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Diagnostic tools for vvIBDV

and characterisation of Australian strains

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Diagnostic tools for differentiation of vvIBDV and characterisation of local IBDV strains
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Foreword

The possible incursion and spread of vvIBDV strains in Australian represents a considerable threat to the local poultry industry. The disease would cause economic losses from mortalities in the order of 5% – 30% and would lead to changes in the existing trade barrier for the importation of poultry products.

The ability to quickly identify and differentiate vvIBDV strains is of particular importance in cases where it is essential to unambiguously establish whether such strains are involved. Currently available methods are lengthy and require a specialised laboratory set-up. Faster methods, such as ELISA which can be done on a larger number of samples, would aid considerably in the speed of diagnosis and prevent the spread of vvIBDV.

The results published in 1999 by a group working in the USA indicated that strains similar to vvIBDV exist in Australia. This result is contrary to the results obtained previously on Australian IBDV strains using different methods. The results obtained by the US group could seriously impact Australia's trade position, therefore it was necessary to show that Australia is free of vv like IBDV strains using the same methods used by the US group.

The aims of the proposed study were to: (a) develop ELISA & RFLP for the rapid detection and differentiation of vvIBDV strains & (b) demonstrate that changes in local IBDV field isolates remain such that they can be clearly differentiated from all overseas strains.

This publication summarises results obtained from the 1 July 2000 to 30 June 2003 at the CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong.

This project was funded in part from the Chicken Meat and Egg industry revenues which is matched by funds provided by the Federal Government. In addition to the funds received from RIRDC, CSIRO Livestock Industry also provided financial, personnel and laboratory support for the project.

This report is an addition to RIRDC's diverse range of over 900 research publications, forms part of our (Chicken Meat and Egg) R&D programs, which aim to support increased sustainability and profitability in the chicken meat industry and to support improved efficiency, sustainability, product quality, education and technology transfer in the egg industry.

The result presented in this report are confidential and for restricted use within RIRDC only, until they have been published.

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Abbreviations

AAHL	Australian Animal Health Laboratory
cDNA	Complementary deoxy ribonucleic acid
CRAb	Chicken recombinant antibody
CRAb-hyper phage	Chicken recombinant antibody expressed on hyperphage surface
CRAb-phage	Chicken recombinant antibody expressed on phage surface
CRAb-Sab	Soluble chicken recombinant antibody
CRAb88	Chicken recombinant antibody specific for vvIBDV strains
CRAb154	Chicken recombinant antibody reacting with all known IBDV strains
ELISA	Enzyme-linked immunosorbent assay
HRP	Horse radish peroxidase
HVR	Hyper variable region (in the VP2 protein of IBDV)
IBDV	Infectious bursal disease virus
IgG	Immunoglobulin class G
Mabs	Monoclonal antibodies
NSW	New South Wales
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
Qld	Queensland
SA	South Australia
SPF	Specific pathogen-free chickens
VP2	Protective antigen of IBDV
Vic	Victoria
vvIBDV	Very virulent infectious bursal disease virus
WA	Western Australia

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Executive Summary

Possible incursion and spread of vvIBDV strains in Australia represents a considerable threat to the local poultry industry. The ability to differentiate vvIBDV strains is of particular importance in outbreaks of IBD where it is required to unambiguously establish wether such strains are involved. Currently, the only methods available for such diagnosis are nucleotide sequencing followed by pathogenicity testing in chickens. These are time consuming and require a specialised laboratory set up. Additional methods, particularly those that are fast, simple and can be done on a larger number of samples in conventional diagnostic laboratories, would aid in diagnosis and prevention of spread of vvIBDV.

During the previous RIRDC funded project (CME97-06) we developed a chicken recombinant antibody (CRAb88) that appeared to be specific for vvIBDV strains reacting only with several vvIBDV strains available in our Laboratory. As vvIBDV have been isolated in many countries around the world, we needed to establish that CRAb88 recognised all vvIBDV isolates regardless of their geographic origin. In this study we confirmed the specificity of CRAB88 for vvIBDV by testing this reagent in two overseas laboratories with a large number of classical, variant and vvIBDV strains. In the course of this testing we also selected another CRAb, CRAb154, that reacted with all know IBDV strains. This enabled us to develop an antigen ELISA that is able to identify if IBDV is present in a test sample, and at the same time determine if any positive sample is vvIBDV. We optimised the test by selecting a hyper-phage expressed CRAb154 (containing multiple copies of detecting antibody) and phage expressed CRAb88 (single copy of detecting antibody) to obtain an ELISA of high sensitivity which is commercially feasible.

The ELISA developed has been transferred to the diagnostic section of our Laboratory and is available to the Australian poultry industry for fast differential diagnosis in the case of an exotic vvIBDV incursion. This CRAb ELISA has also been offered for commercial development to a US based company . A full patent application on CRAbs, including CRAb88, was filed in June 2003.

