H5N3	Dk/Hong Kong/820/80	1,280	160	80
Am-nonGsGD	H5N3	Whis.sw/Shimane/499/83	<10	<10	<10
EA-nonGsGD	H5N1	Md/Hokkaido/24/09	1,280	160	80
	H9N2	Ck/Yokohama/aq/55/01	<10	<10	<10
	H1N1	PR/8/34	<10	<10	<10

Table 2.4 Characterization of the escape mutants

Escape mutant	mAbs	HIT	Mutation in the HA1 protein			
			Position of nucleotide	Nucleotide change	Position of amino acid	Amino acid change
3B5.1 mt*	3B5.1	Negative	167	G to A	56	S to N
		(<1:10)***	485	G to T	162	R to I
3B5.2 mt*	3B5.2	Negative	484	A to G	162	R to G
		(<1:10)	551	C to G	184	A to G
1G5 mt**	1G5	Negative	415	G to A	139	G to R
		(<1:10)				

*selected by the mAbs against Ck/Yamaguchi/7/04

**selected by the mAbs against Ck/Miyazaki/K11/07

*** HI titer

Table 2.5 Comparison of the HA1 epitope at positions 158–170 among the H5 viruses used in this study

Strains	GenBank accession number	Clades	HIT*	Amino acid positions in the HA1 protein (158–170)												
				158	159	160	161	162	163	164	165	166	167	168	169	170
Ck/Yamaguchi/7/04 (H5N1)	AB166862	2.5	+	P	T	I	K	R	S	Y	N	N	T	N	Q	E
Ck/VN-HT/33/03 (H5N1)		1	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Ck/Miyazaki/K11/07 (H5N1)		2.2	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Who.sw./Hokkaido/1/08 (H5N1)	AB436550	2.3.2.1	+	.	.	.	.	K	.	.	.	.	.	.	.	.
Ck/VN-HT/30/10 (H5N1)		2.3.2.1a	+	.	.	.	.	K	G	.	.	.	.	.	.	.
Who.sw./Hamanaka/11 (H5N1)	CY110738	2.3.2.1c	+	.	.	.	.	K	G	.	.	.	.	.	.	.
Mol.dk/VN-HN/77/07 (H5N1)		2.3.4	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Dk/Hong Kong /820/80 (H5N3)	LC042047	EA-non GsGD	+	.	.	.	.	.	.	.	.	.	.	.	K	.
Dk/Japan/11UO0023/11 (H5N2)	KR265592	EA-non GsGD	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Dk/Japan/11OG1032/11 (H5N2)	KR265541	EA-non GsGD	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Dk/Japan/11OG1083/11 (H5N2)	KR265560	EA-non GsGD	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Md/Hokkaido/24/09 (H5N1)	AB530992	EA-non GsGD	+	.	.	.	.	.	.	.	.	.	.	.	.	D
Ck/Ibaraki/1/05 (H5N2)	AB261853	Am-non GsGD	–	K	.	L	.	.	N	.	T	.	.	.	V	.
Whis.sw./Shimane/ 499/83 (H5N3)		Am-non GsGD	–	.	.	.	E	.	T	.	.	.	.	.	V	.

*(+) = HIT-positive to the 3B5.1 and 3B5.2 mAbs; (-) = HIT-negative to the 3B5.1 and 3B5.2 mAbs (HI titers <10)

Table 2.6 Conservation of the HA1 epitope at positions 158–170 among the divergent clades of the H5N1 viruses

Clades	Number of strains (5,366)	Percentage homology of amino acid residues in HA1 protein***												
		P158	T159	I160	K161	**R/K162**	S163	Y164	N165	N166	T167	N168	Q169	E170
0	141	100	99.3	99.3	100	100	100	100	100	100	97.9	100	99.3	100
1	622	99.7	99.7	100	100	99.8	99.8	100	99.8	100	100	100	99.5	100
1.1	68	100	100	100	100	100	100	100	100	100	100	95.6	100	100
1.1.1	25	100	100	100	92	100	100	100	100	100	100	80(D)*	100	100
1.1.2	124	100	96.8	100	100	100	100	100	100	100	100	98.4	99.2	100
2.1.1	66	100	100	100	100	100	100	100	100	100	100	100	100	100
2.1.2	39	100	97.4	100	100	100	100	100	100	100	100	100	100	100
2.1.3	38	100	100	100	100	100	100	100	100	100	100	97.4(E)*	100	100
2.1.3.1	29	100	93.1	100	100	100	69**	100	100	100	100	100	100	100
2.1.3.2	305	100	95.1	100	99.3	98.4	97.1	100	91.8	100	99.3	100	99.7	100
2.1.3.2a	55	100	96.4	100	100	96.4	100(T)*	100	94.6	100	100	100	100	100
2.1.3.2b	69	100	98.6	100	100	100	98.6(T)*	100	100	100	100	97.1	100	100
2.1.3.3	24	100	100	100	100	100	100	100	100	100	100	100	100	100
2.2	609	100	99.5	100	100	91	100	100	100	100	100	100	99.8	100
2.2.1	476	99.8	100	100	100	89.7**	99.4	100	99.8	100	100	99.8	99.6	100
2.2.1.1	95	100	88.4**	100	79**	93.7	90.5	96.8	96.8(H)*	100	97.9	100	92.6	100
2.2.1.1a	73	100	90.4	100	100	78.1(E)*	98.6	100	100 (H)*	100	97.3	100	97.3	100
2.2.1.2	343	99.1	100	100	100	99.4	100	100	99.7	99.7	99.7	100	99.7	100
2.2.2	67	100	100	100	100	100 (I)*	100	100	100	100	100	100	100	100
2.2.2.1	56	100	100	100	100	89.3(I)*	96.4	100	100	100	100	96.4	100	100
2.3.1	18	100	100	100	100	61.1(I)*	94.4	100	100	100	100	100	100	100
2.3.2	104	100	100	94.2	100	100	100	100	100	100	100	100	100	100
2.3.2.1	69	100	100	100	100	100	100	100	100	100	100	100	63.8**	100
2.3.2.1a	264	100	98.1	100	98.1	100	77.3**	100	99.6	100	99.2	100	99.6	100
2.3.2.1b	35	100	100(I)*	100	85.7**	100	97.1	100	71.4**	100	100	100	94.3(K)*	100
2.3.2.1c	521	99.8	98.8	100	99.4	100	91.2(G)*	100	100	100	98.3	100	60.7(R)*	99.8
2.3.3	30	100	100	100	100	100	100	100	100	100	100	100	100	100
2.3.4	512	100	99.8	100	99	94	95.7	99.8	99.8	100	100	98.4	92	100
2.3.4.1	28	100	100	100	100	100	96.4	100	100	100	100	53.6(S)*	100	100
2.3.4.2	45	100	100	100	100	100	100	100	100	100	100	93.3	100	97.8
2.3.4.3	84	100	100	100	100	100	100	100	100	100	100	100	98.8	98.8
2.3.4.4	32	100	100	100	100	46.9(I)*	100	100	100	100	100	100	90.6(R)*	100
2.4	22	100	100	100	95.5	95.5	100	100	100	100	100	100	100	100
2.5	20	100	95	100	100	95	100	100	100	100	100	100	100	100
3	56	100	100	100	100	100	100	100	98.2	100	100	100	100	100
4	26	100	100	100	100	96.2	96.2	100	100	100	100	100	100	100
5	28	100	100	100	100	100	100	100	100	100	100	53.6**	100	100
6	15	100	100	100	100	100	93.3	100	100	100	100	100	86.7**	93.3
7	46	100	69.6**	100	100	76.1**	67.4**	100	73.9**	100	91.3	100	100	100
7.1	18	100	100 (P)*	100	100	94.4(V)*	94.4(N)*	100	100(T)*	100	100	100	100	100
7.2	22	100	95.5(P)*	100	100	100 (V)*	100(N)*	100	100(T)*	100	100(A)*	95.5	100	55.6(K)*
8	4	100	100	100	100	100	100	100	75**	100	100	100	100	100
9	43	100	100	100	100	100	100	100	100	100	100	100	100	100

*Amino acid residues were different and shown in parentheses.

