Jurnal Veteriner Juni 2014 Vol. 15 No. 2 : 156-165
ISSN : 1411 - 8327

Immunological Detection of Newcastle Disease Viral Antigen in the Naturally Infected Chickens by Monoclonal Antibodies against Fusion-2 Protein

(PELACAKAN ANTIGEN VIRUS TETELO PADA AYAM TERINFEKSI SECARA ALAMI DENGAN ANTIBODI MONOKLONAL TERHADAP PROTEIN FUSI-2)

Nyoman Mantik Astawa[1], Anak Agung Ayu Mirah Adi[2]

[1]Virology and Immunology Laboratory, [2]Pathology Laboratory, Faculty of Veterinary Medicine, Udayana University, Jalan PB Sudirman, Denpasar, Bali, Indonesia 80232 Tel. 0361223791
corresponding authors: nyomanmantikastawa@yahoo.com, mirah638@yahoo.co.id

ABSTRAK

Antibodi monoclonal (AbMo) terhadap protein Fusi (F)-2 virus penyakit tetelo/*Newcastle disease* (ND) diproduksi untuk melacak virus tersebut pada ayam yang terinfeksi. Limfosit asal limpa mencit yang sebelumnya telah diimunisasi dengan virus ND galur LaSota difusikan dengan sel myeloma asal mencit. Sel hasil fusi (hibridoma) yang menghasilkan antibodi terhadap virus ND kemudian diisolasi dan dipakai untuk membuat AbMo. AbMo kemudian dipakai untuk melacak virus ND pada ayam atau telur ayam bertunas yang terinfeksi virus ND. Uji *Western Blotting* menunjukkan bahwa 2 dari 5 AbMo yang diproduksi bereaksi dengan protein F2 virus ND dengan berat molekul sebesar 12,5 KDa. Dengan uji *enzyme-linked immunosorbent assay* (ELISA), AbMo tersebut dapat melacak virus dalam cairan korioalantois dengan titer 2^{-2}sampai 2^{-4} unit HA per 0,1 mL. Dengan uji immunoperoksidase menggunakan jaringan ayam terifeksi, antigen virus ND terlacak hampir di seluruh jaringan yang digunakan dalam uji. Namun, antigen virus ND dengan intesitas yang tinggi ditemukan dalam saluran gastrointestinal dan paru.Antigen ND juga terlacak di otak, limpa dan organ lainnya. Hasil ini menunjukkan bahwa AbMo terhadap protein F virus ND dapat diproduksi dan digunakan untuk melacak antigen virus ND dalam cairian alantois telur bertunas dan dalam jaringan ayam terinfeksi.

Kata-kata Kunci : *Tetelo, newcastle disease*, virus, antibodi monoklonal

ABSTRACT

Monoclonal antibodies (mAbs) against Fusion (F)2 protein of Newcastle disease virus (NDV) were produced for the detection of the viral antigen in infected chickens. Cells derived from spleen of Balb/c mice immunized with the virus were fused with mouse myeloma cells to generate hybridomas capable of producing mAbs against the virus. The hybridomas were screened by enzyme-linked immunosorbent assay (ELISA) for anti-NDV specific mAbs using crude viral antigen (allantoic fluid of NDV-infected fertile eggs) and normal uninfected allantoic fluid of fertile eggs as negative control. The NDV proteins reactive with mAbs were then determined by Western Blotting using purified NDV as antigen. The mAbs reactive with F2 (12.5 KDa) protein of NDV were then used for the detection of NDV antigen in both the allantoic fluid of NDV- infected chicken embryos and in organs of naturally infected chickens. The results showed that 2 out of 5 mAbs produced were against F2 protein of NDV. By indirect ELISA, the mAbs were able to detect the viral antigen in allantoic fluid of NDV infected fertile chicken eggs at the titre as low as 2^{-2} to 2^{-4} HA units per 0.1 mL. NDV–antigen was also detected by immunoperoxidase staining in paraffin-embedded tissues of NDV-infected chickens but not in normal uninfected chickens. The most prominent infection was detected in the gastrointestinal tract and the lung. The NDV antigen was also detected in other organs such as the brain, spleen, and several other tissues. It is evident that mAbs produced against F2 protein of NDV were applicable for use in the detection of NDV antigen in infected chickens.

Key words : Newcastle, virus, monoclonal antibodies

INTRODUCTION

Newcastle disease (ND) is a viral disease affecting many avian species. The disease is still endemic in many countries and still causes asignificant death in many avian species. Although prevention and eradication programs such as vaccination and tight biosecurity measures have been conducted intensively, ND outbreaks are still common among poultries especially chickens. ND outbreaksstill cause significant economic losses (Antipas *et al.*, 2012). It is caused by avian paramyxovirus type 1, a virus with non-segmented,single-stranded, and negative sense ribonucleic acid (RNA)genome. The genome codes for six viral structural proteins consisting ofnucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (H/N) and large polymerase (L) proteins(Yusoff and Tan, 2001; Fournier and Schirrmacher, 2013). The NDV genome also codes for non-structural proteins such as V protein of 29KDa (McGinnes *et al.*, 1988)and is found only in the infected cells (Ahmed *et al.*, 2012).Two structural NDV proteins on the surface of the virus, H/N and F proteins, are glycoproteins which play important roles during the infections (Lamb and Park, 2007; Swanson, 2010).

