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The Production and Use of Monoclonal Antibodies for the Detection of Avian Influenza Antigen in the in Infected Chickens

(PRODUKSI DAN PEMANFAATAN ANTIBODI MONOKLONAL UNTUK MELACAK ANTIGEN FLU BURUNG PADA UNGGAS YANG TERTULAR)

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ABSTRAK

Metode diagnostik yang aman dan memadai untuk infeksi virus avian influenza (AI) telah dibuat menggunakan antibodi monoclonal (AbMo) terhadap virus tersebut. Virus yang digunakan dalam pembuatan ABMo ini adalah virus AI-H5N1 isolat Indonesia. Sel mieloma immortal asal mencit difusikan dengan limfosit asal limpa mencit yang sebelumnya telah diimunisasi dengan virus tersebut. Fusi kedua jenis sel tersebut menhasilkan sel hibrid yang disebut hibridoma yang sebagian kecil di antaranya menghasilkan AbMo terhadap virus AI-H5N1. AbMo yang diproduksi kemudian diuji dengan *enzyme linked immunosorbent assay* (ELISA) dan *Western Blotting* (WB). Dalam penelitian ini dibuat 12 AbMo antivirus AI-H5N1 dan 8 AbMo di antaranya digunakan untuk melacak antigen virus AI pada ayam yang terinfeksi. Dengan ELISA indirek, AbMo dapat melacak antigen virus AI pada cairan allantois telur ayam terinfeksi dengan titer 2^{-2} to 2^{-4} HA units per 0.1 ml. Dengan immunoperoxidase, antigen virus AI terlacak pada jaringan ayam yang telah difiksasi dengan formalin dan telah diblok dalam paraffin. Antigen virus AI tidak terlacak pada jaringan ayam yang tidak terinfeksi. Pada ayam terifeksi, sel terinfeksi dengan intensitas yang tinggi dijumpai pada proventrikulus dan usus halus. Antigen virus AI dengan intensitas yang lebih rendah juga terlacak pada paru dan limpa, tetapi sulit terlacak pada otot, otak dan jaringan lainnya. Hasil ini menunjukkan bahwa AbMo yang diproduksi dalam penelitian ini dapat digunakan untuk melacak virus AI pada ayam yang terinfeksi

Kata-kata kunci : flu burung, H5N1, antibodi monoclonal, unggas, virus imuno-peroksidase

ABSTRACT

A safe and appropriate diagnostic method for avian influenza virus (AIV) infection in chickens was established using monoclonal antibodies (mAbs) against the virus. The virus used for the production of the monoclonal antibodies was an Indonesian AIV-H5N1 isolate. Immortal mouse myeloma cells were fused with the lymphocytes derived from the spleen of mice immunized with the virus. The mAbs were tested for their specificity by enzyme linked immunosorbent assay (ELISA) and western blotting using formaldehyde inactivated virus and normal allantoic fluid as antigens. Twelve mAbs specific against AIV were isolated and 8 mAbs were used for immunodetection of AIV antigen in chicken's tissues. By indirect ELISA, the mAbs were able to detect AIV antigen in allantoic fluid at the titre as low as 2^{-2} to 2^{-4} HA units per 0.1 ml. By immunoperoxidase staining AIV–antigen was detected in paraffin embedded tissues of AIV-infected chickens. AIV antigen was not detected in chickens which were confirmed to be AIV negative. In the infected chickens, high intensity of AIV antigen was detected in proventricle gland and small intestine. The AIV antigen with a lesser intensity was detected in lungs and spleen but hardly detected in muscle, brain and several other tissues. This study show clear evidences that mAbs produced in this study are applicable for use in the detection of AIV antigen in infected chickens.

Key words : avian influenza, H5N1, monoclonal antibodies, chickens, virus immunoperoxidase.

INTRODUCTION

Avian influenza (AI) causes the death of millions of domesticated birds and millions others have to be sacrificed in an effort to eradicate the disease (Swayne and Halvorson, 2003; Stageman and Bouma, 2004). Many affected countries suffer a great deal of economic losses brought out by the collapse their poultry industries (Perkins and Swayne, 2002; Perkins and Swayne, 2003; Lewis, 2006). More importantly, the disease also affects human causing a great concern among health authorities in the world (WHO, 2006). The availabilities of accurate and safe diagnostic methods are important in an effort to prevent and to control a future outbreak of AI in both animals and man. Many diagnostic methods developed in the recent years still require expensive facilities and reagents, slow to perform, lack of sensitivity and specificity, unsafe to perform, and unable to determine the virus subtype directly (Gough, 2004).

