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Effect of infectious bursal disease virus on pathogenicity of avian influenza virus subtype H9N2 in broiler chicks

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In this experiment, pathogenesis of H9N2 avian influenza virus (AIV), experimentally infected with infectious bursal disease virus (IBDV) in broiler chicks was examined. Three groups of twenty were randomly selected. Day old chickens in group 1, were infected by 10^3 CID50 of IBDV intrabursaly, and in thirty days of age groups 1 and 2 were challenged with 10^6 EID50 H9N2, intranasaly-intraocular. Chickens in group 3 remained as control (uninfected with neither IBDV or AIV). Tracheal and cloacal swabs, and tissue samples, were collected at 3, 7, and 11 days postinoculation (PI). Serum samples examined for antibodies against avian influenza virus (AIV) by hemagglutination inhibition test (HI). IBD caused lower H9N2 antibody level. IBDV infected chickens (g1) shed AI virus for a longer period than AIV infected birds (g2), from both trachea and cloac. IBDV was related with AIV in brain and liver. Isolation of AIV from trachea, conjunctiva, bursa and lung in IBDV infected group (1), prolonged till 11 days PI. Our study provides evidence that a previous history of IBDV infection in chickens may cause them to be more susceptible to H9N2 low pathogenic avian influenza (LPAI) virus infection and may alter its tissue tropism.

Key words: Infectious bursal disease, avian influenza, virus shedding, broiler chicks.

INTRODUCTION

Avian influenza (AI) is a highly contagious disease caused by type A influenza virus, a genus of the family *Orthomyxoviridae.* Avian influenza viruses are divided into subtypes on the basis of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) (Swayne and Halvorson, 2008). Seventeen HA (H1 to H17) and ten NA subtypes (N1 to N10) have been identified (Tong et al., 2012, Zhu et al., 2012). Avian influenza (AI) has been reported in many countries from the Middle East region and Asia (Alexander, 2002). In 1998, an H9N2 subtype influenza A virus of low pathogenicity has been reported in the industrial poultry populations of Iran (Vasfi Marandi et al., 1999) and thereafter it has caused outbreaks in commercial broiler chickens in Iran (Nili et al., 2002). Outbreaks of H9N2 subtype also occurred in poultry in Pakistan (Bano et al., 2003). Considerable economic loss due to decreased production, increased mortality and the cost of vaccination have occurred following H9N2 infection in Iranian poultry industry (Vasfi Marandi and Bozorgmehrifard, 1999; Nili and Asasi, 2003). H9N2 influenza viruses are also considered to be one of the potential candidates for the next human pandemic (Butt and Smith, 2005). Therefore, it is imperative to understand the pathogenesis and properties of these viruses.

Infectious bursal disease (IBD), initially reported as

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Gumboro disease, is an acute, highly contagious virus infection of young chickens first described by Cosgrove (1962), who found B lymphocytes to be the primary target cells (Kauffer and Weiss, 1980). IBDV is important because it causes clinical disease and mortality in chickens 3 weeks of age or older and prolonged immunosuppression of chickens infected early in life leading to other infections and vaccination failures (Lukert, 1997). Its immunosuppressive effects were reported by others (Allan et al., 1972). Infection with IBDV reduces antibody response to other vaccinations (Faragher, 1974; Giambrone, 1976; Rosenberger, 1977; Muller, 2003, Westbury 2008), but the response against IBDV itself is normal (Skeeles and Lukkert, 1979). The present study was consequently undertaken to evaluate the effects of experimental IBDV infection in chickens by assessing the humoral responses of chickens to influenza virus subtype H9N2 in addition of its effects on H9N2 AIV pathogenicity for broilers.

MATERIALS AND METHODS

Challenge virus

A very virulent strain of Gumboro virus and avian influenza A virus subtype H9N2 were obtained from Razi Vaccine and Serum Research Institute (Iran), influenza virus was passaged in 9 to 11 days old emberyonated chicken eggs and used as a challenge virus in this study. The embryo infective dose (EID50) of infected allantoic fluid was calculated according to the Reed and Muench formula (1938). The virus was diluted 10 fold in sterile phosphate buffered saline (PBS) solution to obtain concentration of 10^6 EID50 in 1 ml. Ten fold serial dilutions of Gumboro virus was inoculated to 10 groups of five 21-day old chickens for evaluation of chicken infectious dose (CID50) by Reed and Muench method and 10^3 CID50 of diluted virus in sterile PBS was used to the trial.

