Research in Veterinary Science 110 (2017) 72–78

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Efficacy assessment of an inactivated Tembusu virus vaccine candidate in ducks

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ARTICLE INFO

Article history:
Received 24 February 2016
Received in revised form 28 October 2016
Accepted 1 November 2016
Available online xxxx

Keywords:
Tembusu virus
Flavivirus
Inactivated vaccine
Duck
Beta-propiolactone (BPL)
Cell culture

ABSTRACT

Duck Tembusu virus (TMUV) is a recently identified pathogen that causes severe egg drop and neurological disease in domestic duck and goose flocks. The infection has spread across the China mainland since its outbreak in 2010. Effective vaccines are needed to fight the disease. In this work, we describe the development and laboratory assessment of a cell culture-derived, inactivated duck TMUV vaccine. The TMUV-JXSP strain was successfully propagated on a baby hamster kidney cell line (BHK-21), inactivated with beta-propiolactone (BPL) and emulsified with mineral oil. The efficacy of different vaccination schedules was assessed in laying ducks and table ducks using virus challenge experiments. Two doses of vaccine provided efficient protection against the virus challenge to avoid the egg production drop in laying ducks. An ELISA demonstrated that 97% (39/40) of ducks seroconverted on day 21 after one dose of the inactivated vaccine and that significant increases in antibody titers against the virus were induced after the second immunization. For table ducks, a single dose of vaccine immunization resulted in a protection index of 87% and significant reduction of viral loads in tissues. Sterilizing immunity can be attained after second immunization. Our results demonstrate that BHK-21 cell culture is suitable for duck TMUV propagation and that BPL-inactivated TMUV vaccine can provide a high level of protection from virus challenge in laying ducks and table ducks. These data provide a scientific basis for the development of an inactivated vaccine for the prevention of duck TMUV infection.

1. Introduction

Tembusu virus (TMUV) is a positive-sense single-stranded RNA virus belonging to the Ntayaantigenic group in genus *Flavivirus*. The virus was first isolated from mosquitoes in Malaysia in 1957 and subsequently detected in Thailand and other areas through the field collection of arthropods for studies of other related viruses (Mackenzie and Williams, 2009). Although there has been serological evidence to suggest that birds may act as reservoir hosts of TMUV, its pathogenicity was to be further investigated (Wolfe et al., 2001). The first case associated with TMUV infection was reported to cause encephalitis and growth retardation in broiler chicks in Malaysia, in which the isolate was named the Sitiawan virus (Kono et al., 2000). TMUV infection in ducks with severe egg production drop was first reported in April 2010 in Southeast China; this infection spread to all of the country's duck production areas, leading to great economic loss for the duck industry (Su et al., 2011). Virus infections were later reported in table ducks, geese, laying chickens and on-farm house sparrows (Liu et al., 2012a, b; Yun et al., 2012; Huang et al., 2013; Tang et al., 2013; Chen et al., 2014). In field conditions, laying ducks were the most strongly associated with natural TMUV infection, which was characterized by a severe decrease in egg production and mortality ranging from 5 to 15%. For table ducks and geese, the infection was characterized by neurological signs such as incoordination and paralysis with high mortality (Yun et al., 2012; Huang et al., 2013; Sun et al., 2014a, b). The virus can be transmitted through the bites of *Culex* mosquitoes (O'Guinn et al., 2013). However, the disease in duck flocks appears to spread horizontally; it remains prevalent during the winter season in north China where the hematophagous arthropod vector activity is low and contact infection was found under experimental conditions (Su et al., 2011; Yan et al., 2011).

Many currently established biosecurity measures are not effective in controlling TMUV infection due to the semi-intensive systems of housing in most waterfowl farms in China. Duck vaccination presents a

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http://dx.doi.org/10.1016/j.rvsc.2016.11.002
0034-5288/

possible solution and has been demonstrated to be effective in the control of West Nile virus infection in geese (Malkinson et al., 2001; Samina et al., 2005). Recent studies reported that chicken embryo-adapted TMUV showed attenuated virulence to ducks with the potential for live vaccine development (Li et al., 2014; Sun et al., 2014a, b). However, no commercial vaccine is currently available. TMUV can infect a range of animals and has a relatively complex transmission cycle (Liu et al., 2013). For environmental safety, variations in the susceptibility of host animals should be considered during live vaccine development. Inactivated vaccines should be a good choice against the emerging disease because of its stability, safety and easy preparation. Recently, a formaldehyde-inactivated vaccine produced by duck embryos and two subunit vaccine candidates containing recombinant envelop protein were reported (Lin et al., 2015; Zhao et al., 2015; Ma et al., 2016). Beta-propiolactone (BPL) was widely applied for the preparation of viral vaccines as an inactivating agent and has been demonstrated significantly higher degree of antigenicity and more protective in some inactivated vaccines compared with other chemicals (Turner et al., 1970; White et al., 1971; LoGrippo and Hartman, 1955; Frazatti-Gallina et al., 2004). Additionally, it could be completely hydrolyzed into nontoxic degradation products. In the present study, we show that duck TMUV can propagate efficiently on a baby hamster kidney cell line (BHK-21). After inactivated with BPL and emulsified with mineral oil adjuvant, the vaccine provides high protective efficacy against the egg production drop in laying ducks and the neurological illness in table ducks, as demonstrated in virus challenge experiments.

