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臺灣鵝源坦布蘇病毒之培養、力價測定與對雛雞之病原性研究

Culture, Titration and Pathogenicity in Chicks of Goose Tembusu Virus Isolated in Taiwan

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中文摘要

坦布蘇病毒，又稱為鴨隻產蛋下降症候群病毒，為一種具封套的正向單股核糖核酸病毒，屬於黃病毒科之黃病毒屬。受感染的鴨隻會出現產蛋急遽下降、進食量下降、生長遲緩與共濟失調、跛行和漸進性癱瘓等神經症狀。近年來，部分國家報告了鴨隻來源之坦布蘇病毒引起的傳染病爆發案例，並在這些地區中受影響的產蛋鴨養殖場造成嚴重的經濟損失。本研究使用 2020 年在台灣所鑑定與分離之鵝源坦布蘇病毒株 NTU/C225/20，以胚胎蛋增殖，進一步將病毒濃縮、測定力價，再感染 1 日齡的無特定病原小雞以進行致病性分析。結果顯示，鵝源坦布蘇病毒株 NTU/C225/20 能生長在最少病原鴨胚胎、無特定病原雞胚胎與 DF-1 細胞株上，並能加以定量，當坦布蘇病毒株 NTU/C225/20 以 10^3 PFU 的劑量透過肌肉注射時，對 1 日齡小雞具有致病性，造成的臨床症狀主要包含生長遲緩、體溫升高和活力降低。肉眼病變主要為肝臟腫大、胸腺充血和脾臟腫大。然而，使用針對 NS5 基因序列增幅之反轉錄聚合酶連鎖反應（RT-PCR）未在實驗小雞的血清和組織（腦、心、肝及脾）中偵測到病毒核酸。本研究為首次針對台灣所分離的坦布蘇病毒株進行接種試驗，未來期盼更進一步探索坦布蘇病毒株 NTU/C225/20 在家禽的致病情形。

Abstract

Tembusu virus (TMUV), also called duck egg drop syndrome virus, is an enveloped, positive-sense, single-stranded RNA virus belonging to the genus *Flavivirus* of the Family *Flaviviridae*. The infected ducks were characterized by heavy egg drop, feed uptake decline, growth retardation, and neurological signs, including ataxia, lameness, and progressive paralysis. In recent years, the infectious disease outbreaks caused by duck TMUV have been reported in some countries and led to severe economic loss on the affected egg-laying duck farms in these areas. In this study, NTU/C225/20, a goose-origin TMUV strain identified and isolated in Taiwan in 2020, was propagated in minimal-pathogen-free duck embryos, specific-pathogen-free (SPF) chicken embryos, and the DF-1 cell line. Following concentration and titration, the virus was further used to infect day-old SPF chicks for pathogenicity analysis. The results revealed that TMUV NTU/C225/20 exhibits pathogenicity in the day-old chicks via intramuscular inoculation (10^3 PFU per chick), characterized by growth retardation, hyperthermia, and slight reduction of activity. Gross lesions in infected chicks were characterized chiefly by hepatomegaly, hyperemic thymus, and splenomegaly. However, none of the sera and selected tissues (brains, hearts, livers, and spleens) were detected positive for TMUV by the NS5-specific RT-PCR assay. This is the first study to investigate the pathogenicity of Taiwan TMUV in chicks. More information on diseases caused by Taiwan TMUV in poultry awaits further investigation.

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Chapter 1 Introduction

Tembusu virus (TMUV), also called duck egg drop syndrome virus, is an enveloped, positive-sense, single-stranded RNA virus belonging to the Ntaya virus group in the genus *Flavivirus* of the Family *Flaviviridae*. As an arthropod-borne flavivirus, TMUV was originally isolated from mosquitoes of the genus *Culex* in peninsular Malaysia in 1955 (Platt et al., 1975). Since 2010, it has been reported that TMUV could also cause infection in ducks (Yan et al., 2011), geese (Yun et al., 2012), chickens (Chen et al., 2014), house sparrows (Tang et al., 2013a), mice (Li et al., 2013), humans (Olson et al., 1983; Tang et al., 2013b), etc. In addition to the spread by mosquitoes, further studies showed possible evidence of contact, aerosol, and vertical transmission routes of TMUV among ducks and ducklings (Li et al., 2015; Zhang et al., 2015). The isolation of TMUV from different natural hosts suggests cross-species transmission might exist. The infectious disease outbreaks caused by duck TMUV had been reported to occur in southeast China (2010), Malaysia (2012), and Thailand (2013), leading to severe economic loss in the affected egg-laying duck farms. Regarding the clinical signs and symptoms, the infected ducks were characterized by heavy egg drop, feed uptake decline, growth retardation, and neurological signs, including ataxia, lameness, and progressive paralysis. Based on statistical results from the disease in China in 2010, infection morbidity in ducks was up to 90%, and mortality ranged from 5 to 15% while occasionally increasing up to 30% due to secondary bacterial infections (Su et al., 2011; Zhang et al., 2017).

In 2019, Tembusu virus strain TP1906 (TMUV-TP1906) was identified and isolated from *Culex annulus* mosquitoes collected from the northern part of Taiwan (Peng et al., 2020). It was the first time that TMUV was found to exist in Taiwan. Analysis of the nucleotide sequence of TP1906 revealed high similarity with Sitiawan virus, which causes encephalitis, growth retardation, and increased blood glucose levels in inoculated chicks (Kono et al., 2000). Concurrently, a TMUV

1080905 isolate was identified from a diseased Pekin duck flock, which is the first report of duck-derived TMUV in Taiwan (Chen et al., 2022). The sequencing of the complete polyprotein gene exhibited 99.3% nucleotide similarity and 99.7% amino acid similarity with mosquito-derived TP1906. The phylogenetic analysis revealed that the Taiwanese TMUVs were grouped with Malaysian TMUVs (chicken-derived Sitiawan virus and TMUV prototype strain MM1775). Next year, TMUV nucleic acid was detected positive in a Roman geese flock in the southern part of Taiwan. TMUV was isolated using minimal-pathogen-free duck embryos successfully, and a novel TMUV strain NTU/C225/20 from geese in Taiwan was then designated and reported, which also shares a high nucleotide identity with strain MM1775 (Chen et al., 2021).

As discussed above, TMUV exhibits a wide range of host species, including ducks, geese, chickens, etc. Currently, various host systems, including different embryonated eggs (duck and chicken) and cell cultures were evaluated and compared for their ability to support duck TMUV isolation and replication in the recently published research. The results showed that duck TMUV yielded higher titers in primary DEF (duck embryo fibroblast) cells, BHK-21 (baby hamster kidney) cells and, embryonated duck eggs than in the other host systems tested. With several methods established, we attempted to multiply TMUV strain NTU/C225/20 on minimal-pathogen-free duck embryos and specific-pathogen-free (SPF) chicken embryos in this study.

Given the duck TMUV outbreaks occurred in neighboring countries and the potential risk of viral adaption to other hosts, investigating the virulence of strain NTU/C225/20 in different animals is required. *In vivo* pathogenicity of various TMUV strains in ducks had been described in some research. However, similar studies in chickens were seldom reported, which may be associated with the absence of devastating TMUV-related epidemic diseases in chicken layer farms so far. The aim of this study was to propagate TMUV strain NTU/C225/20 in embryonated duck eggs and to investigate its pathogenicity in young chickens using an SPF chick infection model.

Chapter 2 Materials and methods

2.1 Cells and viruses

DF-1 cells (chicken embryo fibroblasts; ATCC; CRL-12203) used in this study were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin-amphotericin B solution (PSA). The cells were grown and maintained at 37 °C in a 5% CO_2 humidified incubator.

The Tembusu virus (TMUV) strain used in this study was originally isolated from outbreaks of TMUV-related disease in a flock of 45-day-old Roman geese in southern Taiwan in 2020, characterized by white diarrhea, depression, lameness, prostrate and increased mortality. Viral confirmation was carried out using RNA extraction, TMUV-specific reverse transcription-polymerase chain reaction (RT-PCR) techniques and full-length genome sequencing. The virus strain was certainly isolated using minimal-pathogen-free duck embryos and it was designated as NTU/C225/20 (GenBank accession no. MW821486.1).

2.2 Virus propagation

For virus infection in animals in the later section of this study, enough volume and titer of virus stock were essential to be prepared. The virus is able to replicate during the infection process in target host embryos, and it's also one of the methods of how laboratories produce huge stocks of viruses. Large amounts of viable duck eggs were adopted to make the virus grow and produce themselves inside in this study. The embryonated duck eggs at 13^{th} day of the 28-day embryonation period were disinfected with iodine on the injection site and inoculated with 0.2 mL TMUV strain NTU/C225/20 per egg via the allantoic route. The eggs were sealed and set on incubation at 37 ℃ in the hatchery for 3 days. Embryonic viability was observed daily, and eggs found dead would be

maintained at 4 ◦C until viral harvesting to avoid viral degradation. Embryos that died within 24 hours after inoculation were discarded because they were considered to have a non-specific cause of death, mostly due to bacterial contamination during inoculation. The inoculated eggs that maintained the viability until the 3rd day post-inoculation were euthanized by keeping at 4 ℃ overnight. Ultimately, for both eggs that died during incubation and those that were euthanized, syringe aspiration of allantoic fluid was performed to harvest the virus. The collected fluid would be propagated into another egg for additional passages.

2.3 Virus titration by EID_{50}

Functional titer, also known as the measurement of how much virus infects a target, of TMUV strain NTU/C225/20 suspension used for the following experiments was measured by the EID_{50} (egg infectivity dose 50%) method, the amount of virus that would infect 50 percent of inoculated eggs. Due to the evidence of NTU/C225/20 infection in chicken embryos, in this part, both 12-day-old minimal-pathogen-free embryonated duck eggs and 10-day-old SPF embryonated chicken eggs were used to test by EID_{50} for obtaining which type of host embryos had the higher virus titer. The obtained result might indicate a better embryonated host system for virus replication in the future. Serial ten-fold dilutions of the NTU/C225/20 stock virus were prepared in PBS, and 0.l mL of each dilution was inoculated into allantoic cavities of embryonated eggs. The volumes of virus suspension injected into each egg and the injection sites have to be accurate in the infectivity titration assay. Another group of eggs was injected with the same volume of PBS and used as a control group. Embryonic viability was observed daily, and eggs found dead would be maintained at 4 °C until the last day of the EID_{50} assay. Embryos that died due to bacterial contamination within 24 hours after inoculation were excluded in the calculation and discarded. After incubation at 37 ℃ for one week, observing gross morphology of the embryos was applied as the main approach to identify an infection in each egg. The TMUV-specific RT-PCR techniques with the allantoic fluid in

eggs could be used to detect TMUV nucleic acid, which assisted in determining whether there's an infection or not. Subsequently, the NTU/C225/20 EID_{50} was calculated through numbers of infective eggs by using the Reed-Muench method.

2.4 Virus concentration

The virus harvested in embryonated duck eggs didn't show high enough infectivity to carry out an animal experiment and the obstacles had to be overcome. Instead of continuing the replication and serial passage in duck embryos, the concentration of viral particles was attempted to increase virulence. Currently, there are many concentration approaches for producing high titer viral stocks, and the most common one is ultracentrifugation. However, factors such as viral particle size, viral viability, and sample volume are required to consider to make the correct choice of centrifugation speed, centrifugation time, buffer composition, and other applied methods. The knowledge of the condition is still remarkably poor due to a limited number of related studies on Tembusu virus. Thus, the protocols used in this study mainly referred to those of Dengue virus, which also belongs to *Flaviviridae*.

Ultracentrifugation is frequently used to concentrate viruses from water matrices. In the initial attempt of this study, virus replicates were concentrated by ultracentrifugation at 50,000 × g for 1.5 hours and 4 hours, respectively, at 4 °C layered with a 20% sucrose cushion. After the supernatant was discarded, the virus pellet was gently resuspended with DMEM (20% FBS, 1% PSA) to make a 10-time concentrated stock and stored at -80 ºC. The method to confirm whether the Tembusu virus particles were successfully collected or not was performing a plaque assay.

Another adopted technique to concentrate the virus was polyethylene glycol (PEG) precipitation according to Renner et al (2021) (Renner et al., 2021). Cell-free virus supernatants and 40% PEG-6000/PBS solution (w/v) were respectively sterilized by passing 0.45 um filters and then

mixed to a final PEG concentration of 8% (The PEG should be freshly prepared). The mixture was stored overnight at 4 °C with gentle shaking before centrifugation at 12,000 × g for 30 min at 4 °C. Pelleted viral particles were resuspended in sterile PBS buffer (about 1 mL, based on the pellet size), and cleared by centrifugation at 12,000 × g, 10 min, 4 °C. The pellet was resuspended in sterile DMEM (20% FBS, 1% PSA) buffer and kept overnight at 4 °C.

