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Identification and molecular characterization of a novel flavivirus isolated from geese in China

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ABSTRACT

Since April 2010, a novel contagious disease in ducks and geese, with egg drop, feed uptake decline and neurological signs, caused by a newly emerged virus has spread around Eastern China. Dissection conducted on the dead geese demonstrated hemorrhage in brain, lung, liver, heart, ovary, and enlarged and necrotic spleen. A new virus, named Goose/Jiangsu/804/2010 (JS804) virus, was isolated in Jiangsu area from geese. Then the virus was re-isolated from the affected geese and replicated well in duck embryo fibroblasts and Vero cells, causing the cytopathic effect. The virus was identified as an enveloped positive stranded RNA virus with a size of approximately 40–60 nm in diameter. The full-length genome of this isolated virus was determined, showing that it is closely related to Tembusu virus (a mosquito-borne Ntaya group flavivirus) than other members of the *Flaviviridae* based on the data of phylogenetic analyses. Our systematic studies fulfill Koch's postulates precisely, and therefore, the causative agent of geese occurring in Eastern China is a new flavivirus.

This is the first report that flavivirus infects not only egg-laying and breeder ducks but also geese. The findings extend our understanding of how the virus spreads and causes disease.

1. Introduction

In April 2010, a severe viral disease spread out in most duck-farming and goose-farming regions in China including Zhejiang, Jiangsu, and Fujian provinces. The disease continued to transmit until winter, then the shelducks and geese in the provinces of Shandong, Henan, Hunan, Hubei, and Jiangxi were under a huge threat by this unknown pathogen. The affected egg-laying ducks showed clinical symptom of heavy egg-laying decrease ranging from 20% to 60%, even 90% (Wang et al., 2011), other consistent signs included acute anorexia, antisocial behavior, rhinorrhea, diarrhea, ataxia, and paralysis. The infection rate and morbidity of shelducks was as high as 100% and mortality varied from 5% to 30% possibly due to secondary bacterial infections (Yan et al., 2011). Therefore this viral disease has caused a serious economic loss.

In this research, we performed a systematic investigation, from epidemiology, pathogen isolation, virus characterization, disease reproduction (fulfilling Koch's postulates), to virus genome sequencing. And then, we found that the goose egg drop disease was caused by a new flavivirus, named as JS804 virus, which is closely related to Tembusu virus.

Flaviviruses are single-stranded positive-sense RNA viruses classified in the Genus *Flavivirus* (Hoshino et al., 2009), the Flavivirus genus (family *Flaviviridae*) consists of nearly 70 viruses, which can be grouped into vector-borne (mosquito- and tick-borne) flaviviruses and flaviviruses with no known arthropod vector (NKV) (Chambers et al., 1990; Monath and Heinz, 1996). Many of them, such as Japanese encephalitis virus, yellow fever virus, West Nile virus, and tick-borne encephalitis virus can lead to signs from mild febrile disease, encephalitis, hemorrhagic fever, and shock syndrome to death in both humans and animals (Brault et al., 2011). Flaviviruses particles are spherical and approximately 40–60 nm in diameter. The flavivirus genome, approximately 10.5 kb with a single open reading frame (ORF) encoding a large polyprotein (Zou et al., 2009). The polyprotein can be cleaved by viral and cellular proteases into three structural proteins (capsid [C]; membrane [M], and envelope [E]) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) that function in virus replication, proteolysis, and virus maturation (Lindenbach and Rice, 2003; Mukhopadhyay et al., 2005). The open reading frame (ORF) is flanked by a type-1 capped 5′-terminal non-coding region (NCR) and a 3′-terminal NCR (Zou et al., 2009).

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Up to now, there were few reports about the Tembusu virus isolated from geese (Liu et al., 2012). Here we reported that we isolated the Tembusu virus JS804 from sick goose, characterized and sequenced the whole genome of this virus. In end, our findings will further our understanding of the mechanism of virus spread and disease development in geese, which provides fundamental knowledge that can help in the development of disease control strategies.

2. Materials and methods

2.1. Field samples

Firstly, from the April to November of 2010 clinical examination and the production records were checked for several affected flocks in Jiangsu Province, with the help of Siji Poultry Co., Ltd (Jintan, PR China). Ducks and geese in the affected flocks showing morbidity or death within 6 h were taken to our laboratory for necropsy. Tissues such as brain, liver, ovary and spleen tissues were collected and frozen at −80 °C for further analysis.

2.2. Virus isolation

The strain used in this study was from an affected goose with neurological symptoms in Jiangsu Province. Brain, spleen, and ovary tissues were homogenized in sterile phosphate-buffered saline (PBS, pH 7.2) to give a 20% suspension (w/v). After centrifugation at 12,000*g* for 10 min, the supernatants were filtered through a 0.22 μm filter. The filtered suspension was then inoculated into five 10-day old duck embryonated eggs (0.2 ml/embryo) via the allantoic sac and observed daily. When the embryo died at 6–8 days after inoculation, the allantoic fluid was harvested for another round of inoculations.

