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Hatchery Vaccination using Liposomes as Vaccine Delivery against Infectious Bursal Disease in Broiler Chickens

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Abstract: Infectious Bursal Disease (IBD) is a highly contagious viral disease of chickens. Application of liposomes in IBD vaccine may enhance vaccine delivery and induce high immune response. It was objective of the study to determine the effect of cationic liposomes in induction of IBD antibody. The 85 days old broiler chicks were divided into 4 groups namely the control, IBD, Covac and Sevac groups. The chicks in the control group were not vaccinated. The IBD group was vaccinated with commercial IBD vaccine, MyHatch UPM93 (10^3 EID_{50}/0.1 mL) whilst the Covac group was vaccinated with combination 1:2 ratio of MyHatch UPM93 and cationic liposomes. The Sevac group was vaccinated with combination 1:2 ratio of the IBDV seed virus (10^3 EID_{50}/ 0.1 mL) and cationic liposomes. The commercial and IBD seed virus were used in this study to determine the most effective and practical approach in application of liposomes during vaccine preparation. The chicks were vaccinated via. subcutaneous route (0.1 mL per chick) at day old in hatchery or hatchery vaccination. All groups of chicks were sacrificed at days 7, 14, 21 and 28 post vaccination (pv) for samples collection, except 5 chicks were also sacrificed prior to vaccination at day-old age. The study showed that all chicks did not exhibit any abnormal clinical signs and gross lesions throughout the trial, except reduced in bursal weight were recorded at day 28 pv in the IBD, Covac and Sevac groups. Histologically, lesion scoring of the bursa of Fabricius was slightly increased at days 21 and 28 pv in the Covac and Sevac groups and day 28 pv in the IBD group. The IBD antibody titre for the Covac and Sevac groups were also significantly ($p<0.05$) increased at days 21 and 28 pv and day 28 pv in the IBD group. It was concluded that the application of cationic liposomes can enhance the delivery of IBD vaccine to the bursa of Fabricius and induce high level of IBD antibody with mild bursal lesion.

Key words: Infectious bursal disease, vaccine delivery, liposomes, hatchery or day old vaccination, chickens, antibody, significantly

INTRODUCTION

Infectious Bursal Disease (IBD) or also known as gumboro disease is a highly contagious viral disease in susceptible young chickens (Cosgrove, 1962). The disease results in significant bursal hemorrhage and atrophy, severe immuno suppression and high mortality (Kibenge *et al*., 1988; Whitfill *et al*., 1995; Hair-Bejo *et al*., 2004). Infectious Bursal Disease Virus (IBDV) can be detected in chickens and has a worldwide distribution. This virus is belongs to the family Birnaviridae of the genus Avibirnavirus. It is a double-stranded Ribo Nucleic Acid (dsRNA), very stable and small virus with a diameter approximately 60 nm (Nagarajan and Kibenge, 1997). The virus also has a single capsid shelled non-enveloped virion with icosahedral symmetry (Nick *et al*., 1976; Nagarajan and Kibenge, 1997). The bursa of Fabricius is the main target organ for IBDV (Kaufer and Weiss, 1980).

The virus persist in poultry houses after thorough cleaning and disinfection or remain for long in a contaminated environment. It is more resistant to heat (Alexander and Chettle, 1998; Mandeville *et al*., 2000). Therefore, vaccination programmes are important to prevent IBD outbreak. The objective of vaccination is to stimulate protective immunity while avoiding disease from the vaccine itself. Early vaccination is necessary for protecting the chicks from the disease (Hair-Bejo *et al*., 2004). Nevertheless, maternal antibodies may interfere with early vaccination with IBD vaccines (Muller *et al*., 2003).

