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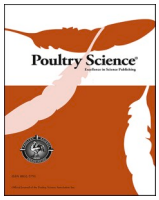

Full-Length Article

Assessing the effect of beta-propiolactone inactivation on the antigenicity and immunogenicity of cluster 2.1 duck Tembusu virus

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ABSTRACT

Duck Tembusu virus (DTMUV), an emerging avian pathogenic flavivirus, causes severe neurological disorders and acute egg drop syndrome in ducks. Currently, several clusters of DTMUV exhibiting significant antigenic variation are circulating in Asia with distinct geographical distributions. Therefore, developing vaccines based on virus clusters specific to regions is essential. To generate an effective inactivated vaccine, the virus inactivation procedure must be optimized for each virus strain. However, no information is available on the optimal inactivation protocol for cluster 2.1 DTMUV, which predominantly circulates in several Asian countries. This study aimed to determine the effect of beta-propiolactone (BPL) inactivation on the infectivity, antigenic integrity, and immunogenicity of cluster 2.1 DTMUV. Our results demonstrated that all conditions of BPL treatment (1:2000, 1:3000, and 1:4000 (vol/vol) concentration; 24, 48, and 72 h of incubation) could completely inactivate cluster 2.1 DTMUV, as evidenced by the absence of cytopathic effect (CPE) and DTMUV antigens after 3 passages in baby hamster kidney (BHK-21) cells. However, BPL at 1:4000 (vol/vol) concentration with 24 h of incubation preserved both the total protein content and the antigenic integrity of cluster 2.1 DTMUV more effectively than other conditions. Furthermore, we found that cluster 2.1 DTMUV inactivated with BPL under this condition was safe and highly immunogenic in ducks. This was evidenced by the absence of clinical signs and the robust induction of DTMUV-specific neutralizing antibodies and T helper lymphocyte responses in immunized ducks. Overall, these findings suggest that a 1:4000 dilution of BPL with 24 h of incubation is the optimal condition for complete inactivation of cluster 2.1 DTMUV without significant loss of antigenicity and immunogenicity. This protocol can serve as a guideline for efficient cluster 2.1 DTMUV inactivation, which is valuable for vaccine and immunoassay development.

Introduction

Duck Tembusu virus (**DTMUV**) is an emerging avian pathogenic flavivirus that causes severe neurological disorders and acute egg drop syndrome in ducks and some other avian species, including chicken and geese (Hamel et al., 2021). At present, DTMUV is widely spread and becomes one of the most economically important pathogens in ducks in Asia, resulting in massive economic losses to the duck producing industry (Hamel et al., 2021; Ninvilai et al., 2019; Qiu et al., 2021). To reduce the significant losses caused by DTMUV, the development of an

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effective DTMUV vaccine to control this emerging virus is urgently needed.

DTMUV is an enveloped, single-stranded, positive-sense RNA virus, which is classified as a new genotype of Tembusu virus (**TMUV**) belonging to the genus *Flavivirus* of the family *Flaviviridae* (Su et al., 2011; Thontiravong et al., 2015; Yun et al., 2012). Currently, DTMUV is genetically classified into three distinct clusters, including cluster 1, cluster 2 (subcluster 2.1 and 2.2), and cluster 3 (Ninvilai et al., 2019). All 3 clusters were found to be circulated in Asia with different geographical distributions (Ninvilai et al., 2019). Among them, cluster 2.1 is currently the predominant strain circulating in several Asian countries, including Thailand (Dong et al., 2024; Ninvilai et al., 2019; Zhou et al., 2023). Interestingly, recent studies revealed that, besides genetic divergence, significant antigenic difference was found among various clusters and subclusters of DTMUV, which potentially affect the cross-protection among DTMUV genotypes (Feng et al., 2020; Tunterak et al., 2023; Yu et al., 2022b). Therefore, the development of vaccine based upon virus genotypes circulating in specific geographical regions is necessary.

