Evaluation of Experimental Inactivated Infectious Bursal Disease Vaccine Using a Local Very Virulent Pathotype Strain

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Summary

By emergence of very virulent infectious bursal disease viruses (vvIBDVs), all classical measures were called into question. In this study an inactivated IBD vaccine with bursal and embryo origins using a recent local vvIBDV isolate, IR499, was prepared. The virus cultivated in both SPF emberyonated eggs and chicks to evaluate the effects of host system on immunogenicity of the vaccine. The results showed that inoculation of broiler breeder chickens with the inactivated IBD vaccine at 20 weeks of age produced significantly greater levels of antibody in comparison with IBD live vaccine and induced antibody titers with a lower variation than the other. The mean antibody titer raised by bursal origin vaccine was significantly greater than other inactivated vaccine and it persisted throughout this study. Our result show that inactivated vaccine with bursal origin induces high level of antibodies, which is essential for protecting of the birds against the vvIBDV strain. Also it shows that including of very virulent strain in inactivated vaccine provides better protection against field vvIBDV strains.

Key words: infectious bursal disease, very virulent, inactivated vaccine, host system

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Introduction

Infectious bursal disease virus (IBDV) is a member of the *Birnavirdae* causes a highly contagious immunosuppressive disease in chickens by targeting developing B-lymphocytes located within the bursa of Fabricius (Lukert & Saif 1997, Nagarajan & Kibenge 1997). Two serotypes of IBDV have been identified. Only strains of serotype 1 are pathogenic for chickens (McFerran *et al* 1980, Lukert & Saif 1997). All IBDV strains of serotype 1 isolated before 1985 (classical IBDV) were antigenically homogenous by both serum neutralization test and monoclonal antibodies. These classical IBDV strains varied in virulence, some were mildly immunosuppressive whereas others were pathogenic causing bursal inflammation and necrosis resulting in immunosuppression with mortality up to 30% (Lukert & Saif 1997). In 1983 variant IBDV strains against which vaccines based on classical strains were not effective, emerged in USA (Jackwood & Saif 1987, Snyder *et al* 1992). So far, occurrence of variant strains of IBDV has been reported from the USA (Snyder *et al* 1992, Vakharia *et al* 1994), Australia (Sapats & Ignjatovic 2000) and China (Cao *et al* 1998). In 1987 very virulent (vv) IBDV strains capable of causing 60 to 100% mortality were isolated in several European, the Middle East, Africa and Asia countries (Reviewed by Van den Berg 2000). The vvIBDV strains are antigenically very similar to classical strains but are able to break through the usually protective levels of maternal antibodies induced by classical IBD vaccines (Chettle *et al* 1989, Van den Berg 1991).

The main procedure of current immunization against IBDV is vaccination of breeder with inactivated vaccine to providing high maternal antibody in their progeny (Lukert & Saif 1997). The chicks are thus protected during the period of susceptibility to IBDV strains. However, the chicks are not protected from other highly pathogenic strains (Van den Berg 2000, Van den Berg *et al* 1991). The higher level of vaccine antibody titers is needed to protection against challenge with the heterologous than with the homologous viruses although induction of high level of

antibody titer can not protect significant bursal atrophy (Van den Berg 1991, Aghakhan 1996). So it seems that providing vaccine homologue to current field strain, is a practical approach to immunization of bird in a limited native area. Keeping this in view the present study was conducted for evaluating immunity induction of an experimental inactivated vaccine using a recently isolated native vvIBDV strain which cultivated in two host systems and comparing it with a commercial inactivated vaccine.

Material and Methods

Virus. The IR499 strain of vvIBDV was isolated in our laboratory used for inactivated vaccine preparation as well as for challenge study.

Inactivated vaccine preparation. To produce an embryo origin vaccine a stock virus of IR499 strain was passaged three times on specific pathogen free (SPF) embryonated eggs (Cuxhaven–Germany). The dead embryo bodies and their chorioallontoic membrane of third passage were collected, homogenized as 25% w/v suspension in sterile phosphate buffer saline (PBS) pH7.4 and clarified by centrifugation at 3000*g* for 30min. The clarified suspension titerated, adjusted on 10^5EID_{50}/ml and inactivated by 0.1% β-propiolactone as stirring 12h at 37°C. The antigen was tested for bacterial and mycological sterility and lack of IBDV infectivity. One part of final suspension mixed and homogenized (Rannie Model Mini Lab Type 8.304) with four parts of a commercial oil adjuvant ISA-70 (SEPPIC, France). To produce a bursal origin vaccine the same stock virus was also passaged two times in SPF originated chickens of 3-4 weeks old. At third days of post second inoculation, the bursas were collected aseptically and vaccine was prepared as described above. The final concentration of virus was 10^5CID_{50}/ml. One ml of each vaccine was inoculated subcutaneously as one dose to each chicken of the related group. The prepared vaccines were stable and no local reaction was

observed after injection. A commercial inactivated oil-emulsion IBDV vaccine, D78 strain, an intermediatestrain was used.

