Original Article

An Indirect ELISA Using a Recombinant Envelope Protein for Detecting Antibody Against Duck Tembusu Virus Infection

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Abstract

Duck Tembusu virus (DTMUV) belonging to *Flavivirus* group, causes severe central nervous system disorders and decreased egg production as found in Thailand. To detect antibody against the DTMUV infection, we developed an indirect enzyme-linked immunosorbent assay (ELISA) based on the recombinant envelop (E) protein for coating antigen. The E protein coding gene of KPS54A61DTMUV was cloned into pPICzαA vector and expressed by using 2% (v/v) of absolute methanol as inducer in *Pichia pastoris* expression. The recombinant E protein was detected in the culture medium at a concentration of 4.55 mg/ml and analyzed a protein expected size by SDS-PAGE at 65 kDa. Western blotting analysis showed specific reactivity of convalescent duck antibodies against DTMUV with the recombinant E protein. Mouse and duck antibody against the recombinant E protein were tested its specificity by immunoperoxidase monolayer assay and immunohistochemistry. The indirect ELISA coated with the recombinant E protein at concentration of 22.75 µg/ml demonstrated a coefficient of variance (%CV) range of 2.8-14.7% and 2.9-14.28% for intra assay and inter assay, respectively. The specificity and sensitivity were 88.24% and 93.22%, respectively with a kappa coefficient measured at 0.81. The indirect ELISA based on the recombinant E protein are routinely useful for DTMUV antibody detection. A retrospective study of serology was done on duck sera collected in different areas and years indicating that DTMUV infection has occurred in Thailand since the first outbreak of HPAI H5N1.

Keywords: ELISA, *Pichia pastoris*, Recombinant Envelop Protein, Duck Tembusu virus

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Introduction

Flaviviruses belonging to the Family *Flaviviridae*, genus *Flavivirus* and are subgrouped into mosquito – borne, tick – borne, unknown vectors and insect specific virus. The most important transmission vector is infected mosquitoes and ticks (Lindenbach *et al*., 2007). Their genome is positive single stranded RNA encoding three structural proteins; nucleocapsid (C), premembrane (PrM), envelop (E) protein and seven non - structural proteins (NS) including NS1, NS2A, NS2B, NS4A, NS4B and NS5 (Franz *et al*., 2012; Liu *et al*., 2012). The E protein on the surface of virus particle that is an important antigen for neutralizing antibody, which serves as a tool for antibody detecting to *Flavivirus* (Erik *et al*., 1990; Yin *et al*., 2013). Flaviviruses could infect avian species include domestic geese, house sparrows, ducks and chickens (Kono *et al*., 2000; Swayne *et al*., 2001; Tang *et al*., 2013; Huang *et al*., 2014). Besides, the Flavivirus, namely Baiyangdian virus which is closely related to TMUV was isolated from ducks in the Southeast China since 2010. Affected ducks exhibited nervous signs and had gross lesions in ovary including ovarian hemorrhage, folliculitis and regression (Cao *et al*., 2011; Su *et al*., 2011; Yan *et al*., 2011). In Thailand, duck TMUV (DTMUV) infection was firstly reported with morbidity of 15 - 25% and mortality was less than 10% (Songserm *et al*., 2014; Chakritbudsabong *et al*., 2015; Thontiravong *et al*., 2015). Based on E and NS5 proteins, there are at least 3 clusters of DTMUVs, including cluster 1, 2.1 and 3 circulating in Thailand (Nilvilai *et al*., 2019). In general, serum neutralization (SN) is a gold standard method to detect the antibody against the DTMUV. However, SN is a time consuming and laborious assay and cannot serve large number of serum samples during extensive disease surveillance. Therefore, indirect ELISA is the best assay to cover these obstacles because of its higher specificity and sensitivity and faster results come out.

The aim of this study was to produce a recombinant E protein of DTMUV-KPS54A61 by *Pichia pastoris* expression system and further to develop an indirect ELISA test kit for antibody detection in ducks. Also, a retrospectively serological study of DTMUV infection in domestic ducks in Thailand was conducted.