**Percentage of the conserved amino acid residues was less than 90.

***The major amino acid residues were shown.

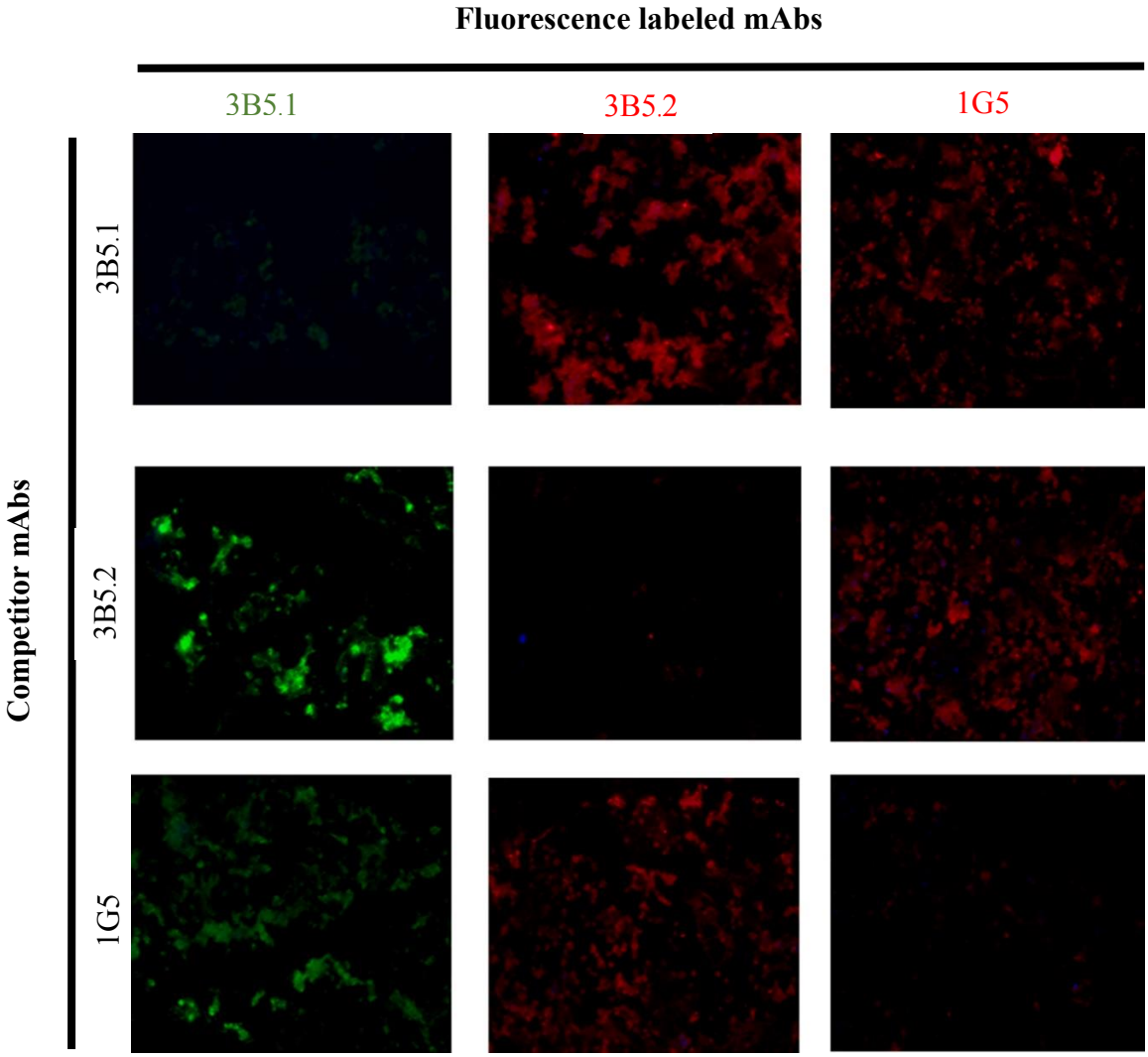

Fig. 2.1 Blocking IFAT. Blocking tests were conducted using anti-H5 mAbs (3B5.1, 3B5.2, and 1G5) as competitors and the mAbs directly labeled with green or red fluorescence. The 3B5.1 mAb was conjugated with Alexa Fluor®488; 3B5.2 with Alexa Fluor® 594; and 1G5 with Alexa Fluor® 594.

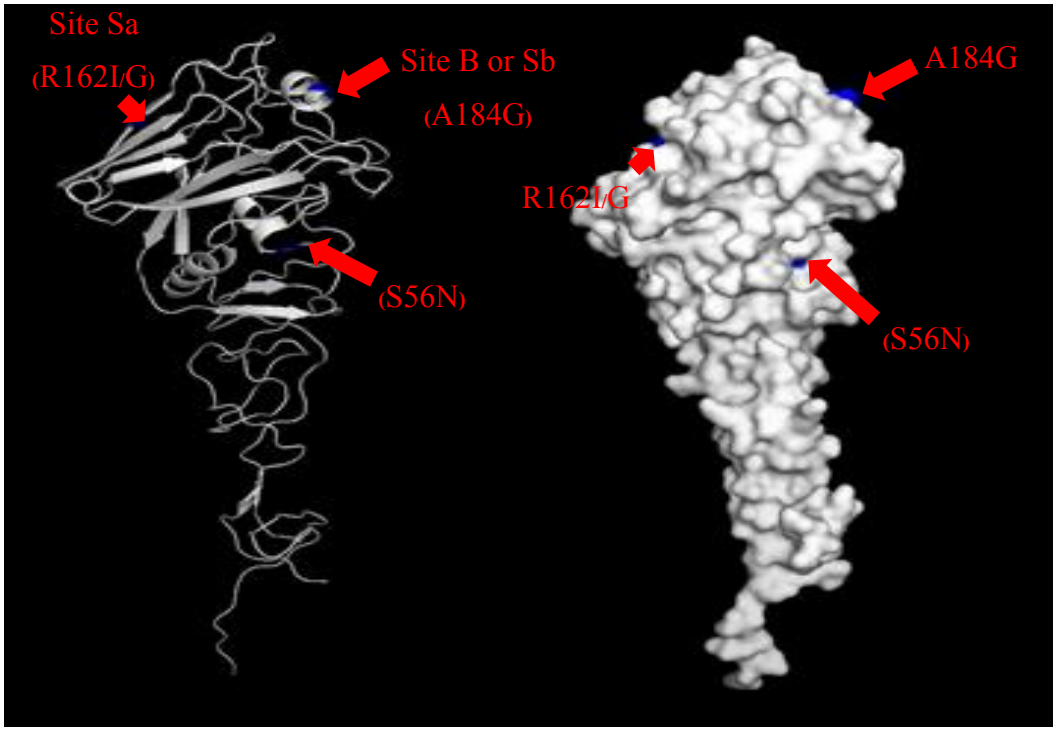

Fig. 2.2 The mutated HA amino acid residues and antigenic sites of the 3B5.1 and 3B5.2 mt of the Ck/Yamaguchi/7/04 strain on the HA1 structural protein model.

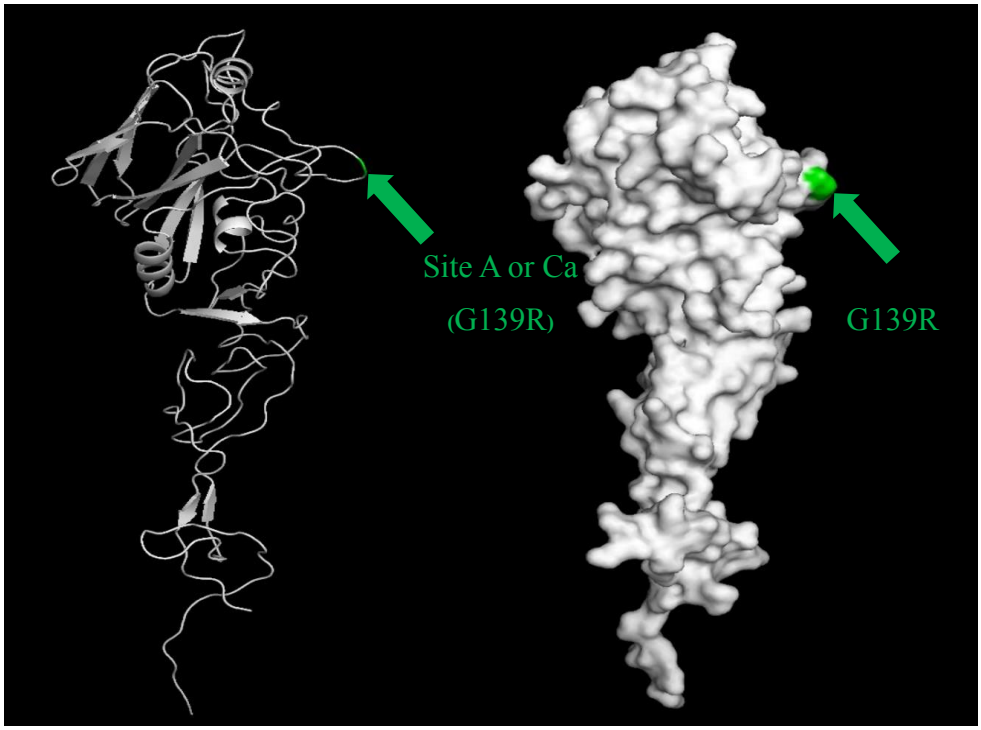

Fig. 2.3 The mutated HA amino acid residues and antigenic sites of the 1G5 mt of the Ck/Miyazaki/K11/07 strain on the HA1 structural protein model

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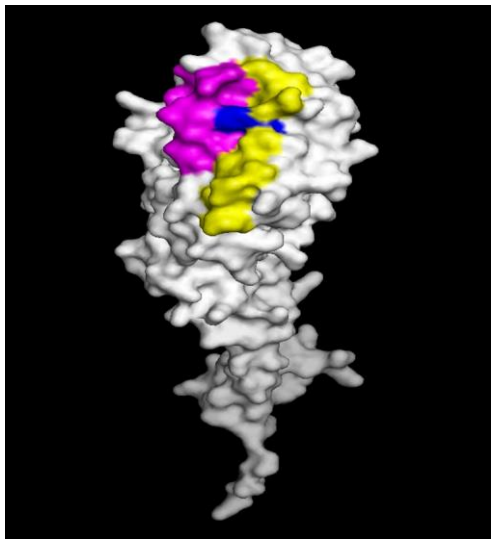