Currently, several types of IBD vaccines are available in commercial chickens industry including killed vaccines, live attenuated vaccines, subunit vaccines and DNA vaccines (Muller *et al*., 2003; Hair-Bejo *et al*., 2000). Inactivated or killed vaccines are typically safe but less effective compared with live attenuated vaccines

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(Van den Berg, 2000; Sarachai *et al*., 2010). The virus is unable to replicate in the chickens. Live attenuated vaccines are usually developed from the field or wild virus attenuated in chicken embyronated eggs or tissue culture (Lauring *et al*., 2010). The vaccine virus could replicate effectively in the target organ of the chickens and could induce protective immune responses similar to natural infection. However, this vaccine may have the risk associated with the potential for reversion. Recent efforts have focused on utilizing technologies such as recombinant DNA methods to develop DNA and subunit vaccines (Park *et al*., 2009). However, the recombinant technologies are costly and complicated. The routes and time of vaccination is always an issue on the efficacy of the vaccine, vaccination failure and economical costs of vaccination programme. Hatchery vaccination could assure a good start of chicken health and performance and will open the door to a real control and prevention of chicken diseases. It guarantees every chick received protection at the earliest age. Vaccine carrier could enhance the efficacy of a vaccine in hatchery vaccination.

It is indeed a critical need for effective delivery vehicles as well as new adjuvants to enhance the efficacy of a vaccine. In many cases, the antigen itself is only very weakly immunogenic, therefore, an adjuvant is needed to intensify the immune response. Adjuvants can also be included in vaccines to guide the type of immune response generated (Peek *et al*., 2008). From the previous studies, among these carriers, liposomes have been proved as efficient drugs and vaccines delivery systems and gaining attention due to their abilities to act as delivery vehicles and adjuvants (Fraley *et al*., 1980; Muderhwa *et al*., 1999; Tardi *et al*., 2000). Liposomes are synthetic spheres composed of lipid bilayers that can encapsulate antigens and was first described by Dr. Alex D. Bangham in 1961. Bangham *et al*. (1965) it was discovered that liposome is essentially bilayer vesicles composed of lipids which form upon exposure to aqueous media. There are several advantages of liposomes as carrier for vaccine delivery. In general, liposome is a tiny empty vesicle made out of the similar material as a cell membrane. Liposomes are composed of relatively biocompatible and biodegradable materials. The liposome size can range from 0.05-5.0 μm (Sharma and Sharma, 1997), 20 nm-20 μm (Taylor and Morris, 1995) or 80 nm-100 μm in diameter (Slabbert *et al*., 2011). These vesicles can be filled with drugs or vaccines and act as carriers to deliver the antigens to the target organs. There are several advantages of liposomes such as sustained release, site specific delivery and useful alternative to reduce dosage amount of antigen (Chrai *et al*., 2011; Slabbert *et al*., 2011). The liposomes composition and preparation can be chosen to achieve desired features. For instance, liposomes can composed in three types; Small Unilammellar Vesicles (SUV), Large Unilamellar Vesicles (LUV) and Multilamellar Vesicles (MLV) with different types of charge such as neutral, positively and negatively charged. The presence of charge lipid is important to reduce the likelihood of aggregation in liposomes (Plessis *et al*., 1996). It was proposed that positively charged or also known as cationic liposomes could deliver antigen to cells by fusion with negatively charged cell membranes and protect antigen from clearance in the body (Onuigbo *et al*., 2012). Positively charged liposomes also allow for efficient electrostatic interactions with negatively charged cell surfaces, resulting in enhanced uptake by cells through adsorptive endocytosis (Tseng *et al*., 2009). In veterinary field, application of liposomes is not well established. Some studies were reported that the combination of commercial Newcastle Disease (ND) vaccine using live La Sota strain with liposomes against ND can induce high antibody titre (Tseng *et al*., 2009). Therefore, the objective of this study was to determine the effectiveness of cationic liposomes in the induction of IBD antibody titre in commercial broiler chickens via. subcutaneous injection at day old of age at hatchery or hatchery vaccination.

MATERIALS AND METHODS

Lipids: A 1,2-Dipalmitoyl-sn-glycero-3-Phosphacholine (DPPC) and 1,2-Dioleoyl-Trimethylammonium-Propane (DOTAP) were commercially purchased from avanti polar lipids (Alabaster, USA) meanwhile, cholesterol was purchased from MP biomedical (California, USA).