Materials and Methods

Virus, Yeast strain and plasmid: The KPS54A61DTMUV was inoculated onto the African green monkey kidney cells (Vero; ATCC® CCL-81™,USA) (Chakritbudsabong *et al*., 2015). Based on E and NS5 proteins, KPS54A61DTMUV was genetically classified cluster 2.1 that is the main cluster found in Thailand. When the cells showed the cytopathic effect, the amount of the virus was estimated by median tissue culture infective dose ($TCID_{50}$). The RNA of the DTMUV was extracted by PureLink® Viral RNA/DNA minikit (Invitrogen®, USA) according to the manufacturer's protocol and used for RT-PCR reaction. The *Pichia pastoris* strain X33 mut^+ and pPICZαA vector (Invitrogen®, USA) were used as the recombinant protein expression host and expression vector, respectively.

Production of recombinant expression vector: The RNA was generated first-strand cDNA by Superscript™ III First-strand synthesis system (Invitrogen®, USA) according to the manufacturer's instructions. The PCR of the envelop gene was amplified by using a pair of PCR primers which were designed according to the published sequence of DTMUV-KPS54A61 (GenBank accession no. KF573582). The Flavi-Ep1 5'-GC**GAATTC**TTCAGCTGTCTGGGGATGC -3'(*EcoR I* site in bold) and Flavi-Ep2 5'-TGC**TCTAGA**GCATTGACATTTACTGCC-3' (*Xba I* site in bold) were forward and reward primers, respectively. The thermal cycling conditions outlined an initial denaturation step at 94°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension 72°C for 40 sec. The final extension step was performed at 72°C for 10 min. PCR products were analyzed by 1.5% agarose gel electrophoresis and purified with FavorPrep™ Gel/PCR purification Kit (Favorgen®). To confirm the E protein encoding gene, the PCR products were analyzed by sanger sequencing. The purified PCR products and the pPICZαA vector were digested by enzymatic digestion by using *EcoR I* and *Xba I* (Thermoscientific), then ligated KPS5A61-E into pPICZαA vector and was designated as pPICZαA/KPS54A61-E.

Host transformation: The pPICZαA/KPS54A61-E was transformed into JM109 by heat shock method and selected zeocin resistant for positive clone on low salt Luria-Bertani agar plates containing 25µg/mL zeocin (low salt LB zeocin). The colony obtained from low salt LB zeocin plates was inoculated into low salt LB zeocin broth and incubated in shaking incubator at 37°C for 16 h. The plasmid containing KPS54A61-E gene was extracted and detected by PCR using a pair of primers for AOXI gene of plasmid. The pPICZαA/KPS54A61-E and pPICZαA that had no insert gene, as negative controls were linearized by *Sac I* (Promega) digestion and transformed into *Pichia pastoris* stain *X33* by electroporation method (Macauley-Patrick *et al*., 2005). The transformed *X33* was inoculated onto YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol, 2% agar and 100 µg/mL zeocin and incubated at 30°C for 3-7 days. Each single colony was picked for determination of methanol utilization plus (Mut^+) in X33 on MM plates (1.34% YNB, 4×10^5% biotin, 0.5% methanol) and MD plates (1.34% YNB, 4×10^5% biotin, 0.5% dextrose). The colonies grown on both of MM and MD plates were then used for the expression of the recombinant protein.

Expression of recombinant envelop protein by P. pastoris: Positive colonies and control colonies were inoculated in 25 ml of buffered glycerol complex medium (BMGY) broth as starter culture and incubated at 30°C overnight with shaking at 250 rpm. Cell pellet and supernatant were separated by centrifuging at 3,000 rpm for 5 min. Cell pellet was then re-suspended in 250 ml of buffered methanol complex medium (BMMY) in flasks. The flasks were covered with 2 layers of sterile gauze and shaken 200 rpm at 30°C overnight. Absolute methanol was used as

inducer by adding in different of concentrations (v/v) at 0.5%, 1% and 2% every 24 h until 120 h. The cell pellet and supernatant were separated from expressed cultures by centrifuging at 13,000 rpm for 10 min. The supernatant was concentrated by using ultrafiltration at 30kDa molecular weight cut off (viva spin 20, GE Healthcare, UK) and cell pellets were ground by using acid washed glass beads (size 0.5 mm). The recombinant protein samples were analyzed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and protein quantification was measured by a spectrophotometer (Nano Drop™, Thermoscientific).