Fig. 2.4 Two HA1 amino acid residue portions of the Ck/Yamaguchi/7/04 strain located adjacent to the HA1 amino acid position 162**.** The HA1 amino acid position 162 is labeled in blue**.** The amino acid residue positions 115QIIPKSSWSDHEAS128 are labeled in magenta**.** The 158PTIKRSYNNTNQE170 residues are labeled in yellow**.**

Chapter III

Development of a latex agglutination test for the detection of H5N1 highly pathogenic avian influenza viruses

3.1 Introduction

Since the outbreak of H5N1 HPAIVs occurred in Hong Kong in 1997 in chicken populations, these H5N1 viruses have continued to cause deadly poultry outbreaks in many countries in Asia, Africa, and Europe. Those outbreaks made economic loss in poultry industry (Davison et al., 1999). Many birds were culled to prevent the spread of HPAIVs. In addition, the viruses have also caused serious diseases highly associated with mortality in humans, leading to an increased awareness of a pandemic outbreak (To et al., 2012). Thus, rapid and specific laboratory diagnosis tests for detecting the H5N1 infection are required for prompt disease control.

Antigen detection using specific mAbs is an appropriate technique for rapid diagnosis of H5N1 infection. Several rapid immunological tests utilizing mAbs against the NP proteins of influenza A virus could be used for screening of H5N1 infection in the field (WHO, 2005); however, those rapid tests had a low sensitivity in detecting H5N1 infections (Chan et al., 2002; WHO, 2005). Although those rapid tests can detect influenza A virus infection, they do not specifically detect the H5N1 infection.

Therefore, even if those tests gave the positive results, additional diagnostic tests such as virus isolation or RT-PCR would be required to confirm the H5N1 infection

Although several rapid tests for H5N1 infection using the anti-H5 mAbs have been reported (Hu et al., 2012; Kobayashi-Ishihara et al., 2014; Wu et al., 2014; Xiong et al., 2015), there is not information on the reactivity of their anti-H5 mAbs against newly circulating H5N1 HPAIVs such as clade 2.3.2.1a or 2.3.2.1c. Moreover, due to their antigenic variation on the HA proteins, not all H5N1 clade strains were detectable by those rapid tests. For example, a clade 2.3.2.1a strain (A/Hubei/1/10), bearing Q119 amino acid residue on the HA protein (H5 numbering), was recognized by a human mAb (FLD1940) (Xiong et al., 2015). However, the other two strains of the same clade 2.3.2.1a (A/chicken/Bangladesh/11RS1984-33/11 and A/chicken/Bangladesh/14VIR2665-23/14), bearing R119 and K119, were not recognized by the same FLD1940 mAb. Therefore, the development of new rapid and reliable tests for H5N1 infection with broader cross-clade reactive anti-H5 mAbs would be useful to perform early diagnosis of H5N1 infection, leading to prompt eradication of HPAIV.

LAT is a well-known rapid diagnostic method to detect antigens or antibodies in a variety of body fluids including urine, blood, saliva and feces. The antigens or antibodies are directly absorbed to the surface of latex beads. Addition of the homologous antibodies or antigens from the body fluids causes the agglutination of the latex beads, and the reaction can be easily seen by naked eyes. The LAT is widely used

for diagnosis with many clinical samples in the field, because of being easy to do, and low costs (Koivunen and Krogsrud, 2006).

In this chapter, a simple and rapid LAT using the anti-H5 mAb (3B5.1) described in the chapter I for the detection of H5N1 HPAIVs was established. The sensitivity and specificity of the test were also evaluated.

3.2 Materials and methods

Viruses

The viruses used in this study included 16 strains of influenza A virus (H5N1 HPAIVs isolated in Japan and Vietnam, LPAIVs, and a human influenza A virus), and 11 strains of avian paramyxovirus (APMV) (Dundon et al., year), including, APMV-1, APMV-6 and APMV-14 (Table 3.1). All viruses were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs. All experiments with the H5N1 HPAIVs were conducted in BSL-3 facilities, whereas the experiments with other viruses were performed in a BSL-2 laboratory.

Preparation of H5N1 HPAIV-spiked chicken fecal samples

One gram of SPF chicken feces was diluted with 1 ml of PBS, and mixed by vortex to prepare the SPF chicken fecal stock solution (1g/ml). H5N1 HPAIV-spiked chicken fecal samples were prepared by mixing 100 µl of the SPF chicken fecal stock solution and an equal volume of AF containing the Ck/Yamaguchi/7/04 or Ck/Miyazaki/K11/07

strains. The final viral concentration of the Ck/Yamaguchi/7/04 or Ck/Miyazaki/K11/07-spiked chicken fecal sample was $10^{9.0}$ and $10^{9.8}$ EID_{50}/ml, respectively.

Preparation of mAb IgG

The anti-H5 mAb (3B5.1, IgG1) that was described in the chapter I was used in this study. The mAb was semi-purified using 50% saturated aluminum sulfate (Harlow and Lane, 1988). Protein concentration of the semi-purified mAb IgG, which was determined by the Lowry method (Lowry et al., 1951), was 10.8 mg/ml.

Preparation of sensitized latex beads

The semi-purified mAb (3B5.1) IgG was coupled to polystyrene latex beads of 1.0 μm in diameter (Polysciences, Inc., Warrington, PA) as described below. Preparation of sensitized latex beads procedures were modified from those described by the manufacturer's instructions. In brief, 0.5 ml of a 2.5% (w/v) suspension of the beads was washed by centrifugation (14,000 x *g*) 3 times with 0.2 M borate buffer (pH 8.5) in 1.5-ml microcentrifuge tubes. Following the final wash, the beads were re-suspended in 1 ml of borate buffer. The semi-purified 3B5.1 mAb IgG was coupled to the latex beads with concentrations of 400 or 1000 μg of the 3B5.1 mAb IgG, and incubated overnight at room temperature with gentle end-to-end mixing. Then, the coupled beads (3B5.1 mAb-beads) were centrifuged (14,000 x *g*) for 10 min to remove the unboud mAb IgG. To reduce non-specific binding, the mAb-beads were blocked with 1.0 ml

of BSA (10 mg/ml) for 30 min at room temperature, and washed with borate buffer by centrifugation (14,000 x *g*). The blocking and washing procedures were done three times. Subsequently, the mAb-beads were re-suspended in 1 ml of storage buffer (1% BSA, 5% glycerol, and 0.1% sodium azide in PBS), and stored at 4°C until use.

LAT

Prior to detecting the H5N1 viruses by the LAT using the 3B5.1 mAb-beads (3B5.1 mAb-LAT), the supernatants of the virus-spiked fecal samples were collected by centrifugation at 6,000 rpm for 10 min.

A mixture containing 5 µl of the 3B5.1 mAb-beads and an equal volume of the AF containing the influenza A viruses and APMVs used in this study (Table 3.1), or the supernatants of the H5N1 HPAIV-spiked fecal samples (clade 2.2 and 2.5 strains) was mixed on a glass slide. Subsequently, the mixture was spread as a circle followed by gentle agitation for 5 min.

The results were scored as antigen-positive (agglutination of mAb-beads were observed) or antigen-negative (no visible agglutination). For evaluation of sensitivity and specificity of the 3B5.1 mAb-LATs, serial two-fold dilutions of the infected AFs and the supernatants of the virus-spiked fecal samples were diluted with 0.89% normal saline starting from undilution until 1:64 dilution.

3.3 Results

Optimization of concentration of anti-H5 mAb for binding to latex beads

Prior to testing the AFs and the virus-spiked-fecal samples containing H5 subtype viruses by the 3B5.1 mAb-LAT, two concentrations of 400 and 1,000 μg of anti-H5 3B5.1 mAb IgG were optimized for obtaining the best sensitivity of the test. The endpoint agglutinations of both concentrations of the 3B5.1 mAb IgG were similar at dilution of 1:8 (data not shown), suggesting that the high concentration of the mAb IgG did not increase the sensitivity of the 3B5.1 mAb-LAT. Thus, the latex beads were coupled with the 400 μg of the 3B5.1 mAb IgG, and used to detect the H5 subtype viruses in the AFs and the virus-spiked fecal samples.

Sensitivity and specificity of 3B5.1 mAb-LAT

The sensitivity and specificity of the 3B5.1 mAb-LAT were examined with a mixture of 5 μl of the AF containing the viruses and an equal volume of the mAb-beads. The agglutinations in the LAT were observed in all seven H5N1 HPAIV strains of the different clades examined (Table 3.2). As shown in Table 3.3, the detection limit of the 3B5.1 mAb-LAT was $10^{8.8\text{-}9.2}$ EID_{50}/ml, or 32−64 HAU for the Ck/Yamaguchi/7/04 (clade 2.5) and Ck/Miyazaki/K11/07 (clade 2.2) strains. In addition, five H5 subtype Eurasian lineage LPAIV strains (H5N1, H5N2, and H5N3 subtypes), but not two Am-nonGsGD strains (H5N2 and H5N3 subtypes), were detected by the 3B5.1 mAb-LAT (Table 3.4). No cross-reactivity was found in any of

the non-H5 subtype viruses of avian (H9N2) and human (H1N1) origins. APMV-1, -6 and -14 strains were also negative in the LAT.

