Vaccine 33 (2015) 1916–1922

Contents lists available at ScienceDirect

Vaccine

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

# Assessing the efficacy of an inactivated chicken anemia virus vaccine

Xinheng Zhang[a,b], Boliang Wu[a,b], Yuanjia Liu[b,c], Weiguo Chen[a,c,d], Zhenkai Dai[a,d], Yingzuo Bi[a,b], Qingmei Xie[a,b,c,d,*]

[a] College of Animal Science, South China Agricultural University, Guangzhou 510642, PR China
[b] Key Laboratory of Chicken Genetics, Breeding and Reproduction, Ministry of Agriculture, Guangzhou 510642, PR China
[c] Key Laboratory of Animal Health Aquaculture and Environmental Control, Guangzhou 510642, PR China
[d] South China Collaborative Innovation Center for Poultry Disease Control and Product Safety, Guangzhou 510640, PR China

ARTICLE INFO

*Article history:*
Received 24 December 2014
Received in revised form 14 February 2015
Accepted 25 February 2015
Available online 8 March 2015

*Keywords:*
Chicken anemia virus
Vaccine development
Pathogenesis
Avian immunology

ABSTRACT

*Background:* Chicken anemia virus (CAV) is an immunosuppressive virus that causes chicken infectious anemia (CIA) which is a highly contagious avian disease. CAV causes major economic losses in the poultry industry worldwide. The current CAV vaccine is a live attenuated strain administered in the drinking water that risks horizontal infection of other chickens. The purpose of this study was to develop a novel vaccine against CAV that can be administered safely using a highly pathogenic isolate inactivated with β-propiolactone hydrolysis that would protect chicks from CAV.
*Methods:* Hens were vaccinated twice intramuscularly with a novel CAV GD-G-12 inactivated vaccine and the humoral immune responses of the hens and offspring were monitored by ELISA. A heterologous intramuscular challenge using the CAV strain GD-E-12 was conducted in the chicks hatched from vaccinated or unvaccinated hens.
*Results:* The vaccine strain, GD-G-12, was shown to be highly pathogenic prior to inactivation evidenced by thymic atrophy and bleeding, and weight loss. The inactivated vaccine was considered safe and showed no signs of pathogenicity. High titers of CAV specific antibodies were detected in the vaccinated hens and in their chicks, indicating vertical transfer of maternal antibodies. Furthermore, the chicks hatched from vaccinated hens were resistant to a heterologous CAV challenge and showed no signs of weight loss and thymic atrophy and bleeding.
*Conclusion:* Our studies are proof of principle that inactivated GD-G-12 might be a novel vaccine candidate to prevent CAV infection, and highlight the utility of using an inactivated virus for this vaccine.


## 1. Introduction

Chicken anemia virus (CAV) is a common immunosuppressive virus that causes thymic atrophy, aplastic anemia, muscle and subcutaneous tissue hemorrhages in young chickens, although chickens of all ages are susceptible to infection [1]. CAV belongs to the genus *Gyrovirus* in the *Circoiridae* family [2]. CAV capsids contain three viral proteins (VP1, VP2, and VP3). VP1 is the major capsid protein [3], VP2 is a scaffolding protein that helps VP1 form the correct conformation and exposes its neutralizing epitopes. Both VP1 and VP2 are targets for neutralizing antibodies [4]. VP3 is a non-structural protein named apoptin. CAV is transmitted through contaminated feathers, and triggers apoptosis in thymocytes and myeloid progenitor cells in young chickens [5]. CAV was first reported by Yuasa from contaminated vaccines in Japan in 1979 [6]. Chicken is the only recognized natural host, but a serological survey demonstrated that the disease is prevalent in domestic and wild birds [7]. CAV is disseminated both vertically and horizontally, and can be present in a latent state in commercial chickens [8].