Restriction fragment length polymorphism (RFLP) has been developed as a diagnostic tool for the discrimination of vvIBDV in the USA (Jackwood & Sommer 1999). Using RFLP, these authors showed that Australian IBDV strains belonged to the same group of viruses as those isolated from Korea since both had the same RFLP profile, and furthermore that Australian IBDV posses a specific cleavage site *Ssp*I found only in vvIBDV. These results therefore suggested that vvIBDV, or similar strains, were present in Australia. In contrast, our published results (Sapats & Ignjatovic 2000; Ignjatovic *et al*., 2002) showed that Australian strains are not closely related to vvIBDV strains, and do not posses the *Ssp*I cleavage site specific for vvIBDV strains. This contradiction of results obviously needed clarification using the same RFLP method. In addition, Jackwood & Sommer's RFLP method has now been marketed as a commercial test for the differentiation of IBDV strains through IDEXX Laboratories, and therefore it was essential to evaluate this method as a tool to differentiate Australia strains. We introduced and evaluated the same RFLP method as used by Jackwood & Sommer's using all available (27 in total) Australian IBDV strains. We also sourced two out of the four Australian samples used in the study of Jackwood & Sommer (1999). We showed that Australian IBDV strains belonged to 12 distinct RFLP groups (A1-A12) which differed from the 6 RFLP groups first identified among vaccine strains used in the USA. In addition, the 12 Australian RFLP groups also differed from the other 38 RFLP groups identified amongst field strains from various r countries including the US (Jackwood & Summers 1997, 1999). Amongst the 12 Australian RFLP groups, it was not possible to correlate particular RFLP groups with know biological properties of IBDV strains, such as genetic or antigenic similarities. Hence in Australia's case , the use of RFLP for strain differentiation is of little value and the effectiveness of this test for strain differentiation is questionable due to the large number of RFLP groups existing world wide .

We were not able to detect a specific *Ssp*I site characteristic of vvIBDV in any of the Australian IBDV strains contrary to the results of Jackwood & Summer (1999). Nucleotide sequencing of Australian strains confirmed that such an *Ssp*I site was not present in Australian strains. Therefore we concluded that this method using RFLP is prone to error, most likely due to cross contamination between samples used in the assay. We detected an *Ssp*I site in one Australian IBDV strain N2/99, but this site was located at another position within the VP2 gene different to that found in vvIBDV. The pathogenicity of strain N2/99 did not differ in comparison to other Australian IBDV strains. The results on RFLP analysis of Australian strains have been published: Sapats S & Ignjatovic J. Restriction fragment length polymorphism analysis of the VP2 gene of Australian strains of infectious bursal disease virus. *Avian Pathology 2002, 31, 559-566.*

We have previously shown that Australian IBDV strains are a diverse group of strains that can be differentiated from overseas IBDV strains and that they are undergoing genetic changes (Sapats & Ignjatovic 2000; Ignjatovic *et al*., 2002). Analysis of an IBDV strain obtained in 1999 from a broiler farm showing elevated mortalities indicated further that unique genetic changes are occurring in local strains possibly leading to an increase in virulence. We therefore proposed to continue to characterise field IBDV strains at the nucleotide level. This would confirm that changes detected in local strains remain unique and in line with other Australian strains, enabling their differentiation from all overseas strains. This would also enable the early detection of local strains with increased virulence and provide industry with an indication of the likelihood of viruses similar to vvIBDV developing in Australia.

A survey of 19 broiler farms was performed in the states of Victoria, NSW, SA & Qld and one layer farm in Qld. Broilers were between 27 - 37 days of age, whereas a layer flock was 48 days-old. IBDV involvement was only suspected on two farms. IBDV was isolated from 10 broiler farms. Antigenic and genetic characterizations of isolated strains indicated that IBDV strains from NSW (01/01, 03/01, 02/02, 03/02, 02/03 & 05/02) and Qld (02/01 & 01/03) were classical like strains that were similar to vaccine strains. The SA isolate (02/00) and two Vic isolates (02/02 & 01/03) were similar to IBDV variants isolated previously in Vic & SA. Overall, the characterization of circulating field strains has indicated that they have not changed significantly in comparison to vaccine and previously isolated strains. Thus Australian IBDV strains remain a unique group of strains that can be differentiated genetically from IBDV strains isolated in other countries. The results have also confirmed that variants are present in Vic and SA whereas in NSW and Qld only classical IBDV strains are present.

The results of the studies, in part, have been published in:

Sapats S & Ignjatovic J. (2002). Restriction fragment length polymorphism analysis of the VP2 gene of Australian strains of infectious bursal disease virus. *Avian Pathology* 31, 559-566

Ignjatovic J & Sapats S.. (2002). Characterisation of additional infectious bursal disease virus field isolates confirms existence of two distinct genetic groups in Australia. *Australian Veterinary Journal* 80, 689-694

Sapats SI, Heine HG, Trinidad L, Gould GJ, Foord AJ, Doolan SG, Prowse S & Ignjatovic J. (2003). Generation of chicken monoclonal antibody fragments that differentiate and neutralise infectious bursal disease virus (IBDV). *Archives of Virology* 148, 497-515

1. Introduction

Australian IBDV strains have been characterized by antigenic analysis and nucleotide sequencing of the hypervariable region (HVR) within the VP2 gene (Sapats & Ignjatovic, 2000; Ignjatovic & Sapats, 2002). Australian field strains could be classified as either classical or variant strains and both groups were genetically distinct from IBDV strains of other countries. Incursion of either US variants or vvIBDV into Australia would have a considerable impact on the local poultry industry. Consequently, considerable effort has been directed towards the development of methods that can differentiate between the various groups of IBDV strains.

