

Characterization of a Highly Virulent Infectious Bursal Disease Virus.

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Summary

A highly virulent infectious bursal disease (IBD) virus strain isolated from the recent outbreaks in Iran was characterized in SPF chickens. The mortality rates caused by the newly emerged virus in the field have been much higher in different genetic lines of chickens than those reported previously for the classic strains. The virus consistently produced a mortality rate of 85-90% in 5-6-week-old SPF birds. Contrary to the standard (classic) viruses, the highly virulent strain caused clinical disease and mortality in SPF chicks under 10 days of age. In virus neutralization tests, cross-reactions were observed in the heterologous systems including the highly pathogenic isolate, the representatives of the standard type I viruses, and selected vaccine strains. These findings, together with the effectiveness of some live IBD vaccines derived from the classic viruses in protecting at least against mortality and gross bursal lesions due to a challenge, probably indicate that, in spite of the dramatic changes in virulence, the newly emerged viruses can still be regarded as belonging to the standard type I IBDV strains. The speculative mechanisms resulting in clinical signs and mortality in very young chicks together with the major changes occurring in the pathogenicity of the virus despite minor antigenic alterations, have been discussed.

Introduction

Infectious bursal disease standard serotype 1 virus strains (IBDV) were first isolated in 1981 in this laboratory (Aghakhan *et al.*, 1994). Those strains were of relatively low to moderate pathogenicity, usually causing less than 5% mortality, the main economical losses being due to immunosuppression and growth impairment. In many instances, the occurrence of a clinically mild form of IBD went unnoticed, being detected as a past infection only at the autopsy of birds which succumbed to other conditions.

This general picture changed dramatically during February to March 1991 when the first cases of IBD , caused by highly virulent strains, were diagnosed and within a few months the disease spread to numerous major poultry-producing areas of the country . Mortality rates have been variable but can be as high as 75% in layer type chickens and 25% in broilers, indicating a possibly lower susceptibility of the heavy breeds. The work presented herein was an investigation into some properties of a prototype of the " new " highly virulent viruses, IBDV strain G 2212/91 , and also an evaluation of selected live vaccine strains concerning their potential to protect chickens against the present situation of the disease.

Materials and methods

Eggs and chickens: Specific pathogen free (SPF) eggs were supplied by Lohmann (Cuxhaven, Germany). These were also the source of the SPF chicks used in this study.

Cell cultures- Primary chick embryo fibroblast (CEF) cell cultures were prepared from SPF embryonated eggs according to the standard methods . Vero , RK13 and Hela cell lines were additionally employed in parts of this work.

Viruses - The British standard serotype 1 IBDV , strain 52/70 (Baygrave and Faragher, 1970) and the cell culture- adapted strain G13 described by McFerran *et al.* (1980), were supplied by the Poultry Department, Central Veterinary Laboratory (CVL), New Haw, Weybridge, Surrey, U.K. G3372/81 was a standard serotype 1 IBDV strain previously isolated in this laboratory (Aghakhan *et al.*, 1994). A highly virulent IBDV strain, designated G2212/91, has been isolated from recent outbreaks of the disease . This strain was subjected to three cycles of limiting dilution technique in SPF embryonated eggs. A stock virus was prepared by inoculating 6-week-old SPF chickens intraocularly with the virus infected egg material. Mortality started three days post-inoculation and peaked on the fourth day. Bursae from the birds that had died and surviving chicks which were killed four days after inoculation were collected and made into a 20% (W/V) homogenate in PBS. The stock virus had an infectivity titre of $\log 10^{5.85}$ EID₅₀/ml. Chicken infective dose 50 (CID₅₀) of the stock virus was determined by inoculating each of the 5-week-old SPF birds, divided into groups of seven, with 0.05 ml. of serial 10 fold dilutions of the virus using eye-drop. Infectivity was assessed on the basis of the bursal lesions in birds that died and the surviving chicks which were killed on the 4th day post-inoculation. This gave an estimate of $\log 10^{4.32}$ CID₅₀/ml. For challenge purposes, 100 CID₅₀ of the G2212/91 strain of IBDV, inoculated intraocularly in 0.05 ml. doses was used.