Preparation inoculum: The commercial freeze-dried live attenuated IBD vaccine (Co) and working IBD seed virus (Se), MyHatch UPM93 strain with virus titre of 10^3 EID_{50}/ 0.1 mL were obtained from Malaysian Vaccine and Pharmaceuticals Sdn. Bhd. (MVP). They were diluted with sterile PBS in dilution 1/100 and stored at -20°C until used.

Preparation of cationic liposomes: Liposomes were prepared by using conventional and most widely used technique in the laboratory, a thin film hydration method (Tseng *et al*., 2009). Briefly, the lipid mixtures; DPPC, cholesterol and DOTAP with molar ratio 7:2:1 were combined in 100 mL round bottom flask. The flask was placed on the rotary evaporator to evaporate the solvents at the temperature 55°C and a thin lipid was formed on the glass wall. The thin film was kept continue under

evaporator around 3 h to ensure the solvent completely removed. The flask which contain dry lipid films were hydrated with diluted IBD vaccine either working IBD seed virus or commercial IBD vaccine based on the ratio 1:2. Then, the hydrated liposome was sonicated in an ultrasonicator water bath at temperature 45°C to homogenize the liposome (Dua *et al*., 2012) then, stored at 4°C.

Characterization of liposomes and IBDV: The measurement of size and zeta potential were performed at 25°C using zetasizer machine (Malvern Instruments Ltd., United Kingdom). First, 0.1 mL of each sample was diluted with 1.0 mL double distilled water. Then, the sample was injected into the cuvex cell before inserted into the machine (Onuigbo *et al*., 2012).

IBD hatchery vaccination: The 85 day old broiler chicks were divided into 4 groups namely the control, IBD, Covac and Sevac groups. Each group contained 20 chicks, except for the control group contained 25 chicks. The chicks in the control group were not vaccinated. About 5 chicks from the control group were sacrificed prior to the vaccination trial. The chicks in IBD group was vaccinated with commercial IBD vaccine, MyHatch UPM93 (10^3 EID_{50}/0.1 mL). The Covac group was vaccinated with combination 1:2 ratio of MyHatch UPM93 and cationic liposomes whilst the Sevac group was vaccinated with combination 1:2 ratio of the IBDV seed virus (10^3 EID_{50}/0.1 mL) and cationic liposomes. The commercial and the IBD seed virus were used in the study to determine the most effective and practical approach in application of liposomes during vaccine preparation. The chicks were vaccinated via. subcutaneous route (0.1 mL per chick) at day old in hatchery or hatchery vaccination. All groups of chicks were sacrificed at days 7, 14, 21 and 28 post vaccination (pv) for samples collection. The body weights of chicks were recorded and blood samples were collected for IBD antibody titre prior to necropsy. On necropsy, the bursa weight was recorded and bursa was fixed into 10% buffered formalin for histological examination and lesion scoring.

Histopathology: All the samples of bursa of Fabricius were fixed in 10% buffered formalin. Each tissue was trimmed to the thickness about 5 mm in size, fixed and dehydrated in a series of alcohol concentration (70, 90 and 100%) and embedded in paraffin wax using an automatic tissue processor (Leica 5500). Sectioning of tissues was done to a thickness of 0.4 μm on a microtome. The samples was mounted on glass slides, dewaxed and stained with Haematoxylin and Eosin (HE) for histological lesion scoring (Tanimura *et al*., 1995).

