Jurnal Veteriner Desember 2010 Vol. 11 No. 4 :196-202
ISSN : 1411 - 8327

Immunological Detection of Rabies Virus in Brain Tissues of Infected Dogs by Monoclonal Antibodies

(PELACAKAN VIRUS RABIES PADA JARINGAN OTAK ANJING TERINFEKSI RABIES DENGAN ANTIBODI MONOKLONAL)

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ABSTRACT

In order to establish an immunological detection of rabies virus in tissues of infected dogs, monoclonal antibodies (mAbs) against rabies virus (RV) were produced. The mAbs were produced by fusion of mieloma cells with the lymphocytes of mice immunized with RV. The mAbs produced were then characterized and used for the detection of rabies virus in brain tissues of infected dogs. Six mAbs designated CC6, EG4, DG10, BB12, CA9 dan EB5 were used in this study. In Western blotting test, some mAbs reacted with 66 KDa which is the glycoprotein of the virus. In immunoperoxidase, 2 mAbs (CC6 and DG10) detected RV in the brain of infected dogs. By direct immunoflourescence, flourescence isotyocyanate (FITC) labelled DG10 mAbs detected RV in fresh and formaldehyde fixed brain tissues. RV was detected in 12 infected dogs but not in normal uninfected dogs. In this study it was confirmed that rabies virus can be detected in the brain tissues of infected dogs by monoclonal antibodies.

Key words : antibody, monoclonal, brain, rabies, dog.

ABSTRAK

Dalam rangka mengembangkan teknik pelacakan virus rabies pada jaringan anjing terinfeksi, antibodi monoklonal (AbMo) dibuat terhadap virus tersebut. AbMo dibuat dengan cara memfusikan sel myeloma dengan limfosit mencit yang telah diimunisasi dengan virus rabies. AbMo yang diproduksi kemudian dikarakterisasi dan dipakai untuk melacak virus rabies pada jaringan otak anjing terinfeksi. Sebanyak enam AbMo yang dinamai sebagai CC6, EG4, DG10, BB12, CA9, dan EB5 dipakai dalam penelitian ini. Dalam uji Western Blotting, beberapa AbMo bereaksi dengan protein 66 kDa yang merupakan protein virus rabies. Dalam uji imunoperoksidase, dua AbMo (CC6 dan DG 10) dapat melacak virus rabies dalam otak anjing terinfeksi. Dengan uji imunofloresens, AbMo yang dilabel dengan floresens isotio sianat (FITC) dapat melacak virus rabies dalam jaringan otak segar mau pun yang difiksasi dengan formalin. Virus rabies juga terlacak pada otak dari 12 anjing terinfeksi, dan tidak terlacak pada jaringan otak yang tidak terinfeksi. Dalam penelitian ini terbukti, bahwa virus rabies dapat dilacak pada jaringan otak terinfeksi dengan AbMo.

Kata-kata kunci: antibodi, monoklonal, otak, rabies, anjing

INTRODUCTION

Rabies is an old zoonotic disease affecting warm-blooded animals including human. According to World Health Organization (WHO) more than 3 millions people are at risk of rabies in the world. The disease is estimated to have killed 50 000–60 000 people in 85 countries where rabies is still endemic (WHO, 2005). Many measures have been conducted in an effort to eradicate the disease in the world but no countries have been able to eradicate the disease completely. The attempts to eradicate the disease have been conducted by vaccination, the control of dog population and others. The disease is however still endemic in many countries and appears to be very difficult to eradicate. In Indonesia, rabies is now endemic in many areas

such as Flores , Sulawesi, Kalimantan, Sumatera Barat dan Bali (Susetya *et al*, 2008).

Rabies is caused by lyssavirus of Rhabdoviridae family (Baloul dan Lafon, 2003). The virion consists of single-stranded RNA (Consales and Bolzan, 2007) which code for viral proteins such as N (nucleocapsid), protein P dan M (membrane), G (envelope glycoprotein) and L (replicase) (Bradame and Tordo, 2001). The disease is transmitted by the bitting of rabid animals such as dog, cat, monkey and others. Dog is however, the most important animals transmitting the disease to human. Detection of dogs carrying the virus is an important step in monitoring rabies in dog population. The development of rapid and accurate diagnostic methods using monoclonal antibodies is therefore important.

