# Characterisation of M2e Antigenicity using anti-M2 Monoclonal Antibody and anti-M2e Polyclonal Antibodies

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**ABSTRAK**

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Protein Matrik 2 ektodomain (M2e) memiliki sifat lestari dan dianggap sebagai antigen potensial untuk mendeteksi infeksi virus influenza A pada unggas yang divaksinasi (DIVA test). Namun studi yang mempelajari antigenisitas M2 dan respon imun pada manusia atau hewan masih sangat terbatas. Pada studi ini sifat antigenik dari masing-masing tujuh belas M2e peptida dan enam belas protein rekombinan M2e (rM2e) yang memiliki variasi asam amino (aa) pada posisi 10, 11, 12, 13, 14, 16, 18 dan 20 dibandingkan dengan metode western blot (WB) dan enzyme-linked immunosorbent assay (ELISA) menggunakan antibodi monoklonal (mAb) 14C2 dari tikus, dan anti-M2e poliklonal antibody (pAb) yang berasal dari ayam dan kelinci. MAb 14C2 memiliki kekuatan pembeda terbaik dan aa posisi ke-11 merupakan imunodominan paling penting yang mempengaruhi ikatan mAb14C2 hingga tingkat yang terbesar. Perubahan pada posisi 14, 16 dan 18 juga mempengaruhi pengikatan mAb14C2, dan perubahan ini terdeteksi pada semua metode (WB atau ELISA) dan antigen yang digunakan (M2e peptida atau protein rM2e). Untuk anti-M2e pAb dari ayam dan kelinci, aa imunodominan ditemukan pada posisi 10 dan perubahan pada posisi 11 tidak mempengaruhi reaksi antibodi. Pengikatan pAb kelinci juga dipengaruhi oleh perubahan pada aa posisi 14 dan 16, hal ini mengkonfirmasi kontribusi posisi tersebut terhadap antigenisitas M2e. Posisi 10 adalah satu-satunya posisi yang penting untuk pengikatan pAb ayam terhadap M2e. Secara keseluruhan penelitian ini menunjukkan antigenik M2e terletak di antara residu 10 - 18 dan perubahan aa pada posisi 10, 11, 12, 14, 16 dan 18, dapat mempengaruhi ikatan antibodi di dalam protein M2e.

**Kata kunci**: Virus Influenza A, epitop M2e, antigenisitas

**ABSTRACT**

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Ectodomain matrix 2 protein (M2e) is a potential antigen for detection of influenza-A-virus infection among vaccinated birds (DIVA test). However, the antigenicity and immune response induced by M2e in either humans or animals are poorly understood. Seventeen M2e peptides and sixteen recombinant M2e (rM2e) proteins with amino acid (aa) changes introduced at position 10, 11, 12, 13 14, 16, 18 and 20 were compared by western blot (WB) and enzyme-linked immunosorbent assay (ELISA) using mouse anti-M2 monoclonal antibody (mAb) 14C2, and chicken- or rabbit-polyclonal antibodies (pAb). The mAb 14C2 had the best discriminating power and aa position 11 was the important immunodominant for mAb14C2, that affected binding to a greatest degree. Changes in the adjacent position 14, 16 and 18 also influenced the binding, and it detected regardless of the method (WB or ELISA), or the antigen used (M2e peptide or rM2e). For chicken pAb and rabbit pAb, the immunodominant aa was position 10 and the antibody reaction was not affected by aa change at 11. The binding of rabbit pAb was also affected by changes at 14 and 16, which confirm the contribution of these positions to the M2e antigenicity. Position 10 was the only important position for the binding of chicken pAb to M2e. Overall, the study showed that the M2e antigenic sites are located between residues 10 – 18 and that aa changes at position 10, 11, 12, 14, 16 and 18 may all affect the antibody binding within the M2e protein.

**Key Words**: Influenza A Virus, M2e epitope, antigenicity

## INTRODUCTION

Highly pathogenic avian influenza subtype H5N1, has continued to be a significant concern for more than a decade globally, and especially in several South East Asia countries where the virus has become endemic in commercial poultry. As fatalities in humans, which have been directly linked to contacts with infected poultry continue to occur, measure that would reduce virus load in the environment, such as vaccination, are

now practiced in several countries. In some countries additional measures are considered such as using the differentiation of infected from vaccinated animals (DIVA) test for use in surveillance of vaccinated flocks in order to estimate the extent of H5N1 challenge in vaccinated flocks.

Ectodomain matrix 2 (M2e) protein is a 24 amino acid long protein located in the Matrix 2 (M2) protein N terminal. It is abundantly express on the cell surface infected by Influenza A virus (IAV), but very few are found in the virion of AIV (Lamb et al. 1985). Previous study, using mouse mAb 14C2 reported that M2e was the most important part for antigenicity of M2 protein as mAb 14C2 could not recognise the M2 lacking the ectodomain (M2e) protein (Zebedee & Lamb 1988). M2e protein is considered to be a potential antigen for DIVA test and high specificity of enzyme-linked immunosorbent assay (ELISA) using synthetic M2e-peptide or recombinant M2e protein has been reported by several studies (Lambrecht et al. 2007; Hemmatzadeh et al. 2013; Tarigan et al. 2015). Although the M2e based DIVA test has been evaluated for possible use in commercial poultry (Lambrecht et al. 2007; Hemmatzadeh et al. 2013; Kim et al. 2010, Tarigan et al. 2015), very little is known about the antigenicity of the M2e and antibody response it induces in infected poultry. Limited studies, however, have been carried on M2e antigenicity and immunogenicity and these studies were mostly focused on the M2e responses in humans, mice, pigs or ferrets (Schotsaert et al. 2009). Those studies concluded that M2e was a poor immunogen and induced antibodies only in a fraction of infected individuals, and antibody titres were low and of short duration (Feng et al. 2006); (Kitikoon et al. 2008); (Bianchi et al. 2001). In addition, antigenic variations in the M2e protein have only been studied using monoclonal antibodies (Zharikova et al. 2005; Zebedee & Lamb 1988).

