Original article

Generation and characterization of a monoclonal antibody against duck Tembusu virus envelope protein

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Abstract

Duck Tembusu virus (DTMUV) is a newly emerging pathogenic flavivirus that has caused massive economic losses to the duck industry in China. Envelope (E) protein of DTMUV is an important structural protein, which is able to induce protective immune response in target animals and can be used as specific serological diagnosis tool. In this study, a novel monoclonal antibody, designated mAb 3E9, was generated against DTMUV E protein. It is positive in indirect ELISA against both His-E protein and the purified whole viral antigen. Also, this mAb showed positive reaction with DTMUV in Western blot and indirect immunofluorescence assay, and the isotype was IgG1. End-point neutralizing assay performed in BHK-21 cells revealed that the neutralization titer of 3E9 against DTMUV JS804 strain reached 1:50. Furthermore, functional studies revealed that 3E9 blocks infection of DTMUV at a step on viral attachment. The anti-E mAbs produced in the present work may be valuable in developing an antigen-capture ELISA test for antigen detection or a competitive ELISA test for antibody detection or therapeutic medicine for DTMUV in poultry.

Key words: duck Tembusu virus, envelope protein, monoclonal antibody

Introduction

In April 2010, a severe duck disease has emerged throughout the main duck-producing regions of eastern China. The infected layer ducks developed a significant reduction in egg production ranging from 20% to 60%, even up to 90%, with mortality rate varying from 5% to 30% (Cao et al. 2011, Su et al. 2011). The diseased ducks developed some nervous system disorders including unsteady standing, falling and quivering (Ti et al. 2015). In addition to ducks, this disease has affected geese, chickens, and sparrows (Sun et al. 2014). The emerging disease was designated duck hemorrhagic ovaritis (DHO) firstly, and further study proved that the causative agent isolated from ducks is duck Tembusu virus (DTMUV). DTMUV is a single-stranded positive-sense RNA virus classified in the Genus Flavivirus, Family

Flaviviridae. As with other flaviviruses, mature virions have a diameter of 45-60 nm, its genome is approximately 11 kb in length. The whole genome is translated into a single polyprotein, which is subsequently processed by viral- and host-encoded proteases into structural and nonstructural proteins (Tang et al. 2012). Three structural proteins (C, prM/M and E) make up the viral particle and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) are required for genome replication and polyprotein processing (Heinz and Stiasny 2012). Similar to the E proteins in other flaviviruses, the envelope (E) protein of DTMUV is the major surface protein of the virion that mediates binding to the cellular receptor and subsequent fusion event between viral and host membranes (Perera et al. 2008). Also, DTMUV E is the primary and majority target of neutralizing antibodies. Crystallographic analysis reveals that the E glycoprotein of flaviviruses folds into three distinct structural domains (I, II and III) (Luca et al. 2012). Domain III of flaviviruses E protein (DIII) is the putative receptor-binding domain and is an important target for neutralizing antibodies and *in vivo* protection (Yu et al. 2013).

However, licensed DTMUV vaccine or drug is currently unavailable. Hence, the development of other technological means against DTMUV is critical for disease control. In viral diseases, for which a specific therapy is not yet available, antibody-based therapy represents a promising alternative strategy. Neutralizing antibodies have been demonstrated to be effective in animal models, such as prophylaxis and as treatments for some flavivirus infections (Morrey et al. 2006, Pierson and Diamond 2008). Also, neutralizing antibodies having strong and specific reactivity to flaviviruses antigens are the most suitable choice for the development of standardized diagnostic tools. In this paper, we described the generation and characterization of a monoclonal antibody, 3E9, specific for the DTMUV E protein. Also, the neutralizing profiles of mAb 3E9 were characterized *in vitro* and *in vivo*. The information provided by this research will facilitate the development of diagnostic tools for the specific serological diagnosis of DTMUV infection, and will contribute to the rational design of vaccines by broadering understanding of the antigenic structure of DTMUV E protein.