Thus, these results suggested the 3B5.1 mAb-LAT was able to specifically detect Euracian H5 HPAIV and LPAIV strains.

Detection of H5N1 viruses in spiked chicken feces

To evaluate the possibility that the 3B5.1 mAb-LAT can be used in the field application, the Ck/Yamaguchi/7/04- and Ck/Miyazaki/k11/07-spiked chicken fecal samples were tested. A mixture of the undiluted and 2-fold diluted supernatants of the viruses-spiked fecal samples and the mAb-beads at the ratio of 1:1, 1:2 and 2.1 were evaluated. The results demonstrated that the agglutination of the 3B5.1 mAb-beads was observed in the undiluted supernatants of the H5N1 virus-spiked fecal samples. However, when the supernatants were diulted at higher than 1:2, no agglutination was found, even in the use of the increased volume (10 μl) of the mAb-latex beads (Table 3.5). As a result, the mAb-LAT could detect the Ck/Yamaguchi/7/04 and Ck/Miyazaki/K11/07 strains in the spiked fecal samples containing $10^{9.0}$ and $10^{9.8}$ EID_{50}/ml ($10^{6.7}$ and $10^{7.5}$ EID_{50}/5 μl/test) , respectively.

3.4 Discussion

Although the rapid diagnosis of AIV infection is frequently performed by the detection of the NP proteins of influenza A virus, this method cannot identify H5

subtype AIV infection. Therefore, methods that can directly detect H5 subtype AIVs would be useful for the rapid diagnosis of HPAI. In this study, the 3B5.1mAb-LAT detecting the H5 subtype AIVs was produced by coupling the anti-H5 neutralizing mAb (3B5.1) with the latex beads. The 3B5.1 mAb-LAT detected the 12 Eurasian H5 subtype AIVs containing HPAIVs (clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1c, 2.3.4 and 2.5) including clade 2.3.2.1a and 2.3.2.1c viruses currently circulating worldwide, and Eurasian LPAIVs (clade EA-nonGsGD) (Tables 3.2 and 3.4), although the test could not recognize the two North American lineage strains (clade Am-nonGsGD). Thus, it was shown that the 3B5.1 mAb-LAT could specifically detect the HA antigens of the Eurasian H5 subtype AIVs. This LAT also could detect the H5N1 viruses contained in chicken feces.

Unfortunately, however, the detection limit of the 3B5.1 mAb-LAT was very low: the test detected viral antigens in the samples containing $10^{8.8-9.2}$ EID_{50}/ml, or 32 to 64 HAU (Table 3.3). Chen et al. (2007b) developed a rapid and simple LAT with mAbs to H5 HA of the clade 9 H5N1 virus for the detection of H5N1 HPAIVs. The test could detect 2 HAU of the H5N1 strain used, indicating that it may be more sensitive than the 3B5.1 mAb-LAT established in this study. In their study, the H5 mAbs were covalently coupled onto the surface of carboxylated latex beads usingcarbodiimide, instead of the plain latex beads used in this study. Carboxylated latex beads are thought to be superior to unmodified beads for use in LATs because they are more hydrophilic, more stable in aqueous solutions, and contain reactive sites for covalent

conjugation of proteins (Chen et al., 2007b). However, it is not always applicable probably since the coupling reaction is affected by various factors in experiments (Personal communication).

IgG concentration bound to latex beads may affect the sensitivity of the LAT. The increased IgG concentration affected the sensitivity of the mAb-carboxylated latex beads, and the best sensitivity was achieved using 1,000 μg of IgG per 0.5 ml of 2.5% bead solution (Chen et al., 2007b). In contrast, according to the manufacturer's guidelines, the recommended IgG concentration for coupling with the plain (unmodified) latex beads used in this study was totally 400 μg of IgG per 0.5 ml of 2.5% beads. In this study, however, the increased concentration of the 3B5.1 mAb IgG used for coupling did not affect the sensitivity of the 3B5.1 mAb-LAT, since coupling of 1,000 μg of IgG with the beads did not significantly enhance the sensitivity of the test (data not shown). The difference in sensitivity seen in both studies might be due to the type of beads (modified or unmodified) and/or characteristics of mAbs used.

According to viral shedding data in H5N1 HPAIV (Ck/Yamaguchi/7/04)-infected chickens, the cloacal swabs contained $10^{3.5-4.5}$ EID_{50}/ml (Tsuda et al., 2007). In this study, the mAb-LAT could detect the Ck/Yamaguchi/7/04 and Ck/Miyazaki/K11/07 strains in the spiked fecal samples containing $10^{9.0}$ and $10^{9.8}$ EID_{50}/ml, respectively (Table 3.5, see also Materials and methods). However, when the supernatants of the spiked fecal samples were diluted higher than 1:2, no agglutination was observed. Therefore, it was suggested that the 3B5.1 mAb-LAT established here

could detect the H5N1 viruses only with high titers shed in clinical samples. Chen et al. (2007b) reported the lowest positive rate (26.8%) of the LAT in the cloacas of experimentally H5N1-infected chickens, while the high positive rates (80–96.7%) were observed in other organs (lung, spleen liver, brain, and pancreas). These results may explain the presence of inhibitors in the cloacas, probably derived from feces, and also explain the reason of the low sensitivity of the 3B5.1 mAb-LAT in the detection of the H5N1 virus contained in the fecal samples. However, since the 3B5.1 mAb-LAT also showed the low sensitivity with the infected AFs, this presumption would not be acceptable.

Taken together, the findings obtained in this study suggest that the 3B5.1 mAb-LAT may not be sensitive enough to detect the H5N1 virus directly from clinical samples collected from the infected birds. The virus titers that were detected by this mAb-LAT were approximately $10^{9.0}$ EID_{50}/ml ($10^{6.7}$ EID_{50}/test). Therefore, an improvement of the sensitivity of the 3B5.1 mAb-LAT would be essential for the field application.

3.5 Summary

Polystyrene latex beads were coupled with a neutralizing 3B5.1 mAb against the H5 HA protein of the clade 2.5 H5N1 strain (Ck/Yamaguchi/7/04). In this study, the LAT using the 3B5.1 mAb bount to beads (designated as 3B5.1 mAb-LAT) was established and evaluated for the detection of various H5 sybtype AIVs. The 3B5.1

mAb-LAT was capable of detecting H5N1 HPAIVs contained in AFs, and the virus-spiked chicken fecal samples. On the other hand, although Eurasian H5 subtype LPAIVs (H5N1, H5N2 and H5N3) were detectable, American H5 LPAIVs (H5N2 and H5N3) were not. The specificity of the 3B5.1 mAb-LAT was evaluated using other subtypes of influenza A viruses (H9N2 and H1N1), and APMVs (serotypes 1, 6 and 14). Nonspecific agglutination and cross-reactivity to those viruses were not observed. Unfortunately, however, the 3B5.1 mAb-LAT showed a low detection limit, since the test could detect viral antigens in the samples with the high virus titers (approximately $10^{8.8\text{-}9.2}$ EID_{50}/ml), or HAU (32 to 64). Therefore, the seinsitivy of the 3B5.1 mAb-LAT shoud be improved for the field application.

Table 3.1 Influenza A viruses and APMVs used in this study

Influenza virus subtypes and APMV serotypes	Strains	Clades	HAU
H5N1	Ck/VN-HT/33/03	1	512
H5N1	Ck/Miyazaki/K11/07	2.2	512
H5N1	Who.sw./Hokkaido/1/08	2.3.2.1	256
H5N1	Ck/VN-HT/30/10	2.3.2.1a	256
H5N1	Who.sw./Hamanaka/11	2.3.2.1c	256
H5N1	Mdk /VN-HN/77/07	2.3.4	256
H5N1	Ck/Yamaguchi/7/04	2.5	256
H5N1	Md/Hokkaido/24/09	EA-nonGsGD	512
H5N2	Dk/Japan/11UO0023/11	EA-nonGsGD	128
H5N2	Dk/Japan/11OG1032/11	EA-nonGsGD	256
H5N2	Dk/Japan/11OG1083/11	EA-nonGsGD	256
H5N3	Dk/Hong Kong/820/80	EA-nonGsGD	64
H5N2	Ck/Ibaraki/1/05	Am-nonGsGD	512
H5N3	Whis.sw/Shimane/499/83	Am-nonGsGD	256
H9N2	Ck/Yokohama/aq/55/01		512
H1N1	A/Puerto Rico/8/34		512
APMV-1	9KS0644		256
APMV-1	10UO343		1024
APMV-1	13OG1235		128
APMV-1	9KS0098		256
APMV-1	11UO040		128
APMV-1	Clone 30		2048
APMV-1	Ibaraki85		128
APMV-6	10EY0002		256
APMV-6	11OG0078		64
APMV-6	11OG0480		32
APMV-14	11OG0352		32

