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# Development and evaluation of indirect ELISAs for the detection of IgG, IgM and IgA1 against duck hepatitis A virus 1

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*Duck hepatitis A virus 1* (DHAV-1) is the principal pathogen that causes duck viral hepatitis (DHV), a highly fatal infectious disease in ducklings. Given the importance of the humoral immune response in the clearance of DHAV-1, indirect enzyme-linked immunosorbent assays (I-ELISAs) to detect immune indices, including IgG, IgM and IgA1, were developed and evaluated in this study. The optimal concentrations of coating-antigen were 1.79 μg/ml, 2.23 μg/ml and 2.23 μg/ml for IgG, IgM and IgA1, respectively. Meanwhile, the optimal dilutions of sera were 1:80, 1:40 and 1:40, respectively; and of the conjugates were 1:300, 1:1800 and 1:800, respectively. Based on these conditions, three linear regression equations, $y = 1.363 + 1.954x$ ($r^2 = 0.983$), $y = 1.141 + 2.228x$ ($r^2 = 0.970$) and $y = 1.103 + 1.559x$ ($r^2 = 0.995$) were derived for IgG, IgM and IgA1, respectively. Analytical sensitivities of the new methods were 1:2560, 1:1280 and 1:640 for IgG, IgM and IgA1, respectively. The concordances between the I-ELISAs and serum-neutralization were 95.2% for IgG and IgA1, and 75% for IgM. Although there was a weak cross-reaction with DHAV-3 positive serum for the IgG and IgA1 tests, it didn't affect the ability to detect DHAV-1 specific antibodies. Thus, these new I-ELISAs were shown to be potentially convenient methods to survey the status of humoral immune response to DHAV-1.



## 1. Introduction

Duck viral hepatitis (DVH) is an acute and highly mortal contagious disease in ducklings. It is characterized by swollen livers mottled with hemorrhages and liver necrosis (Tsai, 2013), and is caused mainly by the globally common duck hepatitis virus (DHV) type 1 that was first reported in Long Island, New York, by Levine and Hofstad in 1945 and that later spread to England, Germany, Canada, Japan and elsewhere (Levine and Hofstad, 1945). In mainland China, the disease was first noted in 1963, while the pathogen was formally identified in 1984 as DHV-1 (Guo and Pan, 1984), more recently rechristened as duck hepatitis A virus 1 (DHAV-1), was categorized into a new genus, *Avihepatovirus*, in the family *Picornaviridae* (Fu et al., 2008; Tsai, 2013). To date, DHAV-1, as the most virulent and widespread cause of duck hepatitis in China, has gravely threatened the duck industry and caused severe economic losses (Hu et al., 2016; Wen et al., 2014).

Interestingly, young ducklings infected with DHAV-1 have a high mortality rate, while adult breeders in infected premises do not become clinically ill and continued to have full production. This observation is mainly based on the fact that the fully developed adaptive immune system of adult ducks that can produce vast quantities of antibodies, including the IgG, IgM and IgA classes, quickly (Lundqvist et al., 2006). IgG and IgM have been reported in various duck secretions (Higgins and Warr, 1993). IgG is the dominate serum antibody class in ducks, which can prevent viral particles from passing from mucosal surfaces into systemic

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circulation (Lundqvist et al., 2006; Magor, 2011). A high titer of virus-specific IgG class often accompanies with continuing viral replication, and can also be observed in chronic infection. IgM is the only class of antibody that is found in all vertebrates and is produced first during an immune response. Although it serves a transient role in an immune response, it can be used as a reliable and sensitive marker of recent and continuous virus infection (Magor, 2011; Seriwatana et al., 2002). IgA, as the second most prevalent serum antibody and the prominent antibody in external secretions, mediates a variety of protective functions through interactions with specific receptors and immune mediators (Woof and Kerr, 2006). The liver plays a central role in the metabolism of IgA, and therefore, the detection of IgA in serum is primarily associated with liver injury and its presence seems to reflect the immunopathological events occurring in DHAV-1 infection. It has been reported that specific IgA in duck sera can be detected (Ferreira et al., 2010). Detection of HAV-specific IgA and IgM antibodies during the acute stage of infection have been reported and applied in the clinical laboratory (Roggendorf et al., 1980; Yoshizawa et al., 1980); however, detection of anti-DHAV-1 IgM and IgA remains to be developed.

Polymerase chain reaction (PCR) has been widely used for the identification of DHAV-1 infection (Anchun et al., 2009; Fu et al., 2008; Kim et al., 2007, 2008); however, a serological method that detects virus-specific antibodies would be of considerable value for monitoring the prevalence of infection. Neutralization tests are generally utilized for virus-specific antibody determinations, but the technique is time-consuming because it needs experienced technicians to examine the cytopathic effects (Hwang, 1969; Sandhu et al., 1992). Since the ELISA was developed (Engvall and Perlmann, 1971), it has been widely used to detect antibodies for surveillance of infectious diseases or to measure antibody titers following vaccination. In recent years, indirect ELISAs using the VP1 and VP3 proteins of DHAV-1 as coating antigens to detect virus-specific IgG have been established (Liu et al., 2010; Shen et al., 2015). This approach is more rapid, simple and practical than the neutralization test (Zhao et al., 1991). Nonetheless, these assays do not contain information regarding the overall humoral responses to DHAV-1. ELISA results are usually expressed as the endpoint titer of a full dilution curve and as a direct OD value at a single dilution. However, obtaining an endpoint titer requires serial dilution of all test samples, which is a time-consuming and labor-intensive process; meanwhile, the precision of a data generated using single dilutions is difficult to determine considering the detection limit (Miura et al., 2008). It has been demonstrated that there is a linear relationship between the absorbance at optimal dilution of samples and the endpoint titers (Hatfield et al., 1987), which could avoid the disadvantages of both endpoint titers and single dilutions.

