ANTIGENIC CONTENT OF VERO CELL ADAPTED INFECTIOUS BURSAL DISEASE VIRUS (IBDV) PRODUCED BY DIFFERENT CULTURE METHODS

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ABSTRACT

Vero cell adapted Infectious bursal disease virus (IBDV) propagated by different culture methods showed that Roller and Microcarrier cultures produced higher titres when compared to stationary culture. The peak titres ($10^{6.5}$ and $10^{7.7}$ tissue culture infective dose-50 ($TCID_{50}$)/ 0.1 ml) were seen by 96 hours post infection (HPI) in Roller and Microcarrier culture methods except in stationary culture where it was seen by 120 HPI. The different inactivated IBD vaccines prepared from different culture methods and one commercial vaccine of Specific Pathogen Free (SPF) embryo origin showed no significant difference in the immune responses assessed by Quantitative Agar Gel Precipitation Test (QAGPT) and Neutralization test. All the vaccinated birds withstood the virulent challenge as demonstrated on 3 days post challenge by the absence of IBDV antigen in the bursa by Agar Gel Precipitation Test (AGPT) while IBD antigen was present in positive control birds. On 10 days post challenge, the bursa:body weight ratio (B:B ratio), bursa:body weight index (B:B index) and bursal scores of vaccinated birds were similar to unvaccinated unchallenged control birds. The unvaccinated challenged birds had bursal atrophy with bursal score of 2.

Key words: Infectious bursal disease; Vero cells; Microcarrier; Antigenic content; Oil adjuvant vaccine

INTRODUCTION

Infectious bursal disease is an acute, highly contagious viral infection of young chickens, often resulting in immunosuppression. It is characterized by marked pathological changes in bursa of Fabricius (BF) (Cosgrove, 1962). The chickens can be vaccinated with live or inactivated IBDV vaccines for protection against IBD,. Live vaccines are used in an attempt to control the virulent form of the disease but with only partial success. The levels of maternal antibodies in day-old chicks often vary widely and in the face of the persistence of IBD virus on most farms, an effective vaccine regimen is difficult to establish. Live vaccine derived from chicken embryo fibroblast (CEF) cultures or embryonated eggs of SPF chicken is expensive. Vaccines derived from non-SPF chicken may contain extraneous avian viruses or microorganisms. Also live vaccine can cause severe immunosuppression, bursal damage and persistence of virus in vaccinated farms.

Mammalian continuous cell lines would be suitable alternatives for vaccine production. They have several advantages over the use of primary cell cultures. Maintaining a continuous cell line is more cost effective than propagating CEF cultures from SPF chicken embryos. Primary cultures have a finite life span *in vitro* and must be propagated from tissues using time consuming and laborious procedures. The aim of this study was to evaluate the growth of vero

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cell adapted IBDV in different culture methods, to prepare oil adjuvant inactivated vaccines and assess immunogenicity in birds.

MATERIALS AND METHODS

Virus passage

The Vero cell adapted IBDV used in this study had a titre of $10^{6.3}$ $TCID_{50}$/ml. Confluent monolayer of vero cells grown in 25-centimetre square (cm^2) tissue culture flask (Nunc,Denmark) was inoculated with vero cell adapted IBDV. The flask was then incubated at 37°C in an atmosphere containing 5 per cent carbon dioxide (CO_2). The monolayer showing 70 per cent CPE was harvested, aliquoted after 3 cycles of freezing and thawing and then stored at -70°C. The cell culture lysates were used as viral inocula in subsequent passages. The IBDV was confirmed by fluorescent antibody test (FAT) and virus neutralization test.

