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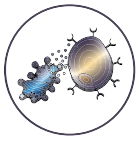

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Research Article

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Seroprevalence of Newly Discovered Duck Flavivirus in Farm Animals

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Abstract

New flavivirus has recently emerged in domestic ducks in China. The virus, provisionally designated duck flavivirus (DFV), causes highly significant economic losses to duck industry in China as a result of reduction in duck meat and eggs. Little is known about the epidemiology of this new viral disease. In this study, we developed a fluorescence-based microneutralization assay to evaluate the seroprevalence of neutralizing antibodies to DFV in domestic farm animals as well as to assess antibody responses in ducks receiving an inactivated DFV vaccine. Screening of duck and pig serum samples found that 54.7% and 1.2%, respectively, of individuals had measurable neutralizing antibody titers to DFV. Interestingly, no serologic evidence of chicken infection was found in a collection of 356 chicken sera from farms where outbreaks of DFV infection among ducks were documented. Thus, results of our serological survey demonstrated for the first time that DFV has a limited host range and duck appears to be the primary host. Furthermore, we showed that a significant portion of vaccinated ducks (approximately 26.7%) had weak or no detectable antibody titers to DFV. The observed suboptimal antibody response in some of vaccinated ducks may help to explain recurring disease outbreaks in duck farms in China, although the region-wide vaccination program has been implemented.

Keywords

Duck flavivirus; Fluorescence-based microneutralization; Seroprevalence

Introduction

New flavivirus has recently been discovered from diseased domestic ducks in China and is provisionally designated duck flavivirus (DFV). DFV infection occurs in laying ducks characterized by a significant reduction in egg production [1,2]. Infection by this new pathogen results in a severe neurological symptom in ducklings and sometimes fatal infection with the reported fatality rate from 10% to 30% [1,3].

DFV is a member of the mosquito-borne Nataya virus complex and is closely related genetically and antigenically to Tembusu and Sitiawan viruses [3]. Tembusu virus infection of mosquitoes occurs predominantly in Malaysia and Thailand where *Culex tritaeniorhynchus* is a major transmission vector. It is reported that Sitiawan virus, a subtype of Tembusu, infects chickens and causes subsequent encephalitis and growth retardation in an experimental setting [4,5]. Interestingly, the recently emerged duck flavivirus in China seems not to show a strong seasonal pattern because in the fall months, the virus still causes epidemics in ducks. The lack of seasonality in infection is also observed in Tembusu virus because sentinel chickens become infected almost year-round.

Since the initial report of DFV-associated disease outbreaks in April 2010, rapid diagnosis methods such as real-time RT-PCR and reverse-transcription loop-mediated isothermal amplification assays (LAMP) have been developed and used to study viral epidemiology [6-9]. However, no specific serological tests for DFV have been developed and consequently little information is available on the seroprevalence of DFV in ducks and other farm animals [10]. The objective of this study was to apply a fluorescence-based microneutralization assay to investigate the seroprevalence of DFV in farm animals including duck, chicken, and pig as well as to evaluate virus-specific antibody responses in ducks receiving an inactivated DFV vaccine.

Materials and Methods

Virus and cell culture

Duck flavivirus (DFV-BZ strain) was isolated from Shandong Province and grown in duck embryo fibroblast (DEF) cells with DMEM (Invitrogen) consisting of 2% calf serum.

Infectivity assay

DFV-BZ infectivity was determined as 50% tissue culture infectious dose ($TCID_{50}$) on DEF cells. In brief, DEF cells were seeded in 96-well plates (2×10^3 cells/well) for 24 h. 200 µL of 10-fold serial dilutions of the stock virus were then added to each of eight replicate wells, and plates were incubated for 1 h. The initial inoculums were removed and the cell monolayers were washed with PBS followed by overlay with DMEM containing 2% heat-inactivated calf serum. The 96-well plates were incubated at 37°C in 5% CO_2 for 5 days, and observed under light microscope for the presence of cytopathic effect (CPE). The $TCID_{50}$ value was calculated using Reed and Muench method.

Serum samples

Serum samples were collected from non-flavivirus-vaccinated domestic egg-laying duck, egg-laying chicken, and pig farms in Shandong, Henan, and Jiangsu provinces of China, respectively. Informed consent was made between Institute of Poultry and Individual Farms in terms of scope of proposed study and any future report of experimental findings involving serum samples from them. Ages of these farm animals during the serum collection were 30 to 32 weeks for ducks, 25 to 27 weeks for chicken, and 30 to 32 weeks for pigs. They were housed in farms close to duck farms where DFV-associated disease outbreaks were observed. Serum samples from vaccinated ducks were collected from ducks receiving two doses of an inactivated DFV vaccine, 4 weeks apart, administered through intramuscular injection route. These ducks had been on vaccination approximately for 10 weeks since the initial immunization. This inactivated vaccine consists of DFV-BZ strain, same strain was used in the seroprevalence study, and aluminum adjuvant. Positive sera

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against duck flavivirus, duck hepatitis virus and duck enteritis virus were prepared using SPF chickens raised and maintained in negative-pressure isolators. All the serum samples were inactivated at 56°C for 30 min for elimination of serum complement.