CAV is an economically important avian pathogen globally due to mortality related to CAV-mediated immunosuppression, which leads to increased mortality due to secondary infections and a poor immune response to other vaccines [9]. CAV outbreaks have been reported in commercial flocks from South Korea [10], Argentina [11], Nigeria [12], Hungary [13], and other countries. In China, CAV was first recognized in 1992 in broilers [14] and is now widely distributed in three Chinese provinces Anhui, Hunan, and Jiangsu [15]. In a survey of domestic poultry farms in five Chinese provinces (Guangdong, Beijing, Zhejiang, Shanghai, and Tianjin) showed that the seroprevalence of CAV was approximately 42%

* Corresponding author at: South China Agricultural University, College of Animal Science, Tianhe Wushan, Guangzhou 510642, Guangdong, PR China.
Tel.: +86 020 85280283; fax: +86 020 85280740.
*E-mail address:* qmx@scau.edu.cn (Q. Xie).

http://dx.doi.org/10.1016/j.vaccine.2015.02.066
0264-410X/

[16], suggesting it is imperative to develop an effective vaccine against CAV.

We have previously analyzed the phylogenetic and molecular characteristics of CAV circulating in Southern China (2011 to 2012), and demonstrated that CAV was highly prevalent in southern China [17]. One of the major determinants of CAV virulence is the amino acid at position 394 in VP1; highly pathogenic isolates tend to have glutamine at this position, while less pathogenic isolates contain histidine [18]. Two of the strains we identified, GD-G-12 and GD-E-12, have a glutamine at position 394. However, the virulence of these viruses *in vivo* has not been characterized. If one of these isolates is pathogenic, *in vivo* it may be useful as a candidate for making an inactivated vaccine that could be protective against virulent CAV strains.

The purpose of this study was to characterize the pathogenicity of GD-G-12, and to develop an inactivated CAV vaccine to prevent transmission. The results of this study may provide significant insights into the use of an inactivated CAV vaccine in SPF hens before eggs are laid, and establish the value of further evaluating the efficacy of the inactivated vaccine.

## 2. Materials and methods

### 2.1. Viruses

Two CAV strains GD-G-12 (GenBank accession number: KF224931) and GD-E-12 (KF224929) were isolated from a commercial farm in southern China and have been previously described [17]. The viruses were propagated in MDCC-MSB1 cells in RPMI-1640 medium supplemented with 10% fetal calf serum plus, 100 μg/mL kanamycin sulfate, and 1 μg/mL Fungizone. The cells were cultured in bottles in an incubator at 37 °C.

### 2.2. Quantifying virus titer using qPCR

To make the assay quantitative a standard was prepared. Two primers JCP1: (5′-CATCAACGGTGTTCAGGC3′) and JCP2: (5′-CCTTGGAAGCGGATAGTCAT-3′) were used to amplify a 535 base pair PCR product from the partial coding regions of CAV [19]. The reaction product was analyzed on 1% agarose gels and ligated into a PMD19-T vector (TaKaRa, Biotechnology, Dalian, China). The PMD19-T vector was then used to transform TOP10 competent cells. The resulting plasmid PMD19-T was purified from the transformed cells using a Plasmid Midi Kit (Omega Bio-Tek, USA). The DNA concentration was calculated by measuring the absorbance at 260 nm. Using the DNA concentration, the copy number of the plasmid was calculated using the following formula: copy/μL = $6.02 \times 10^{23}$ (copy/mol) × DNA concentration (g/μL)/MW (g/mol). Serial 10 fold dilutions from $10^4$ to $10^{10}$ copies of the purified plasmid were prepared in duplicate to produce a standard curve ($y = -3.330 \lg x + 41.99$, $R^2 = 1.000$).

The viruses used in our study are not cytopathic in MDCC-MSB1 cells, therefore, SYBR green-based qPCR was used to determine the number of copies of the CAV genome in the inocula. For the qPCR virus titer assay, the primers QF1 (5′-GAATGTGCCGGACTTGAGGA-3′) and QR1 (5′-GGTCGCAGGATCGCTT-3′) [20], each at a final concentration of 0.6 μL, were used to amplify a 65-base-pair fragment of CAV VP2. After optimization of the qPCR conditions, 10 μL of SYBR Green qPCR Mix (ROX) (Roche, Switzerland) and 1 μL of extracted DNA or dilutions of the plasmid standard were assayed in duplicate. QPCR is a novel strategy for quantifying the virus titer [21].