Avian influenza viruses (AIVs) are a group of viruses with genetic and antigenic diversities in nature. On the basis the antigenic characteristics of their 2 surface glycoproteins, haemagglitinin (H) dan neuraminidase (N), AIVs are grouped into many subtypes. As many as 16 H subtypes which can combine with 9 N subtypes have been identified (Fouchier *et al.* 2005). Such antigenic diversities have often caused difficulties in establishing an appropriate test for an accurate detection of AIV subtypes ((Fouchier *et al.* 2005; Kida dan Sakoda, 2006). Monoclonal antibodies (mAbs) which react only with a single epitope on an antigenic structure have been widely used to detect the viral antigen in the infected hosts and also to differentiate closely related viruses (Zheng *et al.* 2001; Varecova *et al.* 2002, Ohnishi *et al.*2005). In human influenza virus, for instance, the use of mAbs against the H protein of the virus is reported to have 100% sensitivity and 99.1% specificity in determining the H subtype of the virus (Varecova *et al.* 2002). As in human influenza, mAbs against AIV is very likely to have a similar degree of sensitivity and specificity when used in detecting of AIV antigen in the infected hosts including in determining the virus subtype.

Among many different hosts infected by AIVs, avian species especially chicken have been reported to be the most susceptible host for AIV infections. In chickens, highly pathogenic avian influenza (HPAI) virus can cause a severe and a fatal infection with a high morbidity and mortality rates (Perkins and Swayne, 2002; Perkins dan Swayne, 2003). The morbidity and mortality rates can be as high as 100% in susceptible chickens. Incubation period is usually 3 – 7 days and the death of susceptible chickens can occur within 24-48 hours following the onset of clinical signs (Swayne dan Halvorson, 2003). The detection of AIV antigen in the infected chickens is therefore important, especially as confirmative diagnosis of clinically AIV-infected chickens. In our laboratory, several chickens with a severe clinical disease have been confirmed to be due to AIV infection (unpublished data). The chickens were confirmed as AI positive by isolation of the virus in chicken embryonated eggs, identification of the virus by haemagglutination /haemagglu-tination inhibition (HA/HI) test, and detection of viral nucleic acid by reverse transcriptase-polymerase chain reaction (RT-PCR). The availability of mAbs against of AIV-H5N1 is very likely to provide a more appropriate and safer diagnostic methods for the detection of AIV antigen in chickens. We have currently been able produce mAbs against AIV-H5N1 of Indonesian isolate and the applicability of those mAbs for detecting AIV antigen in chicken tissues was examined.

MATERIALS AND METHODS

Cells

Myeloma cells (P3-NS1/1Ag4.1), used for the preparation of hybridomas were obtained from Murdoch University, Australia. The cells were grown in Dubelco's modified essential medium (DMEM) with 10% newborn calf serum (NBCS) and antibiotics penicillin, 200 IU /ml, streptomycin 200 ìg/ml.

Virus

Formaldehyde inactivated AIV-H5N1 used in this study was an Indonesian isolate. The virus was propagated in chicken embryonated eggs and harvested from allantoic fluids. The titer of the virus was determined by haemagglutination (HA) test. The virus has been confirmed as H5N1 subtypes and PCR using H5 and N1 primers (unpublished data).

Production and Characterization of Monoclonal Antibodies

MAbs against the Indonesian isolate of AIV-H5N1 were produced by methods which were similar to those described by Ohnishi *et al* (2005). Six to 7 week-old female Balb/c mice were firstly immunized with 0.2 ml (equivalent to approximately 2^7 HA units) virus emulsified in Freund,s complete adjuvant. Fourteen and 28 days after the first immunization of the mice were respectively immunized with the same antigen but emulsified in Freund's in-complete adjuvant. Seven, 14, 15 and 16 days after the last immunization, the mice were boosted with the same antigen but without adjuvant. The mice were then sacrificed by cervical dislocation. The spleen was removed and used for the preparation of hybridomas.