Antisera - Monospecific antisera were prepared in SPF chickens as described elsewhere (Aghakhan *et al.*, 1994).

IBD vaccine strains- Gumboro vaccine A known as " mild " and vaccines B and C of the " intermediate " strains , were employed in this study. For the purpose of vaccination, these were usually administered in drinking water, unless otherwise stated , according to the manufacturer's recommendation. Infectivity titration, at the time of vaccination, showed that the vaccines contained the bird doses claimed by the producers.

Vaccine testing- The procedures followed were those outlined by the Ministry of Agriculture, Fisheries and Food (1990) Guidelines for the production and control of avian virus vaccines, British Veterinary Medicine Directorate, the British Pharmacopoeia (Veterinary 1985) , and the United States Department of Agriculture (1989) Code of Federal Regulations, Section 9, for testing the efficacy and safety of IBD live vaccines. A vaccine safety test based on antibody response to killed *Brucella abortus* antigen was carried out in these studies (Ministry of Agriculture, Fisheries and Food (1990) Guidelines for the production and control of avian virus vaccines; British Veterinary Medicine Directorate; D.H, Thornton , personal communication).

Serological tests:

The virus neutralization test (VN) - A cell culture, constant virus, decreasing serum dilution microtitre technique was employed. The G13 strain of IBDV described by McFerran *et al.* (1980) was used as antigen for the major part of this work. In addition, vaccine B and C virus strains were used for special purposes at very low passage levels in this laboratory. An antigen dose of 500 TCID₅₀ was considered and virus - serum mixtures were incubated at room temperature for 30 min. before inoculating a volume of 0.05 ml. into each of the microtitre plate wells. Following an absorption period of 75 min., the medium was dispensed in 0.15 ml. volumes and the microplates were incubated at 37°C in a humidified atmosphere. After 96 h., cell cultures were observed for CPE and end-points determined to be the reciprocal (Log₂) of the highest serum dilution which neutralized the virus. A positive IBDV control antiserum, calibrated against the British standard serum (BSS), was included in all VN tests .

The agar gel precipitation test: Preparation of antigen- SPF chickens, 5 weeks of age, each received 10² CID₅₀ of the G2212/91 strain of IBDV using eye drop. Bursae were removed from the birds that died of infection and the surviving chicks which were killed 4 days post-infection. These were homogenized in equal volumes of physiological saline and arcton 113 (Cullen and Wyeth, 1975; Wyeth and Chettle, 1988). The homogenate was

centrifuged at 1300 x g for 20 min. The supernatant was removed and stored in small aliquots at -50°C.

Test procedure- The agar gel consisted of 1% special Noble agar and 8% sodium chloride in distilled water, adjusted to a PH of 7.2. This was autoclaved at 10 lbs. For 15 min. Petri dishes were filled to a depth of 3 mm. and wells , with 6 mm. diameters and 3 mm. interspaces, punched out in a hexagonal pattern i.e. a central well and six peripheral wells. Plates were incubated at room temperature in a moist atmosphere for 48 h. Prior to reading the results in a dark room with an oblique light.

Histopathology- Bursae were removed and fixed in 10% neutral buffered formalin. Sections were stained with haematoxylin and eosin following the conventional methods. Lesions were graded 0 to 5 according to the criteria outlined by Muskett *et al.* (1979) :

0. No damage.

1. Mild necrosis in isolated follicles.

2. Moderate generalized lymphocyte depletion or isolated follicles with severe depletion.

3. Over 50 per cent of follicles with severe lymphocyte depletion.

4. Only outline of follicles remaining with few lymphocytes and increase in connective tissue , cysts and thickened corrugated epithelium.

5. Loss of all follicular architecture with fibroplasia.

Results

Clinical aspects and gross lesions- The first outbreaks caused by the highly virulent strains of IBDV were diagnosed in broiler farms where mortality rates up to 25% were reported. Subsequently the disease occurred in commercial replacement chickens causing mortality rates of up to 75%. The clinical signs, although not specific, were significantly more severe compared to those observed previously with the standard (classic) serotype 1 strains of the virus. There was also a higher incidence of haemorrhagic bursae and haemorrhages, particularly in the thigh muscles.

