RURAL INDUSTRIES RESEARCH
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INFECTIOUS BURSAL DISEASE VIRUS (IBDV):

TO DETERMINE IF CURRENT VACCINATION STRATEGIES PREVENT THE EMERGENCE OF VARIANT IBDV STRAINS IN AUSTRALIA

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FOREWORD

This project has been carried out from July 1995 to June 1997 at the CSIRO Division of Animal Health, initially at the Animal Health Laboratory in Parkville and later at the Australian Animal Health Laboratory in Geelong.

Principal investigators were Drs Sandra Sapats and Jagoda Ignjatovic. Other personal that worked on the project were Ms Fiona Ashton, Som Spiess, Drs Peter Hooper and Stephen Prowse. Project leaders were Drs Ignjatovic & Prowse.

In addition to the funds received from CMRDC & EIRDC, CSIRO Division of Animal Health also provided financial, personal and laboratory support for the project.

The result presented in this report should be considered as confidential and are for restricted use within RIRDC until they have been published.

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CONTENT

ABREVIATIONS

B/B	Bursa/body ratio
CEF	Chicken embryo fibroblast
CID_{50}	Median chick infective dose
ELISA	Enzyme-linked immunosorbent assay
IBD	Infectious bursal disease or Gumboro disease
IBDV	Infectious bursal disease virus
Mabs	Monoclonal antibodies
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SN	Serum neutralisation test
SPF	Specific pathogen free
$TCID_{50}$	Median tissue culture infective dose

EXECUTIVE SUMMARY

Major Objectives of the Project:

(1) To determine if variant IBDV strains are present in Australia by comparing the VP2 proteins of field isolates to that of other IBDV's and vaccine strains.

(2) If variant IBDV strains are present, whether existing vaccination protocols provide protection.

Background & Method

The project was initiated in response to reports during 1995 that some commercial broiler flocks in Victoria and NSW were experiencing problems due to clinically more severe IBDV challenge. It was suspected at that time that variant strains of IBDV might be breaking through the usually protective level of maternal antibodies. We aimed to characterise these strains and determine if IBDV strains in Australia are changing in either antigenicity or pathogenicity. It was also the intention to compare local IBDV strains with those of other countries and if current vaccines provide protection against these recent field isolates.

Strains were characterised by:

(a) nucleotide sequencing to determine if there was a change in the VP2 protective antigen of IBDV;
(b) monoclonal antibodies and serum neutralisation tests to determine if they were antigenic variants;
(c) for severity of histopathological lesions in bursa to determine if there was an increase in strain virulence and
(d) by challenge of commercial broilers from two different sources to determine if chicks were protected by maternally acquired antibodies.

Detection of variant strains of IBDV in Australia

Six isolated field IBDV strains (five from Victoria and one from NSW) and three vaccine strains could clearly be separated into two groups of variant and classical strains. Five field strains from Victoria were all identified as antigenic variants by serum neutralisation tests, monoclonal antibodies and nucleotide sequencing.

The variants also differed from vaccine strains as determined by genetic analysis. All the variants induced histopathological lesions only in Bursae of Fabricius and had no effect on other tissues. All together the results indicate that antigenic variants are present in Australia. Furthermore variants appear to be restricted in distribution to the state of Victoria although this finding needs to be confirmed.

Lack of protection in broilers against challenge with variants

Cross-protection experiments using commercial broilers from two different commercial sources showed that some of the variants from Victoria could break through a level of maternal antibodies that are expected to be protective, and at flock age that would result in immunosuppression.

Comparison of local and overseas strains of IBDV

All Australian IBDV strains could clearly be differentiated from all other IBDV strains at the genetic level. It also appears that Australian IBDV strains are far more genetically diverse than are IBDV strains in other countries. The significance of this genetic diversity, particularly for the field situation, is presently not clear.

Analysis with Australian and USA monoclonal antibodies indicated that USA and Australian variants differ. Overall USA and Australian monoclonal antibodies have some predictive value for identification of antigenic variation in IBDV, however they must be used for discrimination of local strains rather than on a global basis. This is in agreement with the genetic differences detected among strains, since as discussed above, Australian and the USA variants are genetically distantly related.

Detection of antibodies by ELISA in commercial sera

Three commercial ELISA kits for measurement of IBDV antibody, KPL, TropBio and IDEXX were compared with SN and CSIRO ELISA tests. Overall, all three tests accurately detected antibody titres in the median range whereas low and high antibody titres were less well correlated with titres obtained by SN test and CSIRO ELISA. Choice of ELISA system for assessment of antibody titres in commercial flock will therefore have a different predictive value for sera obtained from broilers in comparison to sera obtained from broiler breeders and layers.

Conclusions and final recommendations

Results of nucleotide sequencing, antigenic analysis and challenge trails with IBDV strains isolated during 1995 have all indicated that true variant viruses are present at some operations in Victoria. This appears to be similar to the situation in the USA where antigenic variants have emerged. Current vaccination with strains of intermediate virulence seems to have prevented emergence of pathogenic variants but has possibly facilitated emergence of antigenic variants. We recommend that attention be paid to maintenance of high antibody titres in breeders to minimise virulent challenge of broilers at an early age. The risk of further selection of antigenic variants and exposure to virulent variant challenge be minimised by close attention to husbandry procedures.

CONFIDENTAL

INTRODUCTION

IBD has been endemic in poultry flocks around the world since 1960. It was controlled effectively up until recently by vaccination of parent flocks with live and inactivated oil emulsion vaccines. Progeny chicks from these dams had a high level of maternal antibodies and were protected from bursal damage and immunosuppression induced by field strains of IBDV. In 1985 variant IBDV strains emerged in the USA (Rosenberger *at al*., 1985). Variant strains did not induce overt clinical changes typical of classic IBDV. Instead, prolonged and repeated respiratory problems and poor performance followed early bursal atrophy. Vaccines developed for control of classical IBDV were not effective and variant vaccines were introduced (Snyder *et al*., 1992).

In 1987/88 vvIBDV strains capable of causing of between 30 to 70% of mortalities in broilers and layers were isolated in Holland, Belgium and the UK (Van Der Berg & Meulemans, 1991). Since then outbreaks of vvIBDV have occurred in most European countries as well as Africa, Japan, China and South East Asia. vvIBDV are able to break through maternal antibodies induced by classical, mild IBDV vaccines. For that reason, live vaccines of intermediate virulence have been introduced, as well as various vaccination strategies such as vaccination of broilers and replacement layer chicks by inoculation of live or inactivated oil emulsion vaccines (Claxton & McGavin, 1993; Goddard *et al*., 1994).

In Australia clinical IBD has been successfully controlled by vaccination of parent breeders and commercial layer pullets by priming with Bursavac live and boosting with inactivated Bursavac K or ALP 002/73 vaccines. The high level of maternal antibodies induced has been considered in most operations to be sufficient to protect broilers and commercial pullets against early field challenge. In recent times however, clinically more severe IBD has been seen in young birds with sufficient levels of maternal antibodies. Broilers with regressed bursae were observed between 22 and 34 days of age. Immunosuppression with increased incidence of respiratory disease and elevated mortalities were seen which could not be attributed to any other avian agent**.** It was suspected therefore that variant IBDV might be breaking through the usually protective level of maternal antibodies. Although only two strains, 002-73 and V877, have been available and characterised since the disease was first described in Australia in 1974, prevailing field strains were considered to be classical strains of low pathogenicity.

IBDV has four proteins, VP1, VP2, VP3 and VP4. The VP2 protein is the major host-protective antigen and a region of 130 amino acids in length (between amino acid residues 211 and 340) has been identified in VP2 that induces virus neutralising antibodies. Alterations in this region lead to antigenic variation in IBDV. Virus neutralising antibodies are protective and also differentiate IBDV strains isolated from chicks from those isolated in turkeys, being of serotype I and II, respectively. Within serotype I, IBDV strains can be differentiated into six subtypes, however only strains of the sixth subtype are “true” antigenic variants, as is the case with the USA variants (Jackwood & Saif, 1987).

OBJECTIVES

In this project we aimed to:

(1) Isolate and characterise IBDV strains from flocks with clinical IBD;

(2) Determine if IBDV strains in Australia are changing in either antigenicity or pathogenicity;

(2) Compare local IBDV strains with those of other countries; and

(3) Determine if current vaccines provide protection against these recent field isolates.

METHODOLOGY

Collection and origin of samples

Bursae and spleens were collected aseptically from chicks at between 22 and 32 days of age during an acute phase of disease. Although clinical disease varied, the common feature was that on post mortem all chicks had regressed bursae. Bursae were then collected from hatchmates, which had no signs of clinical disease. Samples were transported frozen to the laboratory, thawed and homogenised in an equal mixture of PBS supplemented with antibiotics and Arclone (fluorocarbon) as a 25% w/v suspension. After freezing and thawing, the suspension was clarified by centrifugation, the water phase collected and tested for the presence of IBDV antigen by ELISA (Fahey *et al*., 1991). Samples positive for IBDV antigen were then inoculated into 3 - 5 weeks old SPF chicks. Three days after infection bursae were collected, homogenised in PBS supplemented with antibiotics (25% w/v suspension) frozen and thawed three times and clarified by centrifugation. Virus stock was then tested for sterility and if contamination was detected, the suspension was passed through 45 μm filter. Virus stocks thus obtained were used for further characterisation. Vaccine strains, 002/73, Bursavac live and Bursavac K (V877 strain) were obtained from the vaccine manufacturer. They were propagated once in chickens, as described above, to obtain working virus stocks.

