Avian Influenza virus M2e protein: Epitope mapping, competitive ELISA and phage displayed scFv for DIVA in H5N1 serosurveillance

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Abstract

Within the avian influenza virus (AIV) history, H5N1 subtype is the most alarming in terms of its spread rate throughout the globe with its demonstrated unusual pattern of evolution. Persistency and constant circulation of this subtype in poultry population in a number of countries have resulted its establishment and declaration as enzootic. The affected countries are commonly characterised by high poultry populations and productions. They are also developing countries which have minimal funding allocated for precaution on disease incursion. Past observations showed that a single AIV epizootic is capable of causing significant economic burden throughout the world. Although epizootic, it still resulted sporadic cases of human infection and mortality. Therefore, H5N1 enzootic countries opt for vaccination strategy (usually with inactivated whole virus) to evade AIV incursions. However, this interferes with the AIV surveillance effort. This is due to the lack of diagnostic tool with the ability to differentiate AIV infected animal from vaccinated animal (DIVA). Following this realisation, several options are made available. Diagnostic tool development which is capable of DIVA requires a highly sensitive and specific target which at the same time is economic, and pose ease of application. In recent years, growing interest on the AIV matrix 2 extracellular domain (M2e) protein has propelled its exploration as the target for AIV serosurveillance diagnostic tool development. It has been demonstrated to be highly sensitive and specific in detection for AIV infection in an indirect enzyme-linked immunosorbent assay (ELISA) setting. The factor which made it highly interesting is its ability for DIVA application. M2e protein can only be found in low concentration on an AIV particle which is used in an inactivated vaccination strategy, while present in high concentration if cells are AIV infected. Therefore, this study has further explores the AIV M2e protein potential for AIV serosurveillance diagnostic tool development and successfully demonstrated an M2e-based test in a competitive ELISA format for DIVA. This particular ELISA format was of interest as it can be potentially used in multiple species application, as AIV is a multispecies pathogen. To ensure the universality of the competitor antibody,

comparative mapping of anti-M2e antibodies from chicken, mouse and rabbit was done. Findings highlighted slight variations in the epitope identified for the M2e antigen by antibodies from different species. Mouse anti-M2e antibodies are more suitable to be used as the competitor antibodies against anti-M2e chicken sera in the M2e-based competitive ELISA test. Consequently, application of the mouse anti-M2e antibodies in the M2e-based competitive ELISA has demonstrated specific and sensitive indication of AIV infection in the H5N1 challenged chicken sera. Biotechnology developments has also introduced the single chain variable fragment (scFv) antibodies as specific and stable bait for antibodies detection against targeted pathogen's protein (antigen). Taking advantage of this knowledge, this study has also successfully isolated reactive and specific anti-M2e scFv antibodies from avian sources. This is critical as an avian sourced antibodies to be used as bait for the targeted pathogen's protein is highly relevant in the setting for AIV serosurveillance application in the poultry industry. These findings are significant in the effort to provide a highly sensitive and specific diagnostic tool, which are also cost effective, easy to apply with high throughput ability. Such ideal diagnostic tool for AIV serosurveillance is highly valuable, as this may hold the key to break the AIV continuous circulation.

Thesis declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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"Life is beautiful when you look back."

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List of Abbreviations

%	percent
°C	degree Celsius
µg	microgram
µl	microlitre
µm	micrometre
2YT	2-yeast-tryptone
aa	amino acid
AGRF	Australian Genome Research Facilities
AIV	avian influenza virus
BDT	Big Dye Terminator
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cfu	colony forming unit
cm	centimetre
DIVA	differentiate infected from vaccinated animal
DNA	deoxyribonucleic acid
dNTP	nucleoside triphosphate
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
g	gram
HA	hemagglutinin protein
HCl	hydrochloric acid
hr	hour
HRP	Horseradish peroxidase
IPTG	isopropylthiogalactosidase
kDa	kilodalton
L	litre
M	molar
M1	matrix 1 protein
M2e	matrix 2 extracellular domain
mAb	monoclonal antibodies
min	minute
ml	millilitre
mm	millimetre
mM	millimoles
mRNA	messenger ribonucleic acid
MW	molecular weight
NA	neuraminidase protein
nm	nanometre
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

rpm	rotation per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
sAb	soluble antibodies
scFv	single chain variable fragment
SDS	sodium dodecyl sulfate
SOE	splicing by overlap extension
TBE	tris-borate-EDTA
V	volt
v/v	volume per volume
V_H	variable heavy chain
V_L	variable light chain

List of Publications

2016 **Hasan NH**, Ebrahimie E, Ignjatovic J, Tarigan S, Peaston A & Hemmatzadeh F. 2016. Epitope mapping of avian influenza M2e protein: Different species recognise various epitopes. PLOS ONE, 11(6), e0156418

2016 **Hasan NH**, Ignjatovic J, Peaston A & Hemmatzadeh F. 2016. Avian Influenza Virus and DIVA strategies. Viral Immunology, 29 (4), 198-211.

Chapter 1 Introduction and Literature review

1.1 Introduction

Highly pathogenic avian influenza (HPAIV) H5N1 is one of the most widespread and highly variant AIV strain with pandemic potential (Fouchier & Guan 2013). Since its major outbreak in 1997, HPAIV H5N1 has become established in five countries, namely People Republic of China, Indonesia, Bangladesh, Vietnam and Egypt (FAO 2011; Fouchier & Guan 2013). This is exacerbated by its concurrent circulation with other low pathogenic AIV (LPAIV), as these viruses evolve through recombination mechanism and the LPAIV may become the source of genetic pool for production of a more fit and virulent progeny (Rohm et al. 1995; Swayne 2007). Potential emergence of a more virulent progeny through such co-circulation is the main reason poultry vaccination is adopted in most of these enzootic countries. This may reduces the rate of virus spread or even act as a barrier for a potential outbreak. Economical and easy to prepare, whole killed virus of heterologous or homologous strain with the field virus is usually used as vaccination strain (Chen, H 2009; Gutierrez et al. 2009). As protection against infection is associated with antigenic relatedness, homologous strain is more preferred to counter H5N1 infection, as its high pool of genetic variants may render heterologous strain vaccination ineffective (Lee & Suarez 2005; Swayne et al. 2000).

The only issue surrounding this option is that the available conventional diagnostic tests are not capable of differentiating serologic reaction from a vaccinated animal from those of virus infected animal. This is because the virus used for vaccination still possess the complete structure of the virus, only it is not capable of replication. Hence, antibodies produced in an infected host is similar to those of vaccinated ones. Due to variety of factors which govern the outcome of vaccination such as the presence of maternal antibodies, and each bird immune response which may differs between species, the available AIV vaccinations are not capable of perfect vaccination (Bublot et al. 2006; Lee, Senne & Suarez 2004). Hence, silent spread of LPAIV within a vaccinated flock is still possible as vaccination may only masked virus infection where very limited sign of disease is observed. Longer circulation within a

population may provide enough time for the virus to evolve and surpass the protection provided by the vaccine strain and resulted vaccine failure (Grund et al. 2011; Lee, Senne & Suarez 2004; Smith, GJD et al. 2006). Thus, it is important for early live virus detection to halt the incursion of new virulent strains. Therefore, differentiating infected from vaccinated animal (DIVA) strategies have been introduced as the counter measure to this issue.

DIVA strategies are aimed to enable vaccination application without compromising diagnostic ability to detect virus infection. Numbers of DIVA strategy options are available for application, namely heterologous vaccination, subunit vaccination, epitope differential of non-structural protein 1 (NS1), ectodomain of matrix 2 (M2e) protein, as well as the hemagglutinin protein 2 (HA2) (Birch-Machin et al. 1997; Boyle & Heine 1993; Capua et al. 2002; Lambrecht et al. 2007; Suarez 2012). An ideal DIVA strategy would present to be easy for application, possess sensitivity and specificity for virus infection detection, and available for large scale screening without unnecessary economic burden.

Among these DIVA strategies, the M2e protein strategy holds the most interest as a part of AIV surveillance. It is demonstrated as sensitive and specific test for DIVA application, where its high epitope density on the surface of infected cells which otherwise is low on the virus particle, is a useful marker for virus infection (Hemmatzadeh et al. 2013; Kim, MC et al. 2010; Lambrecht et al. 2007). As an indirect enzyme-linked immunosorbent assay (ELISA) based system with recombinant M2e protein as the antigen, M2e-based ELISA is proven to be economical with high-throughput capacity. It holds as an ideal test for an AIV surveillance. However, this approach is limited by the weak immunogenicity of the M2e protein (Neirynck et al. 1999). Additionally, AIV is a multispecies infectious agent (Chambers, Dubovi & Donis 2013). Application of an indirect M2e-based ELISA is limited to the availability of species-specific secondary antibodies. Hence, a more universal test format such as competitive ELISA is more relevant for surveillance of AIV.

The key factor for the universality of a competitive ELISA format test lies in the availability of a labelled competitor antibody targeting a specific antigen that identifies dominant epitope, hence, similar epitope in multiple species. Successful attempts on development of competitive ELISA test for AIV have been done targeting the nucleoprotein (NP) (Shafer, Katz & Eernisse 1998; Starick et al. 2006; Zhou, EM et al. 1998). However, an NP-based competitive ELISA is not suitable for application in countries which adapted AIV vaccination in their poultry, since it is does not possess DIVA ability. In the following review, current status of HPAIV H5N1 in Indonesia will be explored as the background of the project development.

1.2 Thesis outline

Chapter 1 includes a brief review on the HPAIV H5N1 status in Indonesia and the availability of DIVA test for surveillance purposes. These two topics are covered in details in Chapter 2 (Avian influenza virus and DIVA strategies), which has been published as a review paper. This is followed by a brief literature review on antigenic mapping, competitive ELISA and phage display technology. This chapter is closed by a short section on the research rationale and aims.

The following chapters are written in a publication format, where Chapter 3 has been published, while the remaining two chapters (Chapter 4 and 5) will be submitted for publication. Briefly, Chapter 3 discusses on the antigenic mapping of the M2e protein using different sources of anti-M2e antibodies, while Chapter 4 describes the evaluation of M2e-based competitive ELISA using monoclonal antibodies as the competitor antibodies against a panel of H5N1 infected chicken sera, as well as vaccinated chicken sera. Chapter 5 describes the isolation of the highly reactive single-chain variable fragment (scFv) anti-M2e antibodies using the phage display technology. Finally, Chapter 6 is the general discussion of the thesis as a whole, which followed by a conclusion at the end of the chapter.

1.3 Avian Influenza Virus (AIV): enzootic H5N1 and DIVA test in Indonesia

Indonesia has been enzootic with H5N1 genotype Z viruses following its first wave of dissemination in 2003. Characterised by high density of poultry industry, continued circulation of H5N1 in Indonesia was mainly attributed to poultry trade and products movement within the country (Smith, GJD et al. 2006). Within the first two years of H5N1 introduction in Indonesia, the virus has evolved rapidly that it can be distinctly grouped based on its geographical trait, spreading vastly across more than 3000 km, from North Sumatra to West Timor. This is especially intensive in the central and eastern Java, the hub of the poultry industry (Smith, GJD et al. 2006). It was later identified that the H5N1 which spread throughout the Indonesian archipelago are closely related to H5N1 originated from Hunan in 2002 and 2003 (Wang, J et al. 2008). This transmission was assumed to have occurred through the route of migratory birds or poultry movement.

Although culling was the primary option for H5N1 control following an outbreak, vaccination option has been implemented in Indonesia in 2004 once H5N1 becoming enzootic. However, reports of vaccine failures has been detected in mid-2005, mainly due to the emergence of antigenic variants of the H5N1 (Bouma et al. 2008; Swayne et al. 2011; Swayne et al. 2015). Study suggested that the widespread use of H5 avian influenza vaccine contributed to the emergence of H5 variants following drift of the virus which overcomes the vaccine-induced immunity (Swayne et al. 2015). This further highlights the need for AI surveillance to monitor the emergence of drift variant virus in the field. The only problem with this is the unavailability of an established method to discriminate between the virus infected sera from those of vaccinated sera. Therefore, strategies which enable the differentiation between infected and vaccinated animal sera (DIVA) have been developed to counter these issues.

Several DIVA options are available, as discussed in Chapter 2 of this thesis. However, further studies are still needed to explore several options in the effort of developing an optimal DIVA test which addresses the weaknesses of the currently available. Here, we look in details the potential of an M2e protein-based ELISA diagnostic kit development.

1.4 AIV genes, M2 and M2e protein

Avian influenza virus is a negative-stranded RNA virus, structurally enveloped and segmented, and belongs to the family *Orthomyxoviridae*, genus *Influenzavirus A* (Lamb & Krug 2001; Nelson & Holmes 2007; Taubenberger & Kash 2010). There are eight gene segments of Influenza A virus (IAV), with each gene segment codes for at least one protein (Table 1.1, Figure 1.1). IAV is known to code for at least 13 viral proteins (Chen, W et al. 2001; Jagger et al. 2012; Lamb & Krug 2001; Steinhauer & Skehel 2002; Wise et al. 2009; Wise et al. 2012; Yamada et al. 2004). Some of the segments encoded more than one protein through mechanisms such as an alternative reading frame (PB1-F2, PB1-N40, PA-X and M2), and mRNA splicing (NS1/NEP) (Chen, W et al. 2001; Jagger et al. 2012; Lamb & Choppin 1981; Lamb & Lai 1980; Wise et al. 2009) (Table 1.1). Each of the IAV gene segments is characterized by 20 – 45 noncoding nucleotides at the 3′ end, and 23 to 61 noncoding nucleotides at the 5′ end (Steinhauer & Skehel 2002). Despite the subtypes variety and high mutation rate of their RNA genome, a total of 12 nucleotides and 13 nucleotides positioned at the 3’ and 5’ end, respectively are fully conserved in all gene segments across all strains of IAV. There is a single exception on position 4 of the 3′ end, which displays U/C heterogeneity (Steinhauer & Skehel 2002).

The M proteins possess relatively slowly evolving genes, especially the M1 gene (Ito et al. 1991). While the M2 gene shows a much faster evolution rate than the M1 gene, it is significantly less rapid than the evolution rate of other surface proteins. The absence of

immune selective pressure, as well as the overlapping reading frame between the M1 and the M2 proteins has been suggested to contribute to the highly conserved region of the M protein (De Filette et al. 2005; Ito et al. 1991).

M2 protein specifically, is a small transmembrane protein of 97 amino acids, coded by an overlapping reading frame (ORF +1) of segment 7 of AIV (Lamb & Choppin 1981; Lamb, Lai & Choppin 1981). It shares a common start codon with the M1 protein, a collinear transcript product of segment 7, up until the ninth aa, while the remaining 88 aa of the M2 continues at the second (+1) ORF (Lamb, Lai & Choppin 1981; Lamb, Zabedee & Richardson 1985) (Figure 1.2). It is a type III integral membrane protein which forms a homotetramer to be functional. It consists of three main parts; a 55 amino acid (aa) cytoplasmic C-terminal, a 19 aa transmembrane protein, and a 24 amino acid (aa) external domain (M2e), exposed on the virion surface.

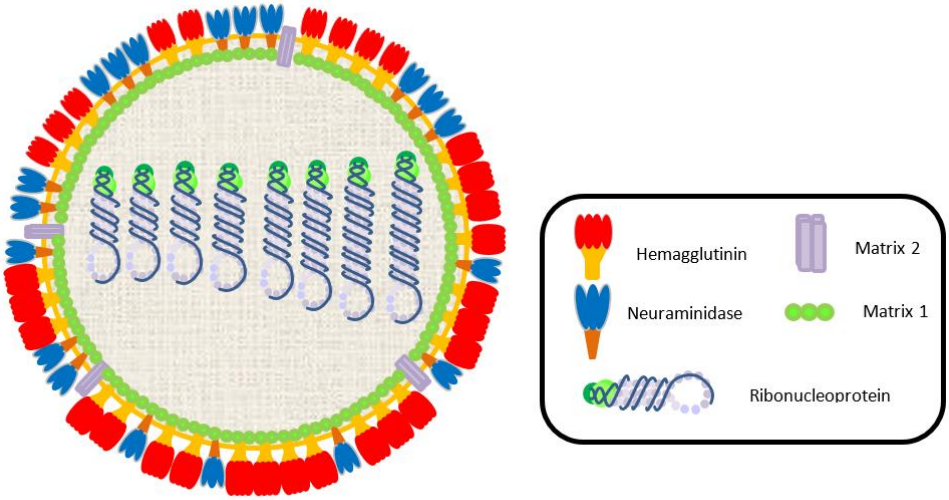

Figure 1.1 An illustration of the avian influenza virus virion. It is generally round in shape, covered with three types of surface proteins, namely, hemagglutinin (HA), neuraminidase (NA), and matrix protein 2 (M2). The rounded structure of the virion is maintained by the matrix 1 (M1) protein which encapsulate eight ribonucleoproteins. Each ribonucleoprotein is responsible for at least one AIV protein, and it is structurally made of nucleoproteins bound together by the virus genomic RNA to make a twisted, self-coil strand. At the opposite end of the loop structure, the strand is associated with the RNA polymerase complex formed by the polymerase-acidic (PA), polymerase basic type 1 (PB1) and polymerase basic type 2 (PB2) proteins. Adapted from Nelson and Holmes (2007).

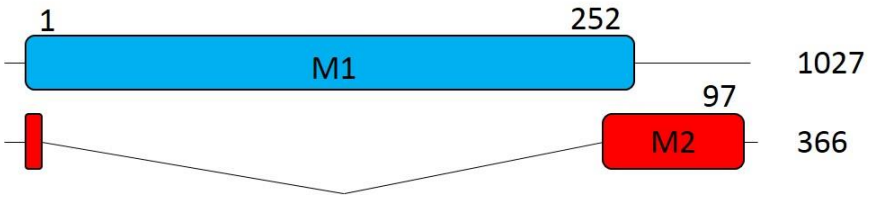

Figure 1.2 An illustration of the M1 and M2 protein mRNA with their coding regions (box) and the number of amino acid for each protein. Non-coding regions are represented with the thin line. Adapted from Lamb et al (1981).

Table 1.1 Summary of Influenza A virus RNA genome segments and functions.

Segment	Protein	Length[a] (nucleotides)	Size[a] (amino acids)	Approximate number of molecules per virion[b]	Function[a]
1	PB2	2,341	759	30-60	Component of RNA polymerase, cap recognition
2	PB1	2,341	757	30-60	Component of RNA polymerase, endonuclease activity, elongation
	PB1-F2		87[c]		Pro-apoptotic activity[c]
	PB1-N40		718		Modulate polymerase function [d]
3	PA	2,233	716	30-60	Component of RNA polymerase, cap-binding[e], endonuclease activity[e], viral RNA binding and replication[e]
	PA-X		61[e]		Repress cellular gene expression, modulate host response to infection[f]
4	HA	1,778	550	500	Surface glycoprotein, receptor binding, fusion activity, major antigen
5	NP	1,565	498	1,000	RNA binding, RNA synthesis, RNA nuclear import, antigen
6	NA	1,413	454	100	Surface glycoprotein, virion release, antigen
7	M1	1,027	252	3,000	Matrix protein, interaction with vRNPs and surface glycoproteins, nuclear export, budding, antigen
	M2	366	97	20-60	Membrane protein, ion channel activity, virus entry and assembly[g]
8	NS1	890	230		Inhibit host mRNA polyadenylation[h], inhibit nuclear export[i], inhibit pre-mRNA splicing[j], regulate viral RNA polymerase activity[k], stimulate translation of specific viral mRNAs[l], interaction with host cell proteins[m], viral IFN antagonist[n] antigen
	NS2 / NEP	418	121	130-200	Nuclear export of vRNPs

[a] Palese and Shaw (2007), [b] Lamb and Krug (2001), [c] Chen, W et al. (2001), [d] Vater (2011)[e] Hara et al. (2006), [f] (Hu, J et al. 2015; Jagger et al. 2012), [g] (Beale et al. 2014; Wise et al. 2012), [h] (Nemeroff et al. 1998), [i] (Fortes, Beloso & Ortin 1994), (Qiu & Krug 1994); [j] (Lu, Qian & Krug 1994); [k] (Shimizu et al. 1994), (Marion et al. 1997); [l] (Enami et al. 1994), (de la Luna et al. 1995); [m] (Wolff, O'Neill & Palese 1996), (Wolff, O'Neill & Palese 1998), [n] (Garcia-Sastre et al. 1998)

1.4.1 **M2 protein**

M2 proteins function as ion channels during virion uncoating at the beginning of cell infection, and regulate the pH of the Golgi apparatus which is essential for HA glycoprotein maturation (Sugrue & Hay 1991). M2 protein is also responsible for the acidification of the viral interior that weakens protein-protein interactions, thus enabling the release of RNP into the cellular cytoplasm, prior to entry into the nucleus (Lamb, Zabedee & Richardson 1985; McCown & Pekosz 2005). Findings also suggested that M2 protein is responsible for preventing premature conformational rearrangement of the HA proteins during their transport in the Golgi lumen to the cell surface for virion formation (Sugrue et al. 1990; Sugrue & Hay 1991), and plays a role in subverting autophagy and thus, contributed to the maintenance of virion stability (Beale et al. 2014).

1.4.1.1 M2 cytoplasmic domain and transmembrane domain

The cytoplasmic C-terminal of M2 protein is amphipathic helix oriented, comprising approximately 54 to 55 amino acids (Hull, Gilmore & Lamb 1988; Schnell & Chou 2008). The C-terminal shows significant diversity at the last 10-21 amino acids (Khurana et al. 2009). It has been suggested that the M2 cytoplasmic tail is responsible for efficiency in genome packaging into the virus particles and virus assembly; and participates in morphogenesis of virions (Chen, BJ et al. 2008; Grantham et al. 2010; Iwatsuki-Horimoto et al. 2006; McCown & Pekosz 2005). It is also crucial for stabilization of the tetramer formation of M2 protein (Salom et al. 2000; Schnell & Chou 2008).

The M2 transmembrane is an α-helix composed of 19 amino acids, which is a part of the ion channel construct (Lamb, Zabedee & Richardson 1985). As mentioned before, the ion channel functions to regulate the pH of proton channel upon virus entry into the host cells, uncoating of viral proteins upon entry, and ensure the proper maturation of HA protein upon virion

formation (Hay et al. 1985; Lamb, Zabedee & Richardson 1985; Pinto, Holsinger & Lamb 1992; Sugrue et al. 1990; Sugrue & Hay 1991).

1.4.1.2 M2 extracellular (M2e) domain

Out of 97 amino acids, approximately 18 to 23 N-terminal amino acids are exposed on the virion surface, nine of which are reported to be highly conserved in all IAV strains (Ito et al. 1991; Khurana et al. 2009; Lamb, Zabedee & Richardson 1985; Liu, Li & Chen 2003; Zabedee & Lamb 1988). The M2 protein can be easily detected due to its abundance on the infected cells (Fang et al. 1981), but only a small amount of this protein has been found on mature virion (14 to 68 M2 molecules per virion) (Zabedee & Lamb 1988). Its small size and low abundance on the virion surface membrane in comparison to the other two membrane proteins (HA and NA), has made it only capable of eliciting a low immune response (Black et al. 1993). Low copy number of the M2e protein on the virion surface membrane is suggested to be related to its pH regulating function (Park et al. 1998). It is suggested that the overabundance of the protein might cause an early disruption of the M1-RNP complex due to rapid and over-acidification in the virus endosome during virus entry to host cell (Martin & Helenius 1991). The low copy number of the M2 protein per virion in turn allows the M2e protein to escape immune selection pressure, thus contributing to the conservation of this region (Black et al. 1993; De Filette et al. 2005; Fiers et al. 2009; Gerhard, Mozdzanowska & Zharikova 2006). It also shares a coding region with the matrix protein, thus limiting its possibility to undergo major changes (De Filette et al. 2005).

1.4.2 **M2e as potential universal vaccine**

The M2 protein has long been the target for the development of influenza universal vaccine due to its highly conserved sequence and its proven ability to significantly reduce morbidity and mortality in various animal models (Fiers et al. 2004; Neirynck et al. 1999; Zharikova et al. 2005). Studies have been carried out to thoroughly evaluate the potential use of M2 protein

for vaccination. Among others were the uses of M2 protein in passive transfer vaccination (Liu, Zou & Chen 2004; Treanor et al. 1990), vaccination with conjugated M2 protein (Fan et al. 2004; Neirynck et al. 1999), vaccination with a complete M2 protein (Ilyinskii et al. 2008), or only with the extracellular domain of the M2 protein (Denis et al. 2008; Frace et al. 1999; Hashemi et al. 2012; Lambrecht et al. 2007; Leung et al. 2015; Liu, Li & Chen 2003). Mostly, these studies demonstrated the ability of anti-M2 antibodies in conferring protection to the non-natural host (mouse) against homologous and heterologous lethal virus challenge, with reduction in the virus titer (Fan et al. 2004; Fiers et al. 2009; Fiers et al. 2004; Mozdzanowska et al. 1999; Neirynck et al. 1999; Slepushkin et al. 1995). Immunization with recombinant virus expressing M2 protein in chicken however, showed no indication of M2 being immunogenic or protective (Nayak et al. 2010). Nevertheless, another study which immunized chicken using recombinant M2 protein and M2e peptide found significant anti-M2 antibodies, but lack the ability to bind the M2 protein on the virus surface or virus infected cells (Swinkels et al. 2013). This means that both M2 protein and M2e peptide were immunogenic, however was likely not protective. Although the currently available M2e vaccine may be applicable in other species, it may not be an optimal vaccine for the poultry industry.

1.4.3 **M2e as DIVA marker**

M2e protein has also been targeted as a DIVA marker due to its epitope differential characteristic (Hadifar et al. 2014; Hemmatzadeh et al. 2013; Kim, MC et al. 2010; Lambrecht et al. 2007). Despite being low in number on a mature virion, the M2e protein exist in a vast amount on an infected cells (Fang et al. 1981; Zabedee & Lamb 1988). A number of studies on M2e-ELISA application using synthetic peptide have indicated that this protein is an effective means of differentiating animals infected with HPAIV strains from vaccinated animals, and is suitable for a long term field application (Kim, MC et al. 2010;

Lambrecht et al. 2007; Nemchinov & Natilla 2007). However, the use of synthetic peptide for a routine surveillance is costly, since the peptide needs to be synthesized *in vitro*.

Alternatively, the use of recombinant protein offers a much lower cost for higher output, with continuous access for a large scale screening (Hemmatzadeh et al. 2013). Apart from being more affordable, a recombinant protein M2e-based ELISA developed for DIVA testing has shown a comparable performance to the synthetic M2e peptide-based ELISA (Hemmatzadeh et al. 2013). It clearly differentiates between chickens which are challenged with live virus or infected, with those which are vaccinated with the whole-killed virus. However, this system tends to generate non-specific reactions when tested with serum from older chickens, and haemolysed serum. It was also noted that monomer form of M2e used in ELISA demonstrated limited antigenicity and this consequently resulted poor diagnostic capability (Hemmatzadeh et al. 2013). Further attempt on presenting M2e in a multimeric form significantly increased efficiency of anti-M2e antibody detection in ELISA (Hadifar et al. 2014; Tarigan et al. 2015). Most importantly, these H5N1 M2e-based ELISA was able to detect positive sera from other AIV strains, namely H5N2, H9N2, H7N7 and H11N6 (Hemmatzadeh et al. 2013). These findings further support M2e applicability in AIV field surveillance with its DIVA ability which is not restricted to H5N1, but also other AIV strains.

1.5 Antigenic mapping

Antigenic or epitope mapping is the identification process of antigen-antibodies binding site on the protein surface (Wang, LF & Yu 2004). It is an important technique developed to understand the correlation of function and structure of protein-protein interactions, such as the elucidation of antigen neutralizing sites (Bannister et al. 2011; Morris 1996). An epitope-based mapping approach is advantageous due to the specificity of immune response produced, while providing a valid and robust basis for potent drug design and vaccine development as a part of disease control measure (Bannister et al. 2011; Irving, Pan & Scott 2001).

1.5.1 **Antibody and Antigenic determinants**

In an operational basis, epitopes are classified into two types, either continuous or discontinuous (Regenmortel 1996; Smith, GP & Petrenko 1997; Wang, LF & Yu 2004) (Figure 1.7). Continuous epitope or also known as linear or sequential epitopes corresponds to short amino acid residues which can bind to antibodies raised against the target protein. Sequence resemblance as minimum as three amino acid residues can be observed between the continuous epitope with the antigen sequence (Bottger & Bottger 2009; Smith, GP & Petrenko 1997).

Meanwhile, discontinuous epitope are made of nonlinear amino acid which are distant from each other and brought together through protein conformation or protein folding (Smith, GP & Petrenko 1997; Wang, LF & Yu 2004) (Figure 1.3). No sequence resemblance can be observed when discontinuous epitope are aligned with the antigen sequence. This is because the epitope structure depends on the conformation of the native protein, where amino acid which are locally apart from each other are brought together (Bottger & Bottger 2009; Regenmortel 2009; Smith, GP & Petrenko 1997).

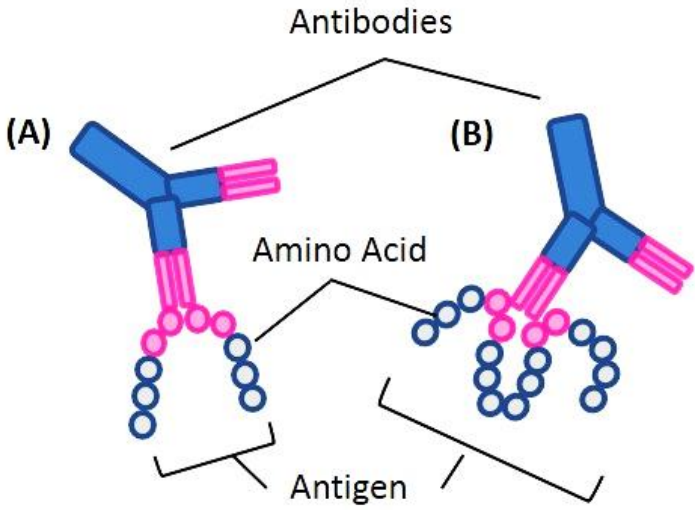

Figure 1.3 Schematic presentation of antibodies interaction with antigen. Epitope is the amino acid residues interacting with the antibodies, where (A) represents the continuous epitope, while (B) represents the discontinuous epitope. Adapted from Hjelm (2011).

Structurally, an epitope (either continuous or non-continuous) are usually located in the protruding regions of proteins or accessible surface regions (Novotny et al. 1986; Thornton et al. 1986). Previous study noted that an epitope is likely to be located at the highest point of hydrophilicity, if not next to it (Hopp & Woods 1981), and are likely to be characterized by moderately conserved residues which are crucial for the stability and protein-protein associations (Keskin, Ma & Nussinov 2005).

Identification of a dominant epitope will provide specific target for vaccine design which may has the potential for pathogen neutralization (Li et al. 2013; Shi et al. 2015). Knowledge on dominant epitope of a target antigen may also be used as basis for diagnostic tool development in an epidemiological surveillance. Therefore, this thesis explores antigenic mapping to identify the dominant M2e antigenic determinant to solidify its choice as the target antigen for a potential competitive ELISA development.

1.5.2 **M2e antigenic determinants**

The first discussion on M2e protein in regards of its protein sequence was due to the anti-M2e monoclonal antibody (14C2) ability to restrict growth of AIV in plaque assay (Zabedee & Lamb 1988). Continuous interest on M2e protein as a universal vaccine drives deeper explorations of its protein sequence, which eventually lead to description of M2e epitopes (Table 1.2). It was described that the N-terminal of M2e (amino acid 1-9), which is highly conserved across AIV strains, is capable of inhibiting AIV replication (Fu et al. 2009; Liu, Li & Chen 2003). Others revealed that aa 6-13 of M2e is responsible for the demonstrated protective immunity in their findings, together with other variations of M2e epitope of aa 4-16 and aa 7-12 (Liu, Zou & Chen 2004; Zhang et al. 2006; Zou, Liu & Chen 2005). Further analyses on M2e protein antigenicity using different types of immunogen (e.g fusion protein, live virus and peptide) and different antibody sources (i.e rabbit, mice, human) revealed a range of identified M2e epitopes encompassing aa 2-16 in general (Grandea III et al. 2010; Pejoski et al. 2010; Wang, R et al. 2008). Other also showed that the N-terminal of M2, aa 2-10 contain immunogenic epitope but not sufficiently protective (De Filette et al. 2011), which contradicted the previously reported findings. It is noted that different species may differ in their germline gene repertoires, antibody generating mechanism and affinity maturing of their antibody molecules (reviewed in Finlay and Almagro (2012). Therefore, different level of M2e antigenicity and slight variations in the described M2e epitopes might have been influenced by the host species. It is the interest of this thesis to further identify any difference between the described M2e epitope with the anti-M2e antibodies from different species.

Table 1.2 Summary of epitopes recognized on influenza A virus M2e protein by different antibodies. Adapted from Hasan et al (2016).

Antibody type and designation	Antibody source	Immunogen	Epitope sequence (Identifying Antibody)	Residue length	References
Polyclonal (AS1, AS2, AS3, AS4)	Rabbit	Fusion-M2e (BSA)	^{2}SLLTEVETPIR^{12}	11	(Liu, Li & Chen 2003)
Monoclonal (8C6, 1B12)	Mice	Fusion-M2e (GST)	^{6}EVETPIRN^{13} ^{2}SLLTEVETPIRNEW^{15}	8 14	(Liu, Zou & Chen 2004; Zharikova et al. 2005; Zou, Liu & Chen 2005)
Monoclonal	Mice	Live virus & synthetic peptide	^{4}LTEVETPIRNEWG^{16}	13	(Zhang et al. 2006)
Monoclonal (L66, N547, Z3G1, C40G1, 14C2)	Human (λ HAC or KM™ mice)	Fusion-M2e (BSA)	^{2}SLLTEVETPIRNEWG^{16} (L66) ^{3}LLTEVETPIRNEWG^{16} (N547) ^{3}LLTEVETPIR^{12} (Z3G1) ^{9}TPIRNE^{14} (C40G1) ^{6}EVETPIRNEW^{15} (14C2)	15 14 10 6 10	(Wang, R et al. 2008; Zabedee & Lamb 1988)
Monoclonal	Mice	Fusion-M2e (BSA)	^{2}SLLTEVET^{9} (M2e8-7) ^{3}LLTEVETPIR^{12} (Z3G1)	8 10	(Wang, Y et al. 2009)
Monoclonal	Mice	Fusion-M2e (BSA)	^{4}LTEVETPIRN^{12} (L18) ^{2}SLLTEVET^{9} (O19) ^{2}SLLTEVETPIRNEWGCRNDSSD^{24} (P6) ^{7}VETPIRN^{13} (S1)	108 23 7	(Fu et al. 2009)
Polyclonal	Mice		^{2}SLLTEVETPIRNEWG^{16}	15	(Pejoski et al. 2010)
Monoclonal	Human		^{2}SLLTE^{6} (TCN-031, TCN-032)	5	(Grandea III et al. 2010)
	Mice	Fusion-M2e (KLH)	^{2}SLLTEVETP^{10}	9	(De Filette et al. 2011)

Difference at residue I11T between the current and previous studies corresponded to the human and swine specific M2e sequence in the former (I11) and avian specific M2e sequence in the latter (T11) (Zhou, C, Zhou & Chen 2012).

1.6 Enzyme-linked immunosorbent assay (ELISA) for AIV surveillance and DIVA

Highly specific and effective conventional serologic diagnostic tests are available for AIV detection, such as the hemagglutination inhibition (HI) and agar gel precipitation (AGP) tests. Although these tests are simple in principle and cost effective, they are also hard on time and impractical (Jenson 2014; Pedersen 2014). Recent developments of diagnostic tests witnessed the increased application and development of enzyme-linked immunosorbent assay (ELISA)-based tool as platform (Aydin 2015; Chen, HW, Wang & Cheng 2011; Ding et al. 2014; He et al. 2013). This is highly likely due to its simple and easy application, yet sensitive and specific enough to detect the targeted antigen.

1.6.1 ELISA principles, components and types

ELISA (direct ELISA) is first introduced as a quantitative assay for detecting antibodies, and is originally based on radioimmunoassay (RIA) principle (Engvall, Jonsson & Perlmann 1971; Van Weemen & Schuurs 1971). Both ELISA and RIA differs in that antigen/antibody in the former is conjugated with enzymes instead of radioactive iodine 125 in the latter. ELISA harbours the same sensitivity as the RIA, yet is simpler and more affordable (Engvall 2010).

Briefly, ELISA is a method which utilises the antigen-antibody specific binding capability to quantitate the presence of antigen or antibody in a fluid sample. Quantification is done through the measurement of colorimetric reading mediated by an enzyme which is conjugated to the secondary antibody. Three main components of this assay are (i) the capture system, which is immobilized on a solid support; (ii) the analyte, which is the substance to be measured, and (iii) the detection system, a chromogenic substrate that changes its colour intensity according to the strength of the immune reaction (Butler 2000; Paulie, Perlmann & Perlmann 2005; Porstmann & Kiessig 1992). Thus, in principle, ELISA uses an enzymatic indicator system for antigen-antibody reactions, with either qualitative or quantitative results

(Butler 2000). Four general types of ELISA are available, namely, direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA (reviewed in Aydin (2015)).

Both direct and indirect ELISA involve the attachment of antigen to a solid surface. However, direct ELISA only requires one antibody (primary) which is enzyme-conjugated to quantify the amount of antigen or antibody (Figure 1.4 (a)). In indirect ELISA, an additional (secondary) antibody tagged with enzyme is used to detect the primary-antibody-antigen complex (Lindstrom & Wager 1978) (Figure 1.4 (b)). It is noted to be more specific in comparison to direct ELISA due to the additional secondary-antibody. Meanwhile, sandwich ELISA is different than the previous two ELISA. Instead of the antigen, sandwich ELISA utilises a capture antibody (binder) to be attached to a solid surface (Kato et al. 1977) (Figure 1.4 (c)). Then, sample containing antigen to be tested is added to the attached antibodies before the addition of a second set of antibody (enzyme-conjugated) specific to the antigen. The targeted antigen are captured in between the capture- and the enzyme-conjugated-antibody used for quantification, hence, sandwich ELISA. This ELISA is also highly specific due to two sets of antigen-specific antibodies used. All three ELISA show high colorimetric intensity upon substrate development if the targeted antigen/antibodies are present.

Finally, competitive ELISA utilises either antigen-specific antibody or antibody-specific antigen to be immobilised on a solid surface (Yorde et al. 1976) (Figure 1.4 (d)). Sample to be tested and enzyme-conjugated antigen/antibody are added simultaneously to the immobilised antibody/antigen. If samples contain antibodies/antigen specific to the binder, it will compete with the positive antigen/antibody to bind to the immobilised antibody/antigen, hence competitive ELISA. Different from the previous ELISA, upon substrate development, presence of specific antibodies/antigen is indicated by low colorimetric intensity as only some binders are occupied by the enzyme-conjugated antigen/antibody. Meanwhile, high

colorimetric intensity indicates absence of specific antibodies/antigen, because all binders are occupied by enzyme-conjugated antigen/antibodies.

Selection between different types of ELISA is generally based on the availability of antigen/antibody-specific antibodies/antigen. This is especially for the secondary antibodies required in both indirect and sandwich ELISA, as well as the competitor antibodies for competitive ELISA. Although both direct and indirect ELISA are simple enough to perform, direct ELISA is prone to false positive with known low sensitivity. Indirect ELISA requires different types of secondary antibodies for testing samples from different species. Both sandwich ELISA and competitive ELISA possess the highest specificity for antigen/antibody detection. However, the setback for sandwich ELISA lies in the availability of a paired antibodies (Jordan 2004), while competitor antibodies needs to be universal yet sensitive enough for it to be applicable across species. Monoclonal antibodies are highly specific for such purposes while peptide can be rapidly available to be used instead of antibodies in recent years. However, both are not feasible options for a large-scale and long-term application, such as in pathogen surveillance. Nevertheless, competitive ELISA in particular is applicable with a large amount of samples and the use of recombinant antigen/antibody is possible (Yang, M et al. 2011).

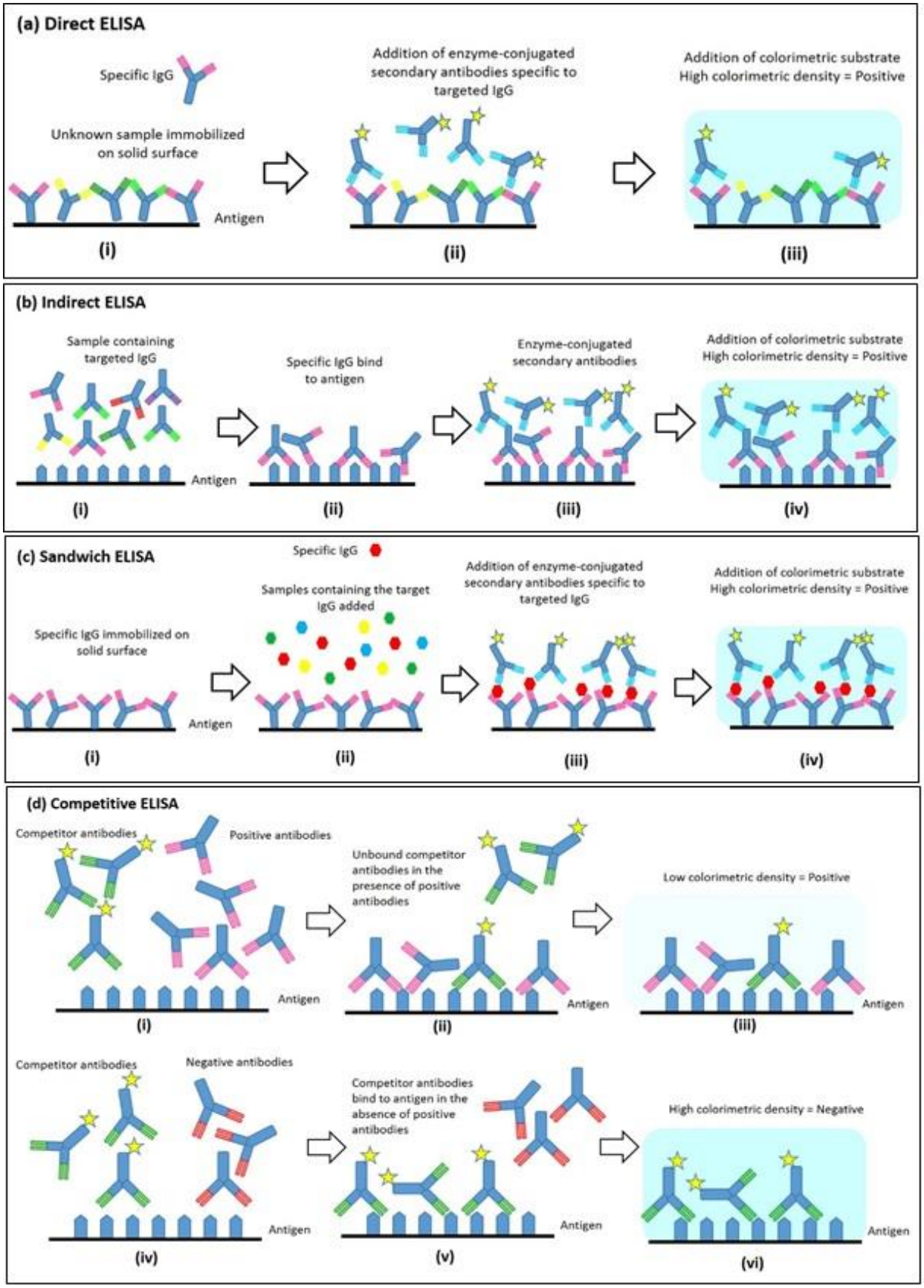

Figure 1.4 Illustrations of different types of ELISA in which arrows represent the wash and rinse step. (a) Direct ELISA, (i) Samples to be tested are immobilised on the solid surface, (ii) enzyme-conjugated antibody are added, before substrate development, (iii) High colorimetric density indicates presence of targeted IgG. (b) Indirect ELISA: (i) Sample containing the targeted IgG added to wells with immobilised antigen, (ii) Specific IgG bind to the antigen, (iii) Addition of enzyme conjugated antibodies to identify the antibody-antigen-complex, (iv) High colorimetric density indicates presence of targeted IgG. (c) Sandwich ELISA: (i) The

first antibody of the target-specific IgG is immobilize on the solid surface, (ii) Addition of samples containing the potential target IgG, (iii) Addition of the second antibody which is target IgG-specific, (iv) High colorimetric density indicator of target IgG presence. (d) (i-iii) Competitive ELISA (cELISA) with the presence of positive antibodies (test sample), where it competes for the antigen with the enzyme-labelled competitor antibodies, thus reduces the color saturation indicating a positive competition; (d) (iv-vi) cELISA with the presence of negative antibodies (test sample) where it gives no competition to the enzyme-labelled competitor antibodies resulting high color saturation indicating a negative competition. Adapted from Gan and Patel (2013).

1.6.2 **AIV and ELISA**

In the context of AIV, comparison studies between conventional test hemagglutination inhibition (HI), agar gel precipitation (AGP), serum neutralization and ELISA for AIV detection found that ELISA demonstrated the most sensitive, specific and accurate results among the tests (Faraz et al. 2010), although precaution is required when monitoring AIV in its early stage of infection (Shiraishi et al. 2012). A number of ELISA targeting the AIV protein such as the hemagglutinin, neuraminidase and nucleoprotein have been developed (Jensen et al. 2013; Jin et al. 2004; Wu, R et al. 2007). Some ELISA kit are also commercially available for rapid screening of AIV infection, such as the ID-Vet IDScreen® (Idvet, Montpellier, France), IDEXX FlockChek™ AI MultiS-Screen Ab Test Kit Idexx, Westbrook, ME), Synbiotics FluDETECT™BE (Synbiotics, Kansas City, MO), and BioCheck AIMSp (BioChek, Reeuwijk, The Netherlands). However, it was reported that these ELISA are not applicable for AIV screening in wild birds due to the nature of AIV in wild birds and different level of pathogenicity demonstrated by AIV strains in different species (Alexander, Parsons & Manvell 1986; Claes et al. 2012; Forman, Parsonson & Doughty 1986). Nevertheless, considerations on the simplicity and easy implementation of ELISA ensures continuous efforts in developing alternative ELISA for AIV screening is still progressing.

In the past, nucleoprotein-based competitive ELISA has demonstrated its reliability and applicability for surveillance use in multispecies (Shafer, Katz & Eernisse 1998; Starick et al. 2006; Zhou, EM et al. 1998). However, these available ELISA are not applicable in countries where vaccination using killed whole virus are used (Chen, H 2009; Marangon, Cecchinato &

Capua 2008). The reason being the targeted antigen are not capable of DIVA application (reviewed in Pantin-Jackwood and Suarez (2013)). Hence, this thesis incorporates an attempt to develop a competitive ELISA based on the isolated anti-M2e-antibody which has been demonstrated as reliable for AIV infection detection and DIVA application.

1.7 Phage display technology

Phage display technology is a method which enable the selection of foreign protein, expressed alongside the bacteriophage protein. This involves the insertion of the gene of interest in a vector either a phage or a phagemid; a plasmid which modified to have the ability to amplify in a bacteria and a bacteriophage (Smith, GP 1985, 1993; Smith, GP & Petrenko 1997). Highlight of this technology are the physically linked phenotype and genotype of the phage, and the ability to select the protein of interest through biopanning – a process of affinity selection to the desired antigen.