We have previously showed that cellular and humoral immune responses played a critical role in controlling virus replication and viral clearance in target organs during DTMUV infection in ducks (Thontiravong et al., 2022). However, both types of immune responses could not completely clear the virus from the brain, leading to severe neurological outcomes in the infected ducks (Ninvilai et al., 2020; Thontiravong et al., 2022). These raise the possibility that early priming of adaptive immune responses prior to infection may reduce viral spreading especially to the brain and thereby severe clinical outcomes. Therefore, vaccine platforms that can induce either viral-specific neutralizing antibodies or both types of adaptive immune responses prior to infection may be efficacious in protection against DTMUV infection in ducks. Inactivated vaccines are among the most widely used vaccine platforms in controlling and preventing viral infections in poultry, including DTMUV (Gao et al., 2020; Lin et al., 2015; Zhang et al., 2017). There are several factors contributing to the immunogenicity, safety, and efficacy of inactivated vaccines, one of which is virus inactivation (Pawar et al., 2015). Formalin and beta-propiolactone (**BPL**) are two of the most commonly used inactivating agents for the preparation of several avian vaccines (Lin et al., 2015; Pawar et al., 2015; Stauffer et al., 2006; Zhang et al., 2017). Compared to formalin, BPL has generally been shown to inactivate a variety of viruses more efficiently while better preserving antigenicity and inducing stronger immunogenicity (Astill et al., 2018; Yu et al., 2022a; Zhang et al., 2017). However, the optimal condition of BPL inactivation can vary with virus type and even the specific strain, suggesting that the inactivation procedure must be carefully optimized for each virus strain to achieve the effective inactivated vaccine (Herrera-Rodriguez et al., 2019; Pawar et al., 2015; Sasaki et al., 2016). Currently, no information is available on the optimal inactivation protocol for cluster 2.1 DTMUV, which predominantly circulates in several Asian countries, including Thailand (Dong et al., 2024; Feng et al., 2020; Ninvilai et al., 2019). Therefore, the objective of this study was to assess the effect of BPL inactivation on the infectivity, antigenic integrity, and immunogenicity of cluster 2.1 DTMUV.

Materials and methods

Virus

DTMUV strain DK/TH/CU-1 isolated from DTMUV-infected ducks in Thailand was used for evaluation in this study (Thontiravong et al., 2015). This virus is classified as DTMUV cluster 2.1, which is the predominant cluster of DTMUV circulating in several Asian countries, including Thailand (Ninvilai et al., 2019). The virus was propagated in baby hamster kidney (**BHK-21**) cells as described previously (Tunterak et al., 2021). Briefly, the cells were inoculated with DK/TH/CU-1 at multiplicity of infection (**MOI**) of 0.01. After absorption for 1 h at 37°C, Opti-MEM (Gibco, NY, USA) supplemented with 5 % fetal bovine serum (**FBS**) and antibiotics was added. Cell culture supernatants were collected, clarified by centrifugation, and then filtered through 0.45 μm filters. To enhance the virus titer for potential use in future inactivated vaccine development, the stock virus was concentrated using Amicon® Ultra centrifugal ultrafiltration devices (Amicon® Ultra 15 ml Centrifugal Filters 100 kDa) (Merck, Darmstadt, Germany) and stored at −80°C until further use. The virus titer was determined by conventional infectious virus titration in BHK-21 cells and expressed as $\log_{10}$ 50 % tissue culture infectious dose (**$TCID_{50}$**)/ml (Reed and Muench, 1983; Tunterak et al., 2021). Virus propagation and handling were performed in a BSL-2 containment facility.

BPL inactivation

To determine the appropriate BPL concentration and incubation time for DTMUV inactivation, DK/TH/CU-1 ($10^{6.33}$ $TCID_{50}$/ml) was treated with different concentrations of BPL (1:2000, 1:3000, and 1:4000 (vol/vol)) and subsequently incubated at 4 °C under constant stirring. The BPL-virus mixtures were collected at 24, 48, and 72 h after the addition of BPL followed by 2-hour incubation at 37 °C for hydrolysis of BPL. The inactivated viruses were immediately examined for virus infectivity in BHK-21 cells and stored at −80 °C for assessing antigenic integrity. An untreated virus suspension that was incubated at the same conditions served as the untreated virus control group.

Confirmation of virus inactivation

Infectivity of both untreated and treated viral samples was tested by cultivating in BHK-21 cells for three consecutive passages as described previously (Tunterak et al., 2021). Briefly, BHK-21 cells were inoculated with viruses treated with or without BPL and cultured at 37 °C for 5 days. Cells were then examined microscopically for the presence of cytopathic effect (**CPE**), followed by immunocytochemistry (**ICC**) staining with anti-flavivirus envelope (**E**) protein (clone D1-4G2-4-15, Merck Millipore, Billerica, MA). The complete inactivation of the viruses was confirmed by both the absence of CPE and DTMUV antigens in three rounds of passages in BHK-21 cells.

Assessment of antigenic integrity

To determine the effect of BPL on protein content and antigenic integrity, untreated and treated viral samples were examined by native polyacrylamide gel electrophoresis (**native PAGE**) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (**SDS-PAGE**) with Western blot analysis, respectively. For native PAGE, 5 μg of protein from untreated and treated viral samples were run on 3-12 % native PAGE Bis-Tris gel (Invitrogen, CA, USA) according to the manufacturer's instructions, using NativeMark Unstained Protein Standard (Invitrogen, CA, USA) as marker. The gels were then stained with Coomassie Blue dye (Thermo Fisher Scientific, DE, USA). For Western blotting, protein from each sample was separated by SDS-PAGE (Invitrogen, CA, USA) and then transferred onto polyvinylidene difluoride (**PVDF**) membrane for 7 min. DTMUV E protein was detected on membrane using mouse anti-flavivirus E protein as a primary antibody and horseradish peroxidase (**HRP**)-conjugated anti-mouse-IgG (Dako Cytomation, CA, USA) as a secondary antibody. The color was developed using Chemiluminescent HRP substrate (Merck Millipore, MA, USA). The protein band pattern and intensity were visualized and measured using ChemiDoc MP Imaging System and Image Lab 6.1 software (Bio-Rad, CA, USA). Densitometric analysis of each band was performed using Image Lab 6.1 software (Bio-Rad, CA, USA) and expressed as relative intensity to untreated viruses.