Histological lesion scoring: The histological lesion scoring for bursa of Fabricius was conducted based on the previous method (Hair-Bejo *et al*., 2000). Briefly, the bursa of Fabricius lesion was scored in a scale of 0-5. No lesion observed (score of 0). Mild degeneration and necrosis of lymphoid cells in a few follicles (score of 1). Mild to moderate degeneration and necrosis of lymphoid cells in a few follicles, oedematous and infiltration of inflammatory cells (score of 2). Moderate follicular degeneration and necrosis involving both the cortex and medulla with obvious interstitial space infiltrated with heterophils, macrophage and a few fibroblasts and erythrocytes (score of 3). Moderate to severe depletion of lymphoid cells in the follicles with necrotic cells and present of cysts in some follicles. The interstitial space is infiltrated with inflammatory cells and well packed with fibrinous connective tissues whilst epithelium was thickened, corrugated and vacuolated in some areas (score of 4). Severe acute cellular necrosis and degeneration in both cortex and medulla with follicular cysts and fibrinous exudates. The interstitial connective tissue is oedematous and infiltrated with mild to moderate inflammatory cells (score of 5) or severe chronic follicle atrophy with cysts formation within the follicles and epithelial lining of the organ with remarkable infiltration of fibroblasts, lymphocytes and macrophages the interstitial tissues (score of 5).

IBD antibody titre (ELISA): The samples of blood serum were submitted to One Point Health Laboratory at Bandar Baru Bangi, Selangor Darul Ehsan for determination of IBD antibody titre using IBD ELISA kit (BioChek, United Kingdom) according to the manufacturer protocol.

Statistical analysis: Data was analysed using one-way Analysis of Variance (ANOVA) to compare the effect of vaccination. The multiple range tests (Tukey, Scheffe, Duncan and LSD) were used as the post hoc using SPSS Version 19.0 for Windows.

RESULTS AND DISCUSSION

Size: There were statistically significant differences ($p<0.05$) between Se (987±140.0 nm) and Co (1333±64.6 nm) groups with the Sevac (1327±185 nm) and Covac (1688±99.6 nm) groups, respectively. However, there were no significant differences ($p>0.05$), between the Se and Co groups and Sevac and Covac groups.

Zeta potential: There were significant differences ($p<0.05$) between the Se (-13.1±1.11 mV) and Co (-12.3±0.68 mV) groups with the Sevac (9.6±1.82 mV) and Covac

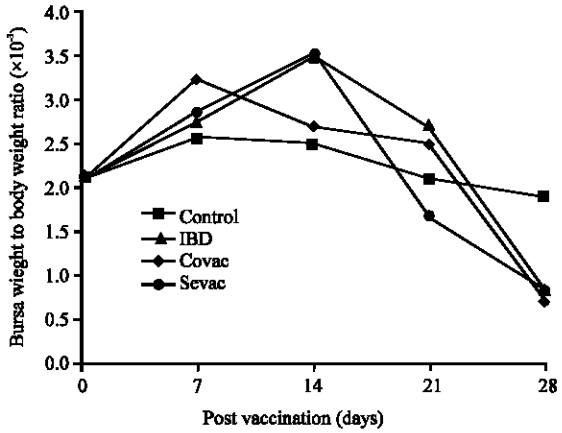

Fig. 1: Bursa to body weight ratio of the chickens throughout the experiment

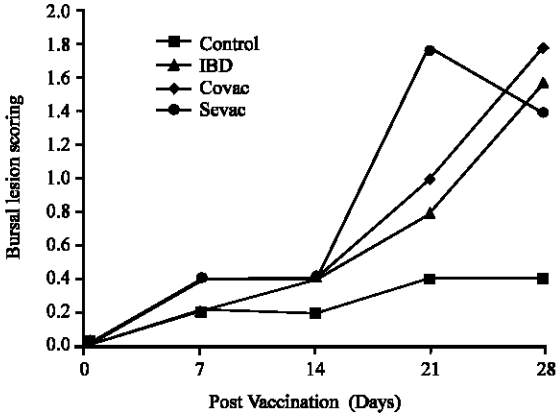

Fig. 2: Histological lesion scoring of the bursa of Fabricius throughout the experiment

