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Diagnostic Investigation of a Tembusu Virus Infection in Broiler Breeders

Keywords: Tambusu Virus; Broiler Breeders; Virus Isolation; Virus Detection; Sequencing

Abstract

This report describes our diagnostic findings on isolation and characterization of a Tambusu virus (TMUV) infection in a broiler breeder flock, which caused a sharp decline in egg production and primary clinical symptoms of lethargy, reduced appetite and watery diarrhea. The main autopsy lesions observed in affected breeder chickens were reproductive system abnormalities, including follicular membrane hemorrhage and follicular liquefaction. This TMUV infection resulted in about 1.8% mortality in eight weeks. The TMUV isolation was made from oviduct specimens and conducted in both embryonated chicken eggs and chicken embryo fibroblast cells cultures. Amino acid analysis of the virus structure of envelope protein (E protein) indicated that the TMUV isolate belonged to the same branch of duck TMUV vaccine strains of FX2010-180P, DF2 and HB, which were widely used in domestic ducks in China. Notably, the chicken TMUV isolate exhibited the highest homology (99.0%) with the TMUV DF2 and HB vaccine strains. The isolation and characterization of chicken-derived TMUV in this study brings an urgent need for further investigations into the impact of TMUV infections on egg-type chickens to enhance prevention and control strategies for better performance of egg-laying hens of commercial layer farms and boiler breeder flocks.

Open Access Case Report

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Introduction

Tembusu virus (TMUV) belongs to the Ntaya virus group of flaviviruses within the Flaviviridae family. It is a single-stranded RNA virus with a genome length of 10,990 bp. The genome encompasses an open reading frame (ORF) that encodes three structural proteins (capsid protein C, PrM protein, and envelope E protein) alongside seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The E protein is the largest structural component of TMUV and acts as the primary virulence antigen. The E protein contains multiple antigenic determinants crucial for viral processes, including adsorption, replication, and biosynthesis [1].

The TMUV was initially detected in ducks in China in 2010 [2]. TMUV infections in ducks cause notable decline in egg production due to primary affecting and damaging the target organs of reproductive system characterized by ovarian hemorrhage, follicular rupture, and follicular membrane hemorrhage. Subsequently, TMUV infections have been diagnosed in chickens, geese, sparrows, and mice [3-5]. In this study, we report a TMUV infection occurred in broiler breeder chickens and findings of isolation and characterization of the TMUV strain to provide new evidence of TMUV infection in chickens.

Materials and Methods

Disease Onset, Sample Collection and Preparation

A disease onset with a sharp decline in egg production occurred in a large-scale broiler breeder flock with over 53,000 broiler breeders at 34-weeks of age, which continued for a period of 8 weeks and caused about 1.8% mortality. Six dead birds during the second week of disease onset were submitted to our laboratory for diagnostic tests. The autopsy examinations showed all the six birds had prominent lesions of follicular membrane hemorrhage and follicular liquefaction; but no observable pathologic lesions were seen in other organs. Tissue specimens of the follicular membranes were collected for laboratory diagnostic tests of possible viral pathogens. Other tissue samples of trachea, lung, liver and spleen were collected for screening tests of avian viruses commonly infected poultry in the region. The same type of tissue specimens from the six birds were pooled and minced with sterile scissors and homogenized at 1:5 dilution (w/v) with sterile phosphate buffered saline (PBS, 8mM NaH_2PO_4, 150mM NaCl, 3mM KCl, and 2mM KH_2PO_4 at pH 7.4). The homogenate underwent three freeze-thaw cycles of freezing at-80°C and thaw at 37°C temperatures, followed by centrifugation at 12,000 rpm for 10 min. The resulting supernatant was filtered through a 0.22 µm syringe filter and stored at -80 °C freezer for various diagnostic tests in this study.