Duck immunization

The immunogenicity of BPL inactivated cluster 2.1 DTMUV, under the most suitable condition, was evaluated in Cherry Valley ducks. Immunogenicity assessment is typically based on measuring immune responses against target antigens or vaccines, including humoral and cellular immune responses (Janssens et al., 2022; Richard-Mazet et al., 2014). In this study, the viruses treated with BPL under the most appropriate condition was initially mixed with Montanide™ ISA 70 VG (SEPPIC, Paris, France) following the manufacturer's recommendation. DTMUV-negative ducks ($n = 5$) were subcutaneously administered with 0.5 ml of inactivated viruses at 7 and 21 days of ages. Another group of five ducks were inoculated with virus-free medium with adjuvant in the same fashion and served as the negative control group. Clinical signs were monitored and recorded daily until 28 days after the first immunization to assess the possible signs of toxicity. To determine DTMUV-specific neutralizing antibody and T cell responses elicited by BPL-inactivated viruses, serum sample were collected for serum neutralization (**SN**) test prior to immunization (day 0) and on days 3, 7, 14, 17, 21, and 28 after the first immunization. Meanwhile, heparinized whole blood samples were collected for DTMUV-specific T cell proliferation assay on days 0, 7, 14, 21, and 28 after the first immunization. The animal experiment was performed in the ABSL-2 containment facility at Chulalongkorn University Laboratory Animal Center (**CULAC**) and was conducted under the approval of Chulalongkorn University Animal Care and Use Committee (Animal use protocol number 2273028).

Serum neutralization (SN) test

The presence of DTMUV-specific neutralizing antibodies in serum samples was determined by SN test using BHK-21 cells as previously described (Tunterak et al., 2018). In brief, triplicate serial two-fold dilutions of heat inactivated sera were incubated with 100 $TCID_{50}$ of DK/TH/CU-1 for 1 h at 37 °C. Following the incubation, the virus-serum mixture was added into a 96-well plate containing BHK-21 cells. The cells were then incubated at 37°C and were examined for the presence of CPE daily for 5 days. Reference DTMUV seropositive and negative sera, uninfected BHK-21 cells and back titration of used virus served as controls. DTMUV-specific neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution that inhibited CPE.

DTMUV-specific T cell proliferation assay

To determine the DTMUV-specific T cell responses induced by BPL-inactivated viruses, peripheral blood mononuclear cells (**PBMC**) obtained from ducks immunized with BPL-inactivated cluster 2.1 DTMUV and from negative control ducks were tested by DTMUV-specific T cell proliferation assay, as described previously with some modifications (Bertram et al., 1997; Nedumpun et al., 2019; Thontiravong et al., 2022). Briefly, PBMC were isolated from heparinized whole blood samples by density gradient centrifugation using Isoprep separation medium (Robbins Scientific Co., CA, USA) according to the manufacturer's protocol. The cells were seeded at a concentration of 2×10^5 cells/well in 96-well plate and cultured in RPMI-1640 medium containing 10 % FBS in the presence of 10 μM Bromodeoxyuridine (**BrdU**) (BioLegend®, CA, USA). The BrdU-cultured cells were then stimulated with homologous DTMUV antigen (DK/TH/CU-1; MOI = 0.1) or the complete RPMI medium alone as an unstimulated control at 37°C in a 5 % CO_2 incubator for 96 h. The cells were harvested and subjected to surface immunofluorescent staining for duck $CD4^+$ and $CD8^+$ *T* cells as described previously (Thontiravong et al., 2022). Following the cell surface staining, the stained cells were fixed and permeabilized using Fixation/Permeabilization Kit (BD Biosciences, CA, USA) and then incubated with PE anti-BrdU monoclonal antibody (BioLegend, CA, USA) diluted in BD cytoperm™ (BD Biosciences, CA, USA) at 4°C for 30 min under the dark condition. The stained cells were run through a CytoFLEX LX (Beckman Coulter, CA, USA) and analyzed with FlowJo software (Tree Star Inc., OR, USA). The percentages of proliferating T helper (Th) and cytotoxic T lymphocytes (CTL) were obtained by gating on $CD3^+CD4^+BrdU^+$ and $CD3^+CD8^+BrdU^+$ subpopulations, respectively. To analyze proliferative data obtained from flow cytometry, unstimulated PBMCs were used for background subtraction in each sample.

