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Original Article

Immune Response to Killed Very Virulent Infectious Bursal Disease Virus by Water-Catholyte-Anolyte in Specific-Pathogenic-Free Chickens

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ABSTRACT

This study aimed to investigate the efficacy, safety and immunogenicity of the attenuated and inactivated Malaysian isolate of vvIBDV (UPM0081) in specific-pathogen-free (SPF) chickens. The UPM0081T15 passage 15 and UPM0081T20 passage 20 of vvIBDV attenuated in Vero cells were inactivated using water-Catholyte-Anolyte (ECA). Complete inactivation of UPM0081T15 with titer of $10^{6.7}$ $TCID_{50}$/0.1 mL and UPM0081T20 with titer of $10^{7.4}$ $TCID_{50}$/0.1mL occurred after 24 hours. The inactivated virus suspension and an equal volume of Freund's incomplete adjuvant were mixed together water-in-oil emulsion and injected subcutaneously into 42-day-old SPF chickens. Neither clinical signs nor gross lesions were observed in chickens before and after vvIBDV challenged. High and protective level of IBD antibody titer was recorded into 2 groups at 2 weeks post infection and 2 weeks post challenged. The study showed that both the inactivated UPM0081T15 and UPM0081T20 was safe and could provide 100% protection against vvIBDV challenged with titer of $10^{7.8}$ EID_{50}/ 0.1 mL.

Keywords: Electrolysed water-Catholyte-Anolyte (ECA), Infectious bursal disease virus (IBDV)

INTRODUCTION

Infectious bursal disease (IBDV) virus is the causative agent of IBD a highly contagious disease of young chickens. The virus infects and multiplies in immature B lymphocyte found in the bursa of Fabricius, which can result in immunosuppression (Faragher et al., 1974). Recent research had indicated the emergence of very virulent IBD virus vvIBDV with difference in virulence and the antigenic variation associated with the strain has been the greatest obstacle for successful control of IBD (van den Berg, 2000).

There is no treatment against IBDV infection and the effect of virus exposure. The induction of active immunity by vaccine is still the effective method of control, but this should include biosecurity measures (Lucio & Hitchner, 1979). The preparation of an effective vaccine is precluded by the isolation and characterisation of an appropriate IBDV strains with subsequent attenuation before the preparation into live or killed vaccines. The inactivation of antigen can be carried out by physical or chemical agents. For physical methods, heat and radiation are involved while for chemical reagents binary ethylenimine (BEI), formaldehyde, aziridines and other derivatives used (Bahnemann, 1990). BEI has been used to inactivate different viruses such as rabies (Larghi & Nebel, 1980), foot-and-mouth disease virus (Dilovski & Tekerlekov, 1983) and Newcastle disease virus (King, 1991). The use of this agent in preparation of IBD vaccine are few in literature (Habib et al., 2006).

On the other hand, Electrolysed water-Catholyte-Anolyte (ECA) is an activated solution with a highly oxidized anolyte solution which functions as a very fast acting antimicrobial agent that destroys viruses and other microorganisms. Studies have suggested that the hypochlorous acid in the course of its production can penetrate microbial cell membranes and in turn exert antimicrobial action through the oxidation of key metabolic systems (Albrich et al., 1986; Barrette et al., 1989). The use of this agent in the inactivation of IBD viruses has not received adequate attention.

Since the purpose of vaccination is the production of a strong immune response to the administered antigen, this feat often requires the addition of an adjuvant. Commonly, water in oil (W/O) emulsions is recommended for small ruminants, bovine, poultry and fishes when long term immunity is required. In the case of IBD, mineral oil based emulsions had been used as adjuvant in order to protect birds against IBDV infection (Benjamin & Hitchner, 1978). The W/O emulsions usually allow for the reduction of the vaccine dose or the antigen

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concentration, which is important to make such vaccines to be cost effective (Aucouturier et al., 2001).