Generally, there are three stages of a phage display experiment, namely (i) the construction of a protein or antibody library, (ii) the selection of targeted protein or antibody through affinity selection, and (iii) the verification of the isolated protein or antibody using biological assay or analyses (Huang, Bishop-Hurley & Cooper 2012) (Figure 1.5). Construction of the desired antibody library requires initial considerations in the phage display properties selection, such as the antibodies gene sources, bacteriophage to be used, recombinant antibody format, vector for phage display and its coat protein selection. Once all of these options has been sorted out and the construction has successfully produced the desired phage library, selection of antibodies with the targeted specificities is done through ‘biopanning’. Briefly, biopanning involves the repetition of the following steps; (a) binding of bacteriophages displayed antibodies against the targeted antigen to capture specific binders, (b) washing to remove the non-specific binders or low affinity binders, and (c) elution of bacteriophages displayed antibodies for further amplification. Additional rounds of biopanning can be done to increase

specificity of the isolated antibodies, with modification in the capture antigen concentration (lower the concentration to target highly specific antibodies), and number of washes which may varies from 10 to 30 cycles (increase wash to target antibodies with high specificity). Finally, the specificity of the isolated antibodies can be verified using bioassays such as ELISA and western blotting.

Due to the ability of phage display technology to isolate specific antibodies to the targeted antigen, it has been mainly used to study protein-protein interaction, especially for health and medical purposes (reviewed in Bazan, Calkosinski and Gamian (2012)), such as producing proteins to be used as therapeutic agents for autoimmune diseases (Farilla et al. 2002; Kim, Y et al. 2011; Klotz, Meuth & Wiendl 2012) and tumour targeting (Cyranka-Czaja et al. 2012; Lin et al. 2012; Yang, J et al. 2011).

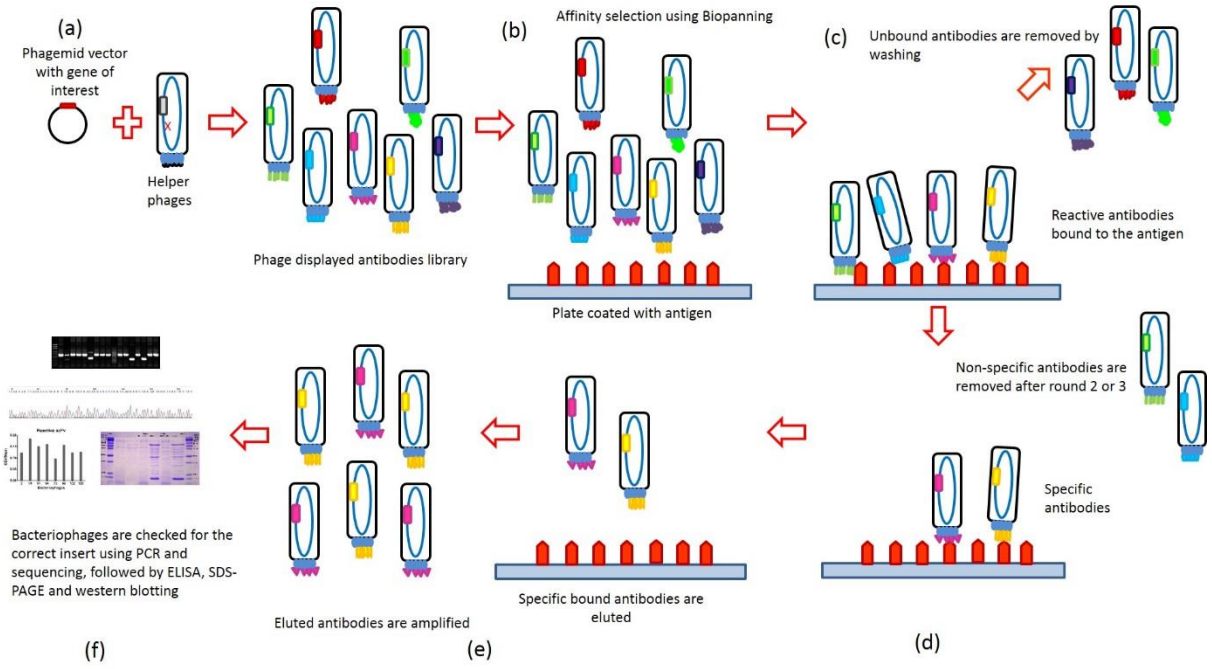

Figure 1.5 Phage display technology involves (a) the insertion of gene of interest into a vector (such as phagemid) and infect it with the helper phage to produce recombinant bacteriophage library which expresses the antibodies of interest. (b) Affinity selection is done to select the bacteriophages with the highly reactive antibody using plate coated with the specific antigen. (c) Unbound bacteriophages are washed away and only bacteriophages expressing the specific antibodies are captured by the antigen. (d) Specifically bound bacteriophages are eluted and amplified. (e) Amplified bacteriophages are checked for positive gene insertion and

expression using (f) PCR, ELISA and SDS-PAGE. Adapted from (Hoogenboom & Chames 2000).

1.7.1 **Naïve and immunized library as a source of phage library**

The immune system variable gene (V-gene) repertoire is the key source of diversity for a phage display library. The V-gene can be obtained from either an immune donor (Clackson et al. 1991; Okamoto et al. 2004) or naïve (non-immune) donor (Marks et al. 1991; Sommavilla et al. 2010) (reviewed in Griffiths and Duncan (1998)). An immune donor would provide a V-gene repertoire which are highly biased towards the antibodies against the immunogen. Therefore, a relatively small library (approximately 10^5 clones) are sufficient to represent the possible antibodies diversity. Also, an immune phage library provides affinity matured antibodies, which enable the selection of antibodies with high affinity. However, an immune donor source may not always possess the antibodies with the desired properties, as immune response may varied from one host to another. Besides, targeted antigen may be toxic and fatal for the potential donor. Tolerance mechanism is a probable issue if antibodies against self-antigen, which is highly valuable as therapeutic agents, is desired.

Meanwhile, a non-immune donor will not require any immunisation, and its non-specific and diverse pool of antibodies library may open up possibilities of retrieving antibodies against a diverse set of antigen (Pansri et al. 2009; Schwimmer et al. 2013). Isolation of antibodies against self-antigen is possible, and a shorter time (less than two weeks) is required for antibodies generation. Nevertheless, such naïve donor will require a large library clones to ensure isolation of high affinity antibodies, and the nature of naïve V-gene repertoire is highly unknown and unpredictable. Therefore, selection of V-gene repertoire is highly dependent on level of antibodies specificity and affinity required, as well as resources and time availability.

1.7.2 **Filamentous bacteriophage for phage display**

Various types of bacteriophage are available for protein or antibody display application, such as the T4, T7 and lambda phage display system (Beghetto & Gargano 2011; Gamkrelidze & Dabrowska 2014; Talwar et al. 2015). However, the widely used bacteriophage for phage display is the filamentous bacteriophages (f1, fd, M13) for it will not lyse the cell it infected while producing its progeny (Bazan, Calkosinski & Gamian 2012). Generally, a filamentous phage is about 900 nm in length with diameter of 6-10 nm, and infects *Escherichia coli* with F pili (Berkowitz & Day 1980; Newman, Swinney & Day 1977). It is composed of a single-strand DNA which encoded nine genes, which are clustered into three main groups, namely genes for replication (g2/g10 and g5), virion structure (g7, g9, g8, g3 and g6), and assembly/secretion (g1/g11, and g4) (reviewed in Rakonjac (2012) and Rasched and Oberer (1986)). Its DNA is encapsulated in a cylindrical capsid made of five types of coat proteins, with p8 coating the whole filamentous phage body; p3 and p6 at one end of the phage, while p7 and p9 at the other end (Endemann & Model 1995; Grant et al. 1981; Henry & Pratt 1969) (Figure 1.6).

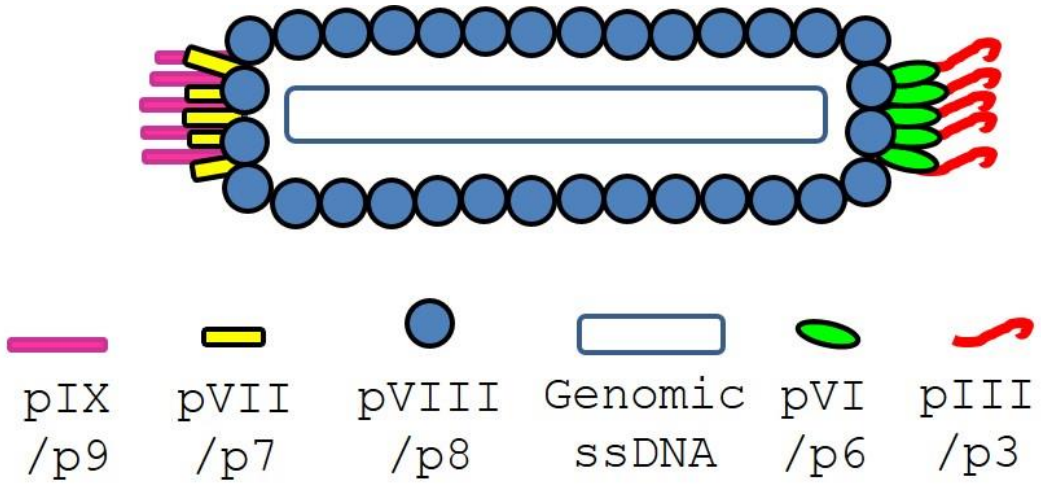

Figure 1.6 An illustration of a filamentous bacteriophages. Structurally, it has a single-stranded DNA encapsulated in a cylindrical capsid, which made of the major coat protein p8 (pVIII); with p9 (pIX) and p7 (pVII) at one end, and p6 (pVI) and p3 (pIII) at the other end. Adapted from Sidhu (2001) and Bratkovic et al (2010).

Majority of phage display used protein fusion with either p3 or p8, dependant on the aims of the target display. If affinity selection is desired, p3 is a better option because it is a monovalent display system which allows identification of high affinity binders. However, p8 is a polyvalent display system, and avidity effect is more dominant than its affinity which usually lead to low affinity ligands (reviewed in Bratkovic (2010) and Huang, Bishop-Hurley and Cooper (2012)).

1.7.3 **Recombinant antibody display format**

Different recombinant antibody display format is also available for selection, including the antigen-binding fragment (Fab), single chain variable fragment (scFv) and its modifications (Carlsson et al. 2012; Shen et al. 2007; Wen et al. 2012). Generally, antibody is a protein with a Y-shaped structure produces by a host to bind foreign or non-self-molecule as a part of the immune defence system (Tizard 2013). The arms structure of the antibody that make up the tip of the Y's (V-shape) determine the versatility and specificity of the host immune responses to an antigen, while the stem structure govern the biological activity that define its response, such as complement-mediated lysis, enhanced phagocytosis or allergy.

An antibody consists of two main fragments, namely the constant fragment (fragment crystallisable, Fc), and the antigen-binding fragment (Fab) (Figure 1.7). The Fab is further characterised by a pair of each of the following: constant heavy fragment (C_H), constant light fragment (C_L), variable heavy fragment (V_H) and variable light fragment (V_L). The H chain is usually 60 kDa in weight, while the L chain is 23 kDa (Bird & Walker 1991; Tizard 2013). Variable region of an antibody is characterised by two features, namely the hypervariable domains and the framework regions (Tizard 2013). The hypervariable regions are the three regions where the sequences are highly variable, while the framework regions are the relatively constant regions in between these hypervariable domains. These hypervariable regions are also known as the complementarity-determining regions (CDRs) (Wu, TT &

Kabat 1970) and are principally the antigen binding site (reviewed in Finlay and Almagro (2012)).

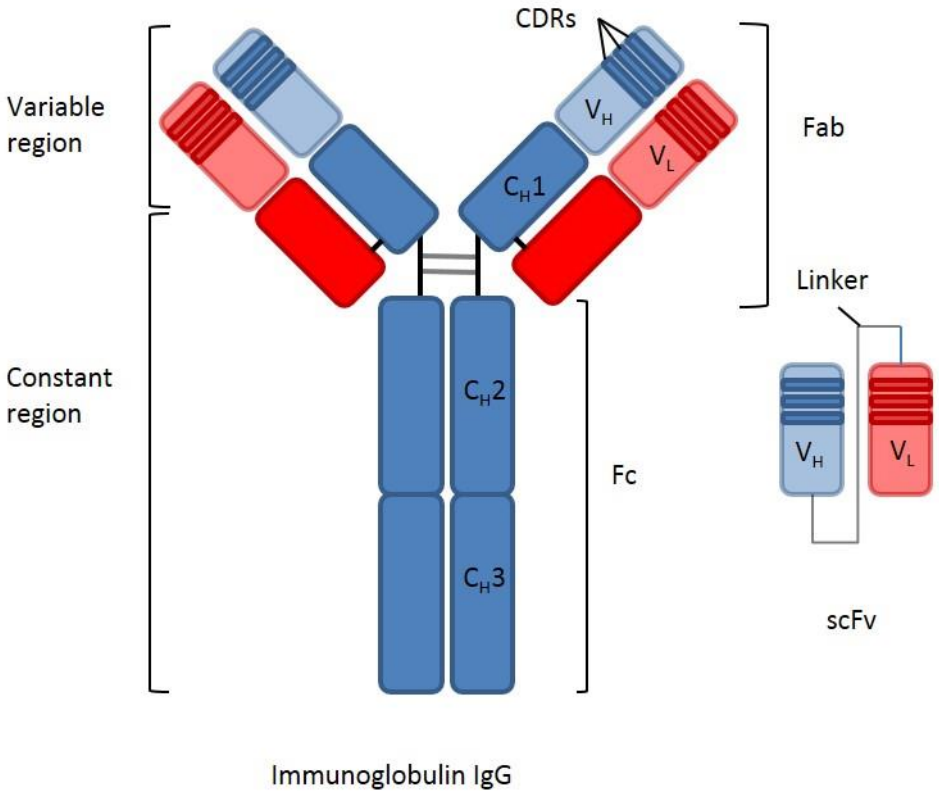

Figure 1.7 Schematic presentation of the immunoglobulin gene, composes of the constant region (Fc) and the variable region (the heavy chain, V_H and the light chain, V_L, each with complimentarity determinant regions, CDRs). Single chain fragment variable (scFv) is composes of the variable chain of heavy and light, connected with a linker, usually $(Gly_4Ser)_3$. Adapted from Tizard (2013).

Fab consists of V_H, C_H1, V_L and C_L, and such association is stabilized through heterodimer formation between V_H/C_H1 and V_L/C_L interfaces (Rothlisberger, Honegger & Pluckthun 2005). Meanwhile, scFv is made of V_H and V_L domains, tethers by a flexible peptide linker such as the glycine-serine $(Gly_4Ser)_3$ linker (Bird et al. 1988; Chen, W et al. 2014; Freund et al. 1993; Glockshuber et al. 1990; Holliger & Hudson 2005). Comparative evaluation of both Fab and scFv showed that Fab is a more functionally stable recombinant antibody format (Quintero-Hernandez et al. 2007; Rothlisberger, Honegger & Pluckthun 2005). Nevertheless, more interest was on scFv due to its small size (30 kDa) in comparison to Fab (50 kDa), and presence of a single polypeptide chain which eases fusion protein construction (Nimmagadda

et al. 2012; Rothlisberger, Honegger & Pluckthun 2005; Weber et al. 2014). ScFv design, construction and expression in *E. coli* enable the demonstration of the gene structure-function relationship in terms of antigen-antibody interactions. Exhibition of its high affinity and stability make the scFv a useful tool for both clinical and medical applications (Chen, W et al. 2014; Min et al. 2011; Singh et al. 2010). Among the successful application of scFv were in hepatitis A antigen quantification for vaccine preparation (Nimmagadda et al. 2012), diagnosis of mycotoxins in field and stored grain or in food (Hu, ZQ et al. 2013), production of antibody for use in treatment against influenza virus (Pissawong et al. 2013), and for use in immunodetection of staphylococcal enterotoxins A, a prevalent causes of foodborne diseases (Chen, W et al. 2014).

1.7.4 **Phagemid as phage display vector**

Two different types of vectors are available for protein display, namely the phage vector and the phagemid – a plasmid-based vector. Both vectors are structurally based on the natural Ff-phage sequence, with phagemid vector is only equipped with fusion protein gene, but lacks other phage genes (reviewed in Russel, Lowman and Clackson (2004)). Phagemid is also modified to carry the plasmid replication origin which allows its replication in *E. coli*. Basically, a phagemid contains replication origin of a plasmid, a selective marker, the intergenic region (IG), a phage coat protein gene, restriction enzyme recognition sites, a promoter, a DNA segment encoding signal peptide, and a molecular tag (Figure 1.8) (Qi et al. 2012). Phagemid is also often modified to have an amber stop codon (TAG) between the displayed sequence and gene III. This allows expression of soluble protein when the vector is transferred into a non-*supE* suppressor strain *E. coli* such as HB2151 (reviewed in Azzazy and Highsmith Jr (2002)). While a phage vector is fully capable to replicate and produce phage displaying the desired protein once it is introduced into *E. coli*, phagemid is converted into a filamentous phage after its co-infection with helper phage such as M13KO7, VCSM13, hyperphage (Rondot et al. 2001) and their derivatives (Baek et al. 2002; Kramer et al. 2003;

Soltes et al. 2003). In terms of its display, phage vector displays protein polyvalently (heterologous protein), while phagemid vector allows monovalent protein display (reviewed in Qi et al. (2012).

Phagemid is more widely used as vectors in phage display due to the following factors; (i) its large capacity in accommodating foreign DNA fragment, (ii) efficient transformation which results in high diversity of phage library, (iii) various selection of restriction enzyme recognition site are available for convenient gene manipulation, (iv) fusion protein expression level can be modulated with ease, and (v) genetically more stable after multiple propagations (Bass, Greene & Wells 1990; Breitling et al. 1991; Qi et al. 2012). As noted in previous section, coat protein III and VIII are the most common coat protein used for expression (Qi et al. 2012). It is also noted that phagemid with coat protein III has a bigger insert size capacity for foreign proteins in comparison to phagemid with coat protein VIII (Smith, GP 1993). However, coat protein III may only be used to express up to five copies of the fusion proteins, while coat protein VIII is capable of expressing up to thousand copies (Veronese et al. 1994).

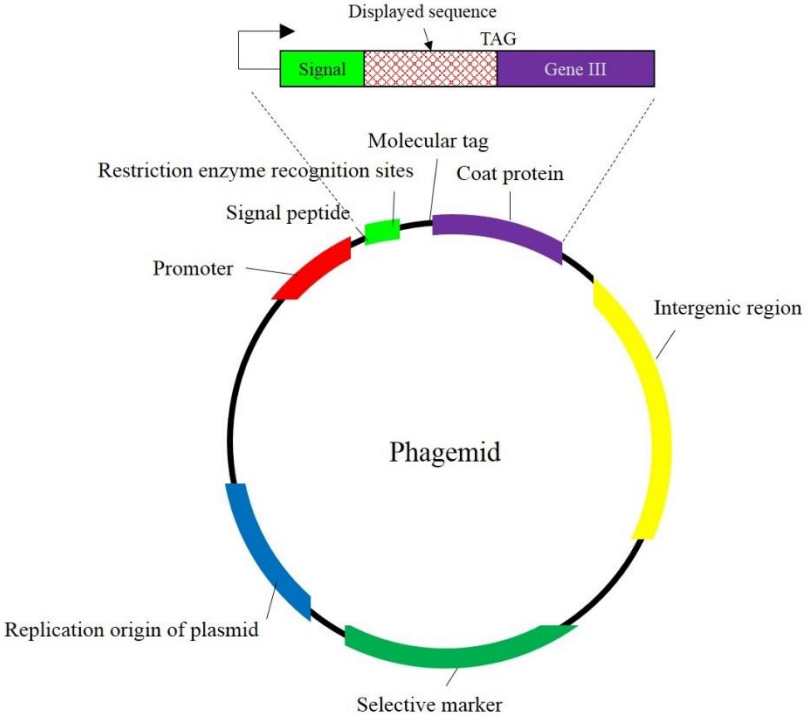

Figure 1.8 An illustration of a phagemid and its main components. Adapted from Qi et al. (2012).

Considering the options available for isolation of anti-M2e antibodies for DIVA and AIV surveillance purposes, this thesis has employed the following selections: H5N1 immunized library from chicken, the natural AIV host, to specifically increase the possibilities of V-gene repertoire harbouring high affinity anti-M2e antibodies; and filamentous bacteriophage which displays an scFv antibody format through coat protein III, while using phagemid to fully exploit the advantages it has to offer to isolate the antibody of interest, for application in an ELISA-based system.

1.8 Research Rationale and Aims

AIV M2e protein has been demonstrated to be capable of DIVA application, which is a critical feature for AIV surveillance in H5N1 enzootic countries. The major issue concerning this protein is that it is a weak immunogen by nature. However, its differential epitope density between virus infected cell and on the virion itself (which used in whole-killed virus vaccination) made it valuable and worthy to explore for DIVA application. It is noted that the first virus challenge experiment after vaccination in chicken may only evoke a low amount of anti-M2e antibodies due to the protective threshold build by killed virus vaccination (Heinen, de Boer-Luijtze & Bianchi 2001). Therefore, a highly sensitive and highly specific detection tool is required to ensure that the detectability of M2e protein is not impaired due to such interference.

M2e-based competitive ELISA will be a highly optimal diagnostic tool for AIV surveillance as it is simple, easy and has a high throughput. Apart from capable of DIVA, an M2e-based competitive ELISA has the potential to be used with more than just one species, as AIV is a multiple host pathogen (Chambers, Dubovi & Donis 2013). However, a competitive ELISA is only applicable if the protein (competitive antibodies) recognizes the same antigenic determinant – the dominant epitope, across species. No known comparison of M2e epitope has been done previously among different species. Therefore, it is the interest of this thesis to

characterize the antigenic determinant of M2e protein from different anti-M2e antibodies sources.

Findings from the M2e mapping are to provide a basic information on the suitability of the available anti-M2e antibodies to be used as the competitor antibodies in a competitive ELISA setting. Therefore, it is also the aim of this thesis to develop a potential M2e-based competitive ELISA using the generated anti-M2e antibodies.

It is noted that scFv antibodies displayed on phage are mostly stable and demonstrated high affinity to the targeted antigen (Chen, W et al. 2014; Min et al. 2011). Therefore, this thesis aims to isolate reactive anti-M2e antibodies using the phage display technology from H5N1 exposed birds for the development of M2e-based competitive ELISA. Presumably, construction of anti-M2e phage displayed antibodies library will enable the selection of the most reactive antibodies to the targeted protein.

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Chapter 2 Avian influenza virus and DIVA strategies

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Avian influenza virus and DIVA strategies

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[Note]In the published work, "endemic" was used in contexts where "enzootic" would be more correct, and that in this thesis "endemic" is marked by inverted commas i.e. 'endemic' where "enzootic' would be more correct.

Abstract

Vaccination is becoming a more acceptable option in the effort to eradicate avian influenza viruses (AIV) from commercial poultry, especially in countries where AIV is 'endemic'. The main concern surrounding this option has been the inability of the conventional serological tests to differentiate antibodies produced due to vaccination from antibodies produced in response to virus infection. In attempts to address this issue, at least six strategies have been formulated, aiming to differentiate infected from vaccinated animals (DIVA), namely (i) sentinel birds, (ii) subunit vaccine, (iii) heterologous neuraminidase (NA), (iv) non-structural 1 (NS1) protein, (v) matrix 2 ectodomain (M2e) protein, and (vi) haemagglutinin subunit 2 (HA2) glycoprotein. This short review will briefly discusses the strengths and limitations of these DIVA strategies, together with the feasibility and practicality of the options as a part of the surveillance program directed towards the eventual eradication of AIV from poultry in countries where highly pathogenic avian influenza is 'endemic'.

Keywords: avian influenza virus, vaccination, DIVA strategies

2.1 Avian influenza virus (AIV)

2.1.1 Gene segments and proteins

Avian influenza viruses are enveloped, segmented, negative-stranded RNA viruses, belonging to the family *Orthomyxoviridae*, genus *Influenzavirus A* (Lamb & Krug 2001; Taubenberger & Kash 2010). Influenza A virus (IAV) is composed of eight gene segments, and each gene segment codes for at least one protein. To date, IAV is known to code for 13 viral proteins (Jagger et al. 2012; Wise et al. 2012). Some of the segments encoded more than one protein through mechanisms such as alternative reading frame (PB1-F2, PB1-N40, PA-X and M2), and mRNA splicing (NS1/NEP) (Chen et al. 2001; Jagger et al. 2012; Lamb & Choppin 1981; Lamb & Lai 1980; Wise et al. 2009).

AIV is classified based on the antigenic variation displayed by the virus surface protein – haemagglutinin (HA) and neuraminidase (NA) (Assaad et al. 1980). A total of 144 possible subtype combinations have been identified for AIV based on the 16 HA subtypes and 9 NA subtypes (Fouchier et al. 2005) found circulating in the aquatic bird population identified as the AIV natural reservoir, predominantly the Anseriformes (particularly ducks, geese, and swans) and Charadriiformes (particularly gulls, terns, and waders) (Munster et al. 2007; Webster et al. 1992). Two new HA subtypes (H17, H18) and NA subtypes (N10, N11) have recently been identified circulating in bats from Central America (Guatemala) and South America (Peru) (Tong et al. 2012; Tong et al. 2013).

2.1.2 **AIV transmissibility**

Observations indicated that movement of AIV from wild to domestic birds occurs relatively frequently due to shared ecosystem, where prolong and repeated exposure of domestic birds to the virus facilitate adaptation of virus to a new host (Swayne 2007). However, virus adaptations for a new host is a complex and a rare event as majority of these transmissions will only cause transient virus infections with limited spread as observed in AIV poultry surveillance (Alexander 2007; Suarez 2010). However, it is important to note that some species such as domestic ducks and geese, turkeys as well as the Japanese quails are more susceptible to AIV infections and may have been the bridging species of wild birds AIV into the chickens and other gallinaceous poultry (Swayne & Slemons 2008).

2.1.3 **AIV evolution**

Continuous outbreaks of AIV infection are driven by two main evolutionary mechanisms used by the virus to evade host immune systems: antigenic drift and antigenic shift (Nelson & Holmes 2007). Antigenic drift occurs in response to the host immune pressure when mutations accumulate in the surface glycoproteins HA and NA, causing minor changes to the antigenic structure of the virus (Nelson and Holmes, 2007). Antigenic shift results from

reassortment of infecting virus subtypes that lead to introduction of strains with completely novel gene combination and often with improvements in the capacity for the production of more viable and fit virus progeny (Holmes et al. 2005).

2.1.4 **AIV pathogenicity**

AIV is classified into low and highly pathogenic avian influenza virus (LPAIV and HPAIV, respectively) based on its lethality in chickens (*Gallus gallus domesticus*) (Swayne 2007; Swayne & Suarez 2000). In domestic poultry, LPAIV generally causes subclinical infection with virus shedding in infected birds, if not mild respiratory disease. In contrast the HPAIV, also formerly known as the fowl plague, causes multiorgan systemic disease, with high percentage of morbidity and mortality in both domestic and wild birds (Alexander 2000; Swayne & Suarez 2000).

The AIV pathogenicity generally relies on the cleavability of the HA0 subunit to HA1 and HA2 by the host cellular proteases (Klenk et al. 1975; Lazarowitz & Choppin 1975; Rott et al. 1980), and HPAIV is characterised by the presence of polybasic amino acids at the HA0 cleavage site instead of a monobasic motif observed for LPAIV (Bosch et al. 1981; Horimoto & Kawaoka 1994; Senne et al. 1996). The monobasic structure of the HA0 cleavage site is only cleavable by the trypsin-like enzymes which are present at limited sites in the host, hence LPAIV infections are confined to respiratory or gastrointestinal tract (Klenk & Garten 1994; Lazarowitz, Compans & Choppin 1973; Rott 1992). In contrast, the polybasic motif found in the HPAIV HA0 is cleaved by ubiquitous proteases present within cells of multiple organs throughout the body, such as furin and subtilisin-related proteases (proprotein convertase 6 – PC6), causing fatal systemic infection (Horimoto et al. 1994; Stieneke-Grober et al. 1992).

2.1.5 **LPAIV and HPAIV in poultry**

Any of the 16 HA subtypes circulating in wild birds reservoirs are considered as LPAIV, while all HPAIV are of H5 and H7 subtypes, although not all of these subtypes are HPAIV (Alexander & Brown 2009; Swayne & Suarez 2000). Apart from the HPAIV H5N3 outbreak in common terns (*Sterna hirundo*) in South Africa in 1961 (Becker 1966) and HPAIV H5N1 outbreak in wild waterfowl in two parks in Hong Kong in 2002 and bar-headed geese (*Anser indicus*) in western China in 2005 (Chen et al. 2005), HPAIV has been rarely isolated from wild bird populations (Swayne & Suarez 2000). Due to the complex pathobiology of AIV, viruses which are highly pathogenic (HP) in domestic birds, generally do not necessarily cause diseases in Anseriformes birds (ducks and geese) in experimental condition (Alexander, Parsons & Manvell 1986; Forman, Parsonson & Doughty 1986). It is important to note that HPAIV usually occurs in domestic gallinaceous poultry (chickens, turkeys, quails and guinea fowls) after exposure to and adaptation of LPAIV from wild birds (Rohm et al. 1995; Swayne 2007). This is usually a unidirectional infection, where the domestic bird-adapted AIV rarely re-infects wild bird's population (Swayne 2007), with the exception of the Asian lineage H5N1 HPAI virus (Chen et al. 2005; Liu et al. 2005).

2.1.6 **Virulence shift of LPAIV to HPAIV**

The LPAIV H5 and H7 subtypes can acquire virulence factors and become HPAIV through several mechanisms focused on the HA protein, which are (i) the substitution and insertion of basic amino acids (aa) in the HA cleavage site (Horimoto et al. 1995; Swayne 1997), (ii) loss of carbohydrate which covers the HA cleavage site through residue mutations (Kawaoka, Naeve & Webster 1984), (iii) recombination of HA with other AIV viral gene such as nucleoprotein (NP) gene (Suarez et al. 2004), or the matrix (M) protein gene (Pasick et al. 2005), or with the 28S ribosomal RNA (Khatchikian, Orlich & Rott 1989), and (iv) polymerase slippage which caused sequence duplication, thus insertion in the HA gene (Garcia et al. 1996; Perdue et al. 1997). Nevertheless, it was suggested that a hidden virulence

potential was readily embedded within the LPAIV strains capable of transformation to a HP strain, where the acquisition of polybasic cleavage site is the key activator for the virulence shift (Bogs et al. 2010; Stech et al. 2009). This assumption is based on observations where alterations in other AIV viral proteins such as deletion of matrix 2 (M2) protein or NP cleavage site reduced AIV pathogenicity (Zhirnov & Klenk 2009); while point mutation accumulation in the NA protein (Deshpande, Naeve & Webster 1985), amino acid deletion in the NA stalk (Munier et al. 2010) and amino acid substitution in the non-structural protein 1 (NS1) (Jiao et al. 2008) and polymerase proteins (basic polymerase 2,PB2 and acidic polymerase, PA) (de Wit et al. 2010; Hatta et al. 2001) promotes virulence of AIV.

2.1.7 **Evolutionary pattern of H5N1**

Within the AIV history, the pandemic potential of Asian lineage H5N1 virus is by far the most alarming due to the rate of its spread and the unusual evolutionary pattern showed by this particular subtype (Fouchier & Guan 2013; Watanabe et al. 2011). Unlike the emergence of other HPAIV which occurs in chickens, the initial outbreak of H5N1 was recorded in domestic geese in Guangdong Province, China in 1996, which then became the primary precursor virus for the major outbreak in chicken farms in Hong Kong in 1997 (HK-97) (Shortridge et al. 1998; Xu et al. 1999). Although the HK-97 genotype had been eliminated through mass poultry depopulation in 1997, the genetic variants of the primary precursor virus (Goose/Gd-like) have continued to circulate exclusively in aquatic poultry until late 2000 (Cauthen et al. 2000; Webster et al. 2002), where the host range expanded to include terrestrial poultry in the following year, providing a larger pool of genetic material for reassortment (Chen et al. 2004; Guan et al. 2002).

The rapid rate of H5N1 evolution was later validated with the identification of six H5N1 reassortants in Hong Kong and mainland China in early 2001, immediately before the outbreak in Hong Kong, mid-May the same year (Guan et al. 2002; Li et al. 2004; Sims et al.

2003). It was identified that this reassortant virus possessed a HA gene that originated from a Goose/GD/96-like virus, while the other seven internal genes were a result of reassortment from other non-H5 avian influenza viruses (Webster et al. 2002). Although no infection with H5N1 was detected from July 2001 onwards, Hong Kong experienced an outbreak caused by the HPAIV H5N1 again in February 2002 (Li et al. 2004; Sims et al. 2003). Eight new H5N1 genotypes were isolated including genotype 'Z', which later become dominant in southern China (Li et al. 2004). Characterized with the deletions of 20 aa in the NA stalk and 5 aa in the NS protein (Guan et al. 2002), genotype 'Z' has been responsible for the emergence of the 2003 and 2004 H5N1 outbreaks, marking the first dissemination wave of H5N1 into eight countries in East and South East Asia, leading to establishment of 'endemicity' in Vietnam and Indonesia (Fouchier & Guan 2013; Wang, Vijaykrishna, et al. 2008).

Although the Asian lineage H5N1 virus was 'endemic' in poultry since 1997, it had later spread and persisted in the wild bird population, evidenced by the H5N1 outbreak in the migratory waterfowl, the bar-headed geese (*Anser indicus*) at Qinghai Lake in western China in 2005 (Chen et al. 2005; Liu et al. 2005). Subsequently, the virus spread rapidly across Asia, Europe, the Middle East, and Africa, marking the second wave of H5N1 dissemination, affecting wild migratory birds and poultry (Gilbert et al. 2006; Wang, Vijaykrishna, et al. 2008). The third wave of H5N1 dissemination to South East Asian countries followed immediately in late 2005. It was characterized by the emergence and predominance of the H5N1 Fujian-like viruses, replacing the multiple H5N1 sublineages in China which were responsible for the previous disseminations (Smith et al. 2006). This event led to the panzootic of H5N1 in poultry, especially in the Asian continent where intermittent outbreaks have been reported, particularly in countries where H5N1 is 'endemic' (China, Vietnam, Indonesia and Bangladesh) (FAO 2011; Fouchier & Guan 2013).

2.2 AIV and vaccination

Following the identification of wild birds as the agent of long distance virus transmission (Artois et al. 2009; Olsen et al. 2006; Tian et al. 2015), and the possible transmission of the virus through domestic animals (Verhagen et al. 2014), culling of the infected birds and the flocks of birds with suspected exposure to the virus have been used as the primary control measures, especially in countries where disease has been recently introduced (Suarez 2005, 2012). However, in countries where infection was already widespread and 'endemic', and other methods were not likely to eradicate the infection, vaccination was chosen as the primary control tool (Domenech et al. 2009; Suarez 2012; Swayne et al. 2011).

To date, AIV vaccination using the inactivated vaccines, and to a smaller portion using the live recombinant vaccine (NDV-H5) has only been exercised as a control or a preventive measure to eradicate HPAI viruses in poultry, either in the event of epidemics, such as seen in Mexico (H5N2, 1994-1995, 1995-2001) (Villareal 2009), Italy (H7N1, 1999-2000; H7N3 and H5N2, 2003-2006) , Hong Kong (H5N1, 2002-2003) (Capua, Mutinelli, et al. 2002; Marangon, Cecchinato & Capua 2008; Sims et al. 2003; Villareal 2009) and others ; or in countries where HPAIV are 'endemic', as is the case for HPAI H5N1 in China, Indonesia, Vietnam and Egypt (Chen 2009; FAO 2011; Marangon, Cecchinato & Capua 2008; Marinova-Petkova et al. 2014).

Vaccination helps to control the spread of infection as vaccinated birds will acquire an elevated level of resistance to infection, thus lower shedding and environmental contamination by virus (Capua et al. 2009; Swayne et al. 2011). Nevertheless, to achieve disease eradication, it is important for a vaccination programme to be implemented in conjunction with adequate biosecurity enforcement and continuous surveillance of infection in vaccinated bird population (Capua et al. 2009). Although vaccination is highly recommended as a control and preventive tool for AIV, silent spread of infection in

vaccinated populations is a major concern, especially where AIV is 'endemic'. This is due to inability of the available inactivated AIV vaccines to provide complete protection to a virulent field challenge, allowing for a small number of birds to become infected and excrete the virus without apparent clinical manifestation of infection. Long term circulation and establishment of AIV in vaccinated population have been reported to cause changes in the genetic and antigenic properties of the virus, producing escape mutants as reported in Mexico (Lee, Senne & Suarez 2004a), China (Smith et al. 2006), and Egypt (Grund et al. 2011). Due to the inability of the available standard serological tests used in disease surveillance to differentiate antibodies produced by vaccination from those that arise by field virus infection, strategies have been developed to differentiate infected from vaccinated animals (DIVA).

2.3 Current understanding of DIVA strategies for AIV

Vaccine development work with the aim to enable DIVA application was first published by Van Oirschot et al. (1986) for Aujeszky's disease virus in pigs; and this investigator later coined the acronym DIVA (Van Oirschot 1999). In parallel growth with the use of vaccine against AIV, advances of DIVA strategies were focused on vaccine developments which are capable of DIVA while permitting the use of the available standard serological tests (DIVA-vaccine approach). Alternatively, DIVA-antigen approach focused more on the serological tests development while allowing the use of conventional vaccines (killed virus).

In this section, six DIVA strategies were discussed in terms of the vaccine format and the available complementary companion diagnostic tests: (i) sentinel birds, (ii) subunit vaccine, (iii) heterologous NA, (iv) non-structural 1 (NS1) protein, (v) matrix 2 ectodomain (M2e) protein, and (iv) hemagglutinin subunit 2 (HA2) glycopolyprotein (gp) (Birch-Machin et al. 1997; Boyle & Heine 1993; Capua, Terregino, et al. 2002; Hemmatzadeh et al. 2013; Lambrecht et al. 2007; Suarez 2012). Summary of these strategies can be seen in Table 2.1.

2.3.1 **Sentinel birds**

The most basic strategy used for detection of live virus infection in a vaccinated flock is the employment of sentinel birds, where approximately 1% of the birds in the monitored farm are left unvaccinated and routinely tested serologically to detect flock exposure to live virus (Suarez 2005, 2012). This strategy offers a sensitive measure of any rising infection within the vaccinated flocks, and monitoring can be done using the available diagnostic tests such as the haemagglutination inhibition (HI) test and the ELISA test detecting NP or HA antibodies. This strategy was successfully employed alongside the heterologous NA emergency vaccination during the HPAI H7N1 outbreak in Italy in 2000 to monitor the field situation (Capua et al. 2009).

2.3.2 **Recombinant subunit vaccines**

As described earlier, HA gene encodes a structural virus protein with important functions for immunity and is one of the key determinants of AIV antigenic properties (Klenk et al. 1975; Lazarowitz & Choppin 1975). Although optimum protection is achieved through the use of vaccination with whole inactivated virus homologous to the circulating strain, studies have indicated that the presence of HA alone in vaccine elicits protective immune response against viral infection (Robinson, Hunt & Webster 1993; Webster et al. 1994). In the subunit vaccine strategy, the AIV HA gene is expressed in bacteria, viruses or yeast system before being purified and prepared for use as a vaccine (Crawford et al. 1999; Davis et al. 1983; Saelens et al. 1999). A variety of different AIV viral vectors have been studied, where protective immunity was demonstrated upon experimental challenges (Table 2.2).

Apart from being efficacious and safe for application, the recombinant subunit vectored-virus vaccines offer immunity through a single vaccination, with the option of vaccination against multiple diseases and the availability of mass vaccine administration (Li et al. 2011; Swayne et al. 2003). Works on recombinant subunit vaccines have expanded significantly following

the advances of reverse genetic technology (Neumann & Kawaoka 2001), where it allows rapid regeneration of reassortant viruses, thus reduces vaccine production time by approximately 2-months (Hoffmann et al. 2002). However, most importantly, the subunit vaccines allow a clear distinction between antibodies produced by vaccination or wild type AIV infection, which is crucial for DIVA surveillance purposes using the standard diagnostic tools. In theory, the vaccinated birds will only produce antibody against the expressed HA protein, but none for internal proteins such as NP and M proteins. Since the vaccinated birds will remain naïve to the internal proteins, infected birds can be identified if antibodies against these proteins are present (Li et al. 2011). Standard diagnostics test available are the agar gel immunodiffusion (AGID) which detects the anti-NP and anti-M antibodies (OIE 2014); and the commercially available enzyme-linked immunosorbent assay (ELISA) kit such the AIV FlockChek ELISA kit (IDEXX labs) (Li et al. 2011), specifically designed for detecting anti-NP antibodies. To date, the recombinant fowlpox-influenza H5 vaccine is licensed and available in El Salvador, Guatemala, Mexico, China and USA (Swayne & Kapczynski 2008), while recombinant herpesvirus turkey (rHVT) is licensed in Egypt and USA, with recombinant duck enteritis virus (rDEV) being licensed in China (OIE 2014; Swayne & Spackman 2013).

2.3.3 **Heterologous NA vaccine**

The heterologous NA vaccine strategy employs an inactivated AIV containing similar HA subtype but different NA subtype to the outbreak strain (Capua, Terregino, et al. 2002). Vaccinated birds are protected against live virus infection by development of anti-HA antibodies, and can be differentiated from infected birds through detection of antibodies against the NA subtype. This strategy allows the use of the standard killed vaccines and screening can be done against anti-NA antibodies using an indirect immunofluorescence assay (Capua, Terregino, et al. 2002), in place of the conventional neuraminidase inhibition (NI) test (Aymard-Henry et al. 1973).

Only three known applications of the heterologous NA vaccine in the field are known. It was first introduced as a measure to differentiate between vaccinated and infected birds during the 1999-2000 H7N1 HPAIV outbreak in Italy (Capua, Mutinelli, et al. 2002). The vaccine was prepared using inactivated H7N3 virus, and infected birds were detected by an indirect immunofluorescent antibody test (iIFAT) specifically developed for anti-N1 antibody (Capua, Terregino, et al. 2002). Similar strategy was implemented during the outbreak of LPAI H7N3 in Italy in 2002-2003, where inactivated H7N1 was used for vaccination, and during the outbreak of HPAI H5N1 in Hong Kong in 2002, inactivated H5N2 virus was used for vaccination (Capua & Alexander 2004).

2.3.4 **AIV nonstructural 1 (NS1) protein: Differential immune response**

The NS1 protein is a multifunctional protein which among its purpose is to regulate viral RNA polymerase activities and viral mRNA translation (Enami et al. 1994; Lamb & Choppin 1979; Shimizu et al. 1994). It is a non-structural protein which is only detectable in infected cells, but not in packaged virions (Skehel 1972). Based on this observation, a DIVA-antigen approach has been suggested which allows the use of conventional whole-killed virus for vaccination (Ozaki et al. 2001). A diagnostic ELISA that targets NS1 antibodies is a simple screening test, as had been previously recognised for foot and mouth disease virus (Neitzert et al. 1991). The first successful demonstration of this strategy for AIV was reported for the equine influenza A virus (Birch-Machin et al. 1997), where NS1 antibodies were identified only in infected ponies but not in the vaccinated ones. Most works on the development of NS1 protein as antigen for DIVA have expressed recombinant NS1 protein in vectors such as pMAL and pET (Brahmakshatriya, Lupiani & Reddy 2010; Tumpey et al. 2005; Zhao et al. 2005).

2.3.5 **Matrix 2 ectodomain (M2e) protein: Highly conserved protein**

M2e protein is the external part of a homotetrameric transmembrane protein encoded by segment 7 of the IAV through an alternative reading frame (+1) mechanism (Holsinger & Lamb 1991; Lamb, Lai & Choppin 1981). This protein forms ion channels on the AIV surface that are crucial for the release of viral genome into the host cell cytoplasm during virus entry (Lamb, Zabedee & Richardson 1985; McCown & Pekosz 2005), and serves as a pH regulator for the Golgi apparatus which is essential for HA glycoprotein maturation (Sugrue & Hay 1991). Two factors have led to the recommendation of M2e protein as DIVA antigen: (i) the relatively invariable nature of M2e protein across AIV strains (Ito et al. 1991; Khurana et al. 2009), where its small size and low abundance in comparison to the other two surface glycoproteins (HA and NA) have allowed M2e protein to escape immune selection pressure and antigenic drift (Fiers et al. 2009); and (ii) the abundance of the M2e protein on the surface of infected cells despite being low in copy number in a mature virion (~3% of the surface glycoprotein population) (Black et al. 1993; Zabedee, Richardson & Lamb 1985). Both of these characteristics have suggested that M2e protein could be a sensitive, specific as well as a universal DIVA antigen. The earliest report on the application of M2e as DIVA antigen in poultry has demonstrated a sensitive M2e peptide-based ELISA for detection of M2e antibodies following infection with HPAIV strains H5 and H7 (Lambrecht et al. 2007). Similar sensitivity of M2e protein as DIVA antigen has also been demonstrated in a challenge study using LPAIV H9N2 (Kim et al. 2010), as well as against multiple AIV reference antisera (Hemmatzadeh et al. 2013).

2.3.6 **Haemagglutinin subunit 2 (HA2) glycoprotein (gp): Highly conserved epitope**

HA2 glycoprotein (gp) is the C-terminal fragment of the cleaved form HA protein (Skehel & Waterfield 1975; Wilson, Skehel & Wiley 1981). It is considerably the more conserved region out of the two HA cleavage products (HA1 and HA2), especially at its N-terminal end, known as the fusion peptide (first 11 residues) which is involved in the fusion activity of IAV (Daniels

et al. 1985; Skehel & Waterfield 1975). The HA2 gp has been suggested as another potential target for DIVA tool based on two key criteria. Firstly, HA2 is highly conserved throughout the 16 HA subtypes of IAV (Fouchier et al. 2005; Nobusawa et al. 1991; Okuno et al. 1993), with only two known epitope variants corresponding to the classical phylogenetic grouping of AIV HA protein (Sui et al. 2009). Four antigenic sites have been identified from HA2, namely site I (aa 1-38, the N-terminal), sites II and IV (aa 125-175) which exhibit different reactivity among IAV subtypes, and site III (aa 38-112) (Vareckova, Mucha & Kostolansky 2013). As observed with the M2e protein approach, detection of antibodies against the highly conserved HA2 gp would theoretically enable a universal detection of all IAV subtypes. Secondly, this conserved region is only accessible to immune recognition following virus infection. It has long been noted that HA0 cleavability is essential for IAV infectivity (Klenk et al. 1975; Lazarowitz & Choppin 1975), where the cleavage of HA0 to form HA1 and HA2 subunits is a prerequisite for membrane binding and virus entry to the host cell (Maeda & Ohnishi 1980; Skehel et al. 1982). HA2 gp is not accessible in the HA0 native form as it is buried in the pocket formed by the stalk of the HA stem trimer (Skehel & Wiley 2000; Vareckova, Mucha & Kostolansky 2013). However, once the HA0 is cleaved, the HA2 gp will be exposed and inserted into the target membrane to allow the conformational change which will lead to membrane fusion and virus entry (Bullough et al. 1994; Chen et al. 1998). Considering these findings, it is reasonable to assume that the presence of antibodies against discrete epitopes on HA2 gp would also be indicative of virus infection.

Table 2.1 List of available strategies for differentiating infected animals from vaccinated animals (DIVA), with some of their advantages and limitations in general.