Materials and Methods

Cells and viruses

The myeloma cell line SP2/0 was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) in a humidified 5% CO_2 atmosphere at 37ºC. BHK21 cells were cultured in the same condition. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Invitrogen), 0.1 mg/ml of streptomycin and 100 IU/ml of penicillin. DTMUV JS804 strain was stored in our laboratory. Also, DTMUV-positive/negative mouse serum were generated and maintained in our laboratory. Female Balb/c mice (5-weeks-old) were purchased from Yangzhou University (Jiangsu, China) and raised in special cages with food and water supplied. All animal experiments were carried out in accordance with the regulations and guidelines of animal experimentation outlined by the people's Government of Jiangsu Province (SYXK (Su) 2010-0005).

Expression of recombinant E protein

The full-length envelope coding sequence was amplified using the primers DTMUV E-*Eco*RI (5'-CACACGAATTCCGAGACTTTGTTGAG-GGAGTGA-3') and DTMUV E-*Xho*I (5'- CACAC-CTCGAGGACATGGATATGGGAACTCTAC-3'). These primers were designed according to the sequence of DTMUV JS804 strain (GenBank No. JF895923) and contained *Eco*R I and *Xho* I sites (shown in underline) to facilitate directional cloning into the pET28a (+) expression vector following amplification, agarose gel purification and restriction enzyme digestion. The recombinant plasmid was verified by restriction enzyme digestion and DNA sequencing, then it was transformed into *E. coli* BL21 cells for expression. After the cells were cultivated to an OD600 (optical density at 600 nm) of 0.6-0.8 in LB media at 37ºC, 0.8 mM isopropyl-b-D-1-thiogalactopyranoside (IPTG) was added into the medium to induce the proteins expression for 5 h. Then the cells were harvested by centrifugation and resuspended in phosphate-buffered saline (PBS, pH7.4). Then cell lysates were prepared by sonication and analyzed by SDS-PAGE using 12% gels to confirm the distribution of the expressed recombinant protein. Finally, the yield of expressed recombinant proteins were analyzed by Western blot.

Preparation and purification of mAb

Monoclonal antibody (mAb) to the recombinant E protein was produced using a standard procedure (Lelli et al. 2012). Five-week-old Balb/c female mice were immunized by subcutaneous injection with 70 μg of purified recombinant E protein emulsified with an equal volume of Freund's complete adjuvant, then

followed by two injections at 2 weeks interval with the protein emulsified in the Freund's incomplete adjuvant. Three days after the last boost, the mice were sacrificed and hybridomas were generated following fusion of splenocytes with NS0 myeloma cells and selected cultures were grown following the standard method (Lelli et al. 2012). The fused cells were cultured and selected in RMPI-1640 medium (HAT medium and HT medium). The mAb-producing hybridoma cells were cloned by limiting dilution of the cells three times. Ascites were derived from the mice primed with a 0.5 ml adjuvant and then injected with 2×10^6 hybridoma cells by intraperitoneal injection. E-specific mAbs were identified by immunostaining of BHK21 cells transfected with plasmids expressing the E protein. The hybridoma-producing mAb 3E9 (IgG1) was cloned twice via limiting dilutions of the cells. mAb 3E9 was purified from ascites by protein A affinity column (GE Healthcare) according to manufacturer's instructions. Briefly, ascites was diluted in $1 \times$ PBS and injected into pre-equilibrated protein A column. The whole antibody was eluted from the column using 25 mM glycine (pH 2.2) elution buffer according to manufacturer's instructions.