Virus isolation (VI) in embryonating chicken eggs (ECE) and cell cultures

Specific-pathogen-free (SPF) chicken fertile eggs, which were obtained from Jinan Sais Poultry Technology Co., Ltd (Pingan Nanqiao District, Jinan, Shandong Province, China), were incubated at 37°C egg incubator in our laboratory for avian VI. When the ECE reached 9-10 days of age, the prepared tissue specimens were each inoculated into 5 ECE via allantoic cavity rote, 0.2 mL per egg. The specimen-inoculated ECE were incubated at 37°C egg incubator and candled daily for 5 days. If embryos perished, they were removed and placed in a 4°C refrigerator. After 5 d incubation, all ECE were removed and placed at 4°C refrigerator for a minimum 5 h or overnight, then allantoic fluid (AF) samples were harvested and embryos were examined. Embryo specimens were collected if specific embryo lesions were observed. The AF samples were used for inoculation of next egg passage for three consecutive passages.

The specimen of follicular membranes was also conducted for VI in UMNSAH/DF-1 cell line (ATCC, CRL-3586). The DF-1 cell cultures were prepared in 25 cm^2 cell culture flasks for specimen inoculation when the DF-1 monolayer cells reached about 80% or

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>80% fluency. Briefly, 1) discard the cell culture medium; 2) add about 2 mL sterile PBS to the flask to wash the monolayer cells gently and then discard the PBS; 3) inoculate 0.5 mL of the filtered specimen homogenate into the 25 cm^2 flask; 4) incubate the flask in a 37°C incubator for 40 min; 5) add 4.5 mL cell culture maintenance medium to the flask. The specimen-inoculated cell culture flask was incubated in a 37°C incubator with 5% CO_2 supplement and was examined daily for a period of 5 days. If cytopathic effects (CPE) were observed and developed about 70% or >70% of the monolayer cells, the CPE positive cell flask was placed in -80°C freezer for harvest. If no CPE were observed at 5 d pi, the cell culture flask was removed from incubator and placed in -80C for harvest. After froze-thaw 3 times, the flask cell culture material was transferred to a 15 mL centrifuge tube and centrifugated at 1200 rpm for 10 min, and then the supernatant was used for inoculation to next cell passage for a total of three serial cell passages.

PCR and One-Step RT-PCR for Virus Detection

VI samples of AF and cell culture materials were processed for detection of avian influenza virus (AIV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), egg-dropping syndrome virus (EDSV), TMUV, and Mycoplasma Synovia (MS) by PCR and One-Step RT-PCR. DNA and RNA extractions were conducted using a fully automated nucleic acid extractor and its automatic nucleic acid extraction kit (the extractor Model No. VNP-96P, Nanjing Vazyme Biotech Co., Nanjing, China) in accordance with the manufacturer's instructions. PCR for MS and EDSV, RT-PCR for NDV, IBV, AIV H9 and TMUV were performed. The resulting products were electrophoresed on a 1% agarose gel, and gel imaging was conducted after electrophoresis for result observation and recording.

Primers sequences for NDV [6], IBV [7], AIV H9 [7], EDSV [8], MS [9], and TMUV [10] were referred from publications (Table 1) and were synthesized by Sangon Biotech Shanghai Co., Ltd. (698 Xiangmin Road, Songjiang District, Shanghai, China). One-Step RT-PCR kit, Taq Master Mix, and TAE electrophoresis buffer were obtained from TransGen Biotech (Catalog No. AT411-02, Zhongguan-cun Dongsheng International Science Park, Haidian District, Beijing, China). The PCR instrument of T100 thermal cycler and gel electrophoresis instruments (PowerPac universal power gel imager ChemiDoc MP) were acquired from Bio-Rad Laboratories (Shanghai) Co., Ltd., Pudong, Shanghai.

The PCR program included pre-denaturation at 95°C for 5 min; denaturation at 95°C for 1 min, annealing at 60°C for 15 s, extension at 72°C for 1.5 min for 32 cycles; and a final extension at 72°C for 10 min.

The One-Step RT-PCR program involved reverse transcription at 50°C for 30 min, pre-denaturation at 95°C for 3 min; denaturation at 95°C for 30 s, annealing at 55°C for 35 s, and extension at 72°C for 1.5 min for 32 cycles; with a final extension at 72°C for 10 min.