2.5 Virus titration by plaque assay

Plaque assays remain one of the most accurate methods for the direct quantification of infectious virions and antiviral substances through the counting of discrete plaques (infectious units and cellular dead zones) in cell culture (Baer and Kehn-Hall, 2014). To evaluate the efficiency of virus concentration methods applied in the replicated NTU/C225/20, the plaque assays were performed to confirm whether the virus titer increased after each concentration attempt. Plaque assays represent one of the most standard methods for the direct quantification of infectious viruses The titer of a virus stock can be calculated in plaque-forming units (PFU) per milliliter.

Before the assay, DF-1 cells were prepared as plate-appropriate host cells for strain NTU/C225/20 at 90-100% confluency in a 12-well cell culture plate. Using the cellular growth media as the diluent to perform a 10-fold serial dilution of the virus. From the serially diluted virus suspension, 0.5 mL of each dilution, a sufficient but low volume of inoculum to cover cells, was then used to infect the cells per well for 1 hour. Gently rock plates every 15 minutes to ensure even coverage. After the infection, the virus suspension was removed and the cells were washed with PBS twice. For an agarose overlay, a 1:1 mixture of warmed 2x cellular growth media and a stock solution of heated 2% low-melting agarose solution was prepared and placed in a 37 °C water bath. Until remained at a proper temperature, the agarose mixture was applied the overlay to the monolayer. When the agarose cooled down and solidified (about 20-30 min), the cells were placed

on incubation at 37 ºC for plaque formation, which could take 3-8 days. Ultimately, fixing the plates with 10% formalin at room temperature for 1 hour and staining the cells with 1% (w/v) crystal violet for 10-20 minutes. Decant the staining solution and gently wash off the crystal violet stain with running water. Once the plate was fixed, stained, and dried, plaques could then be counted to obtain a virus titer.

2.6 Experimental infection of chicks

The infection trials were conducted following the animal welfare guidance and under the approval of the Institutional Animal Care and Use Committee (IACUC) directed by the National Taiwan University (Approval ID: NTU-110-EL-00066). In this part, eighteen 1-day-old chicks were divided into the inoculated group (n=6), the contact group (n=6) and the control group (n=6). For the inoculated group, each chick was intramuscularly injected with 0.03 mL (10^3 PFU) of TMUV NTU/C225/20, and the contact group was housed with the inoculated group. Spirit, appetite, rectal temperature, and body weight were observed and measured daily. Each group of chicks was housed in continuously lit rooms with food and water freely available. On 7- or 14-days post-infection (dpi), blood samples were collected from individuals in each group. Gross lesions were recorded, and organs including brains, hearts, livers, spleens, lungs, kidneys, duodenums, pancreas, thymi, and bursa of Fabricius were harvested. Afterward, blood samples were centrifuged at 3,000 × g for 10 minutes to obtain sera samples, which were then stored at -80 °C for detection of viremia. Tissue samples were also homogenized and collected in DMEM medium (10% FBS, 1% PSA), then stored at -80 °C for the following viral detection.

2.7 Viral RNA extraction and RT-PCR

To make a rapid detection of TMUV infection in the experimental chicks, total RNA was extracted from the prepared chick sera and tissues by using the Total RNA Mini Kit (Geneaid

Biotech Ltd., Taiwan). The performance requires 200 μL of each sample, in which the sera was used only 50 μL and diluted with PBS. The obtained RNA products were stored at -80 °C and part of it was followed with RT-PCR analysis. In a process of PCR, the TMUV-specific sequences were amplified by primers directed against the NS5 gene of Flaviviruses (Forward: 5' –TTT GGT ACA TGT GGC TCG; Reverse: 5' –ACT GTT TTC CCA TCA CGT CC) (Liu et al., 2012). The one-step RT-PCR was performed with the Phire system, consisting of 4 μL of 5× Phire® Reaction Buffer, 3 μL of 2.5 μM dNTP, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 0.5 μL of Phire® Hot Start II DNA Polymerase, 0.1 μL of MMLV reverse transcriptase, 0.1 μL of Ribonuclease Inhibitor, 3 μL RNA products and 7.3 μL H_2O in a 20 μL reaction. Thermal cycling conditions were 40 ºC for 30 min, 98 ºC for 30 s, 35 cycles at 98 ºC for 5 s, 50 ºC for 5 s, and 72 ºC for 10 s. For identifying targets, RT-PCR products were loaded on 2% agarose gels, which were stained with SYBR® Safe DNA gel stain, in 1× TBE (Tris-Borate-EDTA) buffer and were subjected to electrophoresis at 100V for approximately 30 minutes. In the end, using luminescence to exploit the length difference of amplicons. Genome sequencing of positive RT-PCR products was performed to further identify TMUV nucleic acid.

2.8 Statistical analysis

Data derived from animal experiments were calculated as means ± standard error of the mean (SEM). Differences in body weight and body temperature between groups were compared using the student's t-test. The statistical analysis was processed with GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA). A value of $P < 0.05$ was considered statistically significant.

Chapter 3 Results

3.1 Characterization of TMUV strain NTU/C225/20 infection in duck embryos

After the inoculation of the 3rd-passage Tembusu virus (TMUV) strain NTU/C225/20 in the embryonated duck eggs via the allantoic route in EID_{50} (egg infectivity dose 50%) test, the eggs were sealed, incubated at 37 ℃, and observed daily for the viability (Figure 1A-C). Some embryos died at 5th days post-inoculation. The dead eggs would show no vessels and embryo development in the process of egg candling, which means shining a strong light above the egg shelf to view (Figure 1D). The other developing eggs were chilled and opened after one-week-incubation. After egg opening, some embryos presented significantly smaller size, edema and hyperemia with no obvious organ lesions (Figure 2A-B). Besides, liver morphological changes with faint, yellow-to-white spots were seen in some of the embryos with normal outward appearance (Figure 3A-B). The allantoic fluid in the eggs with obvious gross lesions was tested positive by TMUV-specific reverse transcription-polymerase chain reaction (RT-PCR) assay and confirmed by genome sequencing for the presence of TMUV (Figure 4A). Both allantoic fluid and liver tissues of the embryos with normal appearance were also tested by TMUV-specific RT-PCR assay, which showed negative results, however (Figure 4B). The result demonstrated that duck embryos infected with strain NTU/C225/20 would show obvious gross lesions on the outward appearance; therefore, it suggested that the embryo-observing method was enough to distinguish between infection and non-infection, and probably there's no requirement to dissect the embryos or perform RT-PCR test.

3.2 Characterization of TMUV strain NTU/C225/20 infection in chicken embryos

Same as the methods described above, the 2nd-passage TMUV strain NTU/C225/20 was

inoculated into 10-day-old SPF embryonated chicken eggs. After days of incubation, the chicken embryos showed characteristic gross lesions, similar to the observation in the duck embryos (Figure 5). Also, the TMUV infection was confirmed by the NS5-specific RT-PCR and genome sequencing (Figure 6).

3.3 Infectivity titer of 4th-passage TMUV strain NTU/C225/20

The 4th-passage TMUV strain NTU/C225/20, which was planned to use in the pathogenicity experiment, was subjected to a study of infectivity titer. The EID_{50} assay was performed with both embryonated duck eggs and chicken eggs, which were observed daily for viability until the last day of the assay (Figure 7A-C). A series of dilutions of NTU/C225/20 virus suspension from 10^{-1} to 10^{-5} was used to inoculate the SPF embryonated chicken eggs (Figure 8). Three-fifth of the eggs received 10^{-1} diluted virus suspension, one-fifth in 10^{-2} and 10^{-3} dilution groups respectively, and none in 10^{-4} and 10^{-5} dilution groups were dead and determined as infection within one week. However, there were no other eggs displaying gross lesions after egg opening. The infectivity titer in SPF embryonated chicken eggs was $10^{1.48}$ EID_{50}/0.1 mL and this value was considered too low to carry out the following challenge experiment in animals.

As for the same manipulation on embryonated duck eggs, 10^{-2} to 10^{-6} dilution was adopted. Two-fifth of the eggs received 10^{-2} diluted virus suspension and one-fifth in the 10^{-3} dilution group were dead within one week. Later, only one other embryo in the 10^{-2} dilution group was considered as infection by gross observation after egg-opening. None of the embryos were seen infected in the 10^{-4}, 10^{-5} and 10^{-6} dilution groups (Figure 9). After the RT-PCR checks were completed with negative results of the allantoic fluid in the normal-appearance eggs, it's confirmed that the infectivity titer of 4th-passage strain NTU/C225/20 in embryonated duck eggs was $10^{2.32}$ EID_{50}/0.1 mL.

3.4 Evaluation of virus concentration methods for TMUV strain NTU/C225/20

Several methods were attempted to concentrate viable TMUV in this study. At the first attempt, the pellet of virus concentration by ultracentrifugation at 50,000 × g for 1.5 hours at 4 °C with a 20% sucrose cushion was obvious but abnormally light-orange; it might result from impurities present in the virus stock that was used in this condition (Figure 10A). As for ultracentrifugation at 50,000 × g for 4 hours at 4 °C with a 20% sucrose cushion, the pellet with a normal white color was hard to be seen (Figure 10B). For the virus product coming from concentration by ultracentrifugation, the titer was determined too low to cause cytopathic effect (CPE) on DF-1 cells according to the plaque assay result. The agarose overlay was observed for 7 days and showed neither plaque nor cytopathic effect among 10^{-1} to 10^{-4} dilutions of the virus stock (Figure 11A-B).

Another concentration technique by polyethylene glycol (PEG) precipitation was used afterwards. Obvious pellet was seen after centrifugation of virus supernatant/PEG-6000 mixture (Figure 12A-B). For the viruses concentrated to 1,000 times by PEG precipitation, there's a quantifiable result to determine virus titer in the plaque assay. Dilutions of the viral concentrated stock ranging from 10^{-1} to 10^{-6} were made. During the infection period, it could be seen that the cells inoculated with a high concentration of the virus produced cytopathic effects, including cell detachment, disruption, and shrinkage (Figure 13A-B). The plate was fixed and stained at 5^{th} days post-inoculation. 10^{-3} dilution formed clear, big, and countable plaques with ill-defined circular borders on the plate (Figure 14). 10^{-1} and 10^{-2} dilutions showed severe cytopathic effect microscopically and some cellular monolayers were damaged during the process of stain-washing, which is most obvious in the central part of the well. They had numerous small plaques but were difficult to count. 10^{-4} dilution may have 1-2 plaques. 10^{-5} to 10^{-6} dilutions showed no plaque. Given the 17 plaques counted in 10^{-3} dilution, the infectivity titer of the PEG-concentrated virus stock was 3.4×10^4 PFU/mL.

3.5 Clinical signs and gross findings in NTU/C225/20-infected chicks

The NTU/C225/20 suspension concentrated by the PEG-6000 method was experimentally inoculated into 1-day-old chicks to determine the pathogenicity of low-dose TMUV on young chickens. Three (50%) of the inoculated chicks exhibited low activity (Figure 15A) and reluctance to walk (Figure 15B), with abnormal sitting posture (Figure 15B) and curled toes (Figure 15C) during the study, and those were not seen in the contact chicks. One (17%) of the inoculated chicks temporarily showed severe respiratory distress, unstable gait, and lethargy at 4 days post-inoculation (dpi), which improved within several hours (Figure 15D). Throughout the 14-day experiment, there was no death and obvious anorexia in all chicks. Since inoculation with NTU/C225/20, the inoculated chicks grew slower than the chicks without inoculation but housed together. There was a significant difference in percent mean body weight between the inoculated and contact groups from 4 dpi ($n = 6$; $P < 0.001$) till the end of the study ($n = 3$; $P < 0.5$) (Figure 16). The rectal temperature of four (67%) of the inoculated chicks elevated from 2 dpi compared to the contact chicks; that of all inoculated chicks elevated from 3 dpi The hyperthermia began to recover from 6 dpi and occurred again twice at the peaks of 7 dpi and 11 dpi, respectively (Figure 17).

After the sacrifice on the indicated days, a significant difference in body size between inoculated and contact chicks were observed (Figure 18A). Viscous, transparent nasal discharge was seen randomly in some inoculated chicks (three chicks; 50%) (Figure 18B). Necropsies of all the inoculated chicks revealed the same gross lesions characterized by hepatomegaly, subarachnoid hemorrhage, and hyperemic thymus (Figure 19A-C). Splenomegaly was seen randomly in some inoculated chicks (two chicks; 33%) (Figure 19D). Some contact chicks also displayed hyperemic thymus at 7 dpi. No lesions were observable with the naked eye in all of the chicks of control group except for subarachnoid hemorrhage, which could be regarded as postmortem changes.