Virus propagation by different culture methods

Vero cells were grown to confluence in 174-cm^2 surface area tissue culture Roux flask. The growth medium was removed, monolayer was washed with maintenance medium and inoculated with 10 ml of 1 in 10 dilution of seed virus. After one hour incubation at 37°C, 80 ml of maintenance medium was added and incubated at 37°C in presence of 5 per cent CO_2. The Roux flasks were removed at regular intervals of 48, 72, 96 and 120 hours of post inoculation, frozen and thawed three times, aliquoted and kept at -70°C until assayed for total infectious virus. Four similar trials were carried out. Similar trials were also carried out in 603 cm^2 Roller flasks inoculated with 10 ml of 1 in 10 dilution of seed virus.. The bottles were rotated @ five revolutions per hour in roller culture apparatus. Microcarrier culture was performed as described by Berry *et al.* (1999). Cytodex-1 beads (Sigma, USA) were prepared by overnight suspension in 50 ml Calcium magnesium free-phosphate buffered saline (CMF-PBS) pH 7.2. The microcarrier beads were washed twice with CMF-PBS and autoclaved. Microcarrier cultures (1 L) were established by inoculating vero cells (2 x 10^5 cells/ml) into growth medium containing 1 gram (g)/litre Cytodex-1. The cultures were maintained in 5-litre bench top fermenter (B-Braun Biostat, Germany). The incubation was carried out at 37°C. The cultures were stirred continuously at 40 revolutions per minute (rpm) from the point of inoculation. Virus infection was carried out by removing 90% of the medium followed by addition of 100 ml of 1 in 10 diluted seed virus. During viral infection the culture was stirred intermittently at 40 rpm for 1 minute every 12 minutes for 1 hr. Cultures were re-adjusted to their original volume by the addition of fresh maintenance medium and incubated. Samples were taken at regular intervals (48, 72, 96 and 120 hours) for determination of viral titres. Each sample was freeze/ thawed (3 times) to ensure complete release of intracellular virus. Four similar trials were carried out.

Titration of virus

Virus titration was performed as described by Kibenge *et al.* (1988) with slight modifications. Serial 10-fold dilutions of virus were made in maintenance medium and 50 ìl of each dilution was transferred to four wells of a 96-well microtitre plate (Nunc, Denmark) except the last two vertical columns, which served as cell control. In each plate, two samples were processed. Each well then received 100 ìl of a fresh Vero cell suspension in growth medium. Plates were incubated at 37°C in 5 per cent CO_2 for 4 days, after which the monolayers were observed microscopically for typical CPE daily. The virus titre was determined using the procedure described by Reed and Muench (1938).

Virus inactivation and vaccine preparation

IBDV was inactivated with 0.01M Binary ethylene imine (BEI) at 37°C for 10 hours. Then autoclaved sodium thiosulfate was added at 10 times final concentration of BEI for neutralization of residual BEI. After confirming complete inactivation of virus by inoculation in vero cells,oil emulsion vaccines (OEV) were prepared with Montanide ISA 25 (SEPPIC, Paris, Cedex) at oil: aqueous phase ratio of 1:3.

Vaccination trial

Five groups of 4-weeks-old IBD antibody negative White Leghorn chicks, each consisting of 10 birds, were used for vaccination trails. The chicks of group I were vaccinated with vaccine A (Roux - propagated virus), group II with vaccine B (roller - propagated virus), group III with vaccine C (microcarrier - propagated virus), group IV with vaccine D (IBDV inactivated B.P. (Vet), Ventribiologicals, India) and group V was left as unvaccinated control. In all the groups, expect control, the inactivated vaccines were administered subcutaneously at the dose rate of 0.5 ml per bird in the lower neck region. Serum samples were collected after 21 days of vaccination and after 10 days of challenge to assess IBDV antibodies by Quantitative Agar Gel Precipitation test (QAGPT) and by Virus Neutralization test (VNT). All birds of group I-IV were challenged with IBDV (serotype 1, characterized local strain N 35/93) after 21 days of vaccination with a virus titre of $10^{3.5}$ EID_{50}/0.2 ml of CMF-PBS. In control group V, only 5 birds were challenged (unvaccinated) and other 5 birds were housed separately as unvaccinated unchallenged controls. The chicks were inoculated with the virus both ocularly (0.1 ml) and intranasally (0.1 ml). On day 3 post challenge, 3 birds from each group I - IV and 2 birds from group V (control, unvaccinated challenged) were sacrificed and bursae were collected for antigen detection by AGPT. On day 10-post challenge, serum samples from all the remaining birds in each group were collected and then sacrificed. Bursae were collected for the determination of B:B ratio and bursal lesion score as per the method of Muskett *et al*. (1979).