Characterization of mAb 3E9

mAb 3E9's profiles were characterized by Western blot and Indirect immunofluorescence assay (IFA). BHK21 cells were transiently transfected with recombinant plasmids encoding E protein or with empty pVAX1 (control) using Lipofectamine 2000 reagent (Invitrogen). 48 h after transfection, cultured cells were lysed and analyzed by Western blotting using mAb 3E9 as primary antibodies and a HRP-conjugated goat anti-mouse secondary antibody (New England Biolabs, USA). IFA was performed as follows. Briefly, BHK21 cells were transiently transfected with recombinant plasmids encoding E protein or infected with DTMUV JS804 strain, and then fixed with ice-cold acetone. Cells were incubated with mAb 3E9. After 60 min of incubation at 37°C, cells were washed three times with phosphate-buffered saline (PBS). Cells were then treated with a 200-fold dilution of FITC-conjugated anti-mouse IgG (KPL) for 30 min at 37°C and rinsed with PBS. After five washes in PBS, positive cells were detected using a fluorescent microscope.

Indirect enzyme-linked immunosorbent assay

The mAb 3E9 titer of culture supernatant or the ascites was determined by indirect ELISA. Briefly,

a 96-well plate was coated with purified recombinant E protein (2 μg/ml) at 4°C overnight and blocked with 5% skimmed milk dissolved in PBS at 37°C for 2 h. Then, plates were washed five times with PBST and incubated with different concentrations of 3E9 in triplicate for 1 h at 37°C. Plates were washed five times and then incubated with peroxidase-conjugated goat anti-mouse IgG (1:5000) (New England Biolabs, USA) for 1 h at 37°C. Plates were washed five times and then sequentially incubated with TMB substrate (Promega, USA). The reaction was stopped with 2 M H_2SO_4 and the OD value of each well was read at 450 nm using a microplate reader (BioRad 550). The isotype of mAb 3E9 was determined using a Mouse Monoclonal Antibody Isotyping Kit (Promega, USA).

Neutralization assay

The competence of mAb 3E9 to neutralize virus infectivity was carried out in 96-well microplates. Purified mAb 3E9 was serially diluted fourfold in DMEM, and 100 μl aliquots of each dilution were mixed with 100 μl 100TCID$_{50}$ DTMUV JS804 strain. The antibody-virus mixtures were incubated for 1 h at 37°C and then transferred to 96-well plates containing 85%-95% confluent monolayers of BHK-21 cells. After incubation for 72-96 hours at 37°C with 5% CO_2, wells were scored for cytopathic effect and neutralizing titers were expressed as the reciprocal of the final mAb dilution required to neutralize 100% of the inoculated cultures.

For neutralization assay *in vivo*, purified mAb 3E9 was serially diluted fourfold in DMEM, and 100 μl aliquots of each dilution were mixed with 100 μl 200 LD$_{50}$/0.025 ml DTMUV. The antibody-virus mixtures were incubated for 1 h at 37°C. Then 0.025 ml mAb-virus mixture was injected intracerebrally into 3-day-old suckling mouse. The control group only received PBS diluent. The animals were monitored daily for clinical signs of infection, including ruffled hair, a hunched back, paralysis, and death, for 10 days. The neutralization activity was calculated by using Reed and Muench's method.