Fusion protein of NDV initiates infection by fusing the viral envelope with the cellular membrane, which allows the penetration of the virus into of target cells (Lamb and Jardetzky, 2007). The protein is initially translated from NDV mRNA as F0 protein with the molecular weight of 68 KDa. The protein is then cleaved by proteases into F1 (55 KDa) and F2 (12.5 KDa) (Ballagi and Wellmann, 1996, Epsion andHenav, 1987). The cleavage is required in order for the virus to gain its infectivityand to induce cell to cell fusion which facilitates the spread of the virus in both cell cultures and in the infected animals (Fournier and Schirrmacher, 2013)

F protein is also the major determinant of NDV virulence in many infected animals. The amino acid sequence motif at F0 cleavage site determines how the protein is cleaved into F1 and F2 proteins (Dortmans*et al.*, 2011). In the virulent strains of NDV, the presence of the multibasic amino acid motif at its cleavage site enablesthe F0 proteinto be easily cleaved byubiquitous intracellular furin-like and extracellular proteases.This causes systemic and often fatal infection with high mortality (Huang *et al.*, 2004, de Leeuw*et al.*, 2005). In low virulent NDV, however, the presence of monobasic amino acid motif at F protein cleavage site makes such cleavage more difficult as it iscleaved only by extracellular trypsin-like proteaseswhich are normally restricted only in intestinal and respiratory tracts (Merino *et al.,* 2011). The availability of mAbs against F2 protein will enable not only the detection of NDV antigen in the infected chickens, but also will enable further studies regarding the roles of F protein during infection and in the virulence of NDV. The production and use of mAbs against F2 protein for detection of NDV antigen in infected chickens is reported in this article

RESEARCH METHODS

Cells and Viruses

Myeloma cells (P3-NS1/1Ag4.1), were originally obtained from Murdoch University, Australia and have been kept in liquid nitrogen of Biotecnology Laboratory, Disease Investigation Center, Denpasar since 2003. Sensitivity of the myelomas against aminopterin was refreshed by repeatedly growing the cells in DMEM growth medium containing azaguanine. The cells wereculturedin growth Dubelco's modified essential medium (DMEM) with 10% fetal bovine serum (FBS, Gibco, USA), penicillin, 200 IU /mL, and streptomycine 200 ìg/mL).

The virus used for immunization of mice was NDV LaSota strain purchased from a poultry shop. The virus was firstly propagated in 12 day-old fertile chicken eggs. The allantoic fluid of the infected chicken embryos was collected and tested by hemagglutination (HA) test. Virulent field strains of NDV (unpurified and purified) were also used for ELISA and Western Blotting. Following propagation in fertile eggs as above, the virulent field strain NDV was purified by agglutination and elution method using chicken red blood cells. Suspension of washed red blood cells was added to the allantoic fluid containing NDV and incubated for 2 minutes at room temperature to allow the virus to bind with its receptor on the surface of red blood cells. The red blood cells were then washed three times with phosphate buffered saline (PBS) by centrifugation at 1500 rpm. The red blood cells carrying the virus were then resuspended in 0.5 mL PBS and incubated for 60 minutes at 37^{o}C with shaking for complete elution of the virus from red blood cells. The eluted virus in the supernatant fluid was then

collected by centrifugation as above and stored at -20°C.

Immunization of Mice

Six to 7 week-old female Balb/c mice were immunized with allantoic fluid containing LaSota strain of NDV according to the procedures similar to those described by Astawa *et al.,* (2007). The mice were initially immunized with 0.2 mL crude NDV (allantoic fluid containing NDV at the titer of 2^8 HA units per mL) emulsified in Freund's complete adjuvant (SantaCruz, USA). At days 14th and 28thafter the first immunization, mice were re-immunized with the same antigen but the virus were emulsified in Freund's incomplete adjuvant (SantaCruz, USA). The sera of the immunized mice were tested by ELISA to determine the antibody titer against the virus. The immunized mice with high antibody titer were then used for the production of hybridomas. One mouse selected for hybridomas production was then repeated boosted with NDV antigen without adjuvant at days 14, 15 and 16 after the last immunization.Three days after the last booster the spleen of the immune mouse was collected and the lymphocytes derived from the spleen were used for fusion in the preparation of hybridomas producing mAbs against NDV.