Experimental design

Sixty one-day-old commercial broiler chicks were divided randomly into three groups, twenty chicks per group. All animal experiments were kept in separated cages in an isolated room and all biosecurity aspects were considered. Feed and water were available *ad libitum*. Day old chicks in group 1 were inoculated with 10^3 CID50 of infectious bursal disease virus intrabursaly. At the age of 30 days, groups 1 and 2 were challenged with 10^6 EID50/0/1 ml of H9N2 virus intraocculary-intranasaly. Birds in group 3 were not infected with neither IBDV or AIV (Table 1).

Table 1. Program of chickens infection in various groups.

Day of age	1	30
Challenge virus	IBDV	AIV
group1	+	+
group 2	-	+
group 3	-	-

Serology

At the days of 8, 29 and 42 days of age serum samples were collected from 10 birds per group and were tested for evaluation of H9N2 antibody titers by hemagglutination inhibition (HI) test. HI tests were performed following World Health Organization (WHO) recommendations (Webster et al., 2002).

Statistics

The mean titre of chickens antibody response was evaluated by 1-way analysis of variance (ANOVA) followed by $Danet_c$ and Tukey test, allowing for statistical comparisons among the different groups.

Duration of viral shedding

Tracheal and cloacal swabs were collected from three chickens per group on days 3,7 and 11 postinocculation with avian influenza H9N2 and stored at -70°C in sterile microtubes containing 1 ml buffered glycerol medium (50% sterile glycerol, 50% PBS) containing antibiotic-antimycotic.

Isolation of influenza virus from various organs

For studying effect of IBD virus on spread of AI virus in tissues samples, a comprehensive group of organs including trachea, lungs, conjunctiva, brain, liver, pancreas, bursa, thymus and kidney were collected from 3 birds per groups at 3, 7 and 11 dpi and samples from each group were pooled. Tissues were homogenized and 10% suspension was prepared by BHI medium. Suspensions were centrifuged at 1500 × g 10 min in 4°C then the supernatant was collected, and antibiotic (1000 IU/ml and streptomycin 2 mg/ml) and amphotericin B (0.02 mg/ml) (Dennis and Senne, 2008) were added. Suspensions of bursa were filtered before adding antibiotic.

Virus isolation

The influenza virus from various organs and swab samples was investigated by virus isolation method in 10-day-old embryonated chicken eggs. Five eggs were used for each tissue or swab sample and 200 µl/egg was inoculated to 10-day-old embryonated chicken eggs. Infected eggs were incubated for 48 h and then chilled at 4°C for no more than 24 h. Allantoic fluid was collected and a hemagglutination (HA) assay was performed. Samples showing agglutination of fresh chicken red blood cells were scored as positive.

RESULTS

Clinical observations

Results of daily monitoring of all groups showed that all chicks were clinically normal and did not show any abnormality prior to inoculation with influenza virus. From day two post-challenge, birds infected AIV started to show clinical signs such as depression, ruffled feathers, respiratory distress (coughing, sneezing and dyspnea), swelling of the periorbital tissues and sinuses, conjunctivitis, nasal and ocular discharge until day six post-inoculation (PI) that sings reduced. Mortality in IBD + AIV group was 33.3%.

Table 2. Mean titer of serum antibody levels against avian influenzavirus subtype H9N2(log2).

Group/day of age	8	29[a]	42[b]
IBD+AIV	5.8	1.15	8.3
AIV	5.8	1.4	10.14
Control	5.8	1.5	1

a = before inoculation of influenza, b=11 days after inoculation of influenza. Serum sampls were exmined by hemaglutination inhibition test to study the effect of gumboro disease on antibody production in chickens against avian influenza virus subtype H9N2. This test was conducted by WHO manual (webester, 2002).

