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Short communication

# Histamine levels in embryonic chicken livers infected with very virulent infectious bursal disease virus

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ABSTRACT

Histamine is an endogenous nitrogenous compound with extensive effects on immunologic cells and involved in many physiological functions. The current aim was to determine histamine levels in embryonic liver and its association with the pathogenicity of a very virulent infectious bursal disease virus (vvIBDV) isolate serially passaged in chicken embryos. A vvIBDV isolate and the passaged viruses were inoculated into SPF embryonated chicken eggs (0.2 ml per egg) via the chorioallantoic membrane. Embryonic livers were collected at 24, 48, 72, 96, and 120 h post-inoculation and histamine contents were quantified by fluorescence spectrophotometry analyses. Results showed that the histamine content in embryonic livers infected with the original vvIBDV isolate and the early passaged viruses significantly increased 48 h post-inoculation, as compared with the adapted IBDV isolate ($p < 0.01$) and controls ($p < 0.01$), with the concentration peaking from 72 h to 96 h. Most of the infected chicken embryos died from 48 h to 96 h post-inoculation. Moreover, the histamine content in dead embryos was markedly increased compared with live embryos ($p < 0.05$), peaking at 72 h post-inoculation ($p < 0.01$). There was an association between histamine content in embryonic livers and an elevation in histidine decarboxylase activity. Taken together, our results suggest that an excess of histamine correlates with inflammatory responses during vvIBDV infection. This study provides an incremental step in the understanding of the pathogenesis of vvIBDV.



## 1. Introduction

Infectious bursal disease virus (IBDV) is the causative agent of infectious bursal disease (IBD), a highly contagious and immunosuppressive infection among young broiler chickens that results in great economic losses in the poultry industry (Van den Berg et al., 2000). IBDV has two different serotypes: serotypes 1 and 2 (McFerran et al., 1996); only serotype 1 causes IBD in chickens (Ismail and Saif, 1991). Strains within serotype 1 differ in their pathogenicity and antigenicity and are generally classified as very virulent, classical, or antigenic variants. Susceptibility and breed of the chicken flock, types of virus strains, intercurrent primary and secondary pathogens, and environmental and managemental factors all influence the economic impact of IBD (Lukert and Saif, 1997; Van den Berg et al., 2000). Classical virulent IBDV (cvIBDV) strains cause bursal inflammation and severe lymphoid necrosis in infected chickens, resulting in immunodeficiency and 20–30% mortality. Moreover, very virulent IBDV (vvIBDV) strains can cause 60–100% mortality in chickens (Brown and Skinner, 1996). Meanwhile, antigenic variant strains cause rapid atrophy of the bursa without inflammation, hemorrhage, or mortality (Jackwood and Jackwood, 1994; Snyder et al., 1988).

Histamine is an important chemical messenger that causes numerous pathological and/or physiological effects (Shahid et al., 2009). Histamine is classified as an inflammatory mediator (O'Mahony et al., 2011). The large variety of inflammatory and host-defense mechanisms regulated by histamine can be partly explained by the existence of histamine receptors H1R, H2R, H3R, and H4R. By now, the data for chicken histamine receptors are rarely, but histamine receptor H2R was founded in the chicken suppressor T cells, suggesting the histamine receptors are existed and might an important regulator for immune response (Edelman et al., 1987). In addition to its inflammatory actions, histamine induces a variety of effects on immunocompetent cells (Beer et al., 1984; Falus and Merétey, 1992). In the context of the immune system, histamine represent a complex regulatory system (Jutel et al., 2006; O'Mahony et al., 2011), involving the selective recruitment of effector cells into the tissues and the regulation of cellular proliferation, differentiation, maturation, activation and immune response (Bury et al., 1992). As an endogenous biogenic amine,

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histamine is secreted from various cells following the pathogenic challenge (Lojek et al., 2011). Immunohistochemical analyses in chicks demonstrated that histamine was produced in mast cells through the induction of histidine decarboxylase (HDC) in response to inflammatory stimuli and during starvation (Nowak et al., 1997; Endo et al., 1995). Mast cells can be found in all organs and were activated in liver, kidney, spleen, bursa of Fabricius (BF), and thymus of vvIBDV infected chickens (Wang et al., 2008).

Adaptation of IBDV to replication in tissue culture is associated with attenuation (Lange et al., 1987). For instance, the repeated passage of cvIBDV in tissue culture led to the formation of a highly attenuated small plaque phenotype (Müller et al., 1986) that has been used as a live vaccine since. The principal target organ for IBDV is the BF. Although viral antigen has been detected in liver and kidney at early stage of infection, extensive viral replication takes place primarily in the BF (Tanimura et al., 1995; Oladele et al., 2009). Recently we reported on the genetic variability of vvIBDV isolates during serial passage in the chicken embryo (Li et al., 2011), which resulted in increased thickness of the chorioallantoic membrane, hyperemia, and hydrops. As such, the purpose of this study was to assess histamine content associated with inflammatory responses during IBDV infection. This was done by tracking dynamic changes in embryonic liver histamine content among different passages of IBDV field isolates in the chicken embryo and by estimating the correlation of histamine content with pathological changes and pathotypes.

## 2. Materials and methods

### 2.1. Chemical and supplies

L-Histamine was purchased from TakaRa Biotechnology (Dalian) Company (Dalian, China). All reagents were of analytical grade.