Production and Characterization of Monoclonal Antibodies

To produce mAbs against NDV, lymphocytes from the spleen of an immune mouse were fused with immortal myeloma cells. Immortal and actively dividing myeloma cells (20 x 10^6 cells) were fused with 10^8 mouse lymphocytes in a round-bottomed centrifuge tube using 45% polyethylene glycol (PEG) (Sigma Co, USA) following methods as described by Ohnisi *et al.*, 2005. The hybridomas created by such fusion were propagated in selective DMEM-HAT medium (DMEM containing 20% fetal calf serum, hyphoxanthine-aminopterin-thymidine) in 96 wells flat-bottomed tissue culture plates. This selective medium only supported the growth of hybridomas which carried both myeloma and lymphocytes of immune mouse. The growing hybridomas in microplate wells were then tested by indirect ELISA (Campbell, 1991) for the anti-NDV antibodies. Hybridomas producing anti-NDV mAbs were isolated and used for the production of mAbs. When anti-NDV mAbs positive hybridomas were obtained from a microplate well containing more the one clones of hybridomas, the cells were re-cloned by limiting dilution as described by McKearn, (1980). In method, a single clone of stable anti-NDV mAbs secreting hybridomas was obtained by growing them from a single hybridoma cell.

The immunoglobulin class and subclass (isotypes) of mAbs was determined by indirect ELISA using rabbit anti-mouse subtyping isotyper kits (Bio-Rad Laboratory, USA) following the procedures as described by manufacturer (Bio-Rad, USA). As above, wells of ELISA microplate were coated overnight with 100 µL per well of purified NDV antigen. Hybridoma medium containing anti-NDV mAbs was added to wells and incubated for one hour at 37°C. Rabbit anti-mouse Ig isotyper was then added to the wells and incubated as above and 100 mL affinity purified goat anti-rabbit IgG-horse radish peroxidase (HRP) (Bio-Rad, USA, diluted 1:1000 in PBST) was added and incubated at 37°C for one hour. The plates were washed three times between each step with 0.05% Tween-20 in phosphate buffered saline (PBS-T) pH. 7.2. In the final step,wells of the microplate were washed four times with PBS-T and 100 mL substrate solution (3,3',5,5'-tetramethylbenzidine/TMB, KPL, USA) was added. The color development of the substrate was stopped by adding 50 µL 1N H_2SO_4 and the absorbance of substrate color in each well was read by ELISA reader using 450 nm filter. The class and subclass of mAbs was determined by observing which rabbit antimouse Ig isotypers were reactive with anti-NDV mAbs in the microplate wells

The NDV proteins recognized by mAbs were determined by Western Blotting using purified NDV as antigen as described by Astawa *et al.*, (2007). Proteins of purified NDV in sample reducing buffer (1.3% SDS, 5% mercaptoethanol, 0.0625 M Tris-HCl pH. 6.8, 10% glycerol, 0.001% bromophenol blue) were separated by sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) using 3% stacking and 12% separating gels. The separated proteins in the gel were transferred onto nitrocellulose membrane and soaked in 3% skim milk in Tris-buffered saline (TBS/ 100 mM Tris pH.7.4) at room temperature for one hour to block the sticky sites of the membrane The nitrocellulose membrane bearing NDV antigen was then cut into 0.5 cm-wide strips. Each strip was then incubated in hybridomas's supernatant fluid for 24 hours at room temperature. Anti-mouse IgG -alkaline phosphatase (KPL USA, diluted 1:1000 in TBS) was the added to the membrane. After

three times washes with TBS, the NDV proteins reactive with mAbs were visualized by adding 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/ NBT) substrate (KPL, USA).

Detection of NDV Antigen in Egg Allantoic Fluid by Indirect ELISA

ELISA test for detection of NDV in allantic fluid was conducted according to the methods similar to those described by Astawa *et al.*, (2007). NDV antigen used for this study was wild type NDV strain derived from field outbreaks of ND. The viruses were firstly propagated in fertile chicken eggs and titrated by hemaglutination test. A two-fold dilution of the NDV antigen in carbonate-bicarbonate coating buffer (15 mM Na_2CO_3 35 mM NaHCO3 pH.9.6) was prepared and 100 ìL of virus suspension from each dilution starting at the titer of approximately 1 HA unit was coated into wells of ELISA microtitration plate. Following an overnight incubation at 4°C and three times washes with PBST, 100 mL blocking buffer (5% skim milk in PBST) was added. The plate was incubated for another one hour at 37°C. One hundred mL mAb samples diluted 1:10 in PBST were added to each well. The plate was incubated for one hour at 37°C. After three times washes as above, 100 mL anti-mouse IgG-horseradish peroxidase (HRP) (KPL, USA) diluted 1:2000 in PBS-T was added to each well. The microplate was then incubated for one hour 37°C and washed three times as above. The plate was again washed as above and 100 mL substrate solution (3,3',5,5'-tetramethyl-benzidine/TMB, KPL, USA) was added. The color development of the substrate was stopped by adding 50 µL N H_2SO_4 and the intensity of substrate color in each well was read by ELISA reader using 450 nm filter.

