ADAPTATION OF INDIGENOUS INFECTIOUS BURSAL DISEASE VIRUS (IBDV) IN EMBRYONATED CHICKEN EGGS

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ABSTRACT

Infectious bursal disease virus was isolated from bursae of broilers suffering from Gumboro disease and was designated as field virus (FV). The virus was confirmed through agar gel precipitation test (AGPT) and counter current immunoelectrophoresis (CCIE). The virus was titrated by using reverse passive haemagglutination (RPHA) test and egg infective dose fifty (EID_{50}). The FV was inoculated into 9- to 11-day-old embryonated chicken eggs through chorio-allantoic membrane (CAM). At each passage, the virus in the chorio-allantoic fluid (CAF) and embryos was confirmed by AGPT and titrated by RPHA test. Geometric mean titer (GMT) of the virus in CAF was 37 to 64 in 1-3rd passage, 111 to 239 in 4-7th passages. In 8 to 15th passages, virus titer remained from 294 to 588 and in 16-24th passages virus titer ranged from 675 to 2195. Similarly, virus titer in the embryos was 1024 to 512 in 1st -10th passages, while the virus titer in passages 11-24th ranged from 478 to 111. Embryos were monitored for lesions and mortality. Severe lesions were present on the CAM in 1st-7th passages, while moderate to mild haemorrhages were seen in 8th to 16th passages and in 17th – 24th passages no lesions were observed.

Key words: Infectious bursal disease virus, adaptation, embryonated eggs, chorioallantoic fluid.

INTRODUCTION

Infectious bursal disease virus (IBDV) is the etiological agent of an immuno-suppressive disease of young chickens of 3 to 6 weeks of age. The virus causes destruction of lymphoid tissue, especially the bursa of Fabricius (Reddy *et al.,* 1997). Two distinct serotypes (1 and 2) of IBDV have been recognized. The serotype 1, which displays a wide variation in pathogenic potential, is virulent for chickens, whereas serotype 2 is virulent for turkeys (Jackwood and Saif, 1987). IBDV is a member of the genus Avibirnavirus belonging to the family *Birnaviridae*. The virus genome consists of two segments (A and B) and is a double stranded non-enveloped RNA having the size of about 55-65 nm in diameter (Becht *et al.,* 1988).

Strains of IBDV show reduced virulence by passaging in embryonated eggs (Abdel-Alim and Saif, 2002) and chicken embryo fibroblasts (Yamaguchi *et al.,* 2000). Most strains of wild type IBDV recovered from infected bursae do not replicate in CEF cells (McFerran *et al.,* 1980). However, the virus becomes progressively adapted to grow *in vitro*. Passaging of the virus *in vitro* has been associated with attenuation of its virulence, as evidenced by a reduction in its ability to induce bursal lesions (Hassan and Saif 1996). Extended *in vitro* passages of the virus using embryonated chicken eggs has been proposed to be a good approach for the development of attenuated live vaccines (Yamaguchi *et al.,* 1996).

Currently, no report is available on the attenuation of local IBDV strains using embryonated chicken eggs for the production of live vaccine. In an attempt to produce live attenuated vaccine, the present study was conducted to adapt and attenuate the field isolates of IBDV on the basis of the infectivity of the virus to embryos and replication of the virus in chorio-allantoic fluid (CAF) in embryonated chicken eggs (ECEs).

MATERIALS AND METHODS

Collection and processing of samples

Ten isolates of highly virulent IBDV were independently collected from different farms around Faisalabad. Each isolate was experimently injected once into the 18-21-day old chicken and chicken were killed 3-5 days post infection. They produced same pathological lesions in birds as in field. Bursae were collected and homogenized with 10% (w/v) PBS and centrifuged at 5,000 rpm for 15 minutes at 4°C. The supernatant was collected and stored at -20°C. The

supernatant fluid was mixed with chloroform (1:1) and centrifuged at 5000 rpm for 20 minutes. The clear supernatant was collected in the test tube and stored at $-20^{\circ}C$ till used.

Hyperimmune serum

The hyperimmune serum was raised against IBDV (field isolates and D-78 commercial vaccine) in rabbits according to Barnes *et al.* (1982). Serum was inactivated at 56°C for 30 minutes and stored at -20°C.

Confirmation, purification and titration of virus

The IBDV in the supernatant was confirmed with agar gel precipitation test (AGPT) following Sulochna and Lalithakunjamma (1991) and counter current immunoelectrophoresis (CCIE) following Hussain *et al.* (2002). Then the virus was purified with ultra centrifugation for 6 hours by velocity density gradient centrifugation using different sucrose gradients at 4°C (Nagy and Lominiczi, 1984). Pellet obtained was redissolved in PBS having antibiotics (penicillin, streptomycin and neomycin) in it. The titre of the purified virus was checked through reverse passive haemagglutination (RPHA) following Rajeswar and Dorairajan (1999) and egg infectious dose fifty (EID_{50}) following Reed and Muench (1938).