In chickens, only limited studies have dealt with the immune responses to the M2e protein. An M2e-peptide based ELISA was used as a DIVA test to identify chickens and ducks challenged with HPAI viruses H5N1 and H7N7 (Hemmatzadeh et al. 2013: Lambrecht et al. 2007; Marché et al. 2010). Twenty-four and eighteen amino acid long M2e peptides were recognised in ELISA by immune chicken sera indicating that on the M2e peptide at least some, if not all, antigenic domains are correctly presented. Although antibodies to M2e were detectable, they were not consistently detected and were absent in chicks infected with LPAI isolated from water birds. In another study it was also shown that the M2e-peptide based ELISA was able to detect infection with H9N2 strain in vaccinated commercial poultry (Kim et al. 2010). In these studies the synthetic M2e peptide and recombinant M2e (rM2e) coupled to maltose bonding protein (MBP) were used to assess the suitability of M2e as a DIVA reagent (Lambrecht et al. 2007; Hemmatzadeh et al. 2013), and in another study also as a vaccine candidate (Mozdzanowska et al. 2003). Overall, these studies have indicated that synthetic M2e peptide is suitable for DIVA, and rM2e may both be useful for antigenic mapping of the M2e.

To date, limited report is available regarding M2e antigenicity in chickens or recognition of M2e by sera from various AIV infections (Lambrecht et al. 2007; Marché et al. 2010; Kim et al. 2010). For successful implementation of a DIVA test, it is critical to understand if M2e can be used as a universal detecting agent. Therefore, this study aimed to characterise the antigenic epitopes on M2e and identify critical mutations that influence binding of antisera to the M2e antigen. For this purpose, M2e-MBP recombinant proteins were generated as well as synthetic peptides carrying different mutations in the M2e protein, and used to analyse the M2e antigenic determinants. Two different immunological assays, WB and ELISA, were employed to achieve a comprehensive evaluation of M2e antigenicity using polyclonal antibodies (pAb) generated in chicken and rabbit against the M2eC0 peptide. Monoclonal antibody (mAb) 14C2 was also purchased and used for antigenic characterisation and for comparison with the anti-M2e peptide pAb.

## MATERIALS AND METHODS

### Production of recombinant M2e proteins (rM2e)

The synthetic M2e gene (M2eC0) was used to generate fifteen M2e mutant genes (M2eC1 to M2eC15) by introducing various mutations at specific sites (Sumarningsih, 2011). These fifteen M2e genes were cloned into pMAL-P4x expression vector and transformed into *E. coli* BL21 DE3 cell (BioLine Pty Ltd, Alexandria, NSW) to express recombinant M2e-MBP proteins, referred to as rM2e (C1 to C15). After cold osmotic shocked, the expressed proteins were purified with amylose resin beads (New England Biolabs, UK), and analysed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) for the size and purity.

### Synthetic M2e peptide

All M2e peptides (C1 to C18) were synthetized by GenScript (Piscataway, New Jersey, USA) corresponding to the sequences of M2e mutants (Table 1) with purity varied from 79% to 94%. The M2eC15 peptide was not synthesized because it had homolog sequence to that of M2eC14. The M2eC17 peptide was also not included in this study because the synthesis was unsuccessful even after three different attempts by

GenScript. In initial study, M2eC0 peptide was purchased from Peptide 2.0 (Chantilly, VA, USA). All these seventeen peptides (C0 to C18) were dissolved in sterile dd$H_2O$ (1mg/ml) and diluted in 1:1 with sterile glycerol and stored at -20°C.

**Anti-M2 monoclonal antibody 14C2 and anti-M2e polyclonal antibodies**

Mouse monoclonal antibody (mAb) 14C2 was purchased from Abcam, (Sapphire BioScience Pty Ltd, NSW) as ascetic fluid. The mAb 14C2 was generated against the M2 protein of Influenza A Virus A/WSN/33 following live inoculation. In this study, mAb 14C2 was optimised by ELISA titration using M2eC3, M2eC4 and M2eC11 peptides to determine the optimal dilution for mAb 14C2 in WB and ELISA.

Anti-M2e polyclonal antibodies (pAb) were produced in chicken and rabbit immunized using 1 mg of M2eC0 peptide in complete Freund's adjuvant, followed by 2 mg, 4 mg and 8 mg of M2eC0 peptide with incomplete Freund's adjuvant (as a second, third and fourth immunisation, in 3 weeks intervals). Chicken and rabbit were bled prior to each immunisation and the pAb were tested in M2eC0 peptide ELISA to determine the titres. The sera were harvested after the third immunisation when the optimum titres of pAb were found.

**Western blot (WB)**

All sixteen rM2e proteins (C0 to C15) were subjected by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was washed two times using PBS-T (0.5% Tween in PBS) and incubated in blocking buffer (5% skim milk in PBS) for 1.5 hours. All incubations for WB were performed at room temperature. After washing two times, the membrane was incubated with diluted primary antibody for 1 hour, washed five times, and incubated with diluted HRP-conjugated antibody for 1 hour. Different antibody dilution was used for each WB. First WB used dilution at 1/5000 for mAb14C2 and 1/2000 for HRP-rabbit anti mouse IgG. Second WB used dilution at 1/2000 for rabbit pAb and 1/2000 for HRP-goat anti rabbit IgG. Third WB used dilution at 1/1000 for chicken pAb and 1/4000 for HRP-rabbit anti chicken IgG. The antigen-antibody interaction was visualized using Amersham enhanced chemiluminescent (ECL) western blotting detection reagents (GE Healthcare Australia Pty Ltd, Rydalmere, NSW). The membrane was exposed to blue rite autorad film (Astral Pty Ltd, Gymea, NSW) and scanned to measure the band mass by Kodak molecular imaging software.

Value of 100% was given for the band intensity of homologous M2e sequence between antibody and antigen use in each WB. The band intensity for antibody binding to other M2e proteins was then compared to the homologous M2e reaction and expressed as a percentage of binding. Decrease in band intensity of a minimum of 25 to 30% was indicative the reduction in antibody binding.

**Enzyme-linked immunosorbent assay (ELISA)**

All the procedures for ELISA were performed at room temperature. Both ELISA used antigen concentration at 0.6ng/well for rM2e proteins and M2e peptides, which dissolved in carbonate-bicarbonate buffer (pH 9.6) and coated into each well of microtitre plate (NUNC Maxisorb). After coating for overnight, the plate was washed once with PBS-Tween 0.05% (PBS-T). Then 150µl/well of blocking buffer (5% Newborn Calf Serum in PBS-T) was added and incubated for 2 hours. After washing two times, 100µl/well of diluted antibody was added and the plate was incubated for 1 hour. Similar dilution at 1/2000 was used for all antibodies (mAb14C2, chicken pAb and rabbit pAb) in rM2e ELISA. But the dilution used in M2e-Peptide ELISA was different, which was 1/4000 for mAb14C2 and rabbit pAb; and 1/2000 for chicken pAb. After incubated with antibody, the microplate was washed five times, followed by incubation with 100µl/well of diluted HRP-antibody for 1 hour. The dilution at 1/2000 was used for HRP-rabbit anti mouse IgG and HRP-goat anti rabbit IgG, and at 1/4000 dilution for HRP-rabbit anti chicken IgG. After washing five times, 100µl/well of 3,3' ,5,5'-tetramethylbenzidine (TMB) susbtrate (Sigma Aldrich Pty Ltd, Castle Hill, NSW) was added and incubated for 15 minutes. The reaction was stopped by adding 25µl/well of 2M H2SO4. The binding of antibody to antigen was determined based on the optical density (OD) using microplate reader at a wavelength of 450nm (OD450).