### 2.2. Virus strains and their embryo lethal dose$_{50}$

The XA2004 IBDV strain was isolated from field outbreaks in commercial flocks from Xinan County, Henan province, PR China. There were a total of 36 passages performed in embryonated eggs. XA2004-E04, XA2004-E10, XA2004-E16, XA2004-E18, and XA2004-E22 were the passaged viruses with nucleotide sequence variation; the embryo lethal dose$_{50}$ (ELD$_{50}$) of the virus isolates was $10^{-7.00}$/0.4 ml, $10^{-7.40}$/0.4 ml, $10^{-6.35}$/0.4 ml, $10^{-6.00}$/0.4 ml, $10^{-4.77}$/0.4 ml, and $10^{-4.50}$/0.4 ml, respectively (Li et al., 2011). According to the ELD$_{50}$, XA2004-E22 was considered to be an adapted IBDV isolate.

### 2.3. Virus strains infectivity titrations

According to the ELD$_{50}$, the isolate XA2004 and the sequence variations (XA2004-E04, XA2004-E10, XA2004-E16, XA2004-E18, and XA2004-E22) infectivity titers were adjust to $5.0 \times 10^2$ PFU/ml, $5.0 \times 10^2$ PFU/ml, $5.0 \times 10^3$ PFU/ml, $5.0 \times 10^4$ PFU/ml, $5.0 \times 10^4$ PFU/ml, and $5.0 \times 10^5$ PFU/ml, respectively, with normal saline.

### 2.4. SPF eggs

Specific pathogen free (SPF) eggs were purchased from Nanjing Medical University, Nanjing, PR China.

### 2.5. Virus inoculation to SPF chicken embryos

Two hundred and ten 12-day-old SPF-embryonated eggs were separated randomly into seven groups as control and inoculate virus experimental groups ($n = 30$). The embryonated eggs were inoculated with 0.2 ml of the isolate XA2004 and their sequence variations via the chorioallantoic membrane route. Control embryonated eggs were inoculated with equal amounts normal saline. The SPF-embryonated eggs were incubated at 37 °C for 120 h. The number of dead embryos was recorded every 6 h. Six embryonic livers (including dead embryos) were collected at 24 h, 48 h, 72 h, 96 h, and 120 h post-inoculation.

### 2.6. Assay of viral RNA loads in chicken embryonic liver

Viral RNA loads in chicken embryonic livers were assayed by the method of real-time quantitative RT-PCR. Each of the samples (20 mg) were homogenized in a sample tube with 0.5 ml of DEPC-d$H_2O$ and centrifuged at 5000 × *g* for 5 min at 4 °C. The supernatant (0.2 ml) of the homogenate was used to extract total RNA. Total RNA was isolated by Trizol phenol–chloroform extraction (Invitrogen) and ethanol precipitation, following the manufacturer's recommendations. A real-time quantitative RT-PCR was conducted by using a SYBR ExScript$^{TM}$ RT-PCR Kit (Takara Bio, Dalian, China), according to manufacturer's instructions. A pair of primers 5′-GCCGATGATTACCAATTCTCATC-3′ (located at 633–656) and 5′-CATAGTCTGCGGCCACAGCTC-3′ (located at 822–843) were designed based on the consensus sequences of VP2 gene of XA2004 IBDV isolate and the passaged viruses. PCR was performed in an iQ5 Real-Time PCR Detection System (Bio-Rad, USA). Following a denaturation step at 94 °C for 30 s, 35 cycles of amplification were performed at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Data were analyzed according to the threshold cycle (*Ct*) method. To quantitate viral RNA in chicken embryonic livers, concentration of the plasmid pMD19-VP2 was determined using a spectrophotometer. Serial dilutions ranging from $10^9$ copies/μl to $10^1$ copies/μl were used as standard controls. To generate a standard curve, the *Ct* of the standard dilutions was plotted against the number of plasmid copies, which was used as the input.

### 2.7. Assay of histamine contents in chicken embryonic liver

Histidine content in chicken embryonic liver was assayed according to the method described by Ha kanson (Ha kanson et al., 1972). Briefly, each of the embryonic liver samples (50 mg) were homogenized in a sample tube with 1.5 ml of 25% (w/v) trichloroacetic acid, settled for 10 min, and centrifuged at 10,000 × *g* for 10 min at 4 °C. Subsequently, 1.0 ml of the supernatant was diluted to 2.0 ml by the addition of double-distilled water. Then, 0.5 ml of 0.4 N NaOH and 0.1% o-phthaldehyde (OPT) methanol was added, the solution was mixed gently, reacted at 22 °C in darkness for 10 min, and stopped by the addition of 0.5 ml of 0.5 N HCl. A blank was created by combining, in order, 0.5 ml each of 0.5 N HCl, 0.1% OPT methanol, and 0.4 N NaOH. The standard solution of histamine (1.0 mg/ml) was prepared in 0.1 N HCl. Calibration standards were prepared by dilutions of the stock histamine solutions (0–100 μg/ml) with 0.1 N HCl. The fluorescence intensities of the samples were determined by fluorescence spectrophotometry (Shanghai precise scientific instrument Co. Ltd., China) using an activation wavelength of 360 nm and fluorescent monitoring at 450 nm. The histamine concentration was presented as μg/g wet liver sample.

### 2.8. Assay of HDC activity

Histidine decarboxylase (HDC) activity was assayed according to the method described by Shoji (Shoji et al., 2006). Briefly, each of the chicken embryonic liver samples (100 mg) were rapidly removed and placed into a cooled centrifuge tube with phosphorylated cellulose and 1.5 ml of ice-cold 0.02 M phosphate buffer (pH 6.2) containing pyridoxal 5′-phosphate (20 mM) and dithiothreitol