

## Adaptation of Infectious Bursal Disease Virus by Cultivation in Embryonated Chicken Eggs and Evaluation as Potential Candidate for Local Live Attenuated Vaccine

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### Abstract

Infectious bursal disease virus (IBDV) from field outbreaks was purified by cesium chloride gradient centrifugation and collected at density of 1.33. The partially purified IBDV was serially propagated in specific pathogen free embryonated chicken eggs (ECEs). Intensity of lesions of IBDV was high from passage number 11 to 15 and nil after 23<sup>rd</sup> passage. Embryo mortality recorded was highest (100%) at passage number 6 and 7, started decreasing after serial passage and it was zero till passage number 15. All the field isolates were similar in growth pattern and minor differences were observed in geometric mean titers (GMT) of virus recovered from chorio-allantoic membrane (CAM), allantoic fluid (AF) and embryo by reverse passive haemagglutination (RPHA) test. GMT values of IBDV in CAM, AF and embryo at passage number 6 were 194, 168.9 and 222.9 whereas after passage number 13 were 1552.1, 1351.2 and 1782.9 respectively. Twenty fourth passage of IBDV in ECEs ( $10^{3.5}$  EID<sub>50</sub>/ml) was used as live attenuated vaccine in comparison with live commercial vaccine (Bursine-2 at recommended dose) in broiler chicks for immunization. Immune response measured by indirect haemagglutination (IHA) test at day 35 was higher (GMT 2896.3) than Bursine-2 (GMT 891.4).

**Key words:** IBDV, ECEs, Bursa of Fabricius, attenuation, immunization, GMT.

### Introduction

Infectious bursal disease (IBD), an acute immunosuppressive viral malady of young chicks, having lymphoid tissue as predilection site (Tsukamoto *et al.*, 1995) is among major infectious diseases causing huge economic losses in Pakistan (Anjum *et al.*, 1993). Signs of immunosuppression include inability to respond to vaccines with adequate antibodies (Nakamura *et al.*, 1992) and an increased

susceptibility to a range of secondary infections (Saif, 1991). IBD virus is classified into two groups based on pathogenicity to chicken (Lukert and Saif, 1997). Highly virulent IBD virus induces severe clinical signs followed by death, with severe damage to the bursa, thymus and spleen. Classically virulent IBD virus induces low or no mortality, with severe damage to bursa. Attenuated IBD virus causes minimal bursal damage (Lukert and Saif, 1997).

IBD virus actively replicates in the target organs such as bursa of Fabricius, spleen, intestine etc., in the infected chicken (Sluis, 1994), embryonated chicken eggs (ECEs) (Hassan and Saif, 1996) and cell culture (Jackwood and Saif, 1987). The growth of the virus in ECEs is satisfactory for virus isolation from clinical as well as sub-clinical cases. Some of the IBD virus isolates, which cannot grow on cell culture, can be grown on ECEs (McFerran *et al.*, 1980). The highly virulent (HV) strains as well as classical strains are attenuated by in vitro serial passages in ECEs. The ECEs adapted strains did not show gross lesions after eight serial passages (Snedeker *et al.*, 1966). Classical virulent strains were attenuated on ECEs through 13 serial passages by Izawa *et al.*, (1978), which showed reduction of bursal lesions.

The present study was designed to study the growth pattern of local isolates of IBD virus in specific pathogen free embryonated chicken eggs with the aim to attenuate local virus and its evaluation as live attenuated vaccine (local strain) in comparison with live commercial vaccine (Bursine-2 at recommended dose) in broiler chicks for immunization.

### Materials and Methods

#### Sample collection and processing

Infected bursa of Fabricius from outbreaks of infectious bursal disease (IBD) from different broiler flocks (50) were processed following the method of Hussain *et al.* (2003). IBD virus was partially purified by cesium chloride gradient centrifugation and collected at density of 1.33.

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### **Screening of samples for IBD virus**

Preliminary, presence of IBD virus was assessed by indirect haemagglutination (IHA) test (Hussain *et al.*, 2003). Anti-IBD virus antibodies were raised in rabbits by repeated injections of live attenuated commercial (D-78, Intervet). These antibodies were coated on washed sheep erythrocytes by chromium chloride (2% solution) and mixed in equal quantities with field samples (Bensal *et al.*, 1987). Observations were recorded by considering clumps formation as positive and bead formation of erythrocytes at the bottom of titration wells (micro-titration plate) negative (Hussain *et al.*, 2003).

### **Virus inoculation**

Partially purified virus was serially propagated in ten day old specific pathogen free embryonated chicken eggs (ECEs) by chorioallantoic membrane (CAM) as described by Rodriguez-Chavez *et al.* (2002). Lesions and embryo mortality caused by wild field isolates of IBD virus was observed till 24 passages.