As many as 2 x 10^7 immortal mouse myeloma cells prepared as above were fused with 10^8 lymphocytes derived from the spleen of mice immunized with AIV-H5N1. The fusion of the two types of cells was carried out using 50% polyethylene glycol (PEG) (Sigma Co, USA) and produced hybrid cells called hybridomas. The hybridomas were then screened by indirect ELISA (Campbell, 1991) for the anti-AIV antibodies using formaldehyde inactivated AIV-H5N1 and normal allantoic fluid as the antigen. The hybridomas producing mAbs reacted specifically with the virus were cloned by limiting dilution as described by McKearn, 1980, and were then used in the production of MAbs against the AIV-H5N1.

The immunoglobulin (Ig) class and subclass of the mAbs were determined by indirect ELISA using rabbit antimouse subtyping isotyper kits (Bio-Rad Laboratory, USA) according to the procedures described by manufacturer. ELISA microtitration plate was firstly coated overnight with inactivated AIV-H5N1 as described above. Into each well, 100 ul mAb diluted 1:10 in PBST were added and incubated for 1 hour at 37°C. Following three times washes with PBS, rabbit anti-mouse Ig isotyper from the kit was added to the wells and incubated as above. After 3 times washes, 100 ml affinity purified goat anti-rabbit IgG conjugated with HRP (Bio-Rad, USA, diluted 1:1000 in PBST) was added and incubated at 37°C for 1 hour. The plate was again washed as above and 100 ml substrate solution (1 mM 2.2'-azinodi 3-ethylbenzthyazoline-6-sulfonic acid in 0.005 Na citrate, 0.15 Na phosphate and 0.01% H_2O_2) was added. The absorbance of the substrate solution was read in Multiscan spectrophotometer with a 405 nm filter.

The proteins reacted with mAbs were determined by Western blotting assay according to the procedure as described by Zheng *et al.* 2001. Formaldehyde inactivated AIV-H5N1 was diluted in an equal volume of sample loading buffer (1.3% SDS , 5% mercaptoethanol, 0.0625 M Tris-HCl pH. 6.8, 10% glycerol, 0.001% bromophenol blue). The viral proteins were separated by sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) using 3% loading gel and 10% separating gel. The proteins in the gel was then transferred onto nitrocellulose membrane. Following 1 hour blocking at room temperature with 3% skim milk in Tris-buffered saline (TBS/100 mM Tris pH.7.4 adjusted with 1 N HCl) and a brief washing with TBS, nitrocellulose membrane was then cut into 0.5 cm strips. Each strip was then soaked in hybridomas's supernatant fluid containing mAbs and incubated 24 hours at room temperature. Following 3 times washes with TBS, anti-mouse IgG coupled with biotin (Bio-Rad USA, diluted 1:1000 in TBS) was the added to the membrane. After 3 times washes with TBS, streptavidin-alkaline phosphatase (Promega, diluted 1:500 in TBS) was then added to the membrane. The membrane was washed 3 times as above and the reactive AIV proteins in the membrane were visualized by adding 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/ NBT) substrate (Bio-Rad, USA).

Detection of AIV Antigen in Egg Allantoic Fluid by Indirect ELISA

An Indonesian isolate of AIV-H5N1 was propagated in 10 day-old embryonated chicken eggs and the virus was harvested from allantoic fluid. The virus was firstly inactivated with 0.1% formaldehydehyde and then titrated by haemagglutination (HA) assay. A serial two-fold dilution of AIV with a starting titer of approximately 1 HA unit was then prepared in carbonate-bicarbonate coating buffer (15 mM Na_2CO_3, 35 mM NaHCO3 pH.9.6) and 100 ml AIV antigen from each dilution was coated into ELISA microtitration plate. Following an overnight incubation at 4°C and three times washes with 0.05% Tween-20 in phosphate buffered saline pH. 7.2 (PBST), 100 ml blocking buffer (5% skim milk in PBST) was added. The plate was incubated for another 1 hour at 37°C. One hundred ml mAb samples diluted 1:10 in PBST were added to each well. The plate was incubated for 1 hour at 37°C. After three times washes as above, 100 ml anti-mouse IgG-

conjugated with horseradish peroxidase (HRP) (Bio-Rad, USA) diluted 1:2000 in PBS-T was added to each well. The microplate was then incubated for 1 hour 37°C and washed three times as above. The plate was again washed as above and 100 ml substrate solution (1 mM 2.2'-azinodi 3-ethylbenzthyazoline-6-sulfonic acid in 0.005 Na citrate, 0.15 Na phosphate and 0.01% H_2O_2) was added (Bio-Rad, USA. The intensity of substrate color in each well was read by multiscan spectrophotometer using 405 nm filter.