Table 1: Sequences of PCR and RT-PCR primers, target genes and fragment sizes for detection of NDV, IBV, AIV-H9, MS, EDSV and TMUV.

Virus	Primer sequence	Size (bp)	PCR type
NDV	F: ATGGATCCCAAGCCYTCTAC	433	RT-PCR
	R: TGGCTTGTATGAGKGCAGA		
IBV	F: CAGGTAAAGGCGGAAGAAAAC	654	RT-PCR
	R: TGAAGCCCATCTGGTTGAAG		
AIV H9	F: CATCGGCTACCAATCAACAAAC	473	RT-PCR
	R: GATTATTTGTGTATTGGGCGTC		
MS	F: GAAGCAAAATAGTGATATCA	207	PCR
	R: GTCGTCTCCGAAGTTAACAA		
EDSV	F: ACCCGCTTCGTTACACCA	516	PCR
	R: CCCTTCGGAGAAATCCCTA		
TMUV	F: AGACTGCTGGTGCAATGARAC	250	RT-PCR
	R: CGTCGTTCCCARRTTCCA		

Cloning and sequencing analysis of E protein gene of TMUV

RNA samples were extracted from the positive CPE cell culture material, which was tested positive for TMUV, the PCR products of target bands were recovered, ligated with a pMD18-T vector, and transformed into DH5α receptor cells. Positive colonies were selected, verified by colony PCR [11], and submitted to Sangon Biotech for sequencing. The MegAlign program[12] was used for analyzing and comparing sequencing results. The MEGA7 software was used for construction of evolutionary tree graph. Sequence information of 24 TMUV reference strains retrieved from GenBank was provided in (Table 2).

Table 2: Sequence information of 24 TMUV reference strains (#1-24) retrieved from GenBank and 4 vaccine strains(#25-28) commonly used in domestic ducks in China.

Serial No.	TMUV Strains	GenBank Accession No.	Year of Occurrence	Host Species	Source of Origin
1	MM1775	JX477685	1955	mosquito	Malaysia
2	Sitiawan virus	JX477686	2000	chicken	Malaysia
3	D1977/1/MY	KX097989	2012	duck	Malaysia
4	D1921/1/3/MY	KX097990	2012	duck	Malaysia
5	KPS54A61/ THA	KF573582	2013	duck	Thailand
6	DK/TH/CU-1	KR061333	2013	duck	Thailand
7	HZ-2014	KX686580	2014	duck	China
8	CQW1	KM233707	2013	duck	China
9	SD201120	KY623423	2011	duck	China
10	JS201501	KY623428	2015	duck	China
11	lq-1	KF557893	2012	duck	China
12	JS06	KR869106	2014	chicken	China
13	pigeon	JQ920425	2012	dove and pigeon	China
14	SDHS	KF826767	2012	sparrow	China
15	SDMS	KC333867	2012	mosquito	China
16	G23	KT239021	2014	goose	China
17	HZ4-2015	KX686571	2015	duck	China
18	SDLC	KJ740747	2016	duck	China
19	JS-S1	KY810818	2017	duck	China
20	GD06	KX977550	2018	goose	China
21	FQ-C1	KX977555	2018	chicken	China
22	GA	MK907880	2019	duck	China
23	AQ-19	MT708901	2019	goose	China
24	CHN-JL	MN966679	2020	duck	China
25	FX2010-180P	KY623434	2018	duck	China
26	WF100	Not available	2016	duck	China
27	HB	Not available	2016	duck	China
28	DF2	Not available	2021	duck	China

Note: 1) #25-28 are the 4 TMUV vaccine strains currently used in ducks in China. WF100 and FX2010-180P are attenuated live vaccine strains, approved in 2016 and 2018, respectively; HB and DF2 are inactivated vaccine strains, approved in 2016 and 2021, respectively. 2) The nucleotide sequences of the E protein-encoding gene for HB, DF2, and WF100 strains were carried out by Tianjin Ringpu Bio-Technology Co., Ltd., but not deposited to GenBank yet.