Statistical analysis

Data were analyzed using a two tailed, unpaired Student's *t*-test. Correlation was evaluated by Pearson's correlation analysis. All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software Inc., CA, USA). All P-value (**P**) < 0.05 was considered statistically significant.

Results

Effect of BPL inactivation on virus infectivity and antigenic integrity

To determine the effect of various conditions of BPL on virus infectivity, cluster 2.1 DTMUV treated with BPL at different concentrations (1:2000, 1:3000, and 1:4000) and incubation times (24, 48, and 72 h) was examined for virus infectivity by virus isolation and ICC staining in BHK-21 cells for 3 passages. Our results demonstrated that all conditions of BPL treatment could completely inactivate cluster 2.1 DTMUV even at the lowest concentration (1:4000) and shortest incubation time (24 h), as evidenced by the absence of CPE and DTMUV antigens for 3 passages in BHK-21 cells (Fig. 1; Table 1). In addition to complete virus inactivation, the preservation of antigenic integrity after treatment with BPL is required for efficient inactivated vaccine development. The protein content and antigenic integrity of inactivated viruses following BPL treatment at various conditions were examined by native PAGE and SDS-PAGE with Western blot analysis, respectively. Our results showed that the lowest BPL treatment concentration (1:4000) combined with the shortest incubation time (24 h) was most effective in maintaining the total protein content, as evidenced by band intensities in native-PAGE and SDS-PAGE (Fig. 2A-D). Furthermore, based on the band intensities in Western blotting, this treatment also best preserved the antigenic integrity of cluster 2.1 DTMUV compared to other conditions (Fig. 2E and F). These results collectively suggested that a concentration of 1:4000 (vol/vol) of BPL with shaking at 4 °C for 24 h seemed to be the most appropriate condition for efficient cluster 2.1 DTMUV inactivation without a significant loss of antigenicity.

Assessment of the immunogenicity of BPL-inactivated cluster 2.1 DTMUV in ducks

To evaluate the safety and immunogenicity of BPL-inactivated cluster 2.1 DTMUV in ducks, cluster 2.1 DTMUV treated with BPL under the most suitable condition (1:4000, 24 h) was administered to Cherry Valley ducks, and the level of DTMUV-specific neutralizing antibodies and the percentages of proliferating DTMUV-specific Th ($CD3^+CD4^+BrdU^+$) and CTL ($CD3^+CD8^+BrdU^+$) responses were subsequently determined. Following the first immunization, neutralizing antibodies against DTMUV were initially detected at 7 days post immunization in 60 % (3/5) of ducks and the titers gradually increased significantly at a later time point (14 days post first immunization) in all ducks ($P < 0.05$) (Fig. 3A). The second immunization induced a significant increase in neutralizing antibody titers, as measured on days 21 and 28 (7- and 14-days post second immunization) ($P < 0.05$), indicating an effective booster response (Fig. 3A). DTMUV-specific neutralizing antibodies were maintained at a high level until the end of the observation period (Fig. 3A). No neutralizing antibodies against DTMUV were observed in any of the ducks before immunization and in