Cell-binding assay

50 μg/ml mAb 3E9 or 3H11 (another monoclonal antibody without neutralizing activity) or BSA was incubated with 500TCID$_{50}$ DTMUV JS804 strain for 60 min at 4°C. The virus-mAb mixtures were then added to BHK21 cells in 24-well plates for 60 min on ice. Unbound virus was removed after three washes with PBS. Total viral RNA was extracted from infected
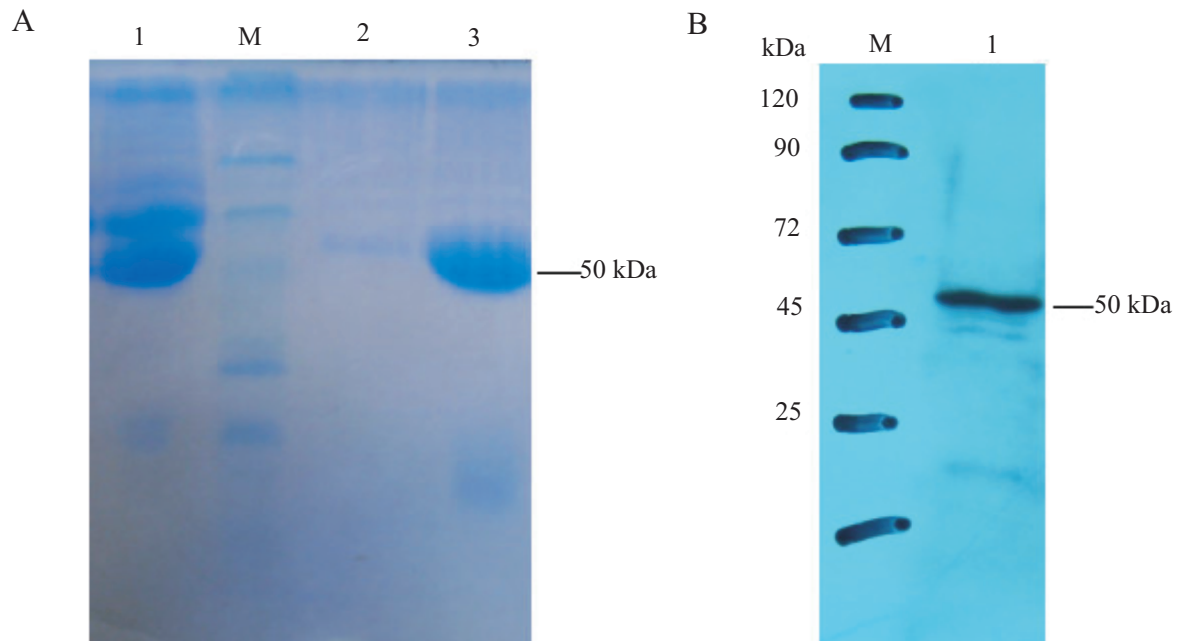
Fig. 1. (A) The expressed and purified recombinant E protein was detected by 12% SDS-PAGE. Lane M: Protein marker; Lane 1: induced pET28a-E; Lane 2: negative control pET28a; Lane 3: induced and purified recombinant E protein. (B) Western blot analysis of the purified E protein. Lane M: Protein marker; Lane 1: purified E protein.
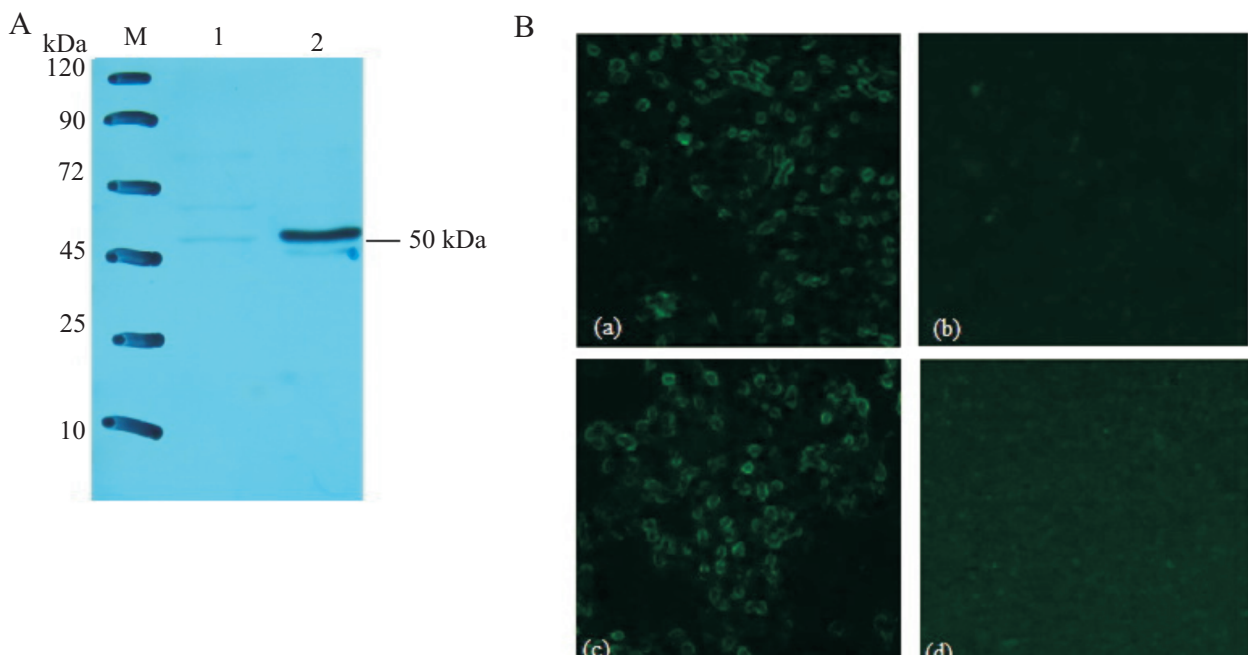

