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STUDIES ON THE INFLUENCE OF THE HOST SYSTEM ON THE
PATHOGENICITY AND IMMUNOGENICITY OF
INFECTIONOUS BURSAL DISEASE VIRUS

DISSERTATION

Presented in partial fulfillment of the requirements for
the degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

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*****

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Department of Veterinary
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To my family
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INTRODUCTION

Infectious bursal disease (IBD) is an acute highly contagious immunosuppressive disease of young chickens that was first recognized by Cosgrove in 1962 in Gumboro, Delaware (10). The most characteristic lesion of IBD is the edematous enlargement of the bursa of Fabricius (BF), followed by bursal atrophy due to necrosis of follicular B lymphocytes (29). Infectious bursal disease virus (IBDV) appears to lyse immature bursal B-lymphocytes expressing IgM molecules (37) which results in suppression of humoral immunity. The immunosuppressive effect of IBD renders the birds more susceptible to many infections, such as colibacillosis, salmonellosis, infectious bronchitis and gangrenous dermatitis (29, 41). Also, IBD has been associated with vaccination failures against other diseases (15).

Severe immunosuppression develops when chicks contract infection during the first two weeks of life either from field exposure or vaccination (29). Older chickens may exhibit a transient decrease in antibody response (12, 17). The disease can be manifested as an acute form when 3 to 6 week-old chickens are exposed to a virulent virus (29). During this age
period, maternal antibody levels usually wane to a nonprotective level and the bursa is at its maximal stage of development, offering an ideal environment for rapid virus replication. The acute form of IBD usually causes significant losses due to retarded growth, increased mortality, and higher condemnation rates due to hemorrhages in the thigh and pectoral muscles. Chickens younger than 3 weeks of age are usually protected by maternal antibody and even in its absence, chicks show resistance to the acute disease at that age (13). Chickens older than 6 weeks of age do not develop clinical disease because of the natural involution of BF (29).

Infectious bursal disease virus (IBDV) is a member of the Birnaviridae family. Viruses belonging to this family have bi-segmented double stranded RNA genome (33). The IBDV genome is formed of two segments (A & B) of double-stranded RNA. Segment A, 3400 base pairs, codes for a 110 K polyprotein N-VP2-VP4-VP3-C (19), while segment B, 2900 base pairs, codes for VP1. The virus has 4 major structural proteins, designated as VP1 to VP4 (4, 11, 38, 46). VP2 (40 K) and VP3 (32 K) are the major capsid proteins, constituting about 51% and 40% respectively of the total protein (4, 11). VP1 (90 K) is considered to be a viral RNA polymerase, while VP4 (28 K) is the viral protease (19, 45). Another protein (VPX) is regarded as a precursor of VP2 (1). A viral protein with molecular weight of 21 K has been demonstrated in bursa samples from
IBDV-infected chickens. The function of this protein is unknown (36). The structural proteins of tissue culture adapted serotype 1 and 2 IBDVs were compared by SDS-PAGE. The molecular weight differences among the structural proteins of serotype 1 viruses were minor and of no value in differentiating viruses belong to serotype 1. However, there were significant differences between serotypes 1 and 2 (47).

An important feature of IBDV is its extreme resistance to physical and chemical agents. The virus is resistant to ether and chloroform and to exposure to .5% phenol for 1 hour at 30 C; it is unaffected by a pH of 2 but can be inactivated by a pH of 12; also it can survive exposure to 56 C for 5 hours (5). The virus infectivity can be reduced by exposure to .5% formalin for 6 hours, or to 1% formalin for 1 hour (5, 9). The stability of IBDV in the environment has resulted in nearly universal presence of the IBD in commercial poultry industry.

A number of host systems has been used to isolate and propagate IBDV. Hitchner (18) reported that both chorioallantoic membrane (CAM) and whole embryo were good for serial passage of the virus and the CAM inoculation route was the most sensitive method. Different primary cell culture systems have been used to propagate IBDV resulting in cytopathic effect (CPE). The virus has been grown in chicken macrophages, lymphocytes (6, 16, 35), and chicken embryo
fibroblasts (8, 39). Also, mammalian cell lines have been used for propagation of IBDV including Vero cells (26, 30), MA-104 and BGM-70 cell lines (26). The virus produced more distinct CPE and higher titers in BGM-70 cells than in Vero cells or MA-104 cell lines (26). However, some researchers reported difficulty in growing some strains in primary cell cultures (31, 32). Also, the virus has the tendency to produce incomplete virus particles when grown in CEF, in contrast to the complete particles produced in the bursal lymphoid cells (34). It was shown that the incomplete particles had a large quantity of a precursor protein (50 K) to the major structural protein (40 K) while the complete particles had a relatively minor proportion of this protein. It was suggested that since incomplete particles are produced in CEF but rarely in bursal cells, the inefficient cleavage may be due to a difference in cellular proteases (34).

Studies showed that group specific epitopes are located on VP2 and VP3; and VP2 has at least two conformational dependent, non-overlapping virus-neutralizing epitopes, one of these is serotype specific and can be used to differentiate between serotypes 1 and 2 (2, 3, 28). It was shown that neutralizing monoclonal antibodies reacted only with the native viral protein, leading to speculation that such epitopes are conformational dependent (3). Another study showed that monoclonal antibodies had reacted with VP2 in
western blots which indicated the presence of non-conformational epitopes on VP2 (14).

Based on virus neutralization tests, there are two serotypes of IBDV designated as 1 and 2 (24, 31). Infection with serotype 1 viruses results in clinical disease in chickens while viruses belonging to serotype 2 are considered non-pathogenic (22). Serotype 2 viruses were first isolated from turkeys, but later were also found in chickens (22). Antibodies against serotype 2 did not protect chickens against challenge with virulent serotype 1 viruses (25). The antigenic diversity of the viruses belonging to serotype 1 was investigated using in-vitro cross-neutralization test (23). It was shown that serotype 1 viruses could be subdivided into 6 subtypes.

During the last decade, several antigenically variant strains of serotype 1 IBDV were isolated (21, 40, 42, 44). Rosenberger et al. (42) isolated 4 viruses, designated A, D, G and E which differed from classic serotype 1 in that they caused rapid bursal atrophy with minimal inflammatory response in three-to-four week-old SPF chickens. These variants were similar to one another but differed from the standard serotype 1 vaccine strains. The classic vaccines provided incomplete protection against the variants while the variant vaccines protected against challenge with other variants or the
standard challenge virus (43). A cross-protection study comparing vaccination with classic and variant strains was conducted in our lab. (20). It was shown that protection is based on the strain and the dose of the vaccine and the challenge viruses. High doses of standard vaccines provided protection against challenge with a low dose of a variant virus but did not protect against a high dose of the same virus. Both low and high doses of variant vaccines protected against either low or high challenge doses of a variant virus. The results indicated that both classic and variant strains share a common neutralizing epitope.

In Europe, a highly virulent strains of IBDV have emerged which resulted in up to 25, 60, and 80-100 % mortality in broiler, laying pullets and specific-pathogen-free (SPF) chickens, respectively (7, 48). These virulent strains were classified as standard serotype 1 IBDV (48).

Currently, vaccination of parents and progeny is the principle method used for control of IBDV. Breeder flocks are vaccinated with live vaccines to prime the immune system followed by inactivated vaccines before laying so that the progeny are protected during the first weeks of life by maternally derived antibodies. The offspring are usually vaccinated with a live vaccine. The timing of vaccination is based on the levels of maternal antibody in the flock, the
ability of live vaccines to overcome maternal immunity and the pathogenicity of the field challenge virus to which the chicks will be exposed (27). In fact, a universal vaccination strategy cannot be adopted because of variability in maternally derived antibody levels, management and the type of vaccines (29).

Since control of IBD is based on the use of vaccines, the effect of the host system used for vaccine production on the pathogenicity and immunogenicity of IBDV is an important consideration. Therefore, the main objective of the work presented in this dissertation was to investigate the influence of different host systems used to propagate the virus on the pathogenicity and immunogenicity of selected IBDVs, the results are summarized in chapter 1. The second objective was to investigate the usefulness of antigen-capture ELISA in titration of IBDV vaccines (chapter 2). The third objective was to compare the pathogenicity of one variant and one classic strain in SPF chickens and the effect of four passages in BGM-70 cell line on the pathogenicity and immunogenicity of the adapted variant strain (chapter 3). Finally, studies presented in chapter 4 dealt with the effect of high passage (40 times) in BGM-70 cells on the pathogenicity, antigenicity and immunogenicity of small and large plaque clones of a variant strain.
The studies presented in this dissertation are of value when considering the host system candidates for vaccine production. Also, it highlights the importance of the choice of the titration system used in comparative studies and adds information about the influence of low and high passage in BGM-70 cells on the pathogenicity and immunogenicity of IBDVs.

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CHAPTER 1

INFLUENCE OF THE HOST SYSTEM ON THE PATHOGENICITY, IMMUNOGENICITY AND ANTIGENICITY OF INFECTIOUS BURSAL DISEASE VIRUS.

SUMMARY. The effect of the host system on the pathogenicity, immunogenicity and antigenicity of infectious bursal disease virus (IBDV) was investigated. One classic (SAL) and one variant strain (IN) of IBDV were passaged separately six times in three host systems, namely BGM-70 continuous cell line, primary chicken embryo fibroblast (CEF) cells, embryonating chicken eggs or one time in SPF chickens bursa of Fabricius (BF). Passage in BGM-70 cells or CEF resulted in loss of pathogenicity, but viruses passaged in embryos or BF maintained their pathogenicity.

For the immunogenicity study, the live and inactivated vaccines, containing $10^3 EID_{50}$ and $10^5 EID_{50}$ respectively, induced different levels of protection. It was concluded that the antigen titration methodology employing embryonating chicken eggs was not suitable for titration of viruses propagated in other host systems because of varying degrees of adaptation and/or pathogenicity of the viruses resulting in variability in antigen mass of the tested vaccines. To test this
assumption, an antigen capture ELISA was used as a titration system to compare the antigenicity of viruses propagated in BGM-70 cells or BF. Preparations containing similar antigen masses were inactivated then inoculated into two age groups of SPF chickens and antibody titers were monitored. During the experimental period, the geometric mean virus-neutralizing (VN) antibody titers of the vaccinated groups did not differ significantly (P>0.05).

INTRODUCTION

Infectious bursal disease virus (IBDV), a member of the birnavirus group, is the etiological agent of a highly contagious immunosuppressive disease of young chickens (11). The bursa of Fabricius is the target organ for infection by IBDV (6). The principal method of IBD control is by vaccination using live attenuated or inactivated viruses. Despite the adoption of biosecurity measures and the intensive use of vaccines, the disease still causes considerable economic losses for the poultry industry worldwide. The resistance of the virus to environmental conditions and the emergence of variant strains are major causes for perpetuation of the infection in most poultry producing regions of the world.