During the previous IBDV project funded by RIRDC (CME97-06) we showed that vvIBDV strains are prevalent in Indonesia and that they are closely related, although not identical, to other vvIBDV strains. This result, together with the recently published sequences for strains isolated in Hong Kong, South China, Korea and Brazil, confirmed that all vvIBDV strains, regardless of the country of origin, are similar by nucleotide sequencing. This has confirmed the value of nucleotide sequencing as a diagnostic method to detect the possible incursion of any exotic IBDV strain into Australia. The usefulness of nucleotide sequencing as a method for IBDV strain differentiation was tested in1999 during an outbreak of IBD in NSW when diagnosis conducted at AAHL showed that a local strain of IBDV was the involved and not a vvIBDV strain.

During the 1999 IBDV outbreak it also became apparent that there were considerable shortfalls in the existing approach for IBDV diagnosis. It took 2 weeks between the outbreak and the diagnosis. In the case of incursion of vvIBDV such a delay in diagnosis would have a major impact on the spread of infection from the original site. The type of diagnostic method used is a significant contributing factor, amongst others, that influence the speed of diagnosis. For example, in the case of the NSW outbreak, the initial diagnosis was made by histopathology performed in a regional laboratory. If at the same time an alternative diagnostic method was available, differential diagnosis could have been done simultaneous avoiding misdiagnosis. Nucleotide sequencing, which is the only method currently available for differential diagnosis, requires a specialised laboratory set-up and takes 3 days to complete. A simple test, such as an antigen ELISA, which can be performed by most diagnostic laboratories on the same day, would aid considerably in the efficiency of diagnosis.

During the previous RIRDC funded project (CME97-06) we generated 42 different chicken recombinant antibodies (CRAbs). In an experimental antigen ELISA, one CRAb, termed CRAb88, was identified that reacted only with vvIBDV strains available at AAHL and showed no reactivity with overseas classical strains 52/70 and 1/68. The results thus indicated that CRAbs have the potential to be used as differential diagnostic reagents in an antigen ELISA. For the development of such an ELISA the following were required: (a) to confirm the specificity of CRAbs using a larger number of overseas strains; (b) to compare different forms of expressed antibodies for their efficacy as diagnostic reagents (c) to replace the existing tag system with a CSIRO owned tag to produce cheaper reagents and (d) to optimise the level of production using different expression systems. Further we proposed to determine to which protein CRAb88 was directed against. This could indicate which part of the IBDV virion is associated with a virulent phenotype.

The USA is also free from vvIBDV strains. A method developed in the USA for the discrimination of vvIBDV is based on restriction fragment length polymorphism (RFLP) (Jackwood & Sommer 1999). This test has been under development and evaluation in Jackwood's laboratory since 1994 [Jackwood & Summers 1994, 1997,1998, 1999). Using this method the authors showed that vaccine strains used in and outside of the USA belonged to one of 6 different RFLP groups. Analysis if strains outside of the USA revealed that a large number of strains fell into RFLP group 6 including vvIBDV, however a unique *Ssp*I site was identified within vvIBDV strains which was absent in classical or variant strains. Consequently, it was concluded that any IBDV isolate which belonged to

RFLP group 6 and possessed an *Ssp*I cleavage site was most likely a vvIBDV strain. Analysis of 184 field strains obtained from 17 countries identified an additional 41 RFLP groups some of which contained an *Ssp*I site. Four Australian strains analysed by this method produced an RFLP pattern which was identical to that obtained with IBDV isolates obtained from Korea and contained the *SspI* site specific of vvIBDV strains.. The results therefore implied the existence of strains similar to vvIBDV in Australia. In contrast, our results obtained by nucleotide sequencing had previously shown that Australian strains were not closely related to vvIBDV strains, nor did they posses the *SspI* cleavage site specific for vvIBDV. In addition, the type of clinical disease observed in the field here indicated that vvIBDV were not present in Australia. . This substantial contradiction in results was disturbing and obviously needed clarification. Without further clarification the results of Jackwood and Sommer would be accepted eagerly by those wishing to show that there were no major differences between IBDV strains isolated in Australia and those occurring elsewhere thus allowing the importation of chicken meat into Australia. Confirmation of the results obtained by Jackwood and Sommer was therefore required to establish that vvIBDV like strains were not present in Australia using the same RFLP method. This would be important in maintaining Australia's current trade position as a country which is free from vvIBDV-like viruses.