Monoclonal antibodies (mAbs) have been widely used in the development of a rapid and accurate diagnostic method for many infectious diseases. Some advantages of mAbs when used in the development of diagnostic methods for viral infection are as follows. They react only with a single epitope in an antigenic structure which provide specific reagent for the detection of viral antigen. They can be produced in a high quatity *in vitro* which enable the production of a cheap reagent (Campbell, 1991; Ohnishi *et al,* 2005). MAbs are therefore very potential to be used in the development of a rapid and accurate laboratory diagnosis for rabies. The use of mAbs for the development of laboratory diagnosis has been reported in many viral infections (Astawa *et al*, 2002, Astawa *et al* 2007; Zheng *et al*, 2001).

MATERIALS AND METHODS

Cells

Myeloma cells (P3-NS1/1-Ag4.1), 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, strptomycine 200 μg/ml.

Production and Characterization of Monoclonal Antibodies

MAbs against the Indonesian isolate of rabies virus 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 undiluted rabies vaccine (Rabvac3). Fourteen and 28 days after the first immunization of the mice were respectively immunized with the same antigen. Seven, 14, 15 and 16 days after the last immunization, the mice were boosted with the same antigen but diluted 1:2 with serum-free media. 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 rabies virus. The fusion of the two types of cells was carried out using 45% 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-Rabies antibodies. The hybridomas producing anti-rabies mAbs were cloned by limiting dilution as described by McKearn, (1980) and were then used in the production of MAbs against the rabies virus.

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 rabies virus. Into each well, 100 ul mAb diluted 1:10 in PBST were added and incubated for 1 hour at 37°C. Following three times washing with PBS, rabbit anti-mouse Ig isotyper from the kit was added to the wells and incubated as above. After 3 times washing, 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.

Viral proteins reacted with mAbs were determined by Western blotting assay according to the procedure as described by Zheng *et al.* 2001. Rabies virus 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 were 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 washing with TBS, anti-mouse IgG coupled with biotin (Bio-Rad USA, diluted 1:1000 in TBS) was then added to the membrane. After 3 times washing 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 RV proteins in the membrane were visualized by adding 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/ NBT) substrate (Bio-Rad, USA).

Purification and Labeling of Monoclonal Antibodies

Some mAbs were purified by affinity chromatography using sepharose 4B labelled with protein (Bio-Rad, USA). The purified mAbs were then labeled with fluorescence isothyocyanate (FITC). Firstly, the purified mAbs were dialyzed with carbonate buffer pH 8,6 overnight at 4°C. FITC was then added to purified mAbs and incubated for 4 hours at room temperature. The FITC labeled-mAbs were passed through sephadex G-25 collum to separate them with unbound FITC.

Detection of Rabies Virus by Direct Immunoflourescence

Touch smear of brain tissues derived from suspected rabbid dogs or thin section of formaldehyde-fixed brain tissues were prepared on poly-L-Lysine coated microscope slides. The brain tissues were fixed with acetone for 15 minutes at room temperature. After washing with PBS, FITC-labeled mAbs were added and incubated for 1 hour at room temperature. The tissues were then washed three times and the presence of rabies virus-infected cells were examined under flourescence microscope.

Detection of Rabies Virus in Brain Tissues by Immunoperoxidase

Thin section of brain tissues derived from suspected rabbid dogs was prepared on poly-L-Lysine coated microscope slides. The tissues were firstly depafinezed twice with xylene, rehydrated with reducing concentration of alcohol. After three times washing with PBS, the tissues were treated with 0,25% typsin for 5 minutes at 37°C to retrieve the rabies virus antigen in the tissues. Then, the tissues were treated with H_2O_2 to inactivate the endogenous peroxidase in the tissues. Anti-RV mAb was then added onto the tissue section on the slide and incubated for 1 hour at room temperature. Following three times washing with PBS, biotinylated anti-mouse IgG (Kappel Lab; diluted 1:100) was added to the tissue section and left for 1 hour at room temperature. Avidin-horse radish peroxidase (HRP) the added and incubated for 20 minutes at room temperature. Diazinobenzidine (DAB) substrates (Sigma Co, USA, 50 mg/50 ml PBS containing 0.07% H2O2). was then added to the tissue sections for 5 minutes. Following staining with Mayer hematoxylin, the presence of rabies virus-infected cells was examined under light microscope.