**Table 1.** Amino acid sequences for the parent and mutant genes of M2e

| M2e | Parent [a] | Amino acid sequence |
|---|---|---|
| | Mutant [b] , Peptide [c] | |
| C1 | C0 [a] | MSLLTEVETP TRNEWECKCS DSSD |
| | C1 [b,c] | ..... ...G..... .... |
| C2 | C1 [a] | MSLLTEVETP TRNGWECKCS DSSD |
| | C2 [b,c] | .....L ..... .... |
| C3 | C1 [a] | MSLLTEVETP TRNGWECKCS DSSD |
| | C3 [b,c] | ..... I..... .... |
| C4 | C3 [a] | MSLLTEVETP IRNGWECKCS DSSD |
| | C4 [b,c] | ..... ..K..... .... |
| C5 | C0 [a] | MSLLTEVETP TRNEWECKCS DSSD |
| | C5 [b,c] | ..... .....R.. .... |
| C6 | C5 [a] | MSLLTEVETP TRNEWECRCS DSSD |
| | C6 [b,c] | ..... ...G.G.... .... |
| C7 | C6 [a] | MSLLTEVETP TRNGWGCRCS DSSD |
| | C7 [b,c] | .....L ..... .... |
| C8 | C6 [a] | MSLLTEVETP TRNGWGCRCS DSSD |
| | C8 [b,c] | .....H ..... .... |
| C9 | C7 [a] | MSLLTEVETL TRNGWGCRCS DSSD |
| | C9 [b,c] | ..... .K..... .... |
| C10 | C5 [a] | MSLLTEVETP TRNEWECRCS DSSD |
| | C10 [b,c] | ..... I..... .... |
| C11 | C10 [a] | MSLLTEVETP IRNEWECRCS DSSD |
| | C11 [b,c] | ..... .....G...N .... |
| C12 | C1 [a] | MSLLTEVETP TRNGWECKCS DSSD |
| | C12 [b,c] | ..... ..S..... .... |
| C13 | C3 [a] | MSLLTEVETP IRNGWECKCS DSSD |
| | C13 [b,c] | ..... .....R.. .... |
| C14* | C5 [a] | MSLLTEVETP TRNEWECRCS DSSD |
| | C14 [b,c] | ..... ...G..... .... |
| C15* | C2 [a] | MSLLTEVETL TRNGWECKCS DSSD |
| | C15 [b] | .....P .....R.. .... |
| C16 | C16 [c] | MSLLTEVETP TRNEWECKCI DSSD |
| C18 | C18 [c] | MSLLTEVETS TRNEWECRCS DSSD |

[a] The M2e parent gene used as a template in mutagenesis
[b] The M2e mutant gene, product of mutagenesis for rM2e protein expression
[c] The M2e sequence for synthetic peptide use in this study
*The sequence of C14 and C15 mutant genes were similar but generated from different parent genes

## RESULTS AND DISCUSSION

The M2e based DIVA test has been considered as the test of choice for use in surveillance of vaccinated flocks in Indonesia in order to estimate the extent of H5N1 challenge in vaccinated flocks and to establish zones that are free from H5N1 (Tarigan et al. 2015; Wibowo et al. 2017). Although the M2e based DIVA test has been evaluated for possible use in commercial poultry, very little is known about the antigenicity of M2e and its specificity to detect infections caused by avian influenza virus (AIV).

The M2e protein has been considered as a highly conserved protein among all influenza A virus (IAV) strains and subtypes. For this reason M2e has been studied as a possible candidate for a universal IAV vaccine (Schotsaert et al. 2009) and also as a potential DIVA diagnostic antigen to detect exposure to different AIV subtypes (Kim et al. 2010; Lambrecht et al. 2007). However, in several studies that have focused on M2e from human IV strains, evolution and mutation at some amino acid positions in the M2e have been reported (Furuse et al. 2009; Ito et al. 1991). Also, data obtained following the emergence and spread of H5N1 and additional surveillance in wild birds have further indicated that the M2e protein may also be under the similar selection pressure as are the HA and the NA proteins (Lam et al. 2008). The M2e amino acid variability has also been reported in other studies to occur in the middle part of M2e that potentially could have an affect on its antigenicity (Liu & Chen 2005; Wang et al. 2009). Ito et al. (1991) reported high M2 variation between positions 10 to 28, whereas the first nine amino acids (1MSLLTEVET10) were highly conserved. In this study, the amino acid variation in the M2e protein of different strains of H5N1 and of other frequently isolated AIV subtypes were determined. The most common mutations were identified for generation of recombinant M2e protein to be used for antigenic mapping of the M2e.

The antigenicity of a protein is strongly associated with the hydrophobicity, 17 M2e amino acid sequence were designed (Table 1) based on the hydrophobicity differences, so that each sequence had between one and five selected mutations in comparison to the rM2eC5 sequence (A/Ck/Indo/BL/03 H5N1 strain). These rM2e proteins had different hydrophobicity values, which indicating the potential of each amino acid position contribute to the M2e antigenicity (result not shown). Peptides analogous to these sequences were synthetized and labeled as M2eC0, M2eC1, etc. Antigenic mapping of M2e was performed by testing the binding of polyclonal and monoclonal antibody to each rM2e proteins in WB and ELISA, and to M2e-peptides in ELISA.