Inoculation of IBDV in embryonated chicken eggs

Embryonated chicken eggs were procured from the University poultry farm and also from rural poultry where vaccination against IBDV was not a routine practice. Eggs were checked for anti IBDV and those having no detectable anti-IBDV antibody were used in this study.

Chicken embryos (9 to 11-day old) were inoculated with 0.1 ml of IBDV suspension via chorio-allantoic membrane (CAM). Eggs were incubated and observed daily for viability. Dead embryos during the first 24 hours post inoculation (PI) were discarded. Mortality was recorded between 2-4 days PI. At the 4th day PI, chorio-allantoic fluid (CAF) was collected. The CAF was centrifuged at 1500 rpm for 20 minutes, supernatant was collected and titre of IBDV in the CAF was determined through reverse passive haemagglutination (RPHA) test. The virus was passaged 24 times in the 9-to 11-day-old embryonated eggs.

Antigen titer in the embryo

After every passage of virus in the embryonated chicken eggs, the embryos and CAM were examined for gross pathological changes. The entire embryos were homogenized with 20% w/v PBS having antibiotics and centrifuged at 5,000 rpm for 15 minutes at 4°C. The resultant supernatant was used to titrate the IBDV in the embryos using RPHA.

RESULTS

The collected bursa samples were processed for the isolation of IBDV and confirmed using AGPT and CCIE. A clear precipitation band was observed between the antigen and antibody. The titre of indigenous virus was 239 through the RPHA and EID_{50} of the virus was $10^{5.4}$.

This virus was serially passaged 24 times in embryonated chicken eggs through CAM route. Following the CAM inoculation in 1st and 2nd passage 100 and 90% mortality of embryos was observed on 3-4 days PI, respectively. The mortality was upto 70% in the first 6 passages. The mortality was reduced from 60% to 0% up to the 16th passage. No mortality was observed between 16th -24th passages (Figure 1).

In the early passages, the titer of the virus was lowered in CAF. On the basis of gross lesions and titer of IBDV, different passages were categorized into 1-3, 4-7, 8-15 and 16-24 passages. In 1-3rd passages, GMT was 37 to 64, while in 4-7th passage titer ranged between 111 to 239 and at 8-15th passages the titer was from 294 to 588. As the number of passages increased, the virus titer also increased and in the 16-24th passages titer of the virus ranged from 675 to 2195. On the other hand, the virus titer in embryo homogenates was high in the initial 10 passages, which was 1024 to 512. The titer of the virus in 11-24th passages ranged from 478 to 111.

The gross pathological lesions were observed 3-4 days PI. The embryos showed oedematous distention of abdomen. Haemorrhages were seen on the CAM, skin, toe joint and cerebrum. Spleen was enlarged, pale and had small necrotic foci. Kidneys were congested and necrotic, lungs also showed congestion. These lesions were severe in the early passages of virus. As the number of passages increased, the lesions reduced on the body surface and CAM, no lesions were observed in 16-24 passages. Severe pathological lesions were seen on entire body surface of the embryo in 1-7 passage. While lesions between 8-15th passages gradually decreased and haemorrhagic spots were also observed on the body surface of embryos. No lesions were observed between 16-24th passages of virus (Plate 1). On the CAM, severe haemorrhages were seen in 1-7th passages of virus, while pathological lesions remained moderate to mild between 8-16th passages and no haemorrhagic spots were observed between 17-24th passages of virus on embryonated chicken eggs (Figure

2).

DISCUSSION

The present work was carried out on IBDV to adapt the field virus on embryonated chicken eggs. The pathogenicity of virus in embryos was also checked. In first passage, 100% embryo mortality was observed. In initial three passages, embryos and CAM were full of lesions. As the number of passages increased, the mortality of the embryo and lesions on the body surface of the embryo and CAM were reduced. No lesions were seen on the embryos from 16-24th passages. Yamaguchi *et al*. (1996) reported that highly virulent IBDV showed severe gross pathological lesions and 100% embryo mortality in the early passages of virus and reduction of lesions were observed in the advanced passages. Izawa