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Results

Clinical symptoms and gross pathologic lesions

The disease onset caused nearly 30% decline in egg production and about 1.8% mortality for a period of 8 weeks. Observations of clinical symptoms included a sudden decline in egg production, lethargy, reduced appetite, and watery diarrhea with green and white feces. Autopsy examinations revealed gross pathologic lesions of myocardial congestion, coronary fat hemorrhage, abdominal fat hemorrhage, follicular membrane hemorrhage, and follicular liquefaction (Figure 1).

Isolation of TMUV

The follicular membrane specimen-inoculated ECE exhibited mortality in 48-120 h pi. Dead chicken embryos displayed edema and hemorrhage on the embryo body (Figure 2) and chorioallantoic membrane. The follicular membrane specimen-inoculated DF-1 cell cultures exhibited CPE lesions of increased refractivity, rounding, and fusion at 48 h pi. As cell death and detachment occurred, many translucent, round cells appeared in the culture medium. The follicular membrane specimen generated VI samples of AF, embryo homogenate and cell culture materials were tested all positive for TMUV, but negative for NDV, IBV and AIV by RT-PCR; and negative for EDSV and MS by PCR. Embryo lesions were not observed on the ECEs inoculated with other tissue specimens, and the harvested AF samples were negative by the PCR/RT-PCR screening tests.

TMUV E protein gene homology and evolutionary tree analysis

This TMUV strain isolated from broiler breeder chickens was named HB202010. Amino acid homology analysis revealed that the HB202010 strain demonstrated a homology ranging from 94.4% to 99.0% with TMUV reference strains. The lowest homology was seen with the live vaccine strain WF100, while the highest homology was seen with the inactivated vaccine strains DF2 and HB. Evolutionary tree analysis positioned the HB202010 strain within the same branch as the live vaccine strain FX2010-180P and inactivated vaccine strains DF2 and HB. All these strains belonged to the Chinese TMUV strains II. Notably, this HB202010 strain did not fall within the same branch as the live vaccine strain WF100 (Figure 3).

Discussion

The newly emerged flavivirus or TMUV infectious disease occurred and spread rapidly in domestic ducks in the East and North regions of China since April 2010. Research studies indicated that TMUV caused a sudden decline in egg production and pathogenic lesions in follicular membranes and folliclesin egg-laying ducks [2, 12-14]. TMUV transmission is unlike the traditional flaviviruses to be primarily transmitted by insect vectors, TMUV remains prevalent even during fall and winter when mosquito populations are scarce. In 2018, researchers in Shanghai Veterinary Research Institute reported that some epidemic strains gained airborne transmission

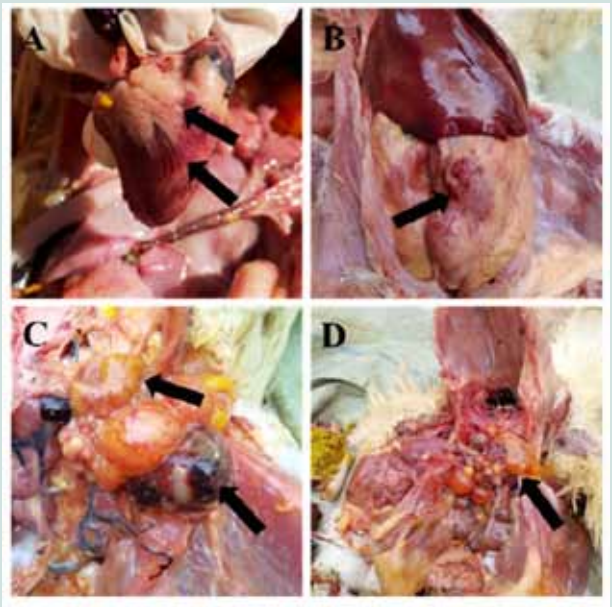

Figure 1: Autopsy lesions in the morbid broiler breeder flock. A: Cardiac congestion and coronary fat hemorrhage; B: Abdominal fat hemorrhage; C: Follicular membrane hemorrhage; D: follicular liquefaction.