Strategy	Sentinel bird	Recombinant subunit vaccines	Heterologous NA	Differential immune response against protein (NS1, M2 and HA2 gp)
Procedure & vaccine used	• Naïve unvaccinated bird are marked and randomly spread in a vaccinated flock • Sentinel birds are routinely tested for influenza virus exposure	• Vaccine using a vector expressing HA and NA proteins • Example: Fowlpox-vectored recombinant vaccine for the H5 subtype	• Vaccines containing the same HA subtype as the field strain, but a different NA subtype. • Example: If the field virus is H7N2, the vaccine is H7N3	• Vaccination using whole-killed virus • Observation of the differential immune responses to the targeted protein (NS1, M2 or HA2)
Available companion diagnostic test	• Hemagglutinin Inhibition (HI) test • Agar gel immunodiffusion (AGID) • Type A-specific ELISA (detect anti-NP)	• Agar gel precipitin (AGP), • ELISA targeting antibodies to the matrix (M) protein or the nucleoprotein (NP) • Fluorescence microsphere immunoassay (FMIA)	• Neuraminidase Inhibition (NI) test • Indirect immunofluorescence assay (iIFAT) • FMIA • Modified NI test	• ELISA-based targeting the antibodies to specified proteins
Advantages	• Low cost • Readily applicable • Sensitive procedure for monitoring in vaccinated flock	• Efficacious in providing protection • Commercially available • Mass administration • The standard diagnostic tests are applicable	• Efficacious in providing protection • Rapidly available through reverse genetics technology	• Conventional inactivated virus can be used for vaccination • Only a single diagnostic test needed
Limitations	• Labor intensive • Time consuming • Naïve birds can potentially act as virus amplifiers and be the source of infection	• Test sensitivity is yet to be determined	• Prior knowledge on circulating strain • Possible introduction of the same NA subtype field strain with the NA subtype used for vaccination • Undetermined sensitivity of serologic testing • Low throughput screening capacity • iIFAT – time consuming, laborious and the result interpretation is subjective	• Risk of false-positive due to the presence of protein contaminant from non-purified vaccine i. e NS1 protein • Risk of false-negative in surinfected host due to the inability of host to seroconvert • HA2 gp approach – need more studies

2.4 DIVA strategies applicability and developments

An ideal surveillance tool is required to be (i) cost effective, (ii) rapid and easily manageable, and (iii) to possess a high sensitivity and specificity in discriminating between naïve-infected host from a vaccinated-only host, as well as a vaccinated-infected host.

Although the sentinel bird strategy is simple to employ, there are concerns that the naïve birds may increase the infection risk for the vaccinated flock following repeated and lengthy exposure to the high load shedding of the virus by the sentinels (Suarez 2012). Acquiring a new infection is still possible in the vaccinated flock due to the continuously evolving nature of AIV, as well as technical vaccination issues, such as ineffective application or insufficient coverage, with poor antigenic match of the vaccine with the field strains (Lee, Senne & Suarez 2004a). Furthermore, this strategy is only capable of detecting virus infection in a naïve host placed in a vaccinated flock, with no direct indication of live virus infection in the vaccinated host itself. This has decisively dismisses it from being an option for a long term application for surveillance purposes.

2.4.1 DIVA vaccine-based strategies: recombinant subunit and heterologous NA

For DIVA vaccines approach, multiple studies have demonstrated the effectiveness of recombinant vaccine strategies in providing the necessary protection against clinical signs, as well as fulfilling its role for DIVA purposes (Table 2.2). However, the fowlpox-HA (H7) vaccine was found to show a reduced protection in chickens which have been previously vaccinated or infected with fowlpox virus (Bublot et al. 2006). Host range restriction may also apply for a particular virus vector such as observed for the infectious laryngotracheitis virus (ILTV) as it replicates poorly in turkeys (OIE 2014). Nevertheless, mass administration and multiple diseases vaccination options offered by the recombinant vaccines highlight the feasible application of recombinant vaccines, as evidenced by the continuous development and application of this particular strategy.

Following the introduction of heterologous NA vaccination application in Italy (Capua, Terregino, et al. 2002), various combinations of HA and NA proteins have been tested and recommended, including the use of rare NA subtypes for vaccine development such as N5 and N8 (Table 2.2) (Beato et al. 2007; Capua et al. 2009). Introduction of the eight-plasmid reverse genetics system which allows rapid *de novo* generation of reassortant live virus has made it possible for the rapid availability of a heterologous vaccine once the NA subtype of the wild type circulating virus is known (Beato et al. 2007; Lee, Senne & Suarez 2004b). Nevertheless, a collection of vaccine with various combinations is necessary to ensure swift implementation in case of outbreak where multiple virus subtypes are present in a single host or population (Swayne et al. 2011).

Since the conventional diagnostic tests are not applicable for the heterologous NA approach, companion tests specific for this strategy, iIFAT have been developed (Capua, Terregino, et al. 2002). Although the test is highly specific and sensitive for application (Cattoli et al. 2006), the iIFAT is also time-consuming and labour intensive assay, as it is with the classical NI test (Aymard-Henry et al. 1973; Capua, Terregino, et al. 2002). It has been suggested that these NA based tests be replaced with a faster, simpler and higher throughput ELISA-based screening system, such as the N2-specific ELISA-based test (Kwon et al. 2009) and truncated-N1-specific ELISA (Wu et al. 2009). Alternatively, a modified version of the NI test is made available where MUN (2′-[4-methylumbelliferyl]-α-D-N-acetylneuraminic acid sodium salt hydrate) was used as the NA substrate in place of the traditional fetuin-based NI test, providing a more rapid analysis and quantitative results where the antibody responses can be measured over time (Avellaneda, Sylte, et al. 2010). Recent developments have revealed a range of refinements on the available known tests (NI and ELISA) (Avellaneda, Sylte, et al. 2010; Wang et al. 2011). However, due to the need for the production of both vaccine and its tailor-made companion test for an optimized performance, limited availability of facilities and

resources are the major drawbacks for this particular strategy. Most importantly, in dealing with H5N1 'endemic' countries, homologous strain is a much preferred option for vaccination as heterologous NA is not an ideal strategy to apply given the diverse genetic variants of H5N1 (Chen 2009; Grund et al. 2011; Gutierrez et al. 2009).

2.4.2 DIVA test-based strategies: NS1, M2e and HA2 proteins

DIVA tests based on NS1, M2e and HA2 proteins are viewed more favourably in terms of their practicality (Table 2.1). These strategies offer a more straightforward approach in comparison to the subunit and the heterologous NA vaccination strategies, where the DIVA test strategy complements the conventional homologous inactivated vaccine administration. Although studies have shown that the presence of HA protein in a vaccine is enough to provide a good protection against live virus infection, in most cases it only reduces the clinical signs, and AIV is still sheds in the faeces of infected birds (Swayne et al. 2001; Swayne et al. 2000). Virus shedding could be in low amount, but the silent spread (asymptomatic) of viral infection is still possible due to the generation of escape mutants in response to vaccination pressure (Lee, Senne & Suarez 2004a). Taken together, homologous strain vaccination is still by far provides the most optimum protection against virus infection, as antigenic relatedness is a significant factor in determining the level of protection induced by vaccination (Lee & Suarez 2005; Swayne et al. 2000).

NS1 protein is highly conserved among AIV subtypes, which is a highly favourable diagnostic property (Tumpey et al. 2005; Wang et al. 2011; Zhao et al. 2005). However, several studies have identified that the NS1 protein also exists in truncated forms in nature (Dundon et al. 2006; Long et al. 2008; Suarez & Perdue 1998), giving rise to concerns that this could affect the overall accuracy of NS1 DIVA test. Also, different level of species susceptibility to AIV infection should be taken into consideration before NS1 DIVA test is adopted for routine use. A study in turkey showed that the NS1 antibodies were only present

for a short time following infection (10 days post-challenged). AIV with a low replication capability in a specific host, either due to low virus adaptability or due to host vaccinal immunity will not be able to produce detectable level of NS1 antibodies despite infection (Avellaneda, Mundt, et al. 2010; Dundon et al. 2007; Soleimani et al. 2012; Takeyama et al. 2011). Similar observation can also be resulted due to the poor immunogenicity of NS1 protein as reported in a challenge study in chickens (Avellaneda, Mundt, et al. 2010).

This strategy also suffers from decreasing specificity with increasing number of vaccination. Low amount of NS1 antibodies were detected in chicken after three times of vaccination with the killed virus contributing to non-specific reactions in the tests, thought to be due to antibody response against leftover NS1 proteins present in the unpurified vaccine (Soleimani et al. 2012; Tumpey et al. 2005; Zhao et al. 2005). This shortcoming however, suggested to be eliminated through the use of vaccination virus with truncated NS1, which remove the possibility of NS1 antibodies detection in vaccinated hosts (Talon et al. 2000; Tumpey et al. 2005). Studies on the truncated NS1 protein (10 nucleotides deletion in the middle of the NS1 protein-coding sequence) demonstrated its capability of providing protective host immunity after influenza virus challenge in mouse, pig and horse models (Quinlivan et al. 2005; Richt et al. 2006; Wang, Suarez, et al. 2008). This has raised the possibility of developing live attenuated virus as vaccine while retaining the capacity of NS1 protein as DIVA marker, although the reversion of the live-attenuated virus to virulent virus is a concern (Wang, Suarez, et al. 2008). This was later vindicated by a study on live mutant NS1 AIV showing its reversion to virulence after five back passages in chicken, thus suggests that a killed vaccine made from a mutant virus with shorter NS1 gene is much safer as well as being practical for DIVA application (Brahmakshatriya, Lupiani & Reddy 2010). Following the occasional detection of NS1 protein antibodies in vaccinated chickens, the NS1-ELISA was suggested to be more suitable for flock monitoring rather than individual birds diagnosis (Takeyama et al. 2011; Wang et al. 2011).

M2e DIVA strategy on the other hand has issues on its specificity and immunogenicity of the M2e antigen. Non-specificity in the recombinant M2e-ELISA was identified to be caused by test serum reactions against the carrier protein used in the M2e expression system (Hemmatzadeh et al. 2013). Although this was not observed in the ELISA system employing synthetic M2e-peptide, the use of recombinant-M2e protein is much preferred as the latter offers a much lower cost for higher output, with continuous access for use in large scale screening (Hemmatzadeh et al. 2013).

Concerns have also been raised where undetectable levels of seroconversion in infected animals may lead to false negative results in M2e-based ELISA. Previous findings indicated that M2e is a weak immunogen (Neirynck et al. 1999), where AIV infections (H1N1 and H3N2, respectively) in mice and humans have engendered poor M2e-specific antibody responses (Feng et al. 2006). A low M2e-antibody response was also observed after a primary infection in pigs with H3N2 or H1N1, but it was increased significantly following challenge infection using H1N1 (Heinen, de Boer-Luijtze & Bianchi 2001). This is hypothesized to be contributed by the small size of the M2e antigenic determinant which limits the number of M2e-reactive B cells for antibody secretion. This is further exacerbated by the antigenic competition posed by the much higher population of HA and NA proteins on the virus surface particle (Feng et al. 2006).

However, in a challenged duck study by Lambrecht et al. (2007), a decreasing trend of M2e antibodies level was reported with the increasing number of vaccination. Increased immunity established by vaccination was assumed to reduce efficient virus replication, hence influencing development of M2e antibody which in turn affected test sensitivity. False negative results have been observed by Kim et al. (2010) where low level of M2e-antibodies was detected despite a H9N2 challenge in chickens vaccinated twice.

Nevertheless, attempts to address these issues have been demonstrated through the improvement in the M2e-ELISA detection efficiency by incorporation of multiple repeats of the M2e protein in the recombinant-M2e-ELISA system (Hadifar et al. 2014; Tarigan et al. 2015) . Otherwise, DIVA application based on M2e protein is proven to have a wide range of reactivity against other IAV subtypes in chickens (Hemmatzadeh et al. 2013).

HA2 peptides were first demonstrated as antigen for H5N1 serodiagnosis using ELISA by Khurana et al. (2011) following identification of one immunodominant epitope through a complete antibody repertoire characterization of H5N1 infection in human (Khurana et al. 2009). Although HA2-specific antibodies have been reported in natural infection in both humans and mice, HA2 is a weak natural immunogen (Stanekova et al. 2012). As observed for the M2e protein DIVA strategy, this factor may also lead to false negative results for the HA2 gp-based antibody detection due to low seroconversion in infected host. However, this approach warrants further study to validate this assumption and to overcome this limitation, as otherwise it offers specificity and universality for surveillance purposes.

Table 2.2 List of selected studies on the development of DIVA strategies for Influenza A virus and the summary of their findings within the last decade. Key: '+' indicates presence of protection by the vaccines or the strategy successfully demonstrated DIVA ability; '-'indicates negative protection by vaccines or unsuccessful DIVA ability; '+/-'indicates partial protection against challenge infection by vaccine or evidence of non-specific reaction for DIVA test results.

Strategy	Vaccination	Challenge Virus Subtype	Animal model	Companion diagnostic test/ DIVA test tool	Protection	DIVA	Comments	Reference
DIVA vaccines: Recombinant vaccine	• Newcastle Disease (NDV) virus expressing HA protein (H5) • Herpesvirus of turkey (HVT) expressing HA (H7) • Virus-like particle (VLP) expressing HA and M1 (H9) • Fowlpox (FP) recombinant expressing HA (H7 and H5) • Infectious laryngotracheitis (ILT) virus expressing HA (H7) • H5N1/PR8-519 (S2 glycoprotein of murine hepatitis virus-MHV replacing NA stalk region)	• H5N1 • H5N2 • H7N1 • H9N2 • H5N8 • H5N3 • H5N2 • H5N9	Chicken, mice	i. NP-GST fusion based ELISA; ii. NP-ELISA (IDEXX Laboratories, Inc., Westbrook, ME)	+/-	+	• Reduced protection shown by FP-HA (H7) in chicken which previously vaccinated or infected with fowlpox	(Bublot et al. 2006; Ge et al. 2007; Kim et al. 2014; Lee et al. 2011; Li et al. 2008; Li et al. 2011; Lozano-Dubernard et al. 2010; Swayne et al. 2001; Swayne et al. 2000; Veits et al. 2003; Veits et al. 2006)

Continued

Table 2.2

Strategy	Vaccination	Challenge Virus Subtype	Animal model	Companion diagnostic test/ DIVA test tool	Protection	DIVA	Comments	Reference
DIVA-vaccine: Heterologous NA	Inactivated wild type: • H7N3 • H5N2 • H7N2 • H9N8 • H9N2 • H7N1 Inactivated reassortant: • H5N1 • H7N8 • H9N8 • H5N8 • H3N4 Inactivated reassortant with truncated NS: • H3N3 Commercial • H5N9	• H7N1 • H5N2 • H7N2 • H9N2 • H7N3 • H3N2	Chicken, turkey	i. Indirect immunofluorescent antibody test (iIFAT) expressing N1, N2 or N3 protein; ii. Micro-NI test with N1, N2 and N8 antigen; iii. Modified micro-NI test with N2, N8 and N9 antigen iv. N2-ELISA	+	+/-	• Micro-NI test is time consuming • Modified version of the micro-NI test provide a more rapid option • ELISA-based test offers a relatively easier and rapid application overall	(Avellaneda, Sylte, et al. 2010; Capua, Terregino, et al. 2002; Cattoli et al. 2006; Cattoli et al. 2003; Kwon et al. 2009; Wang et al. 2011)

Continued

Table 2.2

Strategy	Vaccination	Challenge Virus Subtype	Animal model	Companion diagnostic test/ DIVA test tool	Protection	DIVA	Comments	Reference
DIVA test: Recombinant NS1	Inactivated wild type: • H3N2 • H3N8 • H7N7 • H5N9 • H7N2 • H5N1 • H9N2 Commercial: • H7N2 Live virus: • H5N9 • H7N1 • H7N2	• H3N8 • H3N2 • H7N2 • H9N2	Horse, mice, chicken	i. NS1-ELISA ii. Agar gel precipitin (AGP)	+	+/-	• Non-specific reactions in NS1-ELISA reported is speculated due to the non-purified vaccine used • Incorporation of truncated NS1 in vaccine strain is recommended to address NS1 protein contamination issue • Differences in NS1 immune response between strains and species have been demonstrated i.e. in turkeys and chickens	(Dundon et al. 2007; Ozaki et al. 2001; Soleimani et al. 2012; Tumpey et al. 2005; Zhao et al. 2005)

Continued

Table 2.2

Strategy	Vaccination	Challenge Virus Subtype	Animal model	Companion diagnostic test/ DIVA test tool	Protection	DIVA	Comments	Reference
DIVA test: Truncated NS1	Live virus with truncated NS1: • H7N3 • H3N2	• H7N2 • H3N2	Chicken, turkey	i. NS1-ELISA ii. Fluorescence microsphere immunoassays (FMIA) targeting recombinant NS1	+	+/-	• Low seroconversion in vaccinated-and-challenged turkeys o limited replication site by LPAIV lead to low titer of AIV despite infection (in comparison to HPAIV infection) o could vary between bird species	(Wang et al. 2011; Wang, Suarez, et al. 2008)
DIVA-test: Matrix 2 protein	Inactivated wild type: • H5N9 • H7N1 • H5N1 Commercial: • H9N2	• H7N7 • H5N1 • H9N2	Chickens	i. M2e-peptide-ELISA ii. Recombinant M2e-ELISA iii. Tetrameric-recombinant M2e ELISA	+	+/-	• Recombinant M2e-ELISA is more cost-effective than synthetic peptide-based ELISA • Development of tetramer-M2e as antigen has increased the sensitivity of this strategy, compared with the monomer-M2e based ELISA systems	(Hadifar et al. 2014; Hemmatzadeh et al. 2013; Kim et al. 2010; Lambrecht et al. 2007)

2.5 Recommendations for DIVA programs

For AIV successful monitoring program, DIVA vaccine needs to be (i) effective, (ii) readily distinguishable from the wild type virus, (iii) rapidly available, (iv) cost effective, and ideally (v) applicable by mass administration (by spraying or drinking water); along with companion diagnostic tests or DIVA test which are (i) simple and rapid, (ii) suitable for mass screening, (iii) highly sensitive and specific, and (iv) low cost.

In general, DIVA vaccines (subunit, recombinant and heterologous vaccines) which have been described in the previous section showed high efficiency in providing the optimal protection against AIV infection and capable of DIVA application. Factors affecting vaccine effectiveness such as vaccine strain and target species have to be critically considered to ensure maximum vaccine coverage. Close monitoring of field virus is vital especially where AIV is 'endemic' as continuous infection and circulation of virus promotes immune pressure, thus drifting off the field virus from vaccine seed virus (Swayne & Kapczynski 2008). Availability of vaccine supply particularly in AIV 'endemic' countries should be well managed and maintained as vaccine production is a time consuming process despite its relatively short shelf life (about two years) (Marangon, Cecchinato & Capua 2008). AIV 'endemic' countries usually possess high poultry density, thus cost effectiveness is a critical factor in decision making, which is why advanced vaccines with mass applicability are highly favourable features.

By far, ELISA–based diagnostic test is highly recommended for surveillance and monitoring purposes. However, to ensure the robustness of a DIVA test, field trials using both LPAIV and HPAIV challenge strains still need to be explored in various poultry species model since previous findings have demonstrated that test sensitivity varies between challenge strain and bird species used. Epitope mapping of the DIVA antigens will be an interesting venue to explore as this may aid in scoring a highly sensitive and specific DIVA tool.

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Chapter 3 Epitope mapping of avian influenza M2e protein: different species recognise various epitopes

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Epitope mapping of avian influenza M2e protein: different species recognise various epitopes

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Abstract

A common approach for developing diagnostic tests for influenza virus detection is the use of mouse or rabbit monoclonal and/or polyclonal antibodies against a target antigen of the virus. However, comparative mapping of the target antigen using antibodies from different animal sources has not been evaluated before. This is important because identification of antigenic determinants of the target antigen in different species plays a central role to ensure the efficiency of a diagnostic test, such as competitive ELISA or immunohistochemistry-based tests. Interest in the matrix 2 ectodomain (M2e) protein of avian influenza virus (AIV) as a candidate for a universal vaccine and also as a marker for detection of virus infection in vaccinated animals (DIVA) is the rationale for the selection of this protein for comparative mapping evaluation. This study aimed to map the epitopes of the M2e protein of avian influenza virus H5N1 using chicken, mouse and rabbit monoclonal or monospecific antibodies. Our findings revealed that rabbit antibodies (rAbs) recognized epitope ^{6}EVETPTRN^{13} of the M2e, located at the N-terminal of the protein, while mouse (mAb) and chicken antibodies (cAbs) recognized epitope ^{10}PTRNEWECK^{18}, located at the centre region of the protein. The findings highlighted the difference between the M2e antigenic determinants recognized by different species that emphasized the importance of comparative mapping of antibody reactivity from different animals to the same antigen, especially in the case of multi-host infectious agents such as influenza. The findings are of importance for antigenic mapping, as well as diagnostic test and vaccine development.

Keyword: Antigenic mapping; DIVA test; ELISA; Influenza, M2e protein

3.1 Introduction

Matrix protein 2 (M2) of avian influenza virus (AIV) is a 97 amino acids (aa) protein encoded by RNA segment 7 of the influenza A virus (IAV) (Lamb, Lai & Choppin 1981). It is translated from spliced mRNA and shares a common start codon with the matrix 1 (M1) protein and the first nine aa, while the remaining 88 aa continues at the second (+1) open reading frame (Lamb, Lai & Choppin 1981; Lamb, Zabedee & Richardson 1985). In its native state, M2 is a homotetrameric type III integral membrane protein composed of three domains; namely, a 54 aa cytoplasmic domain located in the viral envelope or cytoplasmic membrane of infected cells, a 19 aa transmembrane domain, and an N-terminal 24 aa ectodomain (M2e) which is exposed on the surface of the virus infected cells and on the viral particles (Holsinger & Lamb 1991; Lamb & Choppin 1981; Lamb, Lai & Choppin 1981; Sugrue & Hay 1991). In the infected cell the M2 protein forms an ion channel which is vital for viral genome delivery into the host cell during virus entry (Lamb & Choppin 1981; Lamb, Zabedee & Richardson 1985; McCown & Pekosz 2005; Pinto, Holsinger & Lamb 1992; Sugrue et al. 1990; Sugrue & Hay 1991). Briefly, M2 ion channel activity is activated by acidification of virus-containing endosomes after internalization of the virus particle into the host cell via clathrin-dependant and –independent mechanisms (Lamb & Krug 2001; Whittaker & Helenius 1998).

Amino acids 1-9 of the M2e protein are highly conserved across AIV strains, while minimal aa variation is observed for residues 10 to 24, making it an attractive target for AIV universal vaccine development (Black et al. 1993; De Filette et al. 2005; Fiers et al. 2009; Gerhard, Mozdzanowska & Zharikova 2006; Ito et al. 1991; Khurana et al. 2009; Lamb, Zabedee & Richardson 1985; Liu, Li & Chen 2003; Neirynck et al. 1999; Pejoski et al. 2010; Zabedee & Lamb 1988). The M2e protein is low in copy number on the virus particle, but it is abundantly expressed on the surface of an infected cells (Lamb & Choppin 1981; Park et al. 1998). This differential epitope density between infected cells (high) and a mature virion (low) (Black et

al. 1993; Zabedee, Richardson & Lamb 1985) is the key feature for its recommendation as a marker for differentiating infected animals in vaccinated population (DIVA), a strategy used in AIV surveillance (Kim et al. 2010; Lambrecht et al. 2007).

The sensitivity and specificity of M2e-based DIVA have been demonstrated in our previous works (Hadifar et al. 2014; Hemmatzadeh et al. 2013; Tarigan et al. 2015). This raised our interest towards the potential use of M2e in a competitive enzyme-linked immunosorbent assay (ELISA) format as a surveillance tool for AIV infection. The principle of competitive ELISA lies in the ability of the test subject antibody (e.g. chicken) to inhibit competitor antibodies, usually produced in rabbit or mouse, from binding to the target antigen. Hence, it is important for the competitor antibodies to react with the same viral epitopes as the antibodies produced by the test species. Such an ELISA format has been successfully demonstrated for the nucleoprotein of AIV, which has been proven to be reliable and applicable for multispecies surveillance (Shafer, Katz & Eernisse 1998; Starick et al. 2006; Zhou et al. 1998). However, M2e-based competitive ELISA is a better alternative DIVA test for an AIV surveillance tool, especially in the highly pathogenic AIV H5N1 'endemic' countries, where poultry vaccination using inactivated AIV is practiced.

It is accepted that due to differences in the germline gene repertoire in different species, accompanied by distinct mechanisms for generation and affinity maturation of antibodies, antigenic determinants recognized by a host can vary from one species to another (Darnule et al. 1980; Finlay & Almagro 2012; Rotter et al. 1983). Earlier studies on M2e protein for vaccine development have reported several antigenic determinants identified by anti-M2e antibodies isolated from rabbit, mouse and human (Grandea III et al. 2010; Pejoski et al. 2010; Wang et al. 2009). In most cases, the M2e epitopes recognized were located in the region that span from the N-terminal to the middle region of M2e, and vary in length from 5 residues (^{2}SLLTE^{6}) (Grandea III et al. 2010), up to 15 residues ^{2}SLLTEVETPIRNEWG^{16}

(Pejoski et al. 2010; Wang et al. 2009). Here, we describe epitope mapping using anti-M2e antibodies from chicken, mouse and rabbit to identify the M2e antigenic determinants for each antibody group, and to assess the most suitable animal source of anti-M2e antibodies in M2e-based competitive ELISA as an advanced DIVA test for H5N1 infections in poultry.

3.2 Material & Methods

3.2.1 Peptides for mouse and rabbit immunization and antigenic mapping

Peptide immunization for mouse and rabbit was done using the 17 amino acid (aa) M2e peptide ($M2e_{2-18}$), corresponding to residues 2 to 18 of HPAIV H5N1 Indonesian strain A/Chicken/West Java/PWT-WIJ/2006, (^{2}SLLTEVETPTRNEWECK^{18}) (Hadifar et al. 2014; Hemmatzadeh et al. 2013; Tarigan et al. 2015). It was conjugated with Keyhole Limpet Hemocyanin (M2e-KLH) at the C-terminal end for the anti-M2e antibodies production in mice (Abmart, Shanghai, China) and rabbits (Peptide 2, Chantilly, Virginia, USA).

M2e-mapping was done using two sets of overlapping short peptides spanning $M2e_{2-24}$. Set 1 included eight peptides of 9-10 aa length (WatsonBio, Houston, Texas) with two aa offsets each; while set 2 included three peptides of 14 aa length (Abmart, Shanghai, China) with three aa offsets each (Table 3.1). $M2e_{2-18}$ was used for anti-M2e antibodies screening in indirect ELISA, as well as the positive antigen control in mapping ELISA, instead of $M2e_{2-24}$, as both showed similar reactivity in previous study (Hemmatzadeh et al. 2013). All peptides used were of >90% purity as determined by high performance liquid chromatography analyses.

Table 3.1 Overlapping peptides covering the full length H5N1 M2e protein ($M2e_{2-24}$), designed with 10 amino acid (aa) with 2 offsets, and 14 aa with 3 offsets each. Peptide $M2e_{2-18}$ was used as a control antigen in place of $M2e_{2-24}$.

Peptide designation	Peptide sequence	Peptide length
M2e 2-11	^{2}SLLTEVETPT^{11}	9-10 aa
M2e 4-13	^{4}LTEVETPTRN^{13}	
M2e 6-15	^{6}EVETPTRNEW^{15}	
M2e 8-17	^{8}ETPTRNEWEC^{17}	
M2e 10-19	^{10}PTRNEWECKC^{19}	
M2e 12-21	^{12}RNEWECKCSD^{21}	
M2e 14-23	^{14}EWECKCSDSS^{23}	
M2e 16-24	^{16}ECKCSDSSD^{24}	
M2e 5-18	^{5}TEVETPTRNEWECK^{18}	14 aa
M2e 8-21	^{8}ETPTRNEWECKCSD^{21}	
M2e 11-24	^{11}TRNEWECKCSDSSD^{24}	
M2e 2-18	^{2}SLLTEVETPTRNEWECK^{18}	17 aa

3.2.2 **Antibodies (sera)**

Three different sources of anti-M2e antibodies were used in this study, namely chicken polyclonal antibodies (cAbs), mouse monoclonal antibodies (mAbs), and rabbit polyclonal antibodies (rAbs) (Table 3.2). CAbs were produced as described previously (Hadifar et al. 2014; Tarigan et al. 2015). In brief, commercial layer chicks were inoculated with inactivated H5N1 AI vaccine (Medivac-AI, PT Medion, Bandung, Indonesia), once (16 weeks of age), twice (12 and 16 weeks of age) or three times (8, 12 and 16 weeks of age). All chicks were challenged with live H5N1 strain (either A/Ck/West Java/PWT-WIJ/2006, or A/Ck/West Java/Sbg-29/2007) two weeks after the last vaccination. All challenge experiments were conducted in the Biosecurity level 3 (BSL3) facilities at the Indonesian Research Centre for

Veterinary Science, Bogor, Indonesia. Collected sera were tested for M2e reactivity using indirect M2e ELISA (Hadifar et al. 2014; Hemmatzadeh et al. 2013). Reference H5N1 sera (A/Chicken/Scotland/59) was obtained from the Veterinary Laboratory Agency (New Haw, Addlestone, UK) as described previously (Hemmatzadeh et al. 2013).

Hybridoma cells producing anti-M2e mAbs were produced by Abmart (Shanghai, China) following immunization of mice with $M2e_{(2-19)}$-KLH peptide. Briefly, six female BALB/c mice were injected subcutaneously at multiple sites with an emulsion contained 0.05 mg KLH-M2e peptide mixed with complete Freund's adjuvant (CFA). Immunization was done four times 14 days apart. Booster injections were given 14 days after last immunization with 0.05 mg KLH-M2e peptide in incomplete Freund's adjuvant (IFA). Serum sampling was done seven days after the third and fourth immunization and sera tested for anti-M2e antibodies using indirect M2e-ELISA. Fusion of myeloma cells and spleen cells was followed by another indirect M2e-ELISA screening. Selected clones of hybridoma cells were expanded and grown in Dulbecco's modified Eagles medium (DMEM) high glucose with L-glutamine (HyClone, GE Healthcare) with 15% foetal bovine serum (HyClone, GE Healthcare) and 1% (v/v) penicillin and streptomycin (Gibco, Thermofisher Scientific). MAb supernatants from cell culture were column purified using Pierce Recombinant Protein A Agarose (Thermofisher Scientific). No significant difference was observed between the column purified and precipitated mAb in indirect ELISA. Thus, for the experiments described here, the mAb supernatants were precipitated using 50% saturated solution of ammonium sulphate and the protein pelleted was resuspended in sterile phosphate saline buffer (PBS) and stored at -20°C until required.

Eight New Zealand White rabbits with the average age of 6 months were chosen to obtain hyperimmune serum against the M2e peptide. Rabbits were inoculated at five subcutaneous sites with an emulsion that contained 0.1 mg of KLH-M2e peptide mixed with CFA. The

rabbits received booster injections containing 0.1 mg KLH-M2e peptide in IFA at day 14 and 28. Blood was collected two weeks after the final immunization and antisera tested using indirect M2e-ELISA.

Table 3.2 Antibody types and animals used for the generation of antibodies either by H5N1 virus challenge, or KLH-M2e$_{2\text{-}19}$ peptide immunization.

Antibody type	Antibody designation	Immunogen
Chicken polyclonal antibodies	PL64 PL80	A/Ck/West Java/PWT-WIJ/2006
	2D10 2B2 2B47 2A17	A/Ck/West Java/Sbg-29/2007: MSLLTEVETPTRNEWECKCIDSSD
	Reference H5N1 sera	A/Chicken/Scotland/59
Mouse monoclonal antibodies	1N5 2D16 2E14 2G14 3D23 3H4	M2e$_{2\text{-}19}$ peptide: SLLTEVETPTRNEWECKC-KLH
Rabbit polyclonal antibodies	rAb-1 rAb-2 rAb-3 rAb-4 rAb-5 rAb-6 rAb-7 rAb-8	

3.2.3 **Indirect M2e-ELISA and antigenic mapping**

All peptides were dissolved in diethylpyrocarbonate (DEPC)-treated water (Bioline) to a final concentration of 1 mg/ml. Peptides were diluted with 0.1 M carbonate-bicarbonate buffer, pH 9.6 (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$) to the final concentration of 10 µg/ml, and 100 µl was added to each well of a 96-well flat bottom microtiter plate (Maxisorp, NUNC) and incubated at 4°C overnight. The coated plates were washed five times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 2% BSA in PBS (200 µl/ well) for 2 hrs at room temperature (RT). The chicken test sera were diluted with the high salt dilution buffer (HS-DB: 0.1 M Tris pH 7.4, 0.5 M NaCl, 1 mM Na_2EDTA, 2% w/v BSA, 3% w/v Triton X-100, 3% w/v Tween 20) (Hadifar et al. 2014; Hemmatzadeh et al. 2013), and mouse and rabbit sera were diluted in PBS containing 1% BSA and 0.05% Tween 20 (PBS-BSA-T) with the dilution of 1:100 for all sera. The blocked plates were washed for five times with PBS-T before the diluted serum was added into wells containing each peptide (100 µl/well). After 1 hr of incubation at RT, the plates were subjected to another five rounds of washing. Species-specific antibodies conjugated with horseradish peroxidase (HRP) enzymes were prepared by dilution of anti-chicken HRP with HS-DB, and anti-mouse HRP (Sigma) and anti-rabbit HRP were diluted with PBS-BSA-T. Diluted secondary antibodies were added to each well (100 µl/well), followed by 1 hr incubation at RT. After washing, the substrate solution [100 µg/ml of tetramethylbenzidine substrate (TMB) (Sigma, St Louis, MO, USA)] in citrate buffer (pH 8) containing hydrogen peroxide (100 µl of 0.6% H_2O_2) was added (100 µl/ well) and incubated at RT for 5 – 20 minutes before the reaction development was stopped with stop buffer (1 M sulphuric acid) (50 µl/ well). The optical density (OD) of each well was determined at OD 450 nm using the BioRad Benchmark Plus Microplate Reader (BioRad, Hercules, USA).

3.2.4 **Statistical and bioinformatics analyses**

Each antigenic peptide was tested in three dilutions with two replicas each. A range of univariate and multivariate analyses were employed in this study as previously described (Ebrahimi et al. 2014), using MINITAB 17 statistical package (*Minitab 17 Statistical Software* 2010). The mean OD_{450} values for the antigen negative wells were subtracted from the mean OD_{450} values of antigen positive wells to get the corrected OD_{450} values. One-way ANOVA and pair-wise mean comparison by Tukey test was used to compare the corrected ELISA values of different antigenic peptides within each type of antibody (chicken, mouse, and rabbit). Antibody reactivity to the M2e peptides was considered as strong (>1.00), medium (0.50-1.00), weak (0.25-0.50) and negative (<0.20) in reference to its OD_{450} value.

Clustering based on Average Linkage algorithm was used to illustrate the similarities/differences between different peptides in reaction with each type of antibody. The same method was used to cluster antibodies against antigenic peptides. Principle Component Analysis (PCA) was used to find the groups of antibody response against antigenic peptides. Also, PCA analysis is a powerful multivariate test which is able to find the differentiating factors in biological characteristics (features) (Mahdi et al. 2014; Zinati et al. 2014). This test was used to identify the antigens that showed discriminating reactivity between mouse, chicken, and rabbit antibodies. Hydrophobicity plot of M2e protein (aa 2-24) was constructed using the BioEdit software (North Carolina State University) and CLC Genomics (QIAGEN) (Hall 1999).

3.2.5 **Ethics statement**

Animal work carried out at the Indonesian Research Centre for Veterinary Science, Bogor, Indonesia was approved by the Research Committee of Indonesian Research Centre for Veterinary Science. The experimental chickens were handled by an expert veterinarian in animal studies based on the guidelines of the National Health and Medical Research Council of

Australia. The animals were checked daily for clinical signs, morbidity, and mortality. All chickens were bled via brachial vein and by cardiac puncture at the terminal step just after CO_2 euthanasia.

3.3 Results

3.3.1 Chicken, mouse and rabbit antibodies selection using indirect-M2e ELISA

Positive anti-M2e cAbs were selected based on findings from previous reports (Hadifar et al. 2014; Hemmatzadeh et al. 2013), where end-point HI antibody titers for all cAbs were approximately 1:512 dilutions (data not shown). Meanwhile, positive anti-M2e mAbs and rAbs showed ELISA titers between 1:1600 to 1:3200, and 1:800 to 1:1600, respectively. As expected, comparison of mean OD_{450} readings for chicken, mouse and most rabbit antibodies showed strong (OD_{450} >1.0) reactivity to the $M2e_{2-18}$ (Table 3.3). All results for statistical analysis can be found in the supplementary data.

3.3.2 Chicken sera recognized at least 2 different epitopes spanning M2e residue 5-18 and 10-17

M2e mapping ELISA results revealed a distinctive reactivity pattern between the chicken sera exposed to the A/Ck/West Java/Sbg-29/2007 (Sbg-29/2007) (*n* = 4) and A/Ck/West Java/PWT-WIJ/2006 (PWT/2006) (*n* = 2). Anti-M2e sera from chickens exposed to Sbg-29/2007 (2A17, 2B2, 2B47 and 2D10) showed a range of medium to strong reactivity to $M2e_{8-21}$, strong reactivity to $M2e_{5-18}$ and weak to strong reactivity to $M2e_{8-17}$ (Table 3.3). With the exception of cAb 2B47, Sbg-29/2007 antisera also showed a range of weak to strong reactivity to $M2e_{10-19}$. Non-reactivity of cAb 2B47 to $M2e_{10-19}$ was not fully understood, but this particular cAb was only reactive to peptides which included residues E8 and T9 (Figure 3.1 and 3.2). Collectively, Sbg-29/2007 antisera showed reactivity to peptides which shared a minimum of eight residues (^{10}PTRNEWEC^{17}) of the M2e (Figure 3.2).

While Sbg-29/2007 antisera were reactive to peptides with 10 residues ($M2e_{8-17}$ and $M2e_{10-19}$), as well as 14 residues ($M2e_{5-18}$ and $M2e_{8-21}$), chicken antisera to PWT/2006 (PL64 and PL80) were only reactive to the 14 residues $M2e_{5-18}$ (Table 3.3). Despite $M2e_{5-18}$ sharing residues with the whole $M2e_{6-15}$ and $M2e_{8-17}$, and most residues in $M2e_{4-13}$ and $M2e_{10-19}$, neither of the PWT/2006 antisera reacted to any of these shorter peptides. This suggested that these 10-residue peptides were inadequate to represent the PWT/2006-strain epitope which elicited antibody responses in the chickens.

Although the reference H5N1 serum (produced against A/chick/Scotland/59 strain) was commercially generated based on its hemagglutinin inhibition titer, it showed strong reactivity to peptide $M2e_{2-18}$ (mean OD_{450} 2.02) (Table 3.3). However, no reactivity was observed between the reference sera and any of the mapping peptides. Alignment of the peptides recognized by the chicken sera showed that at least two epitopes, in addition to the immunogen, were recognized, namely $M2e_{5-18}$ (^{5}TEVETPTRNEWECK^{18}) and $M2e_{10-18}$ (^{10}PTRNEWECK^{18}) (Table 3.3, Figure 3.3). Both epitopes contained residues M2e 10-17, which are recognised by all cAbs and which correspond to the most hydrophilic part of the M2e protein (residues 12 to 20) (Figure 3.3).

Table 3.3 Mean OD_{450} readings for chicken ([a, b, c]), mouse ([d]) and rabbit ([e]) antibodies reactivity to the M2e peptide.

Antibody	OD450 on Peptide																					
	M2e 2-18		M2e11-24		M2e8-21		M2e5-18		M2e16-24		M2e14-23		M2e12-21		M2e10-19		M2e8-17		M2e6-15		M2e4-13		M2e2-11	
2A17[a]	2.11	✓✓✓	0.02	-	0.92	✓✓	1.66	✓✓✓	0.04	-	0.05	-	0.04	-	0.81	✓✓	0.87	✓✓	0.17	-	0.03	-	0.06	-
2B2[a]	2.07	✓✓✓	0.01	-	0.58	✓✓	1.51	✓✓✓	0.02	-	0.04	-	0.05	-	0.26	✓	0.35	✓	0.20	-	-0.01	-	-0.01	-
2B47[a]	1.85	✓✓✓	-0.22	-	1.20	✓✓✓	1.32	✓✓✓	-0.08	-	-0.27	-	-0.12	-	-0.20	-	1.13	✓✓✓	-0.11	-	-0.21	-	-0.13	-
2D10[a]	2.33	✓✓✓	0.04	-	2.14	✓✓✓	2.14	✓✓✓	0.10	-	0.10	-	0.14	-	2.02	✓✓✓	2.24	✓✓✓	0.17	-	0.09	-	0.05	-
PL64[b]	2.29	✓✓✓	0.04	-	0.01	-	0.76	✓✓	0.08	-	0.08	-	0.14	-	0.02	-	0.10	-	0.10	-	0.07	-	0.10	-
PL80[b]	2.34	✓✓✓	0.10	-	-0.05	-	1.13	✓✓✓	0.16	-	0.02	-	0.07	-	-0.02	-	0.08	-	0.13	-	0.01	-	0.04	-
Reference H5N1[c]	2.02	✓✓✓	-0.05	-	-0.01	-	-0.03	-	0.03	-	0.03	-	0.00	-	0.00	-	0.02	-	0.01	-	0.01	-	0.04	-
1N5[d]	2.63	✓✓✓	0.01	-	0.02	-	0.01	-	0.00	-	0.01	-	0.02	-	0.03	-	0.03	-	0.01	-	0.00	-	0.00	-
2D16[d]	3.30	✓✓✓	0.02	-	0.01	-	0.01	-	0.00	-	0.02	-	0.03	-	0.01	-	0.03	-	0.00	-	-0.01	-	-0.01	-
2E14[d]	2.62	✓✓✓	0.35	✓	0.13	-	0.02	-	0.00	-	0.00	-	0.10	-	0.54	✓✓	0.01	-	0.01	-	0.00	-	0.00	-
2G14[d]	2.26	✓✓✓	0.01	-	0.00	-	0.05	-	0.00	-	0.00	-	0.00	-	0.01	-	0.01	-	0.03	-	0.00	-	0.03	-
3D23[d]	1.69	✓✓✓	0.04	-	0.02	-	0.09	-	0.02	-	0.02	-	0.03	-	0.02	-	0.08	-	0.06	-	0.01	-	0.01	-
3H4[d]	2.58	✓✓✓	0.01	-	0.01	-	0.02	-	0.00	-	0.01	-	0.02	-	0.01	-	0.02	-	0.03	-	0.00	-	-0.01	-
Rab-1[e]	1.86	✓✓✓	-0.10	-	0.08	-	1.82	✓✓✓	-0.13	-	-0.19	-	-0.14	-	-0.13	-	0.13	-	1.46	✓✓✓	1.31	✓✓✓	-0.04	-
Rab-2[e]	0.49	✓	-0.48	-	-0.34	-	0.38	✓	-0.53	-	-0.53	-	-0.52	-	-0.52	-	-0.45	-	0.42	✓	0.24	✓	-0.40	-
Rab-3[e]	1.64	✓✓✓	-0.16	-	0.01	-	1.46	✓✓✓	-0.25	-	-0.25	-	-0.23	-	-0.25	-	-0.04	-	1.27	✓✓✓	1.13	✓✓✓	-0.10	-

Continued

Table 3.3

Antibody	OD450 on Peptide																	
	M2e $_{2-18}$		**M2e$_{11-24}$**		**M2e$_{8-21}$**		**M2e$_{5-18}$**		**M2e$_{16-24}$**		**M2e$_{14-23}$**		**M2e$_{12-21}$**		**M2e$_{10-19}$**		**M2e$_{8-17}$**		**M2e$_{6-15}$**		**M2e$_{4-13}$**		**M2e$_{2-11}$**	
Rab-4[e]	0.68	✓✓	-0.43	-	-0.28	-	0.55	✓✓	-0.46	-	-0.46	-	-0.45	-	-0.45	-	-0.35	-	0.60	✓✓	0.39	✓	-0.34	-
Rab-5[e]	1.26	✓✓✓	-0.20	-	-0.04	-	1.45	✓✓✓	-0.22	-	-0.22	-	-0.20	-	-0.20	-	-0.02	-	1.14	✓✓✓	1.01	✓✓✓	-0.14	-
Rab-6[e]	1.41	✓✓✓	-0.26	-	-0.01	-	1.76	✓✓✓	-0.30	-	-0.30	-	-0.28	-	-0.28	-	-0.13	-	0.94	✓✓	1.44	✓✓✓	-0.12	-
Rab-7[e]	1.46	✓✓✓	-0.27	-	-0.03	-	1.25	✓✓✓	-0.31	-	-0.30	-	-0.29	-	-0.29	-	-0.14	-	1.34	✓✓✓	1.01	✓✓✓	-0.13	-
Rab-8[e]	0.68	✓✓	-0.43	-	-0.28	-	0.55	✓✓	-0.46	-	-0.46	-	-0.45	-	-0.45	-	-0.35	-	0.60	✓✓	0.39	✓	-0.34	-

For statistical analysis, please refer to Supplementary (Appendix).

[a]Chickens exposed to A/Ck/West Java/Sbg-29/2007

[b]Chickens exposed to A/Ck/West Java/Sbg-29/2007

[c]Chickens exposed to A/chick/Scotland/59

[d]Mice immunised with KLH-M2e$_{2-19}$

[e]Rabbits immunised with KLH-M2e$_{2-19}$

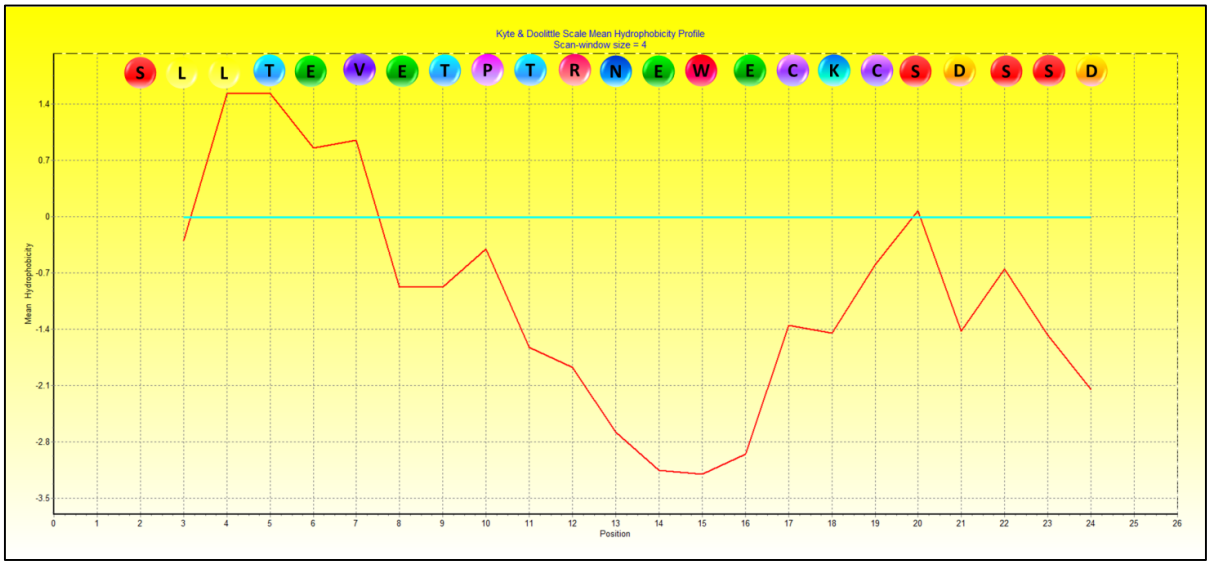

Figure 3.1 Hydrophobicity plot of M2e protein sequence (residue 2 to 24) based on Kyte & Doolittle scale mean of hydrophobicity profile in BioEdit.