Freund's Complete Adjuvant (FCA), a mixture of a non-metabolizable oil, such as a mineral oil, a surfactant (Arlacel A) and mycobacteria (M. tuberculosis) has been used for many years to enhance immunologic response to antigens, and even today is considered to be one of the most effective adjuvants (Jackson & Fox, 1995). Freund's Incomplete Adjuvent (FIA) is similar to FCA but does not contain mycobacterium. FIA is frequently used to boost immune system after the use of live vaccines. The objectives of the study were to determine the safety and immunogenicity of an attenuated and inactivated Malaysian isolate of vvIBDV by using new chemical product ECA in specific-pathogen-free (SPF) chickens.

MATERIALS AND METHODS

Virus and Cells

Vero cell were grow in polystyrene 150 cm^2 (Nunc Easyflasks, Denmark) containing 30 mL RPMI supplemented with 10% FBS. A confluent cell monolayer was infected with vvIBDV (UPM0081) and the virus was allowed to adsorb for 60 minutes at 37ºC in 5% CO_2 with intermittent rotation to allow the virus to adsorb on the surface of Vero cells. After viral adsorption, the inoculums was removed and replaced with 30 mL RPMI containing 1% FBS and retained in an incubator at 37ºC in 5% CO_2 for 4 days.

Harvesting of Virus

The Vero cells infected with the IBDV were harvested when cytopathic effect (CPE) reached 90%. The IBDV-infected cells were centrifuged at 3000 rpm for 20 minutes at 4ºC the resultant supernatant fluids were harvested, filtered through a 0.45 µm filter (Sartorius, Germany), aliquot and stored at -80ºC.

Inactivation of vv IBDV

The inactivation of the two Vero cell adapted and attenuated vvIBDV at passage 15 (P15) and 20 (P20) were carried out using Electrolysed water-Catholyte-Anolyte (ECA) treatment.

Electrolysed water-Catholyte-Anolyte (ECA) Treatment

ECA solution, an anolyte with pH 2.2 was used to inactivate the virus by adding 0.5 mL of viral suspensions at P15 ($TCID_{50}=10^{6.7}$) or P20 ($TCID_{50}=10^{7.4}$) to 4.5 mL of the anolyte solution to make 1/10 dilution. A control group without addition of ECA was included.

Determination of Time Required to Inactivate Virus

Samples from ECA (P15 and P20) groups as the treated viruses and the control group were incubated for different times namely as 6, 12, 24, 30 and 36 hours at 37.5ºC to determine the inactivation time of the virus. After incubation period, the treated virus was inoculated in to 5, 10-day-old SPF embryonated chicken eggs through chorioallantoic membrane (CAM) route. After inoculation, all eggs were sealed with melted wax and were re-incubated at 37.5ºC. Inoculated eggs were candled daily for lesions associated with IBDV.

Perparation of Killed- Virus Oil Emulsion

One volum of killed virus suspecsion (P15 and P20 treated ECA) was mixed with an equal volume of Freund's incomplete adjuvant (Sigma, USA) by using a Waring blender at the highest speed 20000 rpm for 15 minutes. An equal volume of 2% Tween-80 was added to the mixture and the emulsion was mixed again for 15 minutes giving a final 1:3 dilution of virus suspension (Benjamin and Hitchner, 1978).

Experimental Design

A total of 20, 42-day-old SPF chickens, were divided into four groups namely the E1, E2, C1 and C2 with 5 birds in each group (Table 1). E1 group (ECA P15), E2 (ECA P20), C1 (control negative) without inoculation and unchallenged and C2 (control positive) without inoculation and challenged. The inoculum in the groups E1 and E2 were mixed with Freund's incomplete adjuvant, and injected at 42-day-old chickens subcutaneously (0.1mL/dose) with IBDV isolate P15 and P20. At 14 days post inoculation the chicken challenged with vvIBDV field strain (UPM0081) with the titer of $10^{7.8}$ EID_{50}/0.1mL through the oral route. One chicken from each group was sacrificed at day 0 prior to inoculation and all chickens from the groups were sacrificed at day 70 or two weeks post challenged. The clinical signs and mortality were recorded up to 10 days post challenged. Blood samples were collected for detection of IBD antibody. The body weight, bursa weight, bursa to body weight ratio were recorded. Samples from bursa were collected and a part was fixed in 10% formalin for histopathology, and the other was used for IBDV detection using RT-PCR.