After three passages in embryonated duck eggs, the allantoic fluid was inoculated into Vero cells (1.0 ml of a 1:10 dilution in medium) .The cultures were incubated at 37 °C with 5% CO_2 and checked daily for CPE. When 75% of the cells in culture demonstrated CPE (the cells rounding up and floating free from the surface of the flask), the cultures were harvested to infect fresh cells.

2.3. Susceptibility of suckling mice to JS804

The JS804 strain of Tembusu virus at the third Vero cell culture passage was used to study the susceptibility of suckling mice to the virus. Breeder mice of the ICR strain were purchased from Comparative Medicine Centre of Yangzhou University. Uninfected and infected litters were segregated in different rooms throughout the experimental period. Three-day-old suckling mice were infected by intracerebral (i.c.) injection with 25 μl of virus suspension. Meanwhile, mice inoculated with normal saline treated as controls. Animals were observed daily for signs of clinical disease. Fatalities occurring within 2 days of inoculation were considered traumatic, and not included in subsequent data analyses. Brain tissues of the mice appeared clinical signs were harvested to detect the virus nucleic acid by RT-PCR method, and meanwhile inoculated the three-day-old suckling mice again. After sixth passages in suckling mice, serial 10-fold dilutions of the brain tissue extraction were used to measure the mortality of mice. Daily observations of clinical status were made for up to 25 days, and LD_{50} value was calculated according to the Reed–Muench method.

2.4. Virus purification

Vero cells were infected with JS804 and the supernatants of the culture were collected at 5-day post-infection. Cellular debris was removed by centrifugation (12,000 rpm for 30 min at 4 °C), and the precleared supernatants were then subjected to ultra-speed centrifugation; after that, the pellet was resuspended in PBS, loaded onto a discontinuous sucrose density gradient of 30%, 45%, and 60% (W/V), and centrifuged for 2 h at 35,000 rpm. The fraction between the 30% and 45% sucrose gradient was collected and ultra-centrifuged; then the virus pellet was resuspended in PBS and stored at −80 °C until use.

2.5. Electron microscopy

For electron microscopic examination, we followed the procedure described by Hoshino (Hoshino et al., 2007). Generally, cells were fixed in 2.5% glutaraldehyde and 1% osmic acid for 2 h on ice, ultrathin sections were cut and stained with uranyl acetate, and examined with a Hitachi zoom-1 transmission electron microscope. Purified virus pellets were placed onto Formvar-carbon-coated copper grids, negatively stained with 2% phosphotungstic acid (PTA), 2% uranyl acetate (UA), or UA–PTA double staining, and examined with the electron microscope.

2.6. Virus characterization

To determine whether the isolated virus had a lipid envelope, a virus stock of JS804 (2 ml) was mixed with 1 ml of anhydrous ethyl ether and placed at 37 °C for 4 h. For chloroform treatment, 2 ml of JS804 virus stock was mixed with 0.1 ml of chloroform and placed at 4 °C for 20 min. The organic layers were discarded, after that, the infectivity was assayed by inoculating the aqueous phase of the chloroform or ether-treated virus into embryonated duck eggs as descried previously (Miller and Lipman, 1973).

2.7. Animal experiment

To evaluate the pathogenicity of the virus isolate, experimental infection was conducted in 16 8-day-old healthy geese those were tested and found free of the flavivirus. Firstly, eight geese were simultaneously infected by intramuscular injection with 0.5 ml of this virus (10^5 ELD_{50}/ml), additionally, eight geese were mock-infected with sterile PBS in the same manner. The two groups of geese were housed separately in different rooms and were observed daily for 20 days for signs of disease, and the number of geese deaths was recorded. After killed on days 21 post-infection, tissues were collected for pathological examination and virus isolation. Vero cultures were used to recover the virus from brain and ovary samples. The recovered virus was characterized by RT-PCR and subsequent sequencing. All animal experiments were carried out in accordance with the regulations and guidelines of animal experimentation outlined by the people's Government of Jiangsu Province.

2.8. Nucleic acid extraction and identification

In order to determine the nucleic acid type, a convenient method named DNase-SISPA (DNase-Sequence independent single primer amplification) was utilized. 200 μl of cell culture supernatants was filtered through a 0.22 μm filters (Millipore, Billerics, MA) and treated with 100 U of DNase I (Fermentas, Hanover, MD) for 2 h at 37 °C to remove contaminating host DNA. DNase I-resistant nucleic acid was extracted by using AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, PR China) according to the manufacturer's instructions.

Briefly, For DNA virus detection, a second strand of DNA was synthesized from all extracted DNA with 10 pmol of random hexamers (Fermentas, Hanover, MD) and 2 units of Klenow fragment of DNA polymerase (TaKaRa, Dalian, PR China) for 3 h at 37 °C. For RNA detection, first-strand cDNA synthesis was performed using