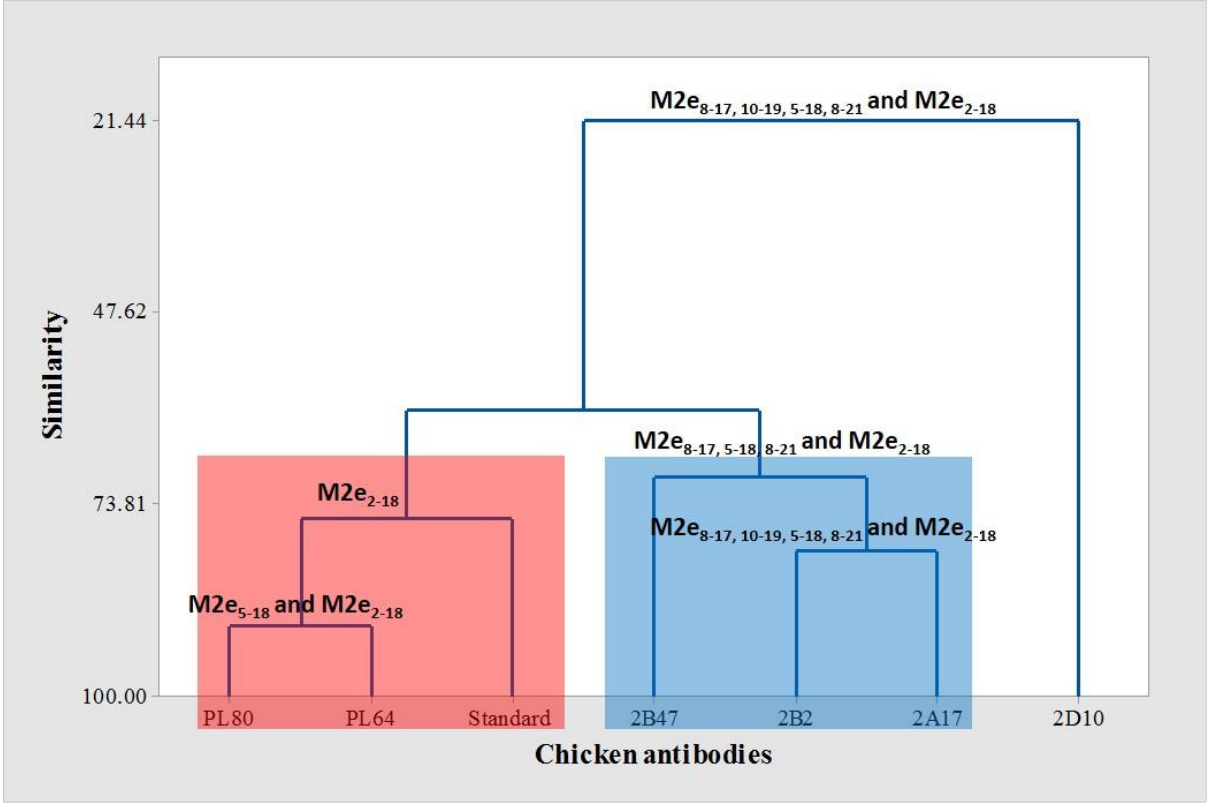

Figure 3.2 Clustering based on average linkage algorithm illustrates the similarity of chicken antibodies reactivity to the M2e peptides as indicated on the nodes of each group. Left to right: Cluster 1 (red box) chicken sera which reacted with $M2e_{5\text{-}18}$ and $M2e_{2\text{-}18}$; Cluster 2 (blue box) chicken sera which reacted with $M2e_{8\text{-}17,\ 10\text{-}19,\ 5\text{-}18,\ 8\text{-}21}$ and $M2e_{2\text{-}18}$; and 2D10 chicken serum which reacted with $M2e_{8\text{-}17,\ 10\text{-}19,\ 5\text{-}18,\ 8\text{-}21}$ and $M2e_{2\text{-}18}$.

3.3.3 Chicken sera reactivity pattern is highly influenced by its immunogen as well as individual chicken immune response

Clustering analysis of chicken antisera based on their reactivity with M2e peptides revealed two major clusters broadly related to the antigen used to immunise the donor chickens (Figure 3.3). Cluster 1 grouped Sbg-29/2007 antisera together, particularly 2B2, 2A17 and 2B47, based on their reactivity to $M2e_{8\text{-}17,\ 10\text{-}19,\ 5\text{-}18,\ 8\text{-}21}$ and $M2e_{2\text{-}18}$; while cluster 2 grouped PWT/2006 antisera (PL64 and PL80), based on their reactivity to $M2e_{5\text{-}18}$ and $M2e_{2\text{-}18}$, along with the reference H5N1 sera (produced against A/chick/Scotland/59) which only reacted to peptide $M2e_{2\text{-}18}$.

Although cAb2D produced against the Sbg-29/2007 strain shared a similar reactivity pattern with cAbs 2B2 and 2A17 ($M2e_{8\text{-}17,\ 10\text{-}19,\ 5\text{-}18,\ 8\text{-}21}$ and $M2e_{2\text{-}18}$), clustering analysis recognized cAb 2D10 sera as the least similar to the other sera. Observation of its OD_{450} readings showed that cAb 2D10 reacted strongly with all five peptides (OD_{450} 2.02 – 2.33) (Table 3.3) which was not observed with the other sera. And uniquely this sera also had high anti-M2e antibodies titre (1:10,240).

3.3.4 Mouse monoclonal antibodies recognized epitopes $M2e_{2\text{-}18}$ and $M2e_{11\text{-}18}$ while rabbit polyclonal antibodies recognized epitope $M2e_{6\text{-}13}$

M2e comparative mapping by mAbs showed minimal variability in their reactivity patterns. While all six mAbs strongly reacted with peptide $M2e_{2\text{-}18}$ (OD_{450} 1.69 – 3.30), only mAb 2E14 showed a weak and medium reactivity to $M2e_{10\text{-}19}$ and $M2e_{11\text{-}24}$, respectively (Table 3.3). Together, mAbs recognized an M2e epitope containing a minimum of eight residues (^{11}TRNEWECK^{18}) to 17 residues (^{2}SLLTEVETPTRNEWECK^{18}), in which the epitopes mostly overlapped with the epitope recognized by cAbs described above (Figure 3.3).

Apart from the similar strong reactivity observed for peptide $M2e_{2\text{-}18}$ (OD_{450} 1.73), rAbs also demonstrated strong reactivity to $M2e_{4\text{-}13,}$ $M2e_{6\text{-}15}$ and $M2e_{5\text{-}18}$ (Table 3.3), a combination

which was not demonstrated in the previous two groups of antibodies. All these peptides shared residues ^{6}EVETPTRN^{13} which indicated that the epitope recognized by rabbit was different from the chicken and mouse antibodies.

Comparison of the M2e epitopes recognized for all three groups of antibodies clearly showed that the chicken, mouse and rabbit sera recognized five epitopes, namely M2e residues 2-18 for all antibodies, with specifically M2e residues 5-18 and 10-17 recognized by the cAbs, M2e residues 11-18 recognized by one mAb, and M2e residues 6-13 by the rAbs (Figure 3.3). The shorter epitopes represented by the different antibodies group was recognized on two different sites of the M2e protein. cAbs and mAbs antibodies recognized epitopes located at the central region of the M2e protein (^{10}PTRNEWECK^{18}), while the rAb antibodies recognized an epitope located at the N-terminal of the M2e protein (^{6}EVETPTRN^{13}) (Figure 3.3).

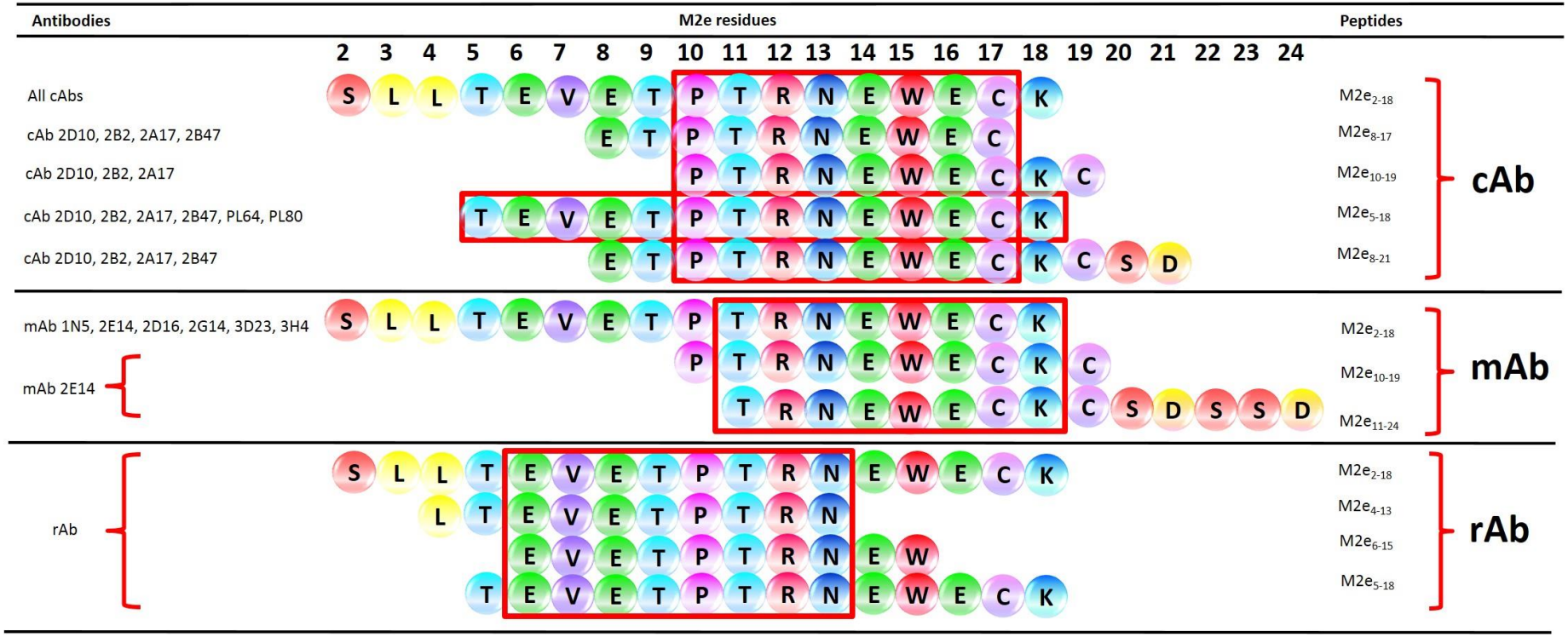

Figure 3.3 The antigenic determinants of M2e protein recognized by chicken, mouse and rabbit antibodies highlighted with the red boxes. In the order from top to bottom, chicken antibodies to Sbg-29/2007 strain that recognized peptides containing residues ^{10}PTRNEWEC^{17}; chicken antibodies to PWT/2006 strain recognized peptides with residues ^{5}TEVETPTRNEWECK^{18}; mouse monoclonal antibodies recognized peptides with residues ^{11}TRNEWECK^{18} and rabbit antibodies recognized peptides with residues ^{6}EVETPTRN^{13}. Tested antibodies are listed on the left, while the peptides corresponding to the residues recognized by each group are indicated on the right.

cAb: chicken antibodies

mAb: mouse antibodies

rAb: rabbit antibodies

3.4 Discussion

Based on our previous success in demonstrating the effective use of M2e protein as a target for DIVA strategy, we attempted to develop a competitive ELISA test targeting the M2e protein. This test was anticipated to possess a broad host species applicability which is capable of DIVA for a simple yet effective AIV surveillance tool in domestic poultry. We have here described the comparative mapping of anti-M2e antibodies from chickens, mice and rabbits. Our findings revealed the occurrence of two separate epitopes on the M2e protein, where one epitope was exclusively recognized by the rAbs antibodies, while the other was recognized by both mAb and cAbs. It is important to note that for development of a competitive ELISA, the test and competitor antibodies need to cross-react with the same, or at least similar epitope, within the same antigen. Such is the case where cAbs are the test antibodies, while mAbs but not rAbs are the potential competitors.

Despite the difference in the immunogen used for anti-M2e antibody production in mice and rabbits (KLH-conjugated peptide) versus chickens (H5N1 live virus), our findings that the five M2e epitopes within the sequence ^{2}SLLTEVETPTRNEWECK^{18} recognised by cAbs, mAbs and rAbs were similar to those of others (De Filette et al. 2011; Fu et al. 2009; Grandea III et al. 2010; Liu, Li & Chen 2003; Liu, Zou & Chen 2004; Pejoski et al. 2010; Wang et al. 2008; Wang et al. 2009; Zabedee & Lamb 1988; Zhang et al. 2006; Zharikova et al. 2005; Zou, Liu & Chen 2005) (Table 3.4). The high frequency of epitope ^{6}EVETPTRN^{13} occurrence in the previous studies suggests that it is likely to be a dominant epitope for M2e protein. Additionally, epitope ^{6}EVETPTRN^{13} is potentially a major epitope for rAbs, whereas a previous study on immunization of rabbits and mice using M2e$_{2\text{-}10}$ ^{2}SLLTEVETP^{10} conjugated with KLH (SP1-KLH) showed to be more immunogenic in rabbits than it was in mice (De Filette et al. 2011).

Minimal variation observed for mAbs was likely due to the double selection using $M2e_{2-19}$-KLH-based ELISA for hybridoma production and final selection. This limited the mAbs reactivity only to the immunogen with low cross reactivity to the other peptides used in the study. Nevertheless, one mAb recognized two other peptides which contain residues M2e 11-18 (Figure 3.3) that overlapped with M2e epitopes recognized for cAbs. Hence, mAb was suggested to be a better competitor in a cELISA-based test for cAbs in contrast to rAbs, as the latter showed fewer overlapping residues (Figure 3.3).

However, it was notable that one mAb and the majority of cAbs showed slight variation in peptide recognition. Although the antigenic determinants recognized by the mAb and cAbs in the current study overlapped with the epitopes found previously (residues 5 to 16 of M2e) (Pejoski et al. 2010; Wang et al. 2008; Zabedee & Lamb 1988; Zhang et al. 2006; Zharikova et al. 2005), they differed in that two of the recognized epitopes (^{10}PTRNEWEC^{17} for cAbs, ^{11}TRNEWECK^{18} for mAb) extended further from the mid-region into the C-terminal end of the M2e protein (Table 3.4). Both were shorter epitopes (8 aa in length) and independent of the N-terminal peptide ($M2e_{2-9}$), with one or two more residues at the epitope C-terminal (C17 and K18) than previously reported epitopes recognized in humans and mice. This suggests that residues ^{2}SLL^{4} is a less important antigenic determinant in chickens and rabbits than it is in humans (Grandea III et al. 2010). Conversely, C17 and K18 may possibly be important residues for cAbs epitope recognition. Importance of K18 for mAb epitope recognition was also suggested by the reported loss of anti-M2e antibody responses following immunization with truncated $M2e_{2-16}$ in a vaccine study in mice (Pejoski et al. 2010). Difference by two to three residues between the M2e epitopes recognized by mAbs has also been described previously (Zhang et al. 2006). Zhang et al. (2006) suggested that such observations could be due to either a true existence of species-related variation in epitope recognition, or difference in assay sensitivity used for epitope recognition, or both (Zhang et al. 2006). Epitope variation was observed in a separate M2e-unrelated study in rabbits using

10 human proteins, where although the epitopes recognized for a single protein were similar, they were not identical (Hjelm et al. 2012). The epitopes recognized by mAbs in the current study represent another species-related variation of the existing recognized M2e epitopes, while this is the first known M2e epitope reported in chickens. Nevertheless, M2e residue C17 and K18 may be of contributing to the antigenic characteristics of M2e.

M2e protein residues S2, T5, E6, P10, I11, E14 and W15 have been identified as critical for antibody interactions (Cho et al. 2015; Grandea III et al. 2010; Huleatt et al. 2008; Tompkins et al. 2007; Wang et al. 2009; Zharikova et al. 2005). Epitope studies have suggested that charged residues (E, K an D), and polar residues (R, N, Q, P and T) are preferred in highly antigenic epitopes (Sun et al. 2011; Tsai et al. 1997), where the hydrophilic amino acids (R, K, N, P, H, D and E) are more prominent (Raghunathan et al. 2011). A recent analysis of the M2e crystal structure complexed with monoclonal antibody has recognized that residues T5, E6, V7, P10, R12 and N13 assist M2e hydrophilic interactions, which contributes to epitope accessibility in antigen-antibody binding (Cho et al. 2015). Amino acid variation at residues P10, E14 and E16 resulted in predicted M2e structural differences between two H5N1 strains, Vietnam/1194/04 and Hong Kong/156/97 (Leung et al. 2015). The latter H5N1 strain showed a folded hairpin structure that limits antigen recognition in comparison to a relatively more accessible structure observed in the former. M2e protein sequence is not available for PWT/2006 strain used in current study. The M2e amino acid sequence of A/chick/Scotland/59 (EMBL accession number CY015082) and A/Ck/West Java/Sbg-29/2007 (H5N1) (GenBank accession number AKI82362.1) only differs by residue E14G for Scotland/59, and K18C for both from the M2e A/Vietnam/1194/04, hence a similar ‘open’ structure is likely for the Sbg-29/2007 M2e protein.

It is noted that antibodies from chickens exposed to two different strains of H5N1 in current study recognized two dominant but overlapping epitopes on the M2e protein. Differences

observed may be related to the M2e membrane-bound protein conformation of these two H5N1 strains. Factors such as degree of protein protrusion from membrane surface (Thornton et al. 1986), as well as its accessibility for binding activities (Novotny et al. 1986) highly influence the whole presentation of the protein to the birds immune system. Reactivity with only the 14 aa mapping peptide ($M2e_{5\text{-}18}$, ^{5}TEVETPTRNEWECK^{18}) observed for sera PWT/2006 may be related to the structural element formed by the protein on the virus particle. Previous study on the human tryptophanyl-tRNA synthetase epitopes using 10 aa and 15 aa peptides has demonstrated similar observations (Hjelm et al. 2010). It was suggested that the 10 aa peptides ($M2e_{4\text{-}13,\ 6\text{-}15,\ 8\text{-}17\ \text{and}\ 10\text{-}19}$) were not sufficient to imitate the functional structure of the epitope since it is located in a loop structure partially characterized by an α-helix. In the case of the M2e protein, its three-dimensional structure showed a compact U-shaped conformation, where a β-turn structure is adopted by residues T5 to E8, and 3_{10} helix from residues I11 to W15 (Cho et al. 2015). Hence, it was likely that although the two epitopes residues overlap, the PWT/2006 sera were only reactive to the 14 aa peptide $M2e_{5\text{-}18}$ due to the lack of complete residue for a functional epitope formed by the 10 aa peptides.

Difference in length of recognized epitopes in anti-M2e cAbs may be related to the different degree of virus virulence between the H5N1 strains and individual chicken immune responses. Strong reactivity to the M2e peptides observed for the 2D chick sera in current study was reasoned to be due to the double boosts vaccination using killed virus, followed by a live virus challenge. Current findings revealed that the Sbg-29/2007 antisera were capable of recognising shorter epitopes in comparison to the PWT/2006 strain. Slight differences in signal intensity for each identified peptide for Sbg-29/2007 antisera were also noted in relation to the number of vaccinations for each individual birds. Previous study on epitope patterns in rabbit's parallel immunizations with a single antigen showed that polyclonal response in individual animal may differs in their affinities (Hjelm et al. 2012). Also, the difference in the immunogen used was implicated in the lack of response to the mapping

peptides observed for the reference H5N1 sera. Temporal and spatial distant origin of the strain used for immunisation (Scotland/59) from the strain used as the basis for the mapping peptide design (PWT-WIJ//2006) has likely influenced this particular cAb reactivity.

Table 3.4 Summary of epitopes recognized on influenza A virus M2e protein by different antibodies.

Antibody type and designation	Antibody source	Immunogen	Epitope sequence (Identifying Antibody)	Residue length	References
Polyclonal (AS1, AS2, AS3, AS4)	Rabbit	Fusion-M2e (BSA)	^{2}SLLTEVETPIR^{12}	11	(Liu, Li & Chen 2003)
Monoclonal (8C6, 1B12)	Mice	Fusion-M2e (GST)	^{6}EVETPIRN^{13} ^{2}SLLTEVETPIRNEW^{15}	8 14	(Liu, Zou & Chen 2004; Zharikova et al. 2005; Zou, Liu & Chen 2005)
Monoclonal	Mice	Live virus & synthetic peptide	^{4}LTEVETPIRNEWG^{16}	13	(Zhang et al. 2006)
Monoclonal (L66, N547, Z3G1, C40G1, 14C2)	Human (λ HAC or KM™ mice)	Fusion-M2e (BSA)	^{2}SLLTEVETPIRNEWG^{16} (L66) ^{3}LLTEVETPIRNEWG^{16} (N547) ^{3}LLTEVETPIR^{12} (Z3G1) ^{9}TPIRNE^{14} (C40G1) ^{6}EVETPIRNEW^{15} (14C2)	15 14 10 6 10	(Wang et al. 2008; Zabedee & Lamb 1988)
Monoclonal	Mice	Fusion-M2e (BSA)	^{2}SLLTEVET^{9} (M2e8-7) ^{3}LLTEVETPIR^{12} (Z3G1)	8 10	(Wang et al. 2009)
Monoclonal	Mice	Fusion-M2e (BSA)	^{4}LTEVETPIRN^{12} (L18) ^{2}SLLTEVET^{9} (O19) ^{2}SLLTEVETPIRNEWGCRNDSSD^{24} (P6) ^{7}VETPIRN^{13} (S1)	108 23 7	(Fu et al. 2009)
Polyclonal	Mice		^{2}SLLTEVETPIRNEWG^{16}	15	(Pejoski et al. 2010)
Monoclonal	Human		^{2}SLLTE^{6} (TCN-031, TCN-032)	5	(Grandea III et al. 2010)
	Mice	Fusion-M2e (KLH)	^{2}SLLTEVETP^{10}	9	(De Filette et al. 2011)
Polyclonal & monoclonal	Chicken, mice, rabbit	Live virus & fusion-M2e (KLH)	^{5}TEVETPTRNEWECK^{18} (cAbs) ^{10}PTRNEWEC^{17} (cAbs) ^{2}SLLTEVETPTRNEWECK^{18} (cAbs, mAbs, rAbs) ^{11}TRNEWECK^{18} (mAb) ^{6}EVETPTRN^{13} (rAbs)	14 8 17 8 8	This study

Difference at residue I11T between the current and previous studies corresponded to the human and swine specific M2e sequence in the former (I11) and avian specific M2e sequence in the latter (T11) (Zhou, Zhou & Chen 2012).

Although the relatively limited number of serum samples available for testing in the current study do not represent the complexity of antibody response to M2e protein, nevertheless, the results presented provided information on differences of M2e epitope recognition by mouse, rabbit and chicken antibodies. Identification of antigenic determinants or epitopes of the target protein will enable us to formulate the most suitable source of anti-M2e antibodies for further development.

In summary, we have identified five epitopes spanning residue 2 to 18 of M2e protein for mouse, chicken and rabbit sera with variations in length (8 to 17 aa) from two localities on the M2e protein (N-terminal and mid-region). We also concluded that mouse anti-M2e antibodies are more suitable to be used as a competitor antibodies than the rabbit anti-M2e antibodies for further work on M2e-based competitive ELISA diagnostic test. This was highly suggestive by the overlapping epitopes (^{11}TRNEWEC^{17}) demonstrated by both chicken antibodies and one of the mouse antibodies.

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Chapter 4 Characterization of monoclonal antibodies to the avian influenza virus H5N1 M2e protein and their potential use in a diagnostic competitive ELISA

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Characterization of monoclonal antibodies to the avian influenza virus H5N1 M2e protein and their potential use in a diagnostic competitive ELISA

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Abstract

A rapid and sensitive serodiagnostic tool which effectively discriminates vaccinated from virus infected animals (DIVA) will simplify surveillance of H5N1 in poultry in vaccinating countries. The reliability of an M2e-based indirect ELISA for DIVA application suggested its potential for use in a competitive based ELISA (cELISA) application. Following our recent findings on similar epitope identified by both mouse and chicken anti-M2e antibodies, we investigated the potential use of mouse anti-M2e monoclonal antibodies (mAbs) in an M2e-based cELISA for H5N1 surveillance in poultry. Field sera from 15 known positive (challenged, infected) and 339 negative (145 vaccinated-non-challenged and 194 non-vaccinated-non-challenged) chickens were used to test four mAbs. Results indicated that the use of mAb 3H4 as competitor antibody significantly differentiated between the H5N1 positive (62%-98% inhibition) and negative sera (5.8%-53.0% inhibition) in chicken. Here, we successfully demonstrated the potential use of mouse mAb in an M2e-based cELISA format as an improvement of the available M2e-based indirect ELISA where it removes the needs for species-specific secondary antibodies. Hence, it can be widely used in species other than chicken for H5N1 surveillance in enzootic countries.

Keywords: highly pathogenic avian influenza virus, M2e protein, competitive ELISA, M2e antibodies, mouse monoclonal antibodies, DIVA

4.1 Introductions

Highly pathogenic avian influenza virus (HPAIV) H5N1 is one of the most widespread AIV subtypes, largely due to its rapid rate of evolution which consequently becoming enzootic in several countries (Guan et al. 2002; Hasan et al. 2016; Watanabe et al. 2011). Vaccination is used as a control measure to mitigate further outbreaks in these countries, but emergence of virus variants is of concern due to drift of the field virus from the seed strain used for vaccine development (Bouma et al. 2008; Swayne et al. 2015). In these situations, an effective

surveillance tool is required to ensure early and rapid detection of active AIV infection in poultry populations (Ahmed et al. 2012; Fouchier & Guan 2013; Grund et al. 2011; Wang et al. 2008).

Conventional serologic tests for AIV detection, such as the hemagglutination inhibition (HI) and agar gel immunodiffusion tests, are simple and economic, yet can be labour intensive and time consuming (Jenson 2014; Pedersen 2014). Faster, simpler methods with high throughput options are much preferred, such as real-time PCR-based methods (Dovas et al. 2010; Gall et al. 2008) for detection of active infection, and enzyme-linked immunosorbent assays (ELISA) targeting anti-AIV protein antibodies for detection of prior exposure.

Two of the most commonly available ELISA-based systems are the indirect ELISA (iELISA) and the competitive ELISA (cELISA). An advantage of cELISA is that, unlike iELISA, species-specific secondary antibodies are not required. A cELISA tests the ability of the test antibodies to inhibit the competitor antibodies from binding to a particular antigen. Thus, it is important that the competitor antibody identifies the same epitope as the test species antibodies. A cELISA based on AIV nucleoprotein (NP) (Shafer, Katz & Eernisse 1998; Starick et al. 2006; Zhou et al. 1998) was proven to be reliable as a species-independent assay with comparable or better sensitivity and specificity to the HI assay (Song et al. 2009). This established cELISA as an ideal primary screening tool for AIV infection surveillance (Marche & Van den Berg 2010; Yang et al. 2011).

However, none of the available tests is suitable for detection of AIV infection in AIV (H5N1) vaccinated animals. This is because the tests do not differentiate antibodies raised in response to exposure to live virus infection from those raised in response to killed virus vaccination (reviewed in Pantin-Jackwood and Suarez (2013)). Although cELISA is ideal as an AIV screening tool, an anti-NP-based cELISA is not suitable for infection surveillance in majority

of H5N1 enzootic countries because inactivated virus vaccination is practised in most of these countries (Chen 2009; Marangon, Cecchinato & Capua 2008). Vaccination usually targets HPAIV, while other low pathogenic avian influenza virus circulation within the population is still possible. Hence, a detection system using anti-NP antibodies as a discriminating system has a serious limitation and vaccination complicates result interpretation when testing for infection (James et al. 2008).

Given the simpler and easier approach of cELISA-based tools in comparison to other available diagnostic tools, the current study was conducted to determine the potential value of a cELISA test based on the AIV external domain of matrix 2 (M2e) protein for H5N1 infection serosurveillance. Advantages in targeting this protein are its sensitivity and specificity in differentiating H5N1 virus infected chickens from vaccinated ones (Hadifar et al. 2014; Hemmatzadeh et al. 2013; Kim et al. 2010; Lambrecht et al. 2007; Tarigan et al. 2015). AIV is known to infect various hosts such as swine, horses, dogs, cats and marine mammals, as well as various avian species (Chambers, Dubovi & Donis 2013). The challenge in setting up a cELISA-based test with a broad-hosted pathogen is in ensuring that the competitor antibodies recognized the same antigenic determinants of the target protein across host species, as this has proven to be not always the case (Darnule et al. 1980; Finlay & Almagro 2012; Hasan et al. 2016; Rotter et al. 1983).Our recent investigation indicated that anti-M2e mouse monoclonal antibodies and chicken polyclonal antibodies recognise the same epitope on the M2e antigen (Hasan et al. 2016). Hence, this study aims to identify and validate the potential development of an M2e-based cELISA as a large-scale serosurveillance diagnostic tool for detecting H5N1 infection, especially in H5N1 enzootic countries.

4.2 Material and Methods

4.2.1 M2e peptide as antigen for indirect and competitive ELISA

M2e peptide ($M2e_{2\text{-}18,}$ ^{2}SLLTEVETPTRNEWECKC^{18}) (Abmart, Shanghai, China) based on HPAIV H5N1 M2e protein was used in all ELISAs. Optimum concentration of 10 µg/ml was used for microtitre plate coating as described previously (Hadifar et al. 2014; Hemmatzadeh et al. 2013).

4.2.2 Monoclonal antibody (mAb)

Mouse monoclonal antibodies 1N5, 2D16 and 3H4 were from hybridoma cell lines derived from mice immunised against HPAIV H5N1 M2e protein peptide (aa 2-19) (Abmart, Shanghai, China) (Hemmatzadeh et al. 2013) (Table 4.1). Briefly, six female BALB/c mice were immunized on six (6) occasions by injections at multiple subcutaneous sites. The first five injections were done using 0.05 mg KLH-M2e peptide with complete Freund's adjuvant, 14 days apart.The final immunization was done with 0.05 mg KLH-M2e peptide in incomplete Freund's adjuvant. Serum was collected on day 7 after the fourth immunization to test for its reactivity in an M2e-based ELISA. Hybridoma clones strongly positive for anti-M2e antibody production were selected using indirect M2e ELISA as previously described (Hadifar et al. 2014; Hemmatzadeh et al. 2013). Clones 1N5, 2D16 and 3H4 were further grown in Dulbecco's modified Eagles medium (DMEM) high glucose (Hyclone, GE Healthcare) supplied with 15% foetal bovine serum (Hyclone, GE Healthcare) and 1% (v/v) penicillin streptomycin (Gibco, Thermofisher Scientific). Using sterile techniques, protein precipitated from cell culture supernatants with ammonium sulphate (1:2) and resuspended in 1X phosphate buffered saline (1:2) was used without further purification (Hadifar et al. 2014; Hemmatzadeh et al. 2013). The M2e-ELISA titer was determined for each mAb in 10-steps two-fold dilutions (from 1:10). Protein concentration of mAb solution was quantified by measuring light absorbance at 280 nm on a NanoDrop 1000 Spectrophotometer (Thermofisher Scientific).

4.2.3 **Sera**

Chicken anti-influenza virus antibodies (cAbs) were produced as described previously (Hadifar et al. 2014; Tarigan et al. 2015). Positive sera (H5N1 vaccinated and challenged chickens, *n*=6) were produced by vaccination with inactivated H5N1 AI vaccine (Medivac-AI, PT Medion, Bandung, Indonesia), followed by live H5N1 virus challenge with strain A/Ck/West Java/PWT-WIJ/2006 or A/Ck/West Java/Sbg-29/2007 (Table 4.1). Commercial layer chicks were vaccinated once (16 weeks of age), twice (12 and 16 weeks of age) or three times (8, 12 and 16 weeks of age) and then challenged with live H5N1 two weeks after the last vaccination. Challenge experiments were conducted in the Biosecurity level 3 (BSL3) facilities at the Indonesian Research Centre for Veterinary Science, Bogor, Indonesia. Sera obtained from the challenged birds were tested for anti-M2e antibodies using indirect M2e-ELISA (Table 4.1) (Hadifar et al. 2014; Hemmatzadeh et al. 2013). The M2e-ELISA titer of the known positive sera was determined in 10-steps two-fold dilutions (from 1:40). Control sera were obtained from chicks which were vaccinated with H5N1 AI vaccine (Medivac-AI, PT Medion, Bandung, Indonesia), but not challenged with live H5N1 virus (chicken vaccinated only, *n*=145), and field sera from chicks which were H5N1 vaccinated using unknown sources and untreated (non-H5N1-challenged) (chicken field sera, presumably H5N1 negative, *n*=194). Vaccinated only chicken sera were included to ensure that the test is able to differentiate between H5N1 vaccinated and H5N1 infected sera. Sera from two unvaccinated specific pathogen free (SPF) chicken were also included as negative control (Table 4.1). Additional known positive chicken sera (n=6, Table 4.1) were obtained from Australian Animal Health Laboratory (AAHL), Geelong, as described previously (Wawegama et al. 2016) to be included in the expanded panel cELISA.

Table 4.1 Anti-M2e positive antibodies generated in response to specific immunogens, either H5 virus challenge (chicken antibodies) or M2e peptide (aa 2-24) immunization (mouse monoclonal antibodies). Negative sera were collected from vaccinated field sera and specific-pathogen free chickens (Indonesian Research Centre for Veterinary Science, Bogor, Indonesia).

Antibody source	**Anti-M2e antibodies**	**Antibody designation**	**Number of samples, *n***	**Immunogen**	**Challenge strain**
Chicken	Positive[a, e]	PL64[a], PL80[a]	2	A/Ck/West Java/PWT-WIJ/2006	H5N1
		2B10[a], 2B2[a], 2B47[a], 2D10[a]	4	A/Ck/West Java/Sbg-29/2007	H5N1
		103[e]	1	A/Ck/West Java/Subang/29/2007	H5N1
		104[e]	1	A/Ck/Indonesia/CSLK-EB/2006	H5N1
		105[e]	1	A/Ck/Wates/1/2005	H5N1
		107[e]	1	A/Ck/Myanmar/295/2010	H5N1
		110[e]	1	A/Ck/Myanmar/1001/1/2006	H5N1
		111[e]	1	A/Ck/West Java/SMI-ENDRI2/2006	H5N1
Chicken	Negative	Vaccinated sera[b]	145	H5N1 AI vaccine (Medivac-AI, PT Medion, Bandung, Indonesia)	na
		Field sera[c]	194	na	na
		SPF[d]	1	SPF	H5N1
Mouse	Positive	1N5, 2D16, 3H4	3	$M2e_{2-19}$ peptide: SLLTEVETPTRNEWECKC-KLH	H5N1

na – not available; [a] – positive chicken sera (experimentally challenged); [b] – vaccinated only chicken sera (negative sera); [c] – chicken field sera (test sera); [d] – known negative chicken sera; [e] – H5N1 positive chicken sera from AAHL (experimentally challenged)

4.2.4 **Indirect M2e-ELISA**

96-well flat bottom microtiter plates (Maxisorp, NUNC) were coated overnight at 4°C with M2e peptide diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6 (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$) to a final concentration of 10 µg/ml (100 µl/well). Plates were washed five times with PBS containing 0.05% Tween 20 (PBS-T) and blocked using 1% BSA in PBS, 200 µl/ well, for 2 hrs at room temperature (RT). After five rounds of washing with PBS-T, test serum diluted to the desired concentration with PBS containing 1% BSA and 0.05% Tween 20 (PBS-BSA-T) for mAbs, and high salt dilution buffer (HS-DB: 0.1 M Tris pH 7.4, 0.5 M NaCl, 1 mM Na_2EDTA, 2% w/v BSA, 3% w/v Triton X-100, 3% w/v Tween 20) for the cAbs (Hadifar et al. 2014), was added into the designated wells (100 µl/well). After 1 hr of incubation at RT, the plates were washed five times with PBS-T. Anti-mouse HRP (Sigma), diluted with PBS-BSA-T (1:1000), was added to each well (100 µl/well), followed by a 1 hr incubation at RT. After a final washing step (five rounds), substrate solution (100 µg/ml of tetramethylbenzidine substrate (TMB) (Sigma, St Louis, MO, USA) in citrate buffer (pH 8) containing hydrogen peroxide (100 µl of 0.6% H_2O_2) was added to the plate (100 µl/ well) and incubated at RT for 5-20 minutes. Reaction development was stopped by adding stop buffer (1 M sulphuric acid) (50 µl/ well) and the optical densities (OD) of each well were determined at OD450 nm using an ELISA plate reader (xMark Microplate Absorbance Spectrophotometer, BioRad). Corrected OD was used for the final result and this was obtained by subtraction of blank well OD reading (well with antigen absent, antibody present) from the test well OD reading to remove non-specific background produced by the antibodies (corrected OD = test well OD – blank well OD).

4.2.5 **Development and standardization of competitive ELISA**

Different positive and negative cAbs with known titers in indirect M2e-ELISA were used as standard test sera and three different monoclonal antibodies (1N5, 2D16, 3H4) were used as competitor antibodies to develop the cELISA.

This protocol was based on the blocking ELISA manual developed for AIV nucleoprotein (Selleck 2010) with modification. 96-well flat bottom microtiter plates (Maxisorp, NUNC) were coated with the M2e peptide (final concentration of 10 µg/ml) and washed prior to addition of test sera as described in the section above. Each cAb was diluted 1:10 with HS-DB, 50 µl/well with duplicate wells per dilution and incubated for 1 hr at RT. Without washing, mAb were added to a desired final concentration (two-fold dilutions, from 1:250 to 1:4000) (50 µl/ well) and incubated at RT for another 1 hr. Wells were washed five times with PBS-T before the addition of anti-mouse HRP (Sigma) (1:1000) and incubation for 1 hr at RT. Wells were washed with PBS-T five times prior to development and OD reading as described above. Corrected OD for each well was done as described above, where blank OD were wells with cAb and mAb present, but lack of antigen to remove non-specific reactivity of mAb with cAb if present.

Percentage inhibition (PI) of competitor mAb binding to the antigen in the presence of cAb was calculated according to the formula below (Song et al. 2009), where the OD of wells with a mixture of cAb and mAb (OD inhibited) was expressed as a percentage of the OD of wells with only mAb (OD noninhibited).

$$\mathbf{PI}\% = \mathbf{100} - \left(\frac{\mathbf{OD\ inhibited} \times \mathbf{100}}{\mathbf{OD\ noninhibited}}\right)$$

4.2.6 Statistical analysis

All data analyses were conducted using MedCalc version 16.8 (MedCalc Software, Ostend, Belgium), while figures were produced using GraphPad Prism® version 6.

Efficiency of the current diagnostic assay was evaluated by calculation of its specificity, sensitivity and both positive and negative predictive value (PPV and NPV, respectively) (Florkowski 2008; Parikh et al. 2008; Stojanovic et al. 2014; Walker et al. 2000). Specificity and sensitivity of the current test were calculated as: specificity = (true positive * 100)/(true positive + false negative); sensitivity = (true negative*100)/(true negative + false positive) (Walker et al. 2000). Cut off titre was established using receiver operating characteristics (ROC) where specificity (x-axis) was plotted against sensitivity (y-axis) using MedCalc version 16.8 (Adhikari et al. 2015).

4.3 Results

4.3.1 Optimization of chicken polyclonal and mouse monoclonal antibodies anti-M2e titers for M2e-based competitive ELISA

To determine the titer for anti-M2e cAbs and mAbs to be tested in cELISA, an indirect ELISA using M2e peptide was conducted. OD readings for cAbs showed that cAb PL80 and cAb 2B47 had the highest anti-M2e end titer (1:2560), with cAb PL64 having the lowest end titre (1:640) (Figure 4.1). OD readings for the mAbs revealed that mAb 1N5 had the highest titer (1:1600), followed by mAb 2D16 (1:400) and mAb 3H4 (1:200) (Figure 4.2). To determine the optimum mAb dilution in setting up the cELISA test, cAb PL80 (strong positive) and cAb PL64 (weak positive) were used as samples in the preliminary testing.

4.3.2 Selection of mAb 3H4 as the competitor in M2e-based competitive ELISA

To select the mAb which provided the optimum inhibition for both high and low positive anti-M2e cAbs, a preliminary cELISA was conducted using three mAbs and the selected strong

and weak cAb representatives. Results for mAb selection showed that only mAb 3H4 demonstrated comparable level of mAb binding inhibition (mAb dilution 1:500, 86.2% - 87.6% inhibition) for both high and low positive cAbs (Figure 4.3), hence the selection of mAb 3H4 in cELISA.

4.3.3 **cELISA using mAb 3H4 distinguished infected from vaccinated chicken sera**

To evaluate the ability of mAb 3H4 to identify H5N1 positive test sera, percentage of inhibition (PI) between mAb 3H4 and known positive sera was measured. cELISA with an expanded panel of sera from H5N1 positive cAbs (*n*=12) showed high PI values with an average of 81.5% (range of 62% - 98%, sd=7.88) (Table 4.2, Figure 4.4). This showed that the anti-M2e antibodies produced by the H5N1 challenged chicken sera successfully inhibit the binding of mAb to the M2e antigen. To verify the M2e-based cELISA is capable of DIVA, chicken sera which were H5N1 vaccinated only (H5N1 negative) sera (*n*=145) were tested. Results showed a mean of 36.3% (range 5.8% - 53.0%, sd=9.06) which indicated low competition for M2e antigen binding between the chicken sera and the mAb. Since experimentally controlled and field chicken sera may show variability in field condition, chicken field sera (H5N1 negative) (*n*=194) were included in the cELISA and showed an average of 5.8% (range 0.0% - 61.6%, sd=13.06) inhibition, while SPF chicken sera (H5N1 negative) (*n*=2) as the negative control showed a mean of 27.6% (25.3% - 30.0%, sd=3.34). The negative chicken sera (vaccinated-non-challenged, n=145; non-vaccinated-non-challenged, n=194; known negative, n=2) showed a mean optical density 18.9 (sd=19.0). Calculation of positive inhibition cut-off value for anti-M2e chicken antisera was 56.8 (mean OD + 2sd).

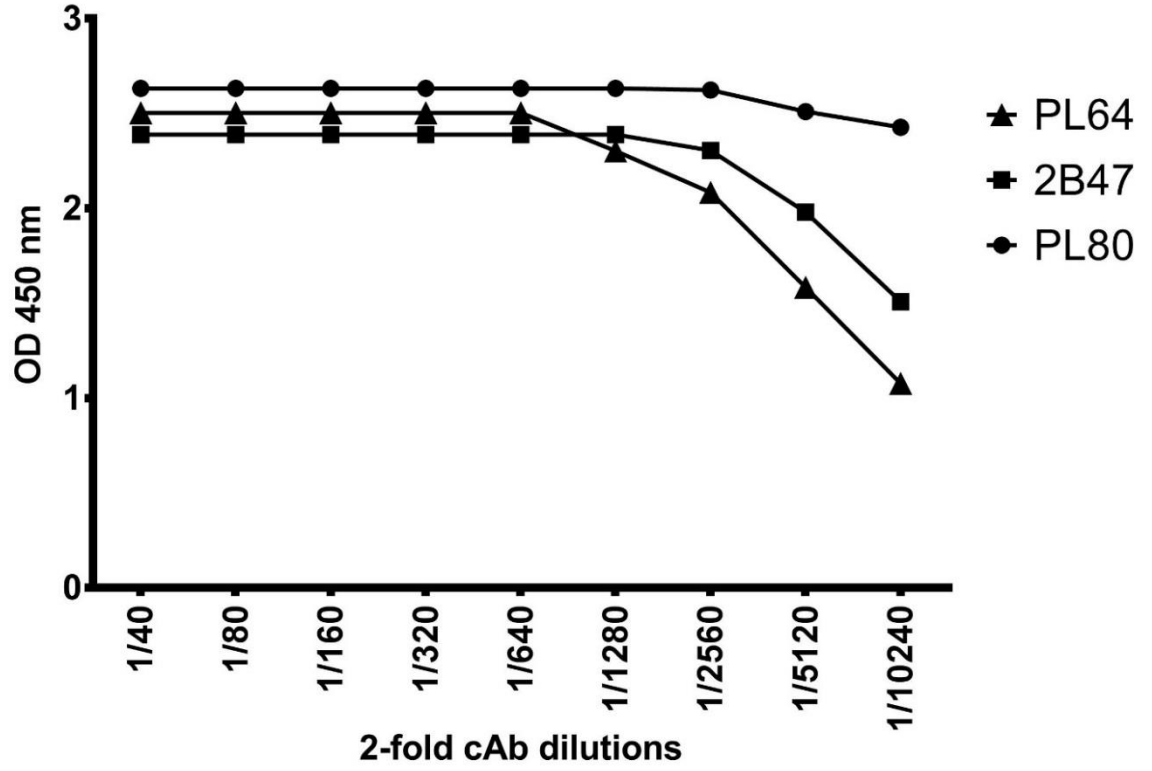

Figure 4.1 OD450 nm of 2-fold dilutions of chicken sera (1:40 – 1:10240 dilutions) incubated with M2e peptide (10 µg/ml) and binding visualised as described in methods.

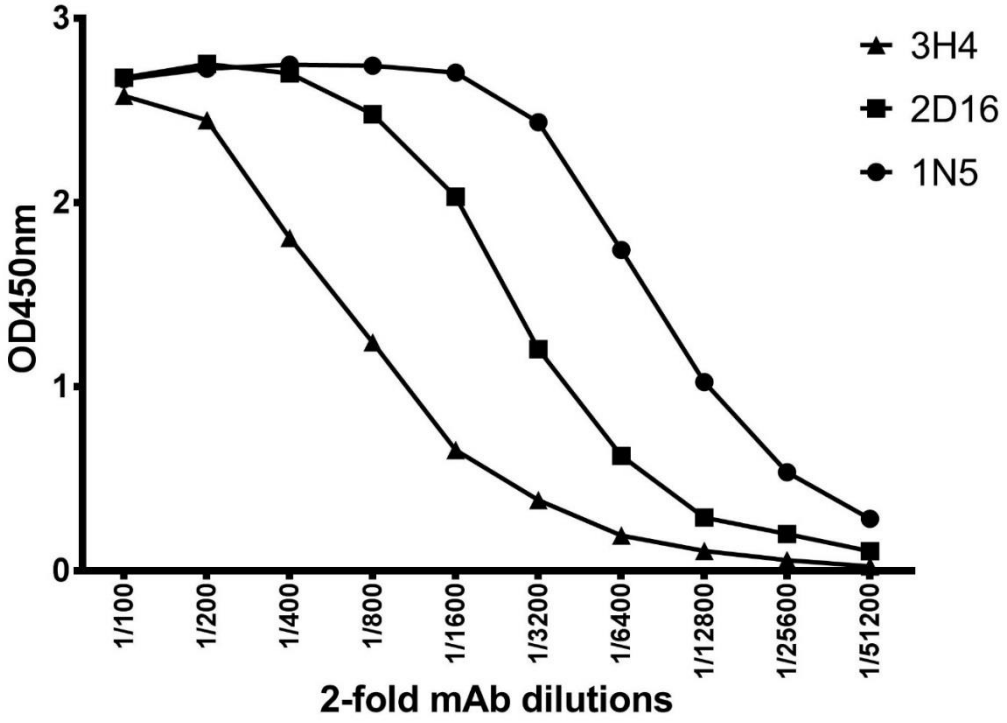

Figure 4.2 Monoclonal antibodies (mAb) of 2-fold dilutions (1:100 – 1:51200 dilutions) anti-M2e ELISA titer tested with M2e peptide (10 µg/ml) in an indirect M2e ELISA.