Production of antibody against the recombinant envelope protein in mice and ducks: Ten 10- day-old-ducks were housed in a clean room with netting for preventing biting of blood suckling flies. Feed and water were provided *ad libitum.* Prior to immunization, serum samples were collected from all ducks for checking antibody against DTMUV by serum neutralization (SN) test and EDTA blood samples were tested for the DTMUV by RT-PCR. Five ducks were intramuscularly injected with the recombinant E protein. The other five ducks were injected with PBS, served as the negative control sample. To obtain mouse antibodies against the recombinant E protein, two 4-wk-old BALB/c mice that had no antibody to DTMUV were immunized with the recombinant E protein by intraperitoneal injection. Clinical sign of illness was daily observed and sera were collected 14 and 28 days after immunization. All procedures of animal experiments were reviewed and approved by Kasetsart University Research and Development Institute, Thailand, no. ID OACKU00160.

Serum preparation: Total of 220 duck sera obtained from different 3 sources, as follows: 1) 5 serum samples obtained from the immunization of the recombinant E protein as described above, 2) 5 serum samples from the negative control group as described above were used as negative control. 3) 210 duck serum samples were collected from DTMUV infected ducks in the central, eastern and western parts of Thailand. In the meantime, 20 duck serum samples that were negative to DTMUV antibody (examined by SN test) kindly received from Bangkok Ranch Company were also used as negative control for Optimization of ELISA test.

Western blotting analysis: Separated bands of protein from 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto 0.45µm nitrocellulose membrane. The membranes were blocked with blocking solution (5% skim milk, 10% Horse serum, 1×PBS-T) at 37°C for 1 h. The primary antibodies obtained from positive DTMUV duck sera were diluted in primary diluent (1% skim milk, 10% horse serum, 0.02% $NaNH_2$, 1×PBS-T) at 1:500 and exposed to membrane for 1 hour at 37°C. There membrane was washed three times with 1XPBS Tween for 5 min. The secondary antibody goat anti-duck IgY conjugated with horseradish peroxidase (HRP) (Bioscience, USA) was diluted (1% skim milk, 10% horse serum, 1×PBS-T) at dilution 1:500 and exposed to membranes at 37°C for 1 h, washed three times and developed color with TMB peroxidase substrate.

Serum neutralization (SN) test: SN test was done in Nunc™ Edge 96 well plates (ThermoFisher scientific). Fifty µl of individual serum was heat-inactivated at 56°C for 30 min and diluted with IMDM medium by two-fold dilution. One hundred $TCID_{50}$ KPS54A61DTMUV was incubated with diluted sera at 37°C for 1 h. Each well was added with Vero cell, approximately 300 cells. Cytopathic effect (CPE) characterized by rounding formation and detachment of the infected cells, was observed daily for 5 days consecutively.

Immunoperoxidase monolayer assay (IPMA): IPMA was performed in 6-well plates containing Vero cells infected with KPS54A61DTMUV. All procedures of IPMA were followed as described earlier (Wellenberg *et al*., 1999). In brief, after infected cells showed 30% CPE, the culture medium was discarded and the infected cells were rinsed with PBS. The cells were fixed with 4% paraformaldehyde, 600 µl in each well at room temperature for 10 min. The fixed cells were then rinsed with distilled water 3 times and added with 1 ml of 2.5% bovine serum albumin (BSA) as a blocking buffer, and incubated at room temperature for 30 min. After removal of the blocking buffer, 800 µl of the primary antibody, mouse serum against the recombinant E protein was added into the wells. The primary mouse antibody was diluted at 1:1,000 or duck serum diluted at 1:500 in 2.5% BSA and incubated for 1 h. After rinsing the wells, a horseradish peroxidase (HRP)-labeled goat anti mouse immunoglobulin G (Bio-Rad, USA) or goat anti- duck immunoglobulin Y (Bioscience, USA) as the secondary antibody was added into the wells and incubated for 1 h. Then, the wells were rinsed with PBS 3 time. DAB (3, 3' – Diaminobenzidine) solution as a substrate (Merck, Singapore), refreshed with 0.1% hydrogen peroxide (H_2O_2), was added and incubated at 37°C for 15 min for color developing. The positive signal of the test was evaluated under an inverted microscope.