Table 3.2 Detection of H5N1 HPAIVs in AFs by 3B5.1 mAb-LAT

Strains	Clades	AF dilution*						
		Undiluted	1:2	1:4	1:8	1:16	1:32	1:64
Ck/VN-HT/33/03	1	+	+	+	+	-	-	-
Ck/Miyazaki/K11/07	2.2	+	+	+	+	-	-	-
Who.sw./Hokkaido/1/08	2.3.2.1	+	+	+	+	-	-	-
Ck/VN-HT/30/10	2.3.2.1a	+	+	+	+	-	-	-
Who.sw./Hamanaka/11	2.3.2.1c	+	+	+	+	-	-	-
Mdk /VN-HN/77/07	2.3.4	+	+	+	+	-	-	-
Ck/Yamaguchi/7/04	2.5	+	+	+	+	-	-	-

*+ = Positive agglutination; – = Negative agglutination

Table 3.3 Sensitivity of 3B5.1 mAb-LAT in detection of H5N1 HPAIV

H5N1 strains	Detection limit	
	EID_{50}/ml	HAU
Ck/Yamaguchi/7/04	$10^{8.8}$	32
Ck/Miyazaki/K11/07	$10^{9.2}$	64

Table 3.4 Detection of H5 subtype LPAIVs and APMVs in AFs by 3B5.1 mAb-LAT

LPAIV subtypes and APMV serotypes	Strains	Clades	AF dilution*						
			Undiluted	1:2	1:4	1:8	1:16	1:32	1:64
H5N1	Md/Hokkaido/24/09	EA-nonGsGD	+	+	+	+	-	-	-
H5N2	Dk/Japan/11UO0023/11	EA-nonGsGD	+	+	+	-	-	-	-
H5N2	Dk/Japan/11OG1032/11	EA-nonGsGD	+	+	+	+	-	-	-
H5N2	Dk/Japan/11OG1083/11	EA-nonGsGD	+	+	+	+	-	-	-
H5N3	Dk/Hong Kong/820/80	EA-nonGsGD	+	+	-	-	-	-	-
H5N2	Ck/Ibaraki/1/05	Am-nonGsGD	-	-	-	-	-	-	-
H5N3	Whis.sw./Shimane/499/83	Am-nonGsGD	-	-	-	-	-	-	-
H9N2	Ck/Yokohama/aq/55/01		-	-	-	-	-	-	-
H1N1	A/Puerto Rico/8/34		-	-	-	-	-	-	-
APMV-1	9KS0644		-	-	-	-	-	-	-
APMV-1	10UO343		-	-	-	-	-	-	-
APMV-1	13OG1235		-	-	-	-	-	-	-
APMV-1	9KS0098		-	-	-	-	-	-	-
APMV-1	11UO040		-	-	-	-	-	-	-
APMV-1	9KS0644		-	-	-	-	-	-	-
APMV-1	Clone 30		-	-	-	-	-	-	-
APMV-1	Ibaraki85		-	-	-	-	-	-	-
APMV-6	10EY0002		-	-	-	-	-	-	-
APMV-6	11OG0078		-	-	-	-	-	-	-
APMV-6	11OG0480		-	-	-	-	-	-	-
APMV-14	11OG0352		-	-	-	-	-	-	-

*+ = Positive agglutination; – = Negative agglutination

Table 3.5 Detection of H5N1 viruses in the virus-spiked chicken fecal samples

Supernatants of H5N1 virus-spiked fecal chicken samples*	mAb-beads	Ratio (Sample:Bead)	Supernatant dilution**				
			Undiluted	1:2	1:4	1:8	1:16
Ck/Yamaguchi/7/04 5 µl	5 µl	1:1	+	−	−	−	−
	10 µl	1:2	+	−	−	−	−
Ck/Miyazaki/K11/07 5 µl	5 µl	1:1	+	−	−	−	−
	10 µl	1:2	+	−	−	−	−
Ck/Yamaguchi/7/04 10 µl	5 µl	2: 1	+	−	−	−	−
	10 µl	1:1	+	−	−	−	−
Ck/Miyazaki/K11/07 10 µl	5 µl	2: 1	+	−	−	−	−
	10 µl	1:1	+	−	−	−	−

* Fecal samples contained the viruses with $10^{9.0\text{-}9.8}$ EID_{50}/ml.

** + = Positive agglutination; − = Negative agglutination

General discussion

AI is a viral disease of birds caused by AIVs belonging to the genus *influenza A virus* of the family *Orthomyxoviridae*. To date, AIV can be classified into16 subtypes of HA (H1−H16) and 9 subtypes of NA (N1−N9) (Webster et al., 1992). Based on pathogenicity of AIVs in chickens, the viruses are divided into 2 pathotypes, LPAIV and HPAIV. All subtypes of LPAIVs are usually circulating and are maintained in aquatic birds (natural host) without any symptoms, and sometime cause either no or mild diseases in poultry. In contrast, H5 or H7 subtype HPAIVs cause severe disease with high mortality up to 100% in poultry.

The HA protein is an antigenic glycoprotein which is found on the surface of influenza A viruses. Function of this glycoprotein is responsible for binding the viruses to the host cells (Wilson et al., 1981). Neutralizing antibodies to the HA protein can bind this glycoprotein on the surface of the viruses, and prevent entry of them to the host cells (Han and Marasco, 2011).

To evade host immune response, influenza A viruses seem to evolve overtime as the viruses replicate. This phenomenon is called “antigenic drift” (Suarez and Schultz-Cherry, 2000). A mutation of the HA gene commonly occurs, and results in antigenic modification of the HA proteins. Consequently, the mutated HA proteins could not be well recognized by the existing neutralizing antibodies. Such antigenic changes of the HA proteins would afford unfavorable effects on diagnosis and protective immunity.

Therefore, characterization of the HA genes or proteins by genetic and antigenic analysis is very important for establishment of adequate diagnosis and disease control.

Since H5N1 HPAIV was first isolated from a goose farm in Guangdong Province, China, in 1996 (Xu et al., 1999), the virus rapidly spread to other countries in Asia, Europe and Africa (WHO 2014, 2015, 2016) and has continued to cause serious economic losses to the poultry industry (Davison et al., 1999). Furthermore, H5N1 HPAIVs have infected humans with high mortality since 1997. To date, 453 deaths of 858 human infected cases have been documented (WHO, 2017). Thus, H5N1 HPAIVs not only threaten animal health but also raise pandemic potential in human population.

Currently, H5 subtype HPAIVs can be classified as clades 0–9 based on the phylogenetic analysis of the HA genes (WHO/OIE/FAO, 2008). Thus, the continuous and rapid evolution of H5 HA has been documented. However, clades 0, 3, 4, 5, 6, 8, and 9 viruses have not been recently detected. Only the clades 1, 2 and 7 viruses have continued evolution of their HA genes, leading to the emergence of new phylogenetic groups (WHO/OIE/FAO, 2008, 2012 and 2014; Smith et al., 2015). Especially, the clade 2 viruses have more diversity than the other clades, and have been classified into second-order (clades 2.1–2.5), third-order (2.1.1–2.1.3, 2.2.1, 2.2.2, 2.3.1–2.3.4), fourth-order (2.1.3.1–2.1.3.3, 2.2.1.1, 2.2.1.2, 2.2.2.1, 2.3.2.1 and 2.3.4.1–2.3.4.4), and fifth-order groups (2.1.3.2a, b, 2.2.1.1a, and 2.3.2.1a, b, c). Some new fifth-order clade strains (2.3.2.1a, 2.3.2.1c) have caused deadly outbreaks in Asia. Recently, the clade 2.3.2.1a and 2.3.2.c viruses have been detected in Africa and Europe (WHO, 2014,

2015, and 2016). However, not all strains of H5 subtype are highly pathogenic: only a small number of H5 LPAIV has sporadically mutated to the HPAIV during the repeated infection and transmission among poultry (Swayne, 2007). Therefore, rapid and specific laboratory diagnosis tests for dectecting both H5 subtype HPAIV and LPAIV infections are required for prompt disease control.

The commonly available laboratory diagnostic tests to identify H5 subtype viruses, e.g., virus isolation followed by HITs or RT-PCR have some disadvantages in terms of high cost, rapidness, necessary expertise, and the need for BSL-3 facilities. On the other hand, rapid diagnostic tests using mAbs against H5 HA proteins (anti-H5 mAbs) to immunologically detect H5N1 viruses have been also reported (Chen et al., 2007; Du et al., 2009; Nguyen et al., 2017; Ohnishi et al., 2012; Sakurai et al., 2013 Wada et al., 2011). However, there have no reports as to whether the mAbs used in the tests could detect currently circulating H5N1 clade viruses, although the test principle was based on the use of broad cross-reactive mAbs. Therefore, it is critical to make efforts to establish anti-H5 mAbs with broad cross-clade reactivity with the epitopes on HA proteins for the development of rapid and specific diagnostic tests of H5N1 HPAIVs, especially the currently circulating and newly emerging H5N1 clade viruses. In the current study, three neutralizing anti-H5 mAbs against clade 2.2 (1G5 mAb) and 2.5 (3B5.1 and 3B5.2 mAbs) HPAIVs were successfully generated from mice immunized with these viruses.