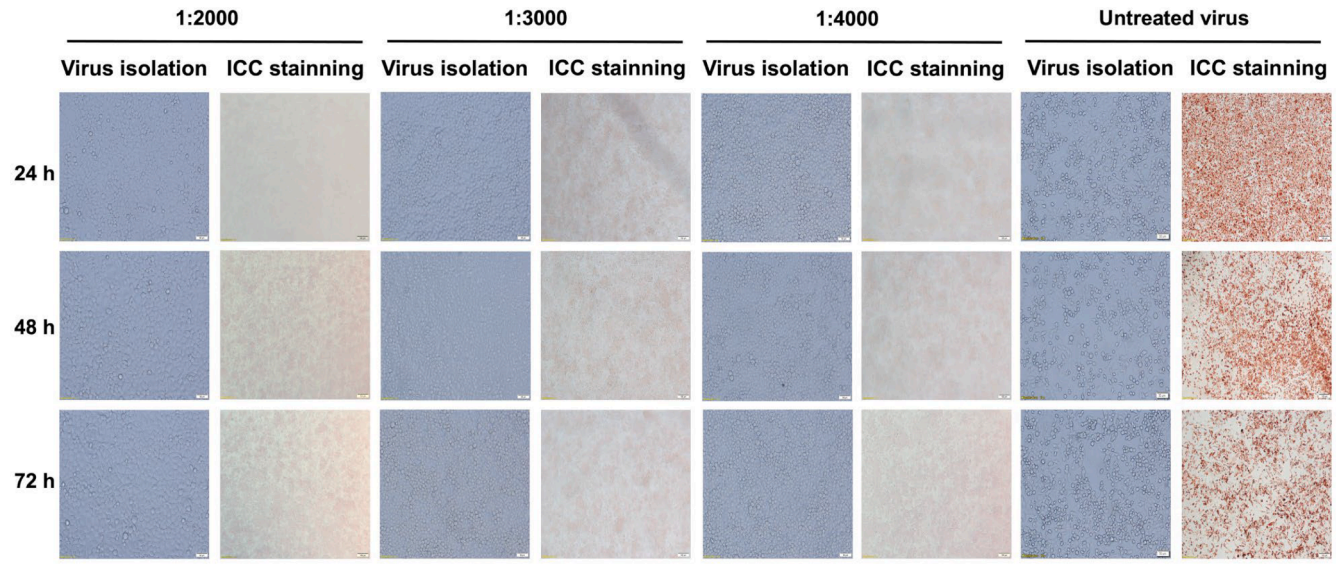

Fig. 1. Confirmation of cluster 2.1 duck Tembusu virus (DTMUV) inactivation by beta-propiolactone (BPL) at various conditions in BHK-21 cells. The cells were inoculated with viruses treated with or without BPL at different conditions (1:2000, 1:3000, and 1:4000 (vol/vol); 24, 48, and 72 h of incubation) and incubated for 5 days. Cells were then examined for the presence of cytopathic effect (CPE), followed by immunocytochemistry (ICC) staining with anti-flavivirus E protein. The absence of CPE and DTMUV antigens after three rounds of passages in baby hamster kidney (BHK-21) cells indicated complete virus inactivation.

Table 1
Comparison of the effect of concentrations and incubation times of beta-propiolactone (BPL) on cluster 2.1 duck Tembusu virus (DTMUV) infectivity.

BPL inactivation condition		Virus isolation[1]			Inactivated (Y/N)[3]
Concentration (vol/vol)	Incubation time (h)	1st passage	2nd passage	3rd passage	
1:2000	24	–[2]	-	-	Y
	48	-	-	-	Y
	72	-	-	-	Y
1:3000	24	-	-	-	Y
	48	-	-	-	Y
	72	-	-	-	Y
1:4000	24	-	-	-	Y
	48	-	-	-	Y
	72	-	-	-	Y

[1] DTMUV infectivity was determined by virus isolation and immunocytochemistry (ICC) staining in baby hamster kidney (BHK-21) cells for three passages.
[2] (-) indicates no cytopathic effect (CPE) and DTMUV antigen observed during passaging in BHK-21 cells.
[3] Y, complete inactivated condition; N, incomplete inactivated condition.

negative control group. Additionally, DTMUV-specific Th and CTL responses elicited by immunization with BPL-inactivated cluster 2.1 DTMUV were further evaluated by DTMUV-specific T cell proliferation assay. Our result showed that the frequency of proliferating DTMUV-specific Th lymphocytes in peripheral blood of ducks immunized with BPL-inactivated cluster 2.1 DTMUV significantly increased on day 21 (7 days post second immunization) ($P < 0.05$) and remained elevated through day 28 (14 days post second immunization) (Fig. 3B). However, BPL-inactivated cluster 2.1 DTMUV did not appear to induce a significant DTMUV-specific CTL response (Fig. 3C). We also observed a significant positive correlation between proliferating Th lymphocyte frequency and neutralizing antibody titers (Pearson correlation coefficient (***r***) ($r = 0.889$, $P = 0.048$), whereas no significant correlation was detected between the frequency of proliferating CTL and the levels of neutralizing antibody ($r = 0.545$, $P = 0.342$). Altogether, these findings indicate that BPL-inactivated cluster 2.1 DTMUV elicits robust neutralizing antibody and specific Th lymphocyte responses in immunized ducks. It should be noted that all ducks from both groups remained healthy, showing no clinical signs throughout the observation period. Taken together, these findings indicated that cluster 2.1 DTMUV, inactivated with BPL at a concentration of 1:4000 (vol/vol) for 24 h, was safe and immunogenic in ducks, confirming that this condition was optimal for cluster 2.1 DTMUV inactivation.

Discussion

Recent studies demonstrated a marked antigenic difference among various clusters and subclusters of DTMUV, which may affect the cross-protection among DTMUV genotypes (Feng et al., 2020; Tunterak et al., 2023; Yu et al., 2022b). Therefore, the development of vaccine based upon virus genotypes circulating in specific geographical regions is essential. Inactivated vaccines are among the most widely used vaccine platforms in controlling and preventing viral infections in poultry, including DTMUV (Gao et al., 2020; Lin et al., 2015; Zhang et al., 2017). Virus inactivation is one of the most crucial steps in the development of inactivated vaccine. BPL is commonly used as an inactivating agent for avian vaccine production (Pawar et al., 2015; Zhang et al., 2017). However, the condition of BPL inactivation must be optimized for each virus strain to achieve the efficient inactivation and protection of antigenicity. No information is available on the optimal inactivation protocol for cluster 2.1 DTMUV, which predominantly circulates in several Asian countries, including Thailand (Dong et al., 2024; Feng et al., 2020; Ninvilai et al., 2019). In the present study, we established the optimal protocol of BPL for efficient inactivation and preservation of the antigenicity and immunogenicity of a cluster 2.1 DTMUV. Our results revealed that BPL at a 1:4000 dilution with 24 h of incubation appeared to be the most suitable condition for the complete inactivation of cluster 2.1 DTMUV without compromising its antigenicity and immunogenicity. To the best of our knowledge, this is the first study to establish an optimal BPL protocol for inactivating cluster 2.1 DTMUV.