et al. (1978) showed reduction of bursal lesions and attenuation of classical virulent strains during 13 serial passages in embryonated eggs. Similarly, Abdel-Alim and Saif (2002) adapted the serotype 2 OH (Ohio) strain of IBDV 10 times and investigated the pathogenic potential of the virus to chicken embryos. Their results showed that OH strain could be pathogenic to chicken embryos, as evidenced by development of pathogenic changes in early passages in embryonated eggs. Jeffrey and Jackwood (2001) also performed 24 passages of IBDV on 9-day old chicken embryos. This study produced lesions in embryos, typically of those previously reported. It was suggested that the attenuation response elicited by the different strains of IBDV might represent a biological distinction.
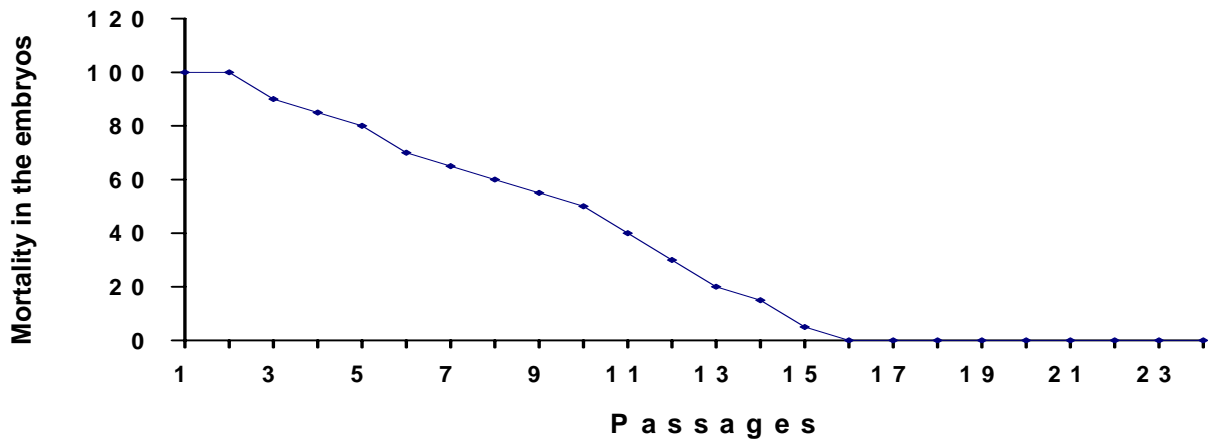
IBDV actively replicate not only in target organ

Fig. 1: Percent mortality in different passages of embryonated chicken eggs inoculated with IBDV.
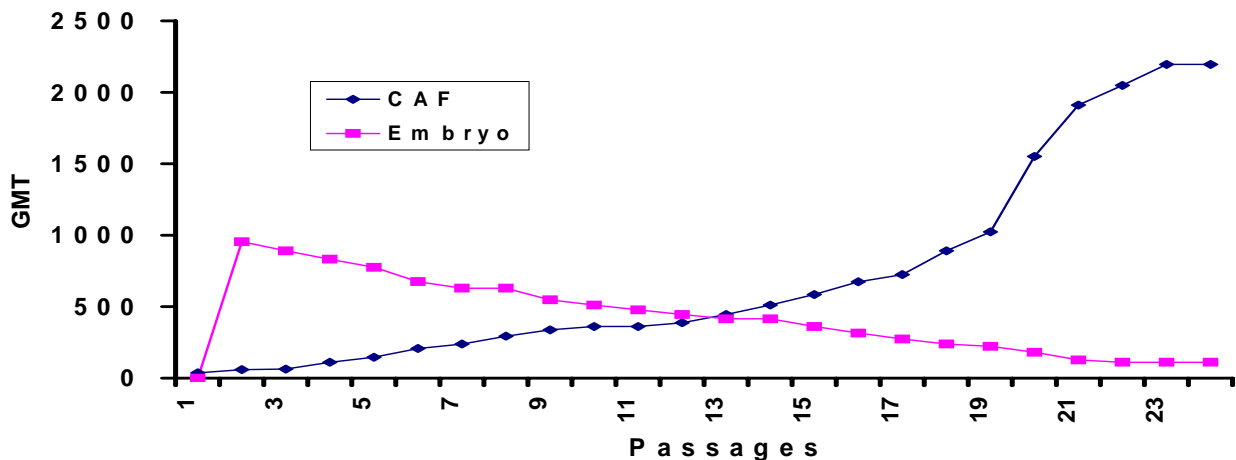

Fig. 2: Titre of the IBDV in chorio-allantoic fluid (CAF) and embryos in different passages of embryonated chicken eggs.

such as bursa of Fabricius in the infected chickens (Stuis, 1994) but also in embryonated eggs (Hassan and Saif, 1996). The IBDV, which was not grown on CEFs, can be grown on embryonated eggs (McFerran *et al.,* 1980). The growth of virus in embryonated eggs is satisfactory for virus isolation for clinical as well as sub clinical cases (Hitchner, 1970). Quantification of IBDV was carried out after every passage in CAF and embryonic tissue by RPHA test. Titer of the virus in CAF increased gradually upto the 20th passage and then remained constant uptil 24th passage. The virus titer in the embryonic tissues was high in the initial passages and decreased gradually upto the 16th passage. The virus titer in the tissues of embryos was constant from 16th to 24th passages. Similar results were described by Hitchner (1970).

The result of present study showed that local IBDV was completely adapted and attenuated in embryonated chicken eggs. The virus titer was low in CAF in early passages but increased with the passages. The lesions were severe on CAM and embryos in initial passages, but their severity decreased in successive passaging. The initial passages of adapted and attenuated virus may be a good candidate for hot vaccine, the middle passages for the intermediate and last passages *i.e.,* 20-24th as mild vaccines, respectively.

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