Virus titration in chickens, production of antisera and identification of virus purity

CID_{50} was determined in SPF chicks at between 3 - 5 weeks of age. Log 10 dilutions of virus stock was made in PBS supplemented with antibiotics and 0.1 ml of each dilution was given intra-ocularly to 15 chicks housed separately in positive pressure isolators. Three days after infection, 5 chicks were killed and weighed, and the bursae removed and weighed. From each bursa a 25% w/v homogenate was obtained as described above and tested for the presence of antigen by ELISA. At 10 days after infection, 5 chicks were removed and body and bursa weight determined. Remaining five chicks were bled at four weeks after infection, sera collected, pooled and stored at -20^0C. The sera represented strain specific sera and each was tested for the freedom of antibodies to other avian pathogens by the protocols used to ascertain SPF flock status.

Cross-protection in SPF chicks

Groups of 20 SPF chicks, each housed in positive pressure isolator, were inoculated at 3 weeks of age with 100 CID_{50} of the 002-73 strain. At 4 weeks after vaccination, all chicks were bled from the wing vain, sera collected and IBDV antibody titres determined in ELISA (Fahey *et al*., 1985). At 4 weeks after vaccination chicks in each group were challenged with 1,000 CID_{50} of each of six isolated field IBDV strains or 002-73 strain. Three days after infection, bursa were

collected, homogenised as described above and tested for the presence of IBDV antigen by ELISA.

Assessment of cross-protection in commercial broilers

Broiler breeder fertile eggs, obtained from local hatcheries, were sprayed with solution of Chickguard® and set for hatching at the CSIRO premises. Following hatching, day old chicks were placed into positive pressure isolators, 25 chick per isolator, and reared under brooding light until 7 days of age. Water and irradiated feed were given *ad lib*. At the time indicated and just before challenge, all chicks were bleed from either jugular or wing vein, sera collected and antibody titres determined in an ELISA. At this time 5 chicks were removed from each group, bursa and body weight determined and bursae stored at -20^{0}C. Chicks in each group were individually infected by intra-ocular inoculation of 1,000 CID_{50} of a challenge virus in 0.1 ml. At 3 days after challenge, 10 chicks were removed from each group, bursa collected, body and bursa weighed and 25% bursal homogenate made as indicated above. The presence of challenge virus was assayed in each bursa by ELISA. At 14 days after infection, remaining 10 chicks were killed, bursa and body weight determined and bursae placed in 10% buffered formalin for histopathological evaluation.

ELISA for IBDV antigen and antibody

Presence of IBDV antigen was determined using an ELISA as described previously (Fahey *et al*., 1991). A CSIRO ELISA, referred to as the antibody ELISA, was used to determine antibody titres in all sera (Fahey *et al*., 1985). End point titres were determined for each sera using $\log_2$ dilutions. Antibody titres were also determined using three commercial antibody ELISA kits, IDEXX, KPL and TropBio. These were obtained from the manufacturers and titres determined according the procedure specified for each kit, using a single dilution of sera.

Monoclonal antibodies (Mabs)

Mabs designated 9-6, 39A, 44-18 and 17-82 are directed against at least two different epitopes on the VP2 antigen. These have been developed in Australia using 002-73 strains and described by Fahey *at al*. (1991). Mabs 17-82, 39A and 9-6 all neutralise virus infectivity *in vitro* and Mabs 17-82 and 39A also protected chickens against challenge with 002-73 strains when given intraperitonealy (Fahey *at al*., 1991).

Mabs R63, B29, BK/9, 57, 67, 44AI and 8, are also directed against different epitopes on the VP2 antigen. These have been developed using the USA classical and variant IBDV strains (Snyder *et al*., 1992). The Mab 21 was also developed by Snyder (unpublished observation) which presumably recognised only vvIBDV strains and thus was directed against vvIBDV specific epitope.

Virus pathogenicity

Groups of 25 SPF chicks, 3 weeks of age, housed in a positive pressure isolators, were inoculated with 1,000 CID_{50} of virus, in 0.1 ml of PBS. At 1, 3, 5, 7, 10 and 14 days after infection, 3 randomly selected chicks were killed, weighed, bursa removed and weighed and placed into 10% buffered formalin. Thin sections were made from each bursa, stained with haematoxylin and eosin and examined for lesions typical of IBDV. Lesions were rated on scale 0 - 4, for no lesions, mild, prominent, severe and extensive lesions, respectively.

Nucleotide sequencing

The gene coding for the VP2 antigen of a number of IBDV strains, including the 002-73 strain, has been previously sequenced (Hudson *et al*., 1986.) From these sequences two regions were identified that were identical in all IBDV strains and are located in the VP2 gene just before and after the protective 211 - 340 region. Primers identical to these two regions were then made and used in PCR to amplify the 211 -340 region in the VP2 of all isolated field IBDV strains, as well as vaccine strain 002-73, Bursavac live and Bursavac K (V877). cDNA that was obtained was then sequenced, translated into the protein (amino acid) sequence and the results obtained compared for all strains. A computer assisted program was then used to analyse genetic relationship of strains and to construct phylogenetic trees.

Serum Neutralisation tests

To determine if isolated IBDV strains are of the same subtype within the serotype I, a SN test was performed in CEF. For this test an IBDV strain needs to be adapted for growth and production of a cytopathic effect in cell culture that is visible as plaques. GT101 strain, which is an Australian cell culture adapted strain already in existence, was used as a representative of classical IBDV. Strain 02/95TC strain was used as a representative of variant strains. It was obtained after two passages of 02/95 strain in CEF (see below). Each sera, in $\log_2$ dilutions, was incubated with between 100 and 500 $TCID_{50}$ of either GT101 or 02/95TC and assayed for plaque inhibition in secondary CEF as described by Fahey *et al*. (1991). Relatedness between viruses was determined using formula of Archetti & Horsfall (1950). An index of 1 indicates that a virus is of the same subtype, whereas an index of 0.4 or less indicates that two viruses are of a different subtype.

Tissue culture adaptation of IBDV strains.

In the past it has proven to be very difficult to obtain tissue culture adapted IBDV strains. Isolated IBDV strains were passaged in cell culture by infection of freshly trypsinized primary and secondary chicken embryo fibroblast with high virus dose. After incubation at 37^0C for 5 - 7 days cells were collected, freeze/thawed and used to infect freshly seeded cells. Each virus was passaged in CEF in such a manner by at least 5 times. In addition to CEF, continuous cell lines BHK and Vero were also used.

RESULTS

Detection of antigenic variants of IBDV in Australia

Six field IBDV strains were isolated during 1994/95 from broilers at four locations in Victoria and at one location in New South Wales (Table 1). Five strains isolated in Victoria (01/94, 02/95, 03/95, 04/95 and 08/95) were antigenic variants when typed by Mabs generated against vaccine strain 002/73. They all lacked two Mab epitopes, 39A and 44-8, present on VP2 antigen of classical vaccine strains 002-73, Bursavac live and Bursavac K. The NSW isolate 06/95 on the other hand was similar by Mab typing to the vaccine strains and had all four Mab epitopes.

Table 1. Origin of IBDV strains and their reaction with Australian Mabs directed against the VP2 antigen

Origin of strains		Isolation		Strain	Reaction with Mab			
State	Farm	Date	Flock age (days)	designation	17-82	39A	44-8	9-6
Victoria	Ga	17/10/94	34	01/94	+	-	-	+
	Gc	4/3/95	29	02/95	+	-	-	+
	Pr	22/5/95	34	03/95	+	-	-	+
	Rd	18/6/95	22	04/95	+	-	-	+
	Gc	28/9/95	32	08/95	+	-	-	+
NSW	Tw	6/7/95	28	06/95	+	+	+	+
	Tw	6/7/95	28	07/95	+	+	+	+
Vaccine Strains				002/73	+	+	+	+
				Bursavac live	+	+	+	+
				Bursavac K	+	+	+	+

By SN test, all Victorian isolates were of one subtype whereas the three vaccine strains and the NSW field strain 06/95 were of a different subtype (Table 2). In the SN test, antisera specific for each of five variants isolated in Victoria had significantly lower SN titres with classical strain GT101 (SN titres were between 4,000 and 32,000) in comparison to SN titres with the variant strain 02/95TC (SN titres were between 64,000 and 128,000). The relatedness index calculated from these titres, indicates that all variants are of one subtype whereas vaccine strains and NSW isolates were related and were of a different subtype. It must be noted however that for the relatedness index, titres for some heterologous reactions were presumed to be the same as they would be for homologous reaction. For example titres of 03/95 antisera were 128,000 in the heterologous reaction with 02/95TC strain (Table 2). It was then presumed that titres of 03/95 antisera would also be 128,000 for the homologous reaction with 03/95 strain. This approach was necessary as we were able to adapt only one strain, 02/95, to grow in cell culture. Other strains failed to grow in any of the cell lines or CEF, after repeated passages. It is therefore possible that additional antigenic differences existed between variants or classical strains, however, these could not be detected due to inability of the majority of local strains to be adapted for growth in cell culture.