We have previously shown that Australian IBDV strains are a diverse group of strains, with existence of classical and variants strains. Nineteen IBDV isolates obtained and characterised during 1996 and 1999 confirmed that Australian strains are heterogenous. All isolates differed from each other and from vaccine strains, with the majority of changes occurring in the same regions of the VP2 protein. It was also demonstrated that in commercial chicks at 14 days of age, maternal antibody did not protect against challenge with some of these strains. When compared with overseas strains all Australian strains could be differentiated as a distinct and separate genetic group. Some of the recent strains however, had amino acid changes not seen before. In addition, three different isolates were circulating on the same location which had not been seen before. When one of these isolates was inoculated into 3-week-old chicks an increase in virulence was observed. There were no mortalities, but for the first time clinical disease was seen in a proportion of the chicks. We therefore proposed to continue to characterise the changes occurring in field strains of IBDV. This would provide further evidence that strains circulating in commercial flocks are, and continue to be uniquely Australian and can be differentiated from all overseas strains. Immunological characterisation of these newly isolated strains would provide assurance that existing vaccination strategies provide sufficient immunity against field challenge. Testing of strain pathogenicity would also enable the early detection of local strains with increased virulence and provide industry with an indication of the likelihood of viruses similar to vvIBDV developing in Australia.

2. Objectives

1. Develop additional methods, ELISA & RFLP, for rapid detection and differentiation of vvIBDV strains

2. Demonstrate that changes in local IBDV field isolates remain such that they can be clearly differentiated from all overseas strains

3. Development of ELISA for differentiation of vvIBDV strains

3.1 Background

Previously we generated a CRAb termed CRAb88 that reacted only with vvIBDV strains available at AAHL (UK strain CS88 & Indonesian strain Tasik94) and showed no reactivity with any Australian IBDV strains. We also generated other CRAbs, some of which showed variable reactivity with other IBDV strains. In order to ensure that CRAb88 was indeed specific for vvIBDV, it was necessary to show that this antibody reacted with all vvIBDV strains isolated from different parts of the world. In addition, we also needed to confirm that CRAb88 did not react with any of other overseas classical, variant, or vaccine strains. Since we have a limited number of overseas IBDV strains available at AAHL (classical strains 52/70, 1/68 and Aphis, variants E and GLS and vvIBDV strains CS88 and Tasik 94, we aimed to perform testing in Europe and USA where we could gain access to the greatest number of IBDV strains.

Since the CRAb88 antibody can be produced in three forms, as a soluble antibody (CRAb88 – SAb), phage-expressed antibody (CRAb88-phage) and hyperphage expressed antibody (CRAb88-hyperphage), we aimed to select the best form of CRAb88 for the development of an ELISA. As there are several differences in reactivity between phage expressed antibodies and soluble antibodies which might be of importance for ELISA development, we evaluated the differences between these forms of antibodies.

Once the most suitable from of CRAb88 was selected, we evaluated all the reagents in an antigen ELISA with a view of developing a test of high specificity, sensitivity and with the potential for commercialisation (reagents that are cheep to produce and stable for a prolonged period of time).

3.2. Method

Production of CRAbs-phage

The method used for the generation of CRAbs has been described in our previous Report (CME97-06) and also in Sapats *et al.* (2003). To produce CRAb-phage, individual colonies of various CRAbs were inoculated into 2xYT-AG media and grown shaking at 30^0C overnight. An aliquot was then added to the same media containing M13KO7 helper phage, incubated at 37^0C for 2 hr, pelleted and transferred to 2xYT media containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. After overnight incubation, cells were pelleted and the phage-containing supernatant removed and stored at 4^0C.

Production of CRAbs-SAb

CRAbs-phage that gave a positive reaction in an IBDV ELISA were used to infect a non-suppressor strain of *E. coli* HB2151 cells to enable the production of soluble CRAbs. The growing cells were plated out, single colonies selected, grown overnight, an aliquots taken and transferred to fresh medium containing ampicillin and glucose, and incubated shaking at 30^0C for 2 hrs. Cells were then pelleted, resuspended in fresh medium containing isopropylthio-β-D-galactoside (IPTG) and ampicillin, and shaken for 6 hr at 30^0C to induce expression of CRAb-SAb. After pelleting the cells, CRAb-SAb was recovered from the periplasm using mild osmotic shock.

ELISA to determine specificity of CRAb-phage & CRAb-SAb

Phage ELISA: Wells of a polystyrene micro titre plates were coated overnight with rabbit anti-IBDV IgG diluted in carbonate-bicarbonate buffer, pH 9.6. After washing with PBS containing 0.1 % Tween 20 (WB), IBDV antigen diluted in PBS containing 5% foetal calf serum (DB) was added and incubated for 1 hr at 37^0C. After washing with WB, CRAb-phage diluted 1/2 using DB was added and incubated for 1 hr. After washing, bound CRAb-phage was detected using anti-M13 IgG-HRP conjugate and absorbances at 405 nm determined after the addition of AZINO-bis 3-Ethylbenz 2, 2'-thiazoline-6-sulfonic acid (ABTS).