Our results demonstrated that BPL at a concentration $\geq$ 1:2000 dilution with $\geq$ 24 h of incubation could completely inactivate a cluster 2.1 DTMUV, which was in line with those observed for cluster 2.2 DTMUV (Zhang et al., 2017). However, apart from complete inactivation, the preservation of antigenicity after inactivation is required for efficient inactivated vaccine development. In this study, we found that the lowest concentration (1:4000) and the shortest incubation time (24 h) of BPL treatment could preserve both the total protein content and the

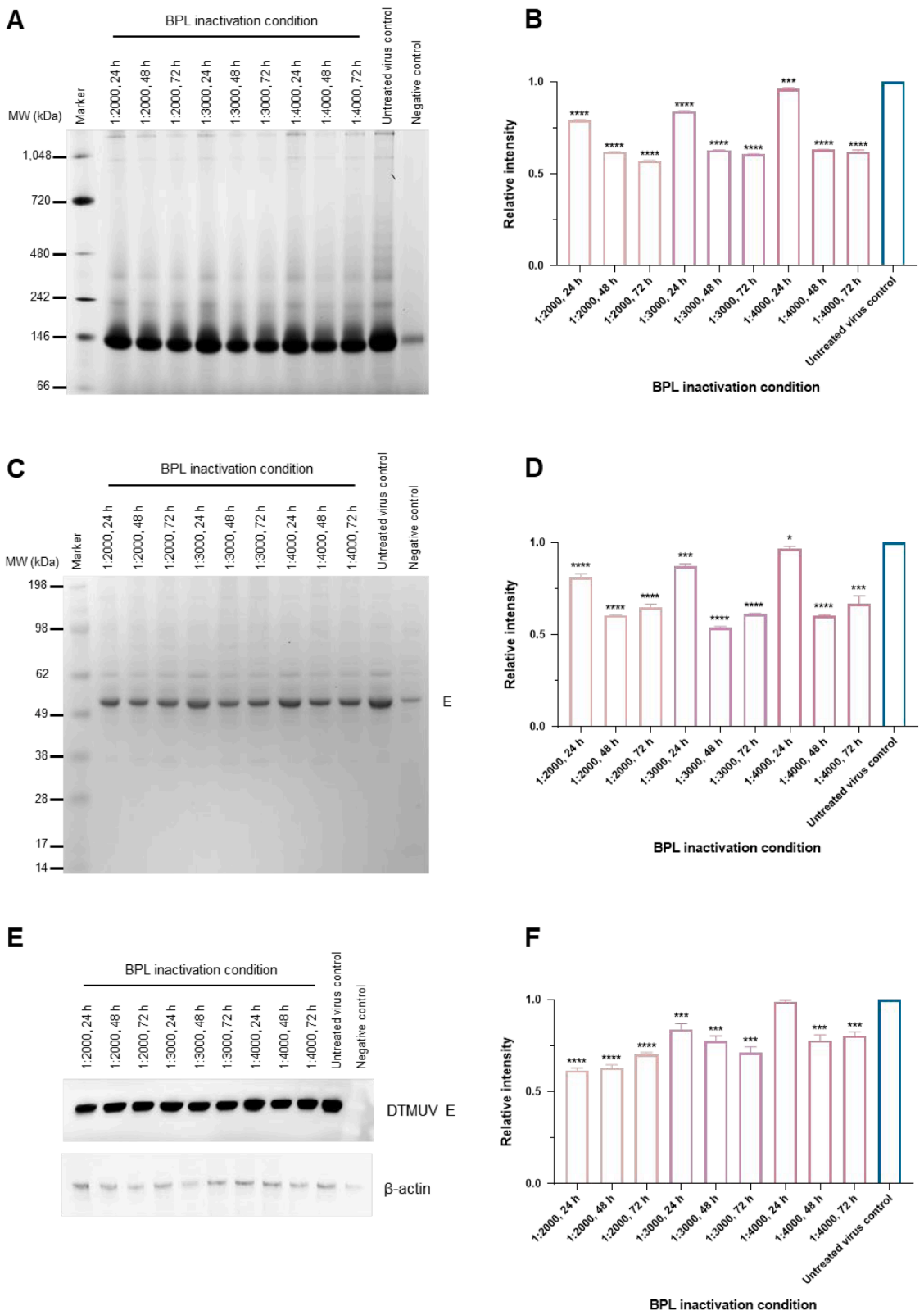

Fig. 2. Assessment of the effect of beta-propiolactone (BPL) treatment on protein content and antigenic integrity of cluster 2.1 duck Tembusu virus (DTMUV). (A) Total protein bands and (B) relative protein content in the viral samples treated with BPL at various conditions on native PAGE. (C) Total protein bands and (D) relative protein content in the viral samples treated with BPL at various conditions on SDS-PAGE. (E) Western blot analysis and (F) relative intensity of DTMUV E protein from the viral samples treated with or without BPL at various conditions. Densitometric analysis of protein bands from native PAGE, SDS-PAGE and Western blotting was performed using Image Lab 6.1 software (Bio-Rad, CA, USA) and expressed as relative intensity to untreated virus control. Data were obtained from three independent experiments and are expressed as mean ± SD. P values were determined by two tailed, unpaired Student's *t*-test. *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ compared to untreated virus control.