Experimental design. One hundred and fifty broiler breeder chicks were hatched and reared in isolation. At two weeks of age, they were divided into five equal groups (P1 to P5) and vaccinated routinely against common avian diseases. Blood samples were taken at second (before first live vaccine), 8th, 20th, 32nd, 36th, 40th and 48th weeks of age and tested for detection of anti IBDV antibody. When breeder chickens were 30 and 31 weeks old, their eggs were collected separately, hatched and chicks of each group were reared in isolation (CH1 to CH5). At one day of age, and then after at intervals of 3 days, 10 chicks from each group were selected and challenged with 0.1ml containing 100 chicks infective doses (CID_{50}) of strain IR499 via eye drop method. Three days after challenge the chicks were sacrificed and their bursa of Fabricius (BF) were examined for the presence of IBDV antigen using the agar gel diffusion (AGP) test. At each interval time 15 chicks were also used for serological studies as explained below. At each sampling time ten specific antibody negative (SAN) of group CH5 chicks (derived from group P5 parents) were also chosen as uninoculated control (group C).

ELISA antigen preparation. D78 strain of serotype I of IBDV was used for seed virus in chicken embryo fibroblast (CEF) culture. CEF cultures were obtained from 10–day-old embryo of SPF chicken. Growth medium was Eagl's minimum essential medium (MEM) (Gibco) supplemented with 10% tryptose phosphate broth (TPB), 5% fetal bovine serum (FBS) and antibiotics (penicillin 100IU/ml and streptomycin 100mg/ml). When the CEF cultures became confluent, seed virus dilutions of 1/10, 1/100 and 1/1000 were prepared and inoculated after washing of these cells with PBS. After 1h adsorption, the culture was supplemented with maintenance medium (MEM containing 10% TPB, 1% FBS, and antibiotic). After 48 to 72h of incubation the dilution with the best cytopathic effect on the culture was chosen and used for final cultivation. The culture fluid was centrifuged at 3000*g* for 30min to remove

cell debris, and supernatant was recentrifuged at 94,500*g* for 1h. The pellet was dissolved in TNE (0.1M Tris–HCl, 0.1M NaCl, 1M EDTA) buffered solution, and this solution was placed on 30% sucrose layer and centrifuged at 115,200*g* for 2h. The pelleted virus dissolved in TNE buffered solution at concentration of 1:400 of original volume.

ELISA procedure. An ELISA system was performed as described by Tsukomoto (1990) with some modifications. Briefly purified viruses were diluted 1: 200 in carbonate and bicarbonate buffer (pH9.6) and diluted viruses were added to each well of plates (Nunc, Denmark). After incubation at room temperature (RT) overnight, the wells washed five times with PBS (pH7.4) containing 0.1% Tween20 (washing solution). The wells were blocked by 300µl of PBS containing 1% bovine serum albumin (BSA), 4% skim powdered milk, 4% glycerin, and 0.5% Tween20 (blocking solution) at 37°C for 1h. A serial two-fold dilution of each sample was prepared and 100µl volume was added to each well. After 40min incubation at RT, the plates were washed three times and a 1:14000 dilution of horseradish peroxidase–conjugated rabbit antichicken IgG (Sigma) was added to the wells. After 40min incubation at RT the wells were washed and 100µl of a freshly prepared solution of substrate was added to each well. This consisted of 125µl of ABTS (0.022%), 12.5ml acid citric (0.96%, pH4) and 15µl of hydrogen peroxide (30%). The color reaction was stopped by adding 50µl of sulfuric acid to each well and plates were left for 20min at RT. The absorbency was measured at 405nm with an automatic ELISA reader (Rosys anthos 2001, Austria).

AGP test. Test was carried out as described in OIE Manuel of Standard (1993).

Preparation of specific antisera and antigen. Five survived SPF chickens of various stages of virus cultivation were inoculated subcutaneously three times in 2 weeks interval with 5ml of prepared vaccines. One week after 3rd injection the birds were exasunguinated and their serum were pooled, aliquated and stored at -20°C until use. The positive antigens were prepared from hemogenizing of bursa of five challenged birds 4 day after inoculation.