SAb ELISA: IBDV antigen was captured onto wells of polystyrene micro titre plates as for the phage ELISA. SAbs were diluted 1/10 in DB, 100 µl added to well of micro titre plates and incubated for 1 hr at 37^0C. After washing, bound SAbs were detected using anti-E tag Mab and goat-anti-mouse IgG-HRP conjugate. Absorbencies were recorded as for the phage ELISA.

IBDV strains

The specificity of CRAb88 for vvIBDV strains, as well as the -specificity of 6 additional CRAbs, was tested at Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, France, in the laboratory of Dr N Eterradoss, an OIE accredited laboratory for IBDV that has the largest number of vvIBDV isolates from around the world and in the laboratory of Dr Jackwood , Ohio State University, Wooster, USA, which has the largest collection of USA IBDV isolates. Viruses included in the study were: vvIBDV strains from the UK (UK661), Belgium (VB849), the Netherlands (DV86), France (8 isolates), Senegal (2 isolates), Ivory Coast (3 isolates), China (1 isolate) and Brazil (1 isolate); classical strains 52/70 and Cu1; variants GLS, Var E and Var A; vaccine strains 228E, TadForte, PBG98, Cu1M, Bursine & D78.

3.3 Results & Discussion

Specificity of CRAb88 and CRAb154

Specificity of CRAb88 for vvIBDV strains was confirmed (Table 1). In Table 1 results for selected strains are only shown. Both CRAb88-SAb and CRAb88-phage reacted in ELISA only with the vvIBDV strains & did not react with any of the other classical, variant or vaccine strains. CRAb88, as shown in Table 1, reacted weakly only with one vvIBDV strain that was from the Ivory Coast. From the 12 other CRAbs that were tested, only CRAb154 reacted with all IBDV strains (Table 1). Results for CRAb154, CRAb66 & CRAb28 are only shown in Table 1. Overall, the results have indicated that CRAb154 recognises all IBDV strains whereas CRAb88 recognises only vvIBDV strains.

Comparison of CRAb-phage & CRAb-SAb in ELISA

CRAb88-phage and CRAb88-SAb, as well CRAb154-phage and CRAb154-SAb were compared for specificity and affinity of binding to various IBDV antigens (Tables 1). CRAb88-phage and CRAb88–SAb had the same specificity of binding to all vvIBDV strains. However, CRAb88-phage gave higher absorbances in ELISA than soluble antibody (Table 1). CRAb154-phage and CRAb154–SAb showed the same specificity of binding to all IBDV strains and CRA154-phage also gave higher absorbances in ELISA (Table 1).

We also determined if CRAb88 & CRAb154 could be expressed as a hyper-phage and used in ELISA instead of phage. In hyperphage, five copies of CRAb88 and CRAb154 are displayed on each phage particle surface, compared to only one copy using the conventional phage system. For this reason CRAb hyperphage can be used at high dilution in ELISA making it a very cheep reagent. Using hyper-phage a considerable increase in the sensitivity of IBDV antigen detection was obtained in comparison to the Mab 9-6 based ELISA with both CRAb88-hyperphage and CRAb154-

hyperphage (Figures 1, 2 & 3). In particular, CRAb154-hyperphage was able to detect variant E with a high degree of sensitivity (Figure 2). However, with CRAb88 this increase in avidity for antigen also lowered the differentiation capacity of hyperphage expressed CRAb88 as it reacted to a low degree with classical pathogenic strain 52/70 (Figure 3). However CRAb88 phage is able to differentiate between vvIBDV strains and 52/70 (Figure 4 & Table1).

As a result of the above testing, we have chosen CRAb88-phage & CRAb154-hyperphage as regents for a vvIBDV ELISA. Both CRAb88-phage & CRAb154-hyperphage were easier and cheaper to produce in larger amounts than the corresponding SAbs, and were equally stable at 4^0C for a prolonged period of time (results not shown). In addition, the ELISA using CRAb-phage/hyperphage was faster and simpler to perform than the ELISA using SAbs due to the omission of one incubation step when using phage/hyperphage antibodies.

Development of ELISA

Further evaluation of the ELISA for differentiation of vvIBDV was undertaken using CRAb154-hyperphage and CRAb88-phage. The following parameters, which all influence an ELISAs' specificity and sensitivity, were evaluated: (1) type of plates, (2) configuration of sandwich ELISA, e.g. type of coating and type of detecting antibody, (3) type of diluents & (4) use of rabbit-anti-M13 IgG. The results showed that: (1) high binder Nunc plates have an advantage over common polystyrene ELISA plates & (2) the highest sensitivity of ELISA was obtained using anti-IBDV polyclonal chicken sera as coating antibodies and CRAbs as detecting antibodies. It was not possible to use CRAbs as coating antibodies and anti-IBDV polyclonal chick sera or -rabbit antisera or Mabs as detecting antibodies. When anti-IBDV polyclonal chick sera was used as the detecting antibody there was non-specific binding in all wells containing CRAbs. This could be explained by the secondary antibody (goat anti-chick IgG-HRP) recognizing "chicken antibody" sequences contained within the CRAbs. When rabbit anti-IBDV sera was used as the detecting antibody the sensitivity of the assay was lost, whereas with Mabs the specificity of the assay was lost (results not shown). The type of diluent used did not influence the specificity of the test but influenced the sensitivity. The most convenient diluents were either 2% skim milk or 5% calf serum in PBS (results not shown). It was not possible to replace commercial anti-M13-IgG-HRP with a locally produced rabbit-anti-M13 IgG (results not shown).

