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Immunogenicity of formaldehyde and binary ethylenimine inactivated infectious bursal disease virus in broiler chicks*

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Abstract: Infectious bursal disease virus (IBDV) was inactivated by two different chemicals—formaldehyde and binary ethylenimine (BEI). Formaldehyde was used at 0.1% and 0.2%, while BEI was used at concentrations of 0.001 and 0.002 mol/L. These four vaccines were tested for their efficiency in generating humoral immune response in different groups of broiler chicks. Both BEI-inactivated vaccines gave relatively higher antibody titers and were almost twice as efficient as formaldehyde-inactivated ones.

Key words: Infectious bursal disease virus (IBDV), Binary ethylenimine (BEI), Formaldehyde, Immune response
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INTRODUCTION

Infectious bursal disease (IBD) is one of the most important naturally occurring viral diseases of commercial reared chickens worldwide (Lukert and Saif, 2003). It is an acute, highly contagious and immuno-suppressive disease of young chicks. The causative agent, infectious bursal disease virus (IBDV), belongs to the family *Birnaviridae*, with its genome composed of two segments of double-stranded RNA (Dobos *et al*., 1979; Lukert and Saif, 1997). It is non-enveloped 60 nm diameter icosahedral particle (Özel and Gelderblom, 1985; Kibenge *et al*., 1988). Serotype I strains are pathogenic, with the target organ being bursa of Fabricius (BF), with markedly different virulence, while studies demonstrated that serotype II strains do not cause disease or protect against infection (McFerran *et al*., 1980; Jackwood *et al*., 1982; Ismail *et al*., 1988; Zierenberg *et al*., 2004).

IBDV is endemic in most poultry producing areas worldwide and may cause high mortality in chickens (Box, 1989). Infectious bursal disease (IBD) is a major global concern to the poultry industry. The economic impact of this disease is related to losses due to mortality, growth retardation or rejection of carcasses (van den Berg, 2000). 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 (van den Berg, 2000). 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

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using different inactivants, for example 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 "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 produces antigenically superior vaccine (Bahnemann, 1990). BEI preserves the conformation and accessibility of epitopes to a much greater extent than formalin and β-propiolactone (Blackburn and Besselaar, 1991; Kyvsgaard *et al*., 1997).

In the present study IBDV was inactivated with formaldehyde and BEI and their comparative immune responses were ascertained in broiler chicks.

MATERIALS AND METHODS

Collection of samples

Infected bursae were collected from an outbreak of infectious bursal disease at a poultry farm near Faisalabad, Pakistan. Complete history of outbreak was taken. These samples were taken from sick birds and 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 containing chloroform. The clear supernatant was harvested and dispensed into aliquots for storage at −20 °C.

Confirmation and titration of virus

The presence of IBDV in the clear fluid was confirmed by agar gel precipitation test as described by Sulochana and Lalithakunjamma (1991). Egg infective dose 50 (EID_{50}) of the virus was calculated by the method of Reed and Muench (1938) in fertile eggs with 9-day-old embryos (Habib *et al*., 2006). The EID_{50} calculated was $10^{3.48}$.

Inactivation of virus

After confirmation and titration of the virus, the supernatant fluid was divided into fractions and subjected to treatment with formaldehyde and binary ethylenimine to inactivate it.

1. Formaldehyde treatment

Thirty-seven percent formaldehyde solution (BDH Chemicals Ltd. Pool, England) was added to viral suspensions (EID_{50}=$10^{3.48}$) to make final formaldehyde concentrations of 0.1% and 0.2%. A control with no added formaldehyde was included. Vials were capped, mixed and incubated at 37 °C for 24 h. The inactivated virus preparation was dialyzed against PBS to remove formaldehyde.

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). The reaction was controlled by following the drop in pH due to the formation of BEI. The BEI preparation was added to the virus suspensions (EID_{50}=$10^{3.48}$) at rate of 1% and 2% to make final BEI concentrations of 0.001 and 0.002 mol/L respectively. A control without addition of BEI was included. Virus suspensions were incubated at 37 °C for 36 h. 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.

Sterility and safety testing

Sterility check of inactivated virus suspensions was made by its inoculation onto different culture media. Safety of the vaccines was ascertained by inoculation into 3-week-old susceptible broiler chicks and by inoculation into 10-day-old embryonated eggs (Habib *et al*., 2006). No unwanted effects were observed over a period of 5 d.

Experimental design

A total number of 150-day-old broiler chicks were purchased from a commercial hatchery. On 14th day of age the chicks were divided into five groups A, B, C, D and E of 30 chicks each.

Immunization of chicks

On 15th day of age birds in groups 'A' and 'B' were immunized by intramuscular route (I/M), with 0.3 ml (150 EID_{50}) of 0.1% and 0.2% formaldehyde-inactivated IBDV respectively. Birds in groups 'C' and 'D' were immunized (I/M) with 0.3 ml (150 EID_{50}) of 0.001 and 0.002 mol/L BEI-inactivated IBDV respectively, whereas birds in group 'E' were kept uninoculated as control.