Since vaccination is currently the most effective control measure of IBDV, there is continuous need to develop efficacious vaccines for commercial poultry production.
Currently, available vaccines are either tissue-culture, egg embryo or bursa of Fabricius propagated. The live vaccines are classified as mild or intermediate based on their pathogenicity, immunosuppressiveness, and immunogenic characteristics (1,7). In a recent study, we investigated the effect of a continuous cell line (BGM-70) on the pathogenicity and immunogenicity of 2 variant strains vaccines (14). When the 2 viruses were passaged 30 times, both lost their pathogenicity but maintained their immunogenicity when used as an inactivated vaccine. When the viruses were used as a live vaccines, they were not protective leading to the speculation that high passage in BGM-70 cells limits the ability of the viruses to replicate in their natural host although they maintained their immunogenic potential. These findings prompted us to investigate the influence of different host systems ,and the passage level on the pathogenicity , antigenicity and immunogenicity of selected classical and variant IBDVs.

MATERIALS AND METHODS

Chickens and embryonated eggs. Specific pathogen free (SPF) eggs (Hy-Vac Laboratory Egg Co., Gowrie, Iowa) were used to obtain the embryos and chickens used in this study. Birds were hatched and raised in a disease containment building then moved into isolators prior to inoculation with
live viruses. The isolators are made of flexible plastic and supplied with filtered intake and exhaust air.

Viruses. Two strains of IBDV, designated SAL and IN, were used. SAL (Bursine 2) is an intermediate commercial vaccine strain (Salisbury Laboratories, Inc., Charles City, Iowa). To verify the pathogenicity of SAL virus, two groups of 2-week-old SPF chickens (10 birds per group) were used. The first group was inoculated via the intranasal and intraocular routes with a dose of $10^3$ EID$_{50}$ of the SAL virus. The birds of the second group were used as uninoculated controls. At 6 days post inoculation, birds in both groups were euthanatized, the bursas were obtained and the mean bursa/body weight ratios were calculated and evaluated statistically. Chickens inoculated with the SAL virus vaccine had significantly (P<0.05) smaller bursas than the uninoculated chickens. The IN virus is a pathogenic variant strain isolated in our laboratory from a commercial layer flock (2).

Adaptation of IBDV to different host systems. The stocks of viruses were obtained from a commercial vaccine or bursal homogenates for the SAL and IN viruses, respectively. Aliquots of each strain were passaged in one of four host systems, namely BGM-70 cells, chicken embryo fibroblast cells (CEF), SPF embryonated eggs (embryos), or chicken bursa of Fabricious (BF).

Adaptation of IBDV to BGM-70 cells. The SAL and IN strains were passaged twice in BGM-70 cells which resulted in
a cytopathic effect (CPE). The viruses were then inoculated into the BGM-70 cells at a multiplicity of infection of 0.001 mean tissue-culture-infective dose (TCID$_{50}$) virus per cell and incubated at 37 C in 5% CO$_2$. At 5 or 6 days post inoculation (PI), when at least 80% of the cells showed CPE, the monolayers were harvested by three freeze-thaw cycles, and the suspension was clarified by low-speed centrifugation. The supernatant fluid was filtered through a 0.45 um filter (Nalgene, Sybron Corp., Rochester, New York). Six passages were made with both SAL and IN strains.

**Adaptation of IBDV to CEF cells.** Primary cell cultures were prepared from 9-to-11-day-old embryos of SPF chicken eggs. The adaptation of IBDVs to CEF cell culture has been described (4).

**Adaptation of IBDV to embryonated SPF eggs.** Chicken embryos (10 day-old) were inoculated with 0.1 ml of virus inoculum via the chorio-allantoic route. Eggs were incubated, observed daily and those with dead embryos during the first 48 hours post inoculation were discarded. On the third day, eggs containing dead embryos and those still surviving were chilled to 4 C. Embryos and chorio-allantoic membranes (CAMs) were harvested and examined for the presence of IBDV characteristic lesions. The CAMs were washed and suspended in phosphate buffer saline (PBS [PH 7.2]). The suspension was inoculated into another batch of SPF embryos and six serial passages were completed in a similar manner.
Passage of IBDV in chicken bursa of Fabricius. SAL and IN viruses were passaged once in 2-week-old SPF chickens. Birds were inoculated via the intranasal and intraocular routes with $10^5$EID$_{50}$ of SAL or IN viruses. Infected bursas were harvested at 4 days post inoculation and processed as described previously (2).

Titration of passaged viruses in SPF embryos. After passage in different host systems, SAL and IN viruses were titrated in 10-day-old SPF embryonated eggs. The virus titer was determined as mean embryo infectious dose 50 (EID$_{50}$) for each of the passaged viruses.

Titration of viruses using antigen capture ELISA. Adjusted titer ($10^5$EID$_{50}$) of the passaged SAL and IN viruses were tested by an antigen capture ELISA to quantitate the amount of antigen present in each virus preparation. A volume of 100 ul/ well was used for all reagents. ELISA plates were coated with a capture antibody which was a polyclonal chicken anti-SAL serum diluted 1:1000 in coating buffer (0.1 M carbonate - 0.1 M bicarbonate buffer [pH 9.6]), and then incubated at 37 C for one hour and 4 C overnight. The plates were rinsed three times with washing buffer (PBS-Tween 20); the blocking solution (5% non fat dry milk in PBS-T) was added and the plates were incubated at 37 C for 1 hour. Virus samples were diluted 1:5 in PBS-Tween 20 , then serially diluted viruses were transferred into the coated wells and incubated for 3 hrs at 37 C. The plates were washed 3 times
with washing buffer (PBS-Tween 20) using an automated ELISA washer and incubated for 1 hr at 37 C with guinea pig anti-SAL virus diluted 1:3000 in PBS-Tween 20. After washing 3 times in PBS-T, goat anti-guinea pig IgG peroxidase-labeled antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1:1000 in PBS-T was added and the plates were incubated at 37 C for 1 hour. The plates were washed 3 times with the washing buffer. A 4 mg substrate, orthophenylene diamine (Sigma Chemical Co. St. Louis, Missouri), and 4 ul of 3% H2O2 were added to 10 ml substrate buffer (0.025 M citrate, 0.05 M phosphate buffer [pH 6.0]) and 100 ul of the substrate was added to each well and incubated, in the dark, for 12 minutes at room temperature. The reaction was stopped by adding 50 ul of 1 N sulphuric acid to each well. The developed color was read at 490 nm (MR 600 Microplate Reader; Dynatech Laboratories). The cut off point was determined by adding 3 standard deviation to the mean absorbance value of known negative samples. Sample titers were expressed as the reciprocal of the highest positive dilution that gave absorbance reading higher than the estimated cut off value (12).

Preparation of inactivated vaccines. Viruses were inactivated with 0.2% beta-propiolactone (Fellows Medical Mfg. Co., Inc., Oak Park, Mich.) for 2 hr at 37 C in a water bath. Water-in-oil-emulsion vaccines were prepared with both Tween 80 (aqueous soluble surfactant) and Arlacel A (oil-soluble
surfactant) added to mineral oil (Drakeol 6 VR high purity light mineral oil, Pennsylvania Refining Co., Los Angeles). The oil : water-phase ratio was 1:1.

**VN test.** The virus-neutralization tests were conducted using a constant-virus varying-serum microtiter procedure in BGM-70 cell culture as described previously (4). The SAL or IN viruses adapted to BGM-70 cells were used as homologous viruses with the tested antisera. A geometric mean titer (GMT) was calculated for each group.

**Experimental design.**

**Influence of the host system on the pathogenicity of SAL and IN strains.** Because of space limitation, three experiments were conducted as shown in Table 1. Two-week-old SPF chickens (15 bird per group) were inoculated each with a dose of $10^3$EID$_{50}$ virus via the intranasal and intraocular routes. In each experiment, a negative control group (uninoculated) was included. At 4, 8 and 12 days post inoculation (PI), five birds from each group were euthanized. The bursas and spleens were excised, and the mean organ/body weight (BW) ratios were calculated (organ weight in grams x 1000/ total body weight in grams).

**Influence of the host system on the immunogenicity of SAL and IN strains.** Three experiments were conducted to evaluate the immunogenicity of each of the live (Table 2) and inactivated (Table 3) vaccines prepared from SAL and IN
strains. Two-week-old SPF chickens were inoculated subcutaneously with a dose of $10^5\text{EID}_{50}$, determined prior to inactivation, of inactivated vaccine; in the live vaccine experiment (Table 2) chickens were inoculated with a dose of $10^3\text{EID}_{50}$ via the intranasal and intraocular routes. Groups of 10 chickens were used for each vaccine and for control groups. Two weeks post vaccination, the birds were challenged with $10^2\text{EID}_{50}$ of a bursal homogenate of the IN strain via the nasal and ocular routes. Five chickens per group were euthanized at 5 and 11 days post-challenge. The bursas and spleens/BW ratios were calculated and evaluated statistically as described in the pathogenicity experiment. Blood samples were collected before vaccination, prechallenge, 5 and 11 days post-challenge. Antibody titers to IBDVs were determined by the VN test.

**Histopathology.** Bursas from the inoculated and control groups were fixed in 10% neutral formalin solution. Sections of the bursas were stained with hematoxylin and eosin (H&E) and evaluated according to the method described by Rosales et al. (10). Bursas were subjectively scored as: 1= no lesion, normal, 2= focal, mild cell necrosis or depletion, 3= multifocal, 1/3 to 1/2 of the follicles show atrophy and 4= diffuse, atrophy of all follicles.

**Statistical analysis.** The average organ/BW ratios of the inoculated birds were compared with those of control groups for statistical significance using analysis of variance.
followed by Fisher's least significant difference.

Criteria for evaluation of protection. Bursa/B W ratio and bursal lesions scoring were used as parameters to evaluate the results of the challenge study. The live and inactivated vaccines were classified into 3 groups: (1) vaccines provided full protection = normal bursa/B.W. ratio and no bursal microscopic lesions. (2) vaccines provided partial protection =normal bursa/BW but bursal microscopic lesions were detected. (3) non protective vaccines = low bursa/B.W. ratio and bursal microscopic lesion were detected.