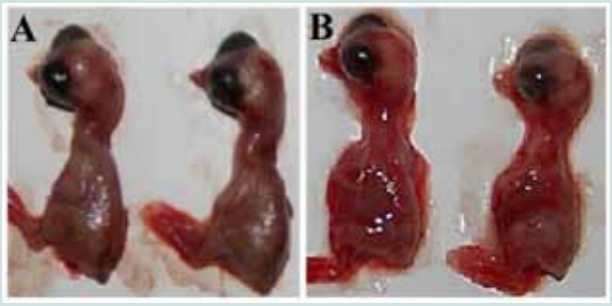

Figure 2: Hemorrhage lesions of SPF chicken embryos in 120 h pi with TMUV positive specimens. A: Control group; B: Virus inoculation group.

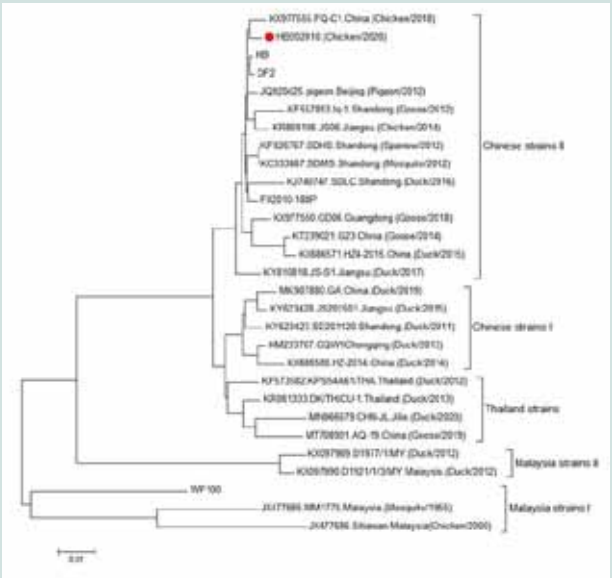

Figure 3: Evolutionary tree analysis of the isolate HB202010 and reference sequences.

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due to a mutation at amino acid position 156 [15]. This discovery confirmed that TMUV transmission is not solely dependent on insect vector of mosquitoes. In terms of pathogenicity, the TMUV strains, which were isolated originally from mosquitoes in Kuala Lumpur of Malaysiain 1955, exhibited low pathogenicity to poultry in Southeast Asia countries [16]. However, its pathogenicity has significantly increased since the TMUV endemic outbreaks in ducks in China in 2010. TMUV infections in ducks caused severe pathologic lesions of hemorrhage of follicular membranes, follicular deformation, atrophy, and liquefaction, resulting in a sharp decline in egg production and substantial economic losses in the duck industry. In regard to host spectrum, ducks were the primary host of TMUV infections, but by 2012, research findings indicated that TMUV infections occurred in other poultry species of chickens and geese [3, 17]. Subsequent studies showed TMUV infections in sparrows, suggesting their potential role as vectors [4]. In 2013, TMUV was found to be able to infect mice, posing a public health risk [5]. Additionally, a study in the same year showed high seropositivity among workers in TMUV-infected duck farms, with 71.9% of workers being seropositive for TMUV antibodies and 47.7% of throat swabs being positive for the virus [18].

In the present study, the TMUV field isolate from broiler breeder chickens caused symptoms of depression, loss of appetite, and a sharp decline of egg production. Autopsy findings showed follicular membrane hemorrhage and follicular liquefaction. Screening tests were negative for other pathogens commonly affecting egg-laying hens, such as IBV, EDSV, and MS. Thus, screening for TMUV should be considered when a layer flock experienced declines in egg production, particularly adjacent to duck farms. TMUV infections in broiler breeders could occur between early and peak egg-laying stages. Manifestations associated with TMUV infections include a slowly increasing or not reaching peak in egg production, or a short peak followed by a substantial 15-30% drop of egg production for 2-3 weeks. In general, our observations on the TMUV affected flocks showed that observable clinical symptoms of TMUV infections could be 4-5 weeks or longer up to 8 weeks within an affected flock, and the spread of TMUV infections to adjacent flocks or poultry premises is relatively slow. The mortality rate could be around 0.1% ~ 0.3% daily in about two weeks of the active infection period.