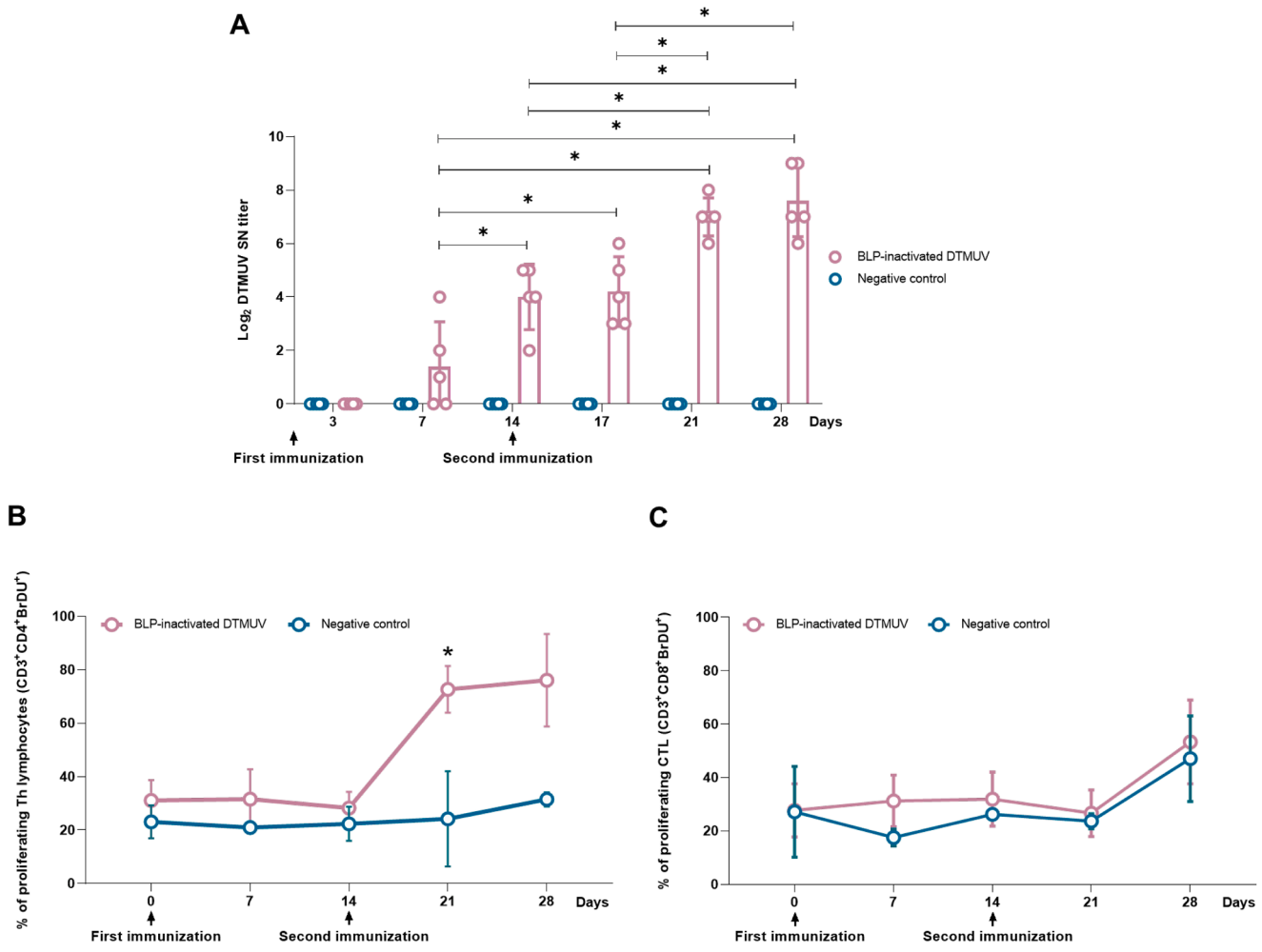

Fig. 3. Humoral and cellular immune responses elicited by immunization with beta-propiolactone (BPL)-inactivated cluster 2.1 duck Tembusu virus (DTMUV) in ducks. (A) Neutralizing antibody responses against DTMUV in ducks immunized with BPL-inactivated cluster 2.1 DTMUV. Ducks were administered with 2 doses of BPL-inactivated cluster 2.1 DTMUV at 2 weeks apart. Serum samples were collected at the indicated times post immunization, and DTMUV-specific neutralizing antibody titers were determined by serum neutralization (SN) test. (B-C) The percentages of proliferating DTMUV-specific T helper (Th) ($CD3^+CD4^+BrdU^+$) (B) and cytotoxic T lymphocytes (CTL) ($CD3^+CD8^+BrdU^+$) (C) in the peripheral blood of ducks immunized with BPL-inactivated cluster 2.1 DTMUV and negative control ducks following ex vivo stimulation with homologous DTMUV antigen. Each data point represents the mean ± standard deviation (SD) of 5 ducks. Asterisks (*) indicate statistically significant differences between time points (for SN test) or between BPL-inactivated cluster 2.1 DTMUV immunized and negative control groups (for DTMUV-specific T cell proliferation assay) ($P < 0.05$, two tailed, unpaired Student's *t*-test).

antigenic integrity of cluster 2.1 DTMUV more effectively than other conditions. Consistent with other viruses (Chida et al., 2021; Gupta et al., 2021; Sasaki et al., 2016;Yu et al., 2022a), we observed that higher BPL concentration with longer incubation time negatively impacted the antigenic integrity of cluster 2.1 DTMUV, thereby potentially affecting the immunogenicity of the viral antigen. Concurringly, previous studies showed that high concentration and long incubation time (>24 h) of BPL caused aggregation of viral particles, disruption of virus structure and loss of viral protein content and antigenic integrity (Chida et al., 2021; Gupta et al., 2021; Sasaki et al., 2016; Yu et al., 2022a). Overall, our findings suggest that a 1:4000 dilution of BPL with 24 h of incubation was the optimal condition for inactivation of cluster 2.1 DTMUV based on a balance between complete inactivation and maintenance of antigenic properties.