Antigenicity of SAL virus propagated in BGM-70 cells versus Bursa of Fabricius. In this study the AC-ELISA was used for titration of the viruses before preparation of oil-emulsion- inactivated vaccines containing equal amounts of virus antigens propagated in BGM-70 cells or BF. The inactivated vaccines, prepared as described earlier, were inoculated subcutaneously into 2 age groups (4 week and 20 week old SPF chickens). Two weeks post vaccination, the chickens were inoculated again via the subcutaneous route with the same dose of the inactivated virus. Blood samples were obtained 4,8 and 14 days post vaccination and 10 days after the second inoculation to evaluate the VN antibody titers.

RESULTS

Adaptation of viruses to BGM-70 cells: Both SAL and IN strains produced CPE after two passages in BGM-70 cells. The
CPE was characterized by cytoplasmic granulation, cell rounding followed by gradual detachment and destruction of the monolayer.

Adaptation of the viruses to CEF primary cell culture. The SAL virus adapted to CEF and produced CPE after two passages, but the CPE was less prominent compared to that in BGM-70 cells. On the other hand the IN strain failed to adapt to this host system after 6 passages.

Adaptation of viruses to egg embryos. Embryos inoculated with SAL and IN strains showed mortalities after 3 days in the first three passages. In subsequent passages, no mortality was observed in inoculated embryos and consequently the embryos were chilled after 72 hr post-inoculation. The CAMs of sacrificed and dead embryos showed congestion and edema. Embryos showed subcutaneous hemorrhages, dwarling, heart paleness, splenomegaly, and necrosis of the liver and spleen.

Pathogenicity studies. Results of the pathogenicity experiments are summarized in Table 1. In the first experiment, chickens inoculated with embryo adapted strains (6 passages) had significantly (P <0.05) smaller bursas than the noninoculated birds. The difference was noticed at 4, 8 and 12 days PI in birds inoculated with the IN strain and only at 8 and 12 days PI in birds inoculated with SAL strain. Marked bursal lymphoid necrosis and depletion was noticed in both groups inoculated with SAL or IN embryo adapted strains. The spleen/BW ratios showed no statistical difference between the
inoculated and uninoculated controls throughout the experimental period. In the second experiment, both the organ (bursa & spleen)/BW ratios and the bursal lesion scores of the birds inoculated with SAL or IN strains, propagated in BGM-70 cell culture, showed no significant differences from the uninoculated birds at any time PI (P>0.05). In the third experiment, chickens inoculated with CEF adapted SAL virus showed no significant differences from the uninoculated controls. In contrast, chickens inoculated with SAL or IN passaged one time in chicken bursas had significantly smaller bursas and larger spleens during the experimental period (P <0.05). Extensive bursal lesions were noticed in both virus-exposed chickens at 4 days PI, and the lesions persisted during the experiment.

**Immunogenicity studies.** The results of the experiments using the SAL and IN live vaccines propagated in different host systems are summarized in Table 2. Chickens vaccinated with either SAL/Embryo, IN/Embryo, SAL/BF or IN/BF live vaccines were not protected as indicated by the lower bursa/BW ratios and higher bursal lesions scores when compared with those of the unvaccinated unchallenged controls (P<0.05). The live vaccines propagated in BGM-70 cell culture elicited satisfactory protection when birds challenged with the bursa derived IN virus, as indicated by the organ/BW ratios and bursal lesions at 5 and 11 days post challenge. Although the bursa/BW ratios of birds vaccinated with SAL/CEF showed no
significant difference from the uninoculated controls at 5 and 11 days post challenge, there was some differences between the two groups in the incidence of histologic lesions in the bursas. The spleen/BW ratios of the vaccinated groups did not show a consistent significant differences from those of the controls. An anamnestic response was noted in all vaccinated groups as shown by the increase of the VN antibody titers.

The results of the immunogenicity studies of the inactivated vaccines of SAL and IN strains are shown in Table 3. After 11 days post challenge, the bursa/BW ratios and bursal lesions scores indicated that the inactivated vaccines made from IN/Embryos, SAL/BGM, IN/BGM and IN/BF vaccines were able to provide substantial protection for the vaccinated chickens against challenge. SAL/Embryo and SAL/CEF provided incomplete protection against challenge. Although the bursa/BW ratio index was not affected, there were variable degrees of microscopic bursal lesions. The SAL/Bf vaccine was not protective against challenge as indicated by the results of both bursa/BW ratios and bursal lesion scores at 5 and 11 days post challenge.

In both experiments of the live and inactivated vaccines, the unvaccinated unchallenged control groups had no antibody response, no bursal lesions, and their bursa/BW ratios were significantly (P<0.05) higher than those of the unvaccinated challenged control chickens which had low VN titers at the
Titration of viruses propagated in different host systems by antigen capture ELISA. The results of ELISA titration of SAL and IN vaccines which contained $10^3 \text{ EID}_{50}$ are shown in table (4). Only viruses propagated in BGM-70 cells had definite titers indicating that vaccines prepared from this host system contained more virus antigen in comparison with those propagated in the CEF, Egg embryos or bursas.

Antigenicity of SAL virus propagated in BGM-70 cells versus BF. The VN antibody titers elicited by SAL vaccines propagated in BGM-70 cells versus BF are illustrated in table (5). The antibody response of the inoculated chickens to the two vaccines were not significantly different ($P>0.05$).

DISCUSSION

The SAL and IN strains were successfully adapted and passaged six times in BGM-70 cells and egg embryos. After six passages in BGM-70 cells, both viruses lost their pathogenicity to SPF chickens as. On the other hand, six passages in embryonated eggs did not affect the pathogenicity of the two viruses. There was considerable bursal atrophy after 4 days PI in birds inoculated with IN virus and after 8 days PI for SAL virus, but the microscopic bursal lesions were detected as early as 4 days PI for both viruses (Table 1). Although SAL virus was adapted and passaged successfully in
CEF, IN virus failed to adapt to this host system after six serial passages. It was observed in other studies that the susceptibility of CEF cell culture to different strains of IBDV varied and some strains were even refractory to grow in this host system (5, 8). The back passage of SAL and IN viruses in SPF chickens maintained or may have increased the virulence of both viruses as indicated by the severe bursal atrophy and damage recorded as early as 4 days PI and persisted throughout the experimental period (9).

The results of the pathogenicity experiment clearly demonstrated the influence of the host system and passage level on the pathogenicity of SAL and IN vaccine strain. Viruses propagated in BGM-70 and CEF cell cultures behaved as mild vaccines since they caused no significant lesions in the bursa, while those propagated in embryos and bursas could be classified as virulent vaccines since they caused marked bursal lesions and bursal atrophy (13).

Based on the results of the immunogenicity studies (table 2&3), the 14 tested vaccines were classified into 3 groups: (1) Vaccines that provided full protection, this group included the live and inactivated vaccines of SAL/BGM, IN/BGM and inactivated vaccines of IN/Embryos and IN/BF vaccines; (2) Vaccines providing partial protection, Included in this group were the live and inactivated vaccines of SAL/CEF and inactivated SAL/Embryos vaccines; (3) Non-protective vaccines, this group included the live vaccines of SAL/Embryos,
IN/Embryos, SAL/BF and IN/BF and inactivated SAL/BF. It is clear that these pathogenic live vaccines already caused considerable damage to the bursas of the vaccinated SPF birds before challenge. These vaccines should be used only in chickens with high maternal immunity which modifies the the adverse effect of the vaccine (7).

Both live and inactivated vaccines propagated in BGM-70 cells offered better protection than those propagated in other host systems. Surprisingly, the SAL/BGM inactivated vaccine offered full protection while the same dose ($10^5$EID$_{50}$) of SAL/BF inactivated vaccine was not protective. In a previous study, it was shown that protection is related to the dose of the vaccine and challenge viruses (3). Hence, we speculated that the observed variability in the immunogenicity of the tested vaccines may be attributed to the lack of correlation between the EID$_{50}$ and the actual amount of the antigen because of varying degrees of adaptation and/or pathogenicity of the viruses to a given host system. Moreover, during the vaccine titration process, we noticed that viruses propagated in BF and embryos produced severe lesions while tissue culture propagated viruses caused mild lesions. Also the results of AC-ELISA (table 4) revealed more antigens in BGM-70 cells derived vaccines than in bursal derived vaccines. Considering the results of egg embryo titration and the AC-ELISA, we point out the influence of egg embryo titration system by the pathogenicity of the inoculated viruses. In fact, in order to
reach a vaccinal dose of $10^5$ EID$_{50}$ of the SAL/BGM vaccine, we had to use a dose of $5 \times 10^7$ TCID$_{50}$.

It could be concluded that the use of egg embryos as a titration system, in this study did not provide a true measure of the amount of antigens in the tested vaccine preparations. Hence, the AC-ELISA was used as a titration system since it is used to detect infectious, noninfectious, complete and incomplete viruses and it is not influenced by the pathogenicity of the titrated viruses. The results of the antigenicity study of SAL vaccines propagated in BF versus BGM-70 cells revealed that the geometric mean virus-neutralizing (VN) antibody titers of the vaccinated groups did not differ significantly ($P>0.05$). A challenge study is required to compare between the immunogenicity of these vaccines.

In conclusion, we noticed marked differences in the pathogenicity and immunogenicity properties of IBDVs propagated in different host systems. The antigenicity of the virus antigens propagated in BF or in BGM-70 cells was not significantly different. The choice of the titration system was highlighted.
1. Influence of the host system on the pathogenicity of SAL and IN strains of infectious bursal disease virus.

<table>
<thead>
<tr>
<th>Virus strain/host/passage No.</th>
<th>Average organ/BW ratio at days PI&lt;sup&gt;ac&lt;/sup&gt;</th>
<th>Bursal lesion score at days PI&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bursa/BW</td>
<td>Spleen/BW</td>
</tr>
<tr>
<td>SAL/Embryo/6</td>
<td>4.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IN/Embryo/6</td>
<td>3.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.50&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>5.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.53&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAL/BGM/6</td>
<td>4.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IN/BGM/6</td>
<td>5.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>4.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAL/CEF/6</td>
<td>5.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAL/BF/1</td>
<td>3.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.63&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IN/BF/1</td>
<td>1.79&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>5.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each bird inoculated with 10<sup>3</sup>EID<sub>50</sub> virus via intranasal and ocular routes at 2 weeks of age. Embryo= 10 day-old SPF chicken embryos, BGM= BGM-70 cells, CEF= chicken embryo fibroblast cells and BF= chickens bursa of Fabricius.

Values within a column followed by different lower-case superscripts are significantly different from controls (P<0.05).