3.6 Detection of TMUV by RT-PCR in NTU/C225/20-infected chicks

Previous research has shown that both replication efficiency and virulence of TMUV in ducks are likely to be reflected by levels of viremia (Yan et al., 2018). To understand the difference in replication efficiency and virulence of NTU/C225/20 in chicks, and to further investigate the replication of NTU/C225/20 in different organs, the chick sera and suspected TMUV-distributed tissues (including brains, hearts, livers, and spleens) collected at 7 or 14 dpi were used to perform TMUV-specific RT-PCR assay. These organs were particularly described in previous reports associated with TMUV pathogenicity in avians. Nevertheless, no positive viral RNA was found in any of the sera and selected tissues samples of both inoculated and contact chicks, while the positive control showed a normal target band, ruling out the potential for false-negative results during the RT-PCR procedure (Figure 20A-E).

Chapter 4 Discussion

Tembusu virus (TMUV) belongs to the *Flavivirus* genus, which is arthropod-borne and able to infect various vertebrates, including birds, rodents, and humans. Other flaviviruses such as dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and Zika virus (ZIKV), pose a significant threat to public health. Some of the diseases caused by flavivirus can also be spread through vector-free transmission, as well as TMUV-related disease was shown to transmit efficiently among ducks without mosquitos in the animal experiments of previous research. In addition, it has been reported that TMUV has the potential to cause antibody-dependent enhancement (ADE) in the mouse model (Liu et al., 2013). ADE occurs when non-neutralizing antibodies present in the host from a primary infection bind to virus particles during a subsequent heterotypic infection. Not only the binding cannot neutralize the virus, but also the virus-antibody complex attaches to the Fcγ receptors (FcγR) on circulating certain cells to facilitate virus entry, leading to exacerbation of the disease. Given the potential threat to both avian and human health, the pathogenicity and transmissibility in different hosts should be understood. In the present study, the replication and pathogenicity of Taiwan goose-origin TMUV strain in chicks were investigated.

It was challenging in the process of harvesting enough viable TMUV to carry out the animal infection experiment. To determine a suitable host system for virus propagation and replication, the DF-1 cell line, primary duck embryo fibroblast (DEF) cells, embryonated chicken eggs, and embryonated duck eggs were used and evaluated. Results showed that all the host systems tested displayed susceptibility to TMUV infection, confirmed by positive results of TMUV NS5 gene-specific RT–PCR. The highest infectivity titer of TMUV was obtained from embryonated duck eggs, while the virus titer generated from embryonated chicken eggs was significantly lower; in other words, chicken embryos had lower susceptibility to TMUV infection compared to duck

embryos. The findings suggest that it would be difficult to prepare strain NTU/C225/20 in chicken eggs. As for replication in cells, TMUV did not produce an apparent CPE in both DF-1 and primary DEF cells. It can be postulated that embryos are more similar to species and provide a favorable environment for virus growth and replication. Based on these reasons, the TMUV field virus underwent 4 passages in embryonated duck eggs to adapt to the host system and thus increase virulence for laboratory use. Nevertheless, on the 4th passage in duck eggs, the virus titer declined instead of elevating as usual. Underlying mechanisms attributed to the reduced virulence could be explored in the future.

Due to the obstacles encountered in the section of virus replication, different concentration methods were applied to 4th-passage TMUV strain NTU/C225/20 and compared. The methods are ultracentrifugation and polyethylene glycol (PEG) precipitation. For the method using ultracentrifugation, which has long been recognized as a common technique to concentrate virus suspension, variable conditions should be considered associated with characteristics of the certain virus, and the process may be time-consuming or may cause virulence loss. The result in the present study probably indicated that the TMUV was not prone to be pelleted or survived through the ultracentrifugation protocol routinely used in flavivirus. On the other hand, high molecular weight polyethylene glycol-6000 (PEG) has also been used for the concentrating of viral samples for decades, especially in arbovirus studies. In comparison to alternative approaches for virus concentration, PEG precipitation has the advantages of its simplicity in protocols and the relative proper environment for viral particles. For the strain NTU/C225/20 concentrated to 10 times with the PEG method, there was slight CPE and plaque formation observed in the plaque assay. Therefore, the 4th passage NTU/C225/20 was attempted to be concentrated 1,000 times in the same manner, and then a concentrated product with virulence of 3.4×10^4 PFU/mL was successfully obtained. Importantly, the DF-1 cells were used in the plaque assay although they seemed not the

ideal selection for virus titration. Additional studies are required to evaluate a type of more liable, susceptible cells for NTU/C225/20 titration. Appropriate host cell selection is one of the factors that the ability of plaque assays to accurately assess viral titers relies upon. According to Tunterak et al (2021), the use of BHK-21 cells as a host system was suggested (Tunterak et al., 2021).

Due to the limited volume of virulent TMUV suspension obtained from the above preparation, low-dose infection targeted in 1-day-old chicks was implemented to evaluate the pathogenicity of NTU/C225/20 in different avian hosts. The results showed that TMUV strain NTU/C225/20 caused delayed growth in a young chicken flock. Apart from growth retardation and hyperthermia seen in all inoculated chicks, only mild signs were seen in part of the chicks (50%) during the observation period, such as low activity, reluctance to walk, and abnormal sitting posture. One (17%) of the inoculated chicks showed more serious signs of respiratory distress, unstable gait, and lethargy at 4 days post-inoculation. It seems that NTU/C225/20-infected chicks were more susceptible to developing acute respiratory signs when receiving human operations, such as rectal temperature measurement, during routine procedures of caring and data collecting. Among the gross abnormalities observed in these experimentally infected chicks, some recognized lesions located in the tissues of several organs with high perfusions, such as livers and spleens. It could be hypothesized that the presence of viremia disseminated TMUV and mediated pathological changes in specific organs. As for the hyperemia found in thymic lobules, as primary lymphoid organs in chickens, it could be a consequence of immune response to viral invasion.

The following molecular analysis did not demonstrate expected results corresponding to the clinical and gross findings. None of the sera and selected tissues (brains, hearts, livers, and spleens) was detected positive for TMUV by NS5-specific RT-PCR assay. The results may come from inappropriate manipulation that occurred during the sample collection, preparation, RNA extraction, and other process leading to RNA degradation or transcription error. It could be the other possible

causes that NTU/C225/20 presents little ability to produce the level of viremia, or that the viral RNA loads in chicks decreased to undetectable levels over the designated time (7 and 14 days post-inoculation). Given the clinical signs and gross lesions we found, it might be a more accountable assumption that NTU/C225/20 induced acute viremia and it was subsequently suppressed rapidly in 1-day-old chicks.

In addition, a previous study demonstrated that the Tembusu virus, similar to other mosquito-borne flaviviruses, can be transmitted without the presence of mosquito vectors. *In vivo* studies indicate that TMUV can be transmitted efficiently among ducks by both direct contact and aerosol transmission (Liu et al., 2012). Among observations from the clinical signs and necropsy, there was no marked evidence indicating infection in chicks by direct contact transmission. However, the data of molecular analysis in this study was unable to present information about viremia and virus distribution of NTU/C225/20 in neither intramuscularly infected chicks nor naïve chicks by direct contact transmission. It remained unknown whether the severity of TMUV-related disease in chicks is associated with the infection routes.

In the final portion of the present study, we focused on the detection of viral nucleic acid in the experimentally infected chicks, which described how TMUV was systemically delivered and replicated in the hosts but not described it possessed virulence to host cells. With the available samples collected in the present study, further studies of immunohistochemistry (IHC) assay and viral titration could be performed in the future to give more detailed information about pathogenicity. It should be discovered whether TMUV can be isolated or not by inoculation of these tissue samples into embryonated duck eggs. In addition, antibody examination for detecting neutralizing antibodies could be used to assess the protective immune response to TMUV after infection in these young chickens.

In conjunction with the relevant evidence in the previous and present studies, it should be noted that although the TMUV-related disease occurred less frequently in chickens than in ducks, the pathogenicity of TMUV in chicks described in this study has raised a concern about the threat to commercial chicken farms and the effect on chicken meat production. The pathogenicity of NTU/C225/20 in adult egg-laying chickens could be further investigated in the future for clarification of both age factor and egg production situation in TMUV-infected chickens.

Chapter 5 Conclusions

In summary, laboratory infection experiments demonstrated that goose-origin Tembusu virus (TMUV) strain NTU/C225/20 isolated in Taiwan in 2020 exhibits pathogenicity in 1-day-old SPF chicks via intramuscular inoculation (10^3 PFU per chick), based on the clinical findings, such as growth retardation, hyperthermia and slight reduction of activity. Gross lesions in TMUV-infected chicks were characterized chiefly by hepatomegaly, hyperemic thymus, and splenomegaly. However, none of the sera and selected tissues (brains, hearts, livers, and spleens) was detected positive for TMUV by the NS5-specific RT-PCR assay. In the procedures of preparing virus stock, we found that duck embryos infected with strain NTU/C225/20 would show obvious gross lesions on the outward appearance with edema and hyperemia of the bodies. EID_{50} (egg infectivity dose 50%) method can be performed in 12-day-old minimal-pathogen-free embryonated duck eggs for virus titration. Appropriate host cell exploration and continued validation of the virus cellular titration protocol with the available TMUV strain are required to ensure optimal sensitivity and accuracy of the virus replication results. More information about the pathogenicity of NTU/C225/20 in chicken flocks, such as detailed viral load/virulence in tissues, production of neutralizing antibodies, age factor, the effect of egg production et al, could be further investigated in the future.

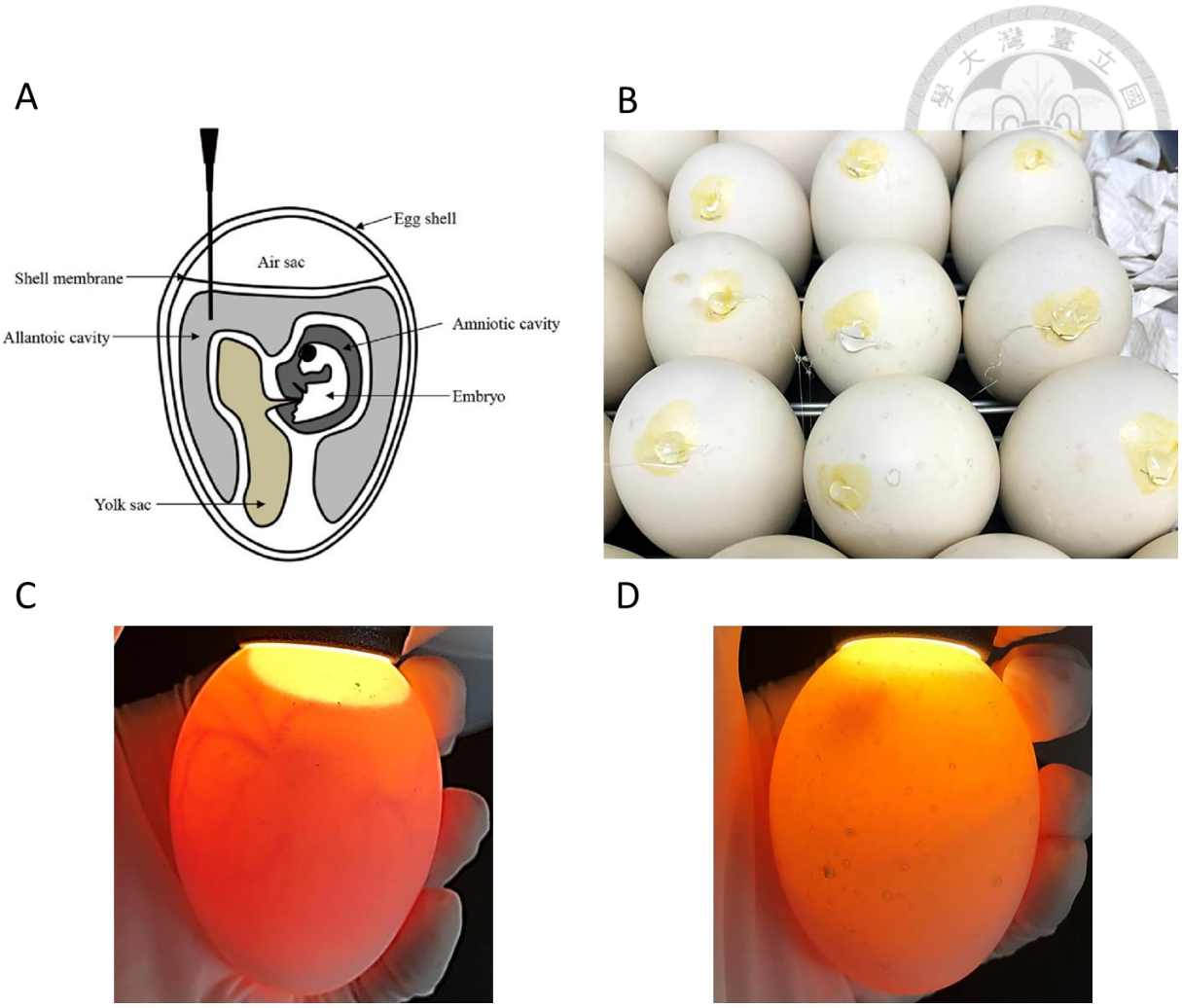


Figure 1. TMUV propagation in embryonated duck eggs. (A) Illustration of the anatomical structure of an embryonated duck egg, indicating injection via allantoic cavity route. (B) The appearance of duck eggs having virus propagation completed. (C) Embryo development with visible vessels at 6 dpi, which occurred in most duck eggs. (D) Failure to develop with no visible vessels and redness in some duck eggs.

