

Monoclonal Antibodies as Ligands for Purification of Rabies virus Proteins from the Brain Tissues of Infected Dogs and Mice

(ANTIBODI MONOCLONAL SEBAGAI LIGAND UNTUK PURIFIKASI PROTEIN VIRUS RABIES ASAL JARINGAN OTAK ANJING DAN MENCIT TERINFEKSI)

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ABSTRAK

Telah dikembangkan teknik pemurnian protein virus rabies berbasis kromatografi imunoafinitas. Dalam teknik ini, antibodi monoklonal (abMo) terhadap virus rabies dilabelkan ke resin CnBr-agarose yang kemudian dipakai sebagai matriks untuk memurnikan protein virus rabies. Homogenat jaringan otak asal anjing dan mencit terinfeksi dan tidak terinfeksi virus rabies dicampur dengan resin abMo-CnBr agarose, dituangkan ke dalam kolom kromatografi dan dicuci secara ekstensif dengan *phosphate buffered saline* (PBS). Setelah dielusi dan dinetralisasi, protein virus rabies dilacak dengan *enzyme-linked immunosorbent assay* (ELISA) and *Western blotting assay*. Dari 3 abMo (BB5, AE11 dan AF6) yang dipakai sebagai ligand, resin abMo AE11-agarose menghasilkan titer protein virus rabies yang lebih tinggi dibandingkan dengan yang menggunakan resin abMo BB5-CnBr agarose dan abMo AF6-CnBr agarose. Pada uji Western Blotting, protein virus rabies yang dimurnikan tampaknya mempunyai berat molekul 65 Kda (glikoprotein) dan 38 kDa proteins. Pada uji ELISA, protein virus rabies yang dimurnikan bereaksi dengan antibodi monoklonal dan poliklonal.

Kata kunci: antibodi, CnBr-agarose, rabies, protein, purifikasi, kromatografi

ABSTRACT

Immunoaffinity chromatography using monoclonal antibodies (mAbs) as ligands has been used to purify rabies virus (RV) individual proteins. In this method, mAbs against RV were firstly purified, coupled to CnBr-agarose resin and used for purification RV individual proteins. Brain tissue homogenates derived from infected and uninfected dogs and mice were mixed with mAbs-CnBr agarose resin and washed extensively phosphate buffered saline (PBS). Following elution and neutralization, purified proteins were detected by enzyme-linked immunosorbent assay (ELISA) and Western blotting assay. Of the three mAbs (BB5, AE11 and AF6) as ligands, mAb AE11-CnBr agarose resin yielded highest protein levels as compared to those of mAb BB5-CnBr agarose and mAb AF6-CnBr agarose resins. In Western Blotting assay, the purified protein appeared to be 65 Kda (glycoprotein) and 38 kDa proteins. In ELISA test, the purified proteins reacted with both mAbs and polyclonal antibodies (pAbs).

Kata kunci: CnBr-agarose, rabies virus, protein, purification, chromatography

INTRODUCTION

Rabies is a zoonotic viral disease and is still endemic in many countries around the world including Indonesia (WHO, 2010). In Indonesia, disease is transmitted by rabid animals such dogs, cats and monkeys. However, dog bites play a major role in rabies transmission among

animals and also from rabid animals to humans (OIE, 2016). One effort in preventing and eradicating the disease from the infected areas is mass vaccination of susceptible animals especially dogs using the appropriate vaccines. The success of such vaccination, however, depends on the protective immune response induced by the vaccine and the coverage of vaccination. The

vaccination coverage to induce herd immunity in susceptible dog population has been suggested to be higher than 70% (Conan *et al*, 2015). To determine the immune response induced by vaccination, the availability of a good serological method such indirect enzyme-linked immunosorbent assay (ELISA) is important. The use purified antigen is required in the development of serological tests with high level of accuracy for rabies virus antibody.