Detection of NDV Antigen in Chicken Tissues by Immunoperoxidase Staining

Detection of NDV antigen in tissues of infected chickens was conducted according to procedures similar to those described by Ohnishi *et al.*, (2005). Organs from six naturally NDV-infected chickens used in this study were provided by Veterinary students undertaking co-assistant training at the Faculty of Veterinary Medicine, Udayanan Univesity, Denpasar Bali during 2012 to 2013. All chickens died from field ND outbreaks and had been confirmed for NDV infection by isolation of the virus in chicken fertile eggs and identification of the virus by hemagglutination inhibition (HI) test. In some cases, NDV infection was also confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers specific to NDV nucleic acid (data not shown).

Organs collected from NDV-infected chickens were preserved and fixed with 4% buffered formaldehyde. Following a routine tissue processing procedures, the organs were embedded in paraffin-blocks and thin (3-5 microns) sections of the organs were prepared by standard methods. NDV infected cells were then detected by immunoperoxidase staining using anti-F2 protein mAbs of NDV following procedures as described by Ohnishi *et al* (2005). Firstly, tissue sections on microscope slides were de-paraffinized twice by xylol and twice with absolute ethanol. The tissue section was washed twice with PBS and heated in citrate buffer at 96°C using microwave oven for 20 minutes to retrieve NDV antigens in the tissue. The tissue sections were then treated with 3% H_2O_2 for 20 minutes at room temperature to inactivate endogenous peroxidase. Hybridoma's supernatant containing anti-NDV mAbs (diluted 1/10 in PBS containing 2% skim milk) were added onto the tissue section and incubated for one hour at room temperature. Biotynilated anti-mouse IgG (Dako-USA) was then added onto tissue section and incubated for one hour at room temperature followed by stretavidin-horse radish peroxidase (DAKO, USA) for 30 minutes. NDV-infected cells in tissues were then visualized by adding diazinobenzidine (DAB) substrates (DAKO, USA) for five minutes and counterstained with Mayer's hematoxyline.

RESULTS AND DISCUSSION

Characteristics of Monoclonal Antibodies

Five clones of hybridomas were isolated and used for the production of mAbs aganst NDV. All those hybridoma clones were confirmed by ELISA to secrete mAbs against NDV antigen as they did not react with normal uninfected allantoic fluid. The mAbs secreted by five clones of hybridomas were then designated as DD10, BF2, BA1, DC5 and EG11 according to the wells on the microplate where the clones were originally found.The isotypes of mAbs were IgG1 (BA1, BF2, DC5 and EG11) and IgG2a (DD10).

In Western Blotting assay, 2 mAbs (DD10, BF2) reacted strongly with the protein band of 12.5 KDa (Figure 1) and 1 mAb (BA1) recognized NDV protein of approximately 84KDa. In this

Table 1. Characteristics of monoclonal antibodies prepared against LaSota strain of Newcastle disease virus

MAbs	Isotypes	Western Blotting		ELISA		IHC
		NDV	NA	NDV	N.A	
DD10	IgG2a	12.5 KDa	—	+++ (2^{-3} HA)	—	+++
BF2	IgG1	12.5 KDa	—	+++ (2^{-4}HA)	—	+++
AA1	IgG1	84 KDa	—	++ (2^{-2}HA)	—	ND
DC5	IgG1	——	—	+++ (2^{-3}HA)	—	ND
EG11	IgG1	——	—	++ (2^{-3}HA)	—	ND

MAbs : monoclonal antibodies
ELISA : enzyme linked immunosorbent assay
NDV : Newcastle disease virus
N.A. : normal allantoic fluid
IHC : immunohistochemistry
ND : not determined
+++ : strong positive
++ : moderate positive
— : negative

assay, two mAbs were not reactive with any NDV protein band (Data not shown).

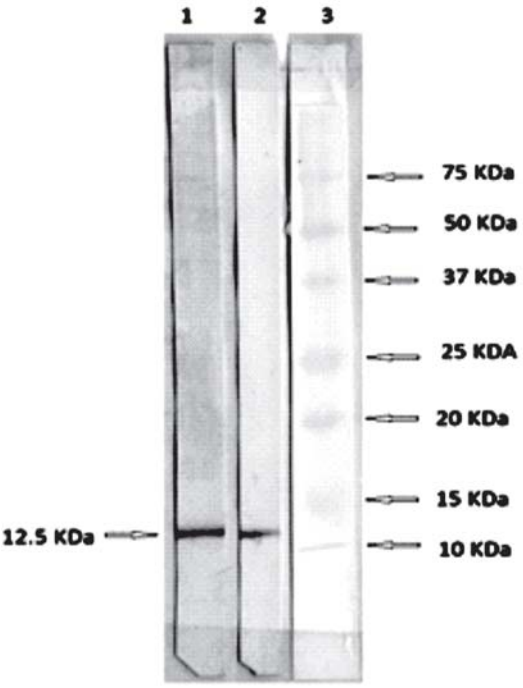

Figure 1. Reactivity of MAbs (DD10 and BF2) with purified NDV antigens analysed by Western Blotting. Antigen : purified NDV. mAbs DD10 (1), BF2 (2), Standard molecular weight markers (3)

Detection NDV Antigen in Allantoic Fluid

AllmAbs detectedNDV antigen in allantoic fluid at the titers as low as $2^{-2}$to 2^{-4}HA units per 0.1 mL. They were not reactive with normal allantoic fluid (Table 1).