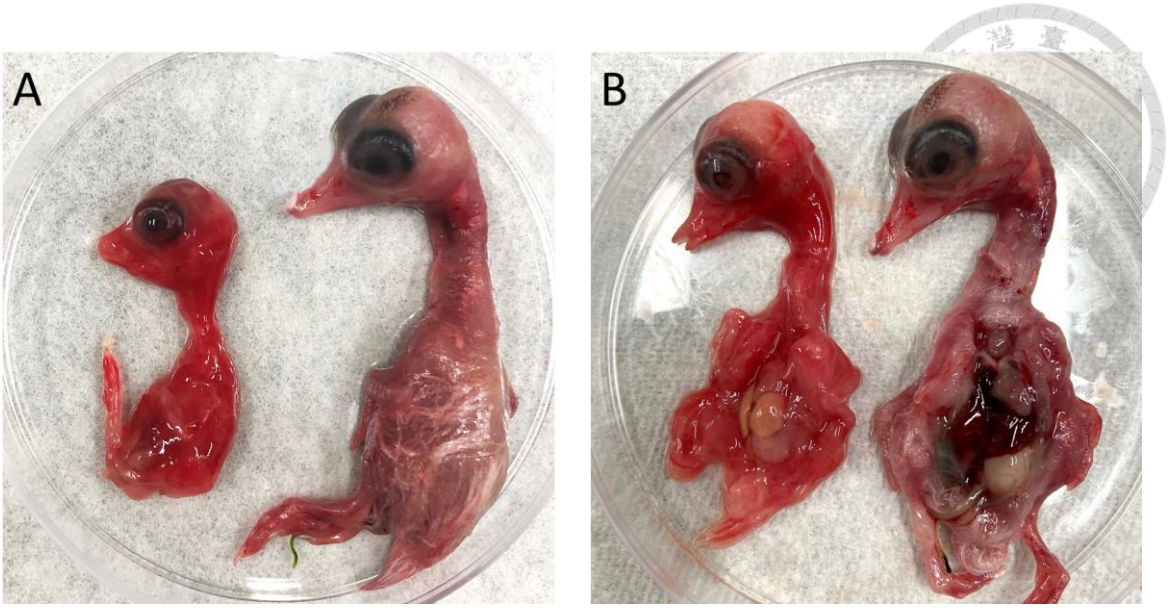


Figure 2. Gross lesions of duck egg embryos inoculated with TMUV strain NTU/C225/20 present at 7 dpi. (A) Outward appearance. (B) Inward appearance. Left, virus-inoculated embryo. Right, control embryo.

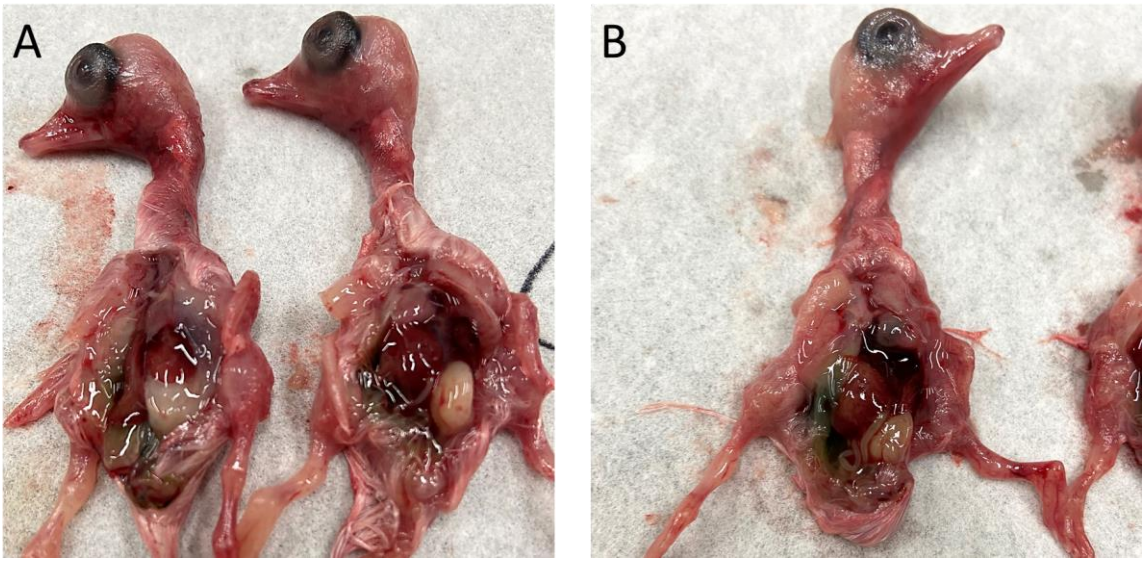


Figure 3. Liver morphological changes of duck egg embryos inoculated with TMUV strain NTU/C225/20 present at 7 dpi with normal outward appearance. (A) Left, virus-inoculated embryo. Right, control embryo. (B) Virus-inoculated embryo.

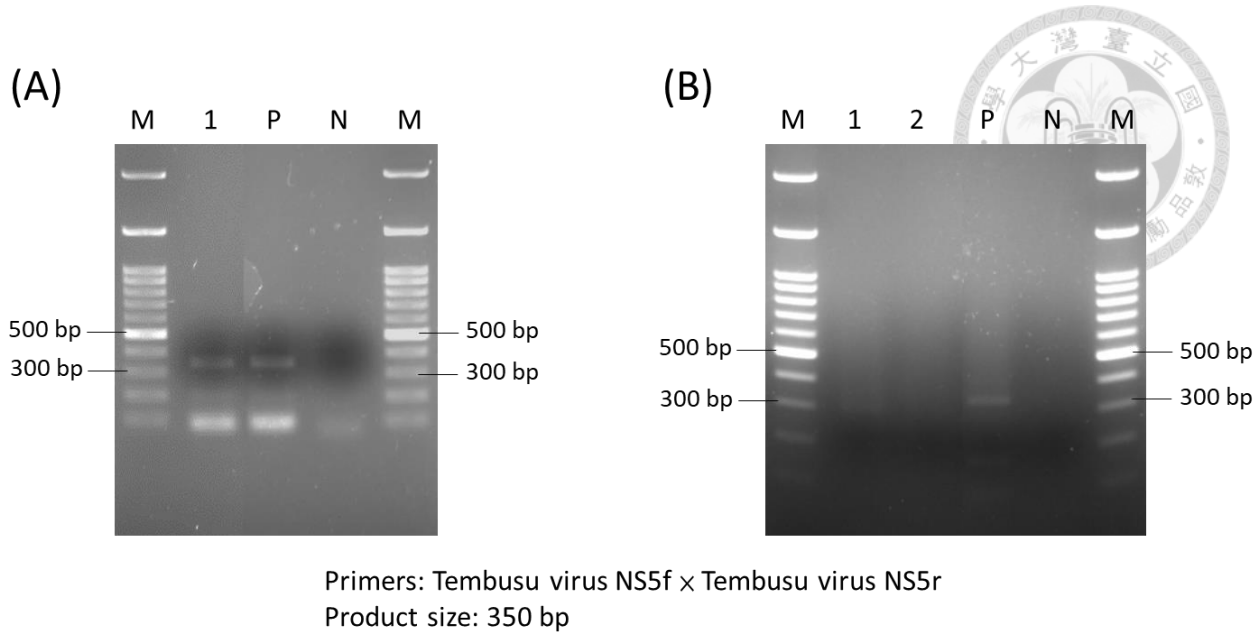


Figure 4. RT-PCR results for the detection of TMUV in TMUV NTU/C225/20-inoculated duck embryos. (A) 1, allantoic fluid of the embryo with obvious gross lesions on outward appearance. (B) 1-2, livers of two individual embryos with normal outward appearance. P, positive control. N, negative control. M, 100 bp DNA Ladder.

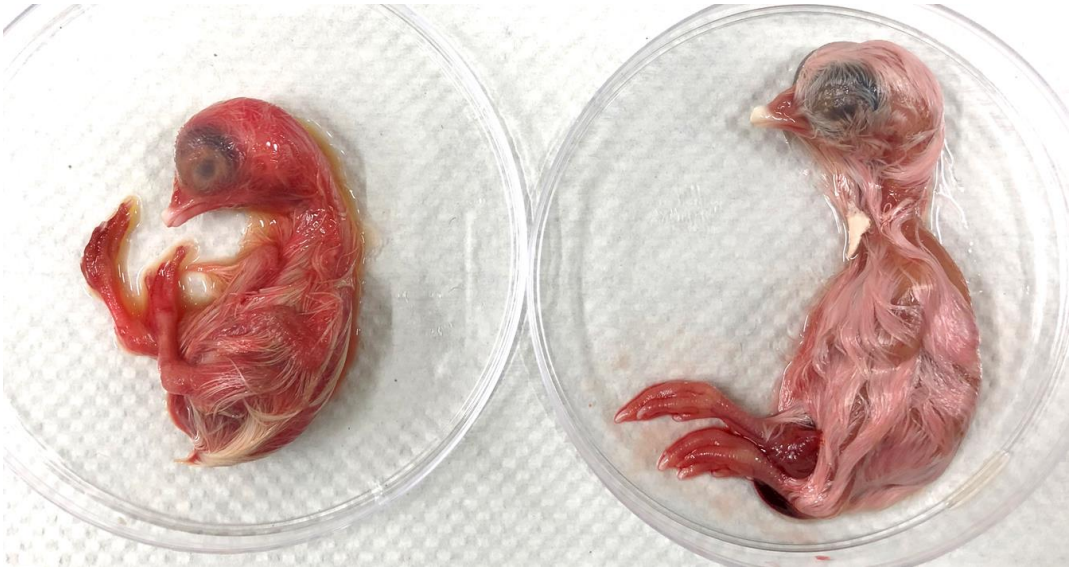


Figure 5. Gross lesions of chicken egg embryos inoculated with TMUV strain NTU/C225/20. Left, virus-inoculated embryo. Right, control embryo.

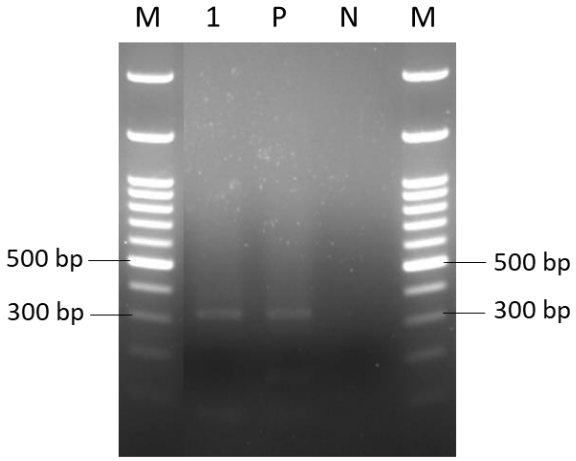


Figure 6. RT-PCR results for the detection of TMUV in TMUV NTU/C225/20-inoculated chicken embryos. 1, allantoic fluid of the embryo with obvious gross lesions on outward appearance. P, positive control. N, negative control. M, 100 bp DNA Ladder.

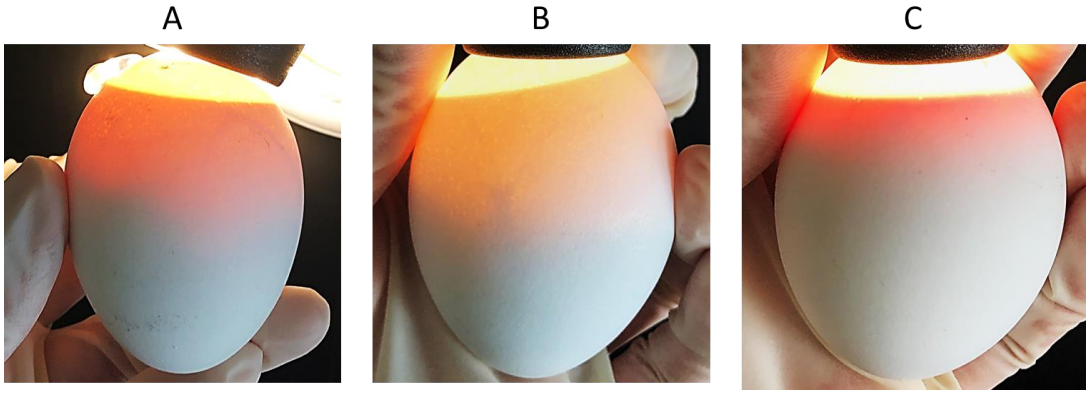


Figure 7. Dead SPF embryonated chicken eggs in EID_{50} test of TMUV strain NTU/C225/20 suspension. (A) Dying egg remains rarely visible vessels. (B) Dead egg with no visible vessels. (C) Dead egg with opaque fluid inside.