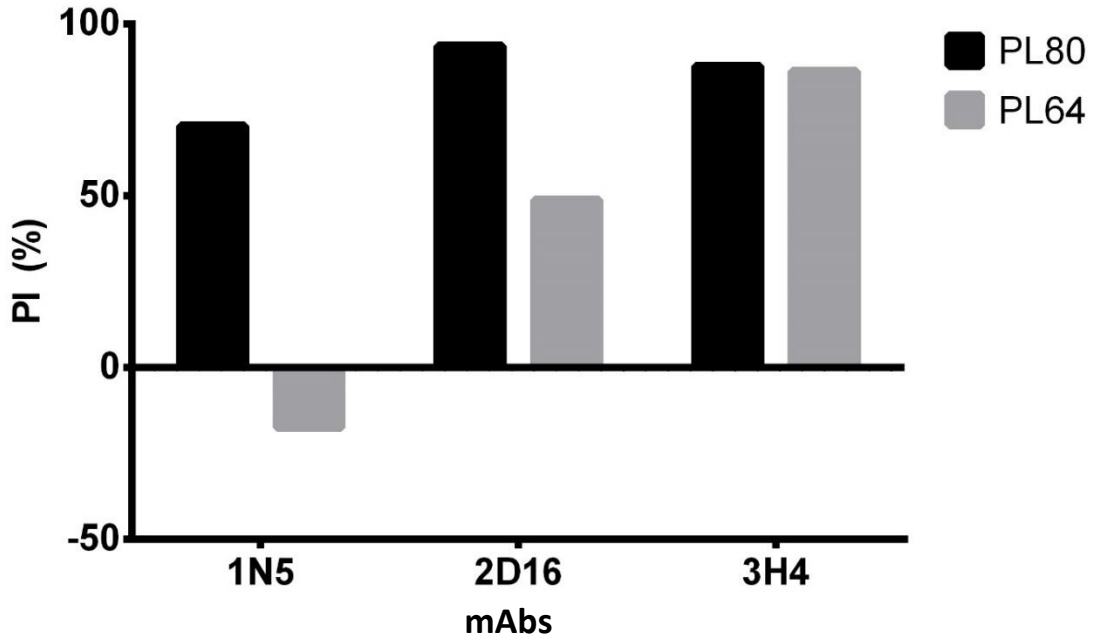

Figure 4.3 Comparison of percentage of inhibition, PI (%) amongst monoclonal antibodies 1N5, 2D16 and 3H4 (final dilution of 1:500) against known high titre anti-M2e sera (experimentally challenged), chicken antibody (cAb) PL80, and known low titre anti-M2e sera, cAb PL64 (both at 1:10 dilution). mAb 3H4 shows comparable competitive level against both chicken antibodies in comparison to the other two monoclonal antibodies.

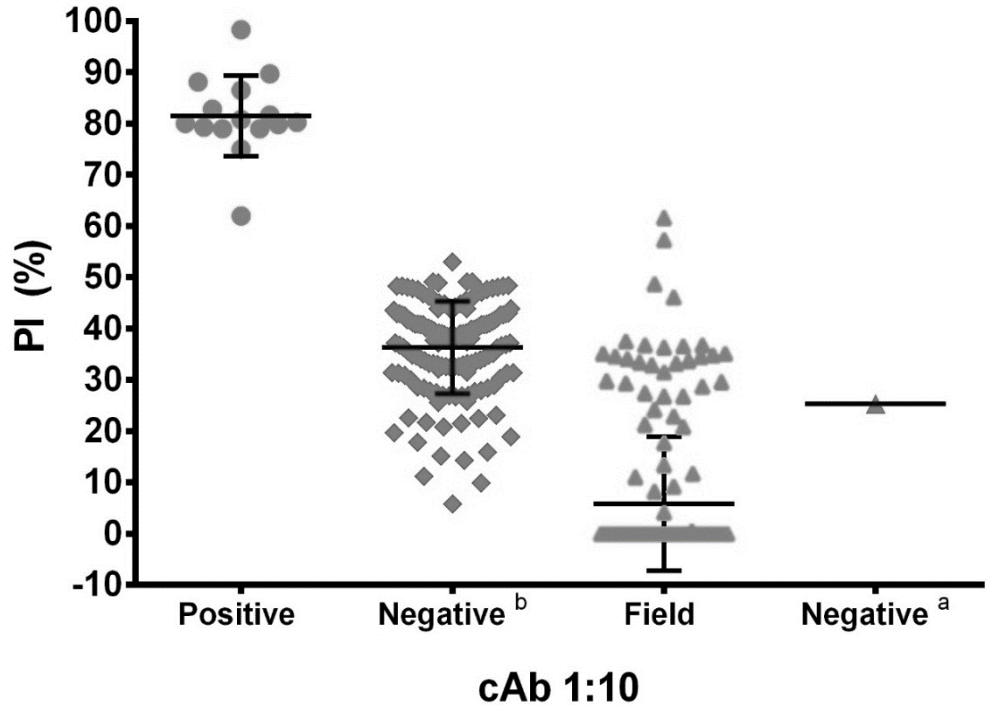

Figure 4.4 Mouse monoclonal antibody 3H4 (1:500) binding inhibition by H5N1 positive, vaccinated only (negative[b]), field and SPF chicken sera (negative[a]) (1:10) in M2e-based cELISA test showing mean and SD of inhibition values.

Each cAb was tested in duplicate of 1:500 mAb dilutions, the optimal dilution based on three separate trial runs (results not shown). A cut-off value for positive inhibition was calculated by using mean OD values of negative sera (including H5N1 vaccinated only, field sera and SPF chicken sera) + 2-fold value the standard deviation (mean negative OD + 2SD) (Table 4.2). Based on the mean OD and SD values, the cut-off value for H5N1 negative in chicken was 56.8%. Therefore, test sera with PI value exceeding these cut-off values were considered as anti-M2e positive, hence infected with H5N1.

Table 4.2. Summary of the H5N1 treatment and infection for chicken sera used in this study, with the average percentage of inhibition (PI) value in the M2e-based cELISA, showing the minimum and maximum values, and standard deviation (sd) for each sera type.

Sera	H5N1 treatment		*n*	Average PI value (min, max)	sd
	Vaccinated	Challenged			
Chicken	√	√	12	81.5% (62.0% - 98.0%)	7.88
	√[a]	-	145	36.3% (5.8% - 53.0%)	9.06
	√[b]	-	194	5.8% (0.0% - 61.6%)	13.06
	-	-	2	27.6% (25.3% - 30.0%)	3.34

[a]: vaccinated with H5N1 AI vaccine (Medivac-AI, PT Medion, Bandung, Indonesia)
[b]: vaccinated using vaccines from unknown sources

Table 4.3. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for H5N1 infection ELISA.

	cAb 95% CI
Sensitivity	100 78.2% to 100%
Specificity	99.4 98.9% to 100%
PPV	88.2 63.6% to 98.5%
NPV	100 98.9% to 100%

4.3.4 Sensitivity and specificity of M2e-based cELISA

M2e-based cELISA demonstrated 100% sensitivity and 99.4% specificity when tested with cAb exposed to H5N1 (Table 4.3). Sera which tested positive showed 88.2% likelihood to be infected with H5N1, while sera that tested negative showed 100% likelihood to be non-H5N1 infected. ROC curve analysis was significantly different from 0.5 (<0.5) for cAb sera group and this indicated that the M2e-cELISA does has an ability to distinguish between the infected and non-infected sera in chicken (Table 4.4, Figure 4.5).

Table 4.4. Area under the ROC curve shows ability of test to distinguish between diseased and non-diseased sera in chicken.

Area under the ROC curve (AUC)	cAb
Area under the ROC curve (AUC)	1.000
Standard Error [a]	0.000
95% Confidence interval [b]	1.000 to 1.000
Significance level P (Area=0.5)	<0.0001

[a] DeLong, M and Clarke-Pearson (1988), [b] AUC ± 1.96 SE

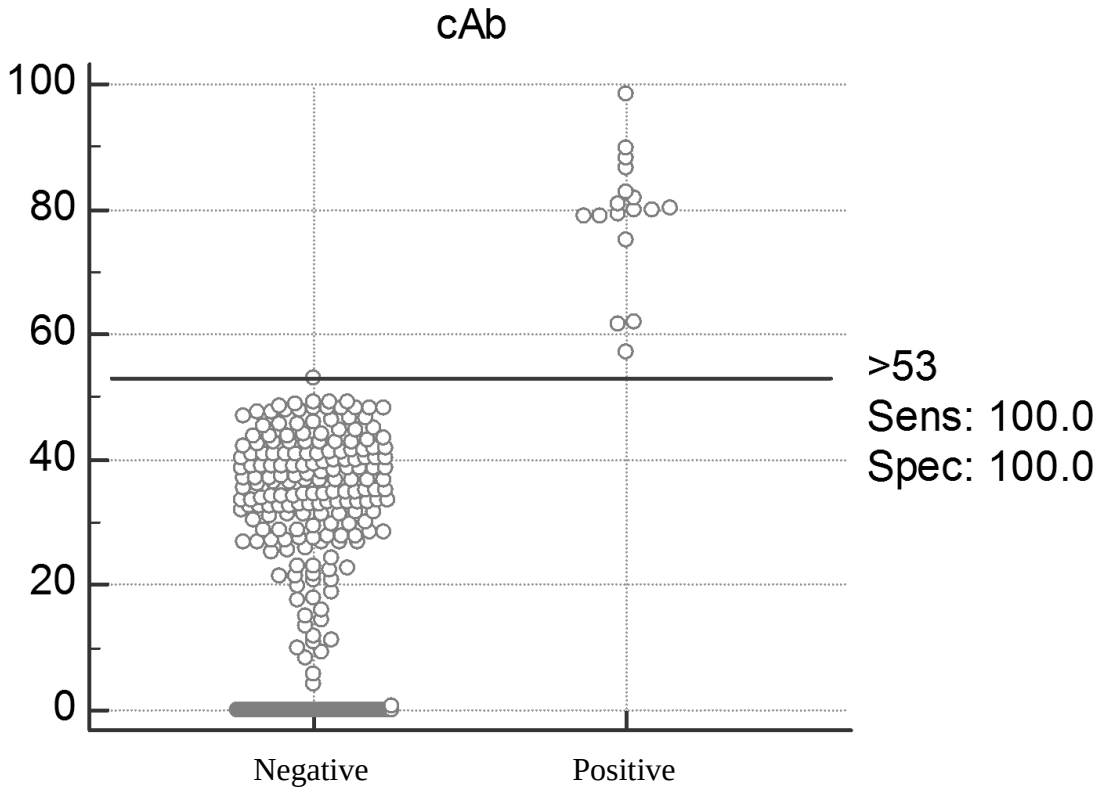

Figure 4.5 Interactive dot diagram on ROC curve evaluation of sensitivity and specificity of M2e-based cELISA using chicken sera from infected (challenged), non-vaccinated and vaccinated chickens.

4.4 **Discussion**

This study demonstrates the ability of M2e-based cELISA to be used as a sero-surveillance and DIVA tool in H5N1 infection detection in chicken. This test successfully identified vaccinated-then-challenged chicken sera as positive, and vaccinated only chicken sera as negative. These are important criteria for H5N1 enzootic countries which practise vaccination using H5N1 killed virus. H5N1 tests using cELISA based on the AIV-NP had similar or better sensitivity and specificity in comparison with agar gel immunodiffusion (AGID) and HI tests in domestic birds (Shafer, Katz & Eernisse 1998; Song et al. 2009; Starick et al. 2006; Zhou et al. 1998). For H5N1 enzootic countries, where vaccination using inactivated virus is practised however, NP-based cELISA is rendered inapplicable for surveillance of AIV infection as it does not distinguish vaccinated from infected birds. Development of an M2e-based cELISA is highly anticipated based on its demonstrated sensitivity and specificity

as a DIVA marker (Hadifar et al. 2014; Hemmatzadeh et al. 2013; Kim et al. 2010; Lambrecht et al. 2007; Tarigan et al. 2015).

Maximising the dynamic range of the M2e-based cELISA for detection of infection required a screening step titrating mAb concentrations to positive chicken sera with low (PL64) and high (PL80) anti-M2e antibody titers. Although mAb 1N5 possessed the highest anti-M2e antibody titer among the three mAbs, it showed no inhibition with the low M2e-antibody titer cAb. Instead, mAb 3H4 with the lowest anti-M2e antibody titer among the mAbs demonstrated the optimum competition with both high and low anti-M2e antibody titer cAbs. Difference in competition ability between mAbs may be related to the high 1N5 titer, which may has hindered or displaced the binding of low M2e titer cAbs to the antigen, resulting in the observed lack of competition between mAb 1N5 and cAb PL64, despite positive inhibition between mAb 1N5 and cAb PL80 (high anti-M2e cAb titer). On the other hand, positive inhibition between mAb 3H4 and cAb PL64 may be explained by the lower anti-M2e antibodies titer of mAb 3H4 in comparison to mAb 1N5. Such condition may has lowered the difference in concentration between the antibodies, thus reduced the binding interference as observed previously.

It is not known if different subtypes of the immunoglobulin may have affected the competition of antibodies in an ELISA setting. Previous studies have shown that chicken sera are generally composed of three immunoglobulin classes, namely IgY (5 to 15 mg/ml), IgM (1 to 3 mg/ml) and IgA (0.3 to 0.5 mg/ml) (Kowalczyk et al. 1985; Rose, Orlans & Buttress 1974), in the order of the highest concentration to the lowest. Meanwhile, the mAb used in this study were of IgG2 subtypes (Hasan et al. 2016). Structurally, both IgY and IgG are relatively similar, with differences in the number of their heavy chain constant regions (four constant regions in IgY, and only three constant regions in IgG), and lack of a hinge structure

between the constant variable (Cv) region 1 and Cv2 in IgY, which made it less flexible than IgG, among others (reviewed in Michael et al. (2010)). Nevertheless, all of these antibodies have demonstrated similar ability to identify the M2e peptide based on our previous epitope mapping experiment (Hasan et al. 2016). It was observed that cAbs PL64 and PL80 mapped to the same epitopes as each other on the M2e peptide used in the cELISA. Similarly, the three mAbs (1N5, 2D16 and 3H4) showed no differences in the epitope to which they bound on the same peptide. Therefore, it was unlikely that differences in the immunoglobulin subtypes may have influenced the different observation made between both mAbs with cAb PL64.

The range of inhibition percentages observed in this study for the negative cAb sera is relatively high in comparison with previously reported AIV-based cELISA, the majority of which reported cut off values <30% (Dlugolenski et al. 2010; Shafer, Katz & Eernisse 1998; Starick et al. 2006; Zhou et al. 1998). It is unlikely that such non-specific readings are caused by non-specific reaction between the sera and the M2e antigen, since the test format was proven to be highly specific and sensitive in an M2e-based indirect ELISA in previous studies (Hadifar et al. 2014; Hemmatzadeh et al. 2013; Tarigan et al. 2015). A caveat for this is the prior observation that non-specific reactions in an M2e-based indirect ELISA format were associated with the use of lipemic or haemolysed sera (Hemmatzadeh et al. 2013). Further investigation is needed to further clarify this observation. Nevertheless, all cAbs were correctly identified for both H5N1 negative (including vaccinated not challenged) and positive sera.

In wild birds serologic surveillance, diverse populations and complicated nature of AIV present difficulties for diagnostic tool development. Serological manifestations observed in domestic birds upon AIV infection do not necessarily reflect those of domestic ducks and

wild birds, as these are affected by the bird species, locality and virus adaptation to the host (Curran et al. 2014; Swayne 2007). Although NP-based cELISA is demonstrated as highly sensitive and specific tool for serologic surveys in several wild bird species (Brown et al. 2009; Curran et al. 2014; Perez-Ramirez et al. 2010), this is not always the case in some others (Claes et al. 2012). Our preliminary works with AIV positive mallard and pheasant sera showed M2e-based cELISA is capable of detecting AIV infection in these wild bird's species. However, duck sera demonstrated a mixed reactivity, where only duck sera which have been vaccinated-and-challenged were identified as AIV positive. Non-vaccinated-and-challenged duck sera demonstrated negative inhibition. Low inhibition value observed for the non-vaccinated and challenged duck sera despite being H5N1 positive is assumed to be due to the low level of anti-M2e antibodies produced from the challenge. Due to the low density of the M2e protein on the surface of the virus particle, and domination of HA and NA proteins on the surface of the infected cells, the duck immune response is likely to be incapable of producing a significant level of M2e-specific antibodies in relation to HA- and NA-specific antibodies for the H5N1 positive duck (Feng et al. 2006; Neirynck et al. 1999). Nevertheless, further investigation is required to be done while considering the challenge virus, duck species use, vaccination protocols, role of maternally derived antibodies as well as the synergies between co-infecting pathogens (Pantin-Jackwood & Suarez 2013).

Although the currently presented M2e-based cELISA possess a limited panel of test sera for known positive H5N1, our findings indicates that M2e-based cELISA is capable of discriminating between H5N1 positive and negative sera in chickens. Further testing with a larger number and variety of animal sera is necessary to aid the capability validation of the M2e-based cELISA system. In conclusion, development of M2e-cELISA based on mAb 3H4 still potentially results in a broad range and species-independent immunodiagnostic assay capable of DIVA application as an alternative H5N1 surveillance tool in enzootic countries.

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Chapter 5 Isolation of reactive single-chain variable fragment antibodies against AIV-M2e protein (scFv anti-M2e) using phage display technology from H5N1-immunized chicken IgY

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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

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Isolation of reactive single-chain variable fragment antibodies against AIV-M2e protein (scFv anti-M2e) using phage display technology from H5N1-immunized chicken IgY

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Abstract

Avian influenza virus (AIV) H5N1 has continuously evolved and caused outbreaks in its enzootic countries. Due to economically impractical option of poultry culling, vaccination has been practiced to cushion the danger of an epizootic which pose concerns to public health. Well-developed diagnostic tools to detect AIV infection are available. However, most are not capable of differentiating infected from vaccinated animals (DIVA), making silent spread of AIV in a vaccinated animal population a major threat. Hence, a rapid and cost-effective diagnostic tool capable of DIVA is critical in AIV enzootic countries. The highly conserved extracellular matrix 2 (M2e) protein, an AIV surface protein has shown high potential for such application, but its small size and naturally low immunogenicity to the host immune system make its detectability an issue when employed as a target for DIVA tools. In this study, we have successfully isolated highly reactive and sensitive single chain variable fragment (scFv) anti-M2e antibodies from AIV-immunized and -challenged chicken using phage display technology to compensate for its low immunogenicity. mRNA isolation was done from spleen lymphocytes of chickens with high anti-M2e ELISA titer, where the immunoglobulin fragments (heavy chain, V_H and light chain, V_L) were then amplified and assembled into a phagemid before being displayed as recombinant bacteriophage to allow reactive antibodies selection. Findings showed that the isolated scFv antibodies possessed high reactivity with M2e antigen, both in soluble and phage-displayed form. Sequence analysis of six selected scFvs from pooled recombinant phages showed that the heavy chain of complementarity determining region 3 (CDRH3) is responsible for 55.6% of the overall variation of the positive scFv antibodies. Although refinement of the scFv anti-M2e antibodies is still required for a viable commercial assay, these antibodies hold potential use as a basis of another M2e-based diagnostic tool development for H5N1 AIV serosurveillance.

Keywords: Phage display, M2e protein, H5N1, DIVA, influenza virus, single-chain variable fragment

5.1 Introduction

Developments in biotechnology have introduced methods to obtain genetically engineered antibodies, specifically the single-chain variable fragment antibody (scFv), a minimal form of a functional synthetic antibody that contains the variable heavy (V_H) and variable light (V_L) chains connected by a flexible polypeptide linker (Bird et al. 1988; Bird & Walker 1991; Wang et al. 2013). It is used as a tool for therapeutic and diagnostic purposes such as detection of antibodies against a wide variety of infectious pathogens (Chen et al. 2014; Nimmagadda et al. 2012), and to investigate the antigen-antibody binding interaction and isolation of the reactive antibodies to an antigen of interest (Guo et al. 2003; Winter et al. 1994). scFv antibodies have accessible coding sequence for further analysis and modification (Hoogenboom et al. 1991; Yajima et al. 2008), can be expressed in high yield bacterial expression systems (Holliger & Hudson 2005), and are also isolatable from the bacterial periplasm space (Nossal & Heppel 1966). In vitro directed molecular evolution can be used to improve the antibody affinity for specific antigens (Fukuda et al. 2006; Liu et al. 2012).

ScFv is usually obtained using phage display technology which uses the bacteriophage ability to express a foreign protein on its surface. Bacteriophages are viruses that feed on bacteria. Bacteriophage used for phage display purposes are modified to support the optimal conditions for the specific protein expression. Advantage of this technology lies in the power to select the most reactive antibodies through a process of affinity selection known as biopanning. This involves repetitive immobilisation of reactive phage using synthetic antigen, thus allows effective selection of antibodies with high affinity and specificity and their rapid generation (Fack et al. 1997; Nimmagadda et al. 2012). Also, phage display technology is a more rapid

and cost effective way to obtain targeted antibodies, circumventing the needs for animals or cell culture experiments and facilities (van Wyngaardt et al. 2004).

Established enzootic of avian influenza virus (AIV) H5N1 in several countries has made vaccination the primary tool to mitigate the risk of avian influenza virus (AIV) infection and outbreaks (Domenech et al. 2009; Suarez 2012; Swayne et al. 2011). Differentiation of antibodies generated from AIV infection versus those from vaccination (DIVA) is still a challenge in monitoring the condition and spread of this virus especially in domestic animals such as the poultry industry. Importance of DIVA tools has been noted as it is critical in ensuring the animals are disease-free. Serosurveillance is important for early detection of potential outbreaks, and diagnostic tools based on AIV proteins such as hemagglutinin (HA), neuraminidase (NA) and non-structural protein 1 (NS1) have been developed to tackle this problem. Although these diagnostic tools are effective in detecting AIV infection in animals, they lack the ability to distinguish the animals which are infected from the vaccinated ones since both conditions produce similar types of antibodies (Hadifar et al. 2014; Hemmatzadeh et al. 2013).

Previous studies on the extracellular domain of the matrix 2 protein (M2e) of AIV have demonstrated its potential use for sero-surveillance in sensitive and specific diagnostic tools with DIVA capabilities (Hadifar et al. 2014; Hasan, Ignjatovic, et al. 2016; Hemmatzadeh et al. 2013; Tarigan et al. 2015). Briefly, the M2e protein is a 24 amino acid (aa) membrane protein on the surface of the AIV particle. It is the N-terminal of M2, a type III homotetrameric, integral membrane protein which further consists of a transmembrane domain and a cytoplasmic tail domain (Rossman & Lamb 2011; Schnell & Chou 2008). Apart from channelling ion exchange during the release of virus genetic material into the host cell (Helenius 1992), M2 protein also plays a role in maturation of hemagglutinin molecules from

the trans-Golgi network (Ciampor et al. 1992). M2 protein is known to inhibit autophagy which may affect the infected cell's survival (Gannage et al. 2009), as well as assisting in virion release (Rossman & Lamb 2011).

Factors driving researchers to take interest of M2e protein are its stable sequence across AIV subtypes and its differential epitope density on infected (high) and non-infected (low) cells (Lamb, Zabedee & Richardson 1985; Zabedee & Lamb 1988). M2e is relatively invariant across AIV subtypes due to its minimal exposure to the host immune response. Its small size and low density on the virus particle in comparison to the other surface proteins i.e hemagglutinin and neuraminidase proteins have partly contributed to this feature. It also tolerates minimal amino acid changes due to its overlapping gene segments with the highly conserved M1 protein.

M2e protein also demonstrated differential epitope density between the surface of the virus particle and on an infected cell, where the latter is significantly higher from the former (Lamb, Zabedee & Richardson 1985; Zabedee, Richardson & Lamb 1985). This characteristic makes M2e protein an attractive target as a marker in DIVA strategies, as this enable the differentiation between an AIV-vaccinated-only animal, from an AIV-vaccinated-then infected animal. Vaccination is usually done using inactivated AIV particle – a whole virus which lacks the ability to replicate. Although M2e protein is present on the inactivated AIV particle, its low density will not elicit any significant antibody response. However, M2e protein is displayed in a large amount on the surface of an infected cells as a part of the AIV progeny assembly and budding strategy (reviewed in Rossman and Lamb (2011)). Therefore, high amount of M2e protein will elicit high level of anti-M2e antibodies, which is indicative of AIV infection. Several studies demonstrated the capability of M2e for chicken and duck DIVA (Hemmatzadeh et al. 2013; Kim et al. 2010; Lambrecht et al. 2007). Improvement in

sensitivity and specificity of the M2e-based DIVA was demonstrated more recently (Hadifar et al. 2014; Tarigan et al. 2015).

However, the main limitation of strategies based on M2e protein is its low detectability, as this may cost the test's sensitivity as well as specificity (Feng et al. 2006). Therefore, using phage display technology to acquire highly specific and reactive anti-M2e antibodies may alleviate this shortcoming, as scFv antibodies are known to be stable and highly reactive (Chen et al. 2014; Min et al. 2011). Following interests on the AIV M2e protein as a target for diagnostic tools development capable of DIVA in AIV sero-surveillance, this study has been developed to isolate the most reactive antibodies against the M2e protein to be potentially used in a competitive ELISA setting. Recombinant Phage Antibody Systems (RPAS) has been showed to efficiently produce scFv through the use of phagemid vector such as pCANTAB5E (Guo et al. 2003; Sapats et al. 2003; Winter et al. 1994). Therefore, an immune scFv library was constructed using RNA isolated from chicken (ck) lymphocytes of an immunized donor vaccinated with H5N1. Specific ck scFv fragments were affinity selected from this library using plate-based biopanning. The selected scFv was expressed in *E. coli*, characterized and used in the development of an ELISA for quantification of reactive M2e antibodies.

5.2 Materials & methods

5.2.1 Plasmids, strains and cells

Phagemid pCANTAB5E (Amersham Biosciences Inc., UK) was kindly donated by Dr Motohiro Ohshima, the University of Shizuaka, Japan. Escherichia coli strains TG1, SOLR, XL-Blue MRF, HB2151 were purchased from Agilent Technologies (Santa Clara, California, USA), while helper phage M13KO7 was purchased from New England Biolabs (MA, USA). Restriction enzymes SfiI and NotI were purchased from New England Biolabs (MA, USA).

Anti-E tag monoclonal antibodies for affinity purification, E tag peptide (GAPVPYPDPLEPR) (Abmart, Shanghai, China), and the M2e peptide (aa 2-18: SLLTEVETPTRNEWECK) were purchased from Abmart (Shanghai, China).

5.2.2 **Chicken serum samples**

Anti-M2e positive chicken sera (n=35) were produced as described previously (Hadifar et al. 2014). Three-weeks-old specific pathogen free (SPF) chickens were immunized one to three times with commercial inactivated AI vaccine (Medivac-AI, PT Medion, Bandung, Indonesia) before challenged with live H5N1 strains (A/chicken/West Java/Sbg-29/2007 or A/chicken/West Java/PWT-WIJ/2006) two weeks after the last vaccination. Challenge experiments were done in the Biosecurity level 3 (BSL3) facilities at the Indonesian Research Centre for Veterinary Science, Bogor, Indonesia. Serum from SPF chicken was used as the negative control and all sera were tested in three duplicates for anti-M2e antibodies using tM2e-ELISA (Hadifar et al. 2014; Hemmatzadeh et al. 2013). The chickens with the higher anti-M2e titre in ELISA were selected for mRNA isolation from spleen lymphocytes. Briefly, chickens were euthanized four weeks after last immunization and immediately, the chicken's spleens were removed for lymphocytes purification.

5.2.3 **cDNA synthesis and V_H-linker-V_L assembly**

Total RNA was isolated from the chicken spleen lymphocytes using GenElute™ Direct mRNA Miniprep Kit (Sigma, St. Louis, Missouri, USA), and cDNA was synthesised using both random hexamers and oligoDT primers using the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). V_H and V_L fragments were amplified with cloned *Pfu* Turbo DNA Polymerase AD (Agilent Technologies), using the primers indicated (Table 5.1; primers *a* – *d*) (GeneWorks, SA, Australia). Linker sequences $(Gly_4Ser)_3$ were incorporated into each of the cDNA fragments to go through a second amplification of V_H and V_L products using

primer *a* and *f* for V_H, and primer *d* and *g* for V_L. A complete insert fragment of V_H-linker-V_L was produced through splicing by overlap extension (SOE) PCR using primers *a* and *d* (Horton et al. 1989; Huston et al. 1988; Sapats et al. 2003; Singh et al. 2010) (Table 5.1). To obtain enough scFv products, five products of V_H-linker-V_L were pooled, precipitated with ethanol and washed. All amplified products were visualized using 1.5% agarose gel electrophoresis run at 100V for 1 hr with 100 bp DNA marker (Axygen, CA, USA) for product size estimation. The approximate size for each primer pair amplification is shown in Table 5.1. PCR product purifications were done using either QIAquick PCR Purification kit (QIAGEN) or QIAGEN Gel Extraction Purification kit (QIAGEN).

5.2.4 **Recombinant phagemid construction**

Generally, recombinant phagemid construction was done according to the expression module/recombinant phage antibody system (RPAS) (Amersham Biosciences Inc., UK) with modifications. Briefly, vector phagemid pCANTAB5E was cultured overnight in 2YT broth (16 g/L bacto-tryptone, 10 g/L Bacto-yeast extract, 10 g/L NaCl) containing ampicillin 20 µg/ml (Sigma) at 37°C with vigorous shaking at 250 rpm. Phagemid was then isolated using QIAprep Spin Miniprep kit (QIAGEN). Both the vector phagemid pCANTAB5E and the amplified insert fragment, V_H-linker-V_L were digested with SfiI at 50°C, and NotI enzymes at 37°C, for three hours each. Digested products were visualized using 1.5% agarose gel electrophoresis and purified using QIAquick PCR Purification kit (QIAGEN) for the insert, and QIAGEN Gel Extraction Purification kit (QIAGEN) for the phagemid. Purified products were quantitated using the NanoDrop 1000 Spectrophotometer (Thermoscientific) with product's purity was checked using sample absorbance ratio at 260 and 280 nm. Both purified products (the insert and the vector) must be at least 30 ng/µl at optical density (OD) 260 nm to be used in the ligation step.

The insert fragment were ligated into phagemid pCANTAB5E using Quick-Stick Ligase (Bioline, London, UK), transformed into competent *E. coli* TG1 cells and grown for 1 hr in 1 ml 2YT broth at 37°C with shaking at 250 rpm. An aliquot of 250 µl of the culture (recombinant phagemid) was plated out on each 2YT plates (four 2YT plates in total) and left overnight at 37°C. Cell lawn contained recombinant phagemid on each 2YT plates were then scraped off and pooled into a new 5 ml 2YT broth containing 2% (w/v) glucose and 100 µg/ml ampicillin (2YTG-A) and cultured overnight at 37°C with shaking at 250 rpm. Before these recombinant phagemid were stored at -20°C for further use, a loop-full of these culture were re-grown in a new 5 ml 2YTG-A for overnight at 37°C and 250 rpm. Recombinant phagemid were then isolated using QIAprep Spin Miniprep kit (QIAGEN) and all recombinant phagemid were confirmed for positive ligation using gene-specific primers (Table 5.1, primer *a* and *d*) and vector-specific primers (Table 5.1, primer *g* and h).

Table 5.1. List of primers, the targeted region and the approximate product size in basepair (bp) used for the amplification of chicken V_H and V_L regions, as well as the primer used for linker $(Gly_4Ser)_3$ incorporation between the amplified genes to produce the scFv (insert). Positive phagemid and insert ligation was screened using vector specific primers (g and h) and gene-specific primers (a and d), while positive recombinant phages with insert was screened using the gene-specific primers.

Primer	Nucleotide sequence (5′ to 3′)	Targeted region	Approximate product size (bp)
	Antibody library construction primers:		
a. HF-*SfiI*	ATG TCT ATG GCC CAG CCG GCC GTG ACG TTG GAC G	VH	~390
b. HR-*XbaI*	GAA CCG CCT CCA CCA TCT AGA GAG GAG ACG ATG ACT TCG G		
c. LF-*SalI*	GGC GGT GGC GGG TCG ACA GCG CTG ACT CAG CCG TCC TCG	V_L	~350
d. LR-*NotI*	AGT TAC TGG AGC GGC CGC ACC TAG GAC GGT CAG GG		
e. Link1	GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT	Linker	~400 (with primer *d*)
f. Link2	CGA TCC GCC ACC GCC AGA GCC ACC TCC GCC TGA		~450 (with primer *a*)
	Vector specific primers:		
g. S1F	CAA CGT GAA AAA ATT ATT ATT CGC	Insert-flanking region on vector	~800 (with the presence of VH -linker-V_L)
h. S6R	GGA GTA TGT CTT TTA AGT AAA TG		
	Gene specific primers:		
a. HF-*SfiI*	ATG TCT ATG GCC CAG CCG GCC GTG ACG TTG GAC G	Insert-flanking region	~750 (VH -linker-V_L without vector)
d. LR-*NotI*	AGT TAC TGG AGC GGC CGC ACC TAG GAC GGT CAG GG		

5.2.5 **Infection of V_H-V_L library with helper phage**

The positive recombinant phagemid cultures were further expanded into a 200 ml volume of 2YTG-A by adding 2 ml of the overnight culture an incubated at 37°C with shaking at 250 rpm until OD_{600} reached 0.6-0.7. A total of 2 ml of $1x10^{12}$ pfu/ml helper phage M13KO7 (NEB) were added to the exponentially grown recombinant phagemid and incubated at 37°C for at least 30 minutes before centrifuged at 4500 rpm (3200 g), 4°C for 30 minutes. Pellets were resuspended in 200 ml of 2YTG-A with 50 µg/ml kanamycin (Sigma) (2YTG-AK) and grown 16-20 hrs at 30°C and 250 rpm. These culture of recombinant bacteriophages (phages) were centrifuged at 4500 rpm (3200 g), 4°C for 30 minutes before its supernatant were filtered using 0.45 µM filter (Millipore) to remove cellular debris. The recombinant phages were precipitated using 1/5 vol of polyethyl glycol (PEG)/NaCl (20% PEG in 2.5 M NaCl) and incubated at 4°C for at least 2 hrs. Recombinant phages pellet were resuspended in 1x phosphate saline buffer (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, pH 7.4) and precipitation were repeated for another two times before the final recombinant phage pellet were resuspended in 1xPBS and filtered using 0.45 µM filter (Millipore). All produced recombinant phages library were checked for their titre using the plaque formation assay. Only recombinant bacteriophages library of at least 1×10^{12} pfu/ml were proceed for biopanning.

5.2.6 **Selection of reactive recombinant phages displaying scFv antibodies and phage rescue**

Biopanning of the recombinant phage displayed scFv library was done based on previous reports (Haque & Tonks 2012; Nimmagadda et al. 2012), with modifications. Briefly, M2e peptide (aa 2-17) (Abmart, Shanghai, China) (final concentration 10 µg/ml) was immobilized in a 25 cm^2 cell culture flask using carbonate-bicarbonate buffer (pH 9) as the plate coating buffer and left at 4°C overnight. Flask was emptied and washed for three times using washing buffer (PBS-0.05% (v/v) Tween20) and incubated with blocking buffer (0.1 g/L PBS, 10

µl/mL of 10% NaN_3) with 0.01% sodium azide at room temperature (RT) for 1 hr. Flask was emptied and washed for another three times using washing buffer before incubated with the recombinant phage library and blocking buffer (8:7 ratio) mixture for 2 hr at 37°C. After the flask was emptied, it was washed 10 times with PBS, and another 10 times washing with PBS-0.1% Tween20. Finally, the bound recombinant phages were eluted with 0.2 M HCl-glycine pH 2.2 which were incubated at 37°C for 10 minutes. Eluted recombinant phages were added to an equal amount of neutralization buffer (1M Tris pH 9.1) and mixed well. The retrieved recombinant phages titre were checked and expanded through phage rescue.

Preparation for phage rescue was done using an overnight culture of *E. coli* TG1 cells in 2YTG medium at 37°C with shaking at 250 rpm. The TG1 cells culture were renewed by adding 1/100 vol of overnight culture into a new 2YT media with 100 µg/ml ampicillin and 1% glucose which incubated at 37°C and 250 rpm, until OD600 reached 0.6-0.7. The retrieved recombinant phages were added to the exponentially grown TG1 cells and further incubated at 37°C with shaking at 250 rpm for 1 hr. This culture was centrifuged at 4500 rpm (3200 g) for 30 minutes before the pellet were resuspended in an equal volume of pre-warmed 2YT media with 100 µg/ml ampicillin and 50 µg/ml kanamycin, and incubate overnight at 30°C with shaking at 250 rpm. The rescued recombinant phages were precipitated using PEG/NaCl as described above and followed by a second and third biopanning and phage rescue. In the second and third biopanning, the number of washes were increased from 10 times washing with PBS and 10 times washing with PBS-0.1% Tween20, to 20 times each washing buffer in the second biopanning round, and 30 times each washing buffer in the third biopanning round.

5.2.7 Screening for recombinant phages and confirmation of its binding specificity using M2e-ELISA

Post-panning recombinant phages were screened for the correct insert size by first infecting an aliquot of the phages with TG1 cells as described above, where the phagemid were retrieved using miniprep kit before amplified using gene-specific primers (Table 5.1, primer *a* and *d*) and vector-specific primers (Table 5.1, primer *g* and h). Recombinant phagemid from the post-panning phages were then sent for sequencing to the Australian Genome Research Facility (AGRF) sequencing services (VIC, Australia, with Sanger sequencing using Applied Biosystems 3730 capillary sequencers using Big Dye Terminator (BDT) chemistry version 3.1 (Applied Biosystems) and analysed for their encoded protein.

Binding specificity of the post-panning recombinant phages were tested in duplicates using an indirect M2e-ELISA as described previously (Hasan, Ebrahimie, et al. 2016), utilising horseradish peroxidase (HRP) mouse anti-M13 antibodies (GE Healthcare, Sweden) as the secondary antibodies. Briefly, M2e peptides were diluted to a final concentration of 50 µg/ml with 0.1 M carbonate-bicarbonate buffer, pH 9.6 (0.1 Na_2CO_3, 0.1 M $NaHCO_3$) to each well of 96-well flat bottom microtiter plates (Maxisorp, NUNC) and incubated overnight at 4°C. The coated plates were washed five times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 2% w/v bovine serum albumin (BSA) at RT for 2 hrs. Recombinant phages were diluted in PBS containing 1% BSA and 0.05% Tween 20 (PBS-BT) in a 4:5 ratio (phage:buffer) and blocked wells were washed for another five times with PBS-T. Diluted phages were added into the wells in three duplicates and incubated for 1 hr at RT before washed another five times with PBS-T. Anti-M13 HRP were diluted to 1:500 with PBS-BT and added to the wells, followed by incubation for 1 hr at RT. After washing, substrate solution [100 µg/ml of tetramethylbenzidine substrate (TMB), (Sigma, MO, USA) in citrate buffer pH 8 containing hydrogen peroxide (100 µl of 0.6% H_2O_2)] was added and incubation at RT for 5-20 minutes was done before reaction was stopped with stop buffer (1

M sulphuric acid). The optical density (OD) of each well was read at OD450 nm using the BioRad Benchmark Plus Microplate Reader (BioRad, Hercules, USA). Graph of the M2e-ELISA readings was produced using GraphPad Prism® version 7.02.

5.2.8 **Expression and purification of soluble scFv**

Soluble scFv was produced by transforming the recombinant phages pCANTAB5E-scFv into the non-suppressor *E. coli* strain HB2151 with 1 mM IPTG induction for overnight in 2YT medium. To obtain enough soluble scFv, four preparations of recombinant phages were pooled, expanded and precipitated as described above. Soluble scFv was isolated using osmotic shock (Liu et al. 2012; Nossal & Heppel 1966) and later purified using affinity column with Protein G HP SpinTrap (GE Healthcare, Sweden). Briefly, recombinant phages were infected into the exponentially grown HB2151 cells in 2YT with 2% glucose (2YTG) and incubated at 37°C for 1 hr. This culture were then centrifuged at 4500 rpm (3200 g) for 30 minutes and resuspended in an equal amount of pre-warmed 2YT medium. A total of 500 µl of this suspension was set aside to be used as control (non-induced scFv expression sample), while the rest of the samples were added with 1 mM isopropylthiogalactosidase (IPTG) for the induction of scFv expression. Culture was incubated at 30°C for overnight with shaking at 250 rpm. Culture was centrifuged at 4500 rpm (3200 g) for 30 minutes at 4°C and the pellet was resuspended in 50 ml ice cold 1X TES (0.2 M Tris/HCl, 0.5 M EDTA, 0.5 M sucrose, pH 8.0) and mixed well. Immediately after, 75 ml ice cold 1/5 TES buffer was added to the mixture to induce a mild osmotic shock and incubated on ice for 30 minutes. This mixture was centrifuged as above and the supernatant was retrieved and incubated in ice for 20 minutes with gentle agitation before being centrifuged again. The supernatant containing the soluble scFv was collected and filtered through 0.45 µM filter (Milipore). The soluble scFv was desalted and concentrated using size exclusion columns Vivaspin 20 (Sartorius, Germany) which collect proteins of <30,000 MW and stored at -20°C.

Affinity purification was employed by cross-linking E tag monoclonal antibodies (E tag mAb) (Abmart, Shanghai) to the binding protein used in the column according to the manufacturer's instructions (Protein G HP SpinTrap, GE Healthcare, Sweden). E tag mAb was prepared from hybridoma cells (Abmart, Shanghai) and checked for positive reactivity in duplicates using indirect ELISA with the E tag peptide and a protocol similar to M2e-ELISA as described previously (Hasan, Ebrahimie, et al. 2016). To ensure compatibility of protein binding used with the E tag antibodies, isotyping of the E tag antibodies was done using Pierce Rapid Isotyping Kits – Mouse (Thermo Scientific, USA).

Briefly, protein purification using Protein G HP SpinTrap was done as the following. The SpinTrap column was equilibrated with 400 µl TBS (50 mM Tris, 150 mM NaCl, pH 7.5) to the column for three times. A total of 200 µl of capture protein (E tag mAb, 1.86 mg/ml), pH 7 was added to the column (0.5 to 1.0 mg/ml in TBS) and fully suspended by manual inversion and incubation of slow, end-over-end mixing for 1 hr on the rotary tube mixer (Ratek, VIC, Australia). The column was centrifuged for 1 min at 150 x g to remove unbound antibody and washed by the addition of 400 µl TBS before another step of centrifugation. A total of 400 µl 200 mM triethanolamine, pH 8.9 (Sigma) was added to the column and centrifuged. Cross-linking was done by the addition of 400 µl of 500 mM dimethyl pimelimidate dihydrochloride (DMP) (Sigma) in 200 mM triethanolamine, pH 8.9 to the column and fully suspended by manual inversion, followed by incubation with slow, end-over-end mixing for 1 hr on the rotary tube mixer. The mixture was centrifuged and washed with 400 µl TBS before another step of centrifugation. This cross-linked mixture was blocked by the addition of 400 µl ethanolamine (100 mM, pH 8.9) (Sigma) and manually mixed and incubated end-over-end on the rotary tube mixer for 30 minutes and centrifuged. Unbound antibodies were removed by the addition of 400 µl elution buffer (0.1 M glycine-HCl, pH 2.7)

and centrifugation at 150 x g for 1 minute. The column was washed for three times with the addition of 400 µl TBS and centrifugation. The targeted protein (soluble scFv), pH 7 was bound by the addition of 200 µl of soluble scFv in TBS to the column and mixed by manual inversion, followed by an end-over-end incubation on the rotary tube mixer for 1 hr. This mixture was washed for five times with wash buffer (TBS with 2M urea, pH 7.5) and centrifuged at 150 x g for 1 min. Finally, elution of bound antibodies was done by the addition of 200 µl of 0.1 M glycine-HCl, pH 2.7, mixed by inversion and centrifugation at 1000 x g for 1 min. Purified soluble scFv was desalted and further concentrated to at least 10 mg/ml of protein using size exclusion columns Vivaspin 20 (Sartorius, Germany).

5.2.9 Soluble scFv binding specificity in M2e-ELISA

Binding specificity of the soluble scFv was tested in duplicates using an indirect M2e-ELISA as described previously (Hasan, Ebrahimie, et al. 2016), utilising anti-E tag mouse antibodies (Abmart, Shanghai, China) as the secondary antibodies. Briefly, M2e-ELISA for soluble scFv was done similar with the post-panning recombinant bacteriophages with differences in the following: M2e peptide was diluted to two final concentrations of 50 µg/ml and 100 µg/ml with 0.1 M carbonate-bicarbonate buffer, pH 9.6 (0.1 Na_2CO_3, 0.1 M $NaHCO_3$). Soluble scFv was diluted in 1:20 v/v in PBS containing 1% BSA and 0.05% Tween 20 (PBS-BT), while anti-E tag antibodies was diluted in 1:10 v/v, also in PBS-BT. The optical density of each well was read at OD450 nm using the BioRad Benchmark Plus Microplate Reader (BioRad, Hercules, USA). Graph of the M2e-ELISA readings was also produced using GraphPad Prism® version 7.02.

5.2.10 Antibody visualization and Western blotting

To visualise soluble scFv and phage displayed scFV anti-M2e antibodies, at least 100 µg/ml of soluble scFv and $1x10^{12}$ pfu/ml of recombinant phages were separated using 12% SDS-

PAGE at 100V. These protein molecular weight were estimated by using the Novel® Sharp Pre-stained Protein Standard (Life Technologies), and the SDS-PAGE were stained using Coomassie Brilliant Blue stain. For Western blotting, soluble scFV anti-M2e antibodies was separated using 12% SDS-PAGE followed by transfer to a nitrocellulose membrane using 1X transfer buffer (0.2 M glycine, 0.025 M tris base, 0.2 L methanol (v/v) in 1L) at 100V. The membrane was firstly washed with PBS-BSA 2%-Tween 0.05% for three times before blocked using PBS-BSA 10% solution for 2 hrs. The blocked membrane was washed with PBS-BSA 2%-Tween 0.05% for another three times and incubated with the primary antibody (anti-E tag mouse antibodies) for 1 hr at RT. Again, the membrane was washed with PBS-BSA 2%-Tween 0.05% for three times before the enzyme-conjugated secondary antibody (anti-mouse HRP antibodies) was added to the membrane and incubated for 1 hr at RT. Membrane was washed with PBS-BSA 2%-Tween 0.05% for another three times and developed using the 3,3′-Diaminobenzidine (DAB) tablet (Sigma) suspended in TBS buffer (0.05 M Tris, 0.15 M NaCl, pH 7.5) with hydrogen peroxide. Development of substrate was stopped after 10 minutes by washing the membrane in PBS and followed by washing in distilled water for three times. The membrane was dabbed dry with blotting paper and left to air dry. Western blotting for phage displayed scFV anti-M2e antibodies was also done as described above, with difference in its primary antibody (anti-M13 mouse antibodies).

5.3 Results

5.3.1 Selection of chicken sera PL80 for mRNA isolation

Following a high M2e-end titer shown in an indirect M2e-based ELISA in a previous study (Hasan, Ebrahimie, et al. 2016), the spleen lymphocytes from PL80 was selected as the candidate for mRNA isolation and construction of M2e phage display antibodies.