RESULTS AND DISCUSSION

Characteristic of Monoclonal Antibodies

As many as 26 clones of stable hybridomas secreting mAbs against the RV were produced in this study. Screening by ELISA showed that all of these 26 clones of hybridomas produced mAbs specific against the virus but not against the normal cells. Six mAbs were further characterized and they were designated as DG10, CC6, EG4, EB5, BB12 and CA9. Isotyping of mAbs using rabbit anti-mouse IgG subtyper subtyping kit showed that the isotypes of the MAbs were CC6, BB12 dan EG4 (IgG1), DG10 (IgG2a) EB5 dan CA9 (IgM) (Tabel 1). In Western Blotting, all mAbs reacted with rabies virus. No mAb reacted with normal uninfected cells. Three mAbs reacted to the viral protein of the molecular weight of 66 kDa (DG10, CC6 and EG4).

Detection of Rabies Virus in Brain Tissues by Monoclonal Antibodies

Some mAbs were used to detect rabies virus in the brain of infected dogs by immuno-peroxidase and immunoflourescence. Using immunoperoxidase staining of formalin fixed tissues, rabies virus was detected in the neuron of brain tissues. The infected cells appeared as brown cytoplasm and bluish violet nuclei (Figure 3). Rabies virus was not detected in the brain

Tabel 1. Characteristics of monoclonal antibodies prepared against rabies virus

MAbs	Isotype	ELISA titer	Reactive protein*	IHC	FAT
CC6	IgG1	2^5	66 KDa	++	±
EG4	IgG1	2^6	66 KDa	++	ND
DG10	IgG2a	2^8	66 Kda	+++	++
BB12	IgG1	2^5	—	+	ND
CA9	IgM	2^5	—	+	ND
EB5	IgM	2^6	—	+	ND

*) Determined by Western Blotting
ND : not determined
+++) : strong positive
++) : moderate positive
+) : weak positive
±) : dubious

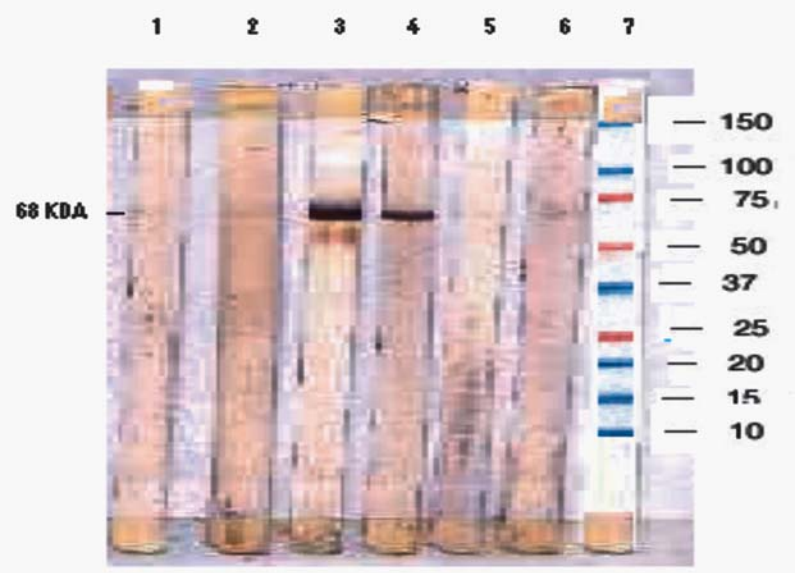

Figure 1. Reactivity of monoclonal antibodies with rabies virus protein in Western blotting assay. 1-6 respectively (EG4, BB12, DG10, CC6, CA9, EA5), 7 : protein markers

tissues of normal uninfected dogs. Similarly, in direct immunoflourescent staining, rabies virus was detected in both formaldehyde-fixed and freshly prepared brain tissues of rabies virus infected dogs but not in normal uninfected dogs. The infected cells appeared as yellowish green and normal uninfected cells appeared as dark when examined under fluorescence microscope (Figure 2). MAb DG10 gave the best result when used in immunoperoxidase and immuno-flourescence.

When used to detect the rabies virus in brain samples of 12 infected dogs, mAb DG10 produced a good result. Rabies virus was detected in all infected dogs but not normal uninfected dogs (Table 3). In the infected dogs, the rabies virus was detected in the neuron of hyphocampus, cerebellum, amnion horn dan cerebrum (Figure 3 and 4). The intensity of infection varied from one to another infected dogs. All infected dogs used in this study have been confirmed to be infected by rabies virus using polymerase chain reaction (PCR) and fluorescence antibody technique (FAT) conducted at the Laboratory of Animal Disease Investigation Centre Denpasar (data not shown). The detection of rabies virus in the brain tissues of 12 rabid dogs is presented in Table 2.