Fluorescence-based microneutralization assay

The fluorescence-based microneutralization assay for assessment of antibody responses against DFV, employed in this study, is similar to the traditional microtiter serum neutralization assay but use the fluorescence-expressing cells as a readout rather than observation of cytopathic effects present in infected cells. Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) was selected for use to monitor the neutralizing ability of serum in our assay. Before CFSE diffuses into cells, it is colorless and non-fluorescent. However, CFSE will produce highly fluorescent carboxyfluorescein succinimidyl ester right after its acetate groups are cleaved by intracellular esterase in metabolically active cells [11]. This cleavage occurs only in viable cells, and thus the amount of fluorescence-expressing cells is proportional to the presence of viable cells that survive killing of replicating viruses due to neutralizing antibody-mediated protection. Presence of neutralizing antibody is determined as a function of protected cells that emit fluorescence.

Seven 2-fold serial dilutions (1:16-1:1024) were made for each serum sample and equal volumes of each dilution were mixed with equal volumes of virus (100 $TCID_{50}$). After an additional incubation at room temperature (RT) for 1 h, 200 µl of the serum-virus mixtures were transferred to 96-well plates containing 2×10^3 DEF cells each well (seeded 24 h ago). Plates were incubated for 1 h at 37°C, inoculums were then removed and the cells were washed with media followed by overlay with DMEM containing 2% heat-inactivated calf serum. The plates were incubated at 37°C in 5% CO_2 for 5 days followed by CFSE staining. 100 µl of staining solution containing 1.0 µM CFSE was added into the wells of 96-well plates. CFSE will undergo acetate hydrolysis in metabolically active cells (i.e., emit fluorescence) so live cells were easily located by fluorescence microscopy. Neutralization titer was defined as the reciprocal of the log2 of the highest antibody dilution that gave a 50% reduction in fluorescence-expressing cells as compared to cell control. Antibody titers were calculated using Reed and Muench method. Each sample was tested in four replicate wells. Virus control (without serum) and cell control (without virus) wells were included on each plate. Serum samples with antibody titer greater than or equal to 16 are considered seropositive.

Results and Discussion

Seroprevalence of DFV in ducks, chickens, and pigs

We employed the fluorescence-based microneutralization assay to investigate the seroprevalence of DFV in ducks, chickens, and pigs. Duck appears to be the primary host of DFV infection but it is still unclear whether other animal species such as chicken and pig are susceptible to virus infection and seroconversion can occur [1,2]. We included pigs in our study because this animal species has served as animal reservoirs for influenza viruses and other pathogens including some flaviviruses. A total of 903 animal serum samples (201 duck sera, 356 chicken sera, and 346 pig sera) were collected from farms in three major duck-producing provinces (Shandong, Jiansu, and Henan) where outbreaks of DFV infection were documented. These farms that derived serum samples for this study are geographically in close proximity to duck farms afflicted by DFV infection.

Neutralizing antibody (NA) titers (range, 16-1024) were detected in approximately 54.7% of duck serum samples (Table 1). Among these, all duck sera (n=74) from Shandong province had neutralizing antibody (NA) titers (≥ 16), which is consistent with previous studies showing DFV was widely distributed among duck farms in this No.1 duck-producing province in China. 33.8% and 21.4% of the serum samples from Henan and Jiangsu, respectively, contained detectable neutralizing antibodies. This analysis revealed that approximately 16% (17/110) of all DFV antibody-positive duck sera exhibited high neutralizing antibody titers (≥ 512) (Table 2). Interestingly, all chicken sera (n=356) had undetectable DFV NA titers (Table 1). These results suggest that DFV widely circulates in ducks, but not in chickens, in the major duck-producing region of China.