Although several broad cross-clade reactive human and mouse mAbs to the H5N1 virus have been reported (Kobayashi-Ishihara et al., 2014; Wu et al., 2014; Zuo et al.,

2015), there is not much information of the reactivity to the recently circulating H5N1 viruses such as clades 2.3.2.1a and 2.3.2.1c that are causing the outbreaks of poultry in Asia, Afria and Europe. The broad cross-clade mAbs reported previously were evaluated based on the H5N1 pseudoviruses, but not live viruses (Kobayashi-Ishihara et al., 2014, Zuo et al., 2015), and were examined only for the reactivity to the H5N1 HPAIVs but not other H5 subtype LPAIVs.

In this study, the 3B5.1 and 3B5.2 mAbs against the clade 2.5 virus reacted to all 26 strains from clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1b, 2.3.2.1c and 2.3.4 isolated in Japan and Vietnam during 2003−2012, suggesting that the epitope(s) recognized by theses mAbs are conserved, and the mAbs may possess broad cross-clade reactivity to H5N1 HPAIV. In additon, the 3B5.1 and 3B5.2 mAbs demonstrated the cross reactivity to Eurasian H5 subtype LPAIVs (clade EA-nonGsGD), but not American H5 subtype LPAIVs (clade Am-nonGsGD).

Although anti-H5 mAbs to the clade 2.5 viruses of the same origin (Ck/Yamaguchi/7/04 and Crw/Kyoto/53/04) isolated in Japan were reported, these mAbs showed the reactivity only to the clade 1 and 2.2 viruses but not clade 2.3.2.1 and 2.3.4 viruses (Uchida et al., 2008; Du et al., 2009). In contrast, the 3B5.1 and 3B5.2 mAbs, which were produced using the same clade 2.5 virus (Ck/Yamaguchi/7/04), showed broad cross reactivity to various clades including clades 2.3.2.1 and 2.3.4 as mentioned above. This result suggests that the previously reported mAbs (Uchida et al., 2008; Du et al., 2009) and the mAbs produced here recognize different epitopes on the H5 HA proteins of the H5N1 virus.

In the present study, I could successfully generate escape mutants of the Ck/Yamaguchi/7/04 strain from the 3B5.1 and 3B5.2 mAbs to analyze cross-reactive epitopes on the HA proteins recognized by these mAbs. An analysis of escape mutants and some clades H5N1 viruses suggested that the 3B5.1 and 3B5.2 mAbs bind to an epitope, including amino acid residues at position 162 (R162 or K162) in the HA1 protein. Unexpectedly, however, when five EA-nonGsGD and two Am-nonGsGD clade H5 LPAIV strains with R162 were examined, the mAbs recognized only EA-nonGsGD clade strains. Although the reason of this discrepancy is unknown, I suspected that the HA1 structure of the Am-nonGsGD clade strains was complex and different from that of the HPAIV and EA-nonGsGD clade strains.

The cross-clade conformational epitope (115QIIPKSSWSDH125 and 158PTIKRSYNNTNQE170, H5 numbering) in the HA1 protein of the H5N1 virus has been reported (Zuo et al., 2015). In the present study, the 3B5.1 and 3B5.2 mAbs could not bind to the mutant strains with the mutation R162I or R162G, which may suggest that the epitope recognized by these mAbs overlapped the epitope (158PTIKRSYNNTNQE170). Next, therefore, I tried to analyze whether the 158PTIKRSYNNTNQE170 epitope was conserved among the 5,366 sequences of various clade H5N1 HAs available in the database. As a result, the R162 or K162 residues in the HA1 protein was highly conserved among 36 clades (84.4%), including clades 2.3.2.1a and 2.3.2.1c, out of the 43 H5N1 clades reported, except for seven clades (2.2.1.1a, 2.2.2, 2.2.2.1, 2.3.1, 2.3.4.4, 7.1 and 7.2). Although the 3B5.1 and 3B5.2 mAbs are expected to fail to react to these 7 clade strains, I am thinking that

clades of the H5N1 HPAIV strains containing the R162 or K162 residue in the HA1 protein could be recognized by these mAbs. Thus, the 3B5.1 and 3B5.2 mAbs may be useful for the rapid and specific detection of H5N1 HPAIVs currently circulating in the field.

Various commercial rapid diagnostic kits utilizing mAbs to the NP protein of nfluenza A virus have been used to detect AIV infections. Early and rapid detection of HPAIV infection is so important for containing HPAI. However, since these commercial kits cannot identify H5 subtypes, further testing is necessary to identify H5 subtypes, such as HITs or RT-PCR. However, since the HIT is not directly applicable to clinical samples to subtype the viruses, virus isolation which needs the well-equiped BSL-3 facilities and takes time is necessarry. Although conventinal and real-time RT-PCRs are highly sensitive and relatively rapid methods, they requires high costs of equipments and reagents, and molecular biological expertises. Therefore, the rapid tests with specific mAbs that can directly detect and identify H5 subtypes would be useful for prompt HPAI control.

In the present study, the 3B5.1 mAb was used to develop the LAT (3B5.1 mAb-LAT) for direct detection of H5 HA antigens. I expected that the 3B5.1 mAb-LAT could detect H5 subtype viruses of various clades, because of the cross-clade reactivity of the 3B5.1 mAb. Actually, the 3B5.1 mAb-LAT could detect and identify the 12 Eurasian H5 subtype AIVs, including HPAIVs (clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1c, 2.3.4 and 2.5) and Eurasian LPAIVs (clade EA-nonGsGD). The results seem so imoportant, because clade 2.3.2.1a and 2.3.2.1c viruses are currently circulating in Asia, Africa, and

Europe and causing deadly outbreaks. Moreover, the test detected the H5N1 virus contained in the virus-spiked fecal samples, suggesting that the test was applicable to clinical samples for the diagnosis of H5N1 infection in the field.

Unfortunately, however, the sensitivity of the 3B5.1 mAb-LAT was not high, compared to that reported in the previous study (Chen et al., 2007). The 3B5.1 mAb-LAT detected the H5 HA antigens in AF samples containing at least 32 HAU, while the LAT reported in the previous study did the antigens in the samples that had titers of 2 HAU (Chen et al., 2007).

The cloacal swabs of chickens inoculated with the H5N1 virus (Ck/Yamaguchi/7/04) was contained $10^{3.5\text{-}4.5}$ EID_{50}/test (Tsuda et al., 2007). The 3B5.1 mAb-LAT may not be sensitive enough to detect the H5N1 virus directly from clinical samples collected from the infected birds, due to its detection limit of approximately $10^{9.0\text{-}9.8}$ EID_{50}/ml ($10^{6.7\text{-}7.5}$ EID_{50}/test).

In this study, I did not compare the sensitivity of the 3B5.1 mAb-LAT with that of other rapid diaganostic tests to detect H5 HA antigens such as immunochromatographic (IC) tests (Marché and van den Berg, 2010; Nguyen et al., 2017; Sakurai et al., 2013; Tsuda et al., 2007). The detection limits of the IC tests were reported to range from approximately $10^{7.0–9.8}$ EID_{50}/ml (Marché and van den Berg, 2010; Tsuda et al., 2007,) or $10^{6.0–7.0}$ pfu/ml (Sakurai et al., 2013). Thus, the the 3B5.1 mAb-LAT seemed to be less sensitive than that of the IC tests. However, since the rapid H5 antigen detection tests that have been used on a commercial or laboratory level

showed the low detection sensitivity, the negative results cannot necessarily granted. Recently, the enhanced sensitivity of the IC tests by silver or fluorescent amplification have been reported, in which the sensitivity was enhanced 10- to 100-fold higher than the original ones (Nguyen et al., 2017; Sakurai et al., 2013; Wada et al., 2011). Nevertheless, however, the improved tests are still less sensitive as compared with the most sensitive techniques such as virus isolation or RT-PCR. Therefore, the rapid antigen detection tests should be mainly used on optimal clinical samples with high virus titers from diseased birds in outbreaks as secreening tests (Chua et al., 2007; Marché and van den Berg, 2010; Okamatsu et al., 2016), and the results always need to be comfirmed by more sensitive laboratory-based tests.

Because of the time limit in the Ph.D. program, I could not try to improve the sentivity of the 3B5.1 mAb-LAT. I am thinking that improvement of the sensitivity of the LAT produced in this study should be considered, because the rapid and direct detection of H5 subtype virus is useful for establishment of prompt HPAI containment measures.

The usefulness of the rapid and direct detection of H5 antigens in clinical samples using broad-reactive mAbs is unquestionable in diagnosis of H5 HPAIV infection. However, H5 viruses have evolved rapidly and extensively, and the continued evolution of the virus has generated new clades. Recently, the new clade 2.3.4.4 viruses (H5N6 and H5N8) with the mutation R162I in the HA1 protein have emerged. Although these clade viruses in addition to other clade ones without R162 or K162 resudue were not available in this study, probably the 3B5.1 and 3B5.2 mAbs could not recognize them.