Immunohistochemistry (IHC): IHC was performed on the brain and spinal cord tissues of the mice experimentally infected with DTMUV from another study conducted earlier (Thiwalai *et al*., 2019). Briefly, the paraffin embedded brain and spinal cord tissues were sectioned and taken up on slides, processed, rehydrated and washed with distilled water. Antigen retrieval was done by incubation of proteinase K at 37 °C for 15 min. After rinsing with distilled water, the slides were added with 1% H_2O_2 for blocking endogenous peroxidase at 37 °C for 10 min. The slides were placed in a humidified box. Non-specific reaction was blocked by 2.5% BSA for 40 min. The duck serum against the recombinant E protein of DTMUV from this study, was used as the primary antibody with ratio 1: 200. After incubated for 1 h, the slides were rinsed with PBS 3 times. A secondary antibody, horseradish peroxidase (HRP)-labeled goat anti- duck immunoglobulin Y (Bioscience, USA) was diluted at 1:1,000 in 2.5% BSA, added onto the slides and

incubated for 45 min. After rinsing with PBS, the slides were incubated with DAB at room temperature for 15 min. Soon after color development, the slides were rinsed with distilled water 3 times, counterstained with hematoxylin and mounted with coverslips. The slides were examined under a light microscope.

Checkerboard titration and indirect ELISA: The optimal dilutions between recombinant E protein and serum sample were analyzed by checkerboard titration as described earlier (Liu *et al*., 2010). The recombinant E protein and control plasmid protein (without insertion of E protein encoding gene) were coated on Nune™ 96-well microplates (Thermo Scientific, UK). The dilution of the recombinant E protein and duck serum samples were started at dilution 1:10 – 1:320. Briefly, 100 µl of coating buffer (pH 9.6) was added, followed by 100 µl of each diluted recombinant E protein and incubated at 37°for 1 h. Each dilution of serum was added and incubated at room temperature for 1 h. After rinsing, 100 µl with dilution 1:5,000 of a horseradish peroxidase (HRP)-labeled goat anti duck IgY (Bioscience, USA) was added and incubated for 45 min and then rinsed with PBS (pH 7.2) containing 0.05% Tween 20 for 3 times. To developed color signal, TMB peroxidase substrate was added into the wells. The color absorbance is measured by ELISA leader at 450 nm. The equation S/P = OD Sample – OD negative control / OD positive control – OD negative control was calculated. The indirect ELISA was calculated sensitivity and specificity by using a two-way (2×2) table (Jacobson, 1998).

The intra and inter assay: The ELISA was tested repeatability and reproducibility by using sixteen DTMUV positive and sixteen negative duck serum samples for both assays. The intra assay tested one serum for 6 times in the same plate at the same period. The inter assay tested one serum for 6 times in each plate and each time. All steps of the ELISA test, dilution and volume per well of buffer in each step were described above. The test results were evaluated as coefficient variance (%CV). Determination of relationship between the SN test and ELISA test was calculated by the correlation coefficient (r) statistics and kappa statistics as described by Landis and Koch (1997) and Hinkle *et al*. (1998).

Retrospective study: Three thousand, four hundred and ninety-two duck serum samples were collected during surveillance of avian influenza in Thailand, in 2004 – 2017. The serum of meat-typed, free-grazing and laying ducks were obtained from central, lower northern, eastern and western parts of Thailand (Table 1). All serum samples kept at -20 °C were filtrated through 0.2-micron filter and then warmed at 56 °C for 10 min before tested by the indirect ELISA.

Table 1 The Indirect ELISA results of retrospective serum group in 2005 – 2017.

Year	Region (Province)	Type of ducks	Positive samples / total samples	% Positive
2005	Central Thailand (Ayuthaya)	Free grazing ducks	9/44*	20.45
2006	Lower Northern Thailand (Phitsanulok and Sukhothai)	Free grazing ducks	57/118*	48.30
2007	Western Thailand (Kanchanaburi)	Laying ducks	44/55*	80
2008	Western Thailand (Kanchanaburi)	Laying ducks	58/60*	96.66
2009	Central Thailand (Nakhonpathom)	Laying ducks	62/74*	83.78
2010	Eastern Thailand (Chachoengsao)	Meat-typed duck	138/150*	92
2013	Central Thailand (Ang Thong)	Laying ducks	30/44	68
	Western Thailand (Ratchaburi)	Meat-typed ducks		
2014	Central Thailand (Nakhonpathom)	Meat-typed ducks	61/77	79.22
2015	Central Thailand (Nakhonpathom)	Laying and Free grazing ducks	101/145	69.65
2016	Central Thailand (Nakhonpathom) (Bangkok)	Meat-typed ducks	70/380	18.42
2017	Central, Lower Northern and Western Thailand (Pathumthani, Nakhonpathom Suphanburi, Chainat, Nakhonsawan,Pitsanulok Pichit, Kanchanaburi)	Free-grazing ducks	1901/2021	94.06