### **Virus Titration**

Quantity of virus in each serial passage was measured by reverse passive haemagglutination (RPHA) test in chorioallantoic membrane (CAM), allantoic fluid (AF) and embryo following the procedure of Nachimuthu *et al.*, (1995). Observations were recorded and continued till the loss of pathogenicity.

### **Immunization of broilers with IBD virus vaccines**

Following 24 passages, an intermediate attenuation of the field virulent strain IBD virus was achieved. Embryo infective dose<sub>50</sub> (EID<sub>50</sub>/ml) was calculated as performed by (Villegas, 1998). Twenty fourth passage of IBDV in ECEs (10<sup>3.5</sup> EID<sub>50</sub>/ml) was used as live attenuated vaccine in comparison with live commercial vaccine (Bursine-2 at recommended dose) in broiler chicks for immunization by two successive inoculations at day 11 and 22. A total of 300 day-old broilers were reared under standard management conditions in three groups (A, B and C) having 100 birds each. Group A was inoculated with embryo adapted local field isolate, B by commercial live attenuated vaccine (Bursine-2, Solvey) and C as un-vaccinated control.

### **Measurement of humoral immune response**

Serum samples collected from each group (n=05) at day zero (pre-vaccination), 14, 21, 28, 35, 42, 49, 56 and 63 were analyzed for quantification of anti-IBDV antibodies using IHA test. Antigen used for titration of antibodies was locally adapted IBD virus.

## **Results**

The present research was designated as preliminary experiment to prepare and find a safe efficacious vaccine to fight against the drastic effects of infectious bursal disease virus (IBDV).

IBDV lesions in specific pathogen free chicken embryos were absent in first four passages. From passage number 5, typical lesions of virus in embryos started and continued till passage number 11 in increasing fashion. Intensity of lesions of IBDV in ECEs remained consistent from passage number 11 to passage 15. Later on from passage number 16 lesions started to reduce in intensity and were absent till 23<sup>rd</sup> passage. Cutaneous hemorrhages in chicken embryos by field virus isolates of IBD were observed in passage no 5 on cerebrum, toes and legs. Later on from passage number 6 to 11 extensive cutaneous hemorrhages were observed on whole body of the embryos.

Mortality caused by IBDV in chicken embryos during different serial passage is presented in table (01). Mortality was not observed during first three passages of IBDV in chicken embryos. In fourth passage embryo mortality recorded was only 12.5% and was maximum at passage number 6 and 7 (100%). Then embryo mortality started decreasing after serial passage and it was zero till passage number 15. Among the field virus isolates there were minor differences in the types of embryo lesions observed and mortality.

The quantity of the IBDV grown in ECEs after each serial passage was measured in chorioallantoic membrane (CAM), allantoic fluid (AF) and embryo by RPHA test. In first 5 passages virus was detected only in CAM and not in AF and embryo. From passage number 6 to onward till passage number 13, titer of IBDV increased gradually and remained almost consistent throughout the experiment. Among the field virus isolates there were no differences in the quantity of virus grown in ECEs by RPHA test. GMT values of IBDV in CAM, AF and embryo at passage number 6 were 194, 168.9 and 222.9 respectively. GMT values after passage number 13 were 1552.1, 1351.2 and 1782.9 in CAM, AF and embryo respectively (table 02).

Antibody titers (IHA test) in all groups at day zero (pre-vaccination) were almost similar and ranged from 16 to 64. Humoral immune response pattern was much different in samples collected from groups A, B and C throughout the experiment. Geometric mean titer (GMT) values of antibodies against in all groups at different time intervals are presented in figure (01).

## **Discussion**

Infectious bursal disease virus (IBDV), do not grow easily in embryonated chicken eggs (ECEs) and takes time for its adjustment. Lesions of growth and embryo mortality were not seen in ECEs observed in first 5 serial passages. Yamaguchi *et al.* (1996) reported that highly virulent IBDV do not show gross

lesions in ECEs after 8 serial passages. Similar findings were also reported by Snedeker *et al.*, (1966). Izawa *et al.* (1978) also showed reduction in bursal lesions and reported attenuation of classical virulent strains during 13 serial passages in ECEs.

It was observed in the present study that after passage number 6, the lesions and mortality of embryo by virulent field IBDV increased and peaked at passage number 11, then declined and finished till passage number 23. So, in comparison virulent IBDV took 23 serial passages for attenuation and adaptation rather than 13 passages as reported by Izawa *et al.*, (1978).