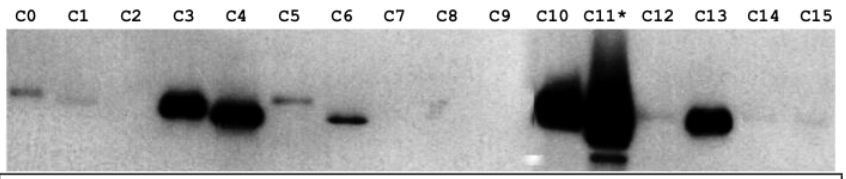
### Antigenic mapping using anti-M2 monoclonal antibody 14C2 (mAb 14C2)

Monoclonal antibody 14C2 was used in this study to compare the M2e antigenicity because it was previously shown to specifically recognise amino acid isoleucine at the position 11 (Zebedee & Lamb 1988). The mAb 14C2 was generated against the M2 protein of human influenza virus A/WSN/33/H1N1 strain, and WB result (Figure 1a) showed that mAb 14C2 reacted strongly with rM2eC11 (homologous reaction), which has similar sequence to M2e of A/WSN/33-H1N1 virus. Introduction of two mutation G16E and N20S into rM2eC10 was used to generated rM2eC11, and the WB of rM2eC10 showed that amino acid different in these positions caused a reduction in mAb 14C2 binding to 60%. MAb14C2 also reacted with rM2eC3, rM2eC4 and rM2eC13, that all having isoleucine at the position 11 (11I), but this reaction was lesser in comparison to the homologous reaction, indicating that changes at other positions (E14G, G16E and R18K) could reduce the binding of mAb14C2. The binding to rM2eC4 and rM2eC13 with additional change (E14G) was further reduced to 40% and 35%, respectively. The reduction to rM2eC3, which similar to rM2eC13 (35%), showed that amino acid change at R18K did not affected the binding of mAb 14C2. Additional mutation (I11T) in rM2eC0, rM2eC1, rM2eC14 and rM2eC15 completely abrogated the mAb 14C2 binding to these M2e proteins.

In WB of rM2eC5, to which mAb 14C2 did not bind, when T11I was mutated back generating the rM2eC10, the binding of mAb 14C2 was restored, but only to 60% of binding, its indicating clearly different contribution of positions 11, 16, and 20 to mAb 14C2 binding. Although four constructs rM2eC2, rM2eC7, rM2eC8, and rM2eC9 had additional P10L change, the contribution for this position to M2e antigenicity was not possible to assess because there was no rM2e proteins containing aa differed from rM2eC11 at position 10 only.

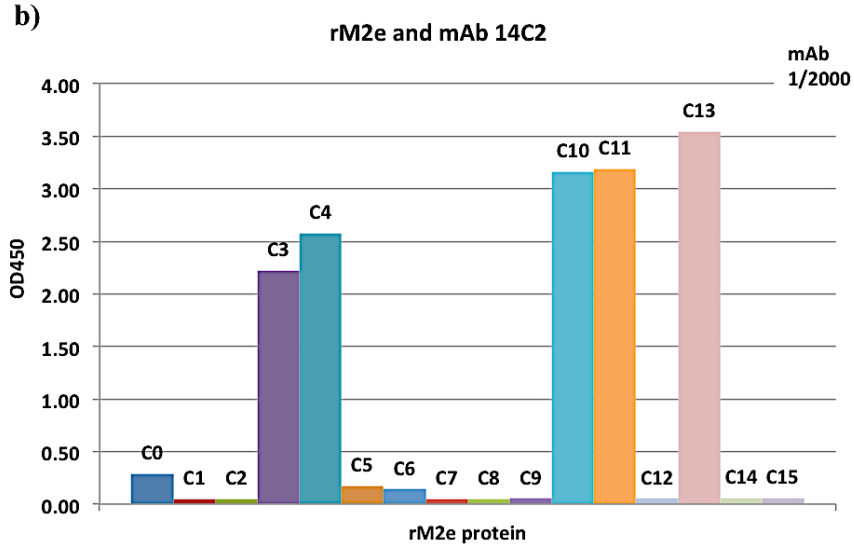
ELISA of mAb 14C2 using rM2e proteins showed similar result as WB (Figure 1.b.). The binding was only found with rM2eC3, rM2eC4, rM2eC10, rM2eC11 and rM2eC13, which contain isoleucine at the position 11 (11I). However, the binding to rM2eC3 and rM2eC4 was reduced in comparison to rM2eC10, rM2eC11 and rM2eC13, indicating that change at position 18 (R18K) could have affected the antibody binding. MAb 14C2 also did not react in ELISA with rM2eC0, rM2eC1, rM2eC5, rM2eC6, rM2eC7, rM2eC8, rM2eC9, rM2eC12, rM2eC14 and rM2eC15, and all these rM2e had amino acid change at position 11 (I11T).

a)




| | | | C0 | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11* | C12 | C13 | C14 | C15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % binding | | | 2 | 1 | 0 | 35 | 40 | 3 | 7 | 0 | 0 | 0 | 60 | 100 | 3 | 34 | 0 | 0 |
| Amino acid position | 10 | P | . | . | L | . | . | . | . | L | H | L | . | . | . | . | . | . |
| | 11 | I | T | T | T | . | . | T | T | T | T | T | . | . | T | . | T | T |
| | 12 | R | . | . | . | . | . | . | . | . | . | K | . | . | . | . | . | . |
| | 13 | N | . | . | . | . | K | . | . | . | . | . | . | . | S | . | . | . |
| | 14 | E | . | G | G | G | G | . | G | G | G | G | . | . | G | G | G | G |
| | 15 | W | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | 16 | G | E | E | E | E | E | E | . | . | . | . | E | . | E | E | E | E |
| | 17 | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | 18 | R | K | K | K | K | K | . | . | . | . | . | . | . | K | . | . | . |
| | 19 | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | 20 | N | S | S | S | S | S | S | S | S | S | S | S | . | S | S | S | S |

b)




c)

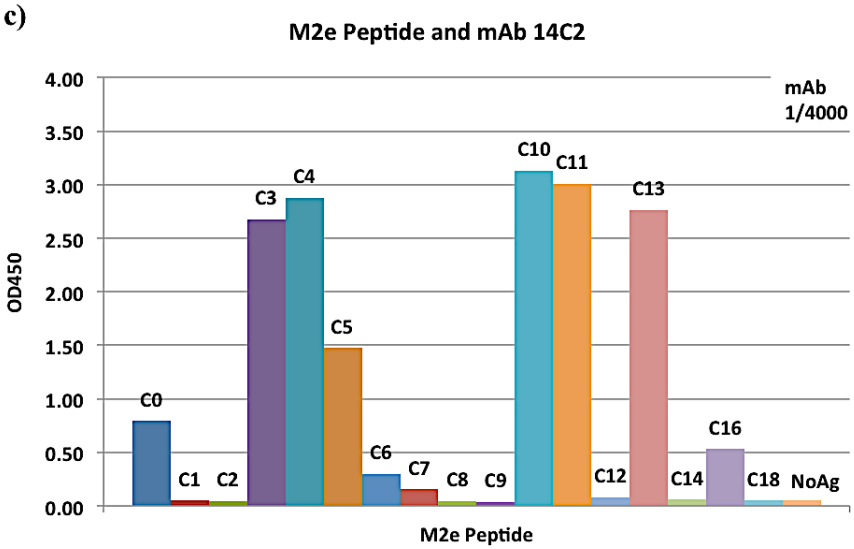



**Figure 1.** Antibody binding analysis of mAb 14C2 to rM2e proteins in WB (a); mAb 14C2 to rM2e proteins in ELISA (b); and mAb 14C2 to M2e peptides in ELISA (c). Amino acid variations between rM2e proteins to rM2eC11 (*) were shown in the table.