All tested serum were positive to HPAI H5N1 by HA-HI test. Data are not available in 2011-2012 because of extensive flood in Thailand

Results

Expression of recombinant E protein: The amplified product of KPS54A61- protein E encoding gene by RT-PCR using specifically designed primers was analyzed by 1.5% agarose gel electrophoresis and showed in length was 1503 base pairs. The positive clone from selected plate was confirmed by PCR using AOXI specific primers after that digestion by *EcoR*I and *Xba*I as restriction enzyme, digested and non- digested fragment were analyzed by agarose gel electrophoresis. The positive clone was inoculated into expression medium and induced by absolute methanol at final concentration (v/v) 0.5%, 1%, 2% at incubation temperature of 30°C for 24, 48, 72, 96 and 120 h. The expression of recombinant E protein was evaluated by 15% SDS-PAGE, which was found in the culture supernatant only. The molecular mass of the recombinant E protein was presented at 65 kDa and 2% (v/v) of absolute methanol at 120 h of expression times.

Immunization of recombinant E protein in mice and ducks: All mice and ducks immunized with the recombinant E protein did not show any clinical signs of illness. They were raised until at the end of the study. Their sera collected at day 28 after immunization, were used for IPMA and IHC as described above.

Protein antigenic testing: Twenty milliliters of the supernatant were concentrated by using ultrafiltration 30kDa molecular weight cut off (viva spin 20, GE Healthcare, UK). The supernatant was also measured for protein quantification by spectrophotometer (Nano drop 2000c, Thermoscientific), with volume of 4.55 mg/mL. Antibodies against KPS54A61DTMUV obtained from infection and from recombinant E protein immunization were used as primary antibodies, which reacted with the recombinant E protein on nitrocellulose membrane and was presented single band at 65 kDa (Fig. 1). IPMA by using the mouse or duck antibody against recombinant E protein as primary antibodies could react positively with the DTMUV antigen of the infected Vero cells cultured at 72 h post infection (Fig. 2). Positive staining by immunohistochemistry was also found in cytoplasm of glia cells and neurons of brain and spinal cord tissues obtained from the former study (Thiwalai *et al*., 2019) (Fig. 3).

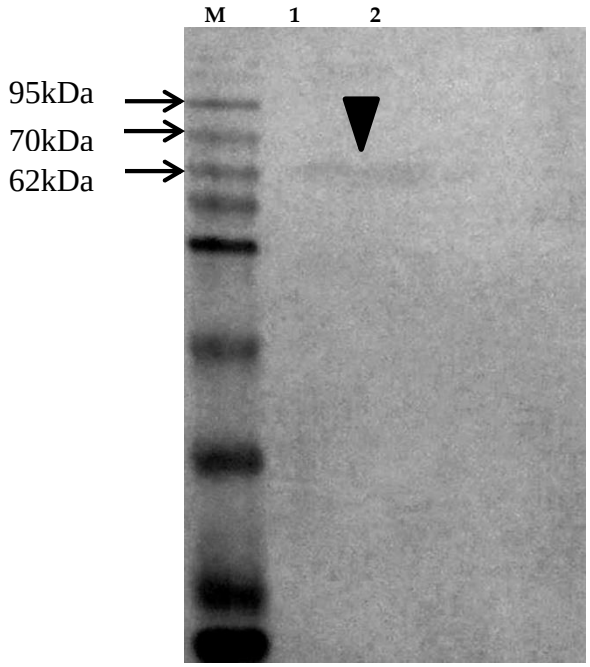

Figure 1 Confirmation of recombinant E protein by western blotting with duck antibodies against DTMUV. Lane M: protein molecular weight marker; lane1: the recombinant E protein, approximately 65 kDa, expressed from *X33* pPICZαA/ KP54A61-E at 120 h; lane2: protein expressed from negative control clone at 120 h. Black arrow head is position of the recombinant E protein.