Screening methods appeared to be an important factor for the selection of hybridomas producing mAbs against rabies virus. In this study, the antigen used for screening of mAbs was rabies virus prepared by infection of cell culture. The virus was inactivated by beta

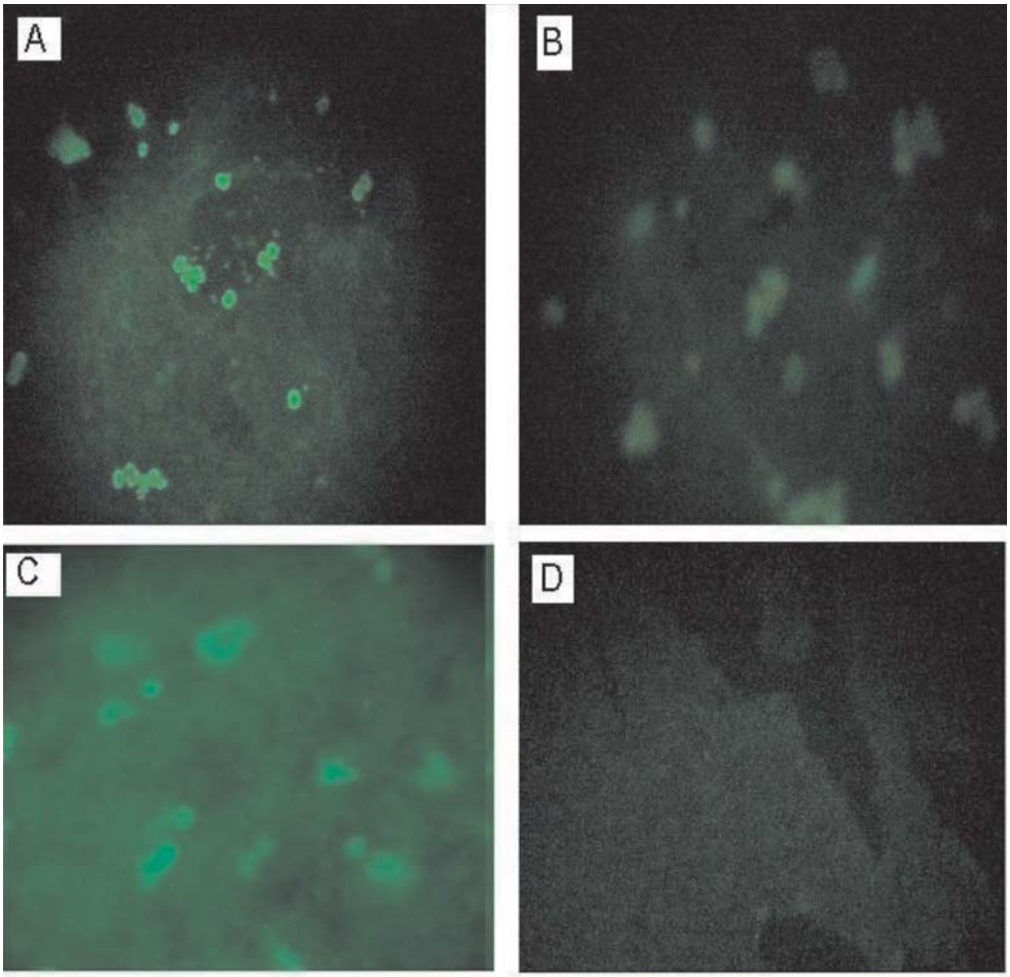

Figure 2. Reactivity of monoclonal antibodies in various brain tissues of rabies virus-infected and normal dogs. The infected cells appared as yellowish green flourescence (A-C) , normal cells appeared as dark background (D).