Table. 1. Different groups of chickens inoculated with inactivated vvIBDV (ECA) and the control groups

Group / Passage	Definition	Time of sampling (Day 0 before inoculation	Time of sampling (2weeks p.c)	No. of chic kens
ECA / P15	Inactivated attenuated vvIBDV inoculated & vvIBDV challenged	1	4	5
ECA / P20	Inactivated attenuated vvIBDV inoculated & vvIBDV challenged	1	4	5
C1	Uninoculated & unchallenged	1	4	5
C2	Uninoculated & challenged	1	4	5

Microscopic Examination and Lesion Score

Sections of bursal tissues from inoculated and control chickens were stained with hematoxylin and eosin staining technique. Bursal histological lesions were scored according to the method of Hair-Bejo et al., 2000). Briefly, bursa of Fabricius lesions was scored in a scale of 0 to 5 based on the presence of lymphoid cell necrosis, degeneration, oedema, hetrophil infiltration, and follicular cysts formation. The score of 0 represented no lesion observed, 1 for mild, 2 for mild to moderate, 3 for moderate, 4 for moderate to severe, and 5 for severe lesions.

Determination of ELISA Titer Against Inactivated IBDV Vaccine

The technique of the test was followed as described by Howie & Thorsen, (1981), and was conducted by One Point Health Company using precoated ELISA kit (BioChek, UK). Briefly diluted test sera (diluted in phosphate buffer at 1:500) were added into the appropriate wells, already coated with IBDV antigens and the plate was incubated at 37°C for 30 minutes. The contents of wells were aspirated and plate was washed five times with 300 µl of ddH_2O. Anti-chicken alkaline phosphates (100 µl) of was added to each well and the plate was incubated at 37°C for 30 minutes. The plate was washed as above. Hundred µl of p-Nitrophenyl phosphate (PNPP) was added to each well and the plate was blanked in the air and the reading was recorded by reading the optical density (OD) spectrophotometrically at 450nm. Positive and negative sera were used as controls as recommended by the manufacturer.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA from bursa of Fabricius was extracted by using the Trizol reagent. Amplification of the hypervariable region of VP2 genes was carried out by using reverse transcriptase polymerase chain reaction (RT-PCR). Primers used for amplification were: (P1) TCA CCG TCC TCA GCT TAC and (P2) TCA GGA TTT GGG ATC AGC (Jackwood et al., 1997).

Statistical analysis

Data was analysed statistically using analysis of variance (two way ANOVA) followed by Duncan's multiple range test was used as the post hoc produced by using SPSS version 15 for windows (Norusis, 2004).

RESULTS

Inactivation of the Virus Attenuated vvIBDV

The two Vero cells adapted vvIBDV and attenuated P15 and P20 were completely inactivated at 24 hours treatment with ECA.

Clinical Signs

No clinical signs and mortality were observed in any chickens in the groups C1, ECAP15, and C2, ECAP20 throughout the experiment, except group C2 had showed clinical signs of severe depression, ruffled feather, and anorexia. There was 100% mortality at day 4 post vvIBDV challenged.

Bursa to Body Weight Ratio ($1x10^{-3}$)

There was no significant difference ($p>0.05$) between the ECAP 15 (3.06 ± 0.04) and ECAP 20 (3.08 ± 0.46) groups, when compared to the group C1 (3.09 ± 0.32).