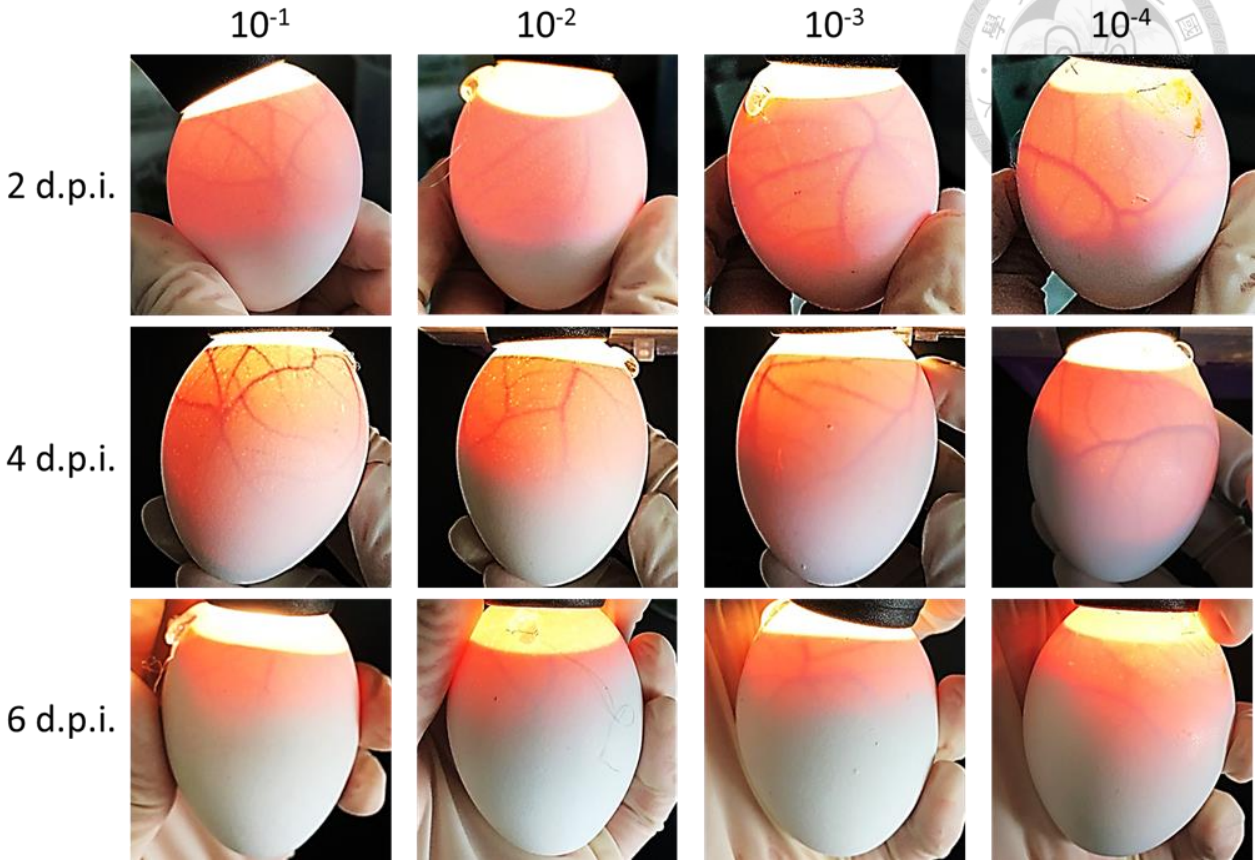


Figure 8. Viable SPF embryonated chicken eggs in EID_{50} test of TMUV strain NTU/C225/20 suspension.

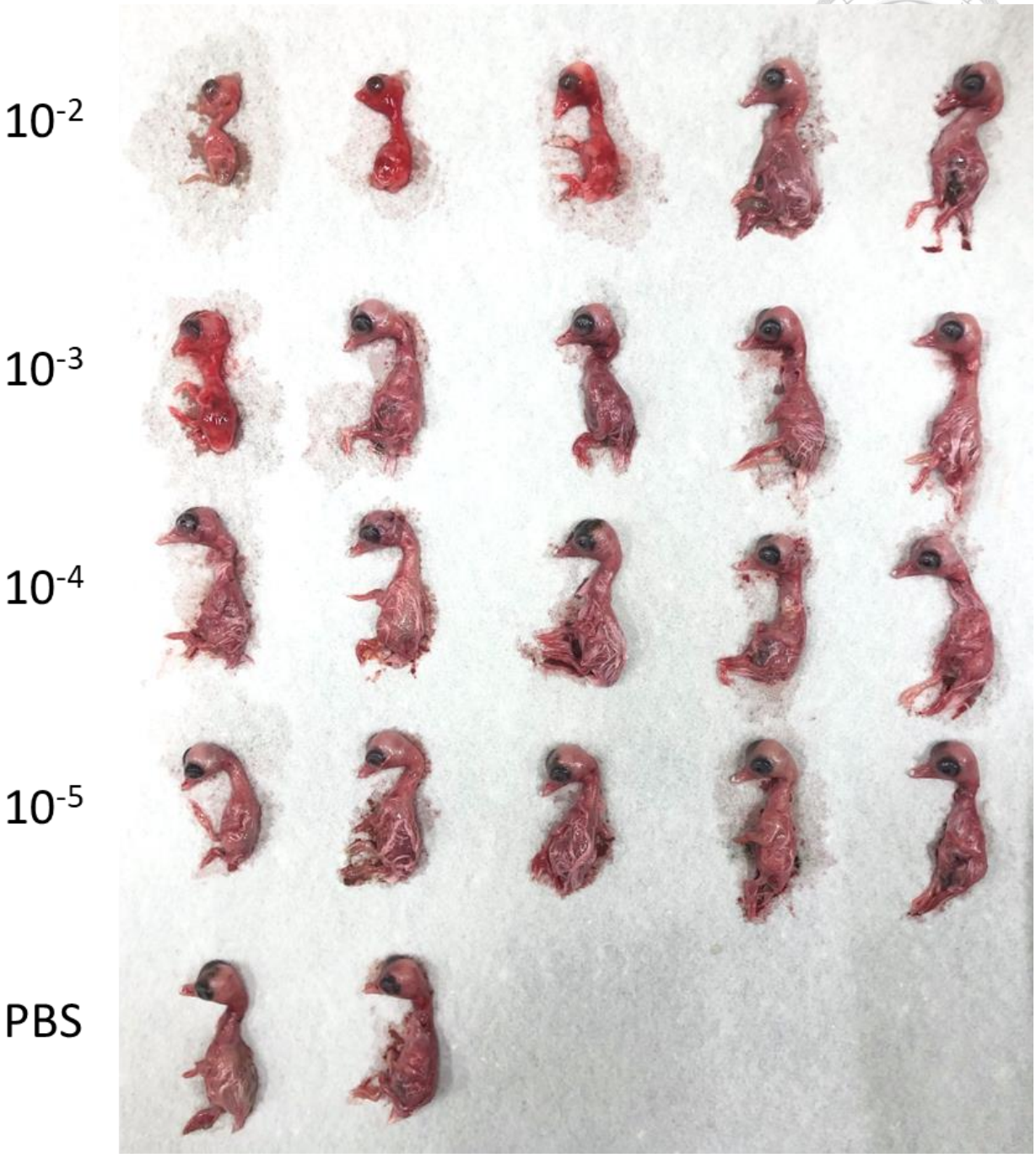


Figure 9. Duck embryos in EID_{50} test of TMUV strain NTU/C225/20 suspension.

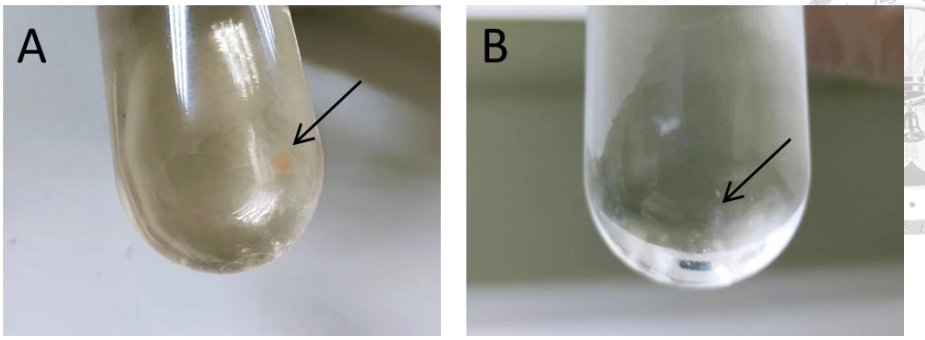


Figure 10. The pellet of TMUV strain NTU/C225/20 suspension after concentration by ultracentrifugation. (A) 50,000 × g for 1.5 hours at 4 °C with a 20% sucrose cushion. (B) 50,000 × g for 4 hours at 4 °C with a 20% sucrose cushion.

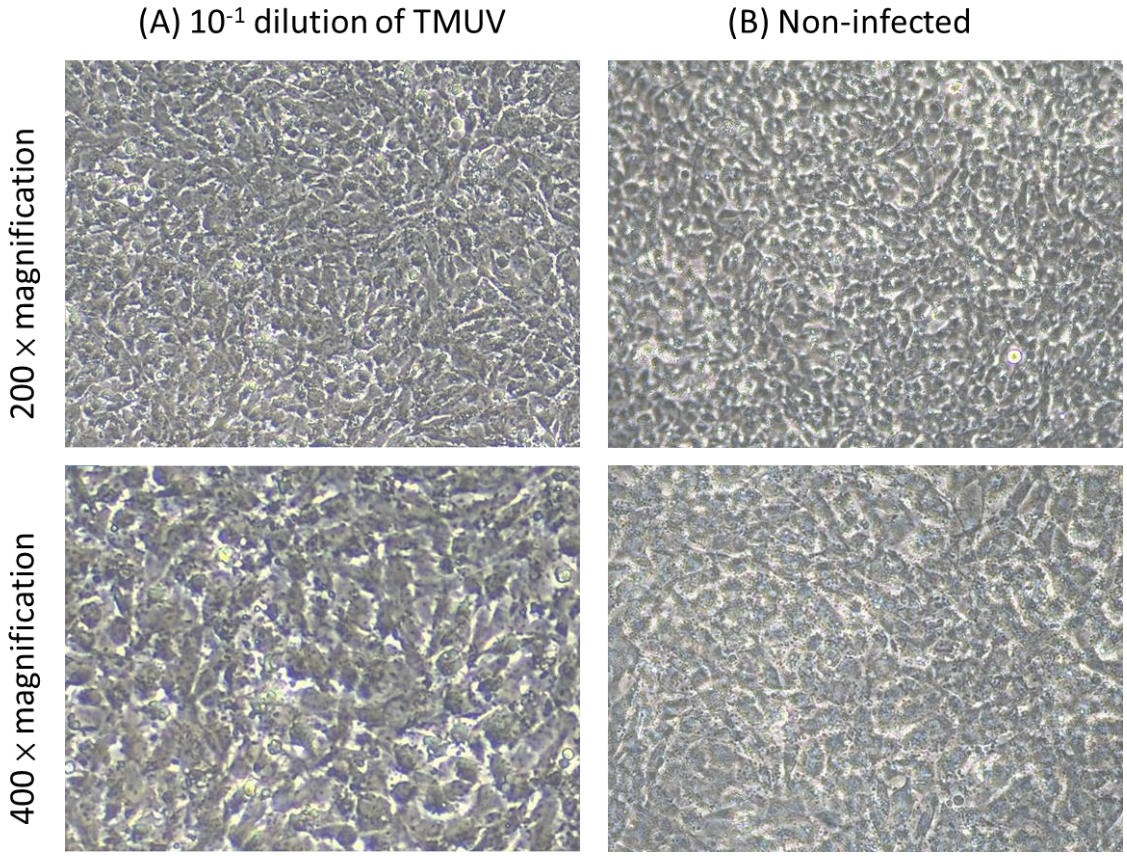


Figure 11. DF-1 cell cultures infected with TMUV strain NTU/C225/20 suspension after concentration by ultracentrifugation. The virus suspension was ultracentrifuged at 50,000 × g for 1.5 hours at 4 °C with a 20% sucrose cushion. (A) The cells infected with 10^{-1} dilution of the virus concentration product at 7 dpi. (B) The non-infected cells as a control group at 7 dpi.