Rabies virus (RV) belongs to lyssavirus of the family Rhabdoviridae (Balaul dan Lafon, 2003). The virion consist of single-stranded RNA genome (Consales and Bolzan, 2007) which codes for viral proteins such as N (nucleocapsid), P (phosphoprotein) dan M (membrane) proteins, G (envelope glycoprotein) and L (replicase) (Bradame and Tordo, 2001). All of those proteins play roles in the infection of virus into target cells. However, glycoprotein (G) is the most important protein for use in the development of both vaccine for preventing rabies and serological test for examining the immune response induced by rabies vaccines. G protein is the virus attachment protein which initiates the binding of the virion with its receptors on the surface of target cells (Kuzmina *et al*, 2013, Mori dan Marimoto, 2014). The antibody against G protein induced by vaccines in hosts will prevent the infection by blocking the viral entry into cells. The use of purified RV protein such as G protein in serological test such as ELISA will ,therefore, increase the sensitivity and specificity of the test.

Many methods can used in the purification of protein used as antigen for the development of ELISA test such as density gradient centrifugation and affinity chromatography (Launa *et al*, 2012; Feng *et al*. 2016). The use of affinity chromatography is particularly interesting as it is simple and can be used to purify a protein from a mixture composed of many proteins (Launa *et al*, 2012). In recent years, one affinity chromatography technique that has been widely used for protein purification is immunoaffinity chromatography using antibody against a protein as ligands (Abdolalizadeh *et al*, 2013). In this method, purified antibody against a protein is coupled into CnBr-activated agarose gel bead and the bead is then used as ligand for protein purification (Kavran and Leahy, 2014). Both polyclonal and monoclonal antibodies can be used as ligands in this purification method. However, monoclonal antibodies (mAbs) appear to be better ligand compared to polyclonal antibodies (pAbs) as mAbs

only bind with a single epitope in a protein molecule (Moser and Hage, 2010) and is therefore likely to obtain a purer individual protein. Currently, mAbs against rabies virus have been produced and some of which reacted specifically with rabies virus antigen (Astawa *et al*, 2015). A study was therefore conducted to examine the potential use of such mAbs as ligands for purification of rabies virus individual proteins.

MATERIALS AND METHODS

Production and Purification of Monoclonal antibodies

In this study, three (BB5, AC11 and AF6)) of eight mAbs against rabies virus produced in the previous study (Astawa *et al*, 2015) were used. The stocks of high titer mAbs were produced by growing hybridomas in culture until they reached a dying stage when most cells were at the stage of dying. The supernatant was then clarified by centrifugation at 1000 x g for 10 minutes. The mAbs were firstly tested and titrated by ELISA as described by Astawa *et al*, 2015. Fifty ml stock of each mAb was used for purification. The mAbs were then purified using rapid antibody purification kit (Cell Biolab, USA) according to the procedures as described by the manufacturer. Briefly, mAbs stock was diluted 1:2 in binding buffer and was passed three times through resin coupled with protein A/G to maximase the binding of IgG to protein A/G. The resin was then washed with 30 ml binding buffer until all unbound components of hybridoma media was washed away from the resin. Bound mAb was then eluted by elution buffer and the pH of mAb elutes were normalized to 7.4 by adding neutralization buffer. Five elutes of 1 ml for each mAbs collected and tested by ELISA according to the procedures as described below.

Coupling of mAbs to CnBr-Activated Agarose Bead

Coupling of mAbs to CnBr-activated agarose was conducted according to procedures as described by Kavran and Leahy, 2014 with modification. The purified mAbs were dialyzed for six hours at roo temperatures in two changes of 500 ml coupling buffer (100 mM NaHCO₃ pH 8.6, 500 mM NaCl). One gram of CnBr-activated agarose resin was then suspended in activation buffer (15 ml 0.1 N HCl) and left at 4°C for two

hours. The activated resin was washed three times with coupling buffer by centrifugation at 500 x g for five minutes. After the last wash, the supernatant was discarded and two ml dialysed mAbs diluted in 2 ml of coupling buffer was mixed with the CnBr activated resin. The coupling of mAbs to CnBr activated resin was conducted by constant shaking of the mAbs and CnBr agarose resin mixture at room temperature for two hours. The mAbs-coupled resin was washed three times with coupling buffer as above. Five ml blocking buffer (100 mM ethanolamine in coupling buffer) was added to the resin and left at room temperature for another one hour with a constant shaking as above. The resin was washed for four times with alternating high pH buffer (100 mM Tris-HCl pH. 9.2, 500 mM NaHCl) and low pH buffer (NaOAc pH 4.0, 500 mM NaCl). Finally the resin was washed twice phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH. 7.4) and kept at 4°C in PBS containing 0.1% NaN₃ (sodium azide).