Detection of AIV Antigen in Chicken Tissues by Immunoperoxidase Staining

The AIV-infected chicken used in this study were cordially provided by co-assistant students in the Faculty of Veterinary Medicine, Udayanan Univesity, Denpasar Bali. All chickens had been tested for AIV infection by virus isolation in chicken embryonated eggs and identification by haemagglutination/haemagglutination (HA/HI) test. The result of the test were then further confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers specific to H5 and N1 subtypes. Two chickens that were confirmed positive to AIV-H5N1 infection and two others that were confirmed negative (unpublished data) were used in this study.

Several organs such as brain, proventricle, small intestine, liver, lung, bursa of Fabricius, spleen, and kidney derived from the chickens were preserved and fixed with 4% buffered formaldehyde. Paraffin embedded organs and thin sections of the organs were prepared by standard methods. Immunoperoxidase staining was then carried out according to the methods similar to those described by Ohnishi *et al* (2005). Thin sections of tissues on microscope slides were de-paraffinized twice by xylol and twice with absolute ethanol. The tissue section was washed twice with PBS and treated with 0.05% trypsin for 10 minutes at 37°C. The endogenous peroxidase of the tissues was then inactivated by 3% H_2O_2 in PBS for 30 minutes at room temperature. After blocking with 20% normal goat serum, mAbs against AIV-H5N1 was added onto the tissue section and incubated for 18 hours at room temperature. The bound mAbs were the detected by biotinylated goat anti-mouse IgG (Biodesign International) and avidin-horse radish peroxidase (Sigma Co, USA). The AIV proteins bound with mAbs were then visualized by adding Diazinobenzidine (DAB) substrates (Sigma Co, USA, 50 mg/50 ml PBS containing 0.07% H2O2).

RESULT AND DISCUSSION

Characteristics of Monoclonal Antibodies

Twelve clones of stable hybridomas secreting mAbs against the AIV-H5N1 of Indonesian isolate were produced. Screening by ELISA using formaldehyde inactivated AIV-H5N1 showed that all of these 12 clones of hybridomas produced mAbs specific against the virus but not against the normal allantoic fluid. Eight mAbs were further characterized and they were designated as AG8, BC12, CC5, CG1, DD9, DF11, EA11, an EE8. Isotyping of mAbs using rabbit anti-mouse IgG subtyper subtyping kit showed 3 mAbs with IgG1 subclass (AG8, DF11, EA11), 1 mAb with IgM subclass (DF9), 3 mAbs with IgG3 subclass (CC5, CG1, DD9) and 1 mAb withIgG2a subclass (EE8) (Table 1). In western blotting, all mAbs reacted with formaldehyde inactivated AIV-H5N1. No mAb reacted with normal allantoic fluid. Two mAbs (reacted with 2 protein bands of the molecular weight of 76 Kda and 55 Kda, 5 mAbs (DF11 and EA11) reacted with a single protein band of 76 Kda. One mAb (DF9) reacted with a diffuse protein band.

AIV antigen in Allantoic fluid

By indirect ELISA, anti-AIV MAbs were able to detect AIV antigen in allantoic fluid at the titers as low as $2^{-2}$to 2^{-4}HA units per 0.1 ml. Different mAbs have different ability in detecting AIV antigen in allantoic fluid. No mAbs reacted with normal allantoic fluid (Table 1).

AIV Antigen Detected in Chicken Tissues

Three (CG1, EE8, AG8) produced a good and a strong result when used for the detection of AIV antigen in chickens. One mAb (DF11) did not react with AIV antigen in chicken tissues. AIV antigen was detected in the two infected chickens but not in uninfected chickens. AIV antigen with a high intensity was observed in proventricle and in small intestine. AIV antigen at a lesser intensity was also observed in other organs such as lung and spleen (Figure 2). AIV antigen was hardly detected in the brain, muscle tissue, and kidney. No clear difference on the distribution of infected tissues was observed between the two infected chickens (Figure 2).