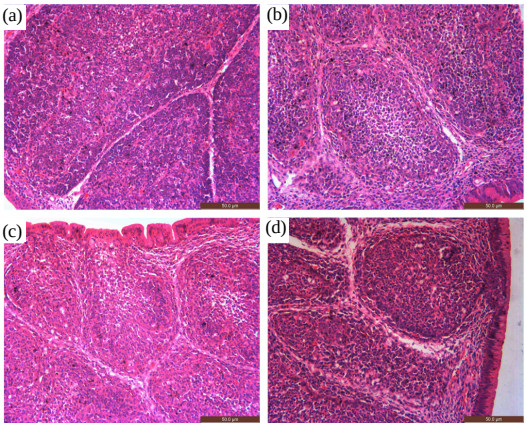

Fig. 3: Histological lesion scoring of the bursa of Fibricius at 28 days post vaccination; a) Control group with lesion scoring of 0; b) IBD group with lesion scoring of 1; c) Covac group with lesion scoring of 1 and d) Sevac group with lesion scoring of 1. HE, Bar = 50 um

(7.4±1.31 mV) groups, respectively. However, there were no significant difference ($p>0.05$) between the Se and Co groups and Sevac and Covac groups.

Clinical signs: Chickens from all groups did not exhibit any abnormal clinical signs throughout the trial.

Gross lesions: No gross lesions were recorded in the chickens in all groups throughout the experiment, except the bursa of Fabricius were atrophied at day 28 pv for the IBD, Sevac and Covac groups.

Bursa to body weight ratio: The bursa to body weight ratio in the IBD group was increased from day 0 until 14 pv and decreased significantly ($p<0.05$) at day 28 pv. The bursa to body weight ratio in the Covac group was increased from day 0 until 7 pv and decreased significantly ($p<0.05$) at day 28 pv. The bursa to body weight ratio for the Sevac group was increased from day 0 until 14 pv and decreased significantly ($p<0.05$) at day 21 and 28 pv. The bursa to body weight ratio of the chickens in the IBD, Covac and Sevac groups were decreased significantly ($p<0.05$) at day 28 when compared to the control group (Fig. 1).

Histopathology: The histological lesion scoring of bursa of Fabricius in the control group remained unchanged throughout the trial. The lesion scoring in the Covac and Sevac groups were slightly increased ($p>0.05$) at day 21 and 28 pv. However, the lesion scoring in the IBD group was significantly increased ($p<0.05$) at day 28 pv. The lesion scoring in all groups at each time of sampling remained unchanged ($p>0.05$) throughout the trial (Fig. 2 and 3).

IBD antibody titre: The antibody titre for the control group was significantly ($p<0.05$) decreased from day 0 pv to a lowest level at day 28 pv. The antibody titre in the IBD, Covac and Sevac groups were significantly decreased ($p<0.05$) at days 7 and 14 pv and increased significantly ($p<0.05$) at day 28 pv. The antibody titre in the IBD, Covac and Sevac groups were increased significantly ($p<0.05$) at day 28 when compared to the control group (Fig. 4).

Liposomes can elicit cell mediated and humoral immunity (Onuigbo *et al*., 2012). Based on the previous study by Rao and Alving (2000) stated that the liposomes can function efficiently in delivery system for entry of antigens into Major Histocompatibility Complex (MHC)

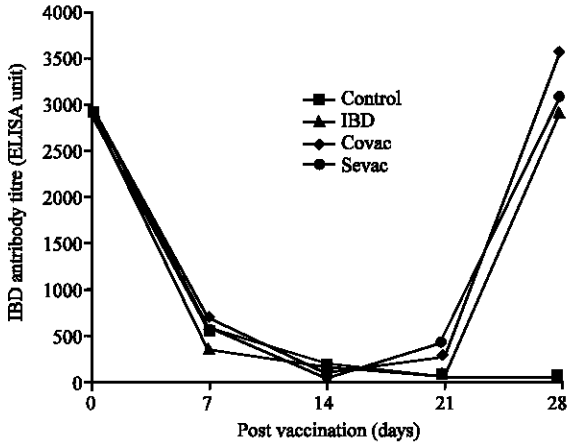

Fig. 4: IBD antibody titre of the chickens throughout the experiment

Class 1 pathway and hence are very effective inducers of cytotoxic T cell responses. Besides, liposomes also have capability to turn antigens into MHC Class 2 pathway of phagocytic Antigen-Presenting Cells (APCs) which can enhance the induction of antigen-specific T cell proliferative as well as antibodies responses (Rao and Alving, 2000). In the present study association of liposomes and IBDV have a significant effect on the immune cells. It appears that the combination both, liposomes and IBDV may allow the antigens to gain access to the both MHC Class 1 and 2 pathways in Antigen Presenting Cells (APC) and therefore, stimulating antibody production as well as cell immune responses.