Quantitative Agar Gel Precipitation test

The QAGPT was performed as per the method described by Thangavelu *et al* .(2000) on 1 percent agarose gels containing 8 percent sodium chloride, with wells of 4 mm diameter and 2 mm interspace. The central well was loaded with known positive antigen and the peripheral wells were loaded with two-fold dilutions of serum. The reciprocal of the highest dilution giving a precipitation line was taken as the titre.

Neutralization test

Neutralization test was carried out by beta method in Vero cell culture. The method described in O.I.E. Manual of Standards (2000) was followed. First, 0.05 ml of virus diluted in tissue culture medium to contain 100 $TCID_{50}$ (50 per cent tissue culture infective doses) per 0.05 ml was placed in each well of a tissue culture microtitre plate (Nunc, Denmark). Serial doubling dilutions of heat-inactivated sera were made in the virus containing wells (100 $TCID_{50}$). Cell controls, serum controls and virus controls were kept in the last two vertical columns. After 30 minutes of incubation at 37°C, 0.1 ml of Vero cell suspension (10^5 cells/ml) was dispensed into each well. Plates were sealed and incubated at 37°C in the presence of 5 per cent CO_2 for 3 days, after which the monolayers were observed microscopically for typical CPE daily up to 6 days. The reciprocal of the highest serum dilution showing no CPE was taken as the titre and the log_2 geometric mean titres were calculated according to method of Brugh (1978).

Bursa : body weight ratio and bursal score

Bursa collected from all the groups after 10 days of challenge were used for estimation of B:B ratio and bursal score. B:B ratio of the vaccinated and control birds were calculated as per the method of Ismail and Saif (1991). Bursa:body weight index was calculated as per the method of Lucio and Hitchner (1979). Bursal lesion score was determined as per the method of Muskett *et al.* (1979).

RESULTS

IBDV antigen specific cytoplasmic fluorescence in infected vero cells was detected at 24 HPI itself and maximum fluorescence was noticed by 48 HPI. The CPE in Vero cultures was not detectable

until 48 HPI. At 72 HPI, the infected cells appeared small, retractile and rounded. This was followed by sloughing-off of the cells from the glass surface leaving empty spaces in the cell sheet by 96 HPI.

In microcarrier vero cell culture grown for 24 hours the cytodex beads were completely covered by vero cells (Fig.1&2). The results of virus titration are given in the Table 1. Since different culture methods had different surface area, the $TCID_{50}$ was calculated per cm^2 for comparison. Statistical analysis was carried out with these titres estimated for 1 cm^2 surface area. The culture methods were compared at 96 HPI by using analysis of variance (Table 2).

Serology

The results of QAGPT and VNT on serum samples collected from vaccinated chicks on 21 days post vaccination and 10 days post challenge are given in Table 3. Titres produced by the commercial vaccine (chick embryo origin) and experimentally prepared vaccine (tissue culture origin) did not differ significantly.

On 3rd day post challenge, the bursae collected from control challenged birds were positive for IBD antigen in AGPT while bursae collected from birds of all the vaccinated groups were negative. This indicated the protection of the vaccinated birds in all the groups against virulent IBD challenge. The bursa:body weight ratio, bursa:body weight index and bursal score assessed on 10 days post challenge are given in Table 4.