### 2.3. Testing the pathogenicity of the GD-G-12 isolate in chickens

These experiments were carried out strictly in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The use of the animals in this study was approved by the South China Agricultural University Committee of Animal Experiments (approval ID 201004152). One day old specific-pathogen-free (SPF) chickens ($n = 40$) confirmed to be free of CAV antibodies were purchased from Guangdong Dahuanong Animal Health Products Co., Ltd. The chickens were randomized to one of two groups. Group I ($n = 20$) was inoculated intramuscularly with 1 mL ($10^{16}$ copy/μL) of GD-G-12 isolated from cell culture inoculum. Group II ($n = 20$) were the negative control chicks and were not inoculated with CAV. Each group was reared separately in different Horsfall-type isolation chambers over a 21-day period. Three randomly chosen chicks were sacrificed from each group at 7, 14, and 21 days post inoculation. The body was measured and the thymus was collected. Thymus samples collected from infected and control chicks were processed for pathology.

### 2.4. Preparation of the inactivated vaccine

MDCC-MSB1 cells were infected with GD-G-12 and maintained in 1640 medium with fetal bovine serum (FBS) at 37 °C then the virus was harvested. The culture was subjected to three freeze-thaw cycles and then clarified by centrifugation at 4500 rpm for 10 min at 4 °C. The supernatant was filtered through a 0.22-μm filter. The virus was inactivated was by treating the virus-containing supernatants with a 4/1000 volume of β-propiolactone hydrolysis (Serva Electrophoresis, Heidelberg, Germany) for 24 h at 4 °C, then incubated at 37 °C for 2 h to allow for β-propiolactone hydrolysis. We chose to use an oil adjuvant to develop the inactivated vaccine.

### 2.5. Assessing the safety of the inactivated vaccine

A total of 60 SPF hens 91 days of age confirmed free of CAV antibodies were purchased from Guangdong Dahuanong Animal Health Products Co., Ltd. and divided into 2 groups ($n = 30$/group). The birds in the experimental groups were inoculated intramuscularly with 1 mL of inactivated vaccine ($7.9 \times 10^{17}$ copy/μL) while the control group received 1 mL of phosphate buffered saline (PBS). Three randomly chosen hens were sacrificed from each group at 7, 14, 21, 28, and 42 days post immunization to collect the thymus. The inactivated vaccine was considered safe if the hens did not have clinical symptoms and no pathological changes were observed in the thymus.

### 2.6. Assessing vaccine efficacy

Vaccine efficacy was tested by measuring the levels of maternal CAV antibodies passed from vaccinated hens to their chicks. Ninety SPF hens at one day old confirmed free of CAV antibodies were purchased and divided into two groups ($n = 30$/group). Hens in the experimental group were vaccinated intramuscularly with 1 mL of the inactivated vaccine ($7.9 \times 10^{17}$ copy/μL). The control group was inoculated with 1 mL of PBS. Fourteen days post-vaccination, the vaccinated hens received a second immunization with 1 mL of the inactivated vaccine ($7.9 \times 10^{17}$ copy/μL). CAV specific antibody production was tested in the sera using an enzyme-linked immunosorbent assay (ELISA) (IDEXX, USA) every 7 days at a 1:100 dilution. The results were analyzed using the xChek software. The optical density was read at 650 nm wavelength on an ELX 800™ microplate reader (BIO-TEK Instruments, USA). Forty-two days post-vaccination, fertilized eggs were collected from each group, incubated, and hatched in an automatic incubator (Yiai, Qingdao) under appropriate conditions. After the chicks had successfully hatched (approximately 21 days), chicks ($n = 80$) from vaccinated hens and control hens were divided into two sub-groups and placed into separate negative pressure chicken disconnectors.