NDV Antigen Detected in Organs of Infected Chickens

In immunoperoxidase staining, twomAbs (DD10 and BF2) were able to detect the viral antigen in formalin-fixed and paraffin-embedded tissues of NDV-infected chickens.NDV-infected cells were visible as brownish cytoplasm with violet nuclei, whereas uninfected cells were visible as clear cytoplasm with violet nuclei. The NDV-infected cells were observed in almost all organs. However, NDV-infected cells with high intensity were detected in gastrointestinal tract (proventricle, small intestines and caecal tonsil), and respiratory tract (trachea and lung) (Figure 2). NDV antigen at a lower intensity was also detectable in other organs such as brain, kidney, bursa of Fabricius (Table 2). No a clear difference on the result was observed between mAb DD10 and mAb BF2 when used in immunochemistry staining. Slight differences on the distribution and intensity of NDV-infected cells were, however, observed among different individual infected chickens (Table 2).

In the intestines, NDV antigen with ahigh intensity was detected in necrotic enterocytes lining the intestinal villi andin some crypts of intestine. The NDV-infected cells were also detected in mucosal and glandular epithelial cells of trachea. In the brain, NDV was detected in cells within perivascular cuffing and in the endothelial cells of blood vessel. NDV antigen was not detected in neural cells such as neuron. Many red blood cells bearing the virus detected in many organs. In lung, NDV antigen was detected in lymphoplasmocytic infiltrates and alveolar macrophages of lung parenchyma (Figure 2)