Following the above evaluations, the following method has been adopted for the differentiation of vvIBDV in ELISA using CRAbs: Nunc MaxiSorb plates are coated with an appropriately diluted polyclonal chicken sera raised against an Australian classical IBDV strain. After overnight incubation, a diagnostic sample is added to four wells, incubated for 1 h, followed by the addition of CRAb154-hyperphage to two wells and CRAb88-phage to two wells. After a 1h incubation, plates are washed and anti-M13 IgG HRP is added, followed by the addition of a substrate. If a sample contains vvIBDV, all four wells will be positive (with colour); if a sample contains any other type of IBDV, only two wells where CRAb154 was added will be positive; samples with no colour are negative for IBDV.

Sensitivity and specificity of this ELISA was evaluated using representative Australian classical and variant strains, overseas classical strain 52/70, USA variant E, and vvIBDV strain CS88. Results showed that the ELISA using CRAb154-hyperphage and CRAb88-phage is highly specific for the detection of vvIBDV strains. Additionally, the sensitivity of the test is much greater in comparison to Mab 9-6 based ELISA enabling the detection and differentiation of IBDV in samples containing small amounts of IBDV antigen (Figure 5).

It was planned that the specificity of the CRAb ELISA be confirmed under field conditions. For this, samples were collected from six different farms with suspected IBDV outbreaks in Indonesia. Samples were tested in Indonesia using the CRAb ELISA. Samples from five farms were positive for IBDV of which three were positive for vvIBDV. It was intended that these samples be transferred to AAHL, re-tested in ELISA and positive samples sequenced to confirm the ELISA findings. However, permission from the Indonesian government to import these samples to AAHL was delayed and

samples were not obtained in time to complete the testing before the end of this project.

Protein specificity of CRAb88

The aim was to determine to which protein of IBDV CRAb 88 is directed towards. For this purpose the two most common methods, western blotting and immuno-precipitaton, were used with CRAb88 expressed as a soluble antibody. In a western blot, CRAb88 was unable to bind to any denatured viral proteins of vvIBDV strain CS88, indicating that the epitope recognized by CRAb88 is highly conformational (result not shown). For this reason immuno-precipitation was employed. A virus preparation of CS88 which had been first solubilized using a non-ionic detergent (viral protein conformation remains intact) was incubated with CRAb88-SAb and immunoprecipitated using two Mabs, one specific for VP2 (Mab 9-6) and another specific for VP3 (Mab17-80). The type of protein immunoprecipitated was then analyzed using a western blot. The results indicated that CRAb88 was specific for a conformational epitope located on VP2. Using the same method, CRAb154 was also found to be specific for VP2 (results not shown).

Table 1. Specificity of various CRAbs, expressed as either SAb or phage for different IBDV strains

		Absorbance							
		CRAb88		CRAb154		CRAb66		CRAb28	
Strain	**Origin**	**SAb**	**Phage**	**SAb**	**Phage**	**SAb**	**Phage**	**SAb**	**Phage**
002/73	Australia	0.05	0.05	1.26	1.44	1.08	1.32	0.05	0.08
V877		0.07	0.04	1.13	1.27	0.64	0.95	0.05	0.07
K3		0.05	0.04	1.07	1.38	0.74	0.83	0.03	0.06
05/5		0.05	0.05	0.91	1.05	0.23	0.64	0.04	0.07
57/70	Classical	0.07	0.13	0.93	2.45	0.66	0.74	0.68	2.3
1/68		0.05		1.14		0.18			
Cu1		0.07	0.11	1.15	2.28	0.81	1.84	0.45	2.2
228	TC vaccines	0.06		0.2		0.15	0.22	0.06	0.1
D78		0.06	0.18	0.6	2.05	0.42	2.34	0.07	0.23
Cu1m		0.06	0.19	0.46	0.77	0.36	0.61	0.08	0.21
VarE	US variants	0.06	0.07	0.76	2.02	0.39	1.35	0.06	0.07
VarA		0.06	0.09	0.28	0.78	0.19	0.53	0.08	0.1
GLS		0.06	0.08	1.OO	2.52	0.72	2.31	0.06	0.1
D86	vvIBDV	0.83	2.35	1.04		0.87	2.41	1.01	2.54
Uk661		0.73	2.28	0.94	2.55	0.93	2.49	1.14	2.47
Brazil-1		0.79	2.35	1.14	2.59	0.86	2.49	1.13	2.5
Senegal		0.74	2.31	0.93	2.44	0.74	2.42	0.08	2.4
IC		0.17	0.38	1.12	2.5	0.84	2.41	1.04	2.53
France		0.75	2.35	1.14	2.31	0.89	2.31	0.91	2.48
China		0.6	2.24		2.51		2.4	0.97	2.5