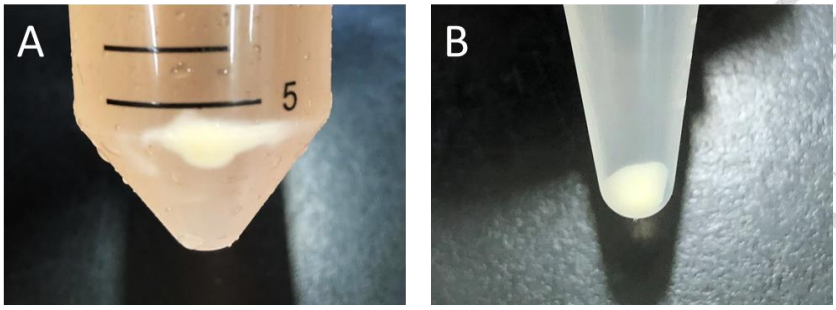


Figure 12. The pellet of TMUV strain NTU/C225/20 after concentration by polyethylene glycol (PEG) precipitation. (A) The virus supernatant/PEG-6000 mixture was processed with centrifugation at 12,000 × g for 30 min at 4 °C to harvest viral particles. (B) The pellet was processed with centrifugation at 12,000 × g for 10 min at 4 °C to remove PEG and other proteins.

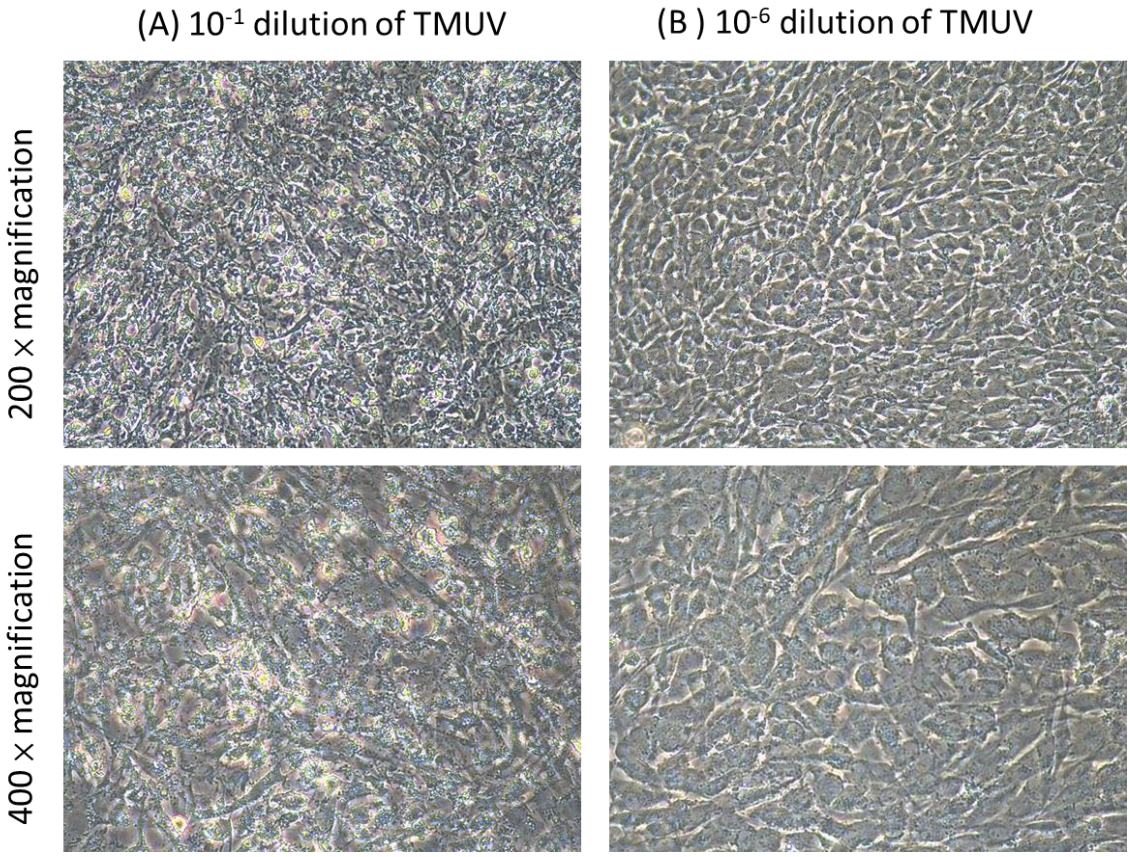


Figure 13. DF-1 cell cultures infected with TMUV strain NTU/C225/20 suspension after concentration by polyethylene glycol (PEG) precipitation. (A) The cells infected with 10^{-1} dilution of the virus concentration product at 5 dpi. (B) The cells infected with 10^{-6} dilution of the virus concentration product at 5 dpi.

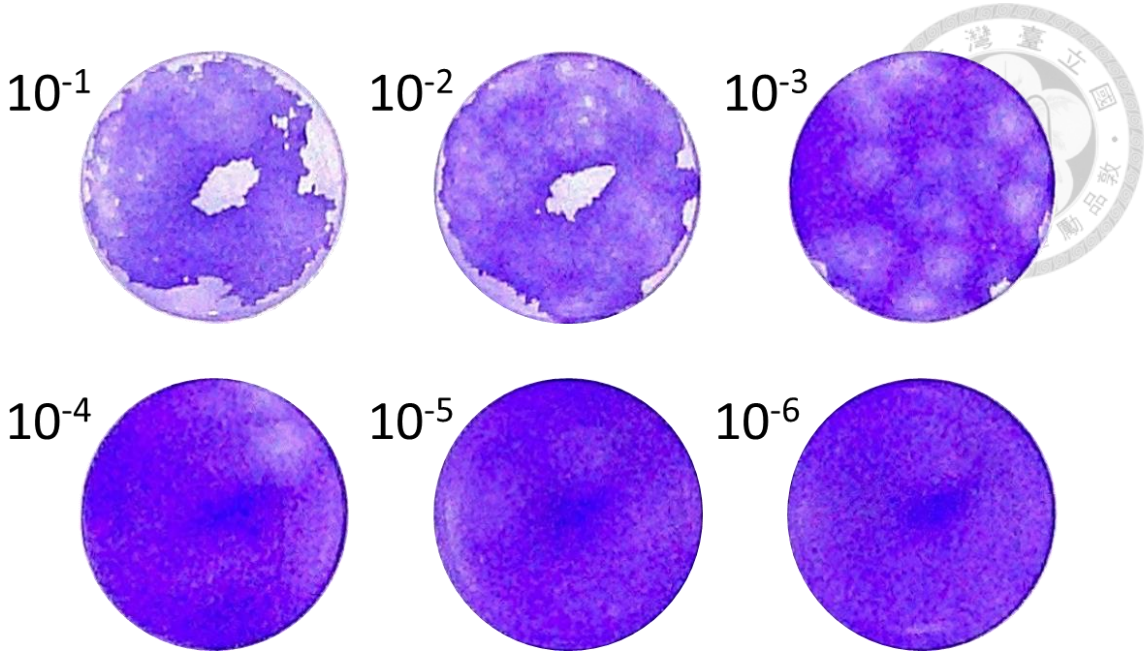


Figure 14. The plaque assay result of TMUV strain NTU/C225/20 suspension in DF-1 cells. The virus suspension was concentrated by polyethylene glycol (PEG) precipitation. The plate was fixed and stained at 5 dpi. Some cellular monolayers of 10^{-1} and 10^{-2} dilutions were damaged during wash steps.

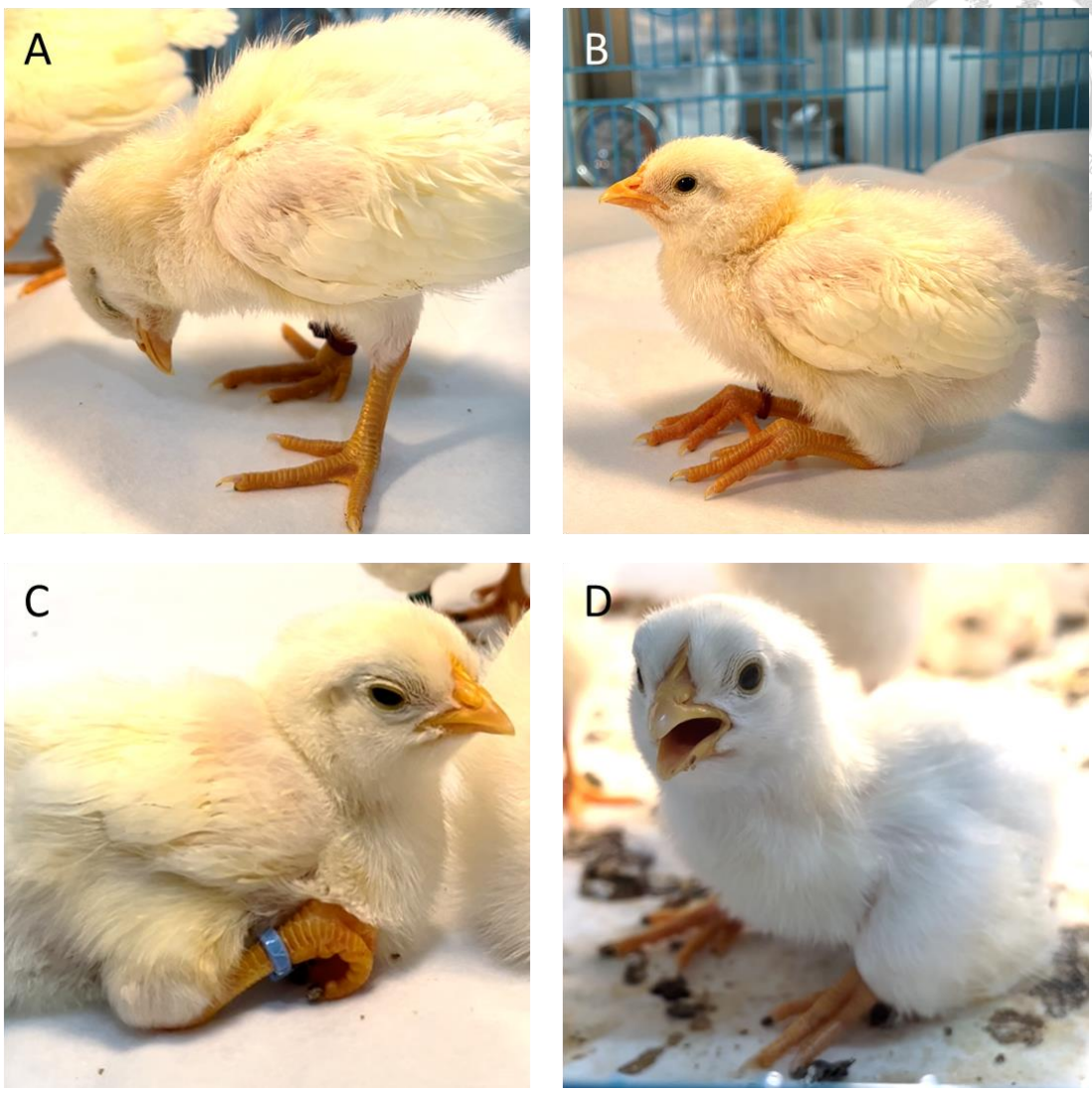


Figure 15. Clinical symptoms in young chickens following experimental infection with TMUV strain NTU/C225/20. (A) Activities were recorded in the inoculated chicks. (B and C) Postures recorded in the inoculated chicks. (D) Abnormal signs in the inoculated chicks were observed at 4 dpi.