Fig. 2. Characterization of mAb 3E9 *in vitro*. (A) The specificity of 3E9 for the DTMUV native E protein. BHK21 cells were transiently transfected with the recombinant plasmid encoding the E protein or with a control vector, empty pVAX1. 48 h after transfection, cultured cells were lysed and analyzed by Western Blotting with mAb 3E9. Lane M: Protein marker; Lane 1: empty vector pVAX1; Lane 2: the recombinant plasmid pVAX1-E.; (B) Reactivity of mAb 3E9 with DTMUV native E protein and duck Tembusu virus determined by indirect immunofluorescence analysis. (a) BHK21 cells were transiently transfected with the recombinant plasmid encoding the E protein; (b) BHK21 cells were transiently transfected with the plasmid pVAX1. (c) BHK21 cells were infected with DTMUV. Three to five days after infection, cells were fixed and analyzed by IFA with mAb 3E9. (d) Uninfected BHK-21 cells were run simultaneously as negative controls.

cells using Axygen Total RNA extraction Kit (Axygen Biosciences, China) according to the manufacturer's instructions and viral RNA was quantified by real-time RT-PCR as previously reported (Zheng et al. 2008).

Pre- and post-adsorption inhibition assay

Neutralization of DTMUV before or after adsorption to BHK21 cells was performed using $100TCID_{50}$ of DTMUV and serial dilutions of 3E9 essentially

as above. In the pre-adsorption assay, the mAb was firstly incubated with BHK21 cells for 60 min at 4°C, then the DTMUV was added and incubated for additional 60 min at 4°C. In the post-adsorption assay, DTMUV firstly were added to BHK21 cells for 60 min at 4°C, then the mAb 3E9 was added and incubated for additional 60 min at 4°C.

Results

Expression and purification of E protein

Recombinant DTMUV E protein was successfully expressed in *E. coli* BL21 (DE3) and purified by Ni-NTA purification system (Merck). The molecular weight of the expressed recombinant E protein was approximate 50 kDa as determined by 12% SDS-PAGE, consistent with the molecular weight of the predicted protein (Fig. 1A). The expression of recombinant E protein was further confirmed by Western blot with mouse anti-serum to DTMUV. The band was in agreement with the predicted size from the prokaryotic expressed protein (Fig. 1B). These results indicated that recombinant E protein, possessing good reactivity with corresponding anti-serum, was correctly expressed and well purified.