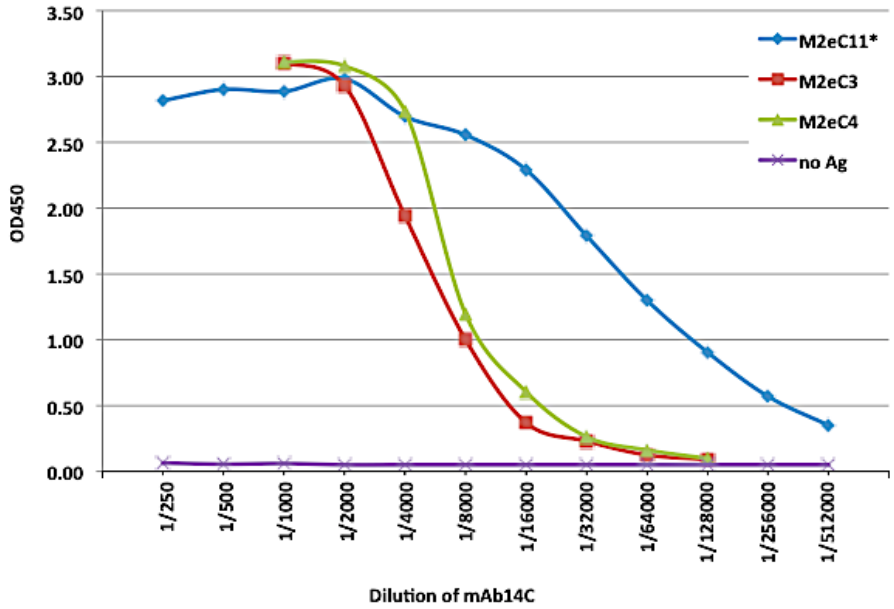



**Figure 2.** Analysis of mAb 14C2 titre in ELISA using three different M2e peptides (M2eC3, M2eC4 and M2eC11). M2eC11 peptide has the homologous sequence to human influenza A virus A/WSN/33/H1N1 strain used to generate mAb 14C2.

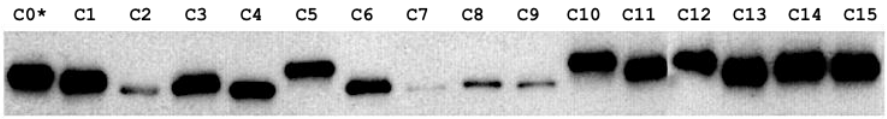
Similar finding was obtained in ELISA with M2e-peptides (Figure 1.c), mAb 14C2 strongly reacted with all five M2e peptides (M2eC3, M2eC4, M2eC10, M2eC11 and M2eC13) that had isoleucine at the position 11, and there was no influence of other amino acid changes to this binding. Positive but low reactions of mAb 14C2 binding was also found with M2eC0 and M2eC5 peptides which had threonine at the position 11 (I11T) and had only one common amino acid change in comparisons to the homologous sequence (rM2eC11) at position 20 (N20S). In this study, mAb 14C2 was titrated on the M2eC11, M2eC3 and M2eC4 peptides (Figure 2) because the binding of mAb 14C2 to M2eC3 and M2eC4 peptide in ELISA differed from the previous result of WB. As shown, there was a difference in binding between these three M2e peptides at the lower concentration of mAb 14C2, indicating that M2eC3 (E14G, G16E, K18R, S20N) and M2eC4 (N13K, E14G, G16E, K18R, S20N) differed antigenically from M2eC11.

## Antigenic mapping using anti-M2e rabbit polyclonal antibody

To investigate if there was any different M2e antibody response between species of animal immunized, rabbit polyclonal antibody to the M2eC0 peptide were used in this study to characterise the M2e antigenicity. Strong reactions of rabbit M2e antiserum were detected in WB (Figure 3.a) with rM2eC0 and rM2eC1 (E14G), rM2eC13 (T11I, E14G, K18R), rM2eC14 (E14G, K18R) and rM2eC15 (E14G, K18R) indicating that changes at the position T11I, E14G, and K18R did not influence its binding. The results also showed a complete absence of reaction of rabbit pAb with M2eC2, M2eC7, M2eC8 and M2eC9 that had either P10L or P10H amino acid change. These two changes at position 10 (P10L or P10H) caused the lack of binding equally. It was of interest that amino acid change at position 11 in rM2eC3, rM2eC4, rM2eC10, rM2eC11 and rM2eC13 did not influence the binding of anti-M2e rabbit pAb, nor did the amino acid changes at the position E14G, G16E and K18R and S20N.