Preparation of Brain Tissue Homogenate of Rabid Dogs and Mice

Brain tissues of infected dogs were obtained from field rabid dogs necropsied in the Pathology section of Animal Disease Investigation Centre Denpasar, Bali Indonesia. The rabies virus-infected mice were obtained by inoculation of suckling mice with the brain homogenate derived from rabid dogs which have previously been confirmed by direct immunofluorescence and/or by mouse inoculation test. The presence of rabies virus in the brain tissue samples of dog or mice was detected direct immunofluorescence assay. Firstly, touch smear of infected brain tissues was prepared on microscope slides treated with poly-L-lysine and fixed with cold acetone for 10 minutes. Fifty µl anti-rabies mAb-FITC conjugate (Bio-Rad) was added to the smear of brain tissues. The smears were left at 37°C for 30 minutes and washed five times with PBS, mounted in 90% buffered glycerol and examined under fluorescence microscope. Brain tissues of infected dogs and mouse with high intensity of RV antigen were then used for purification procedures.

The brain samples of infected mice or dogs were firstly homogenized by grinding in a mortar using piston and passed through 10 ml syringe with 18 G needle. The homogenate was divided into two tubes; one treated with 1% Triton X-100 in PBS and one tube treated with PBS only.

The tissue homogenate was then subjected for three times freezing-thawing to break the cells in the brain tissue homogenate. The homogenates were treated with 0.01% β -propiolacton for 24 hours at 4°C to inactivate the virus, followed by incubation at 37°C for another 24 hours to inactivate the β -propiolacton. Sample of β -propiolacton-treated tissue homogenate was inoculated intracerebrally into suckling mice. When no rabies clinical sign was observed after three week post-inoculation and immunofluorescence test, the brain tissue homogenate was used for RV protein purification. Brain tissue homogenates from uninfected dogs and mice were also prepared using the same procedures as described for those from rabid dogs or mice.

Purification of Rabies Virus Proteins

Tissue homogenate prepared as above was firstly centrifuged at 10000 x g for 15 minutes at 4°C. The supernatant was then collected and mixed with mAbs-CnBr-activated agarose resin and left for one hour at room temperature with a constant gentle shaking. The resin was then poured into 15 ml column and washed with 50 ml PBS until all unbound proteins were washed away from the column. The rabies virus protein bound to resin via mAb ligands was eluted by elution buffer (200 mM glycine pH 2.8) and the pH of the elutes was then normalized by adding neutralization buffer (3 M TRIS-HCl, pH 8.8.). Five elutes from each mAb-CnBr agarose resin were collected and tested by ELISA using mixed mAbs (BB5, AC11 and AF6).

Enzyme-Linked Immunosorbent Assay for Detection of Purified Proteins

Protein samples from each elute was firstly diluted 1/20 in carbonate-bicarbonate coating buffer (50 mM NaHCO₂, 50 mM Na₂CO₃, pH.9.6) and was coated into 96 well ELISA plate (100 µl per well) for overnight at 4°C. Wells of the ELISA plate were washed twice with PBS containing 0.1% Tween-20 (PBST), and blocked with 5% skim milk in PBS for 1 hour at 37°C. Following twice washes as above, 100 µl monoclonal antibody against rabies protein diluted 1:10 was added into each well and incubated for 1 hour at 37°C. Wells on ELISA plate was washed three times with PBS and 100 µl anti-mouse IgG-horseradish peroxidase was added and incubated for another 1 hour as above. After three times washes as above, 100 µl TMB substrate was added and left for 20 minutes at room temperature in dark environment and

stopped with 1 N H²SO₄. The optical density (OD) of the substrate was then read by ELISA reader.