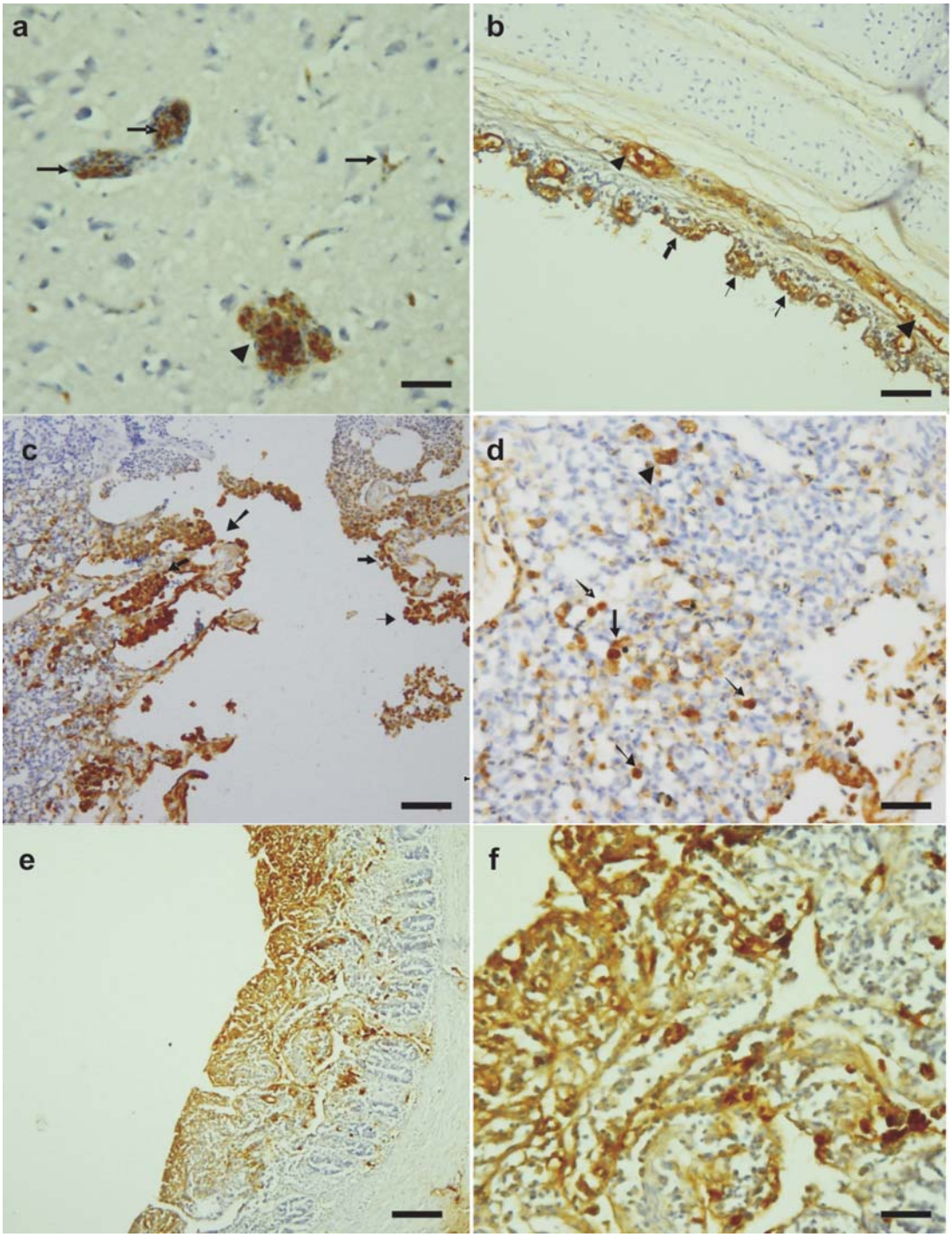

Figure 2. Newcastle disease viral antigen detected by immunoperoxidase staining in several organs/ tissues of NDV-infected chickens. In cells within in perivascular cuffing (arrowhead) and in vascullary and capillary endothelial cells of brain (arrowhead). Bar 30 µm (a). In mucosal epithelial cells (arrow), epithelial glandular cells and submucosal gland of trachea (arrowhead). Bar 50 µm (b). In the aggregate of infiltrating cells and epithelial lining cells of the parabronchus (arrow).Bar 50 µm (c). In lymphoplasmacytic infiltrates (arrow) and in macrophages (arrowhead) of lung parenchyma.Bar 30 µm (d). Heavily infected cells in the diffuse necrotic intestinal villi and some intestinal crypts. Bar 100 µm (e). In necrotic enterocytes of the intestinal villi.Bar 30 µm (f)

Table 2. Newcastle disease virus-infected cells detected by immunoperoxidase staining using mAb DD10 in organs of in several naturally infected chickens

NDV Isolate	Origin	Intensity of NDV infected cells in several organs				
		Intestine	Lung	Brain	Trachea	
500/Bali-1/07	Tabanan/Bali	+++	+++	+	++	++
452/N/2012	Klungkung/Bali	+++	+++	+	++	++
483/N/13	Tabanan/Bali	+++	++	+	++	+
492/N/13	Denpasar/Bali	+++	++	+	++	++
474/N/12	Klungkung/Bali	+++	++	+	++	++
B133	Maros/South Sulawesi	+++	+++	+	++	++

NDV: Newcastle disease virus (+++) : high intensity (++): moderate intensity (+): low intensity

MAbs against F2 protein of LaSota strain of NDV were successfully produced which have enabled the detection of NDV antigen both in allantoic fluid of infected fertile eggs and in organs of naturally NDV-infected chickens. The use of crude NDV (unpurified chorioallantoic fluid containing virus) for immunization of mice appears to be not an hindrance for the production of good and high affinity mAbs against NDV protein. The used such crudeunpurified viruses as immunogen for immunization of mice in the preparation of mAbs have been reported in previous studies (Wickramasinghe *et al.*, 1993; Pantophlet *et al.,* 2001).

Western Blotting assay was used to determine NDV proteins reactive with mAbs. The use of purified NDV for Western Blotting assay produced clear protein bands with the molecular weight of around 84 KDa (mAb AA1) (Data not shown) and 12.5 KDa (mAbs DD10 and BF2) (Figure 1). Proteins bands visible in Western Blotting assay were NDV proteins as all of those mAbs were not reactive with normal allantoids fluid in both ELISA and Western Blotting assays. In addition, the antigen used for Western Blotting assay was purified NDV which further confirmed that the proteins reactive with mAbs were viral proteins. The protein band of 12.5 KDa was likely to be the Fusion (F)2 protein of NDV (Ballagi and Wellmann, 1996; Epsion andHenav, 1987).

The F2(12.5 KDa) protein detected by mAbs (DD10 and BF2) appeared to be cleavage product of F0 protein, the inactive form of F protein. In collaboration with F1 and H/N protein, itfuses the viral envelope with the infected cells andinduces the formation of syncytia by fusing the membrane of NDV-infected cells with the adjacent uninfected cells (Zheng *et al.*, 2013).The ability of NDV to induce syncytium formation is mostly observed in cell cultures (Lamb and Park, 2007). However, studies have shown that the ability of NDV to induce syncitium formation in cell culture is closely related with the ability of the virus to spread systemically in the infected animals (Kim *et al.*, 2011). In the infected animals,fusion of infected and uninfected cells appears tofacilitate the virus to spread from cell to cell without being significantly affected by the presence of antibody which, in turn, facilitates the spread of the virus within the infected animals.The availability of mAbs against F2 protein, will therefore not only enable the detection of NDV, but will also enable further studies on the roles of the F protein in the pathogenesis of ND in chickens and other avian species

In indirect ELISA, the mAbs against F2 protein of NDV was able to detect the virus in allantoic fluid at low concentration which shows that such mAbs are likely to be useful for detection of the virus in other samples such as fecal and nasal swabs as well as in other biological samples. The result also showed that those mAbs have a potential for use in the development of rapid test for ND which useful in both screening and confirmative diagnosis of ND in the suspected infected animals.In confirmative diagnosis,NDV is usually isolated from specimens such as nasal and fecal swabs of both live and dead infected animals. The virus must, however, be firstly propagated in fertile chicken eggs or in cell cultures (OIE, 2012) which is time-consuming and laborious. If mAbs against F2 protein can be used in the development rapid test such as indirect ELISA or sandwich capture ELISA to detect the virus in both nasal and fecal swabs it will certainly simplify the laborious and time-consuming confirmative diagnosis of NDV.