Serology

HI test

There was no evidence of any change in specific antibodies against AIV or IBDV pre and post inoculation of control chickens. Mean antibody titers against influenza virus on the basis of log2 are shown in Table 2. 11 days after inoculation with influenza virus (42th days of age), significant differences were seen between IBD + AIV inoculated birds and AIVs. Results shows that infectious bursal disease can cause significant decrease of antibodys against H9N2 AI virus.

Duration of viral shedding

Chickens co-infected with AIV + IBD (group1) shed H9N2 AIV from day 3 to days 11 PI, while chickens in group 2 (AIV inoculated) shed the virus from day 3 to 7 PI in cloacal and tracheal swabs. Chickens of control group did not shed the virus (Table 3). In addition, chickens in group 1 had more positive sample/total in each time of sampling.

Isolation of influenza virus from various organs

The presence of the virus in various organs obtained from the inoculated and control birds at different days PI was determined by inoculation of 10% tissue suspensions in alantoic fluid of 9 to 11 days emberyonated chicken eggs. The results of the virus detection are shown in Table 4. The results show that most positive samples were detected on days 3 PI. The virus was isolated from the trachea, conjunctiva, lungs, pancreas, bursa, thymus and kidney of all experiment groups at 3 dpi. But in IBD + AIV group brain and liver samples were also positive. All samples except trachea and conjunctiva from other groups were negative at 7 dpi. Trachea, conjunctiva, bursa and lungs samples in IBD + AIV group were also positive till 11 dpi. 11 days PI all samples from AIV group were negative.

Table 3. Results of virus isolation in emberyonated chicken eggs (Tracheal and Cloacal swabs).

Group/day PI	Cloacal swabs			Tracheal swabs		
	3	7	11	3	7	11
1	3/3*	2/3	1/3	3/3	3/3	2/3
2	2/3	2/3	0/3	3/3	2/3	0/3
3	0/3	0/3	0/3	0/3	0/3	0/3

*Number of positive samples/total samples taken.group1 = IBD, g2 = AIV, g3 = Control. On 3, 7 and 11 days after inoculation of avian influenza subtype H9N2, tracheal , cloacal swab were collected using Dacron swabs. Each swab placed in a steriled and antimicotics were added.after 1 hour incubation in environment 200µml of swab medium was inoculated to 9 to 11 days old emberyonated chicken eggs via alantoic sac. Five eggs were used for each swab collected to determine the presence of virus. Infected eggs were incubated for 48 h and then chilled at 4°C for no more than 24 h. Allantoic fluid was collected and a hemagglutination (HA) assay was performed. This table shows positive sample/total sample. According to this table gumboro disease could increase period of virus shedding from trachea and cloaca.

DISCUSSION

In the last decade, frequent incidences of H9N2 AIV outbreaks have caused high mortality in broiler chicken farms in Iran and some other Asian countries, resulting in great economic losses (Nili and Asasi, 2002, 2003). However, the causative virus has not characterized as low pathogenic avian influenza (LPAI) viruses. So far, there has not been any clear explanation for such a definitive differences in mortality and severity of clinical manifestation between affected fields. One of the possible explanations for such a high mortality could be that it is due to mixed infection of the virus (H9N2) with other pathogens. Likewise, It has been declared that the factors such as management, concurrent bacterial or viral diseases, immunosuppression agents, age and strain of chicken, are the main reasons of the pathogenicity variation of H9N2 isolates (Aamir et al., 2007; Capua and Alexander, 2004; Guo et al., 2000; Toroghi and Momayez, 2006; Subler et al., 2008).