Statistical analysis. Significance of differences determined by ANOVA followed by Duncan test. Statistical significance was determined at the 0.05 level of probability.

Results

Serological study of parent. Mean antibody titer (MAT) of serum IgG of all groups has been shown in table 1. There was no significant difference among all groups before first live vaccination. After inoculation of second live vaccine the vaccinated groups had higher MAT than unvaccinated group (P5) (P<0.5).

Table 1. *Mean antibody titers of parent groups at various ages*

Group		Age (week)								
		2	8	20	24	28	32	36	40	48
P1	Mean	933a	8533b	6400b	13653c	11520d	10667d	39813d	9371b6±	8320b±3
	±SD	±455	±3760	±2091	±3799	±2650	±3123	±3304	351	377
P2	Mean	906a	8746b	6661b	15360f	13653c	11947cd	11520d	11093d±	10453d±
	±SD	±533	±4095	±1899	±5300	±5336	±2252	±2650	2930	3519
P3	Mean	875a	8800b	6720b	13227c	11093d	10240d	9387d	9173db±	8533b±3
	±SD	±466	±3642	±1752	±3799	±2930	±3245	±3305	3601	123
P4	Mean	882a	8533b	6661b	5257b	4857b	4240b	3866b	3293ab±	2287ab±
	±SD	±453	±3760	±1899	±3628	±3114	±2262	±2026	2213	1707
P5	Mean	903a	492a	57e	53e	40e	63e	60e	32e	33e
	±SD	±464	±299	-	-	-	-	-	-	-

A, b, c, e, and f: different letters means significant difference (P<0.05), SD:standard deviation

After vaccination with the inactivated vaccines the level of serum antibody increased to highest level in groups P1, P2 and P3 at 24 weeks of age in which the MAT of group P2 was significantly higher than other groups. At the same age there was no significant difference between group P1 and P3. However MAT of these groups were significantly higher than of group P4. Antibody titers of group P4 showed higher variation than other groups. After reaching to the peak at 24 week of age the antibody titer of all vaccinated groups declined. However the antibody titer

of group P2 remained significantly higher throughout the trail. The level of serum antibody of chicks in group P5 did not increased during the experiment.

Serological study of progeny. At first day of age, MAT of groups CH1, CH2, CH3 and CH4 were 8960, 11093, 8533 and 3760 respectively (Table 2). The MAT of group CH2 was significantly higher than other groups ($P<0.05$). There was no significant difference of antibody titer between group CH1 and CH3. The rate of antibody decline was lower in the first four days of age. The reduction of antibody titer in all progeny groups had a similar pattern however the antibody titer of group CH2 was always higher than other groups.

Table 2. *Mean maternal antibody titers of parent groups at various ages after hatch*

Group		Age (day)									
		1	4	7	10	13	16	19	22	25	28
CH1	Mean	8960a	8107a	5170b	3253bc	2077c	1333d	827d	533d	333d	220d
	±SD	±3245	±2930	±1623	±2026	±732	±390	±237	±195	±98	±77
CH2	Mean	11093f	9813a	6187b	3926bc	2560c	1653cd	1220d	580d	467d	313d
	±SD	±2930	±3305	±3721	±10323	±811	±475	±406	±166	±180	±113
CH3	Mean	8533a	7680a	4907b	3093bc	1920c	1314d	843d	507d	328d	193d
	±SD	±3123	±22650	±1652	±403	±662	±390	±328	±183	±168	±70
CH4	Mean	3760b	3280bc	2028c	1378d	853d	360d	384d	242d	230d	150d
	±SD	±2408	±2515	±1957	±939	±532	±262	±226	±213	±1707	-
CH5	Mean	33e	20e	28e	37e	23e	19e	26e	25e	21e	23e
	±SD	-	-	-	-	-	-	-	-	-	-

A, b, c, e, and f: different letters means significant difference ($P<0.05$), SD:standard deviation

Progeny susceptibility. The progeny susceptibility evaluated by AGP test and the results are presented in table 3. In group CH1 the first susceptible case was detected at 16 days of age when MAT was 1333. All of the birds in this group were sensitive at 28 day of age. The first susceptible case of group CH2 was seen at 19 days of age when MAT was 1220. There was no complete susceptibility in group CH2, even at 28 days of age. Two cases of group CH3 were susceptible at 13 days of age when MAT was 1920. The first susceptible case of group CH4 was seen as

early as 7 days of age. All of the birds in this group were susceptible at 16 days of age.