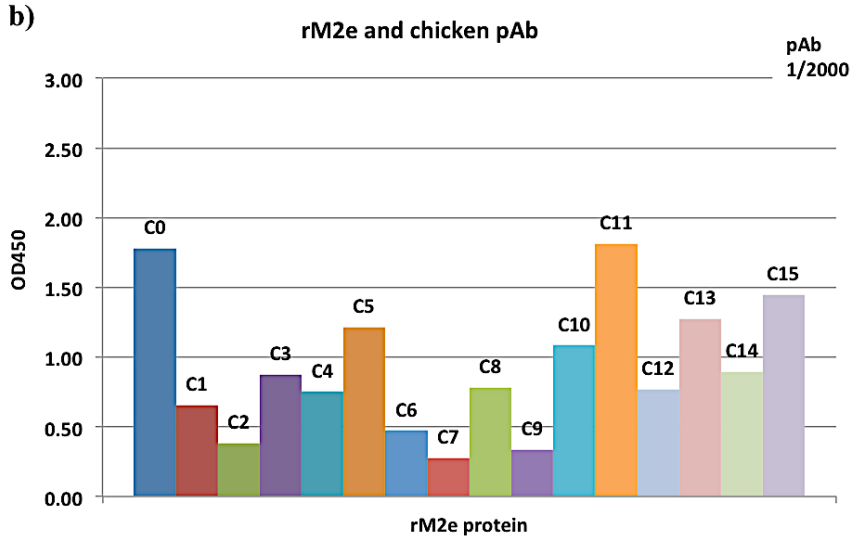
ELISA results using rM2e proteins (Figure 3.b) showed less discriminating value compare to WB. It showed that the highest reaction of rabbit pAb was with the homologous rM2eC0 protein, the binding was reduced to M2eC2, M2eC7 and C9 with changes at position P10L. Result of ELISA M2e-peptide was in agreement with the WB and ELISA rM2e for most peptides (Figure 3.c). Rabbit pAb reacted less with M2eC2, M2eC7, M2eC8, M2eC9 and M2eC18, these all peptides has amino acid different to M2eC0 at position 10 (P10L, P10H and P10S). The differences of antibody binding to M2e were visible only at high dilution (1/4000 concentration) of rabbit pAb indicating the influence of rabbit pAb concentration for differentiation capacity.

a)




| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % binding | | | 100 | 90 | 13 | 75 | 67 | 64 | 47 | 0 | 18 | 13 | 79 | 80 | 71 | 107 | 120 | 104 |
| Amino acid position | 10 | P | . | . | L | . | . | . | . | L | H | L | . | . | . | . | . | . |
| | 11 | T | . | . | . | I | I | . | . | . | . | . | I | I | . | I | . | . |
| | 12 | R | . | . | . | . | . | . | . | . | . | K | . | . | . | . | . | . |
| | 13 | N | . | . | . | . | K | . | . | . | . | . | . | . | S | . | . | . |
| | 14 | E | . | G | G | G | G | . | G | G | G | G | . | . | G | G | G | G |
| | 15 | W | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | 16 | E | . | . | . | . | . | . | G | G | G | G | . | G | . | . | . | . |
| | 17 | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | 18 | K | . | . | . | . | . | R | R | R | R | R | R | R | . | R | R | R |
| | 19 | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | 20 | S | . | . | . | . | . | . | . | . | . | . | . | N | . | . | . | . |

b)




c)

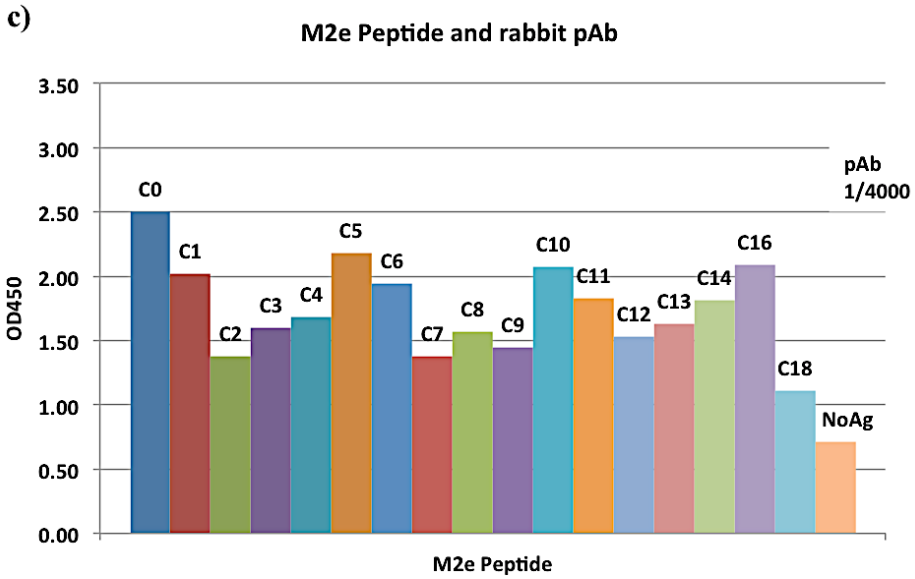



**Figure 3.** Antibody binding analysis of anti-M2e rabbit pAb to rM2e proteins in WB (a); anti-M2e rabbit pAb to rM2e proteins in ELISA (b); and anti-M2e rabbit pAb to M2e peptides in ELISA (c). Amino acid variations between rM2e proteins to rM2eC0 (*) were shown in the table.