Collection of sera

Blood samples were collected from randomly selected five birds of each group at 0, 10, 15, 20 25, and 30 d post-inoculation. Serum was separated, heat inactivated in a water bath at 56 °C for 30 min, to remove non-specific inhibitors, and stored at −20 °C after properly labelling the tubes for further use in indirect hemagglutination test.

Determination of antibody titers against IBDV

Antibody titers against IBDV were measured by indirect hemagglutination (IHA) test after the methods of Rehman *et al*.(1994) and Sawada *et al*.(1982) with some modifications. Briefly, human group O negative red blood cells (RBCs) were separated by centrifugation, washed three times with PBS (pH 7.1) and a 2.5% suspension was prepared in PBS. This erythrocyte suspension was incubated at 37 °C for 10 min with equal volume of tannic acid (1:20000) in PBS. The tannic acid treated RBCs were then washed, resuspended at 2.5% in PBS and mixed with equal amount of virus suspension and PBS (pH 7.1) at a ratio of 1:1:4 at room temperature for 15 min. Sensitized RBCs were washed, spinned down and resuspended at 2.5% in PBS. The IHA test was performed in microtiter plates with 'U' bottomed wells. Serial two-fold dilutions of hyperimmune serum were prepared as a standard control and used to determine the titer of test serum samples collected from various groups. The highest dilution showing haemagglutination was taken as an end point.

Statistical analysis

Significance of differences was determined by analysis of variance (ANOVA) using Duncan's multiple range test. $P<0.05$ value was considered significant.

RESULTS AND DISCUSSION

In this study the vaccines were evaluated on the basis of humoral response. Antibody titers in all the serum samples collected on different days post-vaccination were determined. The results showed that there was a gradual increase in antibody titers till the 20th day post-inoculation, in all vaccinated groups, except the control birds. Afterwards there was a decline in antibody titers. On the basis of results of IHA test we observed that there were slight differences in antibody titers elicited by 0.001 and 0.002 mol/L BEI-inactivated vaccines, but statistically these differences were not significant as was the case in formaldehyde-inactivated vaccines.

In general both BEI-inactivated vaccines gave higher antibody titers than formaldehyde-inactivated vaccines. Statistically this difference was significant. BEI-inactivated vaccines were almost twice as efficient as formaldehyde-inactivated ones in generating antibodies. Antibody titer values of all groups at different days post-inoculation are shown in Table 1.

This study showed that the BEI-inactivated infectious bursal disease virus can be successfully used as an immunogen. BEI has also been widely used to inactivate a number of viruses for vaccine (Bahnemann, 1990; Buonavoglia *et al*., 1988a).

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 for-

Table 1 Antibody response following vaccination

Group	Days post-vaccination					
	0	10	15	20	25	30
A	8.00±0.00[a]	64.00±0.00[a]	115.20±12.80[a]	102.40±15.67[a]	89.60±15.67[a]	64.00±0.00[a]
B	8.00±0.00[a]	64.00±0.00[a]	115.20±12.80[a]	115.20±12.80[a]	115.20±12.80[a]	76.80±12.80[a]
C	8.00±0.00[a]	115.20±12.80[b]	204.80±31.35[b]	230.40±25.60[b]	179.20±31.35[b]	128.00±0.00[b]
D	8.00±0.00[a]	128.00±0.00[b]	230.40±25.60[b]	256.00±0.00[b]	204.80±31.35[b]	128.00±0.00[b]
E	8.00±0.00[a]	8.00±0.00[c]	8.00±0.00[c]	8.00±0.00[c]	8.00±0.00[c]	8.00±0.00[c]

Group A vaccinated with 0.1% formaldehyde-inactivated IBDV; Group B vaccinated with 0.2% formaldehyde-inactivated IBDV; Group C vaccinated with 0.001 mol/L BEI-inactivated IBDV; Group D vaccinated with 0.002 mol/L BEI-inactivated IBDV; Group E unvaccinated control group; Data are expressed as mean±*SE*; Means with no common superscript differ significantly ($P<0.05$)

maldehyde, 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.

The IHA titers in this study were similar to those of Buonavoglia *et al*.(1988b) with Newcastle disease virus (NDV) treated with formalin or BEI and given in an oil vaccine. They observed that the BEI-inactivated vaccine stimulated significantly higher antibody titers.

Based on our current study we recommend using BEI-inactivated IBD vaccines formulated from local strains to achieve higher antibody titers, predictive of better protection against this disease. Addition of adjuvant can further enhance the immune response and improve the efficiency of these vaccines. BEI is inexpensive, easy to prepare and less hazardous to handle and protocol for BEI inactivation is simple and preserves both structural integrity and antigenicity of the virus.

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