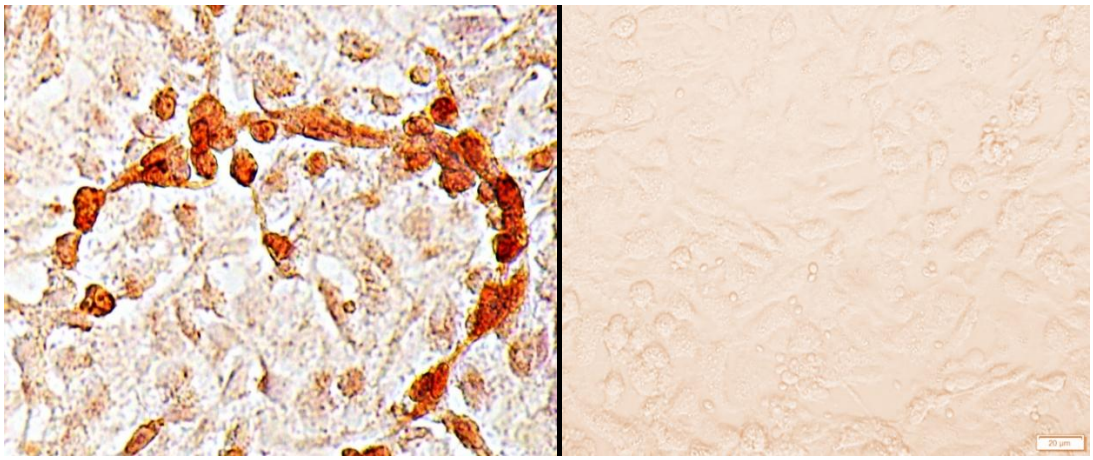

Figure 2 Positive staining of IPMA with the mouse antibody against the recombinant E protein in infected Vero cells (Left). Negative control of non-infected Vero cells (Right).