Titer of virus increased gradually from passage number 6 to 13 and then it remained consistent throughout the experiment. Quantity of the virus in CAM and AF was almost equal; however a little higher amount of virus procured from embryonic tissue. It is evident IBDV has the capacity to grow in CAM, AF and embryo, but it grows more efficiently in embryonic tissue, suggesting that higher amounts of virus could be procured in embryonic tissue culture easily. Twenty four times passaged IBDV in embryonated eggs did not show lesions and that passage was selected for immunization of chicken against IBD. Attenuation of IBDV in eggs was obtained after 43 passages by Lazarus *et al.*, (2008). Variations in the passage numbers for attenuation of IBDV in eggs are attributed to the conditions of

experiment, virulence of virus and availability of nutrients in the eggs for virus multiplication.

Immune response to IBD virus live attenuated vaccine (local) measured by IHA test was much higher in comparison with commercial vaccine as is depicted from the curves of figure (01). This difference might be due to the factor that the antigen used for titration of antibodies in serum samples at different days was local field isolate. Sil *et al.*, (2002) reported that possible reasons for low level of antibodies in commercial broilers may be type, storage, transportation and handling of vaccines. Immune response to vaccines was gradually increasing against the test vaccines being an indicative of vaccine efficacy (Skeels and Lukert, 1979; Adene *et al.*, 1989). Results are comparable with the reports of Winterfield *et al.*, (1979) and Vakharia *et al.*, (1994) but contradictory to Kissling and Henk (1983).

Local isolates of IBDV were successfully adopted on chicken egg embryos and no pathogenic lesions were found after inoculation to the natural host (11 days old chicks). Immune response was much better in limited vaccination trial in comparison with commercial vaccine. Trials on local attenuated vaccine may be extended in field condition to assess the chances for its use as potential candidate for commercial vaccine seed.

**Table 1. Mortality by infectious bursal disease virus in embryonated chicken eggs 6 days post-inoculation**

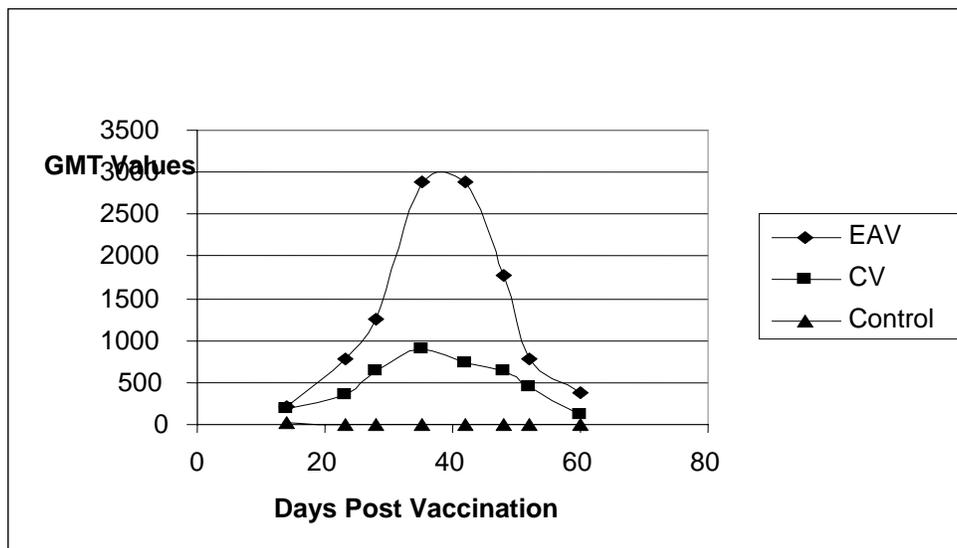
Passage No.	Total eggs	Embryo mortality	Percentage
1-3	30	0	0
4	16	2	12.5
5	20	10	50
6	16	16	100
7	20	20	100
8	20	18	90
9	16	12	75
10	20	10	50
11	20	8	40
12	16	4	25
13	16	2	12.5
14	16	1	6
15-24	16	0	0

**Table 2. Passage wise quantitation of infectious bursal disease virus in chorioallantoic membrane, allantoic fluid and embryo by reverse passive hemagglutination test.**

Passage number	Geometric mean titers of IBD virus		
	CAM	CAF	Embryo
1-4	Not detected by RPHA	Not detected by RPHA	Not detected by RPHA
5	92	92	108
6	194	194	222.9
7	337.8	337.8	338
8	445.7	445.7	445.7
9	675.6	675.6	776
10	891.4	891.4	891.4
11	1176.3	1176.3	1351.2
12	1351.2	1351.2	1552.1
13	1552.1	1552.1	1782.9

CAM= chorioallantoic membrane.

CAF = chorioallantoic fluid.



**Figure 1. Post vaccination antibody profile of embryo adapted and commercial vaccines of infectious bursal disease virus in broilers.**

**EAV:** Embryo adapted vaccine; **CV:** Commercial vaccine; **Control:** Non vaccinated

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