2. Materials and methods

2.1. Experimental animals

Commercial laying ducks and 1-day-old table ducks were purchased from local farms in which no antibody against Tembusu virus was detected by blocking enzyme-linked immunosorbent assay (bELISA) as described below. The laying ducks were housed in an animal facility with screens to prevent contact with flying insects. The table ducks were kept in positive pressure special pathogen-free (SPF) chicken isolators. All ducks were provided with commercial feeds of suitable formulations and water ad libitum. Animal husbandry, infection experiments and biosafety precautions were approved by the China Agricultural University Animal Ethics Committee under the protocol (CAU-AEC-2010-0603) and conducted in accordance with the guidelines of the Beijing Municipality on the Review of Welfare and Ethics of Laboratory Animals approved by the Beijing Municipality Administration Office of Laboratory Animals.

2.2. Virus adaptation and propagation in cell culture

The duck TMUV JXSP strain was isolated from an infected flock via duck embryo inoculation and the genomic characters was described previously (Liu et al., 2012a, b). The infected allantoic fluid collected from the second passage of duck embryos ($JXSP_2$) was used as stock virus for cell culture propagation. The BHK-21 cells were obtained from the China National Platform of Experimental Cell Resources for Sci-Tech. The growth medium for BHK-21 cells was Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, HyClone, Thermo Fisher Scientific Inc.), 100 IU/ml penicillin and 100 μg/ml streptomycin. BHK-21 cells were seeded into 25 cm^2 flasks at a concentration of 2×10^5 cells/ml. When the cell monolayer reached approximately 85% confluence, the growth medium was removed and the cells were infected with 1 ml of the 100-fold diluted stock virus. After 60 min adsorption at 37 °C, DMEM containing 1% FBS was added into each flask and incubated at 37 °C with 5% CO_2. The infected cell culture was monitored for cytopathic effect (CPE) daily, and the virus was harvested when the CPE was greater than 75%. The harvest was subjected to two cycles of freezing/thawing and sequentially passaged in fresh BHK-21 cell cultures.

To assess the adaptation of the virus in cell cultures, the samples collected from the 5th to 20th passages were collected, respectively, for titer quantification via a plaque assay performed as described previously (Gould and Clegg, 1985; Kondo, 2013). Briefly, BHK-21 cells were dispensed into 6-well-plates and incubated at 37 °C to full confluence. A 10-fold serial dilution of the virus sample was prepared in pre-chilled DMEM. Two-hundred microliters of the virus suspension were inoculated into two wells of a BHK cell monolayer and stored at 37 °C for 1 h in a CO_2 incubator with rocking by hand every 15 min. The inoculum was then removed, and overlay medium consisting of 2 × DMEM containing 10% FBS with an equal volume of 2% agarose was added. The plates were stored in the CO_2 incubator for 72 h, and agarose overlay medium containing 0.02% neutral red was added. After staining for several hours, the plaques were counted and the virus titer was recorded as the plaque-forming units per ml (PFU/ml).

According to the titer qualification results, the thirteenth passage of BHK-21 cell-adapted virus ($JXSP_{2\text{-}13}$) was used as working stock virus for vaccine preparation. The growth kinetics of the virus in the BHK-21 cell culture were tested by infecting the cell cultures with different multiplicities of infection (MOI), and the supernatants were collected from the infected cells at 24, 36, 48 and 96 h after infection for plaque assay.

For vaccine preparation, the working stock virus was inoculated onto a cell monolayer in 175 cm^2 cell culture flasks at MOI = 0.001. After 1 h of adsorption at 37 °C, DMEM with 1% FBS was added, and the mixture was incubated for 72 h at 37 °C with 5% CO_2. The virus suspension was harvested via 3 cycles of freezing/thawing and clarified via centrifugation at 1100 × *g* for 15 min at 4 °C. The supernatant was collected and stored at −20 °C for use.

2.3. Virus inactivation and vaccine preparation

To inactivate the virus, different volumes of beta-propiolactone (BPL) (FERAK Berlin Gmbh; NMR ≥ 98.5%) were added to the clarified virus suspensions to the final concentration of 1:2000 (v/v), 1:3000, 1:4000 and 1:5000. After vigorous stirring, the suspensions were stored at 4 °C. Samples were collected at 4, 8 and 24 h after the addition of BPL and were immediately tested for infectivity in BHK-21 cells. Briefly, samples were diluted with DMEM and inoculated BHK-21 cell monolayer in 96-well plates (100 μl/well × 6 wells). After incubation at 37 °C for 1 h, 100 μl of DMEM containing 1% FBS was added to each well. CPE were recorded daily for 7 days, and the 50% tissue culture infectious dose ($TCID_{50}$) was calculated using the method of Reed-Muench. To confirm complete inactivation, the supernatants collected at 24 h after inactivation were transferred to fresh BHK-21 cell cultures in 25 cm^2 flasks for two additional passages.

To obtain the water-in-oil emulsion vaccine, a BPL-inactivated virus suspension was formulated with medicinal-grade white oil (Marcol 52, Exxon Mobil Corporation) at a volume ratio of 2:3 (antigen to oil) and mechanically emulsified using the Blender 800S (Waring Commercial, USA). The virus suspension was inactivated with formalin (formaldehyde ≥37%) to a final concentration of 0.2% (v/v) and emulsified with oil in the same manner to be used as a control in the protection experiment.

2.4. Protective efficacy tests

2.4.1. Laying duck vaccination and challenge

After an acclimation period of 1 week, seventy-five laying ducks were randomly divided into 5 groups. The experimental design was summarized in Table 1. Group L-1 and L-2 were sham-vaccinated subcutaneously in the neck with 0.5 ml of sterile PBS. Group L-3, L-4 and L-5 were vaccinated subcutaneously in the neck with 0.5 ml of BPL- or formalin-inactivated oil-emulsion vaccine as indicated. All birds were