Characterisation of variants by nucleotide sequencing

Molecular analysis also confirmed that the variants isolated in Victoria differed from vaccine strains (Figure 1). All antigenic variants had a number of amino acid substitutions in the protective VP2 antigen in comparison to the vaccine strains 002-73 and Bursavac live. Importantly, amino acid changes were detected in the two regions previously correlated with induction of virus neutralising antibodies and protection (the two regions are boxed in Figure 1).

Table 2. Virus neutralisation titres of sera with classical GT101 and variant 02/95TC strains

Antisera to	Strain GT 101$^{\alpha}$	Strain 02/95TC$^{\alpha}$

IBDV strain	SN titres[β]	RI[χ]	SN titres[β]	RI[χ]
002/73	64,000	1.0	8,000	0.18
Bursavac live	16,000	1.0	4,000	0.35
V877	16,000	1.0	8,000	0.35
06/95	16,000	1.0	8,000	0.35
01/94	4,000	0.08	64,000	1.0
02/95	32,000	0.18	**128,000**[δ]	1.0
03/95	16,000	0.13	128,000	1.0
04/95	8,000	0.13	64,000	1.0
08/95	4,000	0.18	16,000	1.0

[α]GT101 and 02/95 strains adapted for growth in CEF.
[β]Reciprocal of the last dilution of sera that neutralised between 100 - 500 $TCID_{50}$ of virus.
[χ] RI = relatedness index calculated according to the formula of Archetti & Horsfall (1950). Homologous RI is 1.0
[δ]Homologous titre.

The VP2 antigen of 002-73 and Bursavac live were identical. The VP2 of Bursavac K (V877) had only one different amino acid at the position 261 (result not shown). Strain 06/95 from NSW, which was antigenically identical by Mab and the SN test to vaccine strains, differed genetically from them by only two amino acids (Figure 1). Hence the result of nucleotide sequencing agrees with the antigenic analysis confirming also that strains isolated in Victoria are variants.

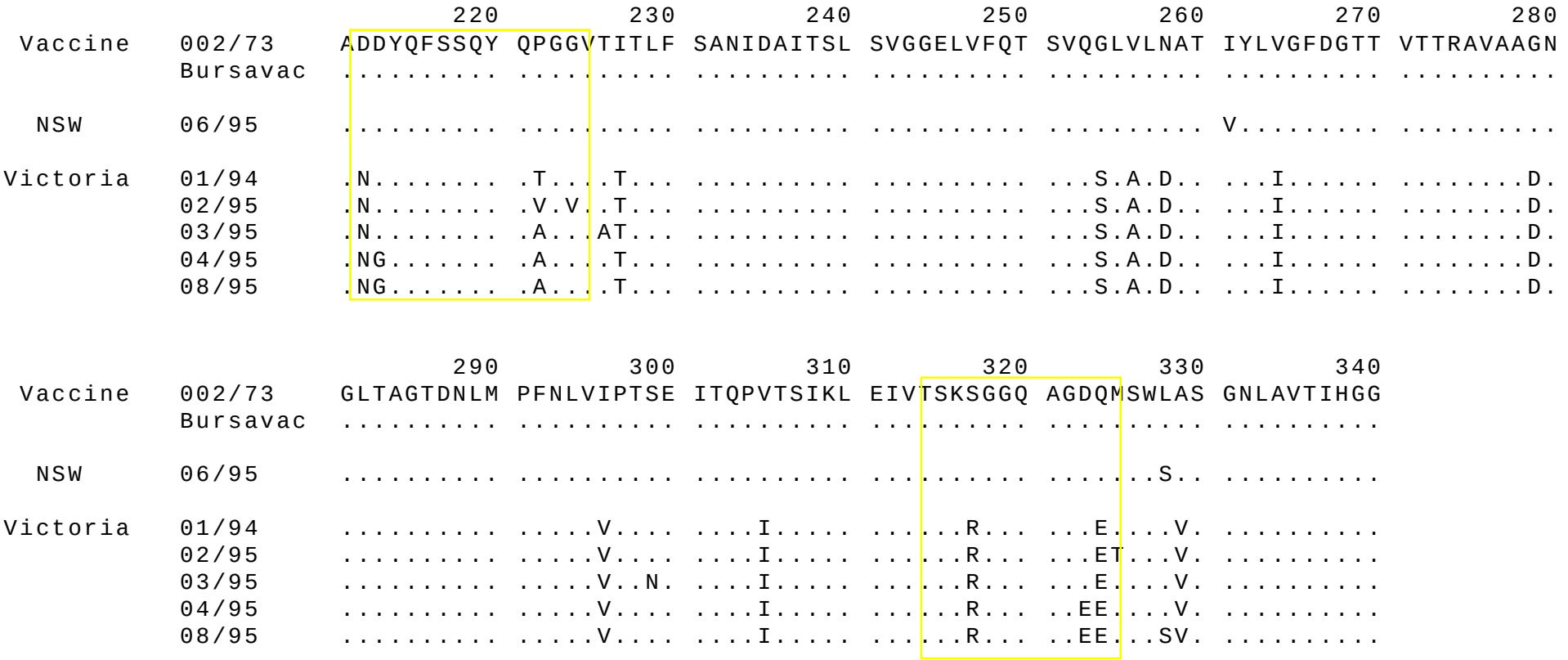
Pathogenicity of isolated field IBDV strains for chicks

Initially all isolated IBDV strains were titrated in chicks to

(i) determine if all strains induced bursal regression,
(ii) determine the CID_{50}, and
(iii) establish if a correlation exists between detection of virus antigen in bursae at 3 days after infection by ELISA and regression of bursae (Table 3).

As shown, all strains caused bursal regression at the limiting dilution of only 1 CID_{50}. For 01/94, 03/95 and 06/95 strains, the same virus titres and CID_{50} were obtained using either antigen detection in bursa at 3 days after infection or B/B ratio at day 10 after infection. B/B ratio was about 10 times more sensitive in the case of 02/95, 04/95 and 06/95 strains.

Figure 1. Comparison of amino acids in VP2 for Australian strains



		220	230	240	250	260	270	280
Vaccine	002/73	ADDYQFSSQY	QPGGVTITLF	SANIDAITSL	SVGGELVFQT	SVQGLVLNAT	IYLVGFDGTT	VTTRAVAAGN
	Bursavac	....	....	....	....	....	....	....
NSW	06/95	....	....	....	....	....	V.........	....
Victoria	01/94	.N........	.T....T...	....	....	...S.A.D..	...I......	........D.
	02/95	.N........	.V.V..T...	....	....	...S.A.D..	...I......	........D.
	03/95	.N........	.A...AT...	....	....	...S.A.D..	...I......	........D.
	04/95	.NG.......	.A....T...	....	....	...S.A.D..	...I......	........D.
	08/95	.NG.......	.A....T...	....	....	...S.A.D..	...I......	........D.

		290	300	310	320	330	340
Vaccine	002/73	GLTAGTDNLM	PFNLVIPTSE	ITQPVTSIKL	EIVTSKSGGQ	AGDQMSWLAS	GNLAVTIHGG
	Bursavac	....	....	....	....	....	....
NSW	06/95	....	....	....	....	.......S..	....
Victoria	01/94	....	.....V....	....I.....	.....R....	...E....V.	....
	02/95	....	.....V....	....I.....	.....R....	...ET...V.	....
	03/95	....	.....V..N.	....I.....	.....R....	...E....V.	....
	04/95	....	.....V....	....I.....	.....R....	..EE....V.	....
	08/95	....	.....V....	....I.....	.....R....	..EE...SV.	....