Monoclonal antibodies against F2 protein of NDV detected the virus-infected cells in many organs. NDV-infected cells at high intensity were observed in gastrointestinal tracts and lung (Figure 2). As the infected chickens used in this study were chickens died fromfield NDoutbreaks, the virus causing the disease was likely to be velogenic NDV. This was then confirmed by the presence of infected cells in almost all organs of infected chickens as shown by the result of immunoperoxidase staining using mAbs against F2 protein.The absence of infected neural cells in the brain and the presence of heavily infected cells within the necrotic areas of intestinal villi further supported the previous finding that the Indonesian isolates of velogenic NDV are mostly viscerotropic (Sudarisman, 2009, Adi *et al.*, 2010). In velogenic-neurotropic NDV which is found in North America and Europe (Ecco *et al.*, 2011), apart from infecting endothelial cells and cells within the peri-vascular cuffing in the brain blood vessel, the virus also infects neuronal cells such as neuron and astrocytes (Nakamura *et al*., 2008; Susta *et al.,* 2011).

In organs with high NDV-infected cells such as gastrointestinal tracts, the availability of cells bearing the receptor for NDV and the presence of abundant proteases seemed to allow the efficient replication of the virus in the organs. Gastrointestinal tract, for instance, is well known as the organ rich in proteolytic enzymes (Banks and Plowright, 2003) and such proteases is responsible for post translational cleavage of F0 into F1 andF2. The cleavage of F protein is required for the virus to gain its infectivity which, in turn, is important for the efficient replication of the virus in gastrointestinal tract.The availability of mAbs against NDVwill enables the development of test for ND diagnosis and for studies on the role F2 protein in NDV infection.

CONCLUSSION

Monoclonal antibodies produced against F2 protein ofNDV are important reagent for use in the detection of NDV antigen in samples derived from both dead and live clinically affected chickens.The mAbs are also applicable for use in detection of NDV antigen in the several tissues/organs of naturally infected chickens which is very useful for histopathogenesis studies of NDV in the infected individuals. The availability of mAbs against F2 protein of NDV will certainly allows further studies on the role of the protein in the pathogenesis of ND, especially its roles in tissue tropism, spread of the virus from cell to cell and other virulence determinant of NDV. For the development rapid test using such mAbs, further studies are still required including purification and manipulation of mAbs. Such purification is required to allow further manipulation of mAbs such as coupling of the mAbs with enzymes, biotin and other probes.

ACKNOWLEDGMENT

The production of monoclonal antibodies used in this study was carried out in Biotechnology Laboratory of Animal Disease Investigation Center (DIC) Denpasar and the authors wish to thanks DIC Director for providing the facilities. We wish also to thank students of the Faculty of Veterinary Medicine, Udayana University for providing organs/tissues used for immunochemistry studies. Many thanks also to Japanese Society for the Promotion of Scince (JSPS) Rohpaku scholarship for providing some reagents and equipments used for the production of monoclonal antibodies.

REFERENCES

Adi AAM, Astawa NM, Putra KS, Hayashi, Matsumoto Y. 2010. Isolation and characterization of a pathogenic Newcastle disease virus from a natural case in Indonesia. *J Vet Med Sci* 72, 313-319.

Astawa NM, Winaya IBO, Agustini LP, Hartaningsih N. 2007. Immunological Detection of Avian Influenza Virus in Infected Ducks by Monoclonal Antibodies gainst AIV-H5N1. *Microbiology Indonesa* 1: 119-124

Ahmed A, Guo X, Hashimoto W, Ohya K.Fukusi H. 2012. Establishment of Nonstructural V and Structural P protein Based ELISA for detection of Newcastle disease virus infection. *J Animal and Vet Adv* 11: 4017-4021

Antipas BB, Bidjeh K, Youssouf. 2012. Epidemiology of Newcastle disease and its economic impact in Chad. *European J Exp Biol 2:* 2286-2292

Banks J, Plowright L. 2003. Additional glycosylation at the receptor binding site of the hemagglutinin (HA) for H5 and H7 viruses may be an adaptation to poultry hosts, but does it influence pathogenicity? *Avian Dis* 47: 942-950.

