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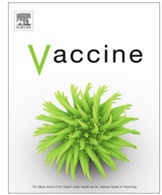

Rapid development and evaluation of a live-attenuated QX-like infectious bronchitis virus vaccine

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ABSTRACT

Infectious bronchitis (IB) is an acute, highly contagious disease, which causes economic losses to the poultry industry worldwide. To control the disease, biosecurity and vaccination are required. In the current research, we rapidly attenuated a QX-like IBV field strain ZYY-2014 using passage in embryos at limiting dilution and tested the safety and efficacy of the attenuated Chinese QX-like IBV strain ZYYR-2014 in 1-day-old specific-pathogen-free (SPF) chickens through spray route. Our result revealed that the attenuated strain presented a decreased pathogenicity in 1-day-old chickens. The strain ZYY-2014 inoculated birds presented typical IBV clinical signs with a mortality of 43%, while the attenuated strain ZYYR-2014 inoculated birds remained healthy. The strain ZYYR-2014 also presented stronger antibody responses and lower viral loads in tracheas, lungs and kidneys. When vaccinated through spray route into 1-day-old SPF chickens, our data suggest a potential of the attenuated ZYYR-2014 strain as a vaccine candidate applied in hatchery, which can contribute in preventing the QX-like IBV infections. Furthermore, attenuation by passage at limiting dilution could be applied for rapid vaccine development against emerging strains.

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1. Introduction

Infectious bronchitis (IB) is an acute, highly contagious disease affecting respiratory, renal, and reproductive systems of chickens, thus resulting in egg production drop, a decline in carcass weight and bird death. Since its first identification in 1931, the disease has been circulating in different regions of the world, causing severe economic losses [1,2].

The characterized pathogen of IB is infectious bronchitis virus (IBV), which belongs to the genus *Gammacoronavirus* in the family *Coronaviridae*, order *Nidovirales*. The virus genome is a linear, single-stranded, positive-sense RNA with a length of approximately 27 kilobases (kb) [3]. Genes encoding four structural proteins are identified in the genome and designated as spike (S), envelope (E), matrix (M), and nucleocapsid (N). Among the four structural proteins, the S protein, consisting of S1 and S2 subunits, plays an important role in receptor binding, virus fusion and antigenic neutralization [4-6]. Furthermore, small changes (<5%) in amino acid sequence of the S1 protein may affect cross-protection [7], thus contributing to complexity of disease epidemiology and control.

Vaccination is considered to be the most effective way to control the disease. Various live attenuated vaccines and inactivated vaccines derived from classical serotypes are often used in the field, showing little or no cross protection against heterologous IBV challenges when used alone [8,9]. Effective strategies including applications of vaccines containing different antigenic types and multiple immunizations could provide broad protection [9]. However, due to high evolutionary rates, virus replication errors and recombination [10,11], emerging IBV strains belonging to different genotypes and serotypes have been reported worldwide [12]. Facing the complex situation of IBV infections, effective vaccines to control the epidemic disease are highly required [13].

Since the first detection of QX-like or GI-19 [14] IBV strain in China in 1996, the QX-like IBV has become the predominant genotype in China. An epidemiological investigation of IBV isolates collected from 2013 to 2015 showed that 46.1% of the IBV strains isolated were characterized as QX-like genotype [15]. Not restricted to China, the QX-like genotype has spread widely to other countries in Asia, Africa and Europe [12]. Current vaccine strains used in China are derived from classical strains of Mass genotype. However, phylogenetic analysis indicates that the QX-like genotype is genetically distant from the Mass-type, which may explain the poor performance of these classical vaccines [16]. In Europe, QX-based vaccines were developed [17] and commercial vaccines such as POULVAC IB QX (Pfizer, France) are produced against endemic IBV

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strains. In China, there were also several attempts to develop an efficient QX-like IBV vaccine through serial passages of field isolates in embryonated specific pathogen free (SPF) eggs, take YX10-D90, aYN and P110 for instance. These vaccine strains were proved to be efficient in SPF chickens through intranasal vaccination [18–20]. However, the process of attenuation requires a long passage time in embryonated eggs, which usually takes up to 1-year, thus is time consuming.

To rapidly develop an effective vaccine, which could be applied in hatchery through spray, we developed a QX-like attenuated strain through rapid attenuation by embryo passage at limiting dilution in the current study. This method was shown to be able to attenuate the virulence of field strain within 5 passages. The attenuated strain presented a reduction of virulence. Furthermore, safety and efficacy tests in 1-day-old SPF chickens through spray route showed that, with a desired level of immunogenicity, the attenuated strain could be used as a promising vaccine candidate against IBV infection.

2. Materials and methods

2.1. Virus isolation

The IBV QX-like strain ZYY-2014 (GenBank accession no.: MG544178) was isolated from an H120 vaccinated flock presenting IB symptoms in Guangdong province, China, 2014, as described before [21]. Briefly, collected kidneys were homogenized and centrifuged at 11.5*g* at 4 °C for 10 min. The supernatant was filtered through 0.22 μm cellulose esters membranes (Merck Millipore Ltd., Ireland) and inoculated into 10-day-old SPF chicken embryonated eggs via allantoic cavity route with a quantity of 0.2 ml and the eggs were incubated at 37 °C for 48 h. Embryos that died within 24 h of inoculation were discarded, and lesions of embryos in the rest of the eggs were monitored. After 48 h, allantoic fluids were harvested under sterile condition for subsequent passages. After five passages, the allantoic fluid was examined using reverse transcription polymerase (RT-PCR) to detect the virus. The embryo 50% infectious doses (EID_{50}) were calculated by the Reed and Muench method.