For the swine cohort, four had detectable antibody titers (1:16, 1:20, 1:50, and 1:79). The relatively low titers observed in these four samples (4/346) are inconclusive in terms of determining exposure to DFV viruses in pigs. To exclude cross-reactivity between duck flavivirus and swine Japanese encephalitis virus, we perform a cross-neutralization test for these positive sera between two viruses and did not observe cross-reactivity (data not shown). Further serologic studies focusing on more field pig serum samples representative of geographic and temporal distributions are required toward the final conclusion of DFV infection of pigs. It should be noted that the assay described here has a strong correlation with the traditional CPE-based serum neutralization assay in measuring antibody response, which is based on the parallel analysis of a subset of serum samples. For example, three duck serum samples with titers of 1:89, 1:354, 1:178 measured in fluorescence-based assay, had titers of 1:79, 1:316, 1:158, correspondingly, in the traditional serum neutralization assay (data not shown).

Evaluation of DFV vaccine-mediated antibody response

Since initial disease outbreaks in April 2010, several inactivated DFV vaccine candidates have been developed and used in the field to immunize ducks. These vaccines consist of inactivated whole viral antigens and aluminum adjuvants. However, recurring outbreaks with DFV infection-associated clinical manifestations such as reduction in egg production and neurological disorder were still frequently observed in duck farms receiving vaccinations, suggesting that vaccines may provide an incomplete protection. We noticed that

Table 1: Summary of antibody prevalence of duck flavivirus in ducks, chickens, and pigs.

Region	Ducks positive/ tested (%positive)	Chickens positive/ tested (%positive)	Pigs positive/tested (%positive)
Shandong	74/74 (100%)	0/124 (0%)	2/116 (1.7%)
Jiangsu	12/56 (21.4%)	0/110 (0%)	1/118 (0.85%)
Henan	24/71 (33.8%)	0/122 (0%)	1/112 (0.89%)
Total	110/201 (54.7%)	0/356 (0%)	4/346 (1.2%)

Table 2: Distribution of neutralizing antibody titers in 110 positive duck sera.

Antibody titers	Number of duck sera
Less than 50	16 (14.5%)
50-128	51 (46.4%)
128-512	26 (23.6%)
More than 512	17 (15.5%)
Total	110

a functional neutralizing assay has not been used for assessment of vaccine's efficacy because the assay was not developed at that time. In this study, we sought to use the functional assay described here to assess antibody titers in a collection of 105 sera from ducks that received two doses of the inactivated DFV vaccine. Despite that approximately 50% (53/105) vaccinated ducks exhibited a robust antibody response (≥ 128), there is a still significant portion of vaccinated animals (about 27%) whose titers were either low (1:16 to 1:50) or undetectable (Table 3). The data suggested that the inactivated vaccines currently used in duck farms might not elicit a robust neutralizing antibody response that is normally associated with a sterilizing immunity. This vaccine-mediated suboptimal antibody response may help to explain the recurring disease outbreaks that still afflict duck industry, despite that the vaccination had been implemented region-wide in China. Our result also calls for a need to generate a more effective vaccine that can provide an optimal protection against infection of ducks by DFV.

Flaviviruses are a group of pathogens that have significant public health and economic impacts worldwide [12]. Most of them including DFV are thought to be mosquito-borne and have the zoonotic potential [2]. Since its discovery, seroprevalance of DFV in farm animals is not clear. With the cell-based fluorescence microneutralization assay, we demonstrated that DFV infection has become widespread in ducks over the past two years. Interestingly, we did not find serological evidence of DFV infection in chicken because all the surveyed chickens were negative in DFV antibody detection. It should be noted that under experimental settings, chickens inoculated with DFV by intramuscular route showed seroconversion to the virus without any clinical signs (data not shown). The observed discrepancy suggests that chicken might not be susceptible to DFV infection under a natural condition. In addition, in a most recent study, Liu and colleagues provided some evidence indicating that a virus; having high sequence identity with DFV, can replicate and cause diseases in chickens and geese [10]. It will be interesting to determine the full-genome sequence of this novel virus and define the genetic basis of the observed difference in viral tropism in domestic birds. Based on this result and the limited exposure of DFV to pigs, we speculate that this novel flavivirus may not have a broad host range. Further studies involving more samples representative of geographic and temporal distributions are needed to confirm this hypothesis. To address this issue will provide novel insights to the transmission mode and mechanism of this newly discovered virus.

Currently, there is no effective vaccine for control and prevention of DFV infection in ducks. The disease has a devastating impact on duck industry in China and its pathogen DFV has the potential to spread to other regions of world. The described cell-based fluorescence microneutralization assay in this study should aid in the development of an effective vaccine and provide a tool for antibody prevalence study.

Table 3: Distribution of neutralizing antibody titers in duck receiving an inactivated duck flavivirus vaccine.

Antibody titers	Number of duck sera
Less than 50	28 (26.7%)
50-128	24 (22.9%)
128-512	30 (28.6%)
More than 512	23 (21.9%)
Total	105

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