Six isolated field strains (01/94, 02/95, 03/95, 04/95, 08/95, 06/95) and two vaccine strains 002-73 and Bursavac live were compared for their pathogencity in bursae in SPF chickens (Table 4). As expected, none of the strains induced mortalities following inoculation of 10,000 CID_{50} into 3 week old chicks. All the strains replicated only in bursa and spleen and antigen was not detected either in thymus or caecal tonsils (results not shown). All variants also induced histopathological lesions in bursae only and had no effect on other tissues such as thymus, caecal

Table 3. Correlation between the presence of antigen in bursa and bursa/body weight ratio following infection of 2 week old SPF chicks with field and vaccine strains

Virus strain	Virus dilution[α]	No of bursae positive for antigen/ No tested[β]	Range of B/B weight ratio (mean)[χ]	No of bursa regressed/ No tested[δ]
01/94	10^{-7}	0/5	2.8 - 4.9 (4.2)	0/10
	10^{-6}	5/5	1.4 - 2.9 (1.9)	10/10
02/95	10^{-6}	0/5	3.2 -7.1 (5.9)	0/8
	10^{-5}	0/5	2.4 - 3.5 (2.7)	9/9
	10^{-4}	5/5	1.3 - 2.9 (2.0)	9/9
03/95	10^{-7}	0/5	3.8 - 5.0 (4.4)	0/9
	10^{-6}	5/5	0.9 - 2.5 (1.8)	9/9
04/95	10^{-7}	0/5	1.0 - 5.9 (5.0)	1/9
	10^{-6}	0/5	1.4 - 2.6 (1.9)	9/9
	10^{-5}	4/5	1.2 - 1.9 (1.6)	9/9
06/95	10^{-7}	0/5	4.8 - 5.9 (5.2)	0/10
	10^{-6}	5/5	0.9 - 2.2 (1.6)	10/10
08/95	10^{-6}	0/5	3.4 - 4.9 (4.2)	0/10
	10^{-5}	0/5	1.5 - 2.0 (1.8)	8/8
	10^{-4}	3/4	1.3 - 2.3 (1.8)	8/8
BV live	1 dose	5/5	1.2 - 2.1 (1.6)	5/5
	0.1 dose	5/5	1.1 - 2.7 (1.7)	5/5
002-73	10^{-6}	0/5	2.7 - 5.1 (3.6)	3/8
	10^{-5}	0/5	1.9 - 3.4 (2.7)	8/9
	10^{-4}	4/5	2.0 - 6.9 (3.1)	7/9

[α]Virus, in 0.1 ml inoculated intra-ocularly to 15 chicks.
[β]Determined by an antigen ELISA in bursae collected at 3 days after infection.
[χ] B/B ratios determined at 7 days after infection.
[δ]Those with B/B ratios that are bellow the mean B/B of non-infected controls.

tonsils or bone marrow (Table 4, results for bursae only shown). Severity of bursal lesions induced by variants was similar to those induced by the 002/73-vaccine strain. Maximum lesion scores of 4 were evident at 3 - 4 days after infection and recovery and bursal repopulation began at between 9 and 14 days after infection. The NSW strain 06/95 caused more severe lesions in the bursa that were evident from day 3 until day 14 after infection and no recovery of bursae was observed during this time.

Table 4. **Histopathological lesions in bursae following infection of 2 week old SPF chicks with 1,000 CID_{50} of field and vaccine strains**

Days after infection	Lesion score in bursa following challenge with IBDV strain[α]								
	002-73	Bursa-vac	01/94	02/95	03/95	04/95	08/95	06/95	None
1	0	0	2	2	0	0	0	1	0
2	3	2	4	3	2	2	3	4	0
3	4	4	3	4	4	3	3	4	0
4	4	4	3	4	4	4	4	4	0
7	4	3	3	2	3	3	4	4	0
9	4	2	3	4	2	2	2	4	0
14	2	3	1	3	3	1	2	4	0

[α]Each lesion score is the average of the score from three bursae.

Lack of protection against challenge with antigenic variants in broilers

Cross-protection experiments with broilers from two different commercial sources showed that three of the five antigenic variants from Victoria and also the strain 06/95 from NSW can break through a level of maternal antibodies that was expected to be protective (Figure 2 - 5 and Table 5).

The variants 01/94 and 04/95 overcame a high titre of maternal antibodies in chicks at 12 days of age and challenge virus was detected by ELISA in bursa 3 days after infection (Figure 2a). Histopathological lesions and bursal regression were also evident in these chicks 14 days after challenge (Figure 2b). At this time chicks were protected against challenge with variants 03/95 and 08/95 as well as classical vaccine strain 002-73 (Figure 2a and 2b).

The variant 02/95 was more virulent and overcame high level of maternal antibodies in chicks as young as 7 days when 20% of challenged chicks had virus detectable in bursa 3 days after challenge (Figure 3). In chicks challenged at 12 days of age virus was detected in 50% of chicks. Histopathological lesions and bursal regression were also evident in greater than 70% of chicks in both groups at 14 days after infection (results for B/B ratios and histopathological lesions not shown). In contrast, chicks only became susceptible to challenge with strain 002/73 at 21 days of age whereas they were resistant to challenge at 12 and 17 days of age (Figure 3).

Figure 2. Percentage of commercial chicks susceptible to challenge with field and vaccine strains of IBDV

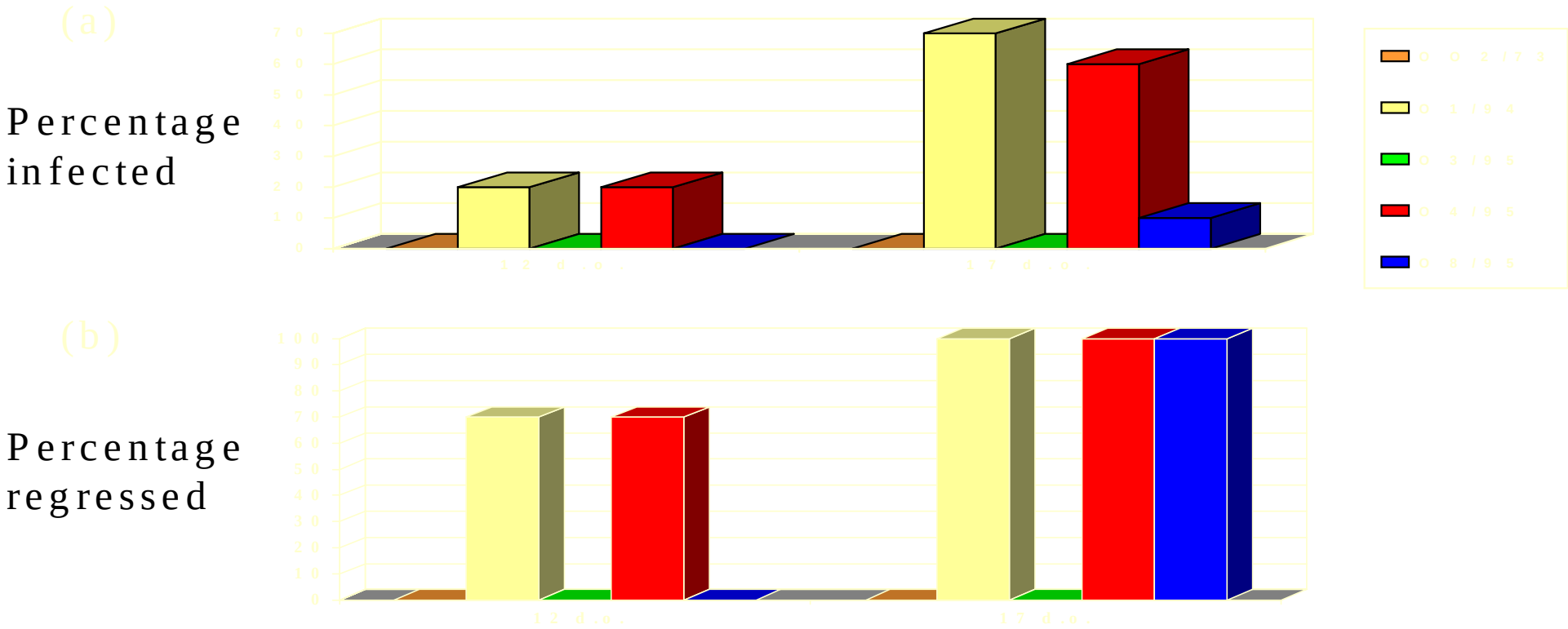
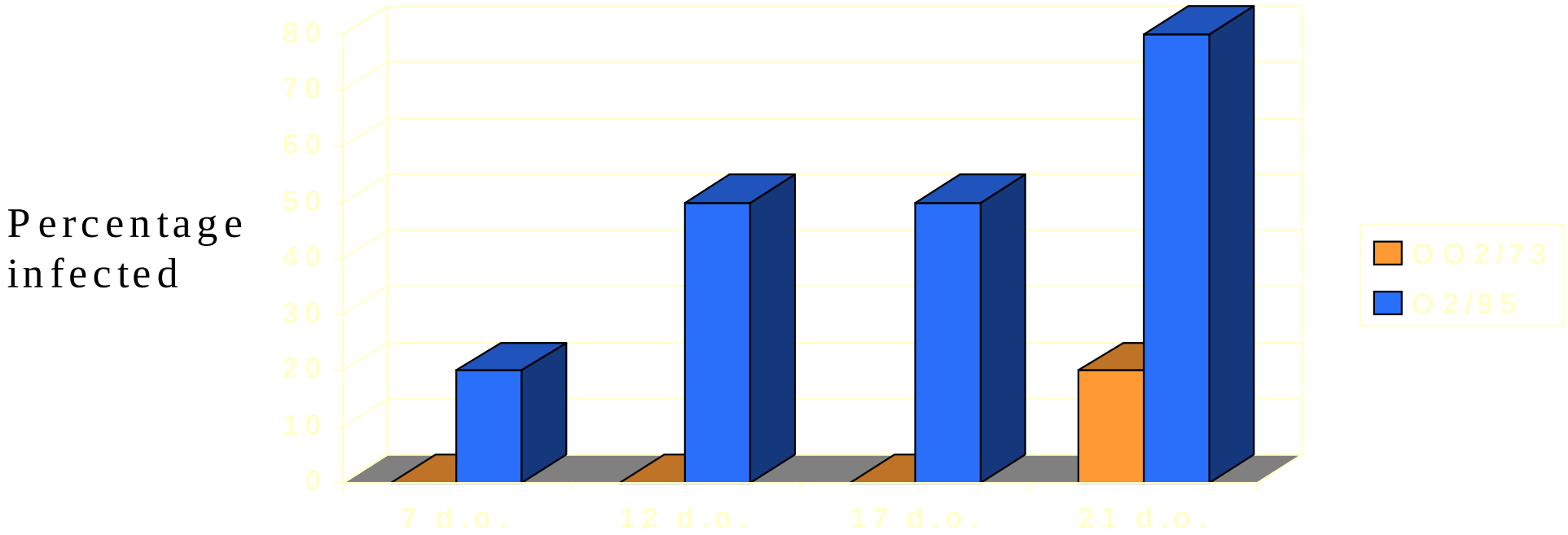