Western Blotting for Detection of Purified Rabies Virus Protein

The purified proteins of rabies virus were the subjected for Western blotting analysis using carbonate/bicarbonate-methanol transfer system (Dunn, 1986). Samples of ELISA positive elutes were diluted in sample reducing buffer (SDS 2,3 %, mercaptoethanol 5%, Tris-HCl 0,0625 M. pH. 6,0, gliserol 10%, bromophenolblue 0,001%) and subjected for SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis) according to Laemli procedures (Laemli, 1970) using mini protean tetra cell electrophoresis system (Bio-Rad, USA). The SDS-PAGE separated RV proteins were transferred onto nitrocellulose ebrane using carbonate/bicarbonate transfer buffer ((10 mM NaHCO₃, 3mM Na₂CO₃, pH 9.9, 20% methanol) and blocked with 5% skim milk in Tris-HCl saline (TBS) buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl. The nitrocellulose membrane was the soaked anti-rabies virus pAb or mAbs for overnight at 4°C. After 3 times washes with TBS, the membrane was soaked in antimouse IgG-alkaline phosphatase (KPL, USA) for 1 hour at room temperature. The proteins reacted with pAb and mAbs were visualised by adding BCIP/NBT substrate (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride).

RESULTS AND DISCUSSION

Titers of mAbs before and after Purification

The titers of mAbs before purification were as follows mAb BB5 (2⁸), mAbs AC11 (2⁶) and AF6 (2⁶). After purification, mAb titers increased

3-4 times as the volumes were reduced from 50 ml to 2 ml after purification (Tabel 1). By ELISA, it was also determined that the elute 1 and 2 of five elute contained purified mAbs (Data not shown).

Rabies Virus Antigen Detected by Direct Immunoflorescen in Brain Tissues

Before they were used as the sources of RV protein purification, brain tissues of infected and uninfected dogs and mice were tested by direct immunoflorescence. Rabies virus antigen was detected in smears of infected dogs and mice and mouse brain tissues, but not in uninfected brain tissues. (Figure 1).

Profiles of Purified RV proteins in ELISA Test

In this study, purified RV proteins eluted from mAbs-CnBr agarose resin were tested by ELISA. Using the resin, purified proteins were detected in elutes 1-2 using mAb AF6-CnBr agarose-resin, in elutes 1-3 using mAbs BB5-CnBr agarose resin, and in elutes 1-5 using mAb AC11-CnBr agarose resin. The use of brain tissue homogente treated with triton-X100 resulted in lower titer of protein. No diference was observed proteins titer obtained from the bain of infected dogs as compared to those obtained from the brain of infcted mice (Figure 2).

Estimated Molecular Weight of Purified RV proteins

Following purification by mAbs-CnBr agarose resins, two bands of purified proteins were detected in elutes of homogenates derived from infected dogs. The molecular weights of the proteins were 65 kDa and and 38 kDa. No protein band was detected in elutes of brain tissue homagenate derived from uninfected dogs were used (Figure 3).

Table 1. Titers of mAbs before and after purification using protein A/G-sepharose resin

mAbs	Isotype	ELISA titers	
		Before purification	After purification
BB5	IgG1	2 ⁶	2 ⁸
AC11	IgG1	2 ⁷	2 ⁹
AF6	IgG1	2 ⁶	2 ⁹