Bano et al. (2003) indicated that H9N2 subtype of AIV as a nonpathogenic virus can cause a severe infection in field condition in presence of opportunist secondary pathogens. They also showed that in chemically bursectomised chickens, H9N2 subtype can cause high mortality. Banani et al. (2002) and Nili and Asasi (2003) suggested that concurrent infections with infectious bronchitis and secondary bacterial infection such as ornithobacterium rhinotracheal, *Escherichia coli* and *Mycoplasma gallisepticum* may be important enhancers of the signs than the other factors in H9N2 infection in chickens. Ramirez et al. (2010) reported that previous infection of IBDV in chickens may render them more susceptible to avian influenza virus (AIV) infection, allowing for the potential introduction of AIVs in an otherwise resistant population. Since various strains of infectious bursal disease viruses and H9N2 AI viruses

Table 4. The results of virus detection from various organs of chickens at different days post inoculation with H9N2 AI virus.

Day PI	Group	Tr	Lu	P	Br	Li	C	K	Bu	Th
	1	+	+	+	+	+	+	+	+	+
3	2	+	+	+	-	-	+	+	+	+
	3	-	-	-	-	-	-	-	-	-
	1	+	+	-	-	-	+	-	+	-
7	2	+	-	-	-	-	+	-	-	-
	3	-	-	-	-	-	-	-	-	-
	1	+	+	-	-	-	+	-	+	-
11	2	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-

Tr= Trachea, Lu= Lung, Th= Thymus, Bu= Bursa of Fabricius, P= Pancreas, K= Kidney, Br= Brain, C=Conjunctiva, Li=Liver. group1=IBD+AIV, g2=AIV, g3= Control suspension. 10% of tissue samples were prepared by adding enough content of BHI(Brain Heart Infusion) to the tissues. After centrifuging at 1500g supernatant was collected and antibiotic-antimycotics were added. after 1 hour incubation in environment 200µml of supernatant was inoculated to 9-11 days old emberyonated chicken eggs via alantoic sac. five eggs were used for each tissue samples collected. eggs were incubated for 48 hr and then chilled at 4° C for no more than 24 hr. Allantoic fluid was collected and a hemagglutination (HA) assay was performed.this table shows that IBDV infection could prolonged presence and isolation period of avian influenza subtype h9n2 from tissues and caused precence of live virus in un common tissues(brain and liver).

commonly circulate in poultry farms in Iran, we carried out experimental coinfection of H9N2 AIV with IBD virus to investigate role of IBDV on some H9N2 pathogenisity factors.

In this experiment, IBDV caused lower AIV antibody levels significantly. Although antibody levels in IBD-infected birds were not severly affected, an observation indicating possible relative resistance, which might be consequence of the age that H9 exposure happened and/or the time between IBDV infection and exposure to H9N2. Otim et al. (2005) reported that Newcastle disease antibody levels after IBDV infection in chickens were lower than those of the control group, but they were still above log mean $2^{5.2}$, the 100% protective titer.

Inoculation of chicks with IBDV prolonged AI virus excretion from cloac and trachea comparing with AIV group, suggesting that this immunosuppressive agent may have also interfered with immune mechanisms that could have prevented virus replication (Otim et al., 2005).

Ramirez (2010) reported that previous history of IBDV infection in chickens may alter host range, tissue tropism or virulence. Results of tissue isolations indicated that prior infection with IBDV prolonged caused altered tissue tropism of H9N2 consequently, isolating the AI virus from liver and brain. There is a question that in which way, AI virus introduced in liver and brain of IBDV infected chickens, from localized infection or by viremia? IBDV might induce prolonged viremia, in AIV infection. Coinfection of IBD promoted the propagation of AIV and increased the pathogenicity and extended the period of H9N2 AIV shedding in broiler chickens and caused mortality under the present experimental conditions.

Conclusions

The results of this study indicated that:

1. Previous infection with infectious bursal disease virus promoted the propagation of H9N2 avian influenza virus and extended the period of its shedding from trachea and cloaca in broiler chickens.
2. It prologed isolation of H9N2 avian influenza virus from tissues and altered tissue tropism of it.
3. It increased the pathogenicity of H9N2 AIV and caused most mortality.

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ABBREVIATION

IBDV, Infectious bursal disease virus; **AIV,** avian influenza virus; **LPAI,** low pathogenic avian influenza; **CID50,** 50% chicken infective dose; **EID50,** 50% emberyo infective dose; **HI,** hemagglutination inhibition.

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