Characterization of a highly virulent IBDV isolate, strain G2212/91 , in SPF chickens *Susceptibility to the highly virulent IBDV* - Unlike the classic strains of the virus, the highly pathogenic strain caused about 10% mortality, even in 9-day- old SPF chicks. Mortality rates of 2%, 36%, 64%, 84%, 92% (the latter two age groups) were recorded for challenges carried out at 7,14,21,28,35 and 42 days of age, respectively.

The serological relationship of the G2212/91 strain to the standard serotype 1 IBDV's and some vaccine strains of the virus. The results of the cell culture microtitre virus neutralization tests are given in table 1.

Table 1- Serological relationship of the G2212/91 strain to the standard serotype 1 and some vaccine strains of IBDV in cell culture virus neutralization tests.

Antisera	Viruses		
	G13	Vaccine strain B	Vaccine strain C
G13	4096	6250	1750
Vaccine B	5600	5600	6250
Vaccine C	220	720	512
G3372/81	5600	4096	1575
52/70	1400	1650	2048
G2212/91	22100	5600	22100

Titres expressed as reciprocal of the final serum dilution which neutralized 500 TCID₅₀ of the virus

Using polyclonal antisera raised against the standard (classic) serotype 1 isolates, the highly virulent virus and the vaccinal strains of IBDV, cross neutralization was observed in all heterologous systems included in this work. In addition, cross reactivity was also observed in a series of VN tests performed in embryonated eggs, employing mainly the non-cell culture adapted strains (Table 2).

Table 2 - The results of virus neutralization tests in embryonated eggs

Antisera	Viruses			
	G13	G3372/81	51/70	G2212/91
G13	1100	670	775	450
G3372/81	1650	1400	1024	580
52/70	670	900	1575	512
G2212/91	2048	1100	1024	2800

Titres expressed as reciprocal of the final serum dilution which neutralized 500 TCID₅₀ of the virus

Growth in mammalian cell lines - There was no evidence of G2212/91 strain multiplying after eight passages in Vero, RK13 and Hela cell lines.

The protective potential of the live IBD vaccines against the highly virulent strains of the virus - The results of studies with three vaccine strains are shown in table 3.

Serology: Two weeks after vaccination, seroconversion, as detected by the AGP test, was found in 85% , 96% and 61% of the birds in vaccine A,B and C groups , respectively.

Mortality rates - All chickens vaccinated with the B and C strains survived challenge, while the vaccine A group of birds experienced a mortality of 20%.

Macroscopic lesions: No grossly visible lesions were observed in the birds vaccinated with strains B and C following challenge whereas 45% of the chickens in vaccine A group showed IBD bursal lesions.

Histopathology: Despite the full protection afforded by vaccines B and C against mortality and the occurrence of gross bursal lesions, microscopic lesions in the organ, generally mild to moderate in severity, could not be prevented by these strains. Comparing the potency of the two vaccines , it seemed that strain B conferred a higher level of immunity than strain C, as demonstrated by slightly more severe challenge - induced bursal lesions in the latter group.

Vaccine safety: Antibody response to *Brucella abortus* , used as antigen in these trials, was not significantly lower in the group vaccinated with the vaccine B strain at one day of age as compared to the control group.

Table 3 - The results of serological, pathological and histopathological studies with three IBD live vaccine strains in SPF chickens.