Gross Lesions

The chickens in the groups C1, ECAP15 and ECAP20 did not show any gross lesions of IBD after two weeks post challenged

Histological Lesions Score

All chickens in the groups C1, ECAP15 and ECAP20 showed normal to mild bursitis with lesion score of 0.45 ± 0.17, 0.62 ± 0.09 and 0.50 ± 0.81, respectively. While the histopathological changes in the group C2 showed severe lymphoid depletion, lymphocytolysis, degeneration and formation of follicular cyst with fibrin exudates in the medulla and mononuclear inflammatory cells, oedema in the interstitial connective tissue at day 2 post-challenged with lesion score of 4.72 ± 0.32.

Antibody Titer (ELISA)

There was no significant difference ($p>0.05$) between the mean antibody titer at two weeks post inoculation ECAP15 12303 ± 4515 and ECAP20 13557 ± 2479.

Detection of the Virus or Viral RNA using RT-PCR

No viral RNA was detected in the bursa homogenates from chickens inoculated with inactivated vvIBDV in groups ECAP15 and ECAP20 at day 14 post challenged.

DISCUSSION

The study showed that UPM0081T15 and UPM0081T20 attenuated vvIBDV were successfully inactivated by ECA. 24 hours after the treatment. The inactivated vvIBDV conjugated with incomplete Freund's adjuvant could provide full protection (100%) against vvIBDV (UPM0081) challenged. Inoculation of inactivated IBDV could give complete protection with no obvious IBD clinical signs, was reported previously (Maas et al., 2001).

The bursa to body weight ratio, were remained unchanged in the ECAP15 and ECAP20 groups. It was reported that no bursa to body weight ratio changes of chicken administered with BGM-70 attenuated and inactivated IN and STC strains of IBDV and the control group (Hassan & Saif, 1996). Histopathological assessment also revealed similar pattern as the bursa lesion score was normal to mild in treatment groups ECAP15 and ECP20 groups

On the basis of humoral immune response, the results showed that the inactivated passage 15 UPM0081T15 and passage 20 UPM0081T20 of vvIBDV used in all the treatment groups were immunogenic with increased in antibody titers in all inoculated groups 2 weeks pi. (Habib et al., 2006). The possible mechanism of inactivation of ECA as regards IBDV needed to be investigated.

The result of this study also showed that a single dose of the inactivated passage 15 UPM0081T15 and passage 20 UPM0081T20 of IBDV in both groups (

ECA) could give 100% protection against vvIBDV challenged, which is in contrast with the report of 100 % protection obtained with the use of two doses of killed IBD vaccines at a week interval in 3 weeks SPF chickens (Hsieh et al., 2007).

Since the current facts showed that the humoral immune response plays the principal role in defense against vvIBDV (Lukert & Saif, 1997), there may be a need to study the cell mediated immune response and the effect of vaccine inactivated by this substances ECA on the cellular mechanism since T cells are also important in the protection against virulent IBDV (Rautenschlein & Sharma, 2002).

The scope of this study did not cover the possible effect of the adjuvant employed but it was assumed that the adjuvant also enhanced the humoral response that was recorded and the use of tween-80 in the combination with Freund's incomplete adjuvant (FIA) made the adjuvant less viscous and this may have reduced the side effects associated with the adjuvant and the possible alteration of the antigenicity of IBDV (Stone et al.,1978).

The chemical interactions between the ECA and incomplete Freund's adjuvant needed to be investigated as this may give a clue to the difference in the humoral and histopathological scores observed in the two treatment groups.

In this experiment, the possibility of replication of the inactivated virus was also investigated by using RT-PCR. No viral genetic material was detected in the bursa of Fabricius of the inoculated birds at two weeks post challenged, indicating that the IBDV was inactivated and there was no replication of viral RNA or inactivated virus in bursa of Fabricius. This further showed that the reversal to virulence as in the case of attenuated live vaccine is not possible in this case.

It was concluded that chickens inoculated once with inactivated ECA attenuated vvIBDV UPM0081 in Vero cell at passages 15 and 20 could adequately protect against vvIBDV challenged.

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