Supporting the *in vitro* findings, cluster 2.1 DTMUV treated with BPL at a 1:4000 dilution for 24 h was shown to be safe and highly immunogenic in ducks. Our results demonstrated that cluster 2.1 DTMUV inactivated with BPL under this condition was able to elicit high levels of DTMUV-specific neutralizing antibodies in all immunized ducks, with titers increasing significantly after the second immunization. Compared to previous findings where DTMUV-specific neutralizing antibody titers induced by viable DTMUV were negatively correlated with viral loads in target organs (Thontiravong et al., 2022), a similar or higher level of neutralizing antibodies against DTMUV was detected in ducks immunized with BPL-inactivated cluster 2.1 DTMUV. In addition to the strong humoral response elicited by BPL-inactivated cluster 2.1 DTMUV, we also observed the expansion of DTMUV-specific Th lymphocyte subpopulation in the peripheral blood of ducks immunized with this BPL-inactivated DTMUV. This expansion significantly correlated with neutralizing antibody titers, suggesting the supportive role of Th lymphocytes in antibody production. In line with most inactivated vaccines (Hoft et al., 2011; Hopf et al., 2016), no significant DTMUV-specific CTL proliferation was observed after BPL-inactivated cluster 2.1 DTMUV immunization. These data suggest that the T cell responses elicited by this BPL-inactivated cluster 2.1 DTMUV are mostly limited to DTMUV-specific Th lymphocyte response, which potentially supports the production of DTMUV-specific neutralizing antibodies. These observations were in accordance with previous studies showing that inactivated vaccines against other flaviviruses predominantly generate a Th lymphocyte response, which have been shown to positively correlate with virus-specific antibody titers (Friberg et al., 2020; Lima et al., 2021; Nayak et al., 2013). We also previously showed that the Th lymphocyte

response significantly correlated with the control of DTMUV replication in infected ducks (Thontiravong et al., 2022). Altogether, these findings indicate that cluster 2.1 DTMUV inactivated with BPL under this condition is probably capable of enhancing protective humoral and Th lymphocyte responses against DTMUV in ducks. However, further studies are required to evaluate the protective efficacy of an inactivated vaccine produced following this protocol against DTMUV infection in ducks.

In conclusion, our data collectively suggests that a 1:4000 dilution of BPL with 24 h of incubation is the optimal condition for complete inactivation of cluster 2.1 DTMUV, while retaining its antigenicity and immunogenicity. This protocol can serve as a guideline for the efficient inactivation of cluster 2.1 DTMUV, which is valuable for the development of immunoassays and inactivated vaccines against cluster 2.1 DTMUV.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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