5.3.2 Chicken phage display scFv library

Amplification of V_H and V_L products showed an approximate size of 400 bp and 350 bp, respectively (Figure 5.1a). Incorporation of linker to obtain the full insert (V_H-linker-V_L) showed amplified products with the approximate sizes of 800 bp for product amplified with vector specific primers (S1F and S6R, Table 5.1) and 750 bp for product amplified using gene specific primers (H*SfiI* and L*NotI*, Table 1) (Figure 5.1b). This was again checked for selected individual clones after cloning of insert to the phagemid vector using the gene specific primers (a and d, Table 5.1), where positive inserts were observed from phagemid, with full and partial insert (the latter resulted from mixed colonies), as well as unsuccessful ligation product (Figure 5.1c). Screening for the insert size was again done from the phagemid of the post-panning (rescued) recombinant phages, where slight variation in the insert sizes was observable (Figure 5.1d).

5.3.3 Biopanning against M2e peptide and selection of M2e-specific chicken recombinant antibodies

Recombinant phagemid clones which are positive (contain the V_H-linker-V_L) were then infected with the helper bacteriophages to enable the production of bacteriophages displaying anti-M2e scFv antibodies. Selection of these bacteriophages was done by repeated binding, washing and elution of the bound (reactive) bacteriophages to the immobilised M2e peptides on the surface of a culture flask. Rescued bacteriophages (post-panning bacteriophages) were then screened to ensure the isolation of specific anti-M2e antibodies. A total of 5 µl of the recombinant phages from each biopanning were PCR-screened for positive inserts containing linked V_H and V_L using primers *a* and *d* (Table 5.1, Figure 5.1d). Visualization of post-panning positive bacteriophages using 12% SDS-PAGE and Coomassie Brilliant Blue stain stained revealed the expected protein bands of ~43 kDa, which was the expressed full-length scFv in comparison to the negative control bacteriophage without insert, pCANTAB5E (Figure 5.2). After the final biopanning cycle, the specificity of the isolated scFv anti-M2e

antibodies was checked by indirect M2e ELISA using anti-M13 as the secondary antibodies. Eight selected recombinant phages pools with positive scFv anti-M2e antibodies gave OD450nm reading of 0.08 to 0.18 (Figure 5.3).

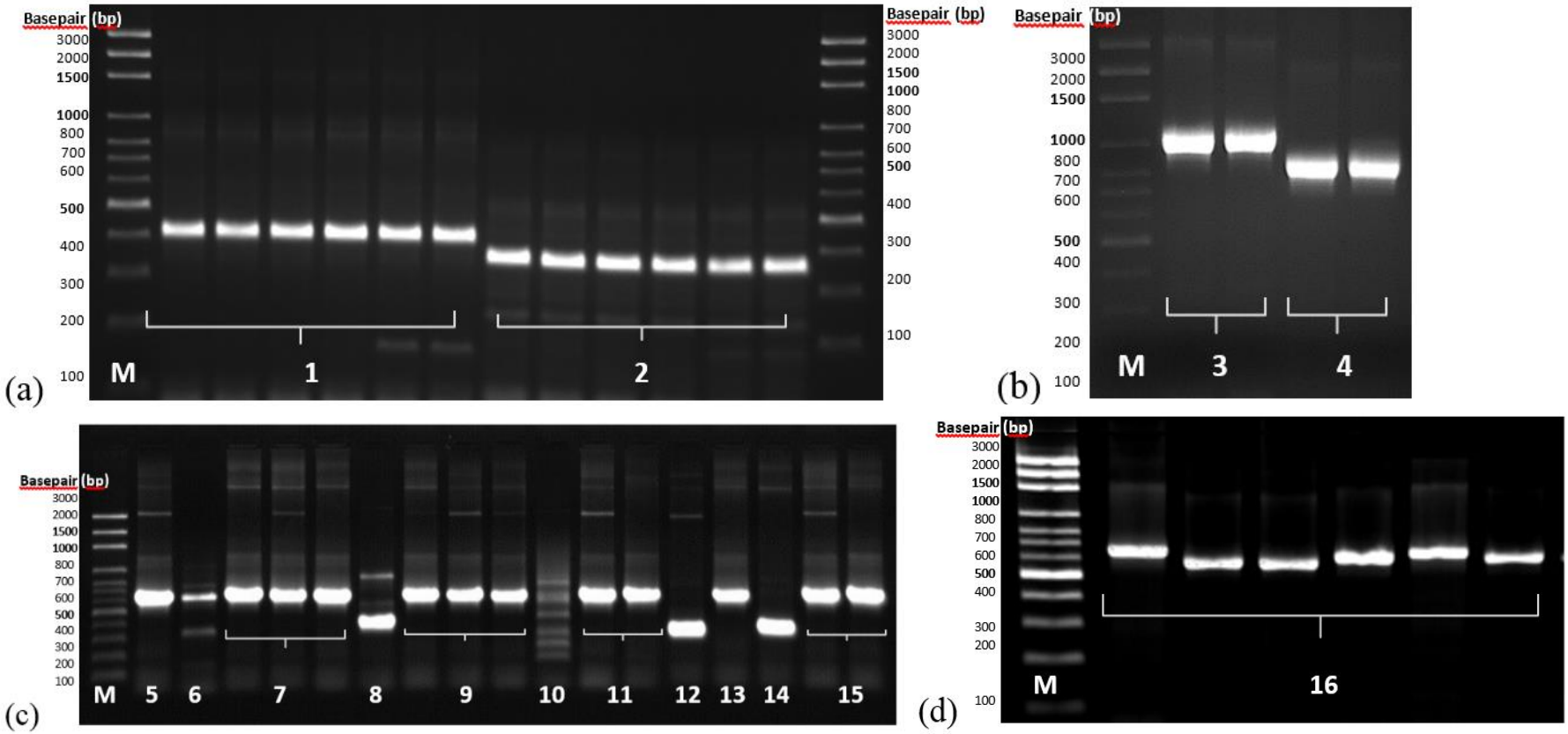

Figure 5.1 DNA products from (a) amplified products of 1: V_H (~400 bp) and 2: V_L (~350 bp), (M: 100 bp marker), (b) amplified products of 3: V_H-Linker-V_L combination after SOE PCR using vector specific primers (S1F and S6R primers, ~800 bp), and 4: V_H-Linker-V_L combination after SOE PCR using gene specific primers (HSfi and LNotI primers, ~750 bp (c) screening for V_H-Linker-V_L from bacterial colonies using gene specific primers, HF-SfiI and LR-NotI after ligation and cloning shows partial insert suspected of mixed colonies (5 and 7), the full insert (6 and 8), and unsuccessful ligate and clone product (9), (d) screening for V_H-Linker-V_L from individual rescued (post-panning) recombinant phages phagemid (~600-700 bp) (10). Slight differences in insert sizes may be due to alteration by the bacteriophages.

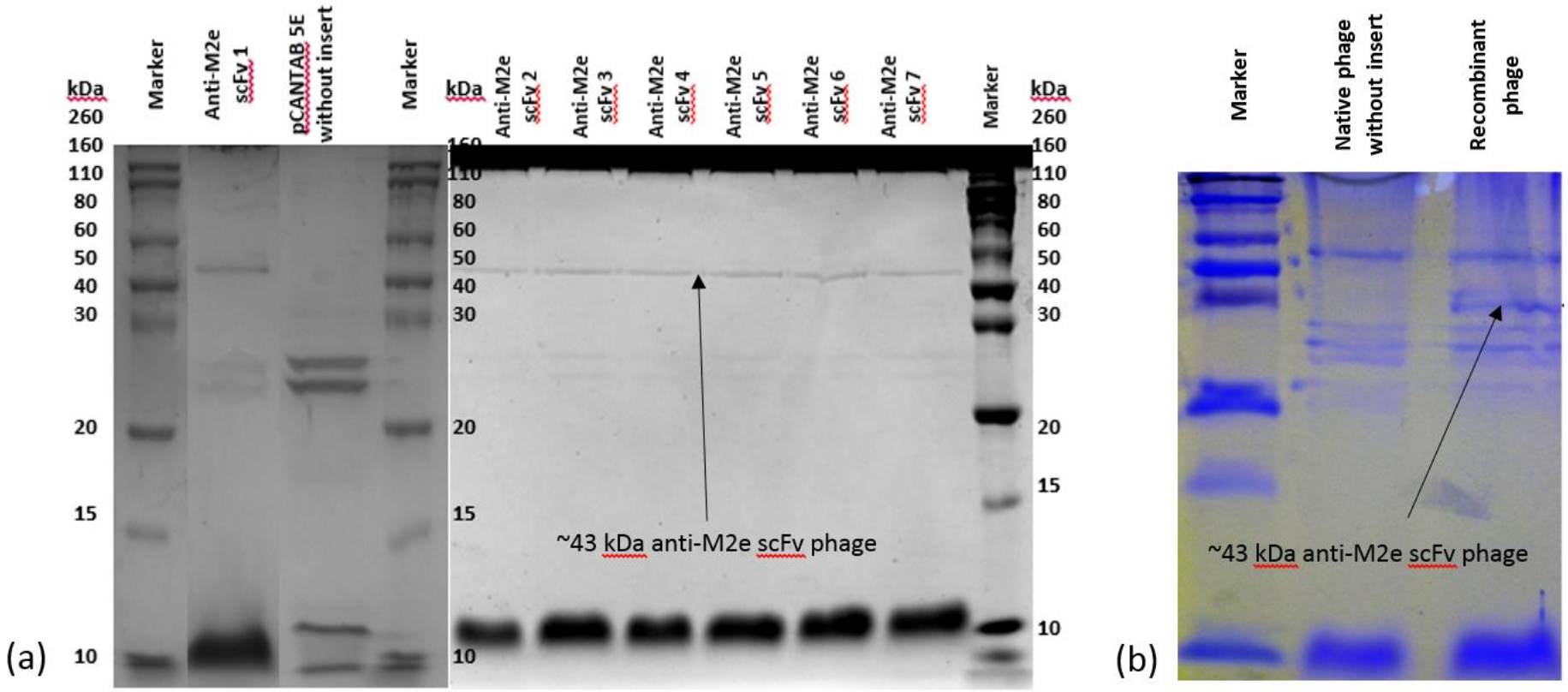

Figure 5.2 (a) Selected post-panning recombinant phages displaying anti-M2e scFv antibodies stained with Coomassie Brilliant Blue (lane 1, 3 to 8) in 12% SDS-PAGE shows protein bands at ~43 kDa, in comparison with negative control pCANTAB5E without any insert (lane 2). (b) Comparison of native phage or recombinant phage with the recombinant antibody visible at ~43 kDa.

5.3.4 **Soluble antibodies showed positive reactivity with indirect M2e-ELISA**

Specificity of the soluble scFv anti-M2e antibodies was again checked using M2e-ELISA and findings showed OD450 nm reading of 0.2 for both soluble scFv with 100 µg/ml final concentration of the M2e peptide (Figure 5.4).

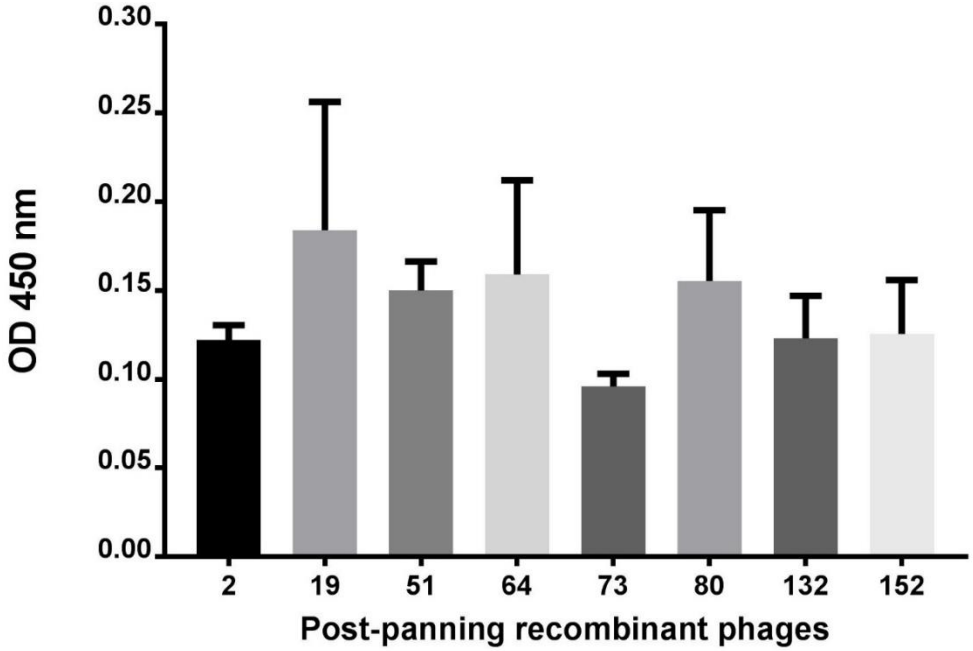

Figure 5.3 Selected post-panning recombinant phages displaying M2e-reactive scFv ($1x10^{12}$ pfu/ml) reading at OD450 nm done in duplicates detected using M2e-based ELISA (50 µg/ml peptide) using anti-M13 HRP (1:500).

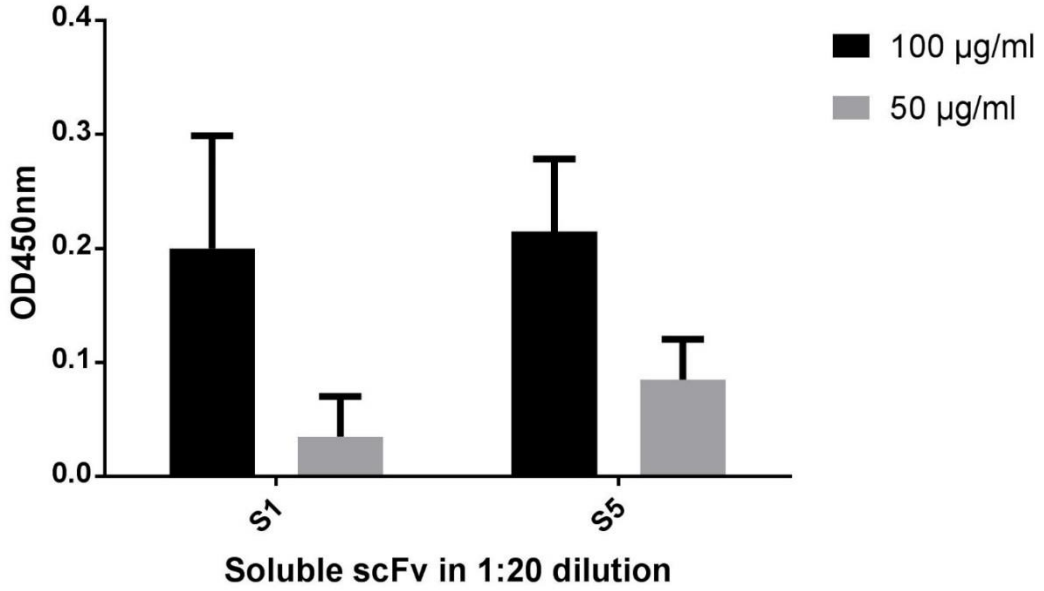

Figure 5.4 Two separate pools of soluble scFv (S1 and S5) reactivity at OD450 nm detected using M2e-based ELISA (100 µg/ml and 50 µg/ml of M2e peptide), with anti-E tag antibodies (1:10) and anti-mouse HRP (1:1000).

5.3.5 **Anti-M2e scFv antibodies visualization and specificity in Western blotting**

To further evaluate the specificity of the M2e soluble scFv anti-M2e antibodies, SDS-PAGE and western blotting were conducted. All soluble scFv anti-M2e antibodies were subjected to 12% SDS-PAGE and stained with the Coomassie Brilliant Blue stain for visualization of protein in SDS-PAGE. IPTG-induced soluble scFv anti-M2e antibodies expression comparison with the non-induced soluble scFv anti-M2e antibodies showed expressed proteins at ~43 kDa (Figure 5.5a), which were then desalted and concentrated using the size-exclusion column (Figure 5.5b).

All positive soluble scFV anti-M2e antibodies from four pools of recombinant bacteriophages were pooled together to increase scFv concentration and subjected to protein purification using Protein G HP SpinTrap (GE Healthcare) which targeted the affinity protein tag, E tag. Anti-E tag subclass was showed to be IgG_3 (Figure 5.6), which known to have a relatively strong binding to the trapping protein G column. ELISA titer showed that the anti-E tag mAb at 1:20 dilution gave OD450 nm reading of more than 1.5 which suggested good reactivity with the E tag peptide (10 µg/ml) (Figure 5.7).

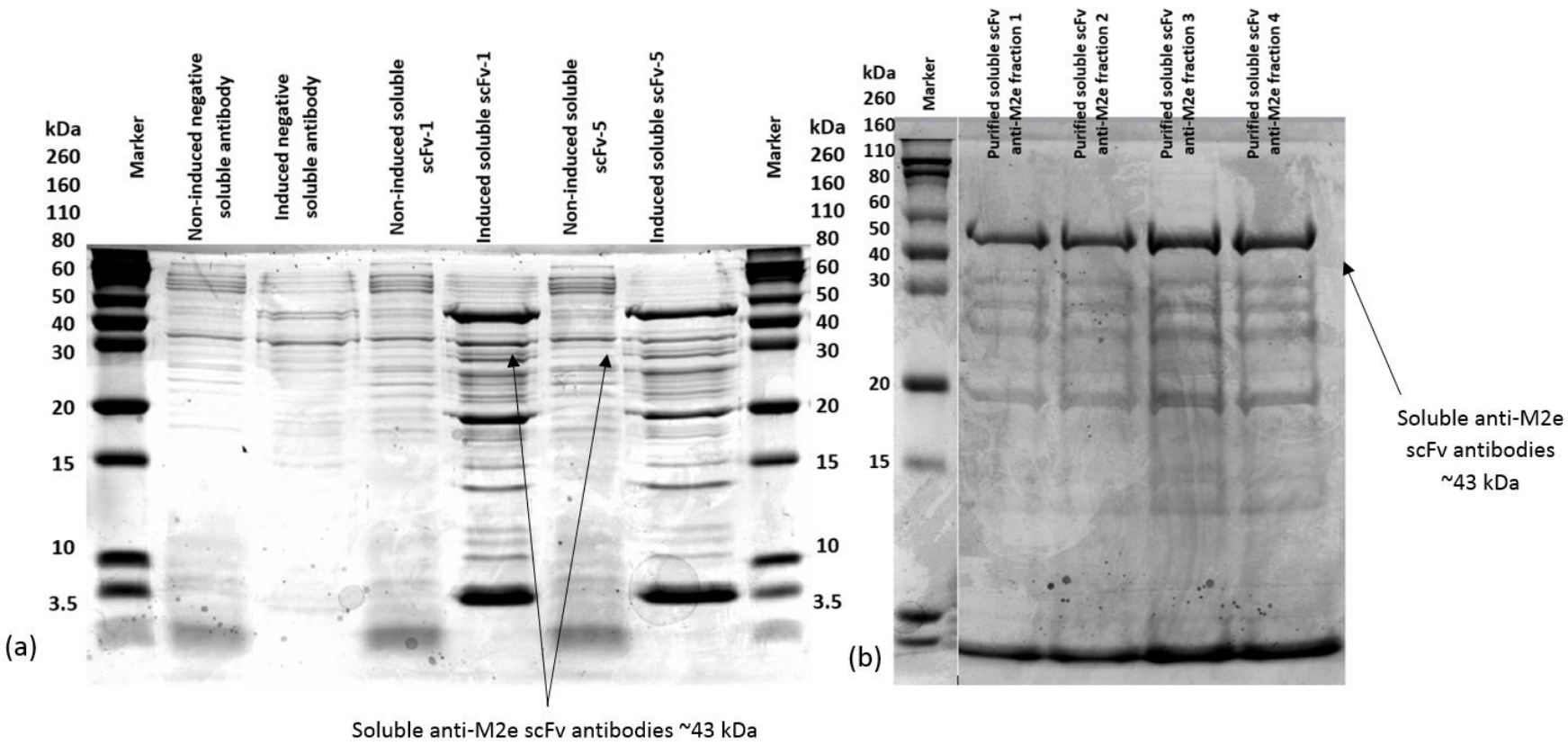

Figure 5.5 Soluble scFv-1 and soluble scFv-5 culture supernatants were run on a SDS-PAGE following induction with IPTG and osmotic shock isolation. Protein marker (M) were located at both end sides of the gel. 1: Negative control – non-IPTG-induced, 2: Negative control – IPTG-induced, 3: scFv sAb-1 – non-IPTG-induced, 4: scFv sAb-1 – IPTG-induced, 5: scFv sAb-5 – non-IPTG-induced, 6: scFv sAb-5-IPTG-induced. The expected product of soluble scFv anti-M2e antibodies is observed at ~43 kDa. (b) Soluble scFv anti-M2e antibodies expression after desalted and concentrated using size exclusion columns to at least 10 mg/ml.

Figure 5.6 Anti-E tag isotyping test shows that it is IgG_3 which showed relatively strong binding to the binding protein G. Left panel: C – positive control; G_1, G_{2a}, G_{2b} - mouse isotypes. Right panel: C – positive control; G_3, A, M – mouse isotypes. Red line indicates positive reaction.

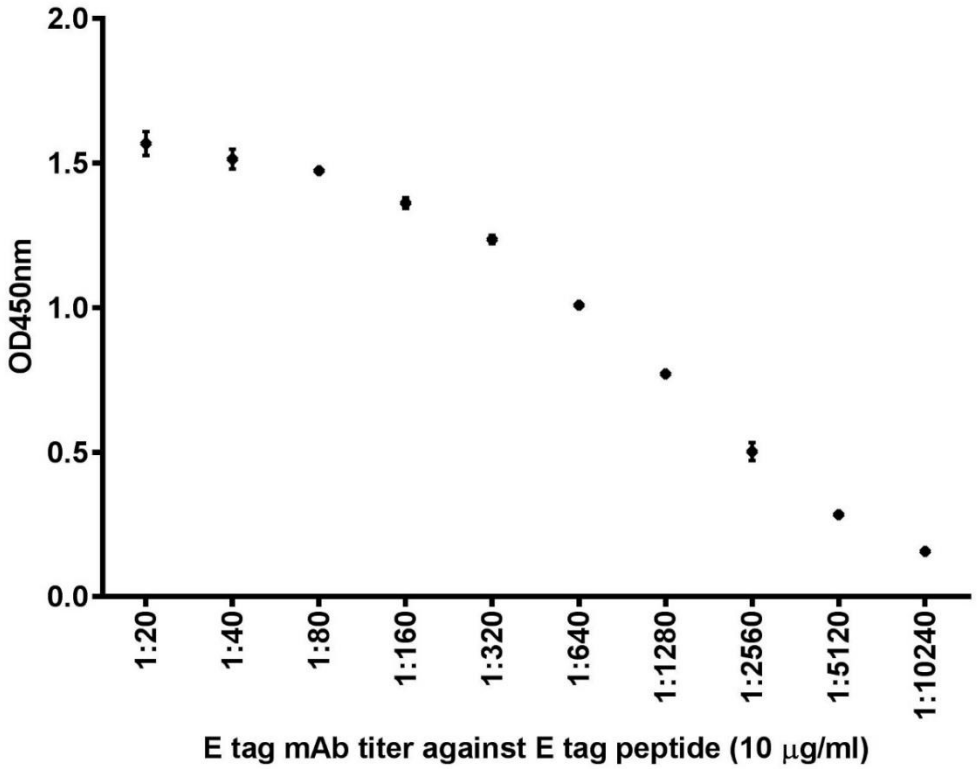

Figure 5.7 Anti-E tag monoclonal antibodies reactivity at OD450 nm against E tag peptide (10 μg/ml) in an indirect ELISA.

Comparison of flow-through, washed and eluted solutions containing antibodies showed recovery of the targeted scFv of ~43 kDa in the washed solution instead of the eluted solution (Figure 5.8a). Further attempt to confirm the specificity of the isolated scFV anti-M2e antibodies was done through Western blotting of the phage displayed form of the scFV anti-M2e antibodies. The targeted M2e scFv antibodies protein band was visible at ~43 kDa. Similar protein band was observed for the scFV anti-M2e antibodies tested with anti-M13 HRP (Figure 5.8b). However, no visible protein band was observed for the soluble anti-M2e scFv developed with anti-E tag monoclonal antibodies (E1, E2, E3).

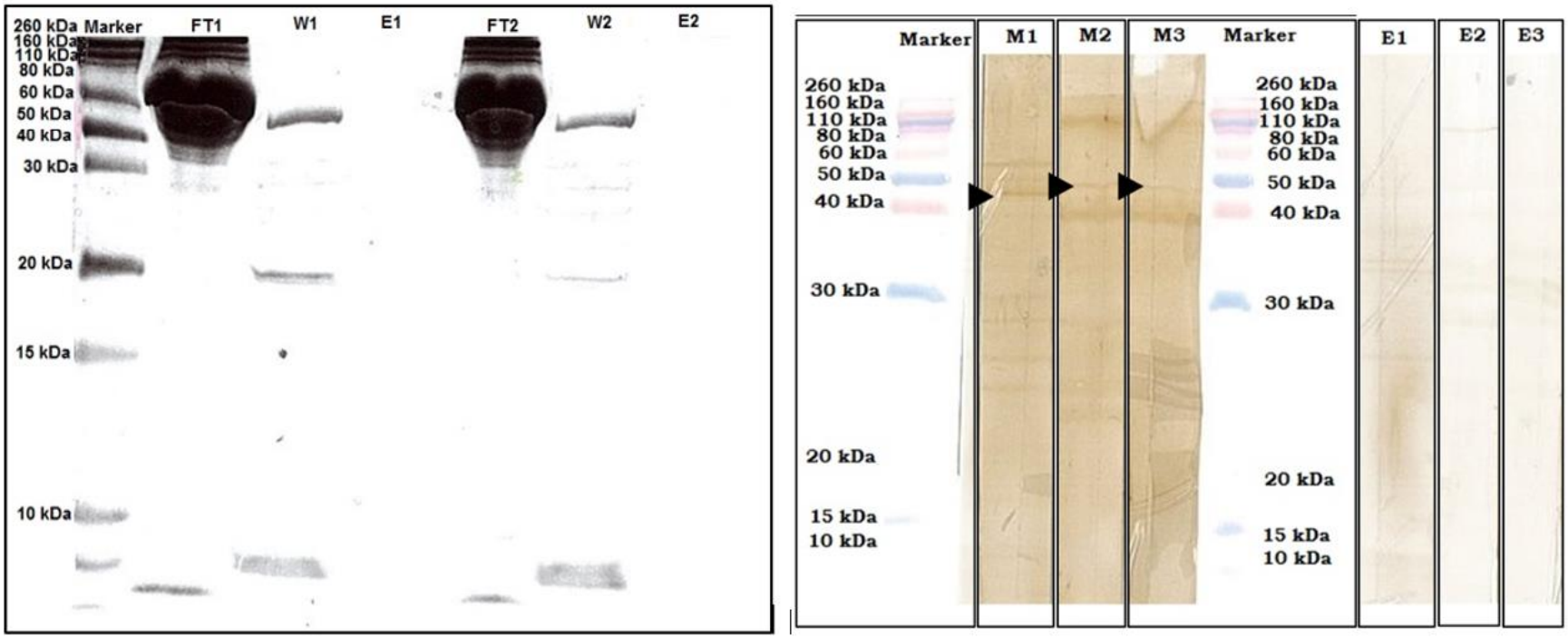

Figure 5.8 (a) Comparison of the flow through (FT) solution, wash (W) and eluted (E) soluble scFV anti-M2e antibodies during protein column purification shows recovery of the targeted protein (~43 kDa) in the washed solution; (b) Phage displayed scFV anti-M2e antibodies shows the targeted protein bands at ~43 kDa in response to anti-M13 HRP (M1, M2, M3), while no visible protein bands are observable for soluble anti-M2e scFv development with anti-E tag monoclonal antibodies (E1, E2, E3).

5.3.6 **Analysis of the isolated chicken recombinant antibodies sequence (crAb)**

To identify the resulted antibodies composition for anti-M2e protein, the post-panning recombinant phages were screened for positive insert using PCR with the insert primers (SfiI and NotI). The amplified insert sequences which encoded the anti-M2e scFv from the pooled positive recombinant phages (crAb-19, -51, -64, -73, -80, -152) (Figure 5.3) were then purified and sent for sequencing, and produced an average of 249 amino acid (Figure 5.9). These crAbs sequences were then aligned and the complementarity determining region (CDR) of the V_H and V_L regions were identified to analyse the variation contributed by each crAb (Figure 5.9). Most amino acid variations can be observed at the CDR regions, especially at the heavy chain CDR (CDRH). It is noted that CDRH is responsible for 55.6% of the overall variation in the crAbs, in comparison to the light chain CDR (CDRL) region, with 42.9% (Table 5.2). Meanwhile, within the V_H region, CDRH3 contributed the highest variation overall with 34.3% (CDRH3 variation (12)/total variation aa H and L (35)), and within the V_L region, CDRL3 contributed 22.8% (CDRL3 variation (8)/total variation aa H and L (35)). It was noted that two types of modifications were observed for the linker sequence in comparison to the original linker sequence. One being the deleted linker repeat (one of three linker repeats), while another was the substitution of glycine (G) to cysteine (C) in one of the anti-M2e scFv clones representative (Figure 5.9).

Table 5.2. Variation percentage calculation per complementarity determining region (CDR), per chain (heavy and light) and overall variation observed in percentage. Calculations were done as follows: variation aa = (variation aa/total aa)*100; variation per chain = (total variation aa for H OR L /overall total aa for H OR L)*100, accordingly; overall variation = (total variation for each chain/total aa for H AND L)*100.

Region		Total aa	Variation aa (%)	Variation %	
				per chain (H/L)	Overall
H	**CDRH2**	18	8 (44.4)	40.0	55.6
	CDRH3	18	12 (70.6)	60.0	
Subtotal		36	20	100.0	
L	**CDRL1**	6	4 (66.7)	26.7	42.9
	CDRL2	9	3 (33.3)	20.0	
	CDRL3	10	8 (80.0)	53.3	
Subtotal		25	15	100.0	
TOTAL		**61**	**35**		

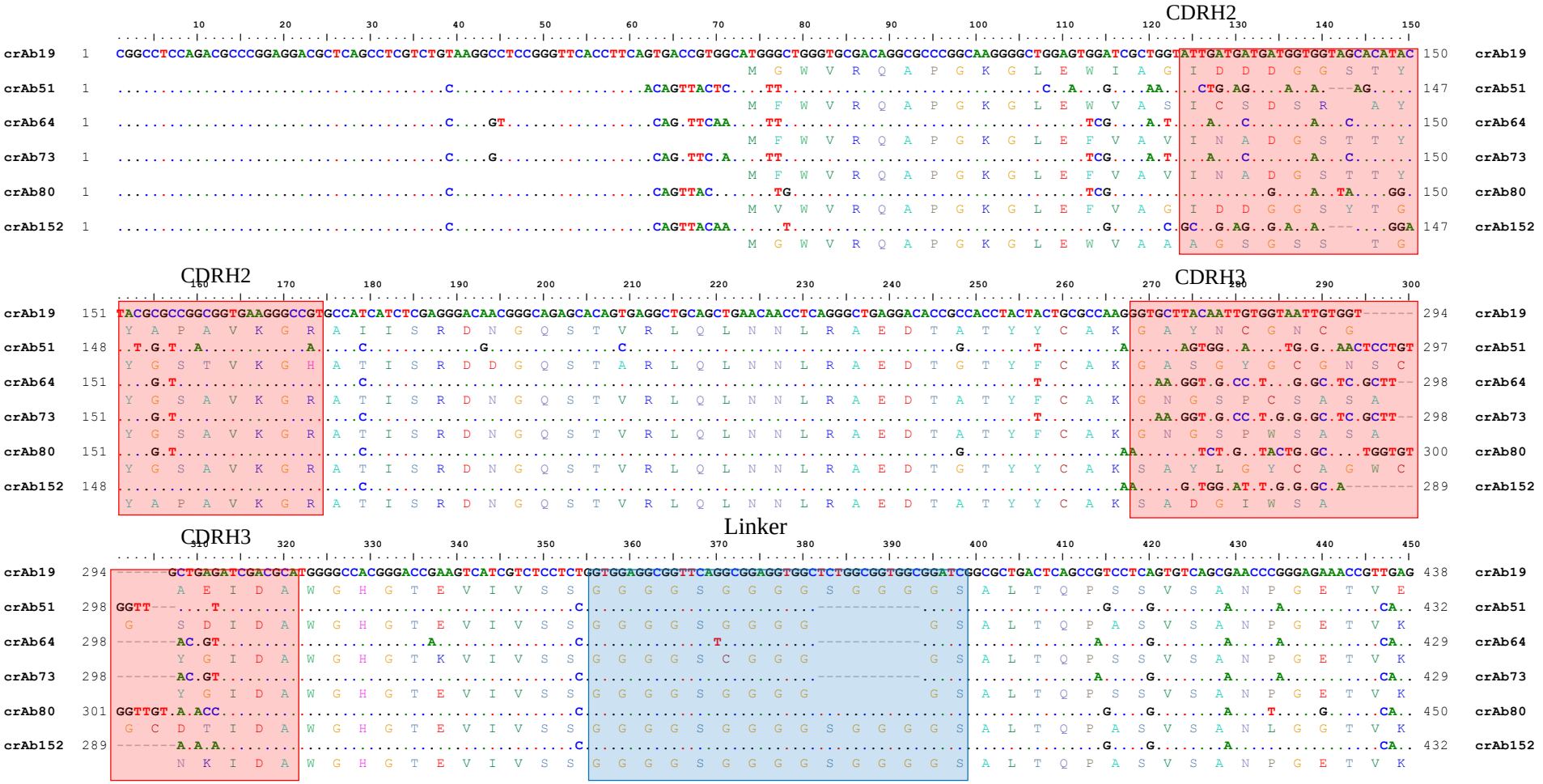

Figure 5.9 Amino acid sequence alignment of the anti-M2e scFv antibodies representatives. Sequences identical to the top-most sequences are indicated by dotted lines (.), while amino acid gaps are indicated by dash (-). Variable heavy (V_H), linker, and variable light (V_L) regions are indicated. Complementarity determining regions 1-3 (CDR1-CDR3) are indicated for both V_H and V_L chains (CDRH, CDRL). Flexible $(Gly_4Ser)_3$ is indicated with the blue shades, while the E tag is in green.

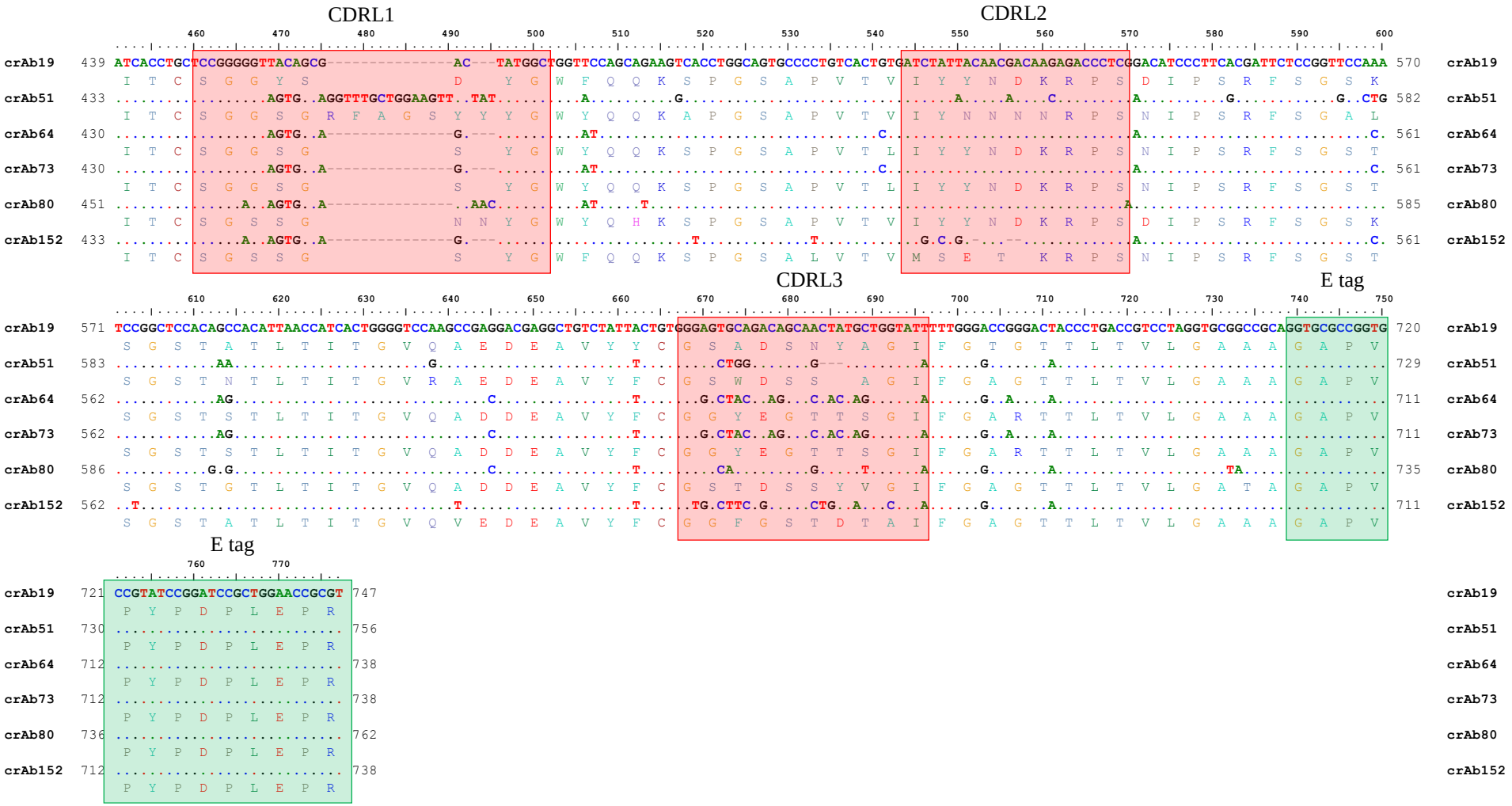

Figure 5.9. Continued.

5.4 Discussions

We described here the construction, expression, purification and immunological characterization of reactive scFV anti-M2e antibodies (scFv) in investigation of its use as the basis for diagnostic tool in AIV sero-surveillance study in H5N1 enzootic countries. To our knowledge, this study is the first attempt in isolating avian origin, recombinant anti-M2e antibodies in scFv form.

The M2e-indirect ELISA results showed that the isolated scFV anti-M2e antibodies bind M2e, following purification of the soluble form of the antibodies. M2e protein itself has a low antigenicity due to its small size (Johansson, Moran & Kilbourne 1987) and relatively low density on the surface of the virus particle in comparison to the other surface proteins, namely hemagglutinin and neuraminidase (Feng et al. 2006). A potential advantage of the phage display system is that four or five copies of the protein product of an introduced gene can be displayed on the pIII part of the bacteriophage (Bazan, Calkosinski & Gamian 2012; Huang et al. 2005; Smith 1993). In the case of scFv anti-M2e, this might lead to enhanced antigen binding ability. Indeed, others have shown that a dimer form of scFv, e.g scFv anti-mycelia for pathogenic fungus *Sclerotinia sclerotiorum* had better antigen binding than the monomeric form (Yajima et al. 2008). Low quantity yield of the purified anti-M2e scFv from this study may have been related to the binding capacity of the tag protein used in the scFv purification in relation to the scFv linker. This was suggested as the targeted anti-M2e scFv was expressed in its soluble form but not detected in the Western blotting.

5.4.1 Minimal detectability of the tag protein and low yield in both Western blotting and protein purification

Relatively low concentration of the scFV anti-M2e antibodies was retrieved after column purification (Figure 5.8a), despite the high intensity of the protein bands observed after protein expression (Figure 5.5). Positive reactivity to M2e antigen has been consistently

observed in ELISA for both phage displayed and soluble forms of the anti-M2e scFV antibodies. It was observed that the anti-M2e scFv (~43 kDa) was retrieved in the washed solution (Figure 5.8a), which indirectly indicated the anti-E tag antibody poorly able to capture the E tag-tagged anti-M2e scFv antibodies during affinity column purification. Therefore, it is assumed that the low yield of the anti-M2e scFv may be related to either biological or physical factors, or possibly both. For example, impaired binding of anti-E tag antibodies to the targeted crAbs, or the non-optimal condition during purification may led to such outcome. Generally, according to the manufacturer, antibodies with isotype IgG_3 (anti-E tag antibodies) are known to have high binding compatibility with the protein G, the binding protein used for purification. Hence, compatibility between the tag protein antibodies and the binding protein is presumably not a problem.

Next, the ability of the anti-E tag antibodies to capture the expressed E tag protein at the C-terminal of the scFV anti-M2e antibodies is in question. Ability of anti-E tag antibodies to bind to E tag peptides has been demonstrated prior to its application (Figure 5.7). This verified reactivity of the anti-E tag antibodies employed to capture the expressed E tag in the affinity column. An alternative explanation for this problem might be the poor accessibility of the E tag on the scFv anti-M2e antibody. This assumption is further suggested with the absence of the targeted protein bands in the Western blotting for the phage displayed scFV anti-M2e antibodies (Figure 5.8b).

Tag proteins can play a significant role in the isolation of the purified protein. In this study, E tag tag protein has been employed as it has shown to be an effectively functional tag protein in previous studies (Abdelkader & Rifaat 2007; Bjerketorp et al. 2004; Wall et al. 2003). Small size of the E tag is desirable since theoretically it will not interfere with the targeted antibody reactivity against the antigen. However, possibly that low expression levels of the

isolated scFv or low intrinsic affinity when the scFv is not in its phage displayed form contributed to the low protein yield in current study (van Wyngaardt et al. 2004). This relates to the cooperative effect of the copies of scFv expressed on the surface of the bacteriophage, which explains the higher intensity detection in ELISA (Sapats et al. 2003).

Gly-Ser linker plays an important role in holding the scFv conformation, imitating a natural antibody. Sequence analysis on the isolated scFv anti-M2e antibodies showed a shortened linker sequence in three of the six representatives obtained for the anti-M2e scFv antibodies (Figure 5.9). Changes in its amino acid was also observed in one of the scFv, where it include a shift of glycine (G) to cysteine (C) (Figure 5.9). It was observed in previous study that a shift from G to serine (S) and vice versa was noted, and this did not significantly affected the stability and flexibility of the linker since the scFv anti-M2e antibodies were still detectable in the ELISA (Finlay et al. 2006). Changes from G to C was relatively unusual, with no known effects. Although the changes in the linker sequences does not affect the reactivity of the scFv itself, position of the E tag protein at the C-terminal of the anti-M2e scFv antibodies may have been partially concealed and poorly accessible to anti-E tag antibodies at the scFv final conformation. This was later showed in the purified protein yield, where only a low amount of the tagged scFv anti-M2e antibodies were isolated. Further study is needed to confirm this assumption.

5.4.2 **M2e-cRABs reactivity and the initial diversity of antibody library**

In our study, the antibodies were derived from immunized birds which showed high reactivity for anti-M2e antibodies. Thus, it is a reasonable assumption that antibody libraries generated from immunized bird would be highly specific and possess a high quality pool of the targeted antibodies. Previous studies had well documented that the V_H region, especially the complementarity-determining region 3 (CDR3), is important for antigen binding and

interaction (Boder, Midelfort & Wittrup 2000; Fermer et al. 2004; Kabat & Wu 1991). This was evidenced by the higher mutation rate in this region in comparison to the others (Boder, Midelfort & Wittrup 2000; Chowdhury & Pastan 1999; Finlay et al. 2006). More than a 1000-fold increase rate of association in monovalent ligand-binding affinity showed to accompany higher mutation rates in the V_H CDR3 region (Boder, Midelfort & Wittrup 2000), while better affinity was observed with increased amino acid length in both V_H and V_L CDR3 regions (Finlay et al. 2006). Also, improvement in anti-mesothelin scFv binding ability of 15- to 55-fold observed to be contributed by random mutations in the V_L CDR3 region (Chowdhury & Pastan 1999). Other findings suggested that the V_L region is also important in determining the specificity and affinity of the isolated antibodies (Hoet et al. 1999; Jang & Sanford 2001; Sapats et al. 2003), where one study isolated a clone which lack entire V_H region but still capable of binding to the antigen (van Wyngaardt et al. 2004). These are in agreement with our isolated anti-M2e scFv antibodies, where high variability of amino acid observed in both V_H and V_L CDR3 regions of the reactive clones. Isolation of six reactive anti-M2e scFv representative clones which resulted from a pooled library of recombinant phages may not be able to represent the actual diversity of the anti-M2e scFv library constructed in this study. It is likely that these representatives were the most dominant clones within the pooled reactive clones to the M2e antigen. Nevertheless, these anti-M2e scFv clones have provided an insight on the diversity demonstrated by the dominant reactive clones isolated from the high quality pool of the targeted antibodies.

5.4.3 **Conclusion and Recommendations**

This work can be further improved by employing different types of tag protein such as His-tag or FLAG (Kirsch et al. 2005; McCafferty et al. 1994). Compatibility of the tag protein and the vector used in this study may also need to be considered. It is noted that scFv expression level varies based on the vector used and the system the scFv is used against (Qi et al. 2012;

Soltes et al. 2007). For example, expression of scFv may be increased by subcloning it into a highly efficient expression system such as pBV220 (Yang et al. 2011). Selection of the helper phage used in complement with a vector of optimal compatibility may contributed to the isolation of better quality antibodies (Baek et al. 2002; Soltes et al. 2007). For instance, KM13 is suggested to perform better than M13K07 as the former showed high discriminatory power manifested by the lower number of eluted phage from the first selection round (Goletz et al. 2002).

Phage display technology is an effective way to isolate pure protein with carefully optimized methods and experience. However, it is rather a long and tedious approach as it is time and energy consuming. As a conclusion, this study has successfully isolated phage displayed and soluble scFV anti-M2e antibodies with high reactivity against the AIV-M2e antigen. Based on these, an H5N1 serosurveillance test based on the anti-M2e scFv antibodies seems promising. Further enrichment and purification of the isolated scFv anti-M2e antibodies is recommended for development of a diagnostic tool which is capable of rapid AIV serosurveillance and DIVA application to prevent further outbreak, especially in H5N1 enzootic countries.

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6.1 General discussions

Overall, this study has demonstrated the promising potential of the AIV M2e protein as the target for diagnostic tool application for AIV infection surveillance. In H5N1 enzootic countries where vaccination using the inactivated AIV virus are practiced, a diagnostic tool with DIVA capability is highly of interest. Indirect M2e-ELISA is proven to be economical with high-throughput capacity, apart from sensitive and specific for AIV infection surveillance. As AIV is a multispecies agent (Chambers, Dubovi & Donis 2013), availability of a more universal test format such as competitive ELISA is ideal. However, the key factor for such universality lies on the ability of the competitor antibody to demonstrate cross-reactivity in multiple species, as previous findings do observed slight variations of antibodies to the same antigen in different species (Almagro 2004; Darnule et al. 1980; Rotter et al. 1983). Identification of the best anti-M2e antibodies source which can be used as the competitor antibodies has brought the success on characterization of the dominant epitope of AIV M2e protein from mouse, rabbit and chicken anti-M2e antibodies.