Bursal lesion scores: 1= no lesions; 2= mild cell depletion in a few follicles; 3= moderate atrophy or cell depletion in 1/3 to 1/2 of the follicles; 4= severe necrosis and atrophy in all follicles.
2. Influence of the host system on the immunogenicity of live vaccines of SAL and IN strains

<table>
<thead>
<tr>
<th>Vaccine Strain/</th>
<th>Challenge Virus</th>
<th>Average organ/BW ratio at days PC*</th>
<th>VN antibody titers (GMT) at days*</th>
<th>Bursal lesions score at days PC**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bursa/BW Spleen/BW</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 11 5 11 Prechallenge 5 11 5 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAL/Embryo/6</td>
<td>IN</td>
<td>2.40b 2.82b 2.58b 2.16b 695 1599 1600 3.0 3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IN/Embryo/6</td>
<td>IN</td>
<td>2.57b 1.25b 3.53b 2.29b 281 198 1606 4.0 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>IN</td>
<td>2.15b 1.17b 3.25b 2.54b &lt;10 126 580 4.0 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>4.80* 5.30* 2.04* 2.00* &lt;10 126 580 4.0 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAL/BGM/6</td>
<td>IN</td>
<td>4.30* 4.40* 1.63* 2.00* 918 1164 2187 1.6 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IN/BGM/6</td>
<td>IN</td>
<td>4.04* 4.40* 1.68* 1.77* 400 764 1602 1.0 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>IN</td>
<td>2.00b 1.50b 1.87* 1.79* &lt;10 100 400 4.0 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>4.25* 4.71* 1.59* 1.78* &lt;10 &lt;10 &lt;10 1.0 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAL/CEF/6</td>
<td>IN</td>
<td>4.78* 4.94* 2.44* 1.96* 263 937 2884 2.5 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAL/BF/1</td>
<td>IN</td>
<td>1.59b 1.57b 1.66* 1.62* 1047 3630 4168 4.0 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IN/BF/1</td>
<td>IN</td>
<td>1.04b 1.89b 2.12* 1.56* 800 5300 9550 4.0 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>IN</td>
<td>1.09b 1.16b 2.09* 2.46* &lt;10 120 280 4.0 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>4.80* 4.95* 2.05* 1.85* &lt;10 &lt;10 &lt;10 1.0 1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Each bird inoculated with $10^6$EID$_{50}$ via intranasal and ocular routes at 2 weeks of age. Embryo= 10 day-old SPF chicken embryo, BGM= BGM-70 cells, CEF= chicken embryo fibroblast cells and BF= bursa of Fabricius

*Each bird was challenged, via the nasal and ocular routes, with $10^6$EID$_{50}$ of bursal homogenate of IN virus at 2 weeks post vaccination.

*Values show averages for 5 chickens. Values within a column followed by different lower-case superscripts are significantly different (P<0.05). BW=body weight. PC= post challenge.

*Homologous virus (SAL or IN) was used as antigen in the virus-neutralization (VN) test. GMT= geometric mean titer for 5 chickens.

*Values show averages for 5 chickens. Bursal lesion scores: 1= no lesions; 2= mild cell depletion in a few follicles; 3= moderate atrophy or cell depletion in 1/3 to 1/2 of the follicles; 4= severe necrosis and atrophy in all follicles.
3. Influence of the host system on the immunogenicity of inactivated vaccines of SAL and IN strains.

<table>
<thead>
<tr>
<th>Vaccine strain/ host/ passage No</th>
<th>Challenge Virus</th>
<th>Average organ/BW ratio at days PC</th>
<th>VN antibody titers (GMT) at days0</th>
<th>Bursal lesions score at days PC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bursa/BW</td>
<td>Spleen/BW</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>SAL/Embryo/6 IN</td>
<td>IN</td>
<td>4.69a</td>
<td>4.24a</td>
<td>2.78a</td>
</tr>
<tr>
<td>IN/Embryo/6 IN IN</td>
<td>4.74a</td>
<td>5.47a</td>
<td>1.73a</td>
<td>1.86a</td>
</tr>
<tr>
<td>None IN</td>
<td>2.15b</td>
<td>1.17b</td>
<td>3.25b</td>
<td>2.54b</td>
</tr>
<tr>
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<td>5.30a</td>
<td>2.04a</td>
<td>2.00a</td>
</tr>
<tr>
<td>SAL/BGM/6 IN</td>
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<td>4.50a</td>
<td>4.58a</td>
<td>1.68a</td>
</tr>
<tr>
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<td>4.53a</td>
<td>1.60a</td>
<td>1.47a</td>
</tr>
<tr>
<td>None IN</td>
<td>2.00b</td>
<td>1.50b</td>
<td>2.80b</td>
<td>1.79b</td>
</tr>
<tr>
<td>None</td>
<td>4.25a</td>
<td>4.71a</td>
<td>1.59a</td>
<td>1.78a</td>
</tr>
<tr>
<td>SAL/CEF/6 IN</td>
<td>IN</td>
<td>4.20a</td>
<td>4.88a</td>
<td>1.95a</td>
</tr>
<tr>
<td>IN/BF/1 IN IN IN IN</td>
<td>1.33b</td>
<td>1.29b</td>
<td>1.99b</td>
<td>1.87b</td>
</tr>
<tr>
<td>None IN</td>
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<td>4.25a</td>
<td>1.76a</td>
<td>1.94a</td>
</tr>
<tr>
<td>None</td>
<td>1.09b</td>
<td>1.16b</td>
<td>2.09b</td>
<td>2.46b</td>
</tr>
<tr>
<td>None</td>
<td>4.80a</td>
<td>4.95a</td>
<td>2.05a</td>
<td>1.85a</td>
</tr>
</tbody>
</table>

Each bird inoculated with 10^5 EID50 subcutaneously at 2 weeks of age. Embryo= 10 day-old SPF chicken embryo, BGM= BGM-70 cells, CEF= chicken embryo fibroblast cells and BF= bursa of Fabricius

Each bird was challenged, via the nasal and ocular routes, with 10^5 EID50 of bursal homogenate of IN virus at 2 weeks post vaccination.

Values show averages for 5 chickens. Values within a column followed by different lower-case superscripts are significantly different (P<0.05). BW= body weight. PC= post challenge.

Homologous virus (SAL or IN) was used as antigen in the virus-neutralization (VN) test. GMT= geometric mean titer for 5 chickens.

Values show averages for 5 chickens. Bursal lesion scores: 1= no lesions; 2= mild cell depletion in a few follicles; 3= moderate atrophy or cell depletion in 1/3 to 1/2 of the follicles; 4= severe necrosis and atrophy in all follicles.
4. Titration of viruses propagated in different host systems using antigen-capture ELISA.

<table>
<thead>
<tr>
<th>Vaccine virus</th>
<th>Host system(^a)</th>
<th>Titer (EID(_{50}))</th>
<th>ELISA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>BGM-70</td>
<td>(10^3)</td>
<td>200</td>
</tr>
<tr>
<td>SAL</td>
<td>CEF</td>
<td>(\cdot)</td>
<td>10</td>
</tr>
<tr>
<td>SAL</td>
<td>Embryo</td>
<td>(\cdot)</td>
<td>0</td>
</tr>
<tr>
<td>SAL</td>
<td>BF</td>
<td>(\cdot)</td>
<td>0</td>
</tr>
<tr>
<td>IN</td>
<td>BGM-70</td>
<td>(\cdot)</td>
<td>100</td>
</tr>
<tr>
<td>IN</td>
<td>Embryo</td>
<td>(\cdot)</td>
<td>20</td>
</tr>
<tr>
<td>IN</td>
<td>BF</td>
<td>(\cdot)</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)BGM-70 = BGM-70 cells, CEF = chicken embryo fibroblast cells, Embryo = 10 day-old SPF chicken embryo and BF = bursa of Fabricius.
5. Antigenicity of SAL virus propagated in BGM-70 cells versus bursa of Fabricius.

<table>
<thead>
<tr>
<th>Blood sample at days</th>
<th>VN titers (GMT)c</th>
<th>20 week old SPF chickens</th>
<th>4 week old SPF chickens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAL/BGM/6*</td>
<td>SAL/BF/1*</td>
<td>SAL/BGM/6*</td>
</tr>
<tr>
<td>4 dpv</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>8 dpv</td>
<td>&lt;100</td>
<td>229</td>
<td>&lt;100</td>
</tr>
<tr>
<td>14 dpv</td>
<td>691</td>
<td>1383</td>
<td>690</td>
</tr>
<tr>
<td>10 dpb</td>
<td>3630</td>
<td>6918</td>
<td>2089</td>
</tr>
</tbody>
</table>

*Days post vaccination (dpv) or boosting (dpb).

Vaccine virus/ host system/ passage level. BGM= BGM-70 cells, BF= bursa of Fabricius. Each bird inoculated via the subcutaneous route with oil emulsion vaccine contains 400 Elisa units. 14 dpv birds were boosted with the same dose of 0.2% Beta-propiolactone inactivated virus.

cValues show averages for 10 chickens. GMT= geometric mean titer
REFERENCES


CHAPTER II

COMPARISON BETWEEN ANTIGEN CAPTURE ELISA AND CONVENTIONAL METHODS USED FOR TITRATION OF INFECTIOUS BURSAL DISEASE VIRUS

SUMMARY. Two antigen-capture enzyme-linked immunosorbent assays (polyclonal & monoclonal AC-ELISAs) were developed and evaluated for titration of infectious bursal disease viruses (IBDV) propagated in different host systems, namely BGM-70 continuous cell line, primary chicken embryo fibroblast cells and chickens bursa of Fabricius.

The polyclonal system was more sensitive than the monoclonal system but both were specific. The results revealed that the conventional systems used for titration of IBDVs (cell cultures & embryonating chicken eggs) were more sensitive than the polyclonal AC-ELISA.

INTRODUCTION

Infectious bursal disease (IBD) is a major economic problem facing the poultry industry worldwide. This highly contagious immunosuppressive disease is caused by a virus
(IBDV) which is classified as a member of the Birnaviridae family (8). The virus has predilection to replicate in the lymphoid cells of the bursa of Fabricius (BF) causing severe inflammatory changes resulting in bursal atrophy (2). There are two serotypes of IBDV, but only serotype 1 is pathogenic to chickens (4). Despite extensive use of vaccines, outbreaks of this disease are still recorded worldwide.

The virus has been adapted to a variety of host systems which subsequently were used for its titration. Traditionally, cell culture, embryonating eggs, or live birds have been used for titration of IBDV. However, there are a number of limitations to this approach such as being time-consuming, labor-intensive, or the possibility of contamination with other avian pathogens.

The antigen capture ELISA (AC-ELISA) has been used for direct detection of a wide range of bacterial, viral, and parasitic agents (17). It is specific, sensitive, simple to perform, and relatively inexpensive to set up. The monoclonal AC-ELISA has been used to verify the presence of IBDV in infected bursal tissues collected from field cases and experimentally infected SPF chickens (12, 14).