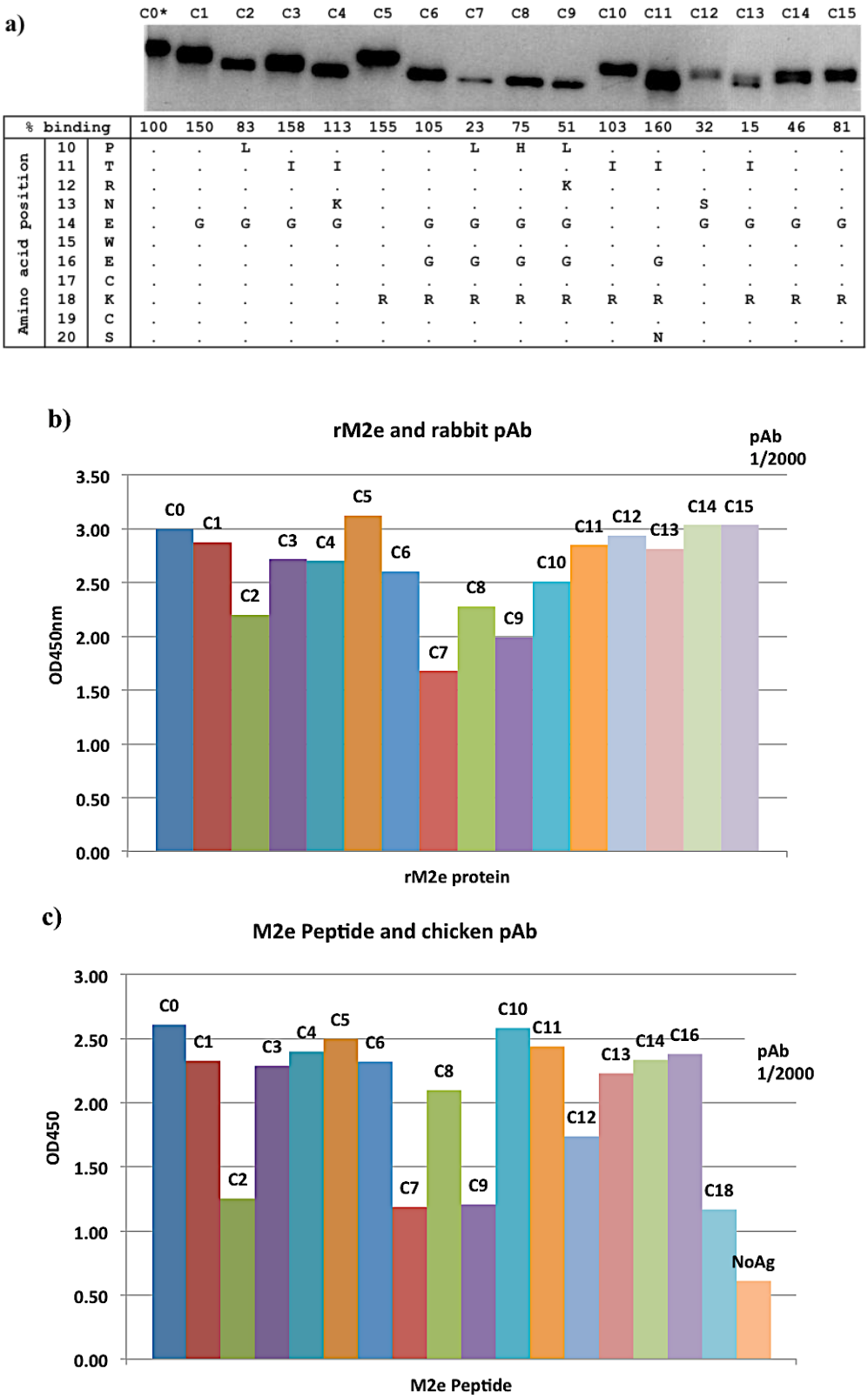



**Figure 4.** Antibody binding analysis of anti-M2e chicken pAb to rM2e proteins in WB (a); anti-M2e chicken pAb to rM2e proteins in ELISA (b): and anti-M2e chicken pAb to M2e peptides in ELISA (c). Amino acid variations between rM2e proteins to rM2eC0 (*) were shown in the table.

**Antigenic mapping using anti-M2e chicken polyclonal antibody**

Anti-M2e chicken polyclonal antibody was also used in this study to analyse the M2e antigenicity. WB result showed chicken pAb (Figure 4.a) was strongly reacted with the homologous rM2eC0 and other rM2e proteins, including rM2eC1 (E14G), rM2eC3 (T11I and N13S), rM2eC5 (K18R) rM2eC10 (T11I and K18R) and rM2eC11 (T11I, E16G, E14G, K18R and S20N). This finding suggested that changes at the position T11I, E14G, E16G, K18R and S20N did not influence the binding of chicken pAb. In contrast, chicken pAb was not reacted or weakly reacted with rM2eC7 and rM2eC9 that had amino acid change at the position 10 (P10L), rM2eC12 (N13S & E14G), and rM2eC13 (T11I, E14G and K18R). There was also some reduction of antibody binding to rM2eC2 (P10L and E14G), rM2eC14 and rM2eC15 (E14G and K18R), which indicate that the change at position 13 (N13S) as well as position 14 and 18 (E14G and K18R) also affected the antibody binding when present simultaneously.

Similar result was obtained from rM2e ELISA, it showed the importance of amino acid changes at position 10 (P10L) and 13 (N13S) (Figure 4.b) for chicken pAb binding. The binding of chicken pAb to rM2eC1 (E14G), rM2eC3 (T11I, E14G), rM2eC4 (T11I, R13K, E14G,) rM2eC6 (E14G, E16G, K18R) and rM2eC14 (E14G, K18R) was reduced significantly (>50%), indicating that changes of amino acid T11I, N13S or N13K, E14G, E16G and K18R, had disturbed antigenicity of the M2e, although it has a lesser degree compare to the changes of P10L and N13S.

Analysis using M2e-peptides ELISA (Figure 4.c) was in complete agreement with WB result, it showed significant reduction of chicken pAb binding to M2eC2, M2eC7 and M2eC12 peptide. These M2e peptides had a common amino acid change at position 10 (P10L), 13 (N13S) and 14 (E14G). The antibody binding to M2eC18, which has P10S change, was also abrogated. The M2e peptide ELISA indicated the important amino acid for chicken pAb were located at position 10, 11, 14 and 18.

**Table 2.** Summary of the important amino acid changes influenced the antibody (mAb 14C2, rabbit pAb, chicken pAb) binding to rM2e in WB, rM2e-ELISA and M2e-peptide ELISA.

| Antibody | rM2e-WB | ELISA | |
|---|---|---|---|
| | | rM2e | M2e-Peptide |
| mAb 14C2 | P10L, H | P10L, H | P10L, H, S |
| | I11T | I11T | I11T |
| | E14G | E14G | E14G |
| | G16E | G16E | G16E |
| Rabbit pAb | P10L, H | P10L, H | P10L, H, S |
| | | | T11I |
| | | | N13K, S |
| | E14G | | E14G |
| | E16G | | E16G |
| Chicken pAb | P10L | P10L, H | P10L, H |
| | | I11T | I11T |
| | N13S | N13S | E14G |
| | | E14G | |
| | | K18R | K18R |

Anti-M2 mAb 142C and anti-M2e pAb (chicken and rabbit) were used in this study to determine the antigenic epitopes on the M2e protein and the result showed that all three antibodies identified the same immunodominant epitopes, although chicken pAb had broader specificity and recognised additional antigenic epitopes on the M2e which were not recognised by rabbit pAb and mAb 14C2. Overall, all antigenic epitopes identified were located between amino acid position 10 and 18 (Table 2). Single amino acid changes at positions 10, 11, 13 and 18 have affected the binding, with amino acid changes at position 14 and 16 possibly acting co-operatively. It was considered that the entire region between amino acid 10 and 18 form a part of a single epitope in which amino acid changes at the position 10, 11 and 13 effect the binding and lead to the lack of recognition by an antibody. Both chicken and rabbit pAb used in this study contained high titres of anti-M2e antibodies and indicating that the synthetic M2eC0 peptide inoculated with Freund adjuvant was immunogenic and could induced high titre of antibodies recognised by both rM2e proteins and synthetic M2e peptides. High antibody titres (>1/32000) for rabbit and chicken M2e pAb were detected in ELISA (result not shown).