Laboratory diagnosis for TMUV cases is commonly conducted by VI using ECE or embryonating duck eggs (EDE). The specimen-inoculated ECE or EDE should develop pathologic lesions of the embryos, such as hemorrhage and edema on the entire embryo, edema of the chorionic allantoic membrane, hemorrhage or enlarge/swollen on embryo organs of liver and kidneys [2, 12-14]. The pathogenicity of duck origin TMUV strains was documented to intensify following around100 consecutive passages in EDE, while its potency weakens after 100 passages in ECE [19]. Research studies demonstrated TMUV strains possess proliferation capabilities in various avian cell lines (DF-1, LMH, CEF, DEF) and mammalian cell lines (Vero, HEK293T, HCT116, A549) [19]. In our present study, DF-1 cells showed distinct cellular lesions in 48 h pi, contributing valuable insights into TMUV pathogenicity and cellular interactions within an avian context.

The E protein, a fundamental surface structural protein of TMUV, serves as the primary antigen for eliciting the production of neutralizing antibodies. Existing evidence underscores the multifaceted role of the E protein, not only mediating viral adsorption to host cells but also facilitating fusion with the host cell membrane and active participation in the invasion process [20]. Consequently, the gene encoding the E protein is conventionally utilized as the principal reference gene for evolutionary analyses of TMUV. In a comprehensive investigation, Yu, et al conducted an evolutionary tree analysis encompassing the open reading frame (ORF), E, NS1, NS3, and NS5 genes of 78 representative TMUV strains originating from Southeast Asia and mainland China [21]. The outcomes of this analysis delineated five major branches, denoted as Malaysian (1955), Malaysian (2012), Thai (2013), and Chinese isolate I and Chinese isolate II. Notably, the Chinese isolate II branch emerged as the prevailing and dominant lineage in China. In alignment with these findings, the evolutionary tree analysis in our study was focused on gene encoding of the E protein, positioning isolate HB202010 within the Chinese isolate II branch. The amino acid homology observed between isolate HB202010 and other reference strains within the same branch ranged from 97.6% to 99.0%, underscoring the limited degree of variation exhibited by this isolate.

Considering the absence of effective pharmaceutical interventions for treating TMUV infection, vaccine immunization stands out as the foremost strategy for impeding TMUV transmission, complemented by robust biosecurity measures. Currently, there are commercially available inactivated vaccines for HB and DF2 strains, and live vaccines for WF100 and FX2010-180P strains. These vaccines have played a pivotal role in shielding ducks from TMUV infection, effecting a transformation in TMUV epidemiology from widespread outbreaks to localized occurrences. Notably, the HB202010 isolate from our present study aligns with the same lineage as the vaccine strains HB, DF2, and FX2010-180P. Given the escalating reports of TMUV-infected chickens in recent years, we advocate for proactive vaccine immunization in breeder farms at elevated risk, administered prior to the onset of laying eggs. This approach ensures that antibody titters during the laying stage remain effective in countering TMUV infection.

References

1. Chen CT, Yi YC, Chiang SC, Liu KC, Lin YL, et al. (2005) Selection of immunodominant fragments from envelope gene for vaccine against Japanese encephalitis virus in DNA priming-protein boosting protocols. Microb Pathog 38: 53-62.

2. Cao ZZ, Zhang C, Liu YH, Liu YH, Ye WC, et al. (2011) Tembusu virus in ducks, China. Emerg Infect Dis 17: 1873-1875.

3. Liu M, Chen SY, Chen YH, Liu CG, Chen SL, et al. (2012) Adapted Tembusu-like virus in chickens and geese in China. J Clin Microbiol 50: 2807-2809.

4. Tang Y, Diao Y, Yu C, Gao X, Ju X, et al. (2013) Characterization of a Tembusu virus isolated from naturally infecte d house sparrows (Passer domesticus) in Northern China. Transbound Emerg Dis. 60: 152-158.