Ballagi PA.Wellmann E. 1996.Identification and grouping of Newcastle disease virus strins by restriction site analysis of s region from the f gene. *Arch Virol* 141: 243-261

Campbell AS. 1991. Monoclonal antibody and immunosensortechnology. Amsterdam: Elsevier, pp 59-93

DeLeeuw OS, Koch G, Hartog L, Ravenshost N. Peeters BP. 2005.Virulence of Newcastle disease virus is determined the cleavage site of Fusion protein and by both the stem region and globular head of the hemagglutinin neuraminidase protein. *J Gen Virol* 86 : 1759-1769

Dortmans J, Koch G, Rottier JM, Peeters PH. 2011. Virulence of Newcastle disease virus: what is known so far? *Vet Res* 42: 122

Ecco R, Susta L, Afonso CL, Miller PJ, Ecco CB. 2011 Neurological lesion in chickens experimentally infected with virulent Newcastle disease virus isolates. *Avian Pathol* 40:145-152

Epsion D, Henav S. 1987. Expression at cell surface of native F protein of the NDV strain Italian from cloned cDNA. *Arch Virol* 95: 79-95

Fournier F, Schirrmacher V. 2013. Oncolytic Newcastle Disease Virus as Cutting Edge betweenTumor and Host. *Biology 2*: 936-975

Huang PA, Elankumaran S, Rockeman DD, Samal SK, 2004. Role of fusion protein cleavage site in the virulence of Newcastle disease virus. *Microb Pathol* 36: 1-10

Kim S, Subbiah M, Samuel AS, Collins PL,Samal SK. 2011 Roles of the fusion and hemagglutinin-neuraminidase proteins inreplication, tropism, and Pathogenicity of Avian Paramyxoviruses. *J Virol* 86 : 8582–8596

Lamb RA, Jardetzky TS. 2007 Structural basis of viral invasion: lessons from paramyxovirus F protein. *Curr Opin Struct Biol* 17: 427–436

Lamb RA, Parks GD. 2007 Paramyxoviridae: The viruses and their replication. In : Knipe, DM; Howley PM., editors. *Fields Virology*. Fifth ed.. Vol. 1. Lippincott WIlliams & Wilkins; Wolters Kluwer: p. 1449-1496.2 vols

McGinnes L, McQuain C, Morrison T. 1988. The P protein and nonstructural 38 K and 29 K of Newcastle disease virus are derived from the same open reading frame. *Virology* 14: 25-24

McKearn TJ. 1980.Cloning hybridomas by limiting dilution in liquid phase: In" *Monoclonal antibodies : new dimension in biological analyisis* (Kennet RH , TJ McKearn Bectol KB Eds), London Plenum Press p 374.

Merino R, Villegas H, Quintana JA, Calderon N.2012.Comparison of the virulence of pathogenic Newcastle disease viruses belonging to the same or different genotypes. *Int J Poult Sci*10 : 713-720

Nakamura K, Ohtsu N, Nakamura T, Yamamoto Y, Yamada M, Mase M, Imai K. 2008. Pathologic and immunohistochemical studies of Newcastle disease (ND) in broiler chickens vaccinated with ND: severe nonpurulent encephalitis and necrotizing pancreatitis.*Vet Pathol* 45 : 928-933.

Ohnishi K, Sakaguchi M, Kaji T, Akagawa K, Taniyama T, Kasai M, Tsunetsugu-Yakota Y, Oshima M, Yamamoto K, , Takasuka N, Hashimoto S, Ato M, Fujii H, Takahashi Y, Morikawa S, Ishii K, Sata T, Takagi H, Itamura S, Odagiri T, Miyamura T, Kurane I, Tashiro M, Kurata T, Yoshikura H, Takemori T. 2005. Immunological Detection of Severe acute respiratory syndrome coronavirus by monoclonal antibodies. *Jpn J Infect Dis* 58 : 88-94

OIE (World Organization for Animal Health). 2012. Chapter 2.3.14.Newcastle disease. OIE Terrestrial Manual online http//www/ OIE.int./international standard setting Newcastle disease, Accessed 16-11-2013)

Pantophlet R, Brade L, Brade H. 2001. Generation and serological characterization of murine monoclonal antibodies against O antigens from *Acinetobacter* reference strains. *Clin Diag Lab Immunol* 8 : 825-827

Sudarisman. 2009. Pengaruh perkembangan system produksi ayam terhadap perubahan genetik dan biologic virus Newcastle disease. *Wartazoa* 9 : 125-133

Susta L, Miller PJ, Afonso CL, Brown CC. 2010. Clinicopathological Characterization in Poultry of Three Strains of Newcastle Disease Virus Isolated From Recent Out breaks *Vet Pathol* 48 : 349-360

Swanson K, Wen X,Leser GP, Paterson RG, Lamb RA,Jardetzky TS. 2010. Structure of the Newcastle disease virus F protein in the post fusion conformation, *Virology* 402 : 372–379.

Wickramasinghe R, Meanger J, Enriquez CE, Wilcox GE. 2003. Avian reovirus protein associated with neutralization of viral infectivity. *Virology* 194 : 688-69

Yussof K, Tan WS. 2001,. Newcastle disease virus: macromolecules and opportunities. *Avian Pathol* 30: 439-455

Zheng J, Fournier P, Schnirrmacher P. 2004. High cell surface expression of Newcastle disease virus protein via replicon vectors demonstrates syncytia forming activity of F and promotion activity of H/N molecules. *Int J Oncol* 25 : 293-302