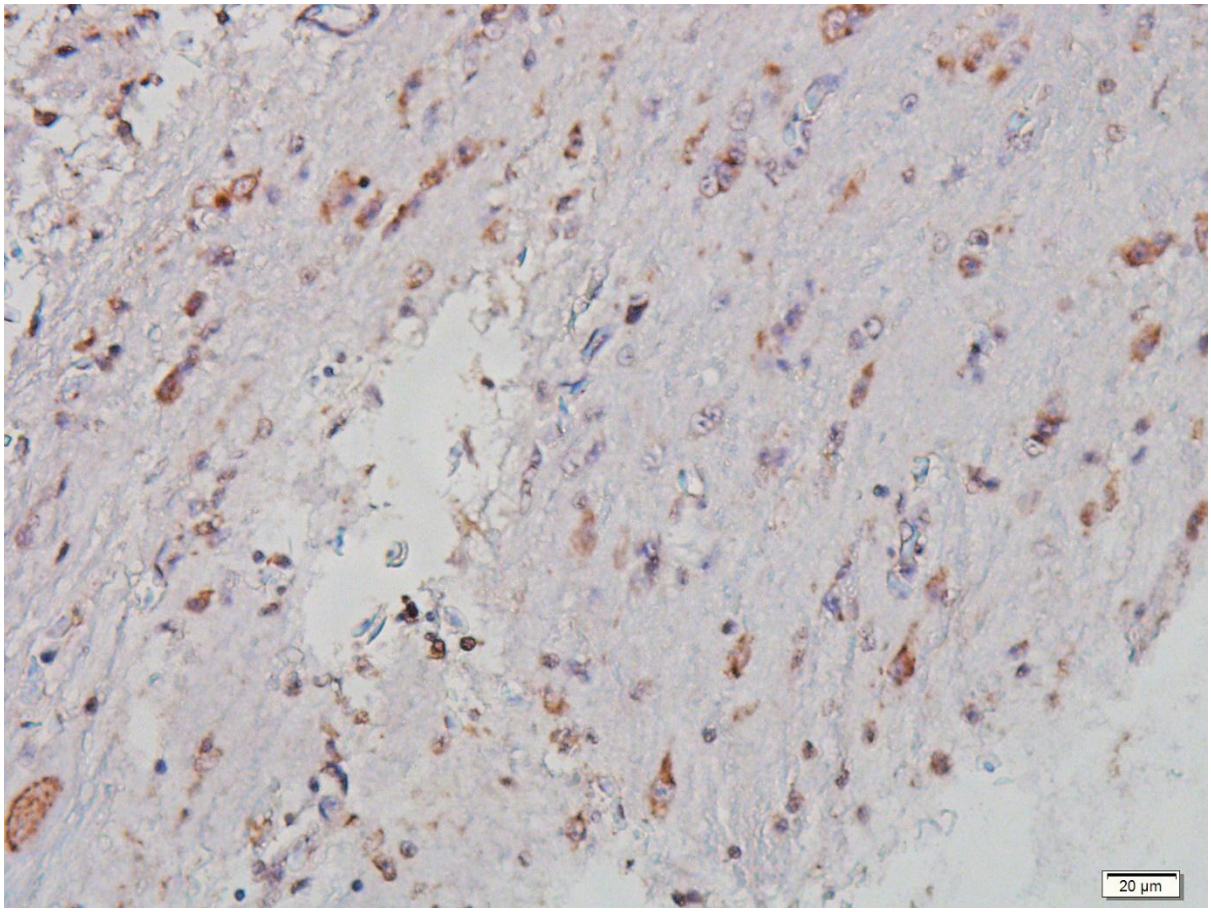

Figure 3 Positive staining (brown color) of DTMUV antigen in spinal cord tissue of an experimentally infected mouse by immunohistochemistry, using duck antibody anti recombinant E protein as the primary antibody.

Optimization of ELISA test: The optimal concentration of the recombinant E protein and serum samples was analyzed by checkerboard titration and the final concentration of coating recombinant E protein was 22.75µg/mL and serum samples diluted at 1:20. The cut-off value were calculated on 20 duck sera from non- infected farms (negative sera) and calculated by the mean of optical density (O.D.) negative plus three times standard deviation of O.D. negative, value of mean (0.0767) and standard deviation (0.0792). The cut off value was 0.31. The serum samples which showed optical density value greater than cut-off value were considered positive. An indirect ELISA, the 130 duck serum samples were found O.D. value ≥ 0.31(positive) and the 90 ducks sera samples were found O.D. value < 0.31 (negative). The O.D. of plasmid protein was used as negative control protein and showed clearly no background.

Intra and Inter assay: The repeatability and reproducibility of ELISA were tested on KPS54A61DTMUV positive and negative serum and calculated by coefficient of variation (%CV). The coefficient variance of intra and inter assay ranged 2.8 - 14.7% and 2.9 –14.28%, respectively (Table 2).

Comparison of ELISA and SN test results: One hundred and eighteen serum samples were positive to SN test (118 out of 220) and their titers ranged 1:40 to 1:5120 meanwhile the ELISA showed the positive results from the samples out of 220. By both methods taken together, 110 serum samples were positive and 90 serum samples were negative. The sensitivity and specificity of the indirect ELISA based on the recombinant E protein were compared with SN test as the gold standard method, the calculated of sensitivity and specificity were 93.22% and 88.24%, respectively (Table 3). Kappa statistic determined concordance between Indirect ELISA test and SN test showed at 0.81 and interpretation level was almost perfect agreement (Landis and Koch. 1997).

Retrospective study: This indirect ELISA was used for analysis of 3,492 duck serum samples. The positive results are shown in Table 1. All provinces where the sera were collected were serologically positive to DTMUV.

Table 2 Repeatability of the developed ELISA test kit for intra and inter-assays with six replications using negative and positive duck sera. The results are shown as the mean of O.D. + S.D. and %CV.

Samples	Mean O.D. + S.D.		Intra assay %CV	Inter assay %CV
	Intra assay	Inter assay		
Negative sera	0.032 ± 0.004	0.030 ± 0.003	12.50	10.00
	0.018 ± 0.002	0.019 ± 0.002	11.11	10.50
	0.020 ± 0.003	0.023 ± 0.002	14.70	8.70
	0.030 ± 0.004	0.029 ± 0.003	13.33	10.34
	0.060 ± 0.007	0.068 ± 0.007	11.67	10.30
	0.025 ± 0.002	0.021 ± 0.003	8.00	14.28
Positive sera	0.383 ± 0.050	0.390 ± 0.048	13.00	12.30
	0.423 ± 0.012	0.395 ± 0.046	2.80	11.64
	0.411 ± 0.040	0.405 ± 0.023	9.70	5.68
	0.344 ± 0.029	0.335 ± 0.010	8.50	2.90
	0.362 ± 0.032	0.329 ± 0.017	8.84	5.16
	0.360 ± 0.039	0.411 ± 0.019	10.83	4.62