Figure 3. Susceptibility of Commercial Chicks to challenge with variant 02/95



Comparison of ELISA antibody titres in chicks challenged with 002-73 and 02/95 strains show that variant 02/95 can break through ELISA titres of 2,000 whereas 002-73 strains can break through titres of 400 (Figure 4).

In Table 5 SN titres of chicks that were susceptible to challenge with 02/95 strains, against classical GT101 and variant 02/95TC strains are compared. The result indicates that all chicks, that did not resist challenge with variant 02/95, had low SN titres against 02/95 strain whereas SN titres against classical GT101 strain were high. The result therefore indicates that at least some of the Victorian isolates are virulent variant strains, that can break through maternal antibodies in young chicks acquired by vaccination of dams with classical vaccine strains. This situation is therefore analogous to the USA variants.

Table 5. Virus neutralisation titres against classical Gt101 and variant 02/95TC strains in sera of commercial broilers susceptible to challenge with 02/95 strain

Chick number	Age at challenge in days[δ]	Titre[α]		
		ELISA[β]	GT101[χ]	02/95TC[χ]
305-1	12	2,000	3,200	100
305-7		8,000	6,400	400
306-8	17	1,000	1,600	>50
322-1	21	800	400	>50
322-5		400	800	>50

[α]Reciprocal of the last dilution giving a positive reaction.
[β]Using classical 002-73 strain as antigen.
[χ]Determined in SN test using between 100 and 500 $TCID_{50}$ of virus.
[δ]Commercial broilers challenged with 1,000 CID_{50} of 02/95 strain of IBDV

Classical 06/95 strain was also able to break through antibody levels at 17 days of age and which are expected to be protective (Figure 5). This result can be possibly explained by the more virulent nature of 06/95 strain, although this conclusion must be confirmed.

Comparison of local and overseas strains of IBDV

Australian strains were compared at the antigenic and genetic level with IBDV strains from other countries.

Mabs developed against the USA variant and classical strains and held at Intervet International B.V., Holland, were used to type Australian IBDV strains. Australian Mabs, in addition, were tested for their cross-reaction with overseas strains in order to confirm their value in predicting antigenic variation in field strains. Some interesting differences and features unique to local isolates were identified (Table 6).

Figure 4. ELISA antibody titres in sera of chicks susceptible to challenge with 02/95 and 002/73 strains

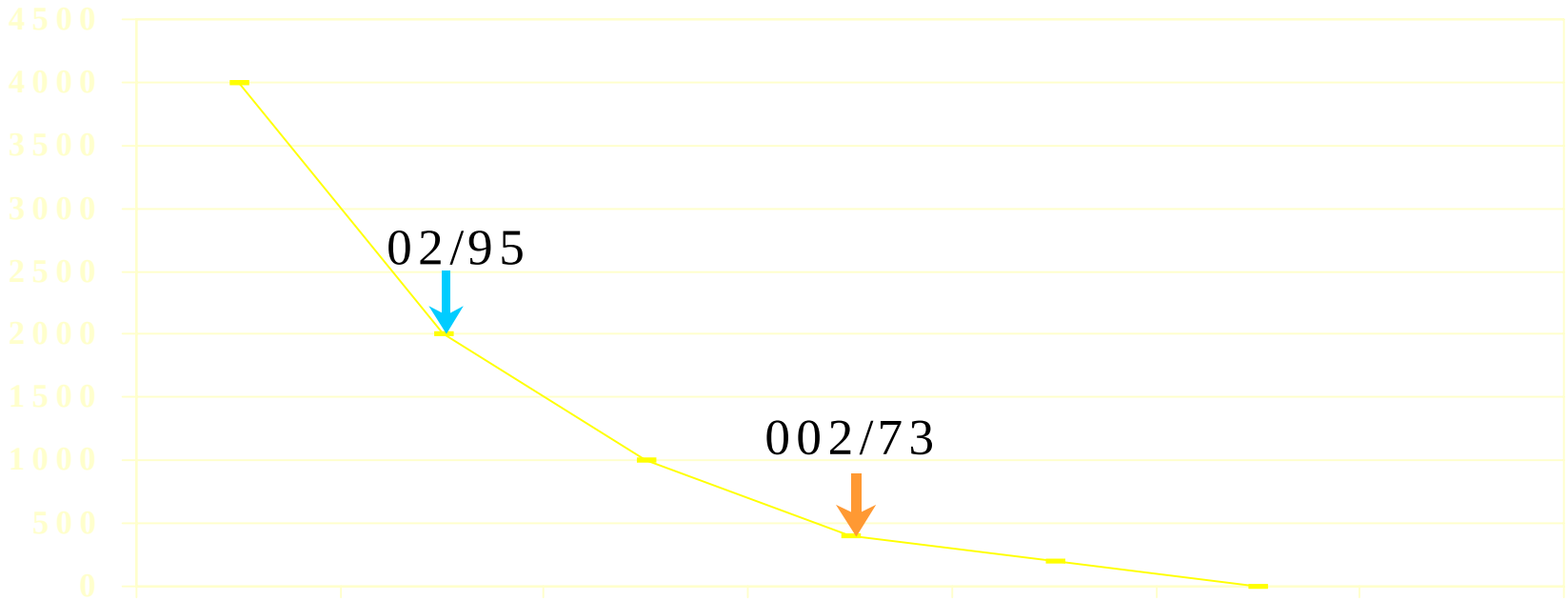
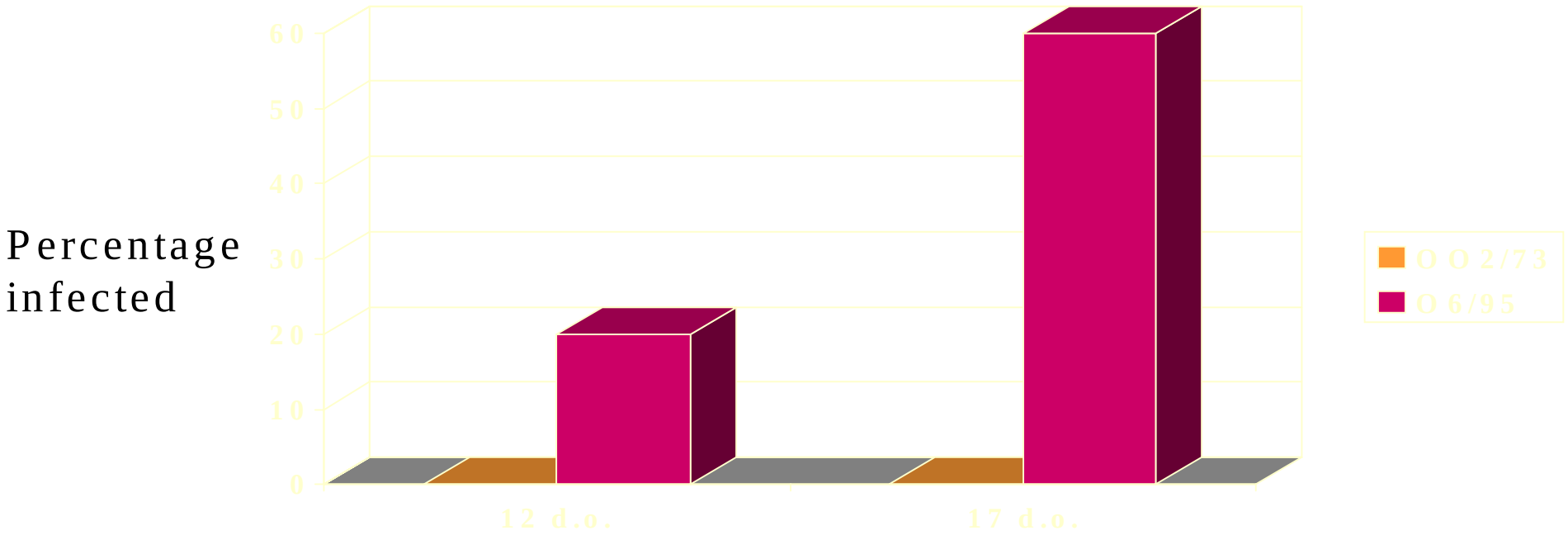


Figure 5. Susceptibility of commercial chicks to challenge with 06/95 classical strain



Using the USA Mabs, all Australian IBDV strains were antigenically identical with all other classical IBDV strains. Mabs B29, R63 BK/9, 44A1 and 8 reacted with all Australian strains as they did with the pathogenic 52/70 strain. Two Mabs, 67 and 57, that are specific for the USA variant E and GLS, respectively, did not react with any of the Australian strains, indicating that Australian and the USA variants differ. Australian Mabs on the other hand, detected antigenic differences among European and USA classical strains and did not react with any of the USA variants. Overall, Australian and the USA Mabs both detected antigenic variations in IBDV strains; however, antigenic changes detected were not the same. This can be expected because Australian and the USA strains differ genetically, as outlined bellow.