Vaccine strain	AGP tests		Mortality rates after challenge	Gross bursal lesions after challenge	Histopathological scores of the bursae after challenge					
	0	1			2	3	4	5		
A	0/20 ^a	16/20	4/20	9/20	-	-	2 ^b	8	5	1
B	0/26	25/26	0/26	0/26	2	13	10	1	-	-
C	0/31	19/31	0/31	0/31	-	4	19	8	-	-
Unvaccinated challenged control	-	-	9/11	11/11	-	-	-	-	1	1
Unvaccinated unchallenged Control	-	-	0/10	0/11	10	-	-	-	-	-

a) Number of positives/total tested

b) Number of chicks

Discussion

The dramatic change in the pathogenicity of the standard IBD viruses and the emergence of the new strains, causing much higher mortality rates in the field and also in SPF birds, was not mirrored in the results of cross-

neutralization tests, employing the highly pathogenic strain, the standard serotype 1 virulent viruses, the vaccine strains, and using polyclonal antisera. This probably indicates an antigenic drift in the neutralization sites on VP2 or perhaps the deletion of an epitope; yet such apparently minor variations exert great influence upon the pathogenicity of the virus. Alternatively, it can be argued that the great divergence of the relatively recent isolates, in terms of virulence, cannot be based solely upon these minor antigenic alterations. If this is true, it may be postulated that, in all possibility more crucial mutational changes have occurred in VP1 and /or VP4 (currently recognised as the viral RNA polymerase and protease, respectively), leading to some sort of modification or enhancement in the virus replication process. Van Den Berg *et al.* (1991) reported that their monoclonal 7C9 recognised an epitope on the mild vaccine strains examined which was missing on the intermediate and highly virulent viruses. However differentiation of the antigenic determinants belonging to the two latter types of strains remained unresolved. Considering that intermediate vaccines currently available are usually derivatives of the classic virulent viruses and the fact that they do not generally confer full protection against the highly virulent strains on a flock basis, it would be extremely valuable to define the neutralization sites on the representatives of these viruses. This is particularly important when attempting to select a vaccine strain and formulate a vaccination strategy. Including in vaccines the strains which exert minimum antigenic selection pressure upon the field virus population would be desirable.

Evaluation of the live IBD vaccines indicated that, taking into account mortality and gross bursal lesions as criteria, vaccines B and C, representatives of the so-called intermediate strains, afforded protection against the highly virulent IBDV strain in SPF birds. On the other hand, vaccine A, used as a mild strain, failed to prevent mortality and gross bursal lesions in all experimental birds. Even in the survivors extensive microscopic bursal lesions could be observed despite an absence of apparently visible damages. Surprisingly, our experiments showed that antibody response to the vaccine A strain was higher than that of the vaccine C strain. This might imply that cellular immune response also plays a role in conferring resistance to a challenge.

Overall evaluation of the vaccines B and C seemed to suggest that the former strain was superior as evidenced by its inducing a significantly higher immune response and also less severe histopathological lesions in the bursa following challenge. Although some vaccine strains derived from the classic IBD viruses can afford protection against mortality and even gross bursal lesions caused by the highly virulent strains, it seems there will always be a proportion of chicks with rather extensive microscopic damages

in the bursa, depending on the antibody levels in a population , though birds with very high titres may be an exception . From these findings it may be deduced that, with the live IBD vaccines currently available and under field conditions, there are few instances where relatively complete protection can be achieved in nearly all individuals within a flock.

The results of the serological tests, the pathological features of the infection in chickens as well as embryos, and the fact that vaccines derived from the classic strains can protect birds, at least against mortality and gross lesions, probably indicate that minor antigenic variations have occurred in the virus and the new strains can still be regarded as belonging to the standard serotype 1 IBD viruses. In this respect, our present situation is entirely at variance with situation existing in the USA, where variant strains have emerged as the result of major antigenic alterations i.e. an antigenic shift of the classic viruses , causing severe losses in some areas (Snyder *et al.*, 1988).

Until very recently, the general concept has been that IBD infections rarely cause clinical disease and mortality in very young chicks up to 3 weeks of age. Clinical signs and mortality were observed in SPF birds, less than 10 days of age, inoculated with the highly virulent strain. At present, the exact mechanism leading to this interesting finding is not understood. Whether different subpopulations of lymphocytes act as target cells for the classic and the more recently emerged highly virulent IBD viruses or whether the same subpopulation now expresses greater susceptibility to the latter strains, needs to be elucidated.

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