OD: Optical density SD: Standard deviation CV: Coefficient of variation

Table 3 Serologic comparison of 220 duck serum samples tested by ELISA and serum neutralization

Number sample of ELISA	Number sample of serum neutralization		
	positive	negative	total
positive	110	12	122
negative	8	90	98
total	118	102	220

Discussion

In this study, we are successful to produce the recombinant E protein of DTMUV by using *Pichia pastoris*, as an expression system. The good quality and adequate amount of the recombinant E protein can be used for ELISA that is a helpful tool of routine diagnosis and surveillance of the disease. We used KPS54A61DTMUV, genetically classified cluster 2.1 because this cluster is the main cluster found in Thailand (Nilvilai *et al*., 2019). Our result of the recombinant E protein production is in accordance with the previous studies in that the recombinant E protein of Flavivirus can be produced in *Pichia pastoris* expression system (Valdés *et al*., 2007; Cardoso *et al*., 2013; Yun *et al*., 2014). In addition, the results of SDS – PAGE indicated that the recombinant E protein was secreted into the culture supernatant. The same result is in accordance with other studies (Sugrue *et al*., 1997; Valdés *et al*., 2007; Yin *et al*., 2013; Yun *et al*., 2014). From the results of western blot analysis, it indicated that our recombinant E protein is highly specific to the antibodies of naturally infected and subsequently recovered ducks. The antibodies obtained from the infected ducks, ducks and mice immunized with the recombinant E protein could obviously react with the antigen coated in the ELISA plates. This indicated that the recombinant E protein was highly specific for antibody raised from DTMUV infection. This ELISA also showed absolutely negative results, without any back ground when tested on duck sera obtained from other diseases including Duck viral enteritis, Riemerellosis, avian influenza and fowl cholera (Data not shown). Positive result of IPMA indicated that the antibodies raised by the mice and ducks immunized with the recombinant E protein, was specific for the viral antigen in the DTMUV infected Vero cells.

An important advantage of *Pichia pastoris* expression is to provide low level of secreted native proteins. Hence, the secreted recombinant protein is the major component of the total protein, as the primary requirement of protein purification (Barr *et al*., 1992; Cardoso *et al*., 2013). In this study, separated bands of protein from 12% SDS – PAGE showed the band of the recombinant E protein at 65kDa indicated that the recombinant E protein was successfully produced.

Immunized mouse or duck antibody could positively react with E antigen of the infected Vero cells and immunized duck antibody reacted with tissue section of experimentally infected mice by IHC, indicating that the recombinant E protein from this system has specific immunogenicity the same as that of the field DTMUV. When compared with SN test, sensitivity and specificity of the indirect ELISA was 93.2% and 88.23%, closely to that of the other study (Yin *et al*., 2013).

The positive results of retrospective serology found in the serum samples collected from all described provinces and years, especially, positive serum collected indicated that the DTMUV has circulated in Thailand since 2004. Since the tested serum samples obtained from the last decade of HPAI H5N1 serological surveillance in Thailand were positive to DTMUV antibody, it might imply whether a combined infection of HPAI H5N1 was occurred since 2004, at the first period of HPAI H5N1 outbreak (Songserm *et al*., 2006). In fact, we had used these HPAI H5N1 positive duck sera obtained from the field for routine diagnosis of HPAI H5 virus in allantoic fluid taken from egg inoculation by HA-HI test. These positive sera were also used as positive control for routine serologic HA-HI test. It is interesting whether or not all the HPAI H5N1 infected ducks exhibiting nervous signs of illness, including ataxia, head trembling and paralysis, may be simultaneously infected with the DTMUV. The result from our retrospective study by ELISA is in accordance with Nilvilai *et al*'s study (2018) in that DTMUV has circulated in Thailand for longer period of time.

In conclusion, the indirect ELISA developed in this study is a helpful tool for surveillance, routine diagnosis and serological monitoring DTMUV infection and future vaccine application. Also, serological study of different clusters of DTMUV infection should be further performed.

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