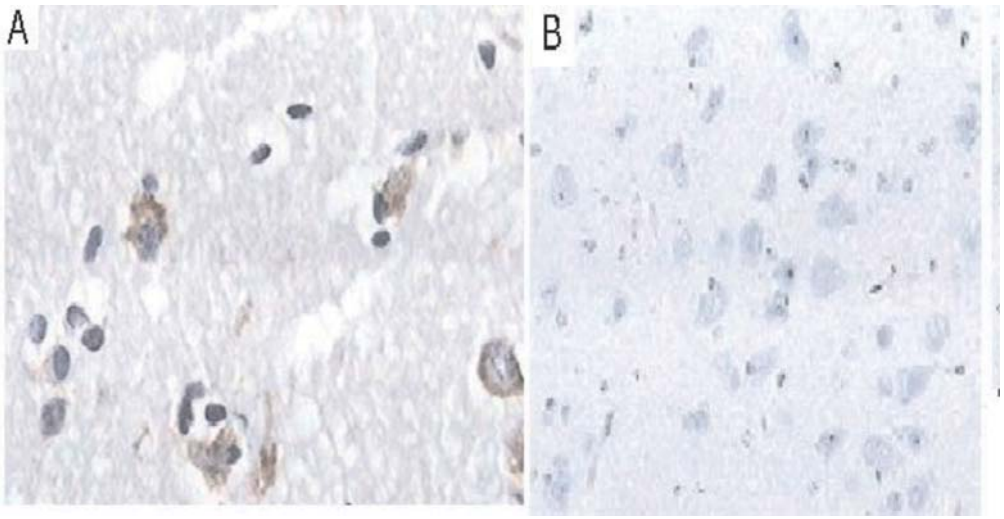

Figure 3. The infected brain tissues detected by immunoperoxidase using monoclonal antibodies. The cytoplasm of infected cells appeared as brown and bluish violet nuclei (A) and nl cells appeared as unstained cytoplasm and bluish violet nuclei (B).

Tabel 2. Detection of rabies virus in 12 infected dogs by monoclonal antibodies

No	Origin	Fresh tissue		Formaldehyde Fixed	
		FAT	IP	FAT	IP
1	Badung 435	+++	ND	ND	ND
2	Tabanan 451	+++	ND	ND	ND
3	Badung 455	+++	ND	ND	ND
4	Badung 455	+++	ND	ND	ND
5	Badung 001	ND	ND	++	ND
6	Badung 148	ND	ND	++	ND
7	Badung 188	ND	ND	++	ND
8	Badung 135	ND	ND	++	ND
9	Badung 097	++	ND	++	++
10	Badung 096	++	ND	++	++
11	Badung 511	++	ND	TD	TD
12	Badung 512	++	ND	TD	TD

TD : not done ++) : moderate positive ±) : dubius
+++) : strong positive +) : weak positive

propiolacton. As the virus was prepared from rabies virus-infected cells, it is very likely that the virus still contains cellular proteins. Using such virus-infected cells, therefore, the ELISA used for screening mAbs against rabies virus was very likely to detect antibodiesagainst both viral and cellular proteins. However, the used of normal cellular antigen as negative control for ELISA has enabled the exclusion of mAbs against cellular proteins. It was then confirmed that all mAbs isolated in this study were those reacted only with viral proteins

The isotypes of mAbs are also important factors in the isolation of a good mAbs. In this study, the isotypes of mAbs against rabies virus was determined by *rabbit antimouse isotyper subtyping kit*. The isotypes of mAbs against rabies virus are required in the selection of techniques used in the development of diagnostic methods. In direct immunoflourescence, for example, the mAbs used need to be purified in order to enable the labeling of the mAbs with fluorescent dyes. Some tests such as capture ELISA also require purified mAbs. The determination of mAbs isotypes is also important for selection of which mAbs are appropriate for a certain test. Monoclonal antibodies with IgG isotype usually produce a more specific reaction as compare to those of IgM (Swayer, 2005; Ohnisi *et al.* 2005).

In Western Blotting, some mAbs react with viral protein and none reacted with cellular proteins (Figure 1). The result showed that all isolated mAbs reacted specifically with rabies virus. The protein band reacted with the mAbs was 66 kDa protein (Figure 1) which appeared to be glycoprotein of rabies virus (Yoneda *et al*, 2008). The glycoprotein of rabies virus plays important role in the infection. It acts as the attachment protein which initiates the first interaction between the virus and host cells. The infection of rabies virus into cells only occurs when the virus binds to its receptor on surface of cells (Consales and Bolzan, 2007).