Overlapping of recognised M2e epitopes has been observed across literatures on M2e protein studies, which is in agreement with findings from this study (De Filette et al. 2011; Fu et al. 2009; Grandea III et al. 2010; Liu, W, Li & Chen 2003; Liu, W, Zou & Chen 2004; Pejoski et al. 2010; Wang et al. 2009; Zabedee & Lamb 1988; Zhang et al. 2006; Zharikova et al. 2005; Zou, Liu & Chen 2005). Generally, we agrees that epitope ^{6}EVETPTRN^{13} is the dominant epitope for the M2e protein. However, this study suggested that rabbit and chicken anti-M2e antibodies showed a slightly different preferences in its epitope. This indicated that different animal species may produce similar antibodies to the same antigen, but with fine difference in epitope recognitions, as observed previously (Hjelm et al. 2012). This knowledge is important especially for diagnostic test development and vaccine development where caution should be applied in using antibodies from different host to the same antigen. Therefore, this ruled out

the possibility of using rabbit anti-M2e antibodies as the candidate for an M2e-based competitive ELISA development.

Findings from the M2e protein antigenic mapping has brought the attempt on the development of a competitive ELISA based on M2e protein for AIV infection screening. As noted earlier, competitive ELISA-based diagnostic tool is highly attractive due to its potential on multispecies applicability (Starick et al. 2006; Zhou et al. 1998). This is especially for a multiple host pathogen, as a competitive ELISA-based tool will remove the needs for species-specific secondary antibodies. Our findings demonstrated the ability of a mouse monoclonal anti-M2e antibodies to be used as the competitor antibodies in the M2e-based competitive ELISA. It also showed the M2e protein ability in differentiating between infected and vaccinated-only animal sera, as noted in previous works using M2e protein in an indirect ELISA-based approach (Hadifar et al. 2014; Hemmatzadeh et al. 2013; Kim et al. 2010; Lambrecht et al. 2007; Tarigan et al. 2015). This finding is significant as the presented competitive M2e-ELISA showed high specificity and sensitivity to AIV infection which can be developed to a full in the field diagnostic tools.

Studies showed that antibodies in a scFv format also possess high sensitivity and specificity for pathogen antigen detection (Chen et al. 2014; Nimmagadda et al. 2012). Therefore, this study has successfully isolated the scFv form of the anti-M2e antibodies from H5N1 immunised chicken. The isolated anti-M2e scFv antibodies are highly reactive and specific in both of its recombinant phage form and soluble antibodies form. This is a critical achievement as this was the first attempt on the isolation of the anti-M2e scFv antibodies, considering the protein's low immunogenicity. High affinity antibodies are highly desirable in its selection for further immunological based application. However, equally important are factors such as the antibody specificity, expression level and stability where chances of securing these are

proportionate to the diversity of the V-gene repertoire from the sample pool (Pansri et al. 2009). Hence, enrichment of the targeted antibodies through the use of immunised donor as the source of immunoglobulin gene isolation coupled with the use of phage display technology is a significant approach to ensure such outcomes.

It is acknowledged that this study is limited in terms of the individual species tested for the anti-M2e antibodies. Future studies which includes antibodies sourced from more animal species may provide additional clues on the intrinsic or extrinsic factors which may have influenced such differences between the epitope recognised by the antibodies from different animal species. Although such differences may have been related to the nature of the immunogen itself, it may have been also dependant on the level of host animal exposure to the immunogen (Hjelm et al. 2012). As mouse has been used in most of the studies which discovered the M2e epitopes, the recognised epitopes must have been biased towards mouse antibodies, while it is not the natural host for AIV, as such is also the case for rabbit. Therefore, epitope recognised by chicken antibodies, the natural host for AIV shows more defined recognition for the M2e epitope. Nevertheless, further research is required to verify this assumptions.

In this study, only chicken sera are available to be tested as its development is meant to be applied for the AIV infections surveillance in the vaccinated poultry industry. Nevertheless, it is noted that the question whether it is applicable for use in other species apart from chicken still remains. Previous study on duck challenge experiment using H5N1 indicated that the detectability of the M2e antibody level varies, and is likely dependent on the specificity of the antigen used in an indirect ELISA (Lambrecht et al. 2007). It is important to keep in mind that for optimum vaccination system in duck, factors that needs serious consideration includes the challenge virus, duck species use, vaccination protocols, role of maternally derived antibodies

as well as the synergies between co-infecting pathogens (Pantin-Jackwood & Suarez 2013). Further study which includes four types of sera (non-vaccinated and non-challenged sera, vaccinated only sera, infected-only sera, and vaccinated-then-challenged sera) from a larger range of animal species will be able to provide answers. It will be interesting to see if the observed findings in this study for chicken, which is a domestic poultry, will be resonated when the same test is to be tested using wild bird's sera.

Although indisputably reactive to the M2e antigen, these anti-M2e scFv antibodies shows minimal detectability in the Western blotting. This may be related with the final conformation of the anti-M2e scFv antibodies in different conditions, such as in an ELISA system, and immobilised on a nitrocellulose membrane during Western blotting. It is noted that different expression detection system may results different behaviour of the antibody as noted in previous studies (Sapats et al. 2003; Van Wyngaardt & Du Plessis 1998), although this was not always true (Muller et al. 1997; Tikunova et al. 2001). Therefore, it would be interesting to explore if there are variations in the protein conformations through the prediction of the 3D structure of the isolated antibodies using x-ray crystallography or nuclear magnetic resonance approaches (Liu, G et al. 2005; Newby et al. 2009).

ELISA are sometimes accompanied by lower analytical sensitivity and are limited to certain types of sample (Hoffmann et al. 2009). Therefore, alternatives technology such as real time PCR (RT-PCR) is one of the highly sorted option and the recommended technology in routine diagnostic work for microorganism infection detection in animal due to its convenience, sensitivity and rapidity (Vidanovic et al. 2016). RT-PCR approach is known to be capable of enabling epidemiological investigation of microorganism apart from its use for the microorganism identification and genotyping (Gwida et al. 2016). It has been highly used for detection of notifiable livestock diseases, namely the foot-and-mouth disease, classical swine

fever, bluetongue disease, avian influenza and Newcastle disease (reviewed in Hoffmann et al. (2009).

In consideration of DIVA application, RT-PCR is suitable for application in disease detection which the targeted gene or combination of gene is credible for differentiating wild-type and vaccine-type virus. Such observation was noted for the canine distemper virus (CDV) in dogs (Dong et al. 2014). Duplex RT-PCR employed primers specific to the highly conserved region of the CDV, and primers which are specific to the wild-type strain CDV. Different size of the amplified products was the indicator for the wild-type virus or vaccine strain genome (Dong et al. 2014). Another example was the targeted group-specific-antigen (*gag* gene) for the feline immunodeficiency virus (FIV) which has been demonstrated to be useful in differentiating between FIV-vaccinated and FIV-infected cats (Wang et al. 2010). Differently labelled probe for different FIV subtypes made the vaccine subtypes to be easily identified. Such application is doable for AIV infection detection, as AIV strain used as vaccine can be easily tagged and differentiated from the circulating AIV strain infecting the host.

Another interesting approach is the multiple microsphere-based assay. It enables the detection of multiple analytes simultaneously through detection by antibodies coupled to different microsphere types (Powell et al. 2013; Wood, O'Halloran & VandeWoude 2011). This was done by internally dyeing the polystyrene microsphere with two or three distinct fluorochromes spectral, so that only unique fluorescence signature are emitted for each microsphere type (Dunbar & Hoffmeyer 2013). This technology also requires a reduced volume of sample to evaluate multiple antibodies, apart from being superior in detecting low level analyte in comparison with ELISA (Powell et al. 2013). Its ability to be multiplexed means that it can be used to detect multiple types of common virus infection in poultry at once, such as Marek's disease, Newcastle disease virus, and infectious laryngotracheitis virus,

apart from influenza virus (Boodhoo et al. 2016; Bulbule et al. 2015; Coppo et al. 2013). A recent development of a microsphere-based assay for detection of antibody to influenza A and Newcastle disease viruses had shown high sensitivity (9.7% and 95.4%, respectively) and specificity (97.3% and 98.5%, respectively). However, such improvement in sensitivity is also accompanied with reduced level of specificity, which means that false positive increased in comparison to ELISA (Powell et al. 2013), especially in detection of analytes at lower concentrations (Dossus et al. 2009; Elshal & McCoy 2006; Nifli et al. 2006).

Nevertheless, these technologies are still costly to support surveillance works in a developing country, which made recombinant technology-based ELISA is still the best option at the moment. It is undeniable that emergence of new technologies will likely bring better options for DIVA purposes, and made the cost for RT-PCR and microsphere-based assay negligible. The RT-PCR would be one of the next best option in consideration of its sensitivity and rapidity for results. Meanwhile, multiple microsphere-based assay would be best in condition where multiple detection of different analytes in one individual is necessary or highly recommended for detection of multiple infections. In the end, what matter is which technology is sensitive and specific enough to detect virus infection during surveillance, with the ability to differentiate between antibodies due to virus infection and vaccination (DIVA), and at the same time is easily accessible and economic for a big scale recurring applications.

6.2 Conclusions

In conclusion, this study has successfully accomplished three tasks, namely (i) identified the most reactive epitope for the M2e protein by mapping the M2e antigen, while (ii) developed a competitive ELISA (cELISA) as a diagnostic tool based on the AIV-M2e protein using a monoclonal antibody as a competing antibody for chicken sera, and (iii) constructed and isolated the reactive anti-M2e recombinant antibodies in a single chain variable fragment

(scFv) format. All these tasks are completed through the usage of M2e-based indirect ELISA for the M2e antigenic mapping, M2e-based cELISA assay development and using phage display technology for the anti-M2e scFv isolation, respectively. These are done in response to the suggestions that an M2e-based diagnostic tool will be an ideal target protein for a rapid, specific and sensitive DIVA tools for AIV infection surveillance, especially in H5N1 enzootic countries. This includes an M2e-based cELISA format which envisioned a rapid and universal, species independent diagnostic tool for AIV infection surveillance. Previous attempt on the development of an M2e-based cELISA using rabbit polyclonal antibodies as the competitor was unsuccessful. Hence, antigenic mapping of antibodies against M2e protein originated from various animal hosts are carried out to finely map the AIV M2e protein dominant epitope. Although an M2e-based indirect ELISA using recombinant M2e protein as antigen shows to be highly sensitive and specific, it is suggested that antibodies in scFv form constructed from a H5N1 immunised chicken will produce anti-M2e library which are highly specific and reactive to M2e protein. Therefore, this study has managed to found the following: (i) epitopes ^{6}EVETPTRN^{13} and ^{10}PTRNEWECK^{18} are identified as the dominant epitope for anti-M2e antibodies in rabbit, and mouse and chicken, respectively, (ii) anti-M2e monoclonal antibodies originated from mouse demonstrates its ability to be used as the universal competitor antibody in a M2e-based cELISA format diagnostic tool for AIV infection surveillance which is capable of DIVA, and (iii) anti-M2e scFv antibodies which are highly reactive to the M2e antigen have been successfully isolated.

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APPENDIX

Supplementary 1

Principal Component Analysis: 1, 2, 3, 4, 5, 6, 7, 8, A, B, C, Full

Eigenanalysis of the Correlation Matrix

Eigenvalue	4.3760	3.3972	2.2004	0.8835	0.5474	0.3408	0.1058	0.0839	0.0620	0.0025
Proportion	0.365	0.283	0.183	0.074	0.046	0.028	0.009	0.007	0.005	0.000
Cumulative	0.365	0.648	0.831	0.905	0.950	0.979	0.988	0.995	1.000	1.000

Eigenvalue	0.0005	0.0000
Proportion	0.000	0.000
Cumulative	1.000	1.000

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
1	0.345	0.021	-0.381	-0.283	-0.111	0.327	0.279	-0.644	0.187	0.022
2	-0.211	-0.107	-0.568	0.222	-0.021	0.019	0.251	0.112	-0.282	0.377
3	-0.218	-0.146	-0.559	0.167	-0.048	-0.067	0.027	0.179	-0.073	-0.293
4	0.063	-0.509	0.193	0.019	0.075	0.075	0.330	-0.065	-0.256	-0.099
5	0.273	-0.405	0.010	0.235	0.261	0.239	0.145	0.208	0.226	-0.484
6	0.457	-0.043	-0.091	0.023	0.048	-0.103	-0.441	-0.170	-0.712	-0.139
7	0.424	0.032	-0.216	-0.087	-0.118	0.403	-0.333	0.522	0.205	0.237
8	0.403	-0.035	-0.013	-0.370	-0.226	-0.555	0.443	0.344	-0.025	0.048
A	-0.062	-0.475	-0.169	-0.029	-0.332	-0.416	-0.448	-0.215	0.376	-0.063
B	0.046	-0.503	0.228	0.106	0.025	0.097	-0.030	-0.052	-0.018	0.635
C	0.324	0.175	-0.136	0.421	0.571	-0.401	0.017	-0.140	0.268	0.201
Full	0.220	0.173	0.167	0.673	-0.638	0.030	0.158	-0.074	0.006	-0.038

Variable	PC11	PC12
1	0.072	-0.063
2	-0.123	0.509
3	0.230	-0.638
4	-0.661	-0.251
5	0.298	0.376
6	0.113	0.066
7	-0.301	-0.128
8	0.148	0.009
A	-0.182	0.180
B	0.462	-0.224
C	-0.169	-0.145
Full	-0.008	-0.040

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Review Article

Avian Influenza Virus and DIVA Strategies

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Abstract

Vaccination is becoming a more acceptable option in the effort to eradicate avian influenza viruses (AIV) from commercial poultry, especially in countries where AIV is endemic. The main concern surrounding this option has been the inability of the conventional serological tests to differentiate antibodies produced due to vaccination from antibodies produced in response to virus infection. In attempts to address this issue, at least six strategies have been formulated, aiming to differentiate infected from vaccinated animals (DIVA), namely (i) sentinel birds, (ii) subunit vaccine, (iii) heterologous neuraminidase (NA), (iv) nonstructural 1 (NS1) protein, (v) matrix 2 ectodomain (M2e) protein, and (vi) haemagglutinin subunit 2 (HA2) glycoprotein. This short review briefly discusses the strengths and limitations of these DIVA strategies, together with the feasibility and practicality of the options as a part of the surveillance program directed toward the eventual eradication of AIV from poultry in countries where highly pathogenic avian influenza is endemic.

Avian Influenza Virus

Gene segments and proteins

AVIAN INFLUENZA VIRUSES (AIV) are enveloped, segmented, negative-stranded RNA viruses, belonging to the family *Orthomyxoviridae*, genus *Influenzavirus A* (77,152). Influenza A virus (IAV) is composed of eight gene segments, and each gene segment codes for at least one protein. To date, IAV is known to code for 13 viral proteins (64,173). Some of the segments encoded more than one protein through mechanisms such as alternative reading frame (PB1-F2, PB1-N40, PA-X, and M2), and mRNA splicing (NS1/NEP) (31,64, 75,78,172).

AIV is classified based on the antigenic variation displayed by the virus surface protein–hemagglutinin (HA) and neuraminidase (NA) (6). A total of 144 possible subtype combinations have been identified for AIV based on the 16 HA subtypes and 9 NA subtypes (46) found circulating in the aquatic bird population identified as the AIV natural reservoir, predominantly the Anseriformes (particularly ducks, geese, and swans) and Charadriiformes (particularly gulls, terns, and waders) (100,168). Two new HA subtypes (H17, H18) and NA subtypes (N10, N11) have recently been identified circulating in bats from Central America (Guatemala) and South America (Peru) (154,155).

AIV transmissibility

Observations indicated that movement of AIV from wild to domestic birds occurs relatively frequently due to shared ecosystem, where prolonged and repeated exposure of domestic birds to the virus facilitate adaptation of virus to a new host (140). However, virus adaptations for a new host is a complex and a rare event as majority of these transmissions will only cause transient virus infections with limited spread as observed in AIV poultry surveillance (2,132). However, it is important to note that some species such as domestic ducks and geese, turkeys, and Japanese quails are more susceptible to AIV infections and may have been the bridging species of wild birds AIV into chicken and other gallinaceous poultry (145).

AIV evolution

Continuous outbreaks of AIV infection are driven by two main evolutionary mechanisms used by the virus to evade host immune systems: antigenic drift and antigenic shift (103). Antigenic drift occurs in response to the host immune pressure when mutations accumulate in the surface glycoproteins HA and NA, causing minor changes to the antigenic structure of the virus (Nelson and Holmes, 2007). Antigenic shift results from reassortment of infecting virus subtypes that lead to introduction of strains with completely novel gene combination and often with improvements in the capacity for the production of more viable and fit virus progeny (58).

AIV pathogenicity

AIV is classified into low and highly pathogenic avian influenza virus (LPAIV and HPAIV, respectively) based on

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its lethality in chicken (*Gallus gallus domesticus*) (140,147). In domestic poultry, LPAIV generally causes subclinical infection with virus shedding in infected birds, if not mild respiratory disease. In contrast the HPAIV, also formerly known as the fowl plague, causes multiorgan systemic disease, with high percentage of morbidity and mortality in both domestic and wild birds (1,147).

The AIV pathogenicity generally relies on the cleavability of the HA0 subunit to HA1 and HA2 by the host cellular proteases (73,82,117), and HPAIV is characterized by the presence of polybasic amino acids at the HA0 cleavage site instead of a monobasic motif observed for LPAIV (15,60,119). The monobasic structure of the HA0 cleavage site is only cleavable by the trypsin-like enzymes, which are present at limited sites in the host, hence LPAIV infections are confined to respiratory or gastrointestinal tract (72,83,116). In contrast, the polybasic motif found in the HPAIV HA0 is cleaved by ubiquitous proteases present within cells of multiple organs throughout the body, such as furin and subtilisin-related proteases (proprotein convertase 6–PC6), causing fatal systemic infection (61,131).

LPAIV and HPAIV in poultry

Any of the 16 HA subtypes circulating in wild birds reservoirs are considered as LPAIV, while all HPAIV are of H5 and H7 subtypes, although not all of these subtypes are HPAIV (3,147). Apart from the HPAIV H5N3 outbreak in common terns (*Sterna hirundo*) in South Africa in 1961 (11) and HPAIV H5N1 outbreak in wild waterfowl in two parks in Hong Kong in 2002 and bar-headed geese (*Anser indicus*) in western China in 2005 (29), HPAIV has been rarely isolated from wild bird populations (147). Due to the complex pathobiology of AIV, viruses that are highly pathogenic (HP) in domestic birds, generally do not necessarily cause diseases in Anseriformes birds (ducks and geese) in experimental condition (4,44). It is important to note that HPAIV usually occurs in domestic gallinaceous poultry (chickens, turkeys, quails, and guinea fowls) after exposure to and adaptation of LPAIV from wild birds (115,140). This is usually a unidirectional infection, where the domestic bird-adapted AIV rarely reinfects wild bird population (140), with the exception of the Asian lineage H5N1 HPAI virus (29,92).

Virulence shift of LPAIV to HPAIV

The LPAIV H5 and H7 subtypes can acquire virulence factors and become HPAIV through several mechanisms focused on the HA protein, which are (i) the substitution and insertion of basic amino acids (aa) in the HA cleavage site (62,139), (ii) loss of carbohydrate that covers the HA cleavage site through residue mutations (66), (iii) recombination of HA with other AIV viral genes such as nucleoprotein (NP) gene (136), matrix (M) protein gene (110), or 28S ribosomal RNA (67), and (iv) polymerase slippage that caused sequence duplication, and thus insertion in the HA gene (47,111). Nevertheless, it was suggested that a hidden virulence potential was readily embedded within the LPAIV strains capable of transformation to a HP strain, where the acquisition of polybasic cleavage site is the key activator for the virulence shift (14,130). This assumption is based on observations where alterations in other AIV viral proteins such as deletion of matrix 2 (M2) protein or NP cleavage site reduced AIV pathogenicity (178), while point mutation accumulation in the NA protein (36), amino acid deletion in the NA stalk (99), and amino acid substitution in the nonstructural protein 1 (NS1) (65) and polymerase proteins (basic polymerase 2, PB2 and acidic polymerase, PA) (35,54) promotes virulence of AIV.

Evolutionary pattern of H5N1

Within the AIV history, the pandemic potential of Asian lineage H5N1 virus is by far the most alarming due to the rate of its spread and the unusual evolutionary pattern showed by this particular subtype (45,167). Unlike the emergence of other HPAIV that occurs in chicken, the initial outbreak of H5N1 was recorded in domestic geese in Guangdong Province, China in 1996, which then became the primary precursor virus for the major outbreak in chicken farms in Hong Kong in 1997 (HK-97) (121,175). Although the HK-97 genotype had been eliminated through mass poultry depopulation in 1997, the genetic variants of the primary precursor virus (Goose/Gd-like) have continued to circulate exclusively in aquatic poultry until late 2000 (26,170), where the host range expanded to include terrestrial poultry in the following year, providing a larger pool of genetic material for reassortment (28,51).

The rapid rate of H5N1 evolution was later validated with the identification of six H5N1 reassortants in Hong Kong and mainland China in early 2001, immediately before the outbreak in Hong Kong, mid-May the same year (51,90,122). It was identified that this reassortant virus possessed a HA gene that originated from a Goose/GD/96-like virus, while the other seven internal genes were a result of reassortment from other non-H5 AIV (170). Although no infection with H5N1 was detected from July 2001 onward, Hong Kong experienced an outbreak caused by the HPAIV H5N1 again in February 2002 (90,122). Eight new H5N1 genotypes were isolated including genotype "Z", which later become dominant in southern China (90). Characterized with the deletions of 20 aa in the NA stalk and 5 aa in the NS protein (51), genotype "Z" has been responsible for the emergence of the 2003 and 2004 H5N1 outbreaks, marking the first dissemination wave of H5N1 into eight countries in East and South East Asia, leading to establishment of endemicity in Vietnam and Indonesia (45,164).

Although the Asian lineage H5N1 virus was endemic in poultry since 1997, it had later spread and persisted in the wild bird population, evidenced by the H5N1 outbreak in the migratory waterfowl, the bar-headed geese (*A. indicus*) at Qinghai Lake in western China in 2005 (29,92). Subsequently, the virus rapidly spread across Asia, Europe, the Middle East, and Africa, marking the second wave of H5N1 dissemination, affecting wild migratory birds and poultry (49,164). The third wave of H5N1 dissemination to South East Asian countries followed immediately in late 2005. It was characterized by the emergence and predominance of the H5N1 Fujian-like viruses, replacing the multiple H5N1 sublineages in China, which were responsible for the previous disseminations (127). This event led to the panzootic of H5N1 in poultry, especially in the Asian continent where intermittent outbreaks have been reported, particularly in countries where H5N1 is endemic (China, Vietnam, Indonesia, and Bangladesh) (41,45).

AIV and Vaccination

Following the identification of wild birds as the agent of long distance virus transmission (5,108,153), and the possible transmission of the virus through domestic animals (162), culling of the infected birds and the flocks of birds with suspected exposure to the virus have been used as the primary control measures, especially in countries where disease has been recently introduced (133,134). However, in countries where infection was already widespread and endemic, and other methods were not likely to eradicate the infection, vaccination was chosen as the primary control tool (37,133,144).

To date, AIV vaccination using the inactivated vaccines, and to a smaller portion using the live recombinant vaccine (NDV-H5) has only been exercised as a control or a preventive measure to eradicate HPAI viruses in poultry, either in the event of epidemics, such as seen in Mexico (H5N2, 1994–1995, 1995–2001) (163), Italy (H7N1, 1999–2000; H7N3 and H5N2, 2003–2006), Hong Kong (H5N1, 2002–2003) (21,96,122,163), and others; or in countries where HPAIV are endemic, as is the case for HPAI H5N1 in China, Indonesia, Vietnam, and Egypt (27,41,96,97).

Vaccination helps to control the spread of infection as vaccinated birds will acquire an elevated level of resistance to infection, thus lower shedding and environmental contamination by virus (22,144). Nevertheless, to achieve disease eradication, it is important for a vaccination program to be implemented in conjunction with adequate biosecurity enforcement and continuous surveillance of infection in vaccinated bird population (22). Although vaccination is highly recommended as a control and preventive tool for AIV, silent spread of infection in vaccinated populations is a major concern, especially where AIV is endemic. This is due to inability of the available inactivated AIV vaccines to provide complete protection to a virulent field challenge, allowing a small number of birds to become infected and excrete the virus without apparent clinical manifestation of infection. Long-term circulation and establishment of AIV in vaccinated population have been reported to cause changes in the genetic and antigenic properties of the virus, producing escape mutants as reported in Mexico (85), China (127), and Egypt (50). Due to the inability of the available standard serological tests used in disease surveillance to differentiate antibodies produced by vaccination from those that arise by field virus infection, strategies have been developed to differentiate infected from vaccinated animals (DIVA).

Current Understanding of DIVA Strategies for AIV

Vaccine development work with the aim to enable DIVA application was first published by Van Oirschot, Rziha, Moonen, Pol, Van Zaane (158) for Aujeszky's disease virus in pigs; and this investigator later coined the acronym DIVA (157). In parallel growth with the use of vaccine against AIV, advances of DIVA strategies were focused on vaccine developments that are capable of DIVA while permitting the use of the available standard serological tests (DIVA-vaccine approach). Alternatively, DIVA-antigen approach focused more on the serological tests development while allowing the use of conventional vaccines (killed virus).

In this section, six DIVA strategies were discussed in terms of the vaccine format and the available complementary companion diagnostic tests: (i) sentinel birds, (ii) subunit vaccine, (iii) heterologous NA, (iv) nonstructural 1 (NS1) protein, (v) matrix 2 ectodomain (M2e) protein, and (iv) hemagglutinin subunit 2 (HA2) glycopolyprotein (gp) (12,16,23,56,81,133). Summary of these strategies can be seen in Table 1.

Sentinel birds

The most basic strategy used for detection of live virus infection in a vaccinated flock is the employment of sentinel birds, where approximately 1% of the birds in the monitored farm are left unvaccinated and routinely tested serologically to detect flock exposure to live virus (133,134). This strategy offers a sensitive measure of any rising infection within the vaccinated flocks, and monitoring can be done using the available diagnostic tests such as the hemagglutination inhibition (HI) test and the ELISA test detecting NP or HA antibodies. This strategy was successfully employed alongside the heterologous NA emergency vaccination during the HPAI H7N1 outbreak in Italy in 2000 to monitor the field situation (22).

Recombinant subunit vaccines

As described earlier, HA gene is a structural virus protein with important functions for immunity and is one of the key determinants of AIV antigenic properties (73,82). Although optimum protection is achieved through the use of vaccination with whole inactivated virus homologous to the circulating strain, studies have indicated that the presence of HA alone in vaccine elicits protective immune response against viral infection (114,169). In the subunit vaccine strategy, the AIV HA gene is expressed in bacteria, viruses, or yeast system before being purified and prepared for use as a vaccine (32,34,118). A variety of different AIV viral vectors have been studied, where protective immunity was demonstrated upon experimental challenges (Table 2).

Apart from being efficacious and safe for application, the recombinant subunit vectored-virus vaccines offer immunity through a single vaccination, with the option of vaccination against multiple diseases and the availability of mass vaccine administration (91,148). Works on recombinant subunit vaccines have expanded significantly following the advances of reverse genetic technology (104), where it allows rapid regeneration of reassortant viruses, and thus reduces vaccine production time by approximately 2 months (57). However, most importantly, the subunit vaccines allow a clear distinction between antibodies produced by vaccination or wild-type AIV infection, which is crucial for DIVA surveillance purposes using the standard diagnostic tools. In theory, the vaccinated birds will only produce antibody against the expressed HA protein, but none for internal proteins such as NP and M proteins. Since the vaccinated birds will remain naïve to the internal proteins, infected birds can be identified if antibodies against these proteins are present (91). Standard diagnostics test available are the agar gel immunodiffusion, which detects the anti-NP and anti-M antibodies (106); and the commercially available enzyme-linked immunosorbent assay (ELISA) kit such the AIV FlockChek ELISA kit (IDEXX labs) (91), specifically designed for detecting anti-NP antibodies. To date, the recombinant fowlpox-influenza H5 vaccine is licensed and available in El Salvador, Guatemala, Mexico, China, and USA (143), while recombinant

TABLE 1. LIST OF AVAILABLE STRATEGIES FOR DIFFERENTIATING INFECTED ANIMALS FROM VACCINATED ANIMALS, WITH SOME OF THEIR ADVANTAGES AND LIMITATIONS IN GENERAL

Strategy	*Sentinel bird*	*Recombinant subunit vaccines*	*Heterologous NA*	*Differential immune response against protein (NS1, M2 and HA2 gp)*
Procedure and vaccine used	Naïve unvaccinated birds are marked and randomly spread in a vaccinated flock Sentinel birds are routinely tested for influenza virus exposure	Vaccine using a vector expressing HA and NA proteins Example: Fowlpox-vectored recombinant vaccine for the H5 subtype	Vaccines containing the same HA subtype as the field strain, but a different NA subtype. Example: If the field virus is H7N2, the vaccine is H7N3	Vaccination using whole-killed virus Observation of the differential immune responses to the targeted protein (NS1, M2, or HA2)
Available companion diagnostic test	Hemagglutinin Inhibition (HI) test Agar gel immunodiffusion Type A-specific ELISA (detect anti-NP antibodies)	Agar gel precipitin ELISA targeting the antibodies to matrix (M) protein or the nucleoprotein (NP) Fluorescence microsphere immunoassay (FMIA)	Neuraminidase Inhibition (NI) test Indirect immunofluorescence assay (iIFAT) FMIA Modified NI test	ELISA-based targeting the antibodies to specified proteins
Advantages	Low cost Readily applicable Sensitive procedure for monitoring in vaccinated flock	Efficacious in providing protection Commercially available Mass administration The standard diagnostic tests are applicable	Efficacious in providing protection Rapidly available through reverse genetics technology	Conventional inactivated virus can be used for vaccination Only a single diagnostic test needed
Limitations	Labor intensive Time consuming Naïve birds can potentially act as virus amplifiers and be the source of infection	Test sensitivity is yet to be determined	Prior knowledge on circulating strain Possible introduction of the same NA subtype field strain with the NA subtype used for vaccination Undetermined sensitivity of serologic testing Low-throughput screening capacity iIFAT–time consuming, laborious and the result interpretation is subjective	Risk of false-positive due to the presence of protein contaminant from nonpurified vaccine i.e NS1 protein Risk of false-negative in surinfected host due to the inability of host to seroconvert HA2 gp approach–need more studies

herpesvirus turkey (rHVT) is licensed in Egypt and USA, with recombinant duck enteritis virus (rDEV) being licensed in China (106,146).

Heterologous NA vaccine

The heterologous NA vaccine strategy employs an inactivated AIV containing similar HA subtype but different NA subtype to the outbreak strain (23). Vaccinated birds are protected against live virus infection by development of anti-HA antibodies, and they can be differentiated from infected birds through detection of antibodies against the NA subtype. This strategy allows the use of standard killed vaccines and screening can be done against anti-NA antibodies using an indirect immunofluorescence assay (23), in place of the conventional neuraminidase inhibition (NI) test (9).

There are only three known applications of the heterologous NA vaccine. It was first introduced as a measure to differentiate between vaccinated and infected birds during the 1999–2000 H7N1 HPAIV outbreak in Italy (21). The vaccine was prepared using inactivated H7N3 virus, and infected birds were detected by an indirect immunofluorescent antibody test (iIFAT) specifically developed for anti-N1 antibody (23). Similar strategy was implemented during the outbreak of LPAI H7N3 in Italy in 2002–2003, where inactivated H7N1 was used for vaccination, and during the outbreak of HPAI H5N1 in Hong Kong in 2002, inactivated H5N2 virus was used for vaccination (20).

AIV nonstructural 1 protein: differential immune response

The NS1 protein is a multifunctional protein that regulates viral RNA polymerase activities and viral mRNA translation (40,76,120). It is a nonstructural protein that is only detectable in infected cells, but not in packaged virions (123). Based on this observation, a DIVA-antigen approach has been suggested, which allows the use of conventional whole-killed

TABLE 2. LIST OF SELECTED STUDIES ON THE DEVELOPMENT OF DIVA STRATEGIES FOR INFLUENZA A VIRUS AND THE SUMMARY OF THEIR FINDINGS WITHIN THE LAST DECADE

Strategy	*Vaccination*	*Challenge virus subtype*	*Animal model*	*Companion diagnostic test/DIVA test tool*	*Protection*	*DIVA*	*Comments*	*Reference*
DIVA vaccines: Recombinant vaccine	Newcastle Disease (NDV) virus expressing HA protein (H5) Herpesvirus of turkey (HVT) expressing HA (H7) Virus-like particle (VLP) expressing HA and M1 (H9) Fowlpox (FP) recombinant expressing HA (H7 and H5) Infectious laryngotracheitis (ILT) virus expressing HA (H7) H5N1/PR8-519 (S2 glycoprotein of murine hepatitis virus-MHV replacing NA stalk region)	H5N1 H5N2 H7N1 H9N2 H5N8 H5N3 H5N2 H5N9	Chicken, mice	NP-GST fusion based ELISA NP-ELISA (IDEXX Laboratories, Inc., Westbrook, ME)	+/−	+	Reduced protection shown by FP-HA (H7) in chicken previously vaccinated or infected with fowlpox	(18,48,71,88,89,91, 94,141,142,160, 161)
DIVA-vaccine: Heterologous NA	Inactivated wild-type: H7N3 H5N2 H7N2 H9N8 H9N2 H7N1 Inactivated reassortant: H5N1 H7N8 H9N8 H5N8 H3N4 Inactivated reassortant with truncated NS: H3N3 Commercial H5N9	H7N1 H5N2 H7N2 H9N2 H7N3 H3N2	Chicken, turkey	Indirect immunofluorescent antibody test (iIFAT) expressing N1, N2 or N3 protein Micro-NI test with N1, N2 and N8 antigen Modified micro-NI test with N2, N8 and N9 antigen N2-ELISA	+	+/−	Micro-NI test is time consuming Modified version of the micro-NI test provide a more rapid option ELISA-based test offers a relatively easier and rapid application overall	(8,23–25,74,84,165)

(*continued*)

TABLE 2. (CONTINUED)

Strategy	*Vaccination*	*Challenge virus subtype*	*Animal model*	*Companion diagnostic test/DIVA test tool*	*Protection*	*DIVA*	*Comments*	*Reference*
DIVA test: Recombinant NS1	Inactivated wild-type: H3N2 H3N8 H7N7 H5N9 H7N2 H5N1 H9N2 Commercial: H7N2 Live virus: H5N9 H7N1 H7N2	H3N8 H3N2 H7N2 H9N2	Horse, mice, chicken	NS1-ELISA Agar gel precipitin	+	+/−	Nonspecific reactions in NS1-ELISA reported is speculated due to the nonpurified vaccine used Incorporation of truncated NS1 in vaccine strain is recommended to address NS1 protein contamination issue Differences in NS1 immune response between strains and species have been demonstrated, that is, in turkeys and chicken	(38,109,128,156, 177)
DIVA test: Truncated NS1	Live virus with truncated NS1: H7N3 H3N2	H7N2 H3N2	Chicken, turkey	NS1-ELISA Fluorescence microsphere immunoassays (FMIA) targeting recombinant NS1	+	+/−	Low seroconversion in vaccinated-and-challenged turkeys limited replication site by LPAIV lead to low titer of AIV despite infection (in comparison to HPAIV infection) could vary between bird species	(165,166)
DIVA-test: Matrix 2 protein	Inactivated wild-type: H5N9 H7N1 H5N1 Commercial: H9N2	H7N7 H5N1 H9N2	Chickens	M2e-peptide-ELISA Recombinant M2e-ELISA Tetrameric-recombinant M2e ELISA	+	+/−	Recombinant M2e-ELISA is more cost effective than synthetic peptide-based ELISA Development of tetramer-M2e as antigen has increased the sensitivity of this strategy, compared with the monomer-M2e-based ELISA systems	(53,56,70,81)

"+" indicates presence of protection by the vaccines or the strategy successfully demonstrated DIVA ability; "−" indicates negative protection by vaccines or unsuccessful DIVA ability; "+/−" indicates partial protection against challenge infection by vaccine or evidence of nonspecific reaction for DIVA test results.

virus for vaccination (109). A diagnostic ELISA that targets NS1 antibodies is a simple screening test, as had been previously recognized for foot and mouth disease virus (102). The first successful demonstration of this strategy for AIV was reported for the equine IAV (12), where NS1 antibodies were identified only in infected ponies but not in the vaccinated ones. Most works on the development of NS1 protein as antigen for DIVA have expressed recombinant NS1 protein in vectors such as pMAL and pET (17,156,177).

M2e protein: highly conserved protein

M2e protein is the external part of a homotetrameric transmembrane protein encoded by segment 7 of the IAV through an alternative reading frame (+1) mechanism (59,79). This protein forms ion channels on the AIV surface that are crucial for the release of viral genome into the host cell cytoplasm during virus entry (80,98), and serves as a pH regulator for the Golgi apparatus, which is essential for HA glycoprotein maturation (137). Two factors have led to the recommendation of M2e protein as DIVA antigen: (i) the relatively invariable nature of M2e protein across AIV strains (63,69), where its small size and low abundance in comparison to the other two surface glycoproteins (HA and NA) have allowed M2e protein to escape immune selection pressure and antigenic drift (43); and (ii) the abundance of the M2e protein on the surface of infected cells despite being low in copy number in a mature virion ($\sim$3% of the surface glycoprotein population) (13,176). Both of these characteristics have suggested that M2e protein could be a sensitive, specific, and universal DIVA antigen. The earliest report on the application of M2e as DIVA antigen in poultry has demonstrated a sensitive M2e peptide-based ELISA for detection of M2e antibodies following infection with HPAIV strains H5 and H7 (81). Similar sensitivity of M2e protein as DIVA antigen has also been demonstrated in a challenge study using LPAIV H9N2 (70), and against multiple AIV reference antisera (56).

Hemagglutinin subunit 2 glycoprotein: highly conserved epitope

HA2 glycoprotein (gp) is the C-terminal fragment of the cleaved form HA protein (125,171). It is considerably the more conserved region out of the two HA cleavage products (HA1 and HA2), especially at its N-terminal end, known as the fusion peptide (first 11 residues), which is involved in the fusion activity of IAV (33,125). The HA2 gp has been suggested as another potential target for DIVA tool based on two key criteria. First, HA2 is highly conserved throughout the 16 HA subtypes of IAV (46,105,107), with only two known epitope variants corresponding to the classical phylogenetic grouping of AIV HA protein (138). Four antigenic sites have been identified from HA2, namely site I (aa 1–38, the N-terminal), sites II and IV (aa 125–175), which exhibit different reactivity among IAV subtypes, and site III (aa 38–112) (159). As observed with the M2e protein approach, detection of antibodies against the highly conserved HA2 gp would theoretically enable a universal detection of all IAV subtypes. Second, this conserved region is only accessible to immune recognition following virus infection. It has long been noted that HA0 cleavability is essential for IAV infectivity (73,82), where the cleavage of HA0 to form HA1 and HA2 subunits is a prerequisite for membrane binding and virus entry to the host cell (95,124). HA2 gp is not accessible in the HA0 native form as it is buried in the pocket formed by the stalk of the HA stem trimer (126,159). However, once the HA0 is cleaved, the HA2 gp will be exposed and inserted into the target membrane to allow the conformational change, which will lead to membrane fusion and virus entry (19,30). Considering these findings, it is reasonable to assume that the presence of antibodies against discrete epitopes on HA2 gp would also be indicative of virus infection.

DIVA Strategies Applicability and Developments

An ideal surveillance tool is required to be (i) cost effective, (ii) rapid and easily manageable, and (iii) possess a high sensitivity and specificity in discriminating between naïve-infected host from a vaccinated-only host, and a vaccinated-infected host.

Although the sentinel bird strategy is simple to employ, there are concerns that the naïve birds may increase the infection risk for the vaccinated flock following repeated and lengthy exposure to the high load shedding of the virus by the sentinels (133). Acquiring a new infection is still possible in the vaccinated flock due to the continuously evolving nature of AIV, and technical vaccination issues, such as ineffective application or insufficient coverage, with poor antigenic match of the vaccine with the field strains (85). Furthermore, this strategy is only capable of detecting virus infection in a naïve host placed in a vaccinated flock, with no direct indication of live virus infection in the vaccinated host itself. This decisively dismisses it from being an option for a long-term application for surveillance purposes.

DIVA vaccine-based strategies: recombinant subunit and heterologous NA

For DIVA vaccines approach, multiple studies have demonstrated the effectiveness of recombinant vaccine strategies in providing the necessary protection against clinical signs, and fulfilling its role for DIVA purposes (Table 2). However, the fowlpox-HA (H7) vaccine was found to show a reduced protection in chicken that have been previously vaccinated or infected with fowlpox virus (18). Host range restriction may also apply for a particular virus vector such as observed for the infectious laryngotracheitis virus (ILTV) as it replicates poorly in turkeys (106). Nevertheless, mass administration and multiple diseases vaccination options offered by the recombinant vaccines highlight the feasible application of recombinant vaccines, as evidenced by the continuous development and application of this particular strategy.

Following the introduction of heterologous NA vaccination application in Italy (23), various combinations of HA and NA proteins have been tested and recommended, including the use of rare NA subtypes for vaccine development such as N5 and N8 (Table 2) (10,22). Introduction of the eight-plasmid reverse genetics system, which allows rapid *de novo* generation of reassortant live virus, has made it possible for the rapid availability of a heterologous vaccine once the NA subtype of the wild-type circulating virus is known (10,86). Nevertheless, a collection of vaccine with various combinations is necessary to ensure swift implementation in case of outbreak where multiple virus subtypes are present in a single host or population (144).

Since the conventional diagnostic tests are not applicable for the heterologous NA approach, companion tests specific for this strategy, iIFAT have been developed (23). Although the test is highly specific and sensitive for application (24), iIFAT is also time-consuming and a labor intensive assay, as it is with the classical NI test (9,23). It has been suggested that these NA-based tests be replaced with a faster, simpler and higher throughput ELISA-based screening system, such as the N2-specific ELISA-based test (74) and truncated-N1-specific ELISA (174). Alternatively, a modified version of the NI test is made available where MUN (2′-[4-methylumbelliferyl]-α-D-N-acetylneuraminic acid sodium salt hydrate) was used as the NA substrate in place of the traditional fetuin-based NI test, providing a more rapid analysis and quantitative results where the antibody responses can be measured over time (8). Recent developments have revealed a range of refinements on the available known tests (NI and ELISA) (8,165). However, due to the need for the production of both vaccine and its tailor-made companion test for an optimized performance, limited availability of facilities and resources are the major drawbacks for this particular strategy. Most importantly, in dealing with H5N1 endemic countries, homologous strain is a much preferred option for vaccination as heterologous NA is not an ideal strategy to apply given the diverse genetic variants of H5N1 (27,50,52).

DIVA test-based strategies: NS1, M2e, and HA2 proteins

DIVA tests based on NS1, M2e, and HA2 proteins are viewed more favorably in terms of their practicality (Table 1). These strategies offer a more straightforward approach in comparison to the subunit and the heterologous NA vaccination strategies, where the DIVA test strategy complements the conventional homologous inactivated vaccine administration. Although studies have shown that the presence of HA protein in a vaccine is enough to provide good protection against live virus infection, in most cases it only reduces the clinical signs, and AIV is still shed in the feces of infected birds (141,142). Virus shedding could be in low amount, but the silent spread (asymptomatic) of viral infection is still possible due to the generation of escape mutants in response to vaccination pressure (84). Taken together, homologous strain vaccination still by far provides the most optimum protection against virus infection, as antigenic relatedness is a significant factor in determining the level of protection induced by vaccination (87,142).

NS1 protein is highly conserved among AIV subtypes, which is a highly favorable diagnostic property (156,165, 177). However, several studies have identified that the NS1 protein also exists in truncated forms in nature (39,93,135), giving rise to concerns that this could affect the overall accuracy of NS1 DIVA test. Also, different level of species susceptibility to AIV infection should be taken into consideration before NS1 DIVA test is adopted for routine use. A study in turkey showed that the NS1 antibodies were only present for a short time following infection (10 days postchallenged). AIV with a low replication capability in a specific host, either due to low virus adaptability or due to host vaccinal immunity will not be able to produce detectable level of NS1 antibodies despite infection (7,38,128,149). Similar observation can also be resulted due to the poor immunogenicity of NS1 protein as reported in a challenge study in chicken (7).

This strategy also suffers from decreasing specificity with increasing number of vaccination. Low amount of NS1 antibodies were detected in chicken after three times of vaccination with the killed virus contributing to nonspecific reactions in the tests, thought to be due to antibody response against leftover NS1 proteins present in the unpurified vaccine (128,156,177). This shortcoming, however, suggested to be eliminated through the use of vaccination virus with truncated NS1, which remove the possibility of NS1 antibodies detection in vaccinated hosts (150,156). Studies on the truncated NS1 protein (10 nucleotides deletion in the middle of the NS1 protein-coding sequence) demonstrated its capability of providing protective host immunity after influenza virus challenge in mouse, pig, and horse models (112,113,166). This has raised the possibility of developing live attenuated virus as vaccine while retaining the capacity of NS1 protein as DIVA marker, although the reversion of the live-attenuated virus to virulent virus is a concern (166). This was later vindicated by a study on live mutant NS1 AIV showing its reversion to virulence after five back passages in chicken, thus suggesting that a killed vaccine made from a mutant virus with shorter NS1 gene is much safer and practical for DIVA application (17). Following the occasional detection of NS1 protein antibodies in vaccinated chicken, the NS1-ELISA was suggested to be more suitable for flock monitoring rather than individual birds diagnosis (149,165).

M2e DIVA strategy on the other hand has issues on its specificity and immunogenicity of the M2e antigen. Nonspecificity in the recombinant M2e-ELISA was identified to be caused by test serum reactions against the carrier protein used in the M2e expression system (56). Although this was not observed in the ELISA system employing synthetic M2e-peptide, the use of recombinant-M2e protein is much preferred as the latter offers a much lower cost for higher output, with continuous access for use in large-scale screening (56).

Concerns have also been raised where undetectable levels of seroconversion in infected animals may lead to false negative results in M2e-based ELISA. Previous findings indicated that M2e is a weak immunogen (101), where AIV infections (H1N1 and H3N2, respectively) in mice and humans have engendered poor M2e-specific antibody responses (42). A low M2e-antibody response was also observed after a primary infection in pigs with H3N2 or H1N1, but it was significantly increased following challenge infection using H1N1 (55). This is hypothesized to be contributed by the small size of the M2e antigenic determinant, which limits the number of M2e-reactive B cells for antibody secretion. This is further exacerbated by the antigenic competition posed by the much higher population of HA and NA proteins on the virus surface particle (42).

However, in a challenged duck study by Lambrecht, Steensels, Van Borm, Meulemans, van den Berg (81), a decreasing trend of M2e antibodies level was reported with the increasing number of vaccinations. Increased immunity established by vaccination was assumed to reduce efficient virus replication, hence influencing development of M2e antibody, which in turn affected test sensitivity. False negative results have been observed by Kim, Choi, Kwon, Kang, Paek, Jeong, Kwon, Lee (70) where low level of M2e-antibodies was detected despite a H9N2 challenge in chicken vaccinated twice.

Nevertheless, attempts to address these issues have been demonstrated through the improvement in the M2e-ELISA

detection efficiency by incorporation of multiple repeats of the M2e protein in the recombinant-M2e-ELISA system (53,151). Otherwise, DIVA application based on M2e protein is proven to have a wide range of reactivity against other IAV subtypes in chicken (56).

HA2 peptides were first demonstrated as antigen for H5N1 serodiagnosis using ELISA by Khurana, Sasono, Fox, Nguyen, Le, Pham, Nguyen, Nguyen, Horby, Golding (68) following identification of one immunodominant epitope through a complete antibody repertoire characterization of H5N1 infection in humans (69). Although HA2-specific antibodies have been reported in natural infection in both humans and mice, HA2 is a weak natural immunogen (129). As observed for the M2e protein DIVA strategy, this factor may also lead to false negative results for the HA2 gp-based antibody detection due to low seroconversion in infected hosts. However, this approach warrants further study to validate this assumption and to overcome this limitation, as otherwise it offers specificity and universality for surveillance purposes.