Figure 1. Comparison of CRAb154-hyper phage & Mab 9-6 ability to detect 002/73 strain

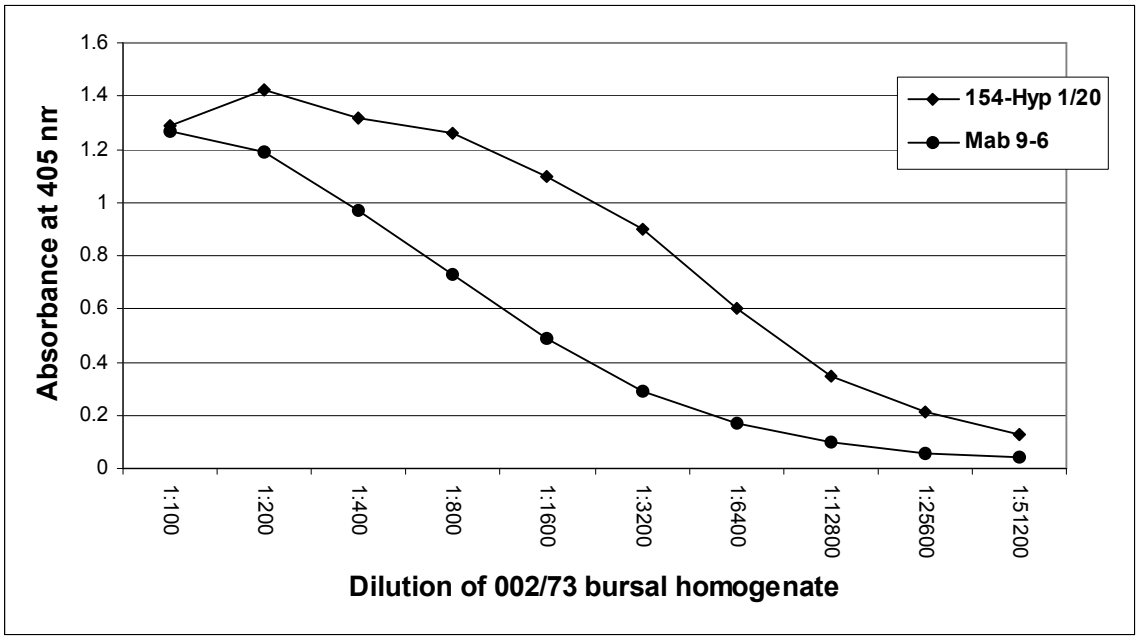


Figure 2. Comparison of CRAb154-hyper phage & Mab 9-6 ability to detect Variant E

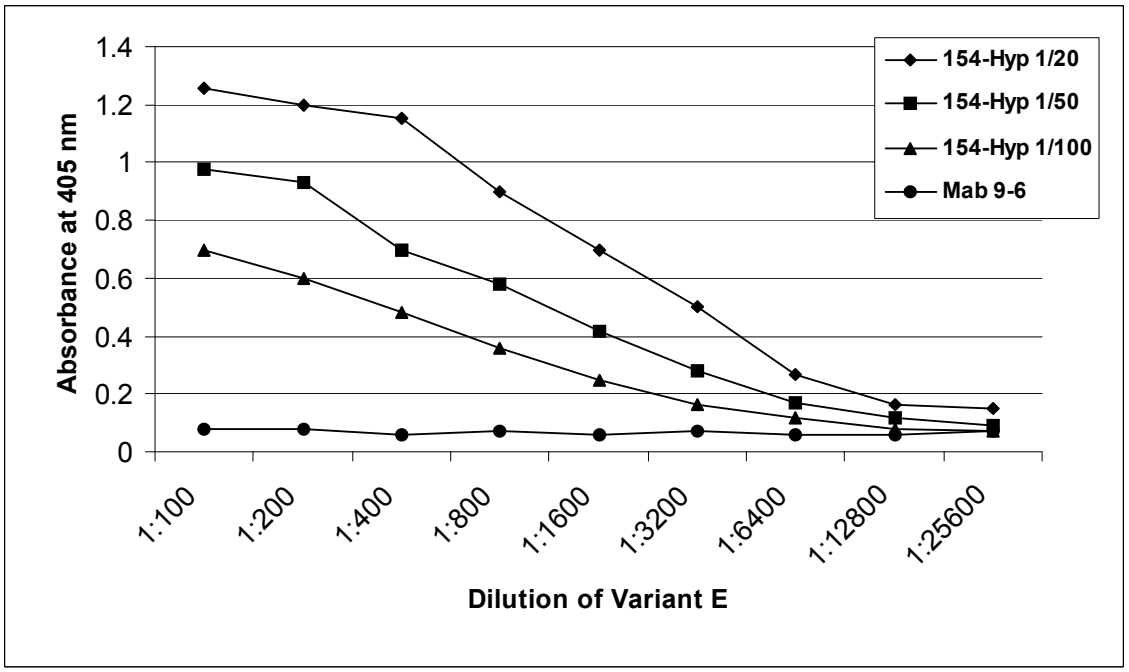


Figure 3. CRAb88 expressed as hyper phage show a degree of cross-reaction with classical strain 52/70