Comparison of the protective VP2 region of Australian and IBDV strains from other countries at the molecular level are shown in Figures 6 and 7.

Comparison of Australian and the USA variants shows that all variants have amino acid changes in the two regions which are considered the most important for virus neutralisation and protection (in Figure 6, two regions are boxed). The changes however are not identical, and Australian variants appear to differ more from Australian vaccine strains than do the USA variants from their vaccine strains. This however might not be of importance, as the protective region of VP2 is highly conformation dependent and not all changes have the same functional implications.

Comparison of Australian and other classical and vvIBDV strains showed that Australian strains have a unique amino acid sequences and that they differ from all other IBDV strains (Figure 7). Notably all Australian stains differ in the region 328 - 322 from other classical strains in that they lack 2 serine residues (S) which are present in all pathogenic classical strains. Existence of the four serine residues has been correlated with the higher pathogencity of overseas classical strains. Notably NSW strain 06/95 is the only Australian strain, which has an extra serine residue. Whether this change is associated with the increased virulence of 06/95 is currently uncertain.

A comparison of the Australian and all other IBDV strains using the nucleotide sequences of the VP2 protein and a computer program that analyses their genetic relationship (phylogenetic analysis) are shown in Figure 8. The USA variants and the majority of European and USA classical strains, are genetically closely related and all belong to the same genetic group (group I). Very virulent IBDV and the SalII intermediate strain belong to group II. However group I and group II strains are still genetically close relatives, differing only in small number of amino acids. All Australian classical strains, vaccines 002-73, Bursavac live and Bursavac K and the NSW 06/95 isolate are in group III, whereas all other Australian isolated variants belong to a separate genetic group (IV). Genetic differences between Australian strains in group III and IV are considerable with many amino acid changes and consequently strains in group III and IV are genetically distant relatives. The significance of this genetic diversity, particularly for the field situation, is presently not clear.

Detection of antibodies by ELISA in commercial sera

Currently, three commercial ELISA kits, KPL, TropBio and IDEXX are available for measurement of IBDV antibody titres. Two of these kits are imported and are based on antigen that differs from that used in the locally developed kit. We compared the efficacy of these ELISAs with SN and CSIRO ELISA tests (Table 7). Each of the three commercial ELISAs performed differently. As shown, titres obtained in ELISA correlated well with SN titres. Antibody titres detected by IDEXX, KPL and TropBio ELISAs correlated well with SN titres

Table 6. Epitopes detected by Mabs on IBDV strains isolated in the USA, Europe and Australia

	Mabs developed against the USA strains*								Mabs developed against Australian 002/73 strain					
Strain	**B29**	**B69**	**R63**	**BK/9**	**67**	**57**	**44A1**	**8**	**17-82**	**3-1**	**39A**	**33-10**	**44-18**	**9-6**
Testing in the USA														
BursaVac3	+	NT	NT	NT	NT	NT	NT	NT	+	-	0	+	+	+
Aphis	+	NT	NT	NT	NT	NT	NT	NT	+	+	+	+	+	+
Univax	+	NT	NT	NT	NT	NT	NT	NT	+	+	-	+	+	+
Arkansas	+	NT	NT	NT	NT	NT	NT	NT	+	+	+	+	+	+
GLS-5	+	NT	NT	NT	NT	NT	NT	NT	-	-	+	-	-	-
Georgia	+	NT	NT	NT	NT	NT	NT	NT	-	-	+	+	-	+
2512	+	NT	NT	NT	NT	NT	NT	NT	+	-	-	+	+	+
Mississipi	+	NT	NT	NT	NT	NT	NT	NT	+	+	+	+	+	+
Variant E	+	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-
Variant A	+	NT	NT	NT	NT	NT	NT	NT	+	-	0	+	-	+
Testing in the Netherlands														
F52/70	+	0/-	+	-/+	-	-	+	+	+	-/+	-/+	+	+	+
Variant E	+	-	+	+/-	+/-	-	+/-	+	-	-	-	-/0	-/0	-/0
GLS	+	-	-	-	-	+	+	-	-	-	-/0	-	-	-
DS326	+	-	ND	ND	-	+	+	+	-	-	-	-	-	-
RS593	+	-	ND	ND	+	-	-	+	-	-	-	-	-	-
002/73	+	-	+	-/+	-	-	+	+	+	0/+	0/+	+	+	+
V877	+	0/-	+	-/+	-	-	+	+	+	0/+	0/+	+	+	+
01/94	+	-	+	-/+	-	-	+	+	+	-	-	+	-	+
02/95	+	-	+	-/+	-	-	+	+	+	-	-	+	-	+
03/95	+	-	+	-/+	-	-	+	ND	+	-	-	+	-	+
06/95	+	+/-	+	-	-	-	ND	+	+	-	-	+	-	+

***Mabs developed by Snyder et al., 1989 and Fahey et al., 1991.**
NL/Aus (only shown when differences between two test systems}