Therefore, a combination of the 3B5.1 and 3B5.2 mAbs and multiple complementary mAbs recognizaing the different epitopes could be useful in the development of a diagnostic test to detect all H5 clades. Acutally, a dot ELISA and IC test using two complementary anti-H5 mAbs were reported for universal detection of H5 subtype viruses (He et al., 2010; Nguyen et al., 20017).

Nevertheless, it may be critical to continuously make efforts to search for anti-H5 mAbs with broad cross-reactivity with all H5 clade viruses in the development of diagnostic systems of H5 subtype HPAIV infection. Moreover, antigenic characterization of H5 viruses using mAbs may help to improve our knowledge on evolution and epidemiology of AIVs, and vaccine designs.

General summary

H5N1 HPAIVs are a threat to both animal and public health, because the viruses have continued to cause deadly poultry outbreaks in many areas of the world, and also caused serious diseases highly associated with mortality in humans, leading to an increased awareness of a pandemic outbreak since the first outbreak in chicken and human populations in 1997, Hong Kong.

Currently, H5 HPAIVs have been classified as clades 0–9 based on the phylogenetic analysis of the HA genes. However, the continued evolution of the H5N1 virus has led to the emergence of new phylogenetic groups. Especially, clade 2 viruses have evolved rapidly and extensively, and the continued evolution of the virus has generated second-order (clades 2.1–2.5), third-order (2.1.1–2.1.3, 2.2.1, 2.2.2, 2.3.1–2.3.4), fourth-order (2.1.3.1–2.1.3.3, 2.2.1.1, 2.2.1.2, 2.2.2.1, 2.3.2.1 and 2.3.4.1–2.3.4.4), and fifth-order groups (2.1.3.2a, b, 2.2.1.1a, and 2.3.2.1a, b, c). Some new fifth-order clade groups (2.3.2.1a and 2.3.2.1c) have caused outbreaks worldwide. It suggests that the newly emerging H5N1 HPAIVs are circulating in many regions. Therefore, an accurate, rapid, and specific diagnostic system for H5 HPAIV infection is essential for the establishment of prompt disease control.

The currently available diagnostic techniques for identifying H5N1 viruses, e.g., virus isolation followed by serological tests to determine HA and NA subtypes or RT-PCR, have some disadvantages in terms of high cost, rapidness, necessary expertise, and the need for a BSL-3 laboratory. Rapid diagnostic tests using specific mAbs against

H5N1 HA proteins (anti-H5 mAbs) to immunologically detect and identify H5 subtype viruses have been reported. However, there have been no reports as to whether the mAbs used in these tests could detect currently circulating H5N1 clade viruses, although the test principle was based on the use of broad cross-reactive mAbs. Therefore, it is important to continuously make efforts to search for anti-H5 mAbs with broad reactivity with the epitopes on HA proteins in the development of highly sensitive and rapid diagnostic systems to detect H5N1 HPAIVs, especially the H5N1 viruses that are currently circulating and the newly emerging H5N1 clades such as clades 2.3.2.1a and 2.3.2.1b.

The first aim of this study is to produce and characterize mAbs against H5 HA (anti-H5 mAbs) of H5N1 HPAIVs isolated in Japan. The reactivities of the produced mAbs with the H5N1 HPAIVs and H5 subtype LPAIVs isolated in Japan and Vietnam were evaluated. In addition, a broad cross-clade epitope(s) on the HA protein of H5N1 HPAIV recognized by the mAbs was predicted based on analysis of the HA amino acids of various calde H5N1 HPAIVs, and escape mutants of the produced mAbs. The second aim of this study is to develop a rapid H5 antigen detection test, LAT, using the produced mAb, and to examine sensitivity and specificity to evaluate its potential of a field application.

In Chapter I, three neutralizing anti-H5 mAb (3B5.1, 3B5.2 and 1G5) using two clades (2.5 and 2.2) of the H5N1 HPAIV isolated in Japan were successfully established. All three anti-H5 mAbs were IgG1 subclass and were able to recognize the HA proteins.

WB analysis indicated that the 3B5.2 and 1G5 mAbs recognized the conformational epitope on the HA proteins (HA0), whereas the 3B5.1 recognized the linear and conformational epitope on the HA proteins (HA0 and HA1). Blocking IFAT showed that each mAb recognized different epitopes. The 3B5.1 and 3B5.2 mAbs against the clade 2.5 virus (Ck/Yamaguchi/7/04) showed cross-reactivity to each strain from the 6 clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1c and 2.3.4 isolated in Japan and Vietnam. I, therefore, expected that the 3B5.1 and 3B5.2 mAbs probably have cross-clade reactivity against H5N1 viruses. In contrast, the 1G5 mAb against the clade 2.2 virus (Ck/Miyazaki/K11/07) showed reactivity only to clades 1 and 2.5 strains. Thus, the 3B5.1 and 3B5.2 mAbs may be useful for specific detection of various H5N1 HPAIVs circulating in the field.

In Chapter II, I further examined whether the mAbs could recognize various H5 clade viruses using the 26 strains from clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1b, 2.3.2.1c and 2.3.4. isolated in Japan and Vietnam. As a result, the 3B5.1 and 3B5.2 mAbs reacted to all the strains tested, but the 1G5 against the clade 2.2 virus showed reactivity only to clades 1, 2.3.4 and 2.5 strains. These results suggested that the epitope(s) recognized by the 3B5.1 and 3B5.2 mAbs are conserved among various H5 clade viruses.

An analysis of escape mutants of the 3B5.1 and 3B5.2 mAbs and the HA1 amino acids of the H5N1 viruses recognized by the mAbs suggested that the mAbs bind to an epitope, including amino acid residues at position 162 (R162 or K162) in the HA1 protein. The amino acid residues including R162 (158PTIK**R**SYNNTNQE170) in the

HA1 protein are thought to be probably one of major cross-clade epitopes. Therefore, the present results suggest that the 3B5.1 and 3B5.2 mAbs have potential to be broad cross-clade reactive mAbs.

Since the H5N1 viruses of all clades 0-9 were not available to evaluate the cross-clade reactivity of the 3B5.1 and 3B5.2 mAbs, the 158PTIKRSYNNTNQE170 epitope in the HA1 protein was aligned and compared among 5,366 strains of clades 0–9 viruses to analyze the conservation of this epitope. The results demonstrated that the R162 or K162 residues were highly conserved among 36 clades, including clades 2.3.2.1a and 2.3.2.1c, out of the 43 H5N1 clades reported, except for seven clades (2.2.1.1a, 2.2.2, 2.2.2.1, 2.3.1, 2.3.4.4, 7.1 and 7.2). This study suggested that H5N1 HPAIVs containing the R162 or K162 residue in the HA1 protein could be recognized by the 3B5.1 and 3B5.2 mAbs.

In Chapter III, a LAT utilizing the 3B5.1 mAb (3B5.1 mAb-LAT) was established and evaluated for the detection of H5N1 HPAIVs. Polystyrene latex beads were coupled with the mAb. The 3B5.1 mAb-beads mixed with AFs containg H5N1 HPAIVs, and H5N1 HPAIV-spiked chicken fecal samples caused agglutination in the LAT. In addition, Eurasian H5 subtype LPAIVs (H5N1, H5N2 and H5N3) but not American H5 LPAIVs (H5N2 and H5N3) were detectable by the test. Although the specificity of the 3B5.1 mAb-LAT was evaluated using other subtypes of influenza A virus (H9N2 and H1N1) and APMVs, nonspecific agglutination and cross-reactivity to those viruses were not observed. Unfortunately, however, I am thinking that the 3B5.1

mAb-LAT may not be sensitive enough to detect the H5N1 viruses, because the test required the virus and HA titers higher than $10^{8.8}$ EID_{50}/ml and 32 HAU, respectively. The test also required the virus titers higher than $10^{9.0}$ EID_{50}/ml in detecting H5 HA antigens directly from H5N1-virus spiked chicken fecal samples. Therefore, the further study shoud be done for improving the test sensitiviy.

The usefulness of the rapid and direct detection of H5 antigens in clinical samples using broad-reactive mAbs is unquestionable in diagnosis of H5 HPAIV infection. However, H5 viruses have evolved rapidly and extensively, and the continued evolution of the virus has generated new clades. Therefore, it is very important to continuously make efforts to search for anti-H5 mAbs with broad reactivity to all H5 clade viruses based on antigenic analysis of the viruses, especially in the development of diagnostic systems.