The IBV QX-like strain HSJ-2016 (GenBank accession no.: MG544176) was isolated from H120 vaccinated broiler flock as well in Guangdong province, 2016. It was used as a homologous IBV stain to determine the efficacy of the attenuated ZYY-2014. The M41 strain was used as a heterologous challenge strain.

2.2. Limiting dilution passage attenuation

The EID_{50} for the strain ZYY-2014 was $10^{-6.39}$ EID_{50}/0.2 ml. According to the EID_{50}, the allantoic fluid of embryonated eggs inoculated with ZYY-2014 was diluted 10^5, 10^6, 10^7 times. The EID_{50} of the virus of the first passage was $10^{-6.65}$/0.2 ml. For the 2nd passage, the allantoic fluid of embryonated eggs inoculated with the virus of the first passage was diluted 10^5, 10^6 and 10^7 times. The diluted allantoic fluid was inoculated for the next passage. For the 3rd passage, the allantoic fluid was diluted 10^6, 10^7 and 10^8 times, since the EID_{50} was $10^{-7.00}$/0.2 ml. The diluted allantoic fluid of each dilution ratio was inoculated into embryonated eggs (5 eggs for each dilution) via allantoic cavity route at a quantity of 0.2 ml/egg and the eggs were incubated at 37 °C for 48 h and the embryos that died within 24 h of inoculation were discarded. After 48 h, the allantoic fluid of each inoculated embryonated egg was collected and examined for the presence of IBV by RT-PCR. The allantoic fluid from the egg inoculated with the highest dilution ratio that was positive for IBV was diluted and used for the next dilution passage. The EID_{50} of the passaged virus was calculated by the Reed and Muench method. After five passages, the allantoic fluid positive for IBV was collected and the virus strain was cultured and designated as ZYYR-2014. The EID_{50} of the strain ZYYR-2014 was calculated by the Reed and Muench method.

2.3. RNA extraction and molecular characterization

Viral RNA was extracted from allantoic fluids applying Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. RT-PCR was performed using a PrimerScript™ one step RT-PCR kit Ver.2 (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instruction. PCR primers for IBV detection were designed specifically according to the conserved sequence of the *S1* gene (forward-primer: TTGAAAACTGAACAAAAGACCG, reverse-primer: TACAAAACCTGCCATAACTAACAT), resulting in a product of about 1740 bp. The amplified *S1* genes of strain ZYY-2014 and ZYYR-2014 were sequenced by BGI (BGI Shenzhen, Shenzhen, China) and analyzed together with *S1* gene sequences of 25 other IBV strains of different genotypes from GenBank (Supple. Table 1) using Clustalx alignment (DNASTAR Inc., Madison, Wisconsin, USA). Phylogenetic tree was constructed by the neighbor-joining method utilizing MEGA6.0 (http://www.megasoftware.net). Bootstrap values were determined from 1000 replicates of the original data.

2.4. Safety test

A total of 120 1-day-old SPF chickens were assigned to 4 groups (30 birds/ group). The experimental groups were intranasally inoculated with 200 μL of $10^{4.5}EID_{50}$ of the ZYY-2014 or ZYYR-2014 strains. Vaccine strain YX10-D90 at a dose of $10^{4.5}EID_{50}$ was used as a positive control. The control group was inoculated with 200 μL of phosphate buffered saline (PBS). All birds were reared in isolators with positive pressure in air-conditioned rooms. After inoculation, all birds were monitored daily for clinical signs such as hunched posture, depression, diarrhea, soiled vent etc. up to 21 days. At day 21, sera of 15 of the birds in each group were collected and antibodies were tested using commercial enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instruction (IDEXX, Westbrook, Maine, USA). Necropsies were performed in all birds. Lesions in trachea like punctate hemorrhages and catarrhal exudates were monitored and noted. Nephritis was characterized by pale and marbled kidneys with urate deposits in the ureters and cloaca. Tracheas and kidneys from birds that died and 3 other randomly selected birds in each group were collected and further processed for histopathology and electron microscopy.

2.5. Virulence reversion test

Ten 1-day-old SPF chickens were divided into 2 groups with 5 chickens per group. At day 1, the inoculated group was intranasally inoculated with 200 μL of $10^{4.5}EID_{50}$ containing the ZYYR-2014 strain and the control group was inoculated with 200 μL of phosphate buffered saline (PBS). At 5 day-post-infection (dpi), the tracheas and kidneys were collected and tissue homogenates were centrifuged at 11.5*g* at 4 °C for 10 min. The supernatant was intranasally inoculated into the next group of 5 chickens at a dose of 0.2 ml/bird via eye-drop. IBV was detected in tissue homogenates using RT-PCR with the primers described before. After 5 passages, chickens inoculated with the supernatant of the tissue homogenates were monitored daily up to 21 days. Tracheas and kidneys of 3 randomly selected birds were collected and processed further for pathological examination. At 5 dpi of each passage, tracheas and kidneys from all birds were collected. Viral RNA was extracted from the collected organs and the *S1* genes was sequenced as

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