Figure 6. Comparison of Australian IBDV strains with USA variants

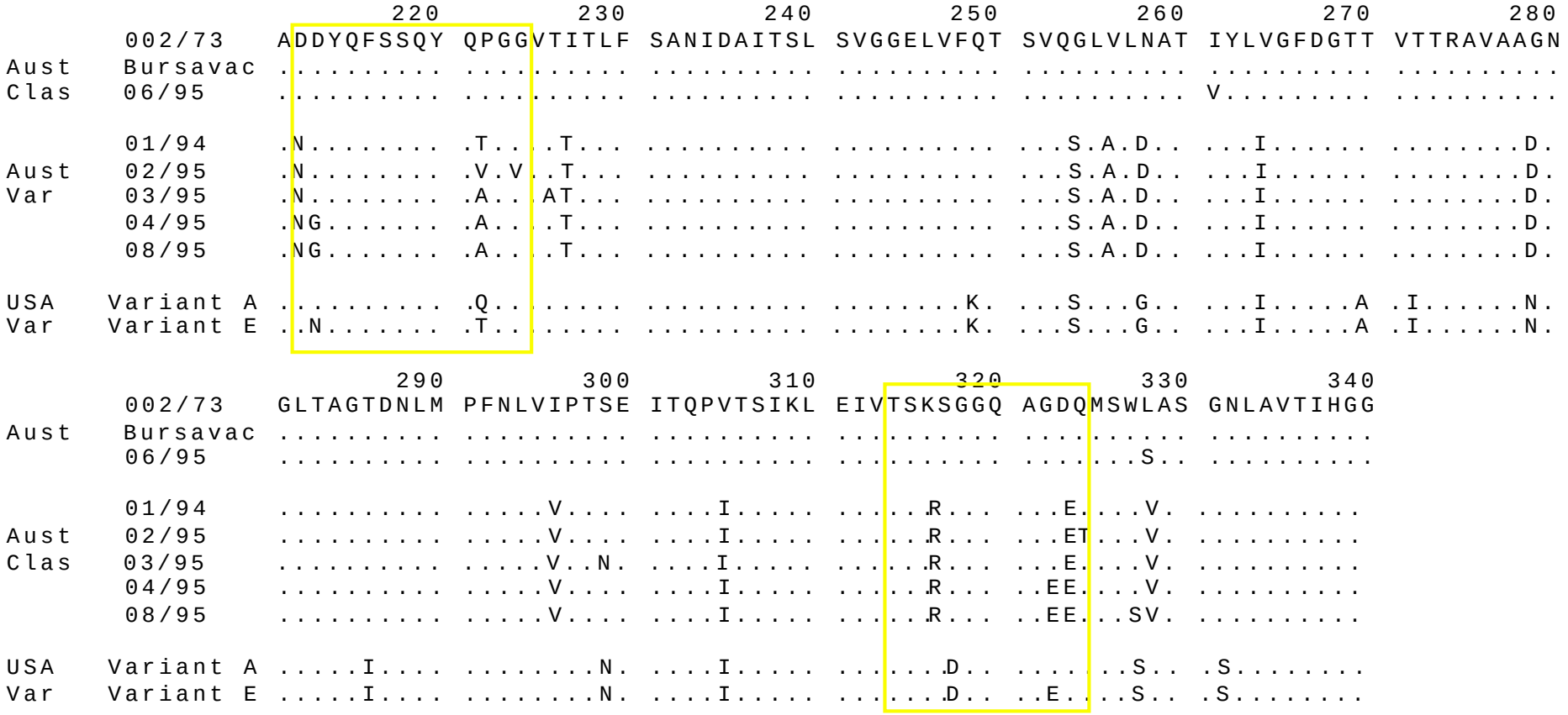


Figure 7. Comparison of Australian and other classical vvIBDV strains

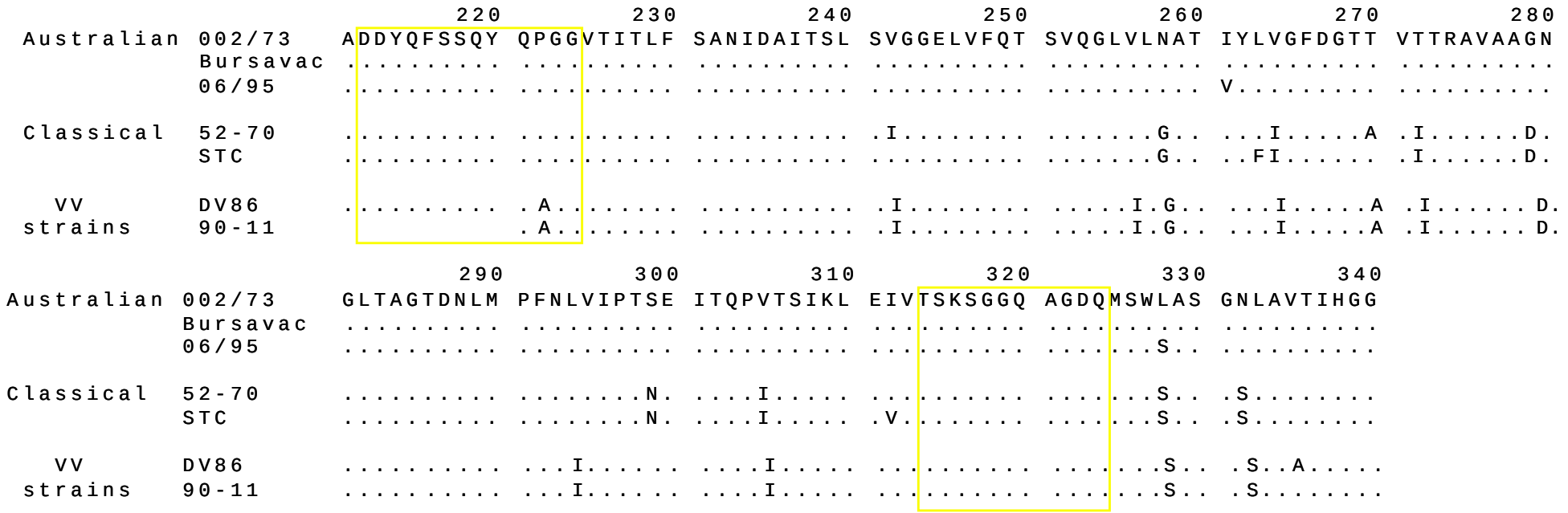


Figure 8. Phylogenetic relationship of Australian strains to overseas strains of IBDV

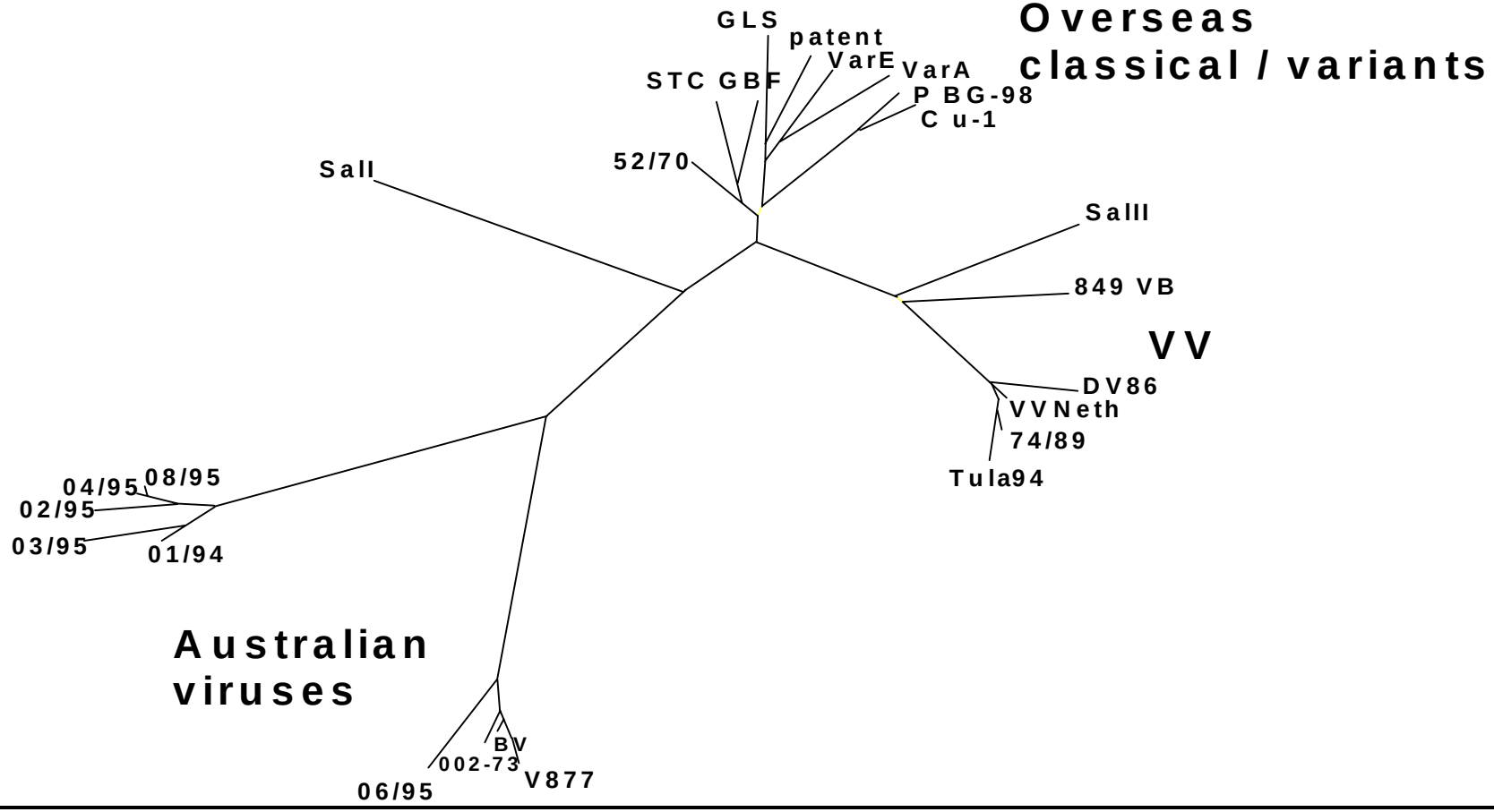


Table 7. Correlation of titres obtained in three commercial ELISA kits with SN titres

Sera from flock age	ELISA titres								SN titres	
	CSIRO		IDEXX		KPL		TropoBio			
		No with	Range	No with	Range	No with	Range	No with		No with/ No tested
21 day	200	13	125 - 521	6	120 - 379	6	2 - 54	10	ND	ND
12 - 21 day	400	20	172 - 688	19	120 - 982	11	3 - 410	20	400	10/10
12 - 17 day	800	19	528 - 1299	16	168 - 1443	16	42 - 1819	19	800	8/19
									400	8/19
									200	1/19
									1600	2/19
12 day	1,600	12	852 - 1782	12	258 - 1997	11	331 - 1659	12	1,600	7/12
									800	5/12
7 - 12 day	3,200	16	932 - 2,878	16	33 - 3,175	16	174 - 6,607	16	3,200	4/8
									1,600	4/8
7 day	6,400	8	1,319 - 3,088	8	335 - 2,745	8	6,310 - 16,000	8	Not tested	
32 weeks	25,600	9	2,291 - 6,573	9	3,801 - 12,864	9	6,310 - >16,000	9	25,000	7/9
									12800	1/9
									51,200	1/9
32 weeks	51,200	11	4,010 - 8,003	11	5,822 - 14,746	11	7,244 - >16,000	11	51,200	6/11
									25,600	1/11
									102,400	4/11
24 weeks	102,000	8	6,275 - 8,079	8	8,771 - 11,439	8	11,482 - >16,000	8	102,400	3/8
									51,200	5/8

only when antibody titres were in the median range between 1600 and 6,400. When titres were low (800 and below) IDEXX and KPL titres correlated better with the SN titres than TropBio titres. However when titres were higher that 6,400 better correlation was obtained with TropBio than with either IDEXX or KPL ELISAs. Therefore choice of ELISA system for assessment of antibody titres in commercial flocks will influence the results and will have a different predictive value for sera obtained from broilers and broiler breeders or layers.