In a previous comparative study (chapter 1), two IBDVs (SAL & IN) were passaged separately in four host systems, namely BGM-70 cells, Chicken embryo fibroblast cells (CEF), embryonating eggs or Bursa of Fabricius (BF). For the virus
titration process, it was noticed that Embryonating eggs and cell cultures were not suitable for titration of viruses propagated in other host systems possibly because of varying degrees of adaptation and/or pathogenicity of the viruses. Therefore, AC-ELISA was used as a titration system to compare the antigenicity of viruses propagated in different host systems.

The aim of this study was to compare two AC-ELISA, a polyclonal versus a monoclonal capture antibody, for titration of IBDVs propagated in different hosts and to evaluate the sensitivity of the AC-ELISA with the conventional titration systems (tissue culture & embryonating eggs).

**MATERIALS AND METHODS**

**Viruses.** Six standard serotype 1 IBDVs from commercial live vaccines were used. They were designated as follows: D-78 (clone-vac D-78), SAL (Bursine 2, Solvay Animal Health, Inc., Charles city, Iowa), BVM (Bursa-Vac-M, Sterwin Laboratories, Millsboro, Delaware), BB (BIO-Burs, formally produced by Agri-Bio Corp. Gainesville, Georgia), UV (Univax-DB, American Scientific Laboratories, Omaha, Nebraska), IBD-BLEN (Sanofi Laboratories, Inc., Overland Park, Kansas). In addition, three serotype 1 IBDVs (NC, LVN and STC) were also used (5). The study also included 5 variant IBDVs, namely IN, MD, A, E and GLS (3, 10, 11, 15). Finally, One serotype 2 IBDV, designated OH (6) and 3 unrelated viruses (Newcastle, Reovirus
and Influenza viruses) were used to test the specificity of the AC-ELISA.

Cell culture. The use of chicken embryo fibroblast (CEF), and an established cell line, BGM-70 cells for adaptation, passage, and titration of IBDV has been described (7).

Titration of IBDVs in embryonating eggs. IBDVs were titrated in 10-day-old SPF embryonating eggs. The virus titer was determined as a mean embryo infectious dose (EID<sub>50</sub>).

Polyclonal AC-ELISA. Reagents and procedures used for the polyclonal AC-ELISA have been described previously (Chapter 1).

Monoclonal AC-ELISA. A virus-neutralizing serotype 1 specific monoclonal antibody (33E8 MAB) was used as a capture antibody in this assay (16). A volume of 100 uL/well was used for all reagents. ELISA plates were coated with the capture mAb diluted 1:900 in coating buffer (0.1 M Carbonate - 0.1 M bicarbonate buffer [pH 9.6]), and then incubated at 37 C for one hour and 4 C overnight. The plates were rinsed three times with phosphate-buffered saline with 0.1% Tween-20 (PBS-T); the blocking solution (5% nonfat dry milk in PBS-T was added and the plates were incubated at 37 C for 1 hour. Virus samples were diluted 1:5 in PBS-T, then serially diluted viruses were transferred into the coated wells and incubated for 3 hrs at 37 C. The plates were washed 3 times with washing buffer (PBS-
T) using an automated ELISA washer and incubated for 1 hr at 37 C with chicken anti-SAL antibody diluted 1:4000 in PBS-T. After washing 3 times in PBS-T, goat anti-chicken IgG peroxidase-labeled antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1:1000 in PBS-T was added and the plates were incubated at 37 C for 1 hour. The plates were washed with the washing buffer 3 times and 100 ul of the substrate 2,2'-Azino-bis(3-ethyl-benzthiazoline) sulphonic acid (ABTS; Sigma Chemical Co., St. Louis, Missouri) in 0.05 M citrate buffer (pH 4.0) and 3% hydrogen peroxide were added to each well. The color reaction was stopped by adding 50 ul of 5% Sodium dodecyl sulphate (SDS) per well, and the absorbance was read at 410 nm (MR 600 Microplate Reader; Dynatech Laboratories). The virus titers were determined by adding 3 standard deviations to the mean absorbance value of known negative samples which represent the cut off point. ELISA titers were expressed as the reciprocal of the highest positive dilutions of the virus samples (13).

Statistical analysis. The correlation coefficient (r) between the polyclonal or monoclonal AC-ELISA titers of the virus samples and other titration systems (cell culture or embryonating eggs) were determined for each host system (BGM-70 cells, CEF or BF).
RESULTS

To evaluate the sensitivity of the polyclonal and monoclonal AC-ELISAs, cell culture adapted IBDVs were titrated in BGM-70 cells or CEF cells and bursal derived viruses were titrated in embryonating eggs, then the virus samples were titrated with the polyclonal and monoclonal AC-ELISA systems.

Results of the titration of IBDVs adapted to BGM-70 cells are summarized in table 6. The polyclonal AC-ELISA titers were consistently higher than those obtained by the monoclonal AC-ELISA. Both systems did not detect IBDV serotype 2 (OH). The correlation coefficient (r) between TCID$_{50}$ and polyclonal or monoclonal AC-ELISAs was 0.61 and 0.66, respectively.

As shown in table 7, only the polyclonal AC-ELISA barely detected SAL virus propagated in CEF with a titer equal to 10$^4$ TCID$_{50}$ (r = 0.80). On the other hand, the monoclonal AC-ELISA detected MD virus in a sample containing 4x10$^4$ TCID$_{50}$ (r = 0.69).

The results of the titration of IBDVs propagated in BF are illustrated in table 8. The polyclonal AC-ELISA detected the GLS virus in a sample containing 1.5x10$^3$ EID$_{50}$, while the monoclonal AC-ELISA did not detect the virus in samples that had less than 5x10$^3$EID$_{50}$. The correlation coefficient between the embryonating egg titration and the polyclonal or
monoclonal AC-ELISAs was 0.86 and 0.89, respectively.

Neither of the AC-ELISA systems detected Newcastle disease virus, Reovirus or Influenza viruses.

DISCUSSION

Conventional titration of IBDV has relied mainly on the growth of infectious virus in cell cultures or SPF embryonating eggs. Such approach requires the presence of live viruses and multiple passages to adapt the virus to the host system used for titration. However, some variant strains of IBDV do not cause embryonial mortalities and do not readily adapt and replicate in primary cell culture (9, 10).

AC-ELISA is a quick and relatively simple assay which can be adapted to titrate viruses propagated in different host systems. The AC-ELISA titer represent both live, infectious or non-infectious viruses and inactivated virus antigens. This could be an advantage over the biological systems of titration (cell culture or SPF embryos) specially when titrating antigens to be used for inactivated vaccines. However, the results of the titration indicated that AC-ELISA was less sensitive than comparable cell cultures or embryonating eggs when the virus was previously adapted to the host system. For example, The polyclonal AC-ELISA did not detect the BF propagated SAL virus which had a titer of 1.25x 10^3 EID_{50} (table 8). Such limited sensitivity of the AC-ELISA may explain the results obtained by Sharma et al. (12) where the
monoclonal AC-ELISA did not detect viral antigens in thymuses and bursas of experimentally infected birds but inoculation of homogenates of bursal and thymic tissues into embryonating chicken eggs revealed the presence of infectious virus from both tissues with titers ranging from $10^1$ to $10^4$ EID$_{50}$. Therefore, the use of AC-ELISA for diagnostic or epidemiological purposes would be limited by its lower sensitivity which necessitates that other viral detection systems should be considered for diagnosis in addition to AC-ELISA.

The results also indicated that the polyclonal system is more sensitive than the monoclonal system when both are used to titrate the same sample. The superiority of the polyclonal system may be attributed to the multiple specificities of binding compared to the mono-specificity of the monoclonal AC-ELISA. Improvements in the sensitivity of the monoclonal AC-ELISA might be achieved by pooling several monoclonals directed against different epitopes on VP2 and VP3 (major structural proteins). Also, the use of avidin-biotin system to immobilize the monoclonal antibodies might result in greater antigen capture capacity compared to monoclonal antibodies which are directly adsorbed on plastic (1). Both ELISA systems were specific for IBDVs since they did not react with unrelated avian viruses tested.
Table 6. Titration of Infectious bursal disease viruses propagated in BGM-70 cell line.

<table>
<thead>
<tr>
<th>Virus^A</th>
<th>Titer^B</th>
<th>Antigen-capture ELISA titer</th>
<th>Polyclonal antibody</th>
<th>Monoclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCID\textsubscript{50}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAL</td>
<td>5x10^5</td>
<td>600</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>SAL</td>
<td>1x10^1</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>IN</td>
<td>1.6x10^4</td>
<td>15</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>IN</td>
<td>1x10^6</td>
<td>100</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>IN</td>
<td>5x10^4</td>
<td>200</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>OH</td>
<td>1.5x10^6</td>
<td>150</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>IN</td>
<td>5.5x10^6</td>
<td>500</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>5x10^5</td>
<td>1000</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>STC</td>
<td>1.5x10^6</td>
<td>500</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>D-78</td>
<td>8x10^6</td>
<td>7000</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>D-78</td>
<td>1.5x10^6</td>
<td>1000</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>BVM</td>
<td>2.5x10^6</td>
<td>1000</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>MD</td>
<td>2.5x10^6</td>
<td>1500</td>
<td>700</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>5x10^6</td>
<td>2500</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>5x10^6</td>
<td>800</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>BB</td>
<td>1.2x10^7</td>
<td>5000</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>5x10^7</td>
<td>6000</td>
<td>600</td>
<td></td>
</tr>
</tbody>
</table>

^A All of the above strains are serotype 1 viruses except for the OH virus which is serotype 2.

^B Viruses were titrated in BGM-70 cells and the titer was determined as a mean tissue culture infectious dose (TCID\textsubscript{50}).

^C The correlation coefficient.
Table 7. Titration of infectious bursal disease viruses propagated in chicken embryo fibroblast cell culture.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titer (TCID&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>Antigen-capture ELISA titer Polyclonal antibody</th>
<th>Monoclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>1x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>SAL</td>
<td>2.8x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>200</td>
<td>25</td>
</tr>
<tr>
<td>SAL</td>
<td>1.6x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D-78</td>
<td>1.2x10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LVN</td>
<td>2.5x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>BVM</td>
<td>2.5x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>MD</td>
<td>4x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>BB</td>
<td>4x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>NC</td>
<td>1.2x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>200</td>
<td>30</td>
</tr>
<tr>
<td>STC</td>
<td>4.8x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>250</td>
<td>20</td>
</tr>
<tr>
<td>UV</td>
<td>5x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>300</td>
<td>70</td>
</tr>
<tr>
<td>E</td>
<td>5x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>350</td>
<td>90</td>
</tr>
</tbody>
</table>

<sup>A</sup> Viruses were titrated in chicken embryo fibroblast cell culture and the titer was determined as a mean tissue culture infectious dose (TCID<sub>50</sub>).