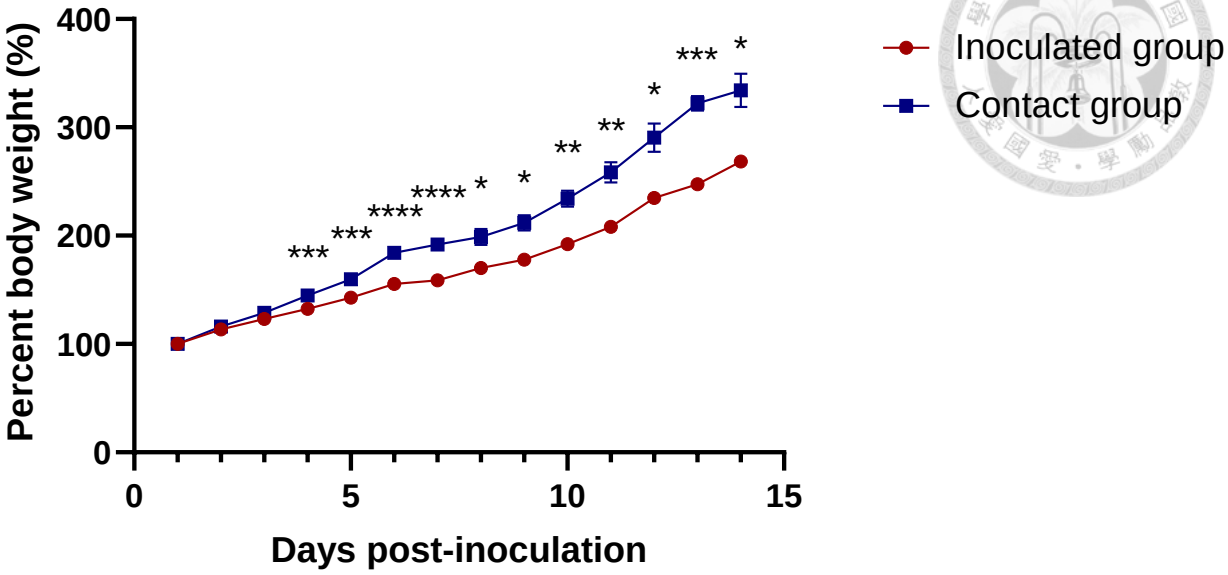


Figure 16. Effect of TMUV NTU/C225/20 on weight gain of young chickens. Comparison of percent body weight change between inoculated and contact chicks from 1 dpi till the end of the study (14 dpi). Body weight of each chick was measured once daily. The data were shown as mean value ± SEM (n = 6, from 1 dpi till 7 dpi; n = 3, from 8 dpi till 14 dpi) and compared with the student's t-test. Asterisks indicate a significant difference between inoculated group and the day-matched contact group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

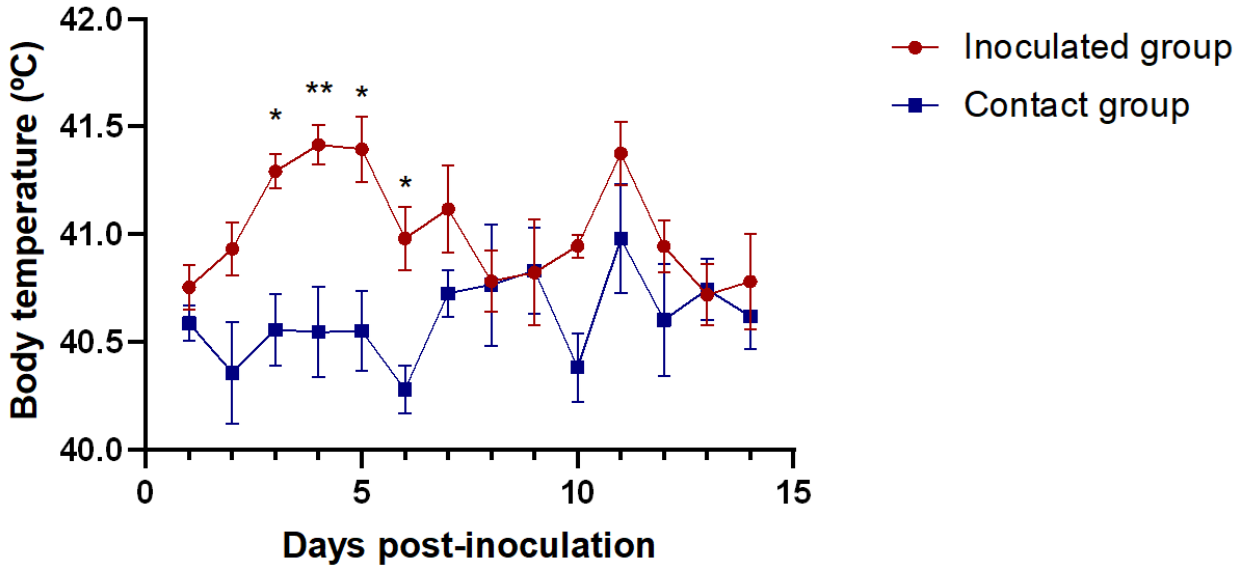


Figure 17. Effect of TMUV NTU/C225/20 on body temperature change in young chickens. Comparison of body temperature change between inoculated and contact chicks from 1 dpi till the end of the study (14 dpi). Each chick had its body temperature once daily. The data were shown as mean value ± SEM (n = 6, from 1 dpi till 7 dpi; n = 3, from 8 dpi till 14 dpi) and compared with the student's t-test. Asterisks indicate a significant difference between inoculated group and the day-matched contact group. *, $P < 0.05$; **, $P < 0.01$.

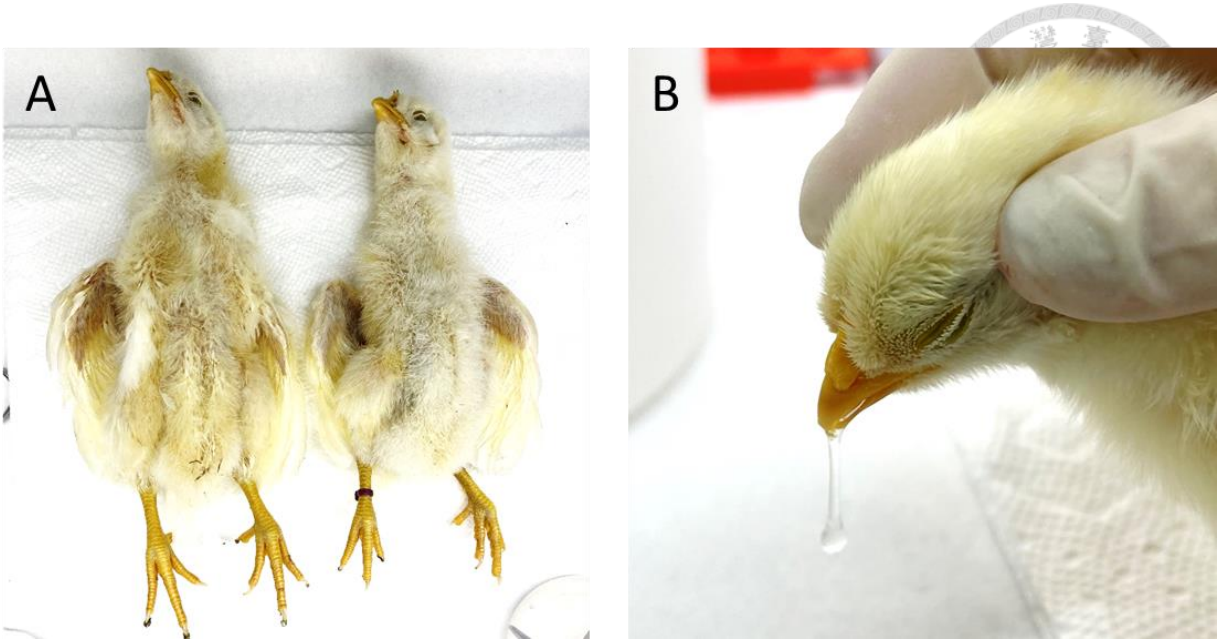
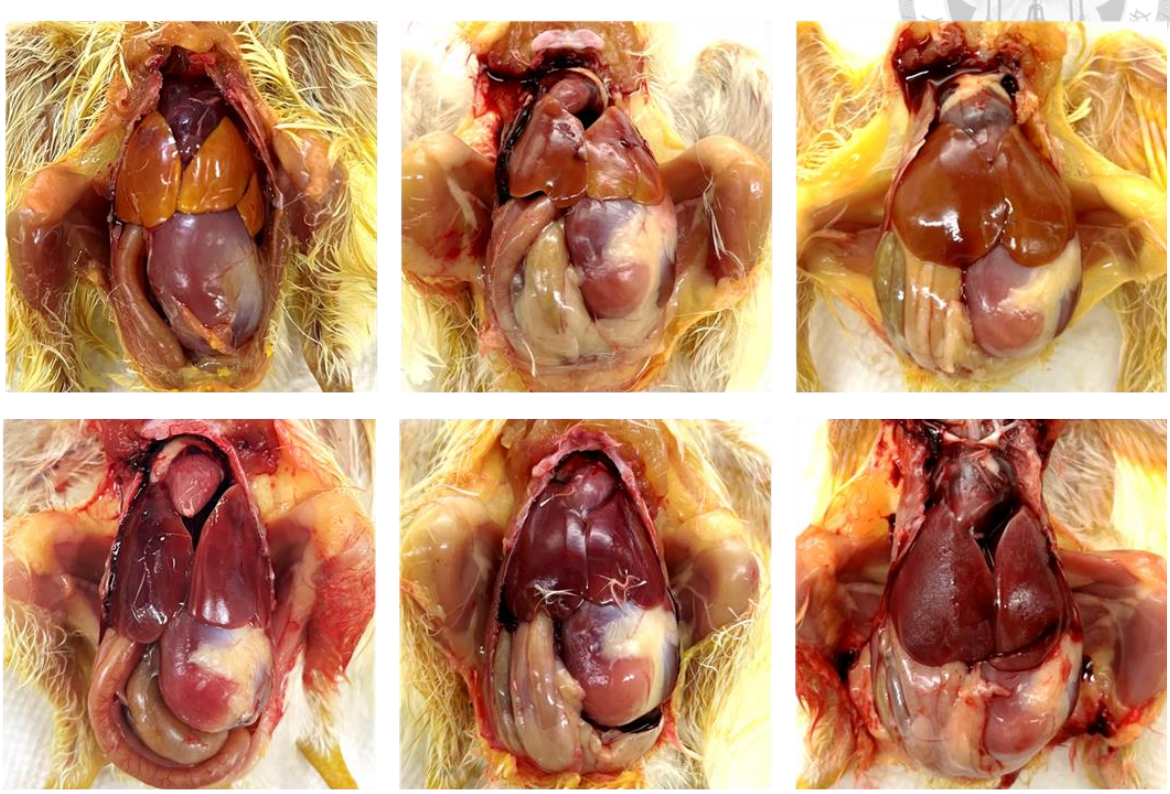


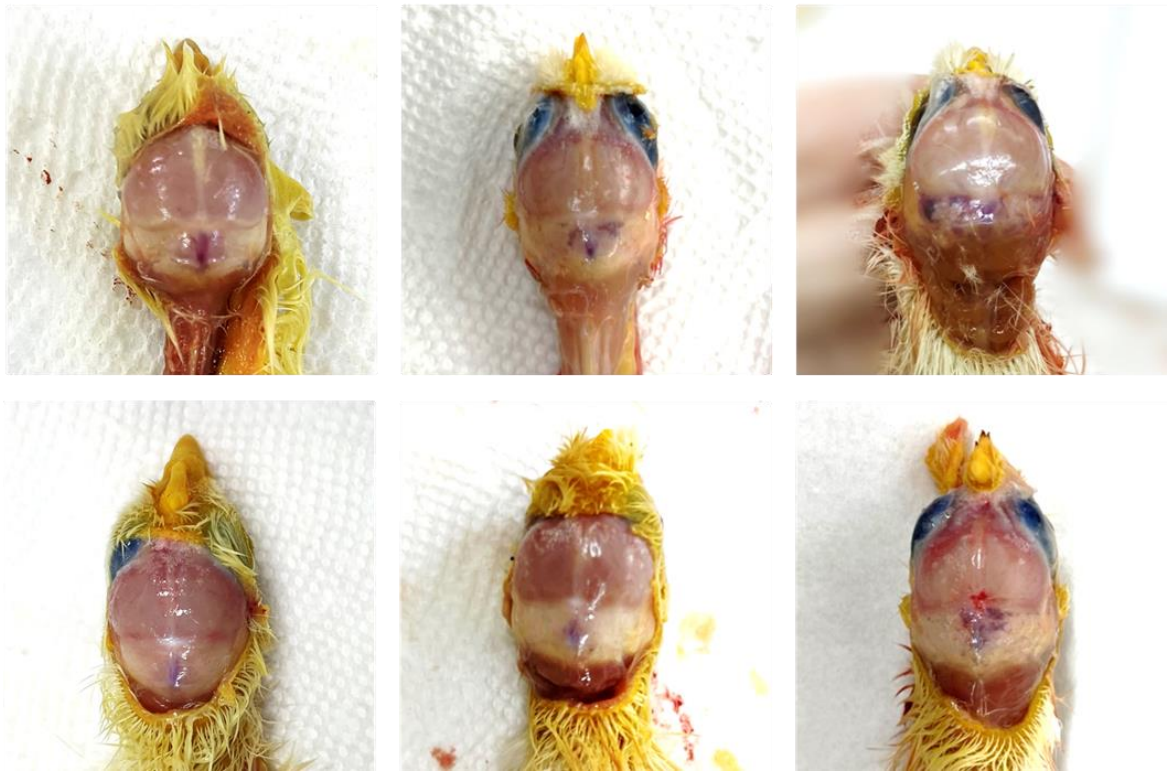
Figure 18. Gross lesions in TMUV NTU/C225/20-inoculated and contact young chickens before dissection. (A) Body size between inoculated and contact chicks at 14 dpi Left, contact group chick weighing 171.0 g. Right, inoculated group chick weighing 128.4 g. (B) Viscous, transparent nasal discharge observed randomly in the inoculated chicks after sacrifice.