Stable anti-AIV-H5N1 mAbs-secreting hybridomas were successfully produced by fusion of immortal myeloma cells with lymphocytes of mice immunized with the virus. The use of crude

Table 1. Characteristics of Monoclonal Antibodies Prepared against Avian Influenza Virus Subtype H5N1 of Indonesian Origin

MAbs	Isotypes	Western blotting		ELISA		IHC
		AIV-H5N1	NA	AIV	N.A	
AG8	IgG1	76 KDa	—	+++ (2^{-3} HA)	—	+++
BC12	IgG1	76 KDa	—	+++ (2^{-4}HA)	—	ND
CC5	IgM	Diffuse	—	++ (2^{-2}HA)	—	ND
CG1	IgG3	76 KDa	—	+++ (2^{-3}HA)	—	+++
DD9	IgG3	Diffuse	—	++ (2^{-3}HA)	—	+++
DF11	IgG1	76/56 KDa	—	++ (2^{-3}HA	—	ND
EA11	IgG1	76/56 KDa	—	++ (2^{-4}HA)	—	+++
EE8	IgG2a	76 KDa	—	+++ (2^{-3}HA)	—	ND

MAbs : monoclonal antibodiesELISA : enzyme linked immunosorbent assayAIV : avian influenza influenzaN.A. : normal allantoic fluidIHC : immunohistochemistry ND : not determed+++ : strong positive++ : moderate positive— : negative

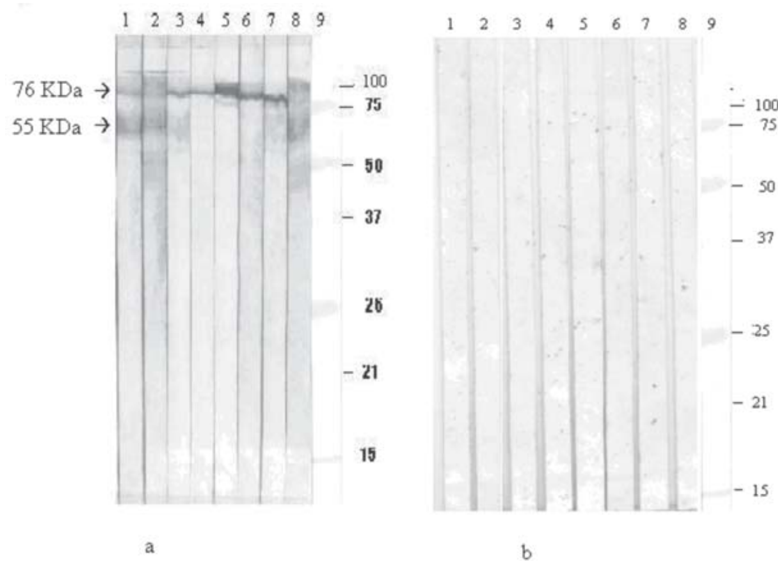

Figure 1. Reactivity of MAbs with AIV-H5N1 and normal allantoic antigens analysed by western blotting. Antigen : formaldehyde inactivated H5N1 (A), normal allantoic fluid. MAbs : DD9, CC5, AG8 CG1, DF11, EA11, EE8 and DF9 (1-8), Standard molecular weight markers (9)

unpurified and formaldehyde inactivated virus for immunization of mice in the preparation of mAbs appeared to be not an important factor for the production of hybridomas stably producing mAbs against AIV. This is evident as all of the isolated hybrodomas consistently produced mAbs against the virus and but not against the normal allantoic fluid. The use of relatively unpurified virus for immunization of mice in the preparation of mAbs has been reported (Wickramasinghe *et al.* 1993; **Pantophlet *et al.* 2001)**.