ELISA: enzyme-linked immunosorbent assay

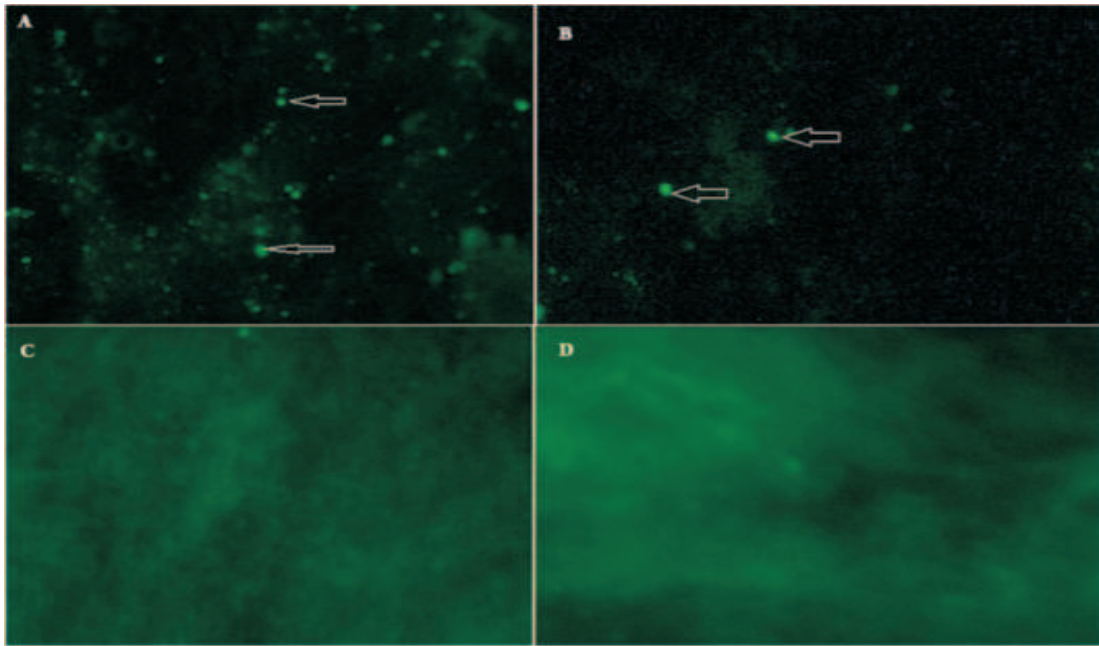


Figure 1. Rabies virus antigen detected by direct immunofluorescent in brain tissues touch smears of infected dog and mice. A: infected dog, B: infected mice C: uninfected dog, D: uninfected mice. Note that rabies virus was detected in the infected but not in uninfected dog or mice