DISCUSSION

Inactivated vaccines play significant role in protection against IBD. Bursa being the predilection site, contains high titres of infectious virus and is used for the production of inactivated bursa-derived vaccines. However, the production of such vaccine is very expensive and cumbersome. The alternate and preferred system could be the cell cultures. The most common culture system used for the growth of IBDV is CEF (Kibenge *et al*., 1988). However, the titres of virus obtained in CEF are not as high as that in bursa-derived vaccine. Thus preparation of inactivated vaccines using cell culture requires bulk culture to be done to achieve the antigenic content required to induce adequate immunity.

Peak titres of $10^{6.5}$ to $10^{7.7}$ were seen between 96 and 120 HPI in all the three culture methods. The Roller and Microcarrier culture methods produced around one log_{10} higher titre than the stationary culture. However no significant difference was seen between Roller and Microcarrier culture. These findings concur with observations made by Lesko *et al.* (1993). They reported that canine distemper virus propagated in vero cells on Gelaspher M gelatin Microcarirer culture had 10 times higher viral titres as compared to the stationary cell culture method.

Although the virus titres of the roller and microcarrier propagated virus vaccine was higher, it is interesting to note that there was no difference in the immune responses evinced by these two vaccines. Thus, it may be argued that these two improved culture methods had no benefit in terms of increased protection. This may be because the threshold value of antigen content may be sufficient to stimulate active immunity and any extra amount of antigen administered may be superfluous. However, it is worth attempting to dilute the vaccines produced by the Roller and Microcarrier culture methods and try to study its potency. If this is successful then the production cost would really come down depending on the dilution permissible. If protection is used as criteria, then these two culture techniques offer no advantage, however, based on virus titres and antigen content they are superior to the conventional stationary culture method.

REFERENCES

Berry, J..M., Barnabe,N., Coombs, K.M. and Butler, M. (1999). Production of reovirus type-1 and type-3 from vero cells grown on solid and macroporous microcarriers. *Biotechnoogy and Bioengineering,,* 62 : 12-19.

Brugh, M. Jr. (1978). A simplified method for recording and analyzing serological data. *Avian Diseases,* 22 : 362-365.

Cosgrove, A.S. (1962). An apparently new disease of chickens - Avian nephrosis. *Avian Dis.,* 6 : 385 - 389.

Ismail,N.M. and Saif,Y.M. (1991). Immunogenicity of infectious bursal disease viruses in chickens. *Avian Diseases,* 35 : 460 - 469.

Kibenge, F.S.B., Dhillon, A.S. and Russell, R.G. (1988). Growth of serotypes I and II and variant strains of infectious bursal disease virus in vero cells. *Avian Diseases.,* 32 : 298-303.

Lesko, J.,Veber,P.and Dusekova, E. (1993). Canine distemper virus replication in cells on microcarriers. *Acta Virologica,* 37 : 412 - 416.

Lucio,B. and Hitchner,S.B.(1979). Infectious bursal disease emulsified vaccine. Effect upon neutralizing antibody levels in the dam and subsequent protection of the progeny. *Avian Dieases.,* 23 : 1037 - 1049.

Muskett,J.C., Hopkins,I.G, Edwards,K.R. and Thomton,D.H.(1979). Comparison of two infectious bursal disease vaccine strains : Efficacy and potential hazards in susceptible and maternally immune birds. *Veerinary. Record,* 104 : 332 - 334.

Office International Des Epizooties Manual. (2000). Manual of standards for diagnostic tests and vaccines for List A and B diseases of mammals, birds and bees, pp. 647 - 656.

Reed, L. J. and Muench, H. (1938). A simple method for estimating 50 per cent end points. *American. Journal of Hygiene.,* 27 : 493 - 497.

Thangavelu,A., Dhinakar Raj,G., Elankumaran,S. and Koteeswaran,A. (2000). Evaluation of a filter paper blood sampling technique for quantitative assessment of antibodies to infectious bursal disease virus. *Tropical Animal Health and Production,* 32: 179-182