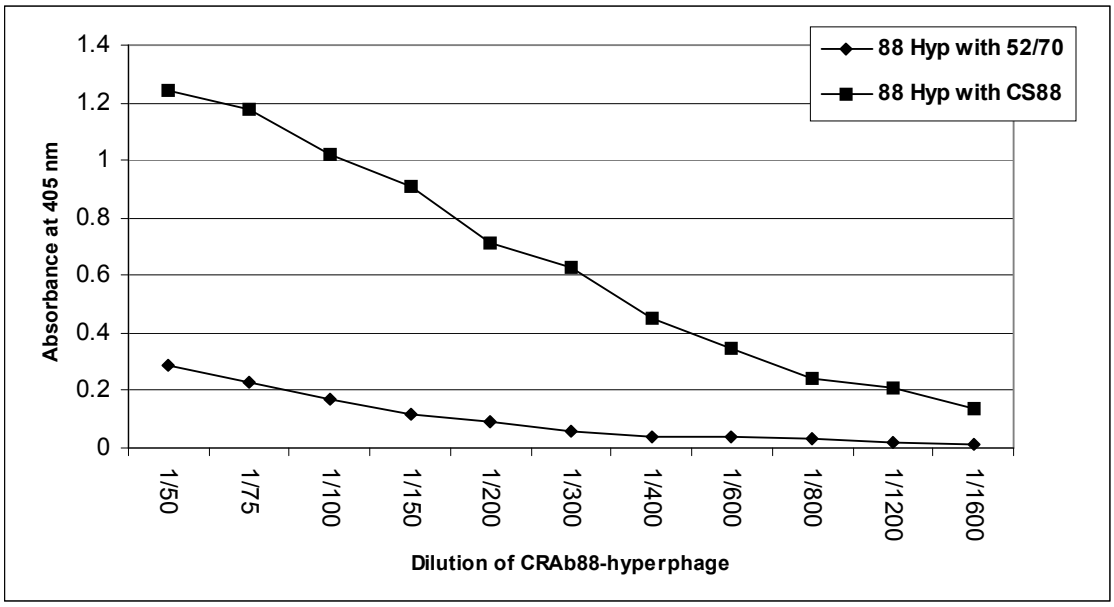


Figure 4. CRAb88-phage is able to differentiate vvIBDV from closely related 52/70 strain

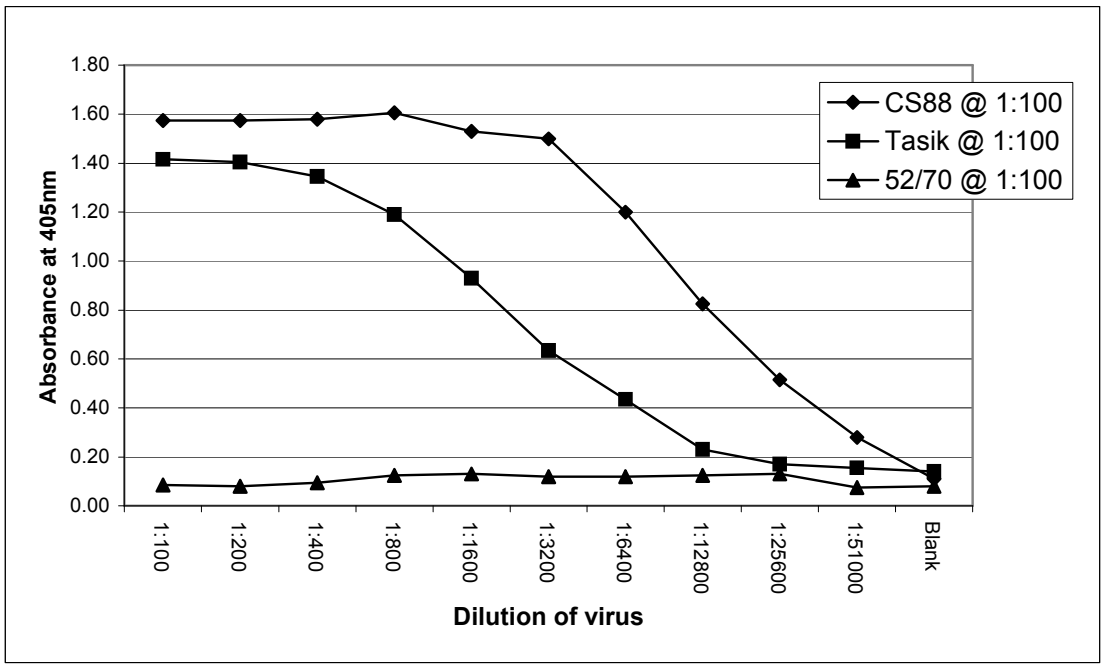
