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Inactivation of infectious bursal disease virus by binary ethylenimine and formalin*

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Abstract: In this experiment conducted to study the inactivation dynamics of infectious bursal disease virus (IBDV) by binary ethylenimine (BEI) in comparison with formalin, IBDV was isolated from the bursa of infected chickens and its confirmation was done by agar gel precipitation test. Viral suspensions were subjected to inactivation with BEI and formalin for pre-set time intervals. BEI was employed at concentrations of 0.001 and 0.002 mol/L while formalin was used at 0.1% and 0.2%. Sampling was done at 6, 12, 24, 36 and 48 h of incubation and samples were tested for their inactivation status in 9-day-old embryonated eggs and 3-week-old broiler chickens. IBDV was completely inactivated by 0.001 and 0.002 mol/L BEI after 36 h of incubation at 37 °C, whereas formalin at 0.1% and 0.2% concentrations inactivated IBDV in 24 h.

Key words: Infectious bursal disease virus (IBDV), Binary ethylenimine (BEI), Inactivation
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INTRODUCTION

Infectious bursal disease virus (IBDV) belongs to the genus *Avibirnavirus*, family *Birnaviridae* (ICTV, 2004), with its genome composed of two segments of double stranded RNA (Lukert and Saif, 1991). It is a non-enveloped 60 nm diameter icosahedral particle (Özel and Gelderblom, 1985; Kibenge *et al*., 1988; ICTV, 2004). IBDV is the causative agent of acute or immunosuppressive disease in chickens. Serotype 1 strains are pathogenic, with the target organ being bursa of Fabricius (BF). While studies demonstrated that serotype 2 strains do not cause disease or protect against infection (Jackwood *et al*., 1982; Ismail *et al*., 1988; Zierenberg *et al*., 2004).

IBDV is responsible for a highly immunosuppressive disease in young chickens and causes significant economic losses to the poultry industry worldwide (Kibenge *et al*., 1988). Due to the high resistance of IBDV to environmental exposure, hygienic measures alone are ineffective and vaccination is thus essential. The economical impact of both clinical and sub-clinical diseases warrants search for and the use of efficient vaccines. Satisfactory protection can be achieved by immunization with live or inactivated vaccines. Classical live vaccines achieve lifelong and broad protection but possess residual pathogenicity and a proportional risk of reversion to virulence. Inactivated vaccines, although costly, are used successfully (Box, 1989). In order to obtain an inactivated immunologic or vaccine composition, the pathogen is harvested and subjected to clarification by chemical treatment and inactivation using different inactivants, for example formalin or formaldehyde, β-propiolactone, ethylenimine, binary ethylenimine or thimerosal.

Most inactivated viral vaccines are prepared by the reaction of viruses with formaldehyde (Brown, 1995). Formalin reacts with many chemical groupings of proteins that lead to the phenomenon of the

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"membrane effect" in which the reaction "closes" the outer protein shell of the virus before the nucleic acid of the infectious genome is destroyed. Even after prolonged incubation of the inactivated antigen infectious nucleic acid can emerge and lead to a replication of the virulent virus. This can cause a sub-clinical infection or even lead to disease. The membrane effect alters the surface proteins of the virus and modifies and reduces the antigenicity of the antigen (Bahnemann, 1990). Binary ethylenimine (BEI), member of a group of alkylating substances "aziridines" reacts very little with proteins and therefore does not alter the antigenic components of the virus. BEI has an inactivation reaction that is more specific for the nucleic acid and it produces antigenically superior vaccine (Bahnemann, 1990).

By the time BEI has been used to inactivate different viruses i.e. rabies (Larghi and Nebel, 1980) foot-and-mouth disease virus (Dilovski and Tekerlekov, 1983), bluetongue virus (Stott *et al*., 1979), porcine parvovirus (Buonavoglia *et al*., 1988a), African horse sickness virus (Soliman *et al*., 1996) and Newcastle disease virus (King, 1991) etc. In the present study IBDV was subjected to inactivation with BEI for comparison with formalin inactivation.

MATERIALS AND METHODS

Collection of samples

Infected bursae were collected from an outbreak of infectious bursal disease at a local poultry farm. Complete history of outbreak was taken. These samples were stored at −20 °C till used.

Field virus isolation and purification

A 10% (w/v) suspension of infected bursae was made by chopping and grinding them in sterilized pestle and mortar with sterilized sand after the method of Reddy *et al*.(1977). The suspension was made in phosphate buffered saline (PBS) containing antibiotics (100 IU penicillin-G/ml and 50 μg gentamicin sulfate/ml). This suspension was later centrifuged at 5000 r/min for 20 min and the supernatant was collected. The supernatant fluid was mixed with chloroform (1:1, v/v) in centrifuge tubes and centrifuged at 5000 r/min for 20 min. Three distinct layers were obtained: top layer containing virus, middle one containing bursal tissue debris and bottom layer was containing chloroform. The clear supernatant was collected in sterilized screw capped test tubes.

Confirmation of virus

The presence of IBDV in the clear fluid was checked by agar gel precipitation test as described by Sulochana and Lalithakunjamma (1991). The hyperimmune serum used in the test was raised against live IBDV vaccine D-78 in rabbits according to the method of Barnes *et al*.(1982).

Titration of virus

Egg infective dose 50 (EID_{50}) of the virus was calculated by the method of Reed and Muench (1938). Fertile eggs with 9 day-of-age embryos were used and injected with virus through chorioallantoic membrane (CAM) route. Ten fold dilutions of the virus suspension were made from 10^{-1} to 10^{-10} in sterile normal saline and each dilution was injected in a batch of five eggs.