In the present study, comprehensive indirect ELISAs were developed and evaluated for the detection of three antibody classes (IgG, IgM and IgA1) specific to DHAV-1. Furthermore, linear regression equations based on these well-developed indirect ELISAs were also generated and assessed. These newly established indirect ELISAs and their associated equations could be used to investigate the systemic immunological responses induced by DHAV-1 and to survey DHAV-1 infection.

## 2. Materials and methods

### 2.1. Antigen preparation

DHAV-1H strain (GenBank: JQ301467.1) was selected for the study and was propagated in 11-day-old duck embryos using standard procedures. The allantoic fluid of duck embryos that died at 36–72 hpi was collected and then purified through three freeze-thawing cycles and differential centrifugation. After low-speed centrifugation (12,000*g*) at 4 °C for 30 min, the supernatant was centrifuged at 67,000 g for 1 h and the 120,000 g for 2 h at 4 °C. Then, the pellets were re-suspended in 1 mL sterile PBS and stored at −80 °C until used.

### 2.2. Duck serum samples

Positive serum specific to DHAV-1 was obtained from breeding Peking ducks (*Anas platyrhynchos domesticus*) that were free of DHAV-1 pathogen, as confirmed by RT-PCR, and were then experimentally infected intramuscularly with DHAV-1H strain suspension. Negative sera were collected directly from DHAV-1-free ducks. These ducks were hatched in isolation under the Laboratory Animal Management Regulations of Sichuan Agriculture University and handled in strict accordance with the Guide for the Committee of Experiment Operations and Animal Welfare of Sichuan Agriculture University, China (approval number: XF2014-18). All of the serum samples were collected from the jugular vein and preserved in a sterile manner at −80 °C until used. Anti-sera against several other common duck-sensitive antigens including duck plague virus (DPV), duck swollen head septicemia virus (DSHSV), *Riemerella anatipestifer* (RA), avian influenza virus (AIV), *Escherichia Coli* (*E. coli*), *Salmonella anatum*, gosling new type viral enteritis virus (NGVEV) and duck hepatitis A virus 3 (DHAV-3) were stored in our lab and were used to confirm the specificity of the new indirect ELISAs.

### 2.3. Preparation of HRP-labeled anti-duck antibodies

The presence of serum-specific IgG, IgM, and IgA1 against DHAV-1 were determined using goat anti-duck IgG-horseradish peroxidase conjugate (KPL, Gaithersburg, Maryland, USA), goat anti-duck IgM-horseradish peroxidase conjugate (Antibodies-online, Shanghai, China) and mouse anti-duck IgA1 (Abd Serotec, Kidlington, UK) labeled with the Lighting-Link$^{TM}$ HRP Conjugation Kit (Innova Biosciences, Cambridge, UK) according to the standard protocol.

### 2.4. ELISAs for detecting anti-DHAV-1 antibodies

A checkerboard titration in 96-well ELISA plates (Corning, New York, USA) was implemented to optimize the coating concentrations of antigen and the optimum dilutions of the serum samples and of conjugates according to the ELISA guidebook (Crowther, 2000), with some modifications. Each well of the plate was coated with 100 μL of purified antigen that had been diluted as 1:200, 1:300, 1:400, 1:500, 1:600, 1:800, 1:1000, 1:1200, 1:1600 or 1:2400 in 0.1 M carbonate buffer (pH 9.6) and incubated at 4 °C overnight. The plate was rinsed with 0.05% Tween 20 in PBS (pH 7.4), 300 μL per well, four times for 5 min each. Identical washing procedures were performed as required throughout the ELISA. The wells were blocked with blocking buffer, 200 μL per well at 37 °C for 2 h. Serum samples that were diluted from 1:20 to 1:10240 by a two-fold serial dilutions in sample diluent were dispensed in triplicates and incubated at 37 °C for 1 h. IgG detection was performed by using a conjugate of HRP-labeled goat anti-duck IgG. The conjugate was optimized with two-fold serial dilutions (1:150 to 1:1200) and incubated at 37 °C for 1 h. The plate was colorized by addition of TMB (Tiangen, Beijing, China) with 100 μL per well and incubated at 37 °C for 15 min, then the colorimetric reaction was stopped by adding 100 μL of 2 M $H_2SO_4$. The absorption of each well was measured at double wavelengths of 450 nm and 630 nm ($OD_{450}$–$OD_{630}$) with a micro-plate spectrophotometer (Model 680, Bio-Rad). A reaction that gave a maximum difference in optical densities between positive and negative samples (P/N) that was no less than 2.1 was considered optimal (Wu et al., 2011).