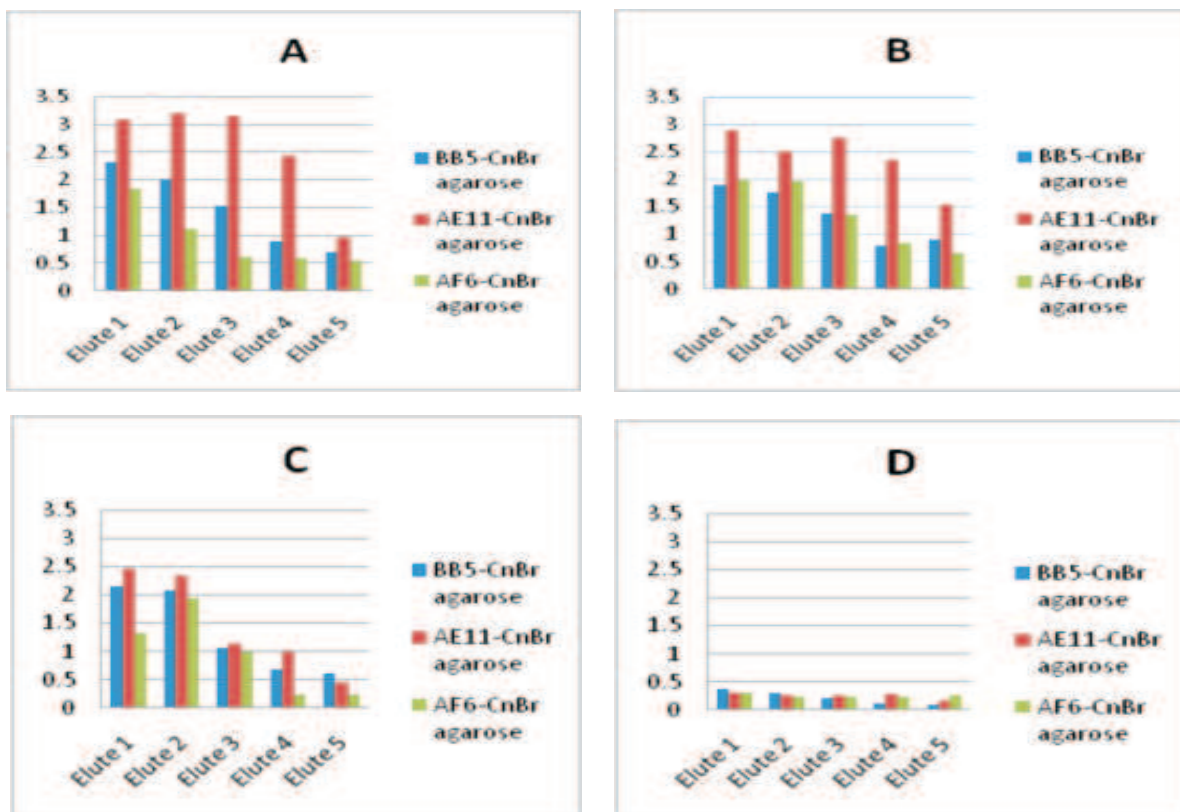


Figure 2. Optical density (OD) of purified rabies virus proteins detected by ELISA using mixed mAbs . A: Untreated brain tissue homogenate of rabid dogs. B: Triton X-100-treated homogenate of rabid dogs. C: Triton X-treated brain tissue homogenate of rabid mouse. D: triton X-100-treated brain homogenate of normal uninfected dogs. Notes that purified proteins were detected in elutes of homogenates derived from infected dogs and mice, but not in uninfected dogs.

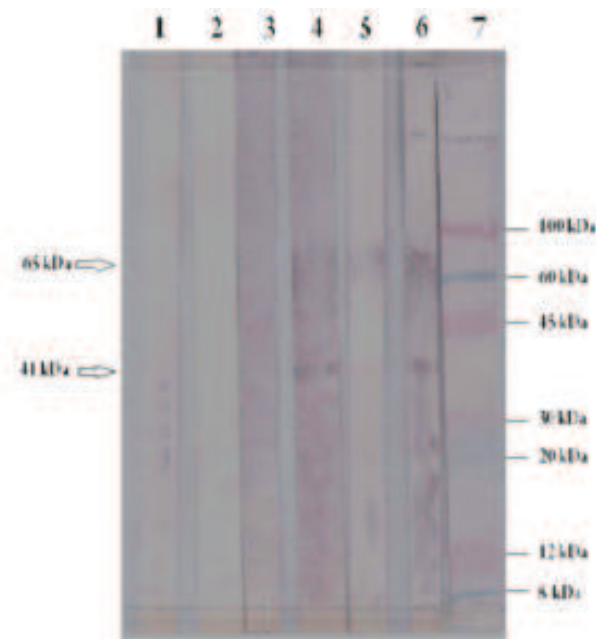


Figure 3. Purified rabies virus proteins detected by Western blotting assay using mixed mAbs. Lanes 1-3: brain tissue homogenate of normal uninfected dogs purified respectively using mAb BB5-CnBr-Agarose, mAb AC11-CnBr agarose and mAb AF6-CnBr agarose resins. Lane 4-6: brain tissue homogenate of infected dog purified respectively using mAb BB5-CnBr agarose, mAb AC11-CnBr agarose and mAb AF6-CnBr agarose resins. Lane 7: molecular weight markers. Note that protein bands were detected only on homogenate of rabid dogs.

Three mAbs (BB5, AC11 and AF6) against rabies virus were used as ligands of immunoaffinity chromatography for purification of rabies virus proteins from brain tissues of infected dogs and mice. Purification of mAbs is required to enable coupling of mAbs to CnBr-activated agarose resin (Read *et al*, 2009; Abi-Ghanem and Berghman, 2012). Purification of antibodies including mAbs is generally achieved by affinity chromatography using agarose matrix coupled with protein A or G which can bind to all mammalian IgGs with different level of affinities (Page and Torp, 2002). A volume of 50 ml hybridoma culture supernatant was used for this purification method. As the concentration of mAbs in hybridoma culture supernatant was reported to be around 25 µg per ml (10-100 µg per ml) (Goding, 1996), the use of 50 ml hybridoma culture supernatant was expected to yield around 1 mg mAb which was sufficient for coupling the mAbs to CnBr activated agarose resin.