The eggs were inoculated with each dilution at rate of 0.1 ml per egg. Ten eggs were inoculated with sterile saline and were kept as control. After inoculation all the eggs were sealed with melted wax and were re-incubated at 37.5 °C. Inoculated eggs were candled daily. Mortality during first 24 h was discounted as non-specific. After 96 h the eggs were opened and embryos were checked for lesions. Signs of infection were characteristic IBDV lesions. Typical lesions in embryos were petechiae and congestion of skin and ecchymotic haemorrhages in the toe joints. The EID_{50} calculated was $10^{3.48}$.

Inactivation of virus

After confirmation and titration of virus, the supernatant fluid was divided into fractions and subjected to treatment with formalin and binary ethylenimine for inactivation.

1. Formalin treatment

Thirty-seven percent formaldehyde solution (BDH Chemicals Ltd. Pool, England) was added to viral suspensions (EID_{50}=$10^{3.48}$) to make final formalin concentration equal to 0.1% and 0.2%. A control with no added formalin was included. Vials were capped, mixed and incubated at 37 °C. Sampling was done at 6, 12, 24, 36 and 48 h of treatment.

2. Binary ethylenimine (BEI) treatment

BEI was prepared as a 0.1 mol/L solution by cyclization of 0.1 mol/L 2-bromoethylamine hydrobromide (Sigma) in 0.175 mol/L NaOH solution at 37 °C for one hour following the method of Bahnemann (1990). BEI was used soon after its preparation. BEI of 0.1 mol/L was added to virus suspension (EID_{50}=$10^{3.48}$) at 1% and 2% for final BEI concentrations 0.001 and 0.002 mol/L respectively. A control without addition of BEI was included. Virus suspensions were incubated at 37 °C and sampling was done at 6, 12, 24, 36 and 48 h of treatment. The residual BEI was hydrolysed in samples by the addition of 1 mol/L sterile Na-thiosulfate (Merck) solution at 10% of the volume of the BEI used.

Pathogenicity tests to check the inactivation of virus

Formalin and BEI treated IBDV was tested for its pathogenicity in: (1) un-vaccinated susceptible chicks of three weeks of age (Allan *et al*., 1972); (2) ten-day-old embryonated eggs (Hitchner, 1970).

1. Inoculation of 3-week-old susceptible broiler chicks

Samples from both formalin and BEI treated virus collected at different time intervals were checked for their pathogenicity by their inoculation into 3-week-old susceptible broiler chicks. Each sample was inoculated in 3 birds at rate of 0.3 ml/bird subcutaneously. Birds were observed for 5 d post inoculation (Helmboldt and Garner, 1964).

2. Inoculation of ten-day-old embryonated eggs

Samples from both formalin and BEI treated viruses collected at different time intervals were also checked for their pathogenicity by their inoculation into ten-day-old embryonated eggs through chorioallantoic membrane (CAM) route. Each sample was inoculated into 3 eggs at rate of 0.1 ml/egg. After inoculation all eggs were sealed with melted wax and were re-incubated at 37.5 °C. Inoculated eggs were candled daily. Mortality during first 24 h was regarded as non-specific. After 96 h the eggs were opened and embryos were checked for lesions (Rosenberger *et al*., 1985).

RESULTS

Vaccine samples that were incompletely inactivated led to development of pathological lesions both in susceptible birds and embryonated eggs upon inoculation, whereas no lesions were observed in the case when vaccine samples were completely inactivated. Typical lesions in birds were observed in bursa, kidney, thymus and muscles while cutaneous congestion was a prominent sign in embryos. BEI of 0.001 and 0.002 mol/L completely inactivated the virus after 36 h, whereas 0.1% and 0.2% formalin inactivated the virus after 24 h of incubation at 37 °C.

DISCUSSION

This study aimed at testing the inactivation ability of BEI for IBDV in comparison with conventional inactivant formalin. Most inactivated viral vaccines are prepared by reacting the viruses with formaldehyde (Brown, 1995). Formalin reportedly reacts with many chemical groupings of proteins that lead to the phenomenon of the "membrane effect" in which the reaction "closes" the outer protein shell of the virus before the nucleic acid of the infectious genome is destroyed. Binary ethylenimine (BEI), a member of a group of alkylating substances "aziridines" reacts very little with proteins and therefore does not alter the antigenic components of the virus (Bahnemann, 1990). In this study BEI was successfully used to inactivate IBDV.

BEI produces antigenically superior vaccine because it does not alter the antigenic components of the virus as compared with other inactivants. Blackburn and Besselaar (1991) used a panel of 23 monoclonal antibodies (mAbs) to study the effect of formalin, beta propiolactone (BPL) and binary ethylenimine (BEI) on the epitopes of the Rift Valley fever virus glycoproteins. After the inactivation period BEI had very little adverse affect on the epitopes whereas BPL significantly altered and formalin partially changed the conformation of most of the epitopes. Larghi and Nebel (1980) studied the inactivation activity of binary ethylenimine on rabies virus and immunogenic properties and stability of the vaccines prepared using this agent. He concluded that rabies tissue culture vaccine inactivated with BEI was highly immunogenic and stable. Pyke *et al*.(2004) also used BEI for inactivation of different viruses.

Buonavoglia *et al*.(1988b) inactivated Newcastle

disease virus (NDV) with BEI. The BEI inactivated vaccine had almost twice the efficiency when compared to a vaccine prepared with formalin.

Keeping in view the above findings promising results can be anticipated by using BEI-inactivated IBDV in chickens. In future studies BEI and formalin-inactivated vaccines will be inoculated in broiler birds to check their comparative efficacy.

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