5. Li S, Li XX, Zhang LJ, Wang YY, Yu X, et al. (2013) Duck Tembusu virus exhibits neurovirulence in BALB/c mice. Virol J 10: 260.

6. Wang JJ, Yu XH, Zheng DX, Zhao YL, Lv Y, et al. (2022) Continuous surveillance revealing a wide distribution of class I Newcastle disease viruses in China from 2011 to 2020. PLoS One 17: e0264936.

7. Wei YD, Gao WH, Sun HL, Yu CF, Pei XY, et al. (2016) A duplex RT-PCR assay for detection of H9 subtype avian influenza viruses and infectious bronchitis viruses. J Integr Agric. 15: 2105-2113.

ISSN: 2325-4645

8. Luan QD, Jiang ZY, Wang DD, Wang SC, Yin YB, et al. (2022) A sensitive triple nanoparticle-assisted PCR assay for detection of fowl adenovirus, infectious bursal disease virus and chicken anemia virus. J Virol Methods 303: 114499.

9. Reck C, Menin Á, Canever MF, Miletti LC (2013) Rapid Detection of Mycoplasma synoviae and Avian Reovirus in Clinical Samples of Poultry Using Multiplex PCR Avian Dis 57: 220-224.

10. Tang Y, Yeh YT, Chen H, Yu CM, Gao XH, et al. (2015) Comparison of four molecular assays for the detection of Tembusu virus. Avian Pathol 44: 379-385.

11. Bergkessel M, Guthrie C (2013) Colony PCR. Methods Enzymol 529: 299-309.

12. Su JL, Li S, Hu XD, Yu X, Wang YY, et al. (2011) Duck egg-drop syndrome caused by BYD virus, a new Tembusu-related flavivirus. PLOS One 6: e18106.

13. Wan CH, Shi SH, Cheng LF, Chen HM, Fu GH, et al. (2010) A newly identified Flavivirus virus causing abrupt egg-laying reduction in ducks. Fujian J Agric Sci 25: 663-666.

14. Yan PX, Zhao YS, Zhang X, Xu DW, Dai XG, et al. (2011) An infectious disease of ducks caused by a newly emerged Tembusu virus strain in mainland China. Virology 417: 1-8.

15. Yan DW, Shi Y, Wang HW, Li GX, Li XS, et al. (2018) A Single Mutation at Position 156 in the Envelope Protein of Tembusu Virus Is Responsible for Virus Tissue Tropism and Transmissibility in Ducks. J Virol. 92: e00427-18.

16. Kono Y, Tsukamoto K, Abd Hamid M, Darus A, Lian TCL, et al. (2000) Encephalitis and retarded growth of chicks caused by Sitiawan virus, a new isolate belonging to the genus Flavivirus. Am J Trop Med Hyg 63: 94-101.

17. Yun T, Zhang DB, Ma XJ, Cao ZZ, Chen L, et al. (2012) Complete genome sequence of a novel flavivirus, duck tembusu virus, isolated from ducks and geese in china. J Virol 86: 3406-3407.

18. Tang Y, Gao X, Diao Y, Feng Q, Chen H, et al. (2013) Tembusu virus in human, China. Transbound Emerg Dis 60: 193-196.

19. Liang YM , Ma Y, Chen ZJ, Wu HG, Liu SW, et al. (2020) Detection of cytotoxicity and proliferative ability of Tembusu virus Du/CH/LSD/110128 strain and chicken - embryo passage attenuated virus in different species cells. Heilongjiang Animal Science and Veterinary Medicine 5: 7.

20. Modis Y, Ogata S, Clements D, Harrison SC. (2004) Structure of the dengue virus envelope protein after membrane fusion. Nature 427: 313-319.

21. Yu GL, Lin Y, Tang Y, Diao YX (2018) Evolution of Tembusu Virus in Ducks, Chickens, Geese, Sparrows, and Mosquitoes in Northern China. Viruses 10: 485.