Generation and characterization of mAb 3E9

Purified E protein was used to immunize Balb/c mice. After cell fusion and screening, several hybridoma cell lines were obtained which produced E-specific mAbs. Among them one cell line, 3E9, was selected for its strongest reactivity against recombinant E proten using indirect ELISA, WB (Fig. 2A), and against native E protein in IFA using DTMUV antigen slides (Fig. 2B). Also, the 3E9 mAb recognized the DTMUV by IFA (Fig. 2B). The subtypes of mAb 3E9 were determined using the Mouse Monoclonal Antibody Isotyping Kit (Promega, USA) according to the manufacturer's instructions. It was shown that the heavy chain of 3E9 was IgG1 and the light chain was κ type. Antibody titers of culture supernatants and the ascites prepared with the hybridoma cell line were measured by indirect ELISA. Antibody titer of the culture supernatants of mAb 3E9 was 1:512, and Antibody titer of the ascite was 1:512,000.

Neutralizing Activity

This was analyzed for mAb 3E9 using the neutralization assay described above. 3E9 supernatant showed strong neutralization activity up to 1:50 dilu-

tion. Since the specific IgG1 concentration in the supernatant was 0.2 mg/ml it can be extrapolated that 3E9 efficiently neutralized DTMUV at concentrations of at least 4 µg/ml. To characterize the neutralizing Activity of 3E9 *in vivo*, an existed suckling mouse model was utilized to analyze the protective efficacy of 3E9 against lethal DTMUV infection. The DTMUV infected group developed clinical symptoms and died 4 to 6 days post-infection. mAb 3E9 treatment showed protection against DTMUV in a dose-dependent manner and strong neutralization activity up to 1:15 dilution. Statistical analysis showed that survival rate of 3E9-treated mice were significantly higher than that of PBS-treated group, indicating that 3E9 confers protection against DTMUV infection *in vivo*.

Cell-binding assay

In order to explore the mechanism of 3E9-mediated neutralization of DTMUV further, cell-binding assay was performed with BHK21 cells. Another E-specific mAb (3H11) without neutralizing activity and BSA were used as controls. The results showed that 3E9 could more significantly ($p<0.01$) inhibit DTMUV binding than 3H11, whereas BSA protein failed to inhibit virus binding (Fig. 3). This phenomenon suggested that the activity of 3E9 may involve the blockade of cell attachment.
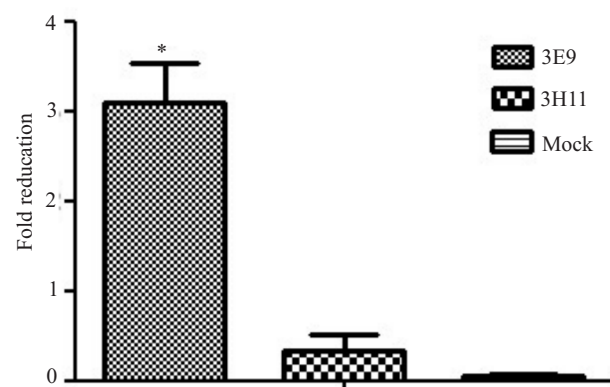

Fig. 3. Mechanism of mAb 3E9-mediated neutralization. Duck Tembusu virus binding to BHK21 cells. The E-specific neutralizing mAb 3E9 blocked cellular attachment significantly more than mAb 3H11 and BSA control. Fold expressions are reported with standard deviations.

Pre- and post-adsorption inhibition assay

In the pre- and post-adsorption inhibition assay (Fig. 4), 3E9 was incubated with DTMUV before or after its binding to a monolayer of BHK21 cells, also
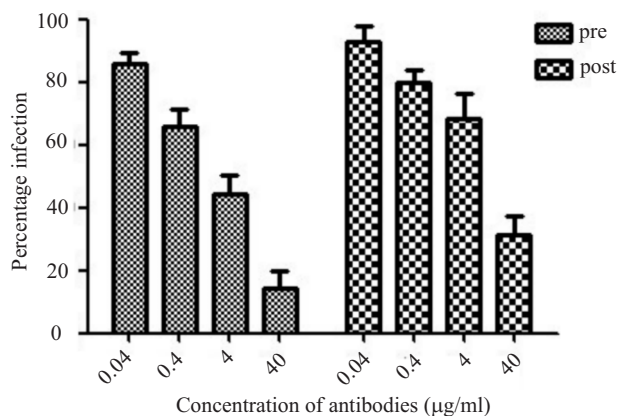
Fig. 4. Pre- and post-adsorption inhibition assays. Pre-binding of DTMUV with 3E9 significantly protected against DTMUV infection (Pre). Infection percentages at the different antibody concentrations are shown with standard deviations.