A

Control group | Contact group | Inoculated group



B



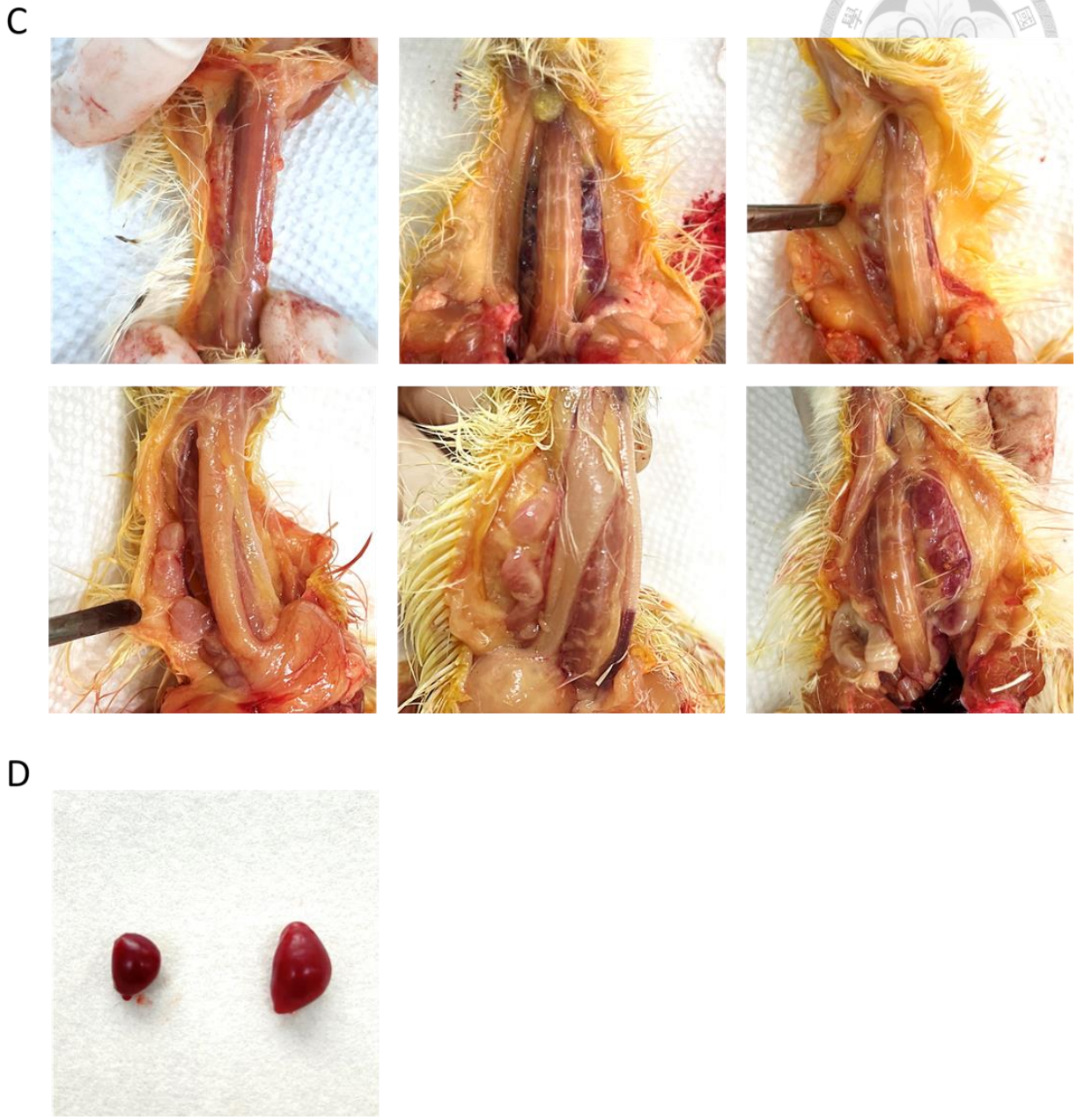
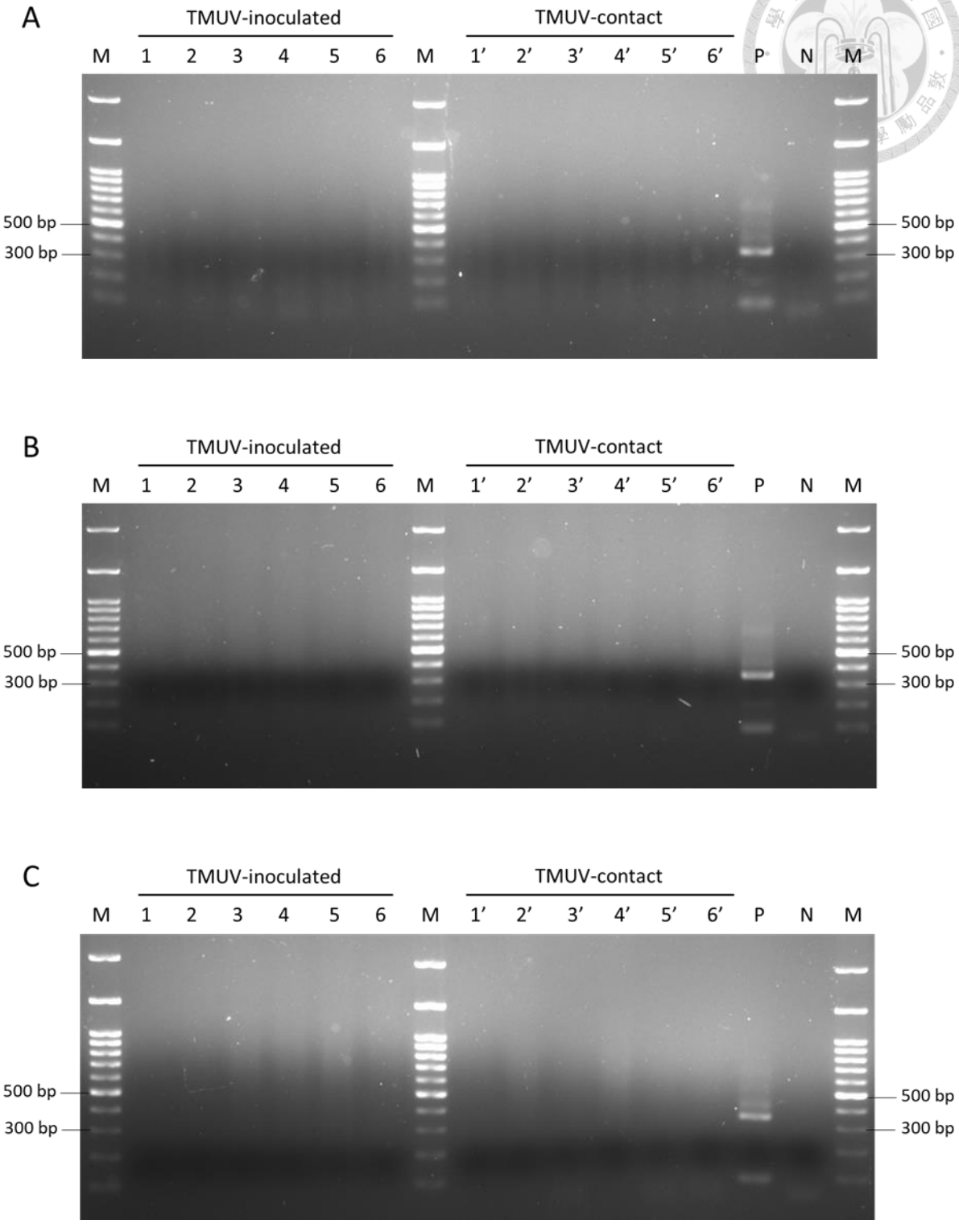


Figure 19. Gross pathologic lesions in TMUV NTU/C225/20-inoculated and contact young chickens. (A) Body cavity and liver morphology at 7 (upper panel) or 14 dpi (lower panel). (B) Skull morphology at 7 (upper panel) or 14 dpi (lower panel). (C) Thymus morphology at 7 (upper panel) or 14 dpi (lower panel). Left, Healthy chicks from the control group. Middle, chicks from the contact group. Right, chicks from the inoculated group. (D) Different spleen size at 14 dpi. Left, contact group chick. Right, inoculated group chick.


A
TMUV-inoculated
TMUV-contact
M 1 2 3 4 5 6 M 1′ 2′ 3′ 4′ 5′ 6′ P N M
500 bp
300 bp
500 bp
300 bp
B
TMUV-inoculated
TMUV-contact
M 1 2 3 4 5 6 M 1′ 2′ 3′ 4′ 5′ 6′ P N M
500 bp
300 bp
500 bp
300 bp
C
TMUV-inoculated
TMUV-contact
M 1 2 3 4 5 6 M 1′ 2′ 3′ 4′ 5′ 6′ P N M
500 bp
300 bp
500 bp
300 bp

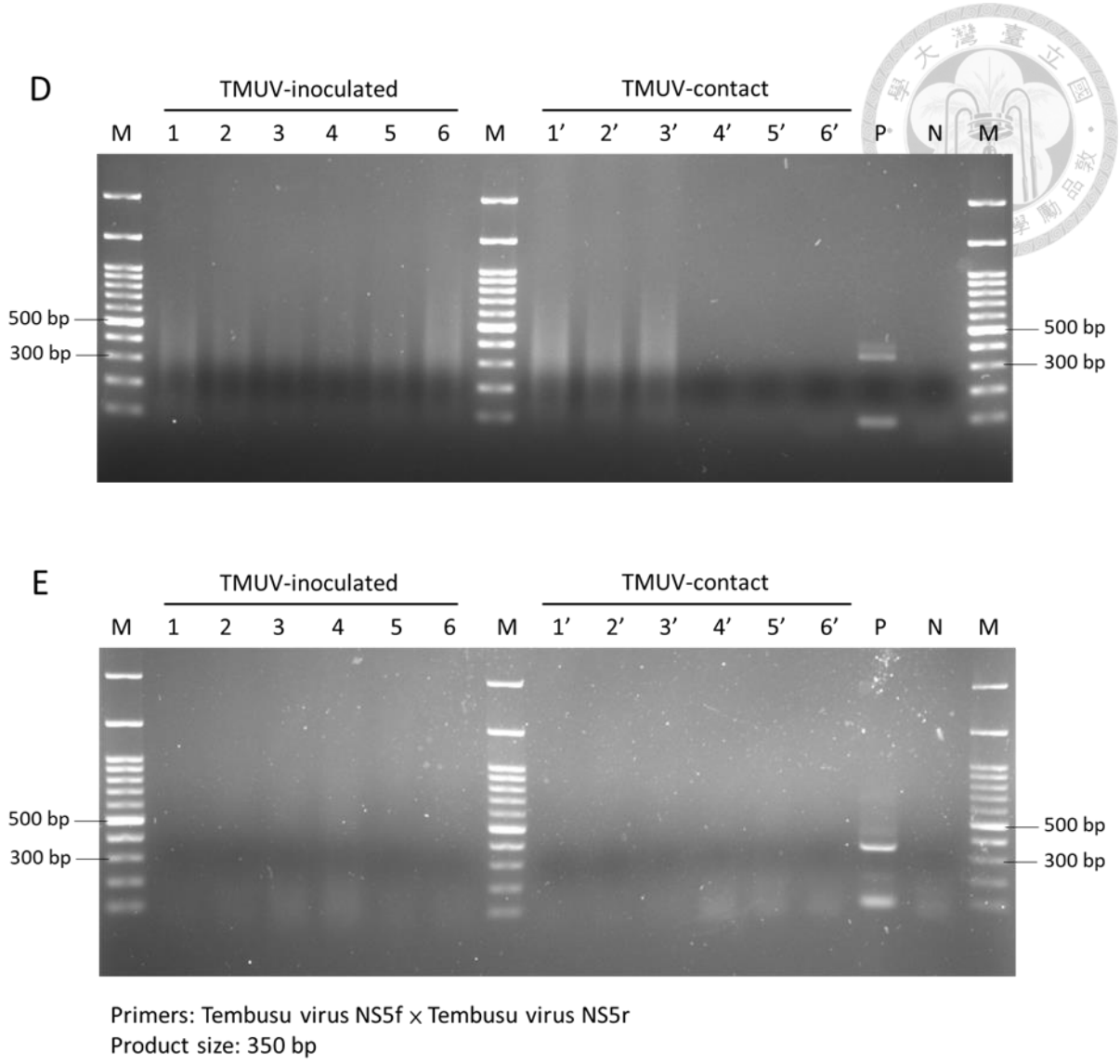


Figure 20. RT-PCR results for the detection of TMUV in different organs of TMUV NTU/C225/20-inoculated and contact young chickens. (A) Serum. (B) Brain. (C) Heart. (D) Liver. (E) Spleen. 1-3, three individual chicks of inoculated group sacrificed at 7 dpi. 4-6, three individual chicks of inoculated group sacrificed at 14 dpi. 1'-3', three individual chicks of inoculated group sacrificed at 7 dpi. 4'-6', three individual chicks of contact group sacrificed at 14 dpi. P, positive control. N, negative control. M, 100 bp DNA Ladder.

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