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学位論文要旨

鳥インフルエンザウイルス(AIV)は、オルトミクソウイルス科 A 型インフルエンザウイルス属に属する鳥類由来のインフルエンザウイルスである。現在、AIV にはウイルス粒子表面にあるヘマグルチニン（赤血球凝集素 HA）とノイラミニダーゼ（NA）という糖蛋白 の抗原性の違いから、16 の HA 亜型と 9 の NA 亜型のウイルスが存在している。また、AIV の病原型は鶏における病原性から特に病原性を示さない低病原性 AIV (LPAIV) と高い死亡率を引き起こす高病原性 AIV (HPAIV)に分類されている。特に、家禽産業で問題となるのは HPAIV であるが、全ての亜型ウイルスが HPAIV になる訳ではなく、H5 あるいは H7 亜型 AIV の中のごく一部の株が、H5 あるいは H7 亜 LPAIV から変異して HPAIV となり、家禽産業に多大な経済的損失をもたらしている。

特に、1997 年の香港の家禽産業における H5N1 亜型 HPAIV の発生以降、本ウイルスは中国本土を含むアジア、アフリカおよび欧州の国々に伝播し、これまでに甚大な経済的な損失を家禽産業に与えてきた。また、特筆すべき点は、人における感染と感染に起因する死亡例がみられた事である。これまでに感染者数 858 名、死亡率は約 62%であることが報告されている。

H5N1 亜型 HPAIV は、家禽間で感染を繰り返している間に急速な進化を遂げてきた。現在、HA 遺伝子の系統樹解析により 10 の clade (0〜9) が認められているが、現在 clade 3〜6 と clade 8〜9 のウイルスは流行していない。一方、clade 1、2 および 7 は進化を続けており、現在さらに新たに second-order (clade 1.1, 2.2 と 7.1)、third-order (clade 1.1.1 と 2.2.1)、fourth-order (clade 2.2.2.1、2.3.2.1 and 2.3.4.1) 、fifth-order clade (clade 2.3.2.1a、2.3.2.1b、2.3.2.1c) が認められている。

日本では、2004 年 から 2016 年に 6 回の HPAI の発生があった。最初の 4 回の発生は、clade 2.5, 2.2, 2.3.2.1 および 2.3.2.1c に属する H5N1 亜型ウイルスの感染によるものであった。しかし、 2014 年の 5 回目の発生は clade 2.3.4.4 に属する H5N8 亜型ウイルスの感染に起因した。また、2016 年の 6 回目の発生は, clade 2.3.4.4 に属する H5N6 亜型ウイルスの感染が原因であった。それ故、正確、特異的かつ迅速な診断は、HPAI の発生を制御する上で重要である。

現在、H5N1 亜型ウイルス感染のウイルス学的診断法としてウイルス分離法（分離後に分離ウイルスの HA および NA 亜型を決定する）あるいは RT-PCR 法が用いられている。しかし、いずれの方法も時間や労力、専門的な知識や技術、コストの問題（高額な試薬や装置など）または BSL-3 施設などの特別な施設が必要であるなどの欠点がある。一方、臨床検体から H5N1 亜型ウイルスを検出する迅速診断法として A 型インフルエンザウイルスの核蛋白（NP）に対するマウスモノクローナル抗体 (mAb) を利用した診断法が応用されている。しかし、本法はインフルエンザウイルスの共通抗原を検出するために亜型を決定できない欠点があることから、H5 亜型ウイルスの HA 蛋白に対する mAb (H5 mAb)を用いた H5N1 ウイルス感染を迅速に診断可能な方法が報告されている。しかし、これまで全ての clade に属するウイルスと反応できる mAb は報告されていない。

そこで本研究では、まず H5N1 亜型ウイルスに対して広い交差反応性を示す H5 mAb の作出とその性状解析を行うことを目的とした。また、二つ目の目的として、作出した H5 mAb を用いた簡便且つ迅速、高感度および高特異性である血清学的診断法の開発を試みた。

第 1 章では、日本で分離された H5N1 亜型ウイルスの HA 蛋白に対する H5 mAb の確立とその性状解析を行った。その結果、Ck/Yamaguchi/7/042 (clade 2.5) に対する中和活性を有する 2 株 (3B5.1 mAb、3B5.2 mAb) の H5 mAb、Ck/Miyazaki/K11/07 (clade 2.2) に対する中和活性を有する 1 株 (1G5 mAb)の H5 mAb が作出された。全ての mAb の subclass は IgG1 であり、赤血球凝集抑制活性を有していた。ウエスタンブロティング法の解析により 3B5.2 および 1G5 mAb は、 HA0 上の conformational epitope を認識していたが, 3B5.1 mAb は HA0 上の linear および HA1 上の conformational epitope の

両方を認識していた。3B5.1 および 3B5.2 mAb は、6 つの clade (1、2.2、2.3.2.1、2.3.2.1a、2.3.2.1c および 2.3.4.) 由来のウイルス株と反応した。これに反して、1G5 mAb は、clade 1 および 2.5 に属するウイルス株のみに反応性を示した。これらの結果から、3B5.1 および 3B5.2 mAb が、種々の clade の H5N1 亜型ウイルスに対して反応性を示す可能性が示唆された。従って、これらの mAb は野外に広く分布している H5N1 亜型ウイルスの検出に応用できる可能性が示された。

第 2 章では、異なる clade に属する多数の H5N1 亜型ウイルス株および H5 亜型 LPAIV (Eurasian-origin : clade EA-nonGsGD、American-origin : clade Am-nonGsGD) に対する 3 つの mAb の反応性の検討と HA 蛋白上に存在するエピトープの解析（エピトープマッピング）を行った。その結果、両 mAbs は clade 1、 2.2、2.3.2.1、2.3.2.1a、2.3.2.1b、2.3.2.1c および 2.3.4 に属する調べられた 26 株全てに対して反応性を示した。これらの clade には、世界各地で流行している clade 2.3.2.1a および clade 2.3.2.1c が含まれていた。一方、1G5 mAb は、clade 1、2.3.4 および 2.5 に属するウイルス株とのみ反応した。これらの結果から、3B5.1 および 3B5.2 mAb によって認識されるエピトープが H5N1 亜型 HPAIV 間で保存されている可能性が示唆された。ブロッキング蛍光抗体法の解析によりそれぞれの mAb は HA 蛋白上の異なるエピトープを認識していることが分かった。3B5.1 および 3B5.2 mAb に対する escape mutant を作製して mAb との反応性を解析したところ、HA1 蛋白の 162 番目のアミノ酸残基 (R162 あるいは K162) を含むエピトープを認識しているものと推測された。次に、R162 あるいは K162 のアミノ酸残基がこれまでに報告されている 43 の H5N1 clade で保存されているか解析したところ、近年アジア、アフリカ、欧州で流行が認められている clade 2.3.2.1a および 2.3.2.1c を含む 36 の clade で保存されていることが明らかになった。一方、予想に反して、3B5.1 および 3B5.2 mAb は HA1 蛋白のアミノ酸残基 R162 を持つ EA-nonGsGD clade の Eurasian-origin LPAIV (H5N1、H5N2 および H5N3) には反応したが、Am-nonGsGD clade の American-origin LPAIV (H5N2 および H5N3) を認識できなかった。この矛盾した結果の理由は不明であった。

これまでに H5N1 HPAIV の HA1 蛋白上に clade 間とウイルス株間で高度に保存されているエピトープ (158 PTIKRSYNNTNQE 170) が報告されているが、本研究で得

られた結果を考慮すると、3B5.1 および 3B5.2 mAb もこのエピトープを認識している可能性が示唆された。

第 3 章では、H5N1 ウイルス検出のための 3B5.1 mAb を用いた簡便・迅速なラテックス凝集試験 (3B5.1 mAb-LAT)を作出し、診断法としての有用性を評価した。

ポリスチレンビーズに中和活性のある 3B5.1 mAb を結合させた (3B5.1 mAb-bead)。3B5.1 mAb-bead と H5N1 亜型 HPAIV および Eurasian-origin の H5 亜型 LPAIV (H5N2 および H5N3) を含む尿腔液と混合させると mAb-bead の凝集が観察された。また、H5N1 亜型 HPAIV を鶏糞便に加えたサンプルを作製し、mAb-bead と混合させたところ mAb–bead の凝集が観察された。一方、H9N2 および H1N1 亜型インフルエンザウイルスや鳥パラミクソウイルス（血清型 1、6 および 14）を mAb-bead と混合させたところ 3B5.1 mAb-bead の凝集は認められなかった。

これまでに報告されている H5 mAb を用いた LAT や免疫クロマトグラフィー診断法と直接検出感度を比較できなかったが、3B5.1 mAb-LAT は、サンプル中に $10^{8.8}$ EID_{50}/ml 以上のウイルス力価あるいは 32 倍以上の HA 価が含まれていなければウイルス抗原を検出できなかったことから、他の方法で報告されている検出感度に比べると感度は低いものと推測された。しかしながら、現在多くの地域で流行し家禽産業に多大な経済的損失をもたらしている clade 2.3.2.1a および 2.3.2.1c に属する H5N1 亜型ウイルスを検出できた。

HPAI の早期の封じ込め対策の実施を容易にする観点から、HPAI 発生時に発症鳥の臨床サンプルから直接 H5 抗原を迅速に検出する方法が有用であることは、疑いの余地がない。しかし、上述したように H5 亜型ウイルスの急速な進化による HA 抗原性の変異が著しいことから、全ての H5 clade ウイルスを検出できるような mAb を作出する継続的な努力が求められるかもしれない。また、本研究で開発した LAT では HA タンパク上の一つのエピトープを認識する 3B5.1 mAb のみを使用したが、複数の異なるエピトープを認識する mAb を組み合わせることにより、より多くの clade ウイルスを検出できると考えられることから、今後このような取り組みも mAb を用いた迅速診断法の開発の上で重要な点になってくると思われる。