Table 3. Progeny susceptibility to challenge at various ages after hatch detected by AGP test three days postinoculation

Group	Age (day)									
	1	4	7	10	13	16	19	22	25	28
CH1	0	0	0	0	0	1	2	4	8	10
CH2	0	0	0	0	0	0	1	2	4	8
CH3	0	0	0	0	2	3	4	8	10	10
CH4	0	0	0	5	8	10	10	10	10	10
CH5	10	10	10	10	10	10	10	10	10	10

Discussion

Shortly after the emergence of vvIBDV in Europe, it appeared that conventional commercial vaccines could not protect the chicken against these viruses (Van den Berg 2000, Van den Berg *et al* 1991). Chicks with maternal antibody have not been protected against a vvIBDV strain and vaccination of breeders with a higher performance oil-emulsion vaccine was proposed (Aghakhan *et al* 1996, Maas *et al* 2001, Vander Berg 2000). In this study effect of different host systems on immunogenicity of a local vvIBDV strain (IR499) was evaluated. The chickens that had been vaccinated with the experimental and commercial inactivated vaccines showed highest level of serum antibody production at the age of 24 weeks, while chickens that received live vaccine alone, showed significantly lower antibody titer. There was no measurable change in serum antibody titer of control chickens. The response of individuals to the vaccine varied, but it was more uniform in the chickens that received inactivated vaccines. The use of control group sera and also pre inoculation sera assured the specificity of the reaction. High titers were observed against bursal origin vaccine when compared to embryo origin and commercial vaccines. These differences were reflected in the maternally derived antibodies of

their chicks and were maintained during the entire experiment. It is not clear why the vaccine should provide high level of antibody. It seems that BF as a selective target for IBDV provides a sufficient system for profound virus propagation which in turn these can be used as an excellent source for vaccine production. Hassan and Saif (1996) noticed that the antigen titration methodology employing embryonating chicken eggs was not suitable for titration of viruses propagated in other host systems. The induction of higher level of antibody resulted in higher level of antibody transmission to their progeny. So it is expected that such breeders can transmit high level of antibody throughout the life. This may result in a practical advantage because the potential antibody transmission declines with age (Wyeth & Cullen 1978).

Preparation of a bursal origin inactivated vaccine provides higher level of antibody, which is necessary for protection against vvIBDV strains. However this does not show that including field strain in inactivated vaccine by self improve immune response qualitatively. A challenge experiment was conducted to evaluate the efficacy of transmitted antibody against a very virulent strain, IR499. The criterion of evaluating of susceptibility to challenge was AGP test three days post inoculation. The chicks with no transmitted antibody, group CH5, were susceptible since first day of age. Despite complete susceptibility of these chicks throughout the study no mortality was observed during a three days course after each challenge. These results are coordinated with other study that the layer breeds have greater susceptibility to IBDV than broiler breeds (Van den Berg 1991). The first susceptible case was observed at 16 (MAT=1220) and 19 (MAT =1333) in CH1 and CH2 groups respectively. There was no significant difference between antibody levels. Therefore, it can be concluded that the critical limit of antibody level for susceptibility or protection in our designed ELISA was between 1330 and 1440. The first susceptible cases of group CH3 were observed at 13 days of age (MAT=1920), this antibody level was significantly higher than that titer in which groups CH1 and

CH2 showed susceptibility. It shows that induced antibody by vaccine strain (as classical strain) could be effective only with very higher level. In fact, the high levels of antibodies (may called cumulative effect of antibodies) induced by classical vaccine strains can provide sufficient protection against vvIBDv strains. On the other hand, even in situation of equal level of antibody titer, the induced antibody by vaccine strain is not effective when compared with the both experimental vaccines. However, the antigenic difference between classical and very virulent strains was not reported (Van den Berg 2000) such difference might exist, which affect the immunogenicity of these strains. Therefore strain used in inactivated vaccine was a main factor, which influenced the protection of the offspring of vaccinated hens (Maas *et al* 2001).

Our results indicate that using an inactivated vaccine with bursal origin high level of antibodies, which is essential for protecting of the birds against vvIBDV strain, was induced. Also it shows that including of very virulent strain of IBDV in inactivated vaccine provides a better protection against the field strains. This approach may be a good way to protect the commercial flocks against vvIBDV at least in limited geographic areas.

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