When examined for their ability to detect rabies virus in brain tissues of rabbid dogs, 2 mAbs produced a very good result. In immunoproxidase using DG10 mAbs, the use of formaldehyde-fixed brain tissues produced a very good result characterized by the presence of brown cytoplasm and bluish violet nuclei (Figure 3). Although, more laborious and time consuming, the use of immunoperoxidase has enabled the detection of rabies virus in brain tissues in which almost likely contains inactivated rabies virus. This is important as rabies virus is a highly contagious which can infect human via biting or wound exposed to infectious rabies virus (Consales and Bolzan, 2007). The use of mAbs in immuno-peroxidase to detect viral infection in tissues or cells has been reported (Ohnishi *et al.* 2005; Astawa *et al.* 2006).

Similarly, the use of mAbs in direct immunofluorescent also produced a very good result. The rabies virus infected cells are characterized by yellowish green fluorescence

whereas uninfected cells appeared as dark field. The advantages of using direct immunofluorescen are follows. In can be used both in fresh and formaldehyde-fixed tissues. It is also a simple and fast test which requires only an hour to get the result. Immunoflourescence has long been considered to be the best laboratory diagnostic method for the detection of rabies virus in fresh brain tissues (Koch *et al*, 1975)

It is clear from this study that mAbs against rabies virus can be produced and used for the detection of rabies virus in infected dogs. They can be used in immunoperoxidase which provide a safe and simple test for the detection of rabies virus in formaldehyde fixed tissues. Indirect immunoflourescence using both fresh and formaldehyde fixed tissues using anti-rabies virus mAbs produced a simple and rapid test for laboratory diagnosis of rabies.

ACKNOWLEDGMENT

We thank to the Programme of "Hibah Penelitian Unggulan Strategis Nasional", Directorate General of Higher Education, Department of Nasional Education, Jakarta for providing the financial support which enables us to conduct this study.

REFERENCES

Astawa NM. 2002. Antibodi monoklonal sebagrabies reagen diagnostik yang spesifik untuk infeksi avian reovirus. Journal Veteriner 3 : 133-139

Astawa et al, 2007. Immunological detection of avian influenza virus in infected ducks by monoclonal antibodies against AIV-H5N1 , Microbiology Indonesia. 1: 119-124

Baloul L, Lafon M. 2003. Apoptosis and rabies virus neuroinvasion. *Biochem* 85: 777-788.

Bradame H., Tordo N. 2001. Host switching in Lyssavirus history from the chiroptera to the carnivora orders. *J. Virol*. 75: 8096-8104.

Campbell AS. 1991. Laboratory technique in biochemistry and molecular biology; Monoclonal antibody and immunosensor technology. Elsevier Amesterdam

Consales CA, Bolzan VL 2007. Rabies review: immunopathology, clinical aspects and treatment. *J. Venom. Anim. Toxins incl. Trop. Dis*., 2007, 13, 1, p. 33

Koch KJ, Sagart JW, Davidson, Lawhaswahs K. 1975. Diagnosis of human rabies by cornea test. *Am J Clin Pathol* 63: 509-15.

McKearn, T.J. (1980). Cloning hybridomas by limiting dilution in liquid phase: In" Monoclonal antibodies : new dimension in biological analyisis. (R..H. Kennet , T.J. McKearn dan K.B. Bectol Eds), Plenum Press. New York and London p 374.

Ohnishi K, Sakaguchi M, Kaji Tohiro, et al. 2005. Immunological Detection of Severe acute respiratory syndrome coronavirus by monoclonal antibodies. *Jpn J Infect Dis* 58: 88-94

Swayer A, 2005. Monoclonal Antibody Core Facility; High through put production of mouse monoclonal antibodies using antigen microarrays. *EMBL research report* 188-191 online http://www.embl.org/ publications accessed 11 November 2006.

Susetya H, Sugiyama M, Inagaki A, Ito N, Mudiarto, MinamotoN. 2008. Molecular epidemiology of rabies in Indonesia. Virus Research 135:144-149

World Health Organization (WHO), 2005 *Expert Consultation on Rabies*. 1. Report. Geneva: WHO Tech. Rep. Series, 931. 121p.

Yoneda A, Tuchiyu K, Takasima Y, Arakawa T, Tsuji, Hayashi, Matsumoto, Y. 2008. Protection of mice from rabies by intranasal immunization with inactivated rabies virus. Exp Anim 57 1-9.

Zheng L, Zhang S, Wood C, Kapil S. Wilcox GE, Loughin T, Minocha AC. 2001. Differentiation of two bovine lentiviruses by a monoclonal antibody on the basis of the epitope specificity. *Clin and Diag Lab Immunol 8* :283-287