Recommendations for DIVA Programs

For AIV successful monitoring program, DIVA vaccine needs to be (i) effective, (ii) readily distinguishable from the wild-type virus, (iii) rapidly available, (iv) cost effective, and ideally (v) applicable by mass administration (by spraying or drinking water); along with companion diagnostic tests or DIVA test that are (i) simple and rapid, (ii) suitable for mass screening, (iii) highly sensitive and specific, and (iv) low cost.

In general, DIVA vaccines (subunit, recombinant, and heterologous vaccines), which have been described in the previous section, showed high efficiency in providing optimal protection against AIV infection and capable of DIVA application. Factors affecting vaccine effectiveness such as vaccine strain and target species have to be critically considered to ensure maximum vaccine coverage. Close monitoring of field virus is vital especially where AIV is endemic as continuous infection and circulation of virus promotes immune pressure, thus drifting off the field virus from vaccine seed virus (143). Availability of vaccine supply particularly in AIV endemic countries should be well managed and maintained as vaccine production is a time consuming process despite its relatively short shelf life (about 2 years) (96). AIV endemic countries usually possess high poultry density, thus cost effectiveness is a critical factor in decision making, which is why advanced vaccines with mass applicability have highly favorable features.

By far, ELISA–based diagnostic test is highly recommended for surveillance and monitoring purposes. However, to ensure the robustness of a DIVA test, field trials using both LPAIV and HPAIV challenge strains still need to be explored in various poultry species model since previous findings have demonstrated that test sensitivity varies between challenge strain and bird species used. Epitope mapping of the DIVA antigens will be an interesting venue to explore as this may aid in scoring a highly sensitive and specific DIVA tool.

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RESEARCH ARTICLE

Epitope Mapping of Avian Influenza M2e Protein: Different Species Recognise Various Epitopes

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Abstract

A common approach for developing diagnostic tests for influenza virus detection is the use of mouse or rabbit monoclonal and/or polyclonal antibodies against a target antigen of the virus. However, comparative mapping of the target antigen using antibodies from different animal sources has not been evaluated before. This is important because identification of antigenic determinants of the target antigen in different species plays a central role to ensure the efficiency of a diagnostic test, such as competitive ELISA or immunohistochemistry-based tests. Interest in the matrix 2 ectodomain (M2e) protein of avian influenza virus (AIV) as a candidate for a universal vaccine and also as a marker for detection of virus infection in vaccinated animals (DIVA) is the rationale for the selection of this protein for comparative mapping evaluation. This study aimed to map the epitopes of the M2e protein of avian influenza virus H5N1 using chicken, mouse and rabbit monoclonal or monospecific antibodies. Our findings revealed that rabbit antibodies (rAbs) recognized epitope ^{6}EVETPTRN^{13} of the M2e, located at the N-terminal of the protein, while mouse (mAb) and chicken antibodies (cAbs) recognized epitope ^{10}PTRNEWECK^{18}, located at the centre region of the protein. The findings highlighted the difference between the M2e antigenic determinants recognized by different species that emphasized the importance of comparative mapping of antibody reactivity from different animals to the same antigen, especially in the case of multi-host infectious agents such as influenza. The findings are of importance for antigenic mapping, as well as diagnostic test and vaccine development.

Introduction

Matrix protein 2 (M2) of avian influenza virus (AIV) is a 97 amino acids (aa) protein encoded by RNA segment 7 of the influenza A virus (IAV) [1]. It is translated from spliced mRNA and shares a common start codon with the matrix 1 (M1) protein and the first nine aa, while the remaining 88 aa continues at the second (+1) open reading frame [1, 2]. In its native state, M2 is a homotetrameric type III integral membrane protein composed of three domains; namely, a 54 aa cytoplasmic domain located in the viral envelope or cytoplasmic membrane of infected cells, a 19 aa transmembrane domain, and an N-terminal 24 aa ectodomain (M2e) which is exposed on the surface of the virus infected cells and on the viral particles [1, 3–5]. In the infected cell the M2 protein forms an ion channel which is vital for viral genome delivery into the host cell during virus entry [2, 3, 5–8]. Briefly, M2 ion channel activity is activated by acidification of virus-containing endosomes after internalization of the virus particle into the host cell via clathrin-dependant and -independent mechanisms [9, 10].

Amino acids 1–9 of the M2e protein are highly conserved across AIV strains, while minimal aa variation is observed for residues 10 to 24, making it an attractive target for AIV universal vaccine development [2, 11–20]. The M2e protein is low in copy number on the virus particle, but it is abundantly expressed on the surface of an infected cells [3, 21]. This differential epitope density between infected cells (high) and a mature virion (low) [15, 22] is the key feature for its recommendation as a marker for differentiating infected animals in vaccinated population (DIVA), a strategy used in AIV surveillance [23, 24].

The sensitivity and specificity of M2e-based DIVA have been demonstrated in our previous works [25–27]. This raised our interest towards the potential use of M2e in a competitive enzyme-linked immunosorbent assay (ELISA) format as a surveillance tool for AIV infection. The principle of competitive ELISA lies in the ability of the test subject antibody (e.g. chicken) to inhibit competitor antibodies, usually produced in rabbit or mouse, from binding to the target antigen. Hence, it is important for the competitor antibodies to react with the same viral epitopes as the antibodies produced by the test species. Such an ELISA format has been successfully demonstrated for the nucleoprotein of AIV, which has been proven to be reliable and applicable for multispecies surveillance [28–30]. However, M2e-based competitive ELISA is a better alternative DIVA test for an AIV surveillance tool, especially in the highly pathogenic AIV H5N1 endemic countries, where poultry vaccination using inactivated AIV is practiced.

It is accepted that due to differences in the germline gene repertoire in different species, accompanied by distinct mechanisms for generation and affinity maturation of antibodies, antigenic determinants recognized by a host can vary from one species to another [31–33]. Earlier studies on M2e protein for vaccine development have reported several antigenic determinants identified by anti-M2e antibodies isolated from rabbit, mouse and human [20, 34, 35]. In most cases, the M2e epitopes recognized were located in the region that span from the N-terminal to the middle region of M2e, and vary in length from 5 residues (^{2}SLLTE^{6}) [35], up to 15 residues ^{2}SLLTEVETPIRNEWG^{16} [20, 34]. Here, we describe epitope mapping using anti-M2e antibodies from chicken, mouse and rabbit to identify the M2e antigenic determinants for each antibody group, and to assess the most suitable animal source of anti-M2e antibodies in M2e-based competitive ELISA as an advanced DIVA test for H5N1 infections in poultry.

Material & Methods

Peptides for mouse and rabbit immunization and antigenic mapping

Peptide immunization for mouse and rabbit was done using the 17 amino acid (aa) M2e peptide ($M2e_{2-18}$), corresponding to residues 2 to 18 of HPAIV H5N1 Indonesian strain A/

Table 1. Overlapping peptides covering the full length H5N1 M2e protein ($M2e_{2-24}$), designed with 10 amino acid (aa) with 2 offsets, and 14 aa with 3 offsets each. Peptide $M2e_{2-18}$ was used as a control antigen in place of $M2e_{2-24}$.

Peptide designation	Peptide sequence	Peptide length
$M2e_{2-11}$	^{2}SLLTEVETPT^{11}	9–10 aa
$M2e_{4-13}$	^{4}LTEVETPTRN^{13}	
$M2e_{6-15}$	^{6}EVETPTRNEW^{15}	
$M2e_{8-17}$	^{8}ETPTRNEWEC^{17}	
$M2e_{10-19}$	^{10}PTRNEWECKC^{19}	
$M2e_{12-21}$	^{12}RNEWECKCSD^{21}	
$M2e_{14-23}$	^{14}EWECKCSDSS^{23}	
$M2e_{16-24}$	^{16}ECKCSDSSD^{24}	
$M2e_{5-18}$	^{5}TEVETPTRNEWECK^{18}	14 aa
$M2e_{8-21}$	^{8}ETPTRNEWECKCSD^{21}	
$M2e_{11-24}$	^{11}TRNEWECKCSDSSD^{24}	
$M2e_{2-18}$	^{2}SLLTEVETPTRNEWECK^{18}	17 aa

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Chicken/West Java/PWT-WIJ/2006, (^{2}SLLTEVETPTRNEWECK^{18}) [25–27]. It was conjugated with Keyhole Limpet Hemocyanin (M2e-KLH) at the C-terminal end for the anti-M2e antibodies production in mice (Abmart, Shanghai, China) and rabbits (Peptide 2, Chantilly, Virginia, USA).

M2e-mapping was done using two sets of overlapping short peptides spanning $M2e_{2-24}$. Set 1 included eight peptides of 9–10 aa length (WatsonBio, Houston, Texas) with two aa offsets each; while set 2 included three peptides of 14 aa length (Abmart, Shanghai, China) with three aa offsets each (Table 1). $M2e_{2-18}$ was used for anti-M2e antibodies screening in indirect ELISA, as well as the positive antigen control in mapping ELISA, instead of $M2e_{2-24}$, as both showed similar reactivity in previous study [26]. All peptides used were of >90% purity as determined by high performance liquid chromatography analyses.

Antibodies (sera)

Three different sources of anti-M2e antibodies were used in this study, namely chicken polyclonal antibodies (cAbs), mouse monoclonal antibodies (mAbs), and rabbit polyclonal antibodies (rAbs) (Table 2). CAbs were produced as described previously [25, 27]. In brief, commercial layer chicks were inoculated with inactivated H5N1 AI vaccine (Medivac-AI, PT Medion, Bandung, Indonesia), once (16 weeks of age), twice (12 and 16 weeks of age) or three times (8, 12 and 16 weeks of age). All chicks were challenged with live H5N1 strain (either A/Ck/West Java/PWT-WIJ/2006, or A/Ck/West Java/Sbg-29/2007) two weeks after the last vaccination. All challenge experiments were conducted in the Biosecurity level 3 (BSL3) facilities at the Indonesian Research Centre for Veterinary Science, Bogor, Indonesia. Collected sera were tested for M2e reactivity using indirect M2e ELISA [25, 26]. Reference H5N1 sera (A/Chicken/Scotland/59) was obtained from the Veterinary Laboratory Agency (New Haw, Addlestone, UK) as described previously [26].

Hybridoma cells producing anti-M2e mAbs were produced by Abmart (Shanghai, China) following immunization of mice with $M2e_{(2-19)}$-KLH peptide. Briefly, six female BALB/c mice were injected subcutaneously at multiple sites with an emulsion contained 0.05 mg KLH-M2e peptide mixed with complete Freund's adjuvant (CFA). Immunization was done four times 14 days apart. Booster injections were given 14 days after last immunization with 0.05 mg KLH-M2e peptide in incomplete Freund's adjuvant (IFA). Serum sampling was done seven days after the third and fourth immunization and sera tested for anti-M2e antibodies using

Table 2. Antibody types and animals used for the generation of antibodies either by H5N1 virus challenge, or KLH-M2e$_{2-19}$ peptide immunization.

Antibody type	Antibody designation	Immunogen
Chicken polyclonal antibodies	PL64	A/Ck/West Java/PWT-WIJ/2006
	PL80	
	2D10	A/Ck/West Java/Sbg-29/2007: MSLLTEVETPTRNEWECKCIDSSD
	2B2	
	2B47	
	2A17	
	Reference H5N1 sera	A/Chicken/Scotland/59
Mouse monoclonal antibodies	1N5	M2e$_{2-19}$ peptide: SLLTEVETPTRNEWECKC-KLH
	2D16	
	2E14	
	2G14	
	3D23	
	3H4	
Rabbit polyclonal antibodies	rAb-1	
	rAb-2	
	rAb-3	
	rAb-4	
	rAb-5	
	rAb-6	
	rAb-7	
	rAb-8	

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indirect M2e-ELISA. Fusion of myeloma cells and spleen cells was followed by another indirect M2e-ELISA screening. Selected clones of hybridoma cells were expanded and grown in Dulbecco's modified Eagles medium (DMEM) high glucose with L-glutamine (HyClone, GE Healthcare) with 15% foetal bovine serum (HyClone, GE Healthcare) and 1% (v/v) penicillin and streptomycin (Gibco, Thermofisher Scientific). MAb supernatants from cell culture were column purified using Pierce Recombinant Protein A Agarose (Thermofisher Scientific). No significant difference was observed between the column purified and precipitated mAb in indirect ELISA. Thus, for the experiments described here, the mAb supernatants were precipitated using 50% saturated solution of ammonium sulphate and the protein pelleted was resuspended in sterile phosphate saline buffer (PBS) and stored at -20°C until required.

Eight New Zealand White rabbits with the average age of 6 months were chosen to obtain hyperimmune serum against the M2e peptide. Rabbits were inoculated at five subcutaneous sites with an emulsion that contained 0.1 mg of KLH-M2e peptide mixed with CFA. The rabbits received booster injections containing 0.1 mg KLH-M2e peptide in IFA at day 14 and 28. Blood was collected two weeks after the final immunization and antisera tested using indirect M2e-ELISA.

Indirect M2e-ELISA and antigenic mapping

All peptides were dissolved in diethylpyrocarbonate (DEPC)-treated water (Bioline) to a final concentration of 1 mg/ml. Peptides were diluted with 0.1 M carbonate-bicarbonate buffer, pH 9.6 (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$) to the final concentration of 10 μg/ml, and 100 μl was added to each well of a 96-well flat bottom microtiter plate (Maxisorp, NUNC) and incubated at 4°C overnight. The coated plates were washed five times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 2% BSA in PBS (200 μl/ well) for 2 hrs at room temperature (RT).

The chicken test sera were diluted with the high salt dilution buffer (HS-DB: 0.1 M Tris pH 7.4, 0.5 M NaCl, 1 mM Na_2EDTA, 2% w/v BSA, 3% w/v Triton X-100, 3% w/v Tween 20) [25, 26], and mouse and rabbit sera were diluted in PBS containing 1% BSA and 0.05% Tween 20 (PBS-BSA-T) with the dilution of 1:100 for all sera. The blocked plates were washed for five times with PBS-T before the diluted serum was added into wells containing each peptide (100 μl/well). After 1 hr of incubation at RT, the plates were subjected to another five rounds of washing. Species-specific antibodies conjugated with horseradish peroxidase (HRP) enzymes were prepared by dilution of anti-chicken HRP with HS-DB, and anti-mouse HRP (Sigma) and anti-rabbit HRP were diluted with PBS-BSA-T. Diluted secondary antibodies were added to each well (100 μl/well), followed by 1 hr incubation at RT. After washing, the substrate solution [100 μg/ml of tetramethylbenzidine substrate (TMB) (Sigma, St Louis, MO, USA)] in citrate buffer (pH 8) containing hydrogen peroxide (100 μl of 0.6% H_2O_2) was added (100 μl/well) and incubated at RT for 5–20 minutes before the reaction development was stopped with stop buffer (1 M sulphuric acid) (50 μl/ well). The optical density (OD) of each well was determined at OD 450 nm using the BioRad Benchmark Plus Microplate Reader (BioRad, Hercules, USA).

Statistical and bioinformatics analyses

Each antigenic peptide was tested in three dilutions with two replicas each. A range of univariate and multivariate analyses were employed in this study as previously described [36], using MINITAB 17 statistical package [37]. The mean OD_{450} values for the antigen negative wells were subtracted from the mean OD_{450} values of antigen positive wells to get the corrected OD_{450} values. One-way ANOVA and pair-wise mean comparison by Tukey test was used to compare the corrected ELISA values of different antigenic peptides within each type of antibody (chicken, mouse, and rabbit). Antibody reactivity to the M2e peptides was considered as strong (>1.00), medium (0.50–1.00), weak (0.25–0.50) and negative (<0.20) in reference to its OD_{450} value.

Clustering based on Average Linkage algorithm was used to illustrate the similarities/differences between different peptides in reaction with each type of antibody. The same method was used to cluster antibodies against antigenic peptides. Hydrophobicity plot of M2e protein (aa 2–24) was constructed using the BioEdit software (North Carolina State University) and CLC Genomics (QIAGEN) [38].

Ethics statement

Animal work carried out at the Indonesian Research Centre for Veterinary Science, Bogor, Indonesia was approved by the Research Committee of Indonesian Research Centre for Veterinary Science. The experimental chickens were handled by an expert veterinarian in animal studies based on the guidelines of the National Health and Medical Research Council of Australia. The animals were checked daily for clinical signs, morbidity, and mortality. All chickens were bled via brachial vein and by cardiac puncture at the terminal step just after CO_2 euthanasia. humane endpoint was not applied in this study.

Results

Chicken, mouse and rabbit antibodies selection using indirect-M2e ELISA

Positive anti-M2e cAbs were selected based on findings from previous reports [25, 26], where end-point HI antibody titers for all cAbs were approximately 1:512 dilutions (data not shown).

Meanwhile, positive anti-M2e mAbs and rAbs showed ELISA titers between 1:1600 to 1:3200, and 1:800 to 1:1600, respectively. As expected, comparison of mean OD_{450} readings for chicken, mouse and most rabbit antibodies showed strong (OD_{450} >1.0) reactivity to the $M2e_{2-18}$ (Table 3). All results for statistical analysis can be found in S1 File.

Chicken sera recognized at least 2 different epitopes spanning M2e residue 5–18 and 10–17

M2e mapping ELISA results revealed a distinctive reactivity pattern between the chicken sera exposed to the A/Ck/West Java/Sbg-29/2007 (Sbg-29/2007) ($n = 4$) and A/Ck/West Java/PWT-WIJ/2006 (PWT/2006) ($n = 2$). Anti-M2e sera from chickens exposed to Sbg-29/2007 (2A17, 2B2, 2B47 and 2D10) showed a range of medium to strong reactivity to $M2e_{8-21}$, strong reactivity to $M2e_{5-18}$ and weak to strong reactivity to $M2e_{8-17}$ (Table 3). With the exception of cAb 2B47, Sbg-29/2007 antisera also showed a range of weak to strong reactivity to $M2e_{10-19}$. Non-reactivity of cAb 2B47 to $M2e_{10-19}$ was not fully understood, but this particular cAb was only reactive to peptides which included residues E8 and T9 (Fig 1). Collectively, Sbg-29/2007 antisera showed reactivity to peptides which shared a minimum of eight residues (^{10}PTRNEWEC^{17}) of the M2e (Fig 1).

While Sbg-29/2007 antisera were reactive to peptides with 10 residues ($M2e_{8-17}$ and $M2e_{10-19}$), as well as 14 residues ($M2e_{5-18}$ and $M2e_{8-21}$), chicken antisera to PWT/2006 (PL64 and PL80) were only reactive to the 14 residues $M2e_{5-18}$ (Table 3). Despite $M2e_{5-18}$ sharing residues with the whole $M2e_{6-15}$ and $M2e_{8-17}$, and most residues in $M2e_{4-13}$ and $M2e_{10-19}$, neither of the PWT/2006 antisera reacted to any of these shorter peptides. This suggested that these 10-residue peptides were inadequate to represent the PWT/2006-strain epitope which elicited antibody responses in the chickens.

Although the reference H5N1 serum (produced against A/chick/Scotland/59 strain) was commercially generated based on its hemagglutinin inhibition titer, it showed strong reactivity to peptide $M2e_{2-18}$ (mean OD_{450} 2.02) (Table 3). However, no reactivity was observed between the reference sera and any of the mapping peptides. Alignment of the peptides recognized by the chicken sera showed that at least two epitopes, in addition to the immunogen, were recognized, namely $M2e_{5-18}$ (^{5}TEVETPTRNEWECK^{18}) and $M2e_{10-18}$ (^{10}PTRNEWECK^{18}) (Table 3, Fig 2). Both epitopes contained residues M2e 10–17, which are recognised by all cAbs and which correspond to the most hydrophilic part of the M2e protein (residues 12 to 20) (Fig 2).

Chicken sera reactivity pattern is highly influenced by its immunogen as well as individual chicken immune response

Clustering analysis of chicken antisera based on their reactivity with M2e peptides revealed two major clusters broadly related to the antigen used to immunise the donor chickens (Fig 3). Cluster 1 grouped Sbg-29/2007 antisera together, particularly 2B2, 2A17 and 2B47, based on their reactivity to $M2e_{8-17,\ 10-19,\ 5-18,\ 8-21}$ and $M2e_{2-18}$; while cluster 2 grouped PWT/2006 antisera (PL64 and PL80), based on their reactivity to $M2e_{5-18}$ and $M2e_{2-18}$, along with the reference H5N1 sera (produced against A/chick/Scotland/59) which only reacted to peptide $M2e_{2-18}$.

Although cAb2D produced against the Sbg-29/2007 strain shared a similar reactivity pattern with cAbs 2B2 and 2A17 ($M2e_{8-17,\ 10-19,\ 5-18,\ 8-21}$ and $M2e_{2-18}$), clustering analysis recognized cAb 2D10 sera as the least similar to the other sera. Observation of its OD_{450} readings showed that cAb 2D10 reacted strongly with all five peptides (OD_{450} 2.02–2.33) (Table 3) which was not observed with the other sera. And uniquely this sera also had high anti-M2e antibodies titre (1:10,240).

Table 3. Mean OD_{450} readings for chicken ([a, b, c]), mouse ([d]) and rabbit ([e]) antibodies reactivity to the M2e peptide.

Antibody	OD450 on Peptide																					
	$M2e_{2-19}$		$M2e_{11-24}$		$M2e_{9-21}$		$M2e_{5-18}$		$M2e_{16-24}$		$M2e_{14-23}$		$M2e_{12-21}$		$M2e_{10-19}$		$M2e_{9-17}$		$M2e_{6-15}$		$M2e_{5-13}$		$M2e_{2-11}$	
2A17[a]	2.11	✓✓✓	0.02	-	0.92	✓✓	1.66	✓✓✓	0.04	-	0.05	-	0.04	-	0.81	✓✓	0.87	✓✓	0.17	-	0.03	-	0.06	-
2B2[a]	2.07	✓✓✓	0.01	-	0.58	✓✓	1.51	✓✓✓	0.02	-	0.04	-	0.05	-	0.26	✓	0.35	✓	0.20	-	-0.01	-	-0.01	-
2B47[a]	1.85	✓✓✓	-0.22	-	1.20	✓✓✓	1.32	✓✓✓	-0.08	-	-0.27	-	-0.12	-	-0.20	-	1.13	✓✓✓	-0.11	-	-0.21	-	-0.13	-
2D10[a]	2.33	✓✓✓	0.04	-	2.14	✓✓✓	2.14	✓✓✓	0.10	-	0.10	-	0.14	-	2.02	✓✓✓	2.24	✓✓✓	0.17	-	0.09	-	0.05	-
PL64[b]	2.29	✓✓✓	0.04	-	0.01	-	0.76	✓✓	0.08	-	0.08	-	0.14	-	0.02	-	0.10	-	0.10	-	0.07	-	0.10	-
PL80[b]	2.34	✓✓✓	0.10	-	-0.05	-	1.13	✓✓✓	0.16	-	0.02	-	0.07	-	-0.02	-	0.08	-	0.13	-	0.01	-	0.04	-
Reference H5N1[c]	2.02	✓✓✓	-0.05	-	-0.01	-	-0.03	-	0.03	-	0.03	-	0.00	-	0.00	-	0.02	-	0.01	-	0.01	-	0.04	-
1N5[d]	2.63	✓✓✓	0.01	-	0.02	-	0.01	-	0.00	-	0.01	-	0.02	-	0.03	-	0.03	-	0.01	-	0.00	-	0.00	-
2D16[d]	3.30	✓✓✓	0.02	-	0.01	-	0.01	-	0.00	-	0.02	-	0.03	-	0.01	-	0.03	-	0.00	-	-0.01	-	-0.01	-
2E14[d]	2.62	✓✓✓	0.35	✓	0.13	-	0.02	-	0.00	-	0.00	-	0.10	-	0.54	✓✓	0.01	-	0.01	-	0.00	-	0.00	-
2G14[d]	2.26	✓✓✓	0.01	-	0.00	-	0.05	-	0.00	-	0.00	-	0.00	-	0.01	-	0.01	-	0.03	-	0.00	-	0.03	-
3D23[d]	1.69	✓✓✓	0.04	-	0.02	-	0.09	-	0.02	-	0.02	-	0.03	-	0.02	-	0.08	-	0.06	-	0.01	-	0.01	-
3H4[d]	2.58	✓✓✓	0.01	-	0.01	-	0.02	-	0.00	-	0.01	-	0.02	-	0.01	-	0.02	-	0.03	-	0.00	-	-0.01	-
Rab-1[e]	1.86	✓✓✓	-0.10	-	0.08	-	1.82	✓✓✓	-0.13	-	-0.19	-	-0.14	-	-0.13	-	0.13	-	1.46	✓✓✓	1.31	✓✓✓	-0.04	-
Rab-2[e]	0.49	✓	-0.48	-	-0.34	-	0.38	✓	-0.53	-	-0.53	-	-0.52	-	-0.52	-	-0.45	-	0.42	✓	0.24	✓	-0.40	-
Rab-3[e]	1.64	✓✓✓	-0.16	-	0.01	-	1.46	✓✓✓	-0.25	-	-0.25	-	-0.23	-	-0.25	-	-0.04	-	1.27	✓✓✓	1.13	✓✓✓	-0.10	-
Rab-4[e]	0.68	✓✓	-0.43	-	-0.28	-	0.55	✓✓	-0.46	-	-0.46	-	-0.45	-	-0.45	-	-0.35	-	0.60	✓✓	0.39	✓	-0.34	-
Rab-5[e]	1.26	✓✓✓	-0.20	-	-0.04	-	1.45	✓✓✓	-0.22	-	-0.22	-	-0.20	-	-0.20	-	-0.02	-	1.14	✓✓✓	1.01	✓✓✓	-0.14	-
Rab-6[e]	1.41	✓✓✓	-0.26	-	-0.01	-	1.76	✓✓✓	-0.30	-	-0.30	-	-0.28	-	-0.28	-	-0.13	-	0.94	✓✓	1.44	✓✓✓	-0.12	-
Rab-7[e]	1.46	✓✓✓	-0.27	-	-0.03	-	1.25	✓✓✓	-0.31	-	-0.30	-	-0.29	-	-0.29	-	-0.14	-	1.34	✓✓✓	1.01	✓✓✓	-0.13	-
Rab-8[e]	0.68	✓✓	-0.43	-	-0.28	-	0.55	✓✓	-0.46	-	-0.46	-	-0.45	-	-0.45	-	-0.35	-	0.60	✓✓	0.39	✓	-0.34	-

For statistical analysis, please refer to S1 File.

[a]Chickens exposed to A/Ck/West Java/Sbg-29/2007

[b]Chickens exposed to A/Ck/West Java/Sbg-29/2007

[c]Chickens exposed to A/chick/Scotland/59

[d]Mice immunised with KLH-$M2e_{2-19}$

[e]Rabbits immunised with KLH-$M2e_{2-19}$

doi:10.1371/journal.pone.0156418.t003

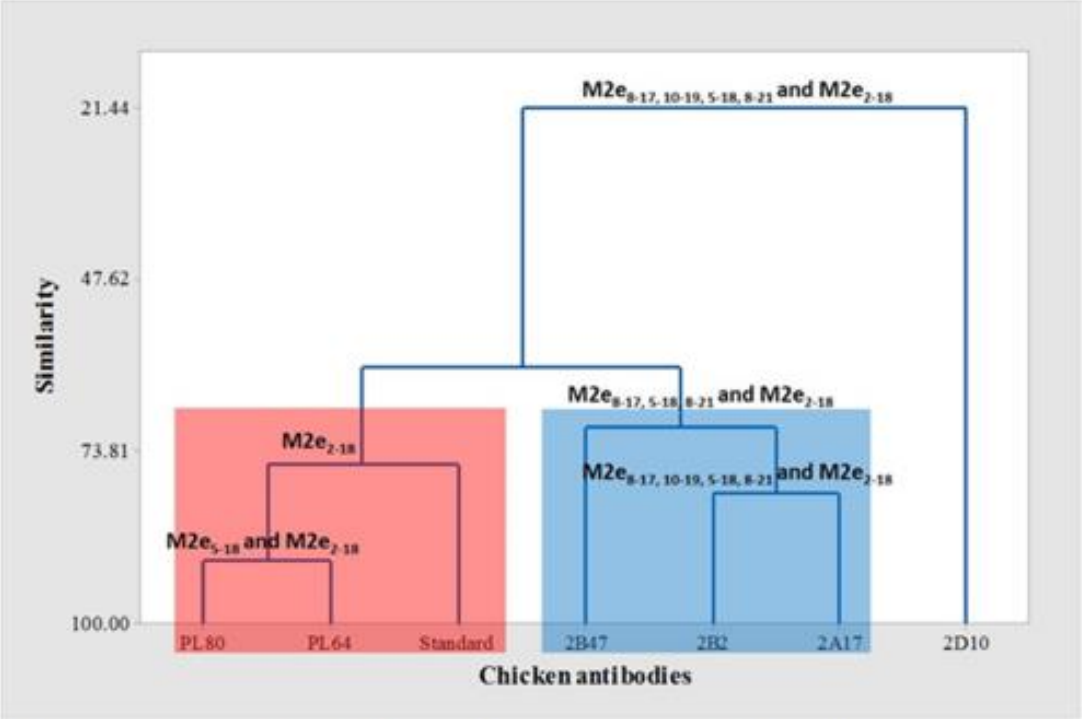

Fig 1. Clustering based on average linkage algorithm illustrates the similarity of chicken antibodies reactivity to the M2e peptides as indicated on the nodes of each group. Left to right: Cluster 1 (red box) chicken sera which reacted with $M2e_{5-18}$ and $M2e_{2-18}$; Cluster 2 (blue box) chicken sera which reacted with $M2e_{8-17,\ 10-19,\ 5-18,\ 8-21}$ and $M2e_{2-18}$; and 2D10 chicken serum which reacted with $M2e_{8-17,\ 10-19,\ 5-18,\ 8-21}$ and $M2e_{2-18}$.

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Mouse monoclonal antibodies recognized epitopes $M2e_{2-18}$ and $M2e_{11-18}$ while rabbit polyclonal antibodies recognized epitope $M2e_{6-13}$

M2e comparative mapping by mAbs showed minimal variability in their reactivity patterns. While all six mAbs strongly reacted with peptide $M2e_{2-18}$ (OD_{450} 1.69–3.30), only mAb 2E14 showed a weak and medium reactivity to $M2e_{10-19}$ and $M2e_{11-24}$, respectively (Table 3). Together, mAbs recognized an M2e epitope containing a minimum of eight residues (^{11}TRNEWECK^{18}) to 17 residues (^{2}SLLTEVETPTRNEWECK^{18}), in which the epitopes mostly overlapped with the epitope recognized by cAbs described above (Fig 2).

Apart from the similar strong reactivity observed for peptide $M2e_{2-18}$ (OD_{450} 1.73), rAbs also demonstrated strong reactivity to $M2e_{4-13}$, $M2e_{6-15}$ and $M2e_{5-18}$ (Table 3), a combination which was not demonstrated in the previous two groups of antibodies. All these peptides shared residues ^{6}EVETPTRN^{13} which indicated that the epitope recognized by rabbit was different from the chicken and mouse antibodies.

Comparison of the M2e epitopes recognized for all three groups of antibodies clearly showed that the chicken, mouse and rabbit sera recognized five epitopes, namely M2e residues 2–18 for all antibodies, with specifically M2e residues 5–18 and 10–17 recognized by the cAbs,

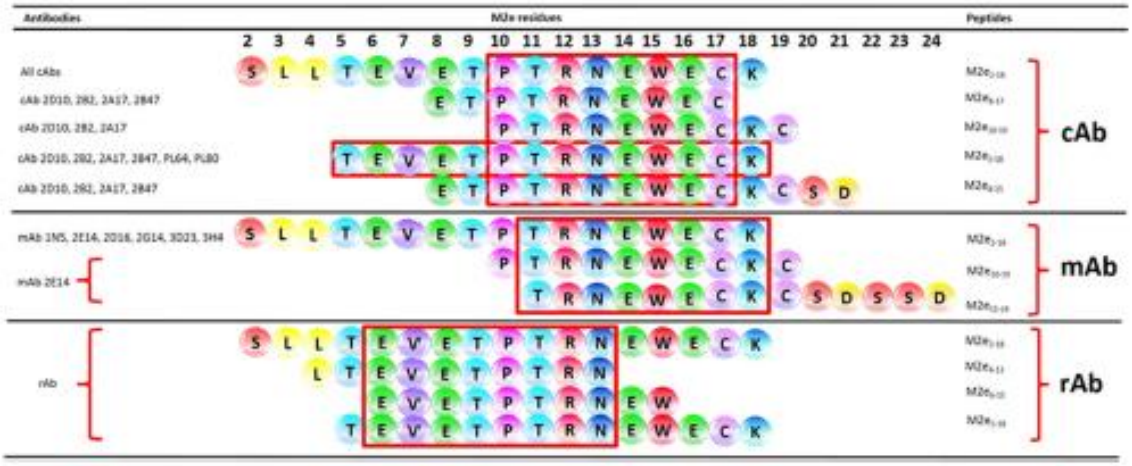

Fig 2. The antigenic determinants of M2e protein recognized by chicken, mouse and rabbit antibodies highlighted with the red boxes. In the order from top to bottom, chicken antibodies to Sbg-29/2007 strain that recognized peptides containing residues ^{10}PTRNEWEC^{17}; chicken antibodies to PWT/2006 strain recognized peptides with residues ^{6}TEVETPTRNEWECK^{19}; mouse monoclonal antibodies recognized peptides with residues ^{11}TRNEWECK^{19} and rabbit antibodies recognized peptides with residues ^{6}EVETPTRN^{13}. Tested antibodies were listed on the left, while the peptides corresponding to the residues recognized by each group are indicated on the right.

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M2e residues 11–18 recognized by one mAb, and M2e residues 6–13 by the rAbs (Fig 2). The shorter epitopes represented by the different antibodies group was recognized on two different sites of the M2e protein. cAbs and mAbs antibodies recognized epitopes located at the central region of the M2e protein (^{10}PTRNEWECK^{18}), while the rAb antibodies recognized an epitope located at the N-terminal of the M2e protein (^{6}EVETPTRN^{13}) (Fig 2).

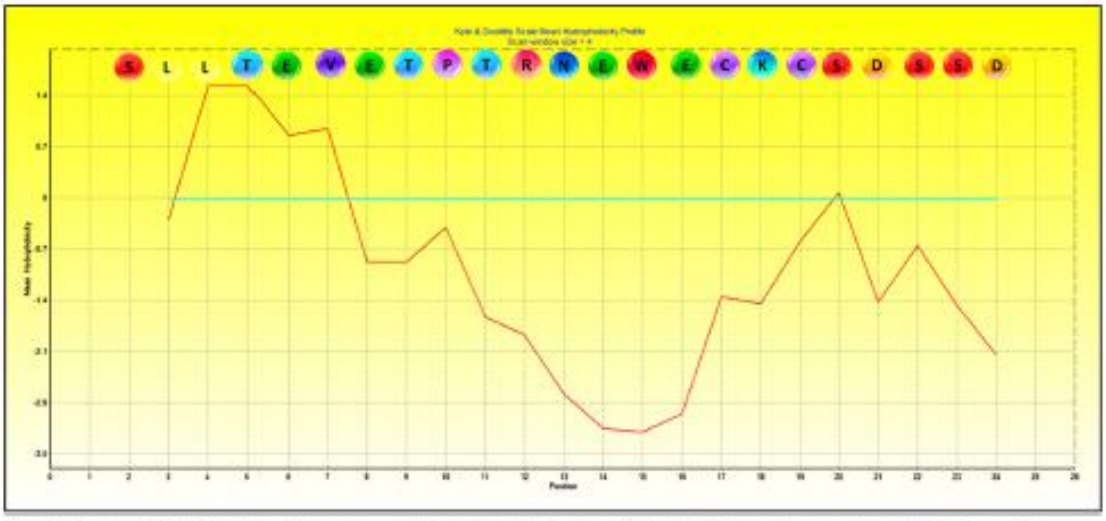

Fig 3. Hydrophobicity plot of M2e protein sequence (residue 2 to 24) based on Kyte & Doolittle scale mean of hydrophobicity profile in BioEdit.

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Discussion

Based on our previous success in demonstrating the effective use of M2e protein as a target for DIVA strategy, we attempted to develop a competitive ELISA test targeting the M2e protein. This test was anticipated to possess a broad host species applicability which is capable of DIVA for a simple yet effective AIV surveillance tool in domestic poultry. We have here described the comparative mapping of anti-M2e antibodies from chickens, mice and rabbits. Our findings revealed the occurrence of two separate epitopes on the M2e protein, where one epitope was exclusively recognized by the rAbs antibodies, while the other was recognized by both mAb and cAbs. It is important to note that for development of a competitive ELISA, the test and competitor antibodies need to cross-react with the same, or at least similar epitope, within the same antigen. Such is the case where cAbs are the test antibodies, while mAbs but not rAbs are the potential competitors.

Despite the difference in the immunogen used for anti-M2e antibody production in mice and rabbits (KLH-conjugated peptide) versus chickens (H5N1 live virus), our findings that cAbs, mAbs and rAbs recognized five M2e epitopes within the sequence ^{2}SLLTEVETPTRNEWECK^{18} was similar to those of others [13, 14, 20, 34, 35, 39–45] (Table 4). The high frequency of epitope ^{6}EVETPTRN^{13} occurrence in the previous studies suggests that it is likely to be a dominant epitope for M2e protein. Additionally, epitope ^{6}EVETPTRN^{13} is potentially a major epitope for rAbs, whereas a previous study on immunization of rabbits and mice using $M2e_{2-10}$ ^{2}SLLTEVETP^{10} conjugated with KLH (SP1-KLH) showed to be more immunogenic in rabbits than it was in mice [40].

Table 4. Summary of epitopes recognized on influenza A virus M2e protein by different antibodies.

Antibody type and designation	Antibody source	Immunogen	Epitope sequence (Identifying Antibody)	Residue length	References
Polyclonal (AS1, AS2, AS3, AS4)	Rabbit	Fusion-M2e (BSA)	^{2}SLLTEVETPIR^{12}	11	[13]
Monoclonal (8C6, 1B12)	Mice	Fusion-M2e (GST)	^{6}EVETPIRN^{13} ^{2}SLLTEVETPIRNEW^{15}	8 14	[39, 44, 45]
Monoclonal	Mice	Live virus & synthetic peptide	^{4}LTEVETPIRNEWG^{16}	13	[43]
Monoclonal (L66, N547, Z3G1, C40G1, 14C2)	Human (A HAC or KM™ mice)	Fusion-M2e (BSA)	^{2}SLLTEVETPIRNEWG^{16} (L66) ^{3}LLTEVETPIRNEWG^{16} (N547) ^{3}LLTEVETPIR^{12} (Z3G1)/^{8}TPIRNE^{14} (C40G1) ^{6}EVETPIRNEW^{15} (14C2)	15 14 10 6 10	[14, 42]
Monoclonal	Mice	Fusion-M2e (BSA)	^{2}SLLTEVET^{9} (M2e8-7) ^{3}LLTEVETPIR^{12} (Z3G1)	8 10	[34]
Monoclonal	Mice	Fusion-M2e (BSA)	^{4}LTEVETPIRN^{12} (L18) ^{2}SLLTEVET^{9} (O19) ^{2}SLLTEVETPIRNEWGCRNDSSD^{24} (P6) ^{7}VETPIRN^{13} (S1)	108 23 7	[41]
Polyclonal	Mice		^{2}SLLTEVETPIRNEWG^{16}	15	[20]
Monoclonal	Human		^{2}SLLTE^{6} (TCN-031, TCN-032)	5	[35]
	Mice	Fusion-M2e (KLH)	^{2}SLLTEVETP^{10}	9	[40]
Polyclonal & monoclonal	Chicken, mice, rabbit	Live virus & fusion-M2e (KLH)	^{5}TEVETPTRNEWECK^{18} (cAbs) ^{10}PTRNEWEC^{17} (cAbs) ^{2}SLLTEVETPTRNEWECK^{18} (cAbs, mAbs, rAbs)/^{11}TRNEWECK^{18} (mAb) ^{6}EVETPTRN^{13} (rAbs)	14 8 17 8 8	This study

Difference at residue I11T between the current and previous studies corresponded to the human and swine specific M2e sequence in the former (I11) and avian specific M2e sequence in the latter (T11) [5,7].

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Minimal variation observed for mAbs was likely due to the double selection using $M2e_{2-19}$-KLH-based ELISA for hybridoma production and final selection. This limited the mAbs reactivity only to the immunogen with low cross reactivity to the other peptides used in the study. Nevertheless, one mAb recognized two other peptides which contain residues M2e 11–18 (Fig 2) that overlapped with M2e epitopes recognized for cAbs. Hence, mAb was suggested to be a better competitor in a cELISA-based test for cAbs in contrast to rAbs, as the latter showed fewer overlapping residues (Fig 2).

However, it was notable that one mAb and the majority of cAbs showed slight variation in peptide recognition. Although the antigenic determinants recognized by the mAb and cAbs in the current study overlapped with the epitopes found previously (residues 5 to 16 of M2e) [14, 20, 42–44], they differed in that two of the recognized epitopes (^{10}PTRNEWEC^{17} for cAbs, ^{11}TRNEWECK^{18} for mAb) extended further from the mid-region into the C-terminal end of the M2e protein (Table 4). Both were shorter epitopes (8 aa in length) and independent of the N-terminal peptide ($M2e_{2-9}$), with one or two more residues at the epitope C-terminal (C17 and K18) than previously reported epitopes recognized in humans and mice. This suggests that residues ^{2}SLL^{4} is a less important antigenic determinant in chickens and rabbits than it is in humans [35]. Conversely, C17 and K18 may possibly be important residues for cAbs epitope recognition. Importance of K18 for mAb epitope recognition was also suggested by the reported loss of anti-M2e antibody responses following immunization with truncated $M2e_{2-16}$ in a vaccine study in mice [20]. Difference by two to three residues between the M2e epitopes recognized by mAbs has also been described previously [43]. Zhang et al. (2006) suggested that such observations could be due to either a true existence of species-related variation in epitope recognition, or difference in assay sensitivity used for epitope recognition, or both [43]. Epitope variation was observed in a separate M2e-unrelated study in rabbits using 10 human proteins, where although the epitopes recognized for a single protein were similar, they were not identical [46]. The epitopes recognized by mAbs in the current study represent another species-related variation of the existing recognized M2e epitopes, while this is the first known M2e epitope reported in chickens. Nevertheless, M2e residue C17 and K18 may be of contributing to the antigenic characteristics of M2e.

M2e protein residues S2, T5, E6, P10, I11, E14 and W15 have been identified as critical for antibody interactions [34, 35, 44, 47–49]. Epitope studies have suggested that charged residues (E, K an D), and polar residues (R, N, Q, P and T) are preferred in highly antigenic epitopes [50, 51], where the hydrophilic amino acids (R, K, N, P, H, D and E) are more prominent [52]. A recent analysis of the M2e crystal structure complexed with monoclonal antibody has recognized that residues T5, E6, V7, P10, R12 and N13 assist M2e hydrophilic interactions, which contributes to epitope accessibility in antigen-antibody binding [47]. Amino acid variation at residues P10, E14 and E16 resulted in predicted M2e structural differences between two H5N1 strains, Vietnam/1194/04 and Hong Kong/156/97 [53]. The latter H5N1 strain showed a folded hairpin structure that limits antigen recognition in comparison to a relatively more accessible structure observed in the former. M2e protein sequence is not available for PWT/2006 strain used in current study. The M2e amino acid sequence of A/chick/Scotland/59 (EMBL accession number CY015082) and A/Ck/West Java/Sbg-29/2007 (H5N1) (GenBank accession number AKI82362.1) only differs by residue E14G for Scotland/59, and K18C for both from the M2e A/Vietnam/1194/04, hence a similar 'open' structure is likely for the Sbg-29/2007 M2e protein.

It is noted that antibodies from chickens exposed to two different strains of H5N1 in current study recognized two dominant but overlapping epitopes on the M2e protein. Differences observed may be related to the M2e membrane-bound protein conformation of these two H5N1 strains. Factors such as degree of protein protrusion from membrane surface [54], as well as its accessibility for binding activities [55] highly influence the whole presentation of the

protein to the birds immune system. Reactivity with only the 14 aa mapping peptide ($M2e_{5-18}$, ^{5}TEVETPTRNEWECK^{18}) observed for sera PWT/2006 may be related to the structural element formed by the protein on the virus particle. Previous study on the human tryptophanyl-tRNA synthetase epitopes using 10 aa and 15 aa peptides has demonstrated similar observations [56]. It was suggested that the 10 aa peptides ($M2e_{4-13,\ 6-15,\ 8-17\ and\ 10-19}$) were not sufficient to imitate the functional structure of the epitope since it is located in a loop structure partially characterized by an α-helix. In the case of the M2e protein, its three-dimensional structure showed a compact U-shaped conformation, where a β-turn structure is adopted by residues T5 to E8, and 3_{10} helix from residues I11 to W15 [47]. Hence, it was likely that although the two epitopes residues overlap, the PWT/2006 sera were only reactive to the 14 aa peptide $M2e_{5-18}$ due to the lack of complete residue for a functional epitope formed by the 10 aa peptides.

Difference in length of recognized epitopes in anti-M2e cAbs may be related to the different degree of virus virulence between the H5N1 strains and individual chicken immune responses. Strong reactivity to the M2e peptides observed for the 2D chick sera in current study was reasoned to be due to the double boosts vaccination using killed virus, followed by a live virus challenge. Current findings revealed that the Sbg-29/2007 antisera were capable of recognising shorter epitopes in comparison to the PWT/2006 strain. Slight differences in signal intensity for each identified peptide for Sbg-29/2007 antisera were also noted in relation to the number of vaccinations for each individual birds. Previous study on epitope patterns in rabbit's parallel immunizations with a single antigen showed that polyclonal response in individual animal may differs in their affinities [46]. Also, the difference in the immunogen used was implicated in the lack of response to the mapping peptides observed for the reference H5N1 sera. Temporal and spatial distant origin of the strain used for immunisation (Scotland/59) from the strain used as the basis for the mapping peptide design (PWT-WIJ//2006) has likely influenced this particular cAb reactivity.

Although the relatively limited number of serum samples available for testing in the current study do not represent the complexity of antibody response to M2e protein, nevertheless, the results presented provided information on differences of Me2 epitope recognition by mouse, rabbit and chicken antibodies. Identification of antigenic determinants or epitopes of the target protein will enable us to formulate the most suitable source of anti-M2e antibodies for further development.

In summary, we have identified five epitopes spanning residue 2 to 18 of M2e protein for mouse, chicken and rabbit sera with variations in length (8 to 17 aa) from two localities on the M2e protein (N-terminal and mid-region). We also concluded that mouse anti-M2e antibodies are more suitable to be used as a competitor antibodies than the rabbit anti-M2e antibodies for further work on M2e-based competitive ELISA diagnostic test. This was highly suggestive by the overlapping epitopes (^{11}TRNEWEC^{17}) demonstrated by both chicken antibodies and one of the mouse antibodies.

Supporting Information

S1 File. Detailed statistical analysis performed in this study.
(DOCX)

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Author Contributions

Conceived and designed the experiments: NHH EE JI FH. Performed the experiments: NHH ST AP FH. Analyzed the data: NHH EE JI FH. Contributed reagents/materials/analysis tools: NHH JI ST AP FH. Wrote the paper: NHH EE JI AP ST FH.

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