M2e protein has been reported as a poor immunogen and induced low or no detectable antibody titres following live infection in humans (Fiers et al. 2004). The antibodies titres from animals or humans infected with IAV were found to be in low titres and of short duration (Feng et al. 2006). However, it has been shown that the M2e synthetic peptide or recombinant M2e protein could induce high titre of antibody in mice and it can bind to the M2e in the surface of MDCK cells infected by PR8 IAV (Wu et al. 2007). When inoculated as a free peptide, M2e induced low (<100) antibody response in mice (Xia et al., 2011), but this immunogenicity was improved by pairing M2e with certain carrier proteins, such as hepatitis B virus core protein, Freund's adjuvant, the Norovirus P particle and other immunomodulators (Wu et al. 2007; Xia et al. 2011; Li et al. 2011). The number of M2e polyclonal antibodies have been produced and used to study M2e antigenicity (Frace et al. 1999; De Filette et al. 2006).

From direct comparisons of binding to rM2e, two antigenic sites were associated with the positions 10 and 13. Amino acid change at position 10, either P10L or P10S, reduced the antibody binding significantly, indicating that significant proportion of antibodies is directed towards this epitope. Therefore, position 10 is considered as an immunodominant epitope for M2e. At the same position, amino acid change P10H had less impact on antibody binding. It was evident that by introduction of a single mutation P10H in M2eC6 to generate M2eC8, the binding of antibody to the mutant M2eC8 did not change significantly. The amino acid change at the position 13, either N13K or N13S, also reduced antibody binding significantly and could therefore be considered as an immunodominant epitope. When comparisons were made between the parent and the mutant M2e, contribution of other amino acid changes to the antigenicity of the M2e became evident. In particular, the change at the position 14 (E14G) was the most common change between M2e of AIV and caused reduction in antibody binding. In M2e protein with amino acid E14G, additional E16G mutation further reduced antibody binding indicating these two positions might be the part of the same epitope. Another common amino acid change K18R was also reduced the antibody binding, but this was not a consistent finding. In M2e-peptide ELISA using chicken pAb, antigenic differences between proteins were less evident, with amino acid changes P10L and P10S significantly influencing the binding of antibody. Two other amino acid changes that marginally affected the binding of antibody were E14G and K18R. It is consider that the antigenicity differences between rM2e proteins and M2e peptide are due to the higher affinity of anti-M2e pAb for peptide than for rM2e protein, resulting in antibody having high titres and less discriminating ability for minor antigens present in a peptide.

Analysis using rabbit pAb indicated that the only amino acid changes that influence the binding were P10L, P10H or P10S. These changes caused the lack of binding equally, which was contrary to the results with chicken pAb. The reason for lesser discriminating value of rabbit pAb in comparison to chicken pAb obtained by the same immunisation is speculative. Both sera have been obtained by immunisation with the same peptide and adjuvant, and titres were comparable. While antigenic presentation of immunizing M2e peptide should be the same in both rabbit and chicken, it is possible that chicken, as a natural host for AIV, is more capable to recognise minor antigenic differences and mount an effective immune response. Rabbits have been often used to produce antibody against avian pathogen, including against purified antigens, in which case usually it generate broadly reactive antibody response.

Unlike for chicken and rabbit pAb, the dominant epitope detected by mAb 14C2 was isoleucine at the position 11. The highest reaction of mAb 14C2 was found with the M2eC11 (figure 1.a), which has isoleucine at position 11 (11I) and homologous sequence to M2e of A/WSN/33 IAV. However, less reaction was found with other M2e (M2eC3, M2eC4, M2eC10 and M2eC13), which also had isoleucine at position 11. This indicates that changes at other positions (E14G, G16E and R18K) could also have affected and reduced the binding of mAb 14C2 to M2e. Previously, the M2e protein antigenic sites have been

determined to be located in the middle part of M2e, which was in the first ten amino acids at the N-terminal end of M2e (Fu et al. 2009). Also, study using mouse mAb 8C6 suggested that the M2e antigenic determinant was located between amino acid residues 8 to 12 (8-ETPIR14) (Zou et al. 2005). MAb 8C6 was also reported can not recognise 7-mer M2e peptide containing amino acid substitution either at position 9 (T9A), 12 (R12A) or both positions 9 and 12 (T9A; R12A) (Zou et al. 2008). A panel of M2e human recombinant monoclonal antibodies have also been used for M2e antigenic study and showed that different M2e epitopes, SLLTEVETPIRNEWG, LLTEVETPIRNEWG, LLTEVETPIR, and TPIRNE were recognised by monoclonal antibody L66, N547, Z3G1, and C40G1, respectively (Wang et al. 2008). Hence, different results could have been obtained since every monoclonal antibody could only recognise one often-discrete epitope (Zhang et al. 2006).

In the present study, the M2e specific mouse mAb 14C2 was used to analyse the M2e antigenicity. WB and ELISA results showed that amino acid substitution at the position 11 (I11T) could destroy the M2e antigenicity. This result was similar to the earlier study (Zebedee & Lamb 1988) used mAb 14C2 on immunoprecipitation and immunoblot assays, and it showed eight different M2 proteins from heterologous IAV containing different amino acids at the position 11 (I11T) could not be recognised by mAb 14C2. This finding indicates that mAb 14C2 specifically binds to isoleucine at the position 11. The important of isoleucine at position 11 as immunodominant epitope for M2e also reported by study using human mAb 8C6, which specific for the M2e with sequence 5-EVETPIRN-14, it showed that mAb 8C6 weakly reacted with GST-5- EVETPTRN-14 (Liu & Chen 2005). The same study also reported that the residue 10 was the most important amino acid for the M2e antigenicity, that human mAb 8C6 could not bind to GST-EVETLTRN (Liu & Chen 2005). This finding was supported by previous study, which reported the influenza A virus escape mutant with mutation at position 10 (P10L and P10H), found in mice infected using PR8 influenza A virus and treated with mAb 14C2 (Zharikova et al. 2005).

## CONCLUSION

In summary, the study showed that the M2e antigenic sites are located between residues 10 – 18 and amino acid changes at these sites may all affect the antibody binding to M2e protein. It also identified that the capacity for antigenic mapping of the M2e protein was different between antibody raised in chicken and rabbit. Isoleucine position 11 is crucial for antibody binding of mAb 14C2 to M2e. However, the critical amino acid changes for rabbit pAb and chicken pAb binding was proline at position 10. Therefore, these positions 10 and 11 can be considered as the important immunodominant epitopes for M2e.

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