By using direct immunofluorescence, the rabies virus antigen was detected in the brain smears of infected but not in uninfected dogs and mice (Figure 1), but not in the brain smear of uninfected dogs and mice. Such detection is important to determine the intensity and severity of rabies infection in the brain tissues. Direct immunofluorescence assay has been considered as confirmatory standard for detection of rabies virus in tissues of infected animals or human (Jackson, 2013). By this technique, it appears that rabies virus infection in brain tissues of infected dogs was more intense than those of infected mice. Other test which is also used to detect rabies virus in brain tissues is mouse inoculation test (MIT). Compared to the direct immunofluorescence test, MIT is higher in sensitivity and is lower in specificity (Kadam *et al*, 2011). MIT has been used as both confirmatory and gold standard of rabies diagnosis. Combination of both mouse inoculation and direct fluorescence assay are often required for accurate detection of rabies virus infection in the infected tissues. Rabid mice used in this study also the result of MIT as further confirmatory test for the detection of rabies virus in the brain of infected dogs.

When the brain tissue homogenates of infected dogs or mice were used as sources of rabies virus proteins for purification, the RV proteins bind to their homologous mAbs in mAb-CnBr agarose resin. The rabies virus individual protein is therefore trapped in the resin. Following several times washes to remove the unbound proteins, only proteins bound to mAbs retained in the resin and elution by low pH buffer can yield rabies virus protein with high level of purity. In this study, both untreated and triton X100-treated homogenates were used as the sources of rabies proteins. It appeared that the ELISA optical density of proteins purified from homogenates of rabid dog brain tissues treated with triton-x 100 was slightly lower than those untreated homogenates. Triton X is a detergent widely used in lysing cells used for protein extraction. Triton X-100 disrupts the cellular integrity by interfering with polar head of lipid bilayers in cell membrane (Koley and Bard, 2010). By lysing the infected cells using triton X, it was expected that the level of viral proteins obtained was higher than those in untreated homogenate. Similarly, OD of rabies virus proteins obtained from homogenates of rabid mouse brain homogenates was similar to those of rabid dogs. No immunoreactive protein was

detected in the brain homogenate of uninfected dogs and mice which indicated that mAbs-CnBr agarose resin only binds and therefore trapped the rabies virus proteins but not normal cellular proteins.

The purified rabies virus proteins detected by ELISA were further confirmed by Western blotting assay. However, two bands of purified rabies virus proteins were detected by Western blotting assay from elutes of brain homogenates of rabid dogs and none was detected from non rabid dogs. The result confirmed that the purified proteins were rabies virus proteins with minimum contaminant of cellular proteins. The molecular weights of proteins detected by Western blotting assay were around 65 kDa and 38 kDa. The two proteins appeared to be glycoprotein and phosphoprotein respectively as it has been reported that the molecular weight of rabies virus glycoprotein and phosphoprotein were 64-68 kDa and 37-40 kDa respectively (Jackson, 2010). However, the two proteins bands detected by Western blotting assay need further confirmation using polyclonal antibody which is not available at this stage.

Purification of rabies virus proteins by immunoaffinity chromatography using mAbs as ligands is expected to yield purer proteins as compared to those using other purification methods. This immunoaffinity chromatography technique has been used to purify cytokines such as TNF α (Abdolalizadeh *et al.*, 2013), enzymes (Karimi *et al.*, 2012) and other biological markers (Mousavi and Nasiri, 2015). For rabies virus, affinity chromatography using DEAE cellulose resin has been used to purify the virus for use in vaccines or serological tests (Frazatti-Gallina *et al.*, 2004). The availability of immunoaffinity chromatography using mAbs as ligands for purification of rabies virus proteins provides an alternative method of antigen preparation which can be performed when individual proteins are required. In addition, the individual proteins obtained from these purification methods are expected to be purer and free from contaminating cellular proteins.

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