<sup>B</sup> The correlation coefficient.
Table 8. Titration of infectious bursal disease viruses propagated in bursa of Fabricius.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titer&lt;sub&gt;A&lt;/sub&gt;</th>
<th>Antigen-capture ELISA titer</th>
<th>Polyclonal antibody</th>
<th>Monoclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>1.25x10³</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SAL</td>
<td>1.25x10⁶</td>
<td>600</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>SAL</td>
<td>1x10²</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IN</td>
<td>1.25x10²</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IN</td>
<td>3.75x10²</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GLS</td>
<td>1.5x10³</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>2.5x10³</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>STC</td>
<td>5x10³</td>
<td>50</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>LVN</td>
<td>4x10⁴</td>
<td>100</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>A</sup> Viruses were titrated in 10-day-old SPF embryonating eggs and the titer was determined as a mean embryo infectious dose (EID<sub>50</sub>).

<sup>B</sup> The correlation coefficient.
REFERENCES


CHAPTER III
PATHOGENICITY, ATTENUATION AND IMMUNOGENICITY OF INFECTIOUS BURSAL DISEASE VIRUS

Summary. An investigation was conducted in specific pathogen free chickens on the pathogenicity of bursa-derived and tissue culture attenuated classic (STC) and variant (IN) serotype 1 strains of infectious bursal disease virus. The IN bursa-derived virus caused bursal inflammation, necrosis, and atrophy earlier than the bursa-derived STC virus. Both viruses lost their pathogenicity after four passages in BGM-70 cells. A statistically significant level of protection was observed in SPF chickens vaccinated with the attenuated IN virus used as a live or inactivated vaccine followed by homologous (IN) and heterologous (STC) challenge with bursa-derived viruses.

INTRODUCTION

Since it was described by Cosgrove in 1962 (1), infectious bursal disease (IBD) has been considered as one of the important viral diseases threatening the poultry industry worldwide. The virus (IBDV), a member of the Birnaviridae
family, replicates primarily in the bursa of Fabricius and causes severe inflammation and bursal atrophy which results in immunosuppression in young chickens exposed to the virus during the first few weeks of life (5).

Effective control of IBD was accomplished by the use of live modified or inactivated vaccines, prepared from classic serotype 1 (5). However, during the last decade, IBD has reemerged as a significant economic problem which was attributed to variants of serotype 1 in the USA and the emergence of the very virulent classic serotype 1 viruses in Europe. These events led to changes in vaccination strategies adopted to control the disease (7,10).

Variant strains are classified as subtypes of serotype 1 IBDV based on the virus neutralization assay (3) and they are distinct from the classic serotype 1 viruses in terms of pathogenicity and immunogenicity. They have been reported to overcome immunity induced by classic serotype 1 vaccines and cause rapid bursal atrophy but with minimal or no inflammatory response (7,8).

The IN virus is a variant strain isolated in our laboratory from commercial Leghorn pullets (2). Similar to other variants, it induces severe bursal atrophy by 5 and 10 days post inoculation in 3 week-old SPF chickens. The in vitro cross-neutralization assay and cross protection studies indicated that the IN strain is antigenically related to one
This study was conducted to compare the pathogenicity of two serotype 1 viruses, the classic STC and the variant IN viruses. These viruses were derived from tissue-culture, BGM-70 cells or the bursa of Fabricius (BF). In addition, the immunogenicity of live and inactivated vaccines prepared from the IN virus adapted to BGM-70 cells was investigated.

**MATERIALS AND METHODS**

**Chickens and embryonated eggs.** Specific pathogen free (SPF) eggs (Hy. Vac Laboratory Egg Co., Gowrie, Iowa) were incubated at our facilities and the hatching Chicks were raised in a disease containment building. Before inoculation with live viruses, the chicks were moved into plastic isolators supplied with sterile intake and exhaust air under positive pressure.

**Viruses.** Two serotype 1 strains of IBDV, the classic STC and the variant IN viruses were used in this study. Both viruses were propagated in SPF chicken bursas (bursa-derived) and were titrated in SPF egg embryos.

**Adaptation of viruses to BGM-70 cells.** Bursal homogenates of the IN and STC viruses were used for adaptation in BGM-70 cells as described previously (chapter 1). Both viruses were passaged in the BGM-70 cells for 4 times.
Virus-neutralization (VN) assay. The VN titers were determined using the constant-virus diluting-serum procedure in BGM-70 cells (4).

Preparation of oil emulsion vaccines. The IN and STC viruses were inactivated with 0.2% beta-propiolactone (Fellows Medical Mfg. Co., Inc., Oak Park, Mich.) for 2 hours at 37 C in a water bath. Water-in-oil-emulsion vaccines were prepared with both Arlacel A (oil-soluble surfactant) mixed with mineral oil (Drakeol 6 VR high purity light mineral oil, Pennsylvania Refining Co. Los Angeles). The oil to water-phase ratio was 1:1.

Histopathology. Sections of bursal tissues from inoculated and control birds were stained using the hematoxylin-and-eosin-staining technique. Bursal histological lesions were scored according to the method described by Rosales et al. (6). Bursas were subjectively scored as: 1 = no lesion, 2 = focal, mild cell depletion, 3 = multifocal, 1/3 to 1/2 of the follicles show atrophy, and 4 = diffuse, atrophy of all follicles.

Statistical analysis. The average bursa/body weight ratios and spleen/body weight ratios were compared with those of control groups using an analysis of variance (ANOVA) followed by Fisher's Least significant difference test.

Experimental design.

Pathogenicity of IN and STC strains (bursa derived versus BGM-70 cells adapted viruses). Five groups of two-week-old SPF
chickens (20 birds per group) were used in this experiment (table 9). The first 2 groups were inoculated via the intranasal and intraocular routes with bursal homogenates containing the IN or STC viruses (10^2EID_{50}/bird). The birds in the third and fourth groups were inoculated via the intranasal and intraocular routes with the BGM-70 cells adapted viruses (10^5TCID_{50}/bird). Chickens of the fifth group were used as uninoculated controls. At 2, 4, 8 and 16 days post inoculation (PI) five birds from each group were euthanatized. The bursas and spleens were obtained, and the mean organ/body weight (BW) ratios were calculated (organ weight in grams x1000/total body weight in grams).

**Immunogenicity of the IN virus passaged in BGM-70 cells.**

One experiment was conducted to evaluate the immunogenicity of both live and inactivated vaccines prepared from the IN strain which was passaged 4 times in BGM-70 cells (table 10). Two-week-old SPF chickens (10 birds/group) were vaccinated, via the intranasal and the intraocular routes, with the live vaccine (10^5TCID_{50}/bird). The inactivated vaccine (10^5 TCID_{50}/bird) was inoculated via the subcutaneous route. Two weeks post vaccination, each chicken was challenged with 10^2EID_{50} of a bursal homogenate of either IN or STC viruses via the nasal and ocular routes. Five birds per group were euthanatized at 5 and 12 days post-challenge. The means of organ/BW ratios were calculated and evaluated statistically. Sections of the bursas of the challenged and control groups
were processed for microscopic examination as described earlier. Antibody titers were determined by the VN test on blood samples collected before vaccination, prechallenge, and 5, 12 days post-challenge.

RESULTS

Adaptation of viruses to BGM-70 cells. Both IN and STC strains caused CPE after two passages in BGM-70 cells. The CPE was characterized by cell rounding, granulation of the cytoplasm followed by detachment of the infected cells from the monolayer.

Pathogenicity studies. Results of the pathogenicity study are summarized in table 9. Chickens inoculated with IN or STC bursa-derived viruses had significantly (P<0.05) smaller bursas and larger spleens than the noninoculated control birds. These differences were noticed at 4 days post inoculation (PI) in chickens inoculated with the IN strain and at 8 days PI in birds inoculated with STC strain.

A difference in the onset of bursal changes between the two viruses was also observed microscopically. At 2 days PI, the IN strain induced moderate to severe necrosis and marked lympholysis with prominent heterophilic infiltrations. Whereas, the STC strain induced only mild multifocal necrosis and lympholysis with scattered lymphocytic infiltrates and few heterophils (fig.1). By 4 days PI, the inflammation in the IN
infected bursas had already subsided and follicular atrophy was evident while severe extensive necrosis and inflammation with heterophilic infiltrates were observed in bursas of the STC infected chickens (fig. 2). From 8 to 16 days PI, bursas of both the STC and IN inoculated birds had abundant interlobular fibrous connective tissue septa separating small shrunken lymphodepleted follicular remnants (ghost follicles) with little to no inflammation.

The bursa/BW and spleen/BW ratios of chickens inoculated with the cell-culture passaged IN or STC strains were not significantly different from those of the uninoculated controls at any time PI (P>0.05). Microscopically, the bursas of birds inoculated with the cell-culture passaged IN or STC strains had intact, large follicles with normal architecture comparable to the uninoculated control bursas (fig. 3).

**Immunogenicity studies.** Results of the immunogenicity studies are depicted in table 10. Chickens vaccinated with live or inactivated cell-culture passaged IN vaccines had significant protection when challenged with IN or STC bursa-derived viruses as indicated by the organ/BW ratios compared to those of the controls at 5 and 12 days post challenge (P>0.05). The challenge viruses elicited high VN antibody titers in the vaccinated birds indicating that a secondary immune response was mounted. The unvaccinated challenged birds had low VN antibodies titers after challenge. The
unvaccinated, unchallenged birds had no detectable VN antibodies titers throughout the experimental period.

Bursal lesion scores of the vaccinated birds at 5 and 12 days post challenge ranged from 1 (normal, no lesions) to 2 (mild, scattered cell depletion in few follicles). Severe bursal necrosis and atrophy were observed in the nonvaccinated, challenged groups, while the nonvaccinated, nonchallenged groups had normal bursas.

**DISCUSSION**

Early reports indicated that variants are different from classical IBDV strains in that they caused a very rapid bursal atrophy within 72 hours PI associated with a minimal inflammatory response in susceptible 3-to 4-week-old SPF leghorn chickens (7). Similar results were reported by Sharma et al. (8) who compared the pathogenicity of the serotype 1 classic IM and variant A isolates of IBDV in SPF chickens. Both viruses caused extensive lesions in the bursas but at three days PI, bursal necrosis induced by the IM isolate was accompanied by an inflammatory response whereas inflammation was lacking in the lesions induced by the A variant. It was suggested that the pathologic response elicited by the two viruses may represent a biological distinction between the classic and variant serotype 1 viruses.