Screening method appeared to be the more important factor for the success selection hybridomas producing anti-AIV-H5N1 mAbs. As in the immunization, the antigen used in the ELISA test for screening mAbs was formaldehyde inactivated AIV-H5N1. The virus was originally propagated by in the allantoic cavity of chicken embryonated eggs. The virus was then harvested from the allantoic fluid of the infected chicken embryo and was therefore expected to contain a plenty of normal allantoic fluid. It was therefore very likely that the immunization of mice with

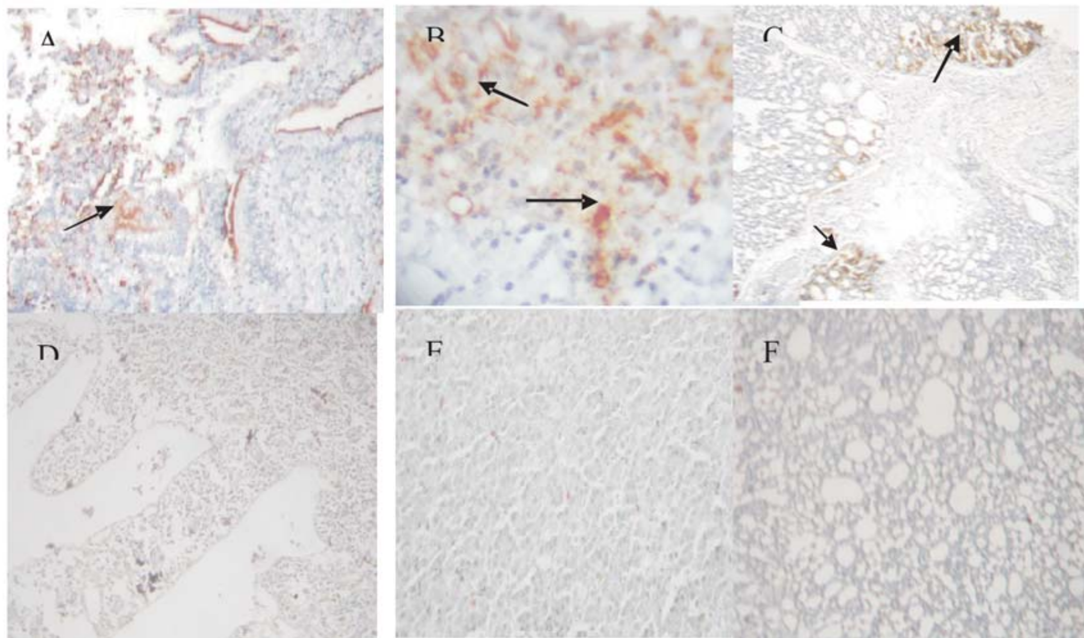

Figure 2. Detection of Avian Influenza Viral Antigen in Various Chicken Tissues by ImmunoperoxidaseTechnique using monoclonal antibodies anti-AIV H5N1 Infected small intestine (A), spleen (B) and lung (C). Normal small intestine (D), spleen (E) and lung (F). Infected cells ('!)

such crude antigen will stimulate the production of antibodies against both AIV and normal allantoic fluid. This then was confirmed when several mAbs which reacted with normal ascitic fluid were detected by ELISA using the unpurified AIV-H5N1(data not shown). Such mAbs were excluded by retesting with ELISA using normal allantoic fluid as an antigen.

Isotyping showed that mAbs produced in this experiment were of IgM, IgG1, IgG2a and IgG3 subclasses. The information on the isotype of mAbs is important in the selection serological techniques used for purification of mAbs. In addition, the determination of mAbs isotype is also important in the selection of techniques to be developed using mAbs. The availability of mAbs of IgG isotypes will also enable the purification of the mAbs using Protein A or protein G (Sawyer, 2005) which is often required for the development of a particular test such as capture ELISA (Ohnisi *et al.* 2005).

In western blotting assay, all isolated mAbs reacted specifically only with viral antigen. None of them reacted with blots of normal allantoic fluid which were used as the negative control in this experiment. The result confirmed that MAbs specific to AIV-H5N1 can be produced by immunization of mice with relatively unpurified virus. The protein bands recognized by mAbs were 76 KDa, 55 KDa and several diffuse bands. The protein band with 76 KDa detected by most mAbs (CG1, AG8, EA11, EE8 , BC12) is likely to be uncleaved haemagglutinin (HA0) of AIV-H5N1. The HA protein of AIV is a surface glycoprotein encoded by segment 4 (HA) of the viral segmented RNA genomes. The protein is initially translated as uncleaved precursor of HA protein with the molecular weight of around 76 KDa. It is then post-translationally cleaved by host cellular proteases into two subunits, HA1 (56 KDa) and HA2 (25 KDa) (Skehel and Waterfield, 1975; Zhirnov *et al.* 2002). The protein contains sialic acid which plays an important role in the binding of the virus into the receptor molecules on the surface of susceptible cells (Hulse *et al.* 2004) and such cleavage step is necessary for the infection of the virus into susceptible cells (Lazarowitz *et al.* 1973; Zhirnov *et al.*2002). The availability of mAbs against this protein will enable further investigation on the function of this protein in the infection process.