DISCUSSION

In an attempt to understand the epidemiology of the current IBD situation in Australia, we have characterised local IBDV strains at the antigenic and molecular level. We aimed also to correlate these changes with vaccinal immunity in commercial flocks in order to provide insurance that the best possible strategies are being applied for control of endemic strains.

Characterisation of a number of recently isolated strains isolated between 1994 and 1995 and their comparison with existing vaccine strains revealed some of the features of local IBDV strains previously unknown.

Firstly, Australian IBDV strains are uniquely heterogenous. The vaccine strains and the 06/95 strain isolated in NSW, were all antigenically similar as determined by nucleotide sequencing. They are classical like strains. On the other hand, strains isolated in the state of Victoria differed from these classical strains by nucleotide sequencing, and also antigenically. Antigenic changes detected in these strains occurred in one virus neutralising epitope (39A) in the protective VP2 antigen. Two virus neutralisation epitopes were identified in local IBDV strains, these are defined by Mabs 39A and 17-82 (Fahey *at al*., 1991). Virus neutralising antibodies are protective and both Mabs, 39A and 17-82 protected chicks against virulent IBDV challenge (Fahey *et al*., 1991). Therefore, the loss of the 39A epitope in Victorian strains indicates that this change might be significant for protection.

Antigenic change in variant strains was also detected with immune chick sera in the SN test. Victorian variants were of different subtypes than vaccine strains and the NSW isolate 06/95. The IBDV strains isolated in the USA were possible to differentiate into six subtypes, however only strains that belonged to the sixth subtype were "true" antigenic variants as determined by cross-protection (Jackwood & Saif, 1987; Rosenberger *et al*., 1985; Snyder *et* al., 1992). We were however restricted in antigenic characterisation of local IBDV strains using the SN test, as we were unable to adapt any strain, to grow in CEF except for 02/95.

In vivo cross-protection experiments using commercial broilers of 7 - 21 days of age also confirmed that 3/5 isolated variants can break through maternal antibodies at an early age that could result in immunosuppression. Antigen was found in the bursa of these chicks 3 days after infection and B/B ratio and histopathological changes indicative of IBDV infection were visible at 14 days after challenge.

We did not determine if isolated strains induced immunosuppression, however all strains were virulent, causing regression of bursa in SPF chicks at 7 days after infection and with a very low (minimum) dose of virus.

Although we showed that isolated Victorian variants can break through maternal antibodies as early as 12 days of age, the implications of this for the field situation in less well defined. The majority of variants, except for one isolated at 22 days of age, were isolated from flocks at

between 28 - 32 days of age. This would imply that field challenge has occurred in the majority of these flocks at an age that would not result in immunosuppression. However, in all instances, regressed bursa and respiratory disease or diarrhoea were detected in flocks from which IBDV was isolated indicating existence of a susceptible population.

Commercial chicks challenged in the laboratory situation that did not succumb to infection all had ELISA and virus neutralising antibody titres to classical strains that were in excess of 2,000 whereas their antibody titres to variants were below 400. Antibody titres of 400 and above are protective against challenge with classical strains (Fahey *et al*., 1987). These results therefore imply that broilers should have maternal ELISA antibody titres well above 2,000 until 14 day of age, in order to be fully protected against challenge in the field by both classical and variant strains. Therefore if hygiene and correct vaccination of breeders with the full antigenic dose of currently used classical Webster's Bursavac K vaccine is maintained, challenge with variants should not represent a major problem.

Comparison of Australian strains with overseas strains at the genetic level has indicated that our strains are a distinct group of strains, which can be separated from all other classical, variant and vvIBDV strains. These genetic differences enable the differentiation of most if not all overseas IBDV strains from Australian strains by nucleotide sequencing, which therefore can be used as a differentiation tool. On the other hand, the result of these genetic differences means that reagents and vaccines developed overseas might not always have the same value in comparison to those generated against the local strains. The mutations that are occurring in local strains, although of the same nature as those seen in other countries, give rise to strains that differ from the overseas strains. Therefore, it is not unexpected that Australian and the USA variants differ, because their parent strains differ. Also, that the Mabs developed against the USA strains, as shown, will not detect differences between Australian strains.

Antigenic comparisons using Mabs developed in the USA identified all Australian strains, as classical strains. This antigenic homogeneity is in contrast to the results obtained by nucleotide sequencing that shows that strains differ. However in spite of these differences at the nucleotide level, strains still have considerable degree of antigenic conservation which is detected by Mabs.

The significance of the clear division of Australian strains at the antigenic and genetic level into classical and variant group of strains is presently not obvious. It was not possible to ascertain from the present study whether the heterogeneity in Australian IBDV strains has always been present or is of recent origin. We were not able to obtain any other strains isolated between 1974 and 1994, except for three vaccine strains. This could be important for the future epidemiology of IBDV in Australia because if changes were of recent origin they could indicate a favourable environment for genetic variation leading to “true variants”. Secondly, as genetic analysis indicates that the two groups of Australian strains differ more from each other than do USA variant and classical strains it would be expected, in analogy with the USA situation, that considerable problems should be encountered in the field. This however appears not to be the case at this time and could imply that current vaccines are antigenically superior to those used in other countries.

IMPLICATIONS

Overall the current IBDV situation in Australia resembles that in the USA where antigenic variants emerged a number of years ago. The isolation of similar antigenic variants in Australia strengthens the view that vaccination with strains of intermediate virulence, such as are the Australian vaccine strains, prevents the emergence of pathogenic variants, but possibly facilitates the emergence of antigenic variants.

RECOMMENDATIONS

We recommend that attention be paid to the maintenance of high antibody titres in breeders and broilers in order to minimise virulent challenge of broilers at an early age. Also, the risk of further selection of antigenic variants and exposure of broilers to a virulent challenge with variants might be minimised by adopting husbandry procedures aimed at reducing field challenge.

GLOSSARY

B/B	Bursa/body ratio = weight of bursa divided by body weight x 1,000. Bursa regresses following infection with IBDV resulting in significantly lower B/B ratios in infected in comparison to non-infected chicks.
Classical IBDV	All IBDV strains that infect chicks and belong to the serotype I, as opposed to strains that infect turkeys and are of serotype II. Strain vary in virulence, the majority are immunosupressive and some may cause up to 30% of mortality in SPF chicks.
Epitope	A small region in the protein which is antigenic and induces antibody response.
Polymerase chain reaction	A reaction in which a small portion of RNA or DNA is copied to produce multiple copies of DNA. Such DNA copies are then used to obtain sequence of the selected portion.
vvIBDV:	Pathogenic variant of IBDV, cause between 30 and 70% of mortality in commercial chicks.
Variant IBDV	Antigenic variants of IBDV, cause immunosuppression only.

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ATTACHMENT 2

Project title: INFECTIOUS BURSAL DISEASE VIRUS (IBDV): TO DETERMINE IF CURRENT VACCINATION STRATEGIES PREVENT THE EMERGENCE OF VARIANT IBDV STRAINS IN AUSTRALIA

Objectives

(1) To determine if variant IBDV strains are present in Australia by comparing the VP2 proteins of field isolates to that of other IBDV's and vaccine strains.

(2) If variant IBDV strains are present, whether existing vaccination protocols provide

Background

Clinical IBDV has been control well in Australia. In late 1994 and early 1995 there have been some indications that commercial flocks at a number of sites are experiencing problems with IBDV and that these might be caused by variant IBDV strains. The existence of variant strains however might not be recognised because of the effectiveness of currently used vaccines. It was of importance for the to the poultry industry to understand if there were recent changes in the strains predominating in Australia and insure that more virulent viruses do not emerge. Also to insure that the best possible vaccination strategies are applied for the control of endemic strains.

Research

Research carried our aimed to: (1) isolate and characterise IBDV strains from flocks with clinical IBD; (2) determine if IBDV strains in Australia are changing in either antigenicity or pathogenicity; (2) compare local IBDV strains with those of other countries; and (3) determine if current vaccines provide protection against these recent field isolates.

Outcome

Variant strains were isolated from commercial sites in two states. Those isolated in the state in Victoria were conformed to be true antigenic variants by a number of means, including cross-protection studies. Chicks that lacked virus neutralising antibodies to variants were not protected and were susceptible to a challenge at an early age that could lead to the immunosuppression.

Implications

The results indicate that, as in other countries, IBDV strains in Australia are also changing and that correct vaccination strategies and attention should be given to maintaining the adequate level of antibodies in the flocks such that resistance to challenge with variants is obtained.

Publications

Results of this work are currently being prepared for publication.

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