virus titer was measured as above described. The results showed 3E9 neutralized infection when applied before the binding of the virus. These findings indicated that mAb 3E9-mediated neutralization activity occurs primarily before DTMUV cellular attachment.

Discussion

In the present study, we generated and characterized a monoclonal antibody (3E9) against DTMUV E protein. mAb 3E9 has strong neutralizing activity with duck Tembusu virus *in vitro* and could protect mice against DTMUV infection *in vivo*. Furthermore, functional experiments suggested that mAb 3E9 neutralized viral infectivity at a pre-attachment step in the viral life cycle.

E protein is an important structural protein of flaviviruses. By analogy with well-studied flaviviruses, the E protein of DTMUV is the major surface protein of the virion that mediates binding to the cellular receptor and subsequent fusion event between viral and host membranes (Mukhopadhyay et al. 2005). Similar to the E proteins in other flaviviruses, DTMUV E is the primary target of neutralizing antibodies (Liu et al. 2012). The functional and immunobiological characteristics of the E protein make it a good candidate for development of a prophylactic vaccine (Azevedo et al. 2011, Coller et al. 2011). In our study, we injected mice with recombinant E protein and isolated a mAb that recognized a protein band of around 50 kDa in Western blot, corresponding to E protein. By IFA, we found the mAb 3E9 could recognize the E protein expressed in eukaryotic cells or whole duck Tembusu viron, indicating that the epitope recognized by the mAb 3E9 is exposed on the E protein surface. In our study, we also found that mAb 3E9 could sig-

nificantly inhibit cellular attachment of DTMUV to BHK21 cells, and it inhibited infection when added before virus binding, suggesting that mAb 3E9 neutralizes viral infectivity at a pre-attachment step in the viral life cycle. Generally, antibodies have the potential to neutralize the infectivity of flaviviruses by interfering with several steps of the virus entry pathway, such as attachment, internalization and fusion, or depending on the locations of their binding sites (Oliphant and Diamond 2007). Our finding is similar to Oliphant's research, which reported mAb E53 of West Nile virus inhibited infection primarily by blocking viral attachment to some cells (Oliphant et al. 2006).

DTMUV is an important poultry pathogen that causes substantial egg production drop in infected ducks (Cao et al. 2011, Su et al. 2011, Ti et al. 2015). DTMUV-induced egg production drops also observed in other domestic birds, including hens and geese, caused lots of economic losses to the poultry industry in China. But up to now, specific antiviral therapy or vaccines are absent for DTMUV. Vaccination is efficient and fast to DTMUV control, but no TMUV successful commercial vaccine has been developed and used in the field. Several inactivated TMUV vaccine candidates have been developed and used in some areas but these inactivated vaccines might not elicit a robust neutralizing antibody response (Sun et al. 2014). Therefore, the need for development of alternative ways against TMUV appeared to gain high priority. Monoclonal antibodies have become a powerful tool to study protein structure and have been widely used to diagnose and treat a variety of infectious agents (Denisova et al. 2008, Shen et al. 2009, Sukupolvi-Petty et al. 2010). The mAb 3E9 presented herein may advantageously be used to treat or prevent DTMUV infection in mice, for this reason, mAb 3E9 may further be developed as a therapeutic antibody to treat DTMUV in ducks after further investigation.

Acknowledgements

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