In indirect ELISA, mAbs were able to detect AIV antigen in the allantoic fluid of infected chicken embryonated eggs with varying degrees of sensitivity from 2^{-2} to 2^{-4} HA units. HA test is widely used to quantify AIV antigen derived from the allantoic fluid of chicken embryonated eggs.

The result of HA test is usually confirmed by HAI test using referent sera specific to AIV virus (Gough, 2004). The titer of AIV which is still detectable by HA test is 1 HA unit. In this study, it is clear that detection of AIV antigen in allantoic fluid by indirect ELISA is 4 to 16 times more sensitive than HA test. This result may indicate that indirect ELISA using mAbs may be used to detect AIV antigen in other biological samples such as feces, discharges, and plasma with a significantly higher sensitivity as compared to HA test. However, this indication needs further investigation.

When examined whether the mAbs were applicable for use in the detection of AIV antigen in infected chicken' tissues, several mAbs (CG1, AG8 and EA8) produced a strong positive results (Figure 2). One mAb did not react with AIV antigen in the infected chickens, suggesting that this MAbs did not recognized the AIV epitope in formaldehyde fixed and paraffin embedded tissues. The reason behind this is unknown. It is however possible that the epitope recognized by this mAb might have been destroyed or hidden during the tissue processing. When the 3 mAbs were used to stain tissues or organs of normal uninfected chickens, none of them produced a positive result. This showed that all of the three the selected mAbs reacted specifically with AIV antigen and are therefore applicable for use in development of specific test for the detection AIV infection in chickens. The use of mAbs in the immunochemistry staining for the detection of viral antigen in the infected host has been widely reported (Ohnishi *et al.* 2005; Astawa. 2002).

In the infected chickens, high intensity of AIV antigen was detected in organs such as preventricle and intestine villi (Figure 2), suggesting that the virus replicates efficiently in these two gastrointestinal organs. This is in accord with the finding that influenza viruses replicate preferentially in the intestinal tract, resulting in the excretion of high titer viruses in the feces (Horimoto and Kawaoka, 2001). The combination of the availability of cells bearing the receptor for AIV and the presence of abundant proteolytic enzymes may contribute to the efficient replication of the virus in the small intestine and proventricle of chickens. In addition, gastrointestinal tract is rich in proteolytic enzymes (Banks and Plowright, 2003) which are responsible for post translational cleaving of HA0 into HA1 and HA2 (Garten and Klenk, 1999). The cleavage of HA protein is required for the efficient replication of the virus in the two organs. It is therefore expected that the two organs contain plenty of virus.

The availability of mAbs against AIV-H5N1 has enabled the detection of AIV antigen in chicken tissues. The use of mAbs on formaldehyde fixed and paraffin embedded tissues may enable the development of a relatively more appropriate and safer test which can be performed in laboratory with simple facilities and low biosecurity levels. The immunological detection system developed in this experiment also safe to perform on daily basis as it uses formaldehyde fixed tissues which inactivate the AIV. As chickens and other birds are susceptible hosts for AIV infection (Matrosovich *et al.* 1999), the availability of test to detect chickens carrying the virus is important in preventing the future outbreaks AI in susceptible hosts.

It is also important to note that, using mAbs produced in this experiment, it may be possible to determine H5 and N1 subtype of AIV. At this stage, however, which mAbs react specifically to H5N1 subtypes and which mAbs cross-react with other AIV subtypes need further invest-tigation. If mAbs which react specifically only with AIV of H5N1 subtype are available, then determination this subtypes can be carried out directly by mAbs. This is important as AIV of H5N1 subtype is one that causes most fatal infection in avian species and in mammal including human (Swayne and Suarez, 2000).

CONCLUSSION

It is clear that monoclonal antibodies produced against AIV-H5N1 are important reagent for use in the detection of AIV antigen in clinically affected chickens. The mAbs are applicable for use in detection of AIV antigen in the allatoic fluid of inoculated chicken embryonated eggs and also in the several tissues/ organs of naturally infected chickens.

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