In this study, we compared the microscopic lesions
induced by the pathogenic serotype 1 classic (STC) and variant (IN) viruses that were propagated in BF. Microscopic examination of the infected bursas beginning at 2 days PI rather than 3 days PI as reported earlier (7,8) enabled us to assess the sequence of microscopic changes caused by the IN and STC strains. The IN strain triggered a very rapid inflammatory response which subsequently subsided by 4 days PI. By comparison, the STC strain did not elicit an overt inflammatory response until 4 days PI which subsequently subsided by 8 days PI. It would be of interest to compare the pathogenicity of other classic and variant strains of the IBDV with observations made as early as the first day PI.

Passage of the IN and STC strains 4 times in BGM-70 cells resulted in loss of their pathogenicity as indicated in table 9. The use of BGM-70 cell line for isolation and/or propagation of IBDV has several advantages over cells of avian origin. This cell line is easier to handle and maintain, and free from vertically transmitted avian viruses. Both classic and variant strains adapted readily and produced CPE after 2 passages on that cell line.

In an earlier study (9), it was shown that high passage (30 times) of the IN strain in BGM-70 cells had an adverse effect on the immunogenic potential of the live vaccine made from that strain. It was speculated that such prolonged passage affected the ability of the virus to replicate in the
bursa, resulting in lack of protection. The results of the immunogenicity study (table 10) indicated that the live IN vaccine caused no lesions in the bursa of vaccinated birds and induced a significant level of protection (P>0.05). The presence of mild to moderate lesions in some bursas of challenged birds may indicate that the dose of the vaccine should be higher than $10^5$TCID$_{50}$/bird. A lower level of passage in BGM-70 cells e.g. 2 passages may be required.

The results of this study indicate that the pathology induced by both the variant and classic viruses examined is similar. It is concluded that variant IBDVs might not be homogenous as a group in terms of their pathogenicity or different from the classic viruses as previously described. The usefulness of the BGM-70 cell line for the attenuation of the IBDV without loss of immunogenicity was clarified and confirmed.
9. Pathogenicity of bursa-derived and tissue-culture-derived IN and STC strains of infectious bursal disease virus

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Average organ/BW ratio at days post inoculation&lt;sup&gt;†&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bursa/BW</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>IN/BF&lt;sup&gt;†&lt;/sup&gt;</td>
<td>5.40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>STC/BF</td>
<td>6.70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IN/BGM&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>5.03&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>STC/BGM</td>
<td>5.62&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>5.13&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>†</sup>Each bird was inoculated via the intranasal and intraocular routes with a bursal homogenate of the virus (dose = 10<sup>8</sup>EID<sub><sub>50</sub></sub>). BF = bursa of Fabricius.

<sup>‡</sup>Each bird was inoculated via the intranasal and intraocular routes with a dose of 10<sup>6</sup>TCID<sub><sub>50</sub></sub> of the virus passaged four times in BGM-70 cells.

<sup>§</sup>Mean values for five birds. Values within a column followed by different lower case superscripts are significantly different from controls (P<0.05). BW = body weight.
10. Immunogenicity of the live and inactivated IN vaccines after 4 passages in BGM-70 cells.

<table>
<thead>
<tr>
<th>Vaccine virus strain</th>
<th>Challenge virus strain</th>
<th>Bursa/BW ratio at days post challenge&lt;sup&gt;5&lt;/sup&gt;</th>
<th>Spleen/BW ratio at days post challenge&lt;sup&gt;5&lt;/sup&gt;</th>
<th>VN antibody titers at days&lt;sup&gt;4&lt;/sup&gt; Prechallenge</th>
<th>Bursal lesion score at days PC&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
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<td>4.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>5.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each bird inoculated via the intranasal and intraocular routes with a dose of 10<sup>10</sup>TCID<sub>50</sub>.  
<sup>b</sup>Each bird inoculated via the subcutaneous route with a dose of 10<sup>10</sup>TCID<sub>50</sub>.  
<sup>c</sup>Each bird was challenged via the intranasal and intraocular routes with 10<sup>4</sup>EID<sub>50</sub> of the bursal homogenate of IN or STC wild viruses at 4 weeks of age.  
<sup>d</sup>Values are averages for five chickens. Values within a column followed by different lower-case superscripts are significantly different (P<0.05). BW= body weight.  
<sup>e</sup>IN virus was used as antigen in the virus-neutralization assay (VN). Values are the geometric mean titer for five chickens.  
<sup>f</sup>Bursal lesion scores: 1= no lesions; 2= mild cell depletion in a few follicles; 3= moderate atrophy or cell depletion in 1/3 to 1/2 of the follicles; 4= severe necrosis and atrophy in all follicles. Values are average for 5 chickens.
Figure 1. Bursas of 2-week-old SPF chickens euthanatized on the second day post inoculation with bursa derived viruses. (A) IN virus. Moderate to severe necrosis and lympholysis with heterophilic infiltrations are observed within and around all bursal follicles. (B) STC virus. No inflammation or necrosis is observed in this lobule. H&E. 25X.
Figure 2. Bursa of 2-week-old SPF chickens euthanatized on the fourth day post inoculation with bursa derived viruses (A) IN virus. Note generalized collapse of the bursa with abundant fibrous connective tissue surrounding small lymphodepleted follicles. (B) STC virus. Severe necrosis and heterophilic inflammation admixed with cellular debris and macrophages are present within and around collapsing follicles. H&E. 25X.
Figure 3. Bursa of 2-week-old SPF chickens euthanatized on the fourth day post inoculation with BGM-70 cells derived viruses (A) IN virus after 4 passages in BGM-70 cells. Follicles remain large and intact. (B) Uninoculated control. Normal structure of the bursal follicles. H&E. 25X.
REFERENCES


CHAPTER IV

SMALL AND LARGE PLAQUE VIRUS CLONES OF A VARIANT INFECTIOUS BURSAL DISEASE VIRUS: ISOLATION, PATHOGENICITY, ANTIGENICITY AND IMMUNOGENICITY STUDIES.

Summary. A variant strain of infectious bursal disease virus was passaged forty times in BGM-70 cell line. Small plaque (Sp) and large plaque (Lp) clones were isolated and plaque purified four times. The Sp and Lp formed circular plaques about 0.5 and 6 mm in diameter, respectively. Both clones lost their pathogenicity for specific-pathogen-free (SPF) chickens and did not elicit significant levels of virus-neutralizing antibody titers. However, the Sp and Lp clones were shown to maintain their immunogenicity when used as inactivated vaccines in SPF chickens.
INTRODUCTION

Infectious bursal disease virus (IBDV) is known to form small plaque (Sp) and large plaque (Lp) clones when passaged in Chicken embryo (CE) cells. Lange et al. (4) reported that Sp and Lp developed after serial passage of the highly pathogenic Cu-1 strain in CE cells. The Lp virus was pathogenic and caused clinical manifestations and destruction of the bursa of Fabricius (BF), while the Sp virus was not pathogenic and caused few necrotic foci in the BF of the inoculated chickens. In another report (6), the RF-1 virus was passaged 25 times in chicken embryos followed by 15 passages in chicken embryo fibroblast (CEF) cells then plaque purified. Both Lp and Sp clones were significantly less pathogenic for 1 and 28-day-old chickens than the parent strain. Both clones, especially the Lp clone, were immunogenic in 28-day old chickens when inoculated as a live vaccines.

The aim of the present report was to isolate and characterize Sp and Lp clones of a variant strain passaged forty times in BGM-70 cells.
MATERIALS AND METHODS

Chickens and housing. Two-week-old chickens were obtained from our SPF flock and housed in plastic cages maintained in positive-pressure plastic isolators with filtered intake and exhaust air. The chickens were provided with feed and water ad libitum.

Virus. The IN virus is a variant serotype 1 strain isolated in our laboratory from a commercial layer flock (1). The adaptation of the IN strain to BGM-70 cells has been described previously (chapter #1). The virus was passaged forty times in BGM-70 cells.

Plaque purification. A confluent monolayer of BGM-70 cells was grown in six-well cell-culture plates (Corning, Corning Works, Corning, New York). A volume of 0.2 ml of 10-fold serial dilutions of the 40-passaged IN virus was absorbed onto the monolayers at 37 C for 60 minutes. The cultures were overlayed with maintenance medium containing 1.6% Nobel agar (Difco, Detroit, Michigan). The plates were incubated at 37 C for 5 days and then stained with 0.05% neutral red. Small and large plaques were plaque-to-plaque purified 4 times. The plaque-purified clones were propagated in BGM-70 cells for
further characterization.

Antigen-capture enzyme-linked immunosorbent assay (AC-ELISA). The Sp and Lp clones were tested by the AC-ELISA to verify the presence of a neutralizing epitope detected with 33E8 monoclonal antibody. The AC-ELISA test was performed as described previously in chapter 2.

Inactivated vaccine preparation. Inactivated vaccines from the Sp and Lp clones were prepared as described earlier in chapter 1.

Virus neutralization (VN) test. The VN test was carried out using the constant-virus diluted-serum procedure in BGM-70 cells (3).

Histopathology. Bursas of the inoculated and control groups were sectioned, processed and examined for microscopical lesions. Scoring of the bursal lesions, ranged from 1 to 4, were subjectively conducted according to the method described by Rosales et al. (5).

Experimental design.

Pathogenicity and antigenicity of the small and large plaque clones. Two-week-old SPF chickens were divided into three groups of 20 birds each. Each chicken in the first and the second groups was inoculated via the intranasal and intraocular routes with a dose of $10^4 \text{ TCID}_{50}$ in 0.1 ml of the
small and large plaque clones. The third group was left as uninoculated controls. Five birds from each group were euthanatized at 2, 6, 14 and 21 days post inoculation. The bursas and spleens were collected, and the average organ/body weight ratios were calculated. Also, blood samples were collected at the time of euthanasia, and the antibody titers were determined by the VN test.

**Immunogenicity of inactivated vaccines of the small and large plaque clones.** Forty 2-week-old SPF chickens were divided equally into 4 groups. Each bird in the first and the second groups was vaccinated via the subcutaneous route with inactivated vaccines \(10^5\text{TCID}_{50}/\text{bird}\) prepared from the small or large clones. The third and the fourth groups served as positive and negative controls. Two weeks post vaccination, each bird in the first three groups was challenged via the intranasal and intraocular routes with \(10^5\text{EID}_{50}\) of the bursa derived IN virus, the fourth group was left uninoculated. At 5 and 11 days post challenge, five chickens from the vaccinated and control groups were euthanatized. Evaluation of protection was based on VN antibody titers, organ/body weight ratios and bursal microscopic lesion scoring.

**Statistical analysis.** The average organ/body weight ratios were examined statistically using analysis of variance and Fisher's Least significant difference test.
RESULTS AND DISCUSSION

Isolation of the Sp and Lp clones. The IN strain was passaged 40 times on BGM-70 cells. The virus induced CPE characterized by cell granulation and detachment from the monolayer which was subsequently destroyed. The forty passaged viruses produced circular plaques with different sizes ranging from 0.5 to 6 mm in diameter (Figure 4). The small and large plaques were plaque to plaque purified 4 times which led to the isolation of small and large plaque clones (figure 5& 6). Five days after inoculation of the virus into tissue culture, the diameters of the small and large plaques were 0.5 and 6 mm, respectively.

Both of the small and large clones reacted with the monoclonal AC-ELISA which indicates that both clones maintained the neutralizing site detected by the 33E8 monoclonal antibody.

Pathogenicity and antigenicity of the Sp and Lp clones. The average organ/body weight ratios from birds inoculated with the small or large plaque viruses showed no significant difference (P>0.01) from those of the uninoculated controls throughout the experimental period (Table 11). Also, both of
the small and large plaque clones elicited low levels of VN antibody titers. These results indicate that both clones lost their pathogenicity to SPF chickens, and their ability to induce an immune response was adversely affected when inoculated as live vaccines.

**Immunogenicity of inactivated vaccines of the Sp and Lp clones.** Both inactivated vaccines offered significant protection (P>0.01) against challenge with the bursa derived IN virus as indicated by the bursa/body weight ratios (Table 12). However, mild to moderate lesions were detected in the bursas of the challenged birds which indicates that both clones induced partial protection. Since protection is based on the dose of the vaccine, complete protection may be achieved by increasing the vaccine dose higher than $10^5\text{TCID}_{50}$ (2).

This study indicates that the Sp and Lp clones maintained their antigenicity and immunogenicity as shown by the results of AC-ELISA and the significant protection induced by the inactivated vaccines prepared from both clones. However, the low VN titers elicited by the live preparations of the Sp and Lp clones indicates that prolonged passage on BGM-70 cells may affect the ability of the Sp and Lp clones to replicate efficiently in its natural host and diminish their ability to induce a satisfactory immune response. Similar observations were reported when the uncloned IN strain was passaged 30 times on BGM-70 cells (7).
No differences were detected between the Sp and Lp viruses in terms of pathogenicity, antigenicity or immunogenicity. It would be of interest to conduct a back passage study to investigate whether these clones will regain their pathogenicity or maintain their phenotypic characteristics.
11. Pathogenicity and antigenicity of the small and the large plaque clones of the IN strain.

<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>Virus*</th>
<th>Average bursa/b.w ratios$</th>
<th>Average spleen/b.w ratios$</th>
<th>VN antibody' titers</th>
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<tbody>
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<td>IN/Sp</td>
<td>4.66*</td>
<td>1.22*</td>
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</tr>
<tr>
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<td>&lt;10</td>
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<td></td>
<td>control</td>
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<td>0.90*</td>
<td>&lt;10</td>
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<td>6</td>
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<td>4.82*</td>
<td>1.14*</td>
<td>10</td>
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<tr>
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<td>1.18*</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
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<td>1.34*</td>
<td>&lt;10</td>
</tr>
<tr>
<td>14</td>
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<td>1.30*</td>
<td>30</td>
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<td>1.58*</td>
<td>10</td>
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<td>1.42*</td>
<td>&lt;10</td>
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<td>1.37*</td>
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<td>10</td>
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<td>control</td>
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<td>1.69*</td>
<td>&lt;10</td>
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</table>

*Each bird was inoculated via the intranasal and intraocular routes with $10^6$ TCID$_{50}$ at 2 weeks of age. Sp= small plaque clone. Lp= large plaque clone.

$Values are average for 5 chickens. Values followed by different lower-case superscripts are significantly different from controls (p<0.01).

'IN virus (5 passages in BGM-70 cells) was used as antigen in the virus-neutralization test. Values are geometric mean titer for five chickens.
12. Immunogenicity of inactivated vaccines of the small and the large plaque clones of the IN strain.

<table>
<thead>
<tr>
<th>Vaccine\ Challenge\ Virus</th>
<th>Bursa/BW ratio at days post</th>
<th>Spleen/BW ratio at days post</th>
<th>VN antibody\ titers</th>
<th>Bursal lesions score at days post challenge</th>
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</thead>
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<tr>
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<td>5</td>
<td>11</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>IN/Sp IN/wt</td>
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<td>4.16*</td>
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<td>6.22*</td>
<td>2.10*</td>
<td>1.90*</td>
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</table>

*Each chicken was inoculated via the subcutaneous route with inactivated vaccine containing $10^5$ TCID$_{50}$ at 2 weeks of age. Sp= small plaque clone, Lp= large plaque clone.

*Each chicken was challenged via the intranasal and intraocular routes with $10^5$EID$_{50}$ of bursal homogenate of IN virus at 2 weeks post vaccination. wt= wild type.

*Values show average for 5 chickens. Values within a column followed by different lower-case superscripts are significantly different from controls (P<0.01).

*IN virus (5 passage on BGM-70 cells was used as antigen in the virus-neutralization (VN) test. Values show geometric mean titer for 5 chickens.

*Values show average for 5 chickens. Bursal lesion scores: 1= no lesions; 2= mild cell depletion in a few follicles; 3= moderate atrophy or cell depletion in 1/3 to 1/2 of the follicles and 4= severe necrosis and atrophy in all follicles.
Fig. 4. Mixed plaque formation of the IN strain after 40 passages in BGM-70 cells. The monolayer of the BGM-70 cells was incubated at 37°C for 5 days, and stained with neutral red.
Fig. 5. Small plaque of the IN strain after 44 passages in BGM-70 cells. The monolayer of the BGM-70 cells was incubated at 37°C for 5 days, and stained with neutral red.
Fig. 6. (A) large plaque of the IN strain after 44 passages in BGM-70 cells. (B) Negative control. The monolayer of the BGM-70 cells was incubated at 37 C for 5 days and stained with neutral red.
REFERENCES


GENERAL DISCUSSION

Since vaccination is the principle method used to control IBD, there are continuous efforts aimed at developing effective and economic vaccines for commercial poultry production. The work presented in this dissertation was initiated to investigate the effect of the host systems used to propagate infectious bursal disease viruses (IBDV) on the biological characteristics of the virus. Most importantly, changes in the pathogenicity, antigenicity and immunogenicity of the virus are of significance for the control of the disease. The introduction of bursa-derived inactivated vaccines for the control of IBD was an important motive to investigate the influence of the host systems considering reports indicating that bursa-derived vaccines were superior to embryo-derived vaccines (6, 69).

In Chapter 1, a series of experiments were conducted to investigate the effect of the host system on the pathogenicity, antigenicity and immunogenicity of the IBDV in SPF chickens. Tissue culture-derived, embryo-derived and bursa-derived viruses were compared. A classic (SAL) and
variant (IN) serotype 1 viruses were passaged separately six times in three host systems namely, embryonating chickens eggs, BGM-70 continuous cell line, primary chicken embryo fibroblast (CEF) cells or one time in SPF chickens bursa of Fabricius (BF). Chickens inoculated with the live and inactivated vaccines made from the above viruses, containing $10^3\text{EID}_{50}$ and $10^5\text{EID}_{50}$ respectively, resulted in varied immune responses which was attributed to possible differences in antigen mass that was not accounted for by the employed titration method. To study that assumption, SPF chickens were vaccinated with inactivated vaccines produced in BGM-70 cells or bursa and standardized to contain similar antigen mass as measured by the antigen capture ELISA. The virus neutralizing antibody titers were not significantly different. It would be of interest to conduct a challenge study to extend this observation.

In chapter 2, a study was conducted to compare two antigen-capture ELISAs (AC-ELISA) and the conventional methods used for titration of IBDV. In this study, the AC-ELISA was used to titrate IBDVs propagated in different host systems, namely BGM-70 cells, CEF, or BF. Although the AC-ELISA is simple to perform, time-saving and relatively inexpensive to set up, the results revealed that conventional systems were more sensitive than the AC-ELISA for titration of IBDVs. Despite its limited sensitivity, the use of AC-ELISA
represents a simple alternative method for titration of viruses which do not cause embryonal mortalities and do not readily adapt and replicate in cell cultures. Moreover, the AC-ELISA enabled us to compare the antigenicity of the SAL virus propagated in BGM-70 cells or BF as shown earlier in chapter 1.

The objectives of the work presented in the 3rd chapter were to compare the pathogenicity of bursa-derived and tissue culture-derived classic (STC) and variant (IN) strains of IBDV, and to investigate the influence of low passage (4 times) in BGM-70 cells on the immunogenicity of live and inactivated vaccines prepared from the IN virus. Early reports indicated that variants are different from classic IBDV strains in that variants caused a very rapid bursal atrophy with no or minimal inflammatory response. The results of this study indicated that both viruses induced similar pathological changes in terms of bursal inflammation and atrophy, however the onset and severity of such events were different.

The BGM-70 cell line is routinely used in our laboratory for isolation and propagation of IBDV. The use of this cell line has several advantages over cell cultures of avian origin. It is easier to handle, maintain, and free from vertically-transmitted avian viruses. Moreover, both classic and variant strains readily adapt and produce characteristic
CPE after a few passages. The results presented in chapter 3 indicated that the STC and IN viruses lost their pathogenicity after four passages in BGM-70 cells. Significant protection was noticed when the live and inactivated vaccines prepared from the attenuated IN virus were inoculated into SPF chickens.

The influence of high passage in BGM-70 cells on the biological characteristics of the IN strain was investigated in chapter 4. The virus was passaged 40 times in BGM-70 cells followed by isolation and purification of small and large plaque clones. Both clones maintained a neutralizing site detected by monoclonal AC-ELISA and the inactivated vaccines prepared from the small or large plaque clones induced significant protection against challenge with the bursa-derived IN virus. On the other hand, both clones lost their pathogenicity and their ability to elicit marked virus-neutralizing antibody titers when inoculated as live vaccines. These results may indicate that prolonged passage in BGM-70 cells adversely affects the ability of the virus to replicate in its natural host. A back passage study would be useful to investigate whether these clones will regain their pathogenicity or maintain their phenotypic characteristics.
BIBLIOGRAPHY