The physical characteristics of liposomes have been shown to be a factor that can influence the relatively levels of antibody expression against microorganisms (Tseng *et al*., 2009). Characteristics of liposomes such as size and charge have been shown to affect liposomal uptake by macrophages. Cellular uptake of liposomes is generally believed to be mediated by the adsorption of liposomes onto the cell surface and subsequent internalization and endocytosis. It is widely accepted that positively charged liposomes can be taken up in large quantities (Tseng *et al*., 2009).

The particle sizes for the combination of positively charged liposomes with IBDV either commercial or seed virus used in the present study were <2000 nm which still within the range size of liposome that was reported previously (Sharma and Sharma, 1997). It was suggested before that the MLV liposomes acts as a carrier function to bring the antigens in the hydrophilic core and the antigens are then released from the lipid bilayers (Tseng *et al*., 2009). The slow release of antigens acts as a depot effect in which the presence of the antigen may be maintained for a longer period of time compared to soluble antigen. Knowledge of zeta potential of liposomes would help to predict its fate *in vivo*. The value of zeta potential of the liposome in the present study was low (<30 mV) and hence, it will reduce tendency toxicity of the liposomes to occur (Smistad *et al*., 2007). Besides, positively charged liposomes will normally fuse with negatively charged cells. Once internalized, the particles are offered to the continuous lymphoid tissue. The adsorption of the negatively charged particles at the positively charged sites via. electrostatic interaction can lead to localized neutralization and a subsequent bending of the membrane favouring in turn endocytosis for cellular uptake. Thus, the formulation of liposomes with positively charged surface properties can influence their cellular uptake and intracellular distribution and it is possible to localize the liposomes to specific intracellular targets (Honary and Zahir, 2013). Because of many proteins, vaccines and cell membrane surface are slightly anionic, a positive zeta potential not only has benefits for enhanced vaccine loading efficiency but it also might provide the effective accumulation in the target cells (Honary and Zahir, 2013).

The present study also demonstrated that MyHatch UPM93 either with or without liposomes is safe with good body weight performance and without causing any gross lesions or abnormal clinical signs to the chickens. The bursa weight and bursa to body weight ratio of the chickens in the IBD, Covac and Sevac groups were remained unchanged when compared to the control group, except at day 28 pv the organ was smaller due to proliferation of the IBDV in the bursa of Fabricius and therefore, leading to the induction of high level of IBD antibody titre. A similar pattern of lesions scoring was recorded in the bursa of Fabricius. This clearly showed that the liposomes in the Covac and Sevac groups play a role in accelerate the movement of IBDV vaccine to the target organ. However, the mild lesion scoring recorded in this study demonstrated that the IBD vaccine either with our without liposomes is safe to apply in the commercial broiler chickens.

The induction of high and protective level of IBD antibody (>1000 ELISA unit) is directly related with the lesion scoring in the bursa of Fabricius. It is interesting to note that despite of low concentration of IBDV in the Covac and Sevac groups (2/3) when compared to the IBD group the induction of IBD titre was remained high. This demonstrated that the encapsulation of IBDV in liposomes could enhance the induction of IBD antibody titre (Tseng *et al*., 2009; Onuigbo *et al*., 2012).

CONCLUSION

This study demonstrated that the application of cationic liposomes can enhance the deliver IBD vaccine

to the target organ, the bursa of Fabricius and induce high and protective level of IBD antibody titre with mild bursal lesion. Hatchery vaccination using UPM93 IBDV strain either with or without cationic liposomes is effective and could induce high and protective level of IBD antibody against IBDV challenged. These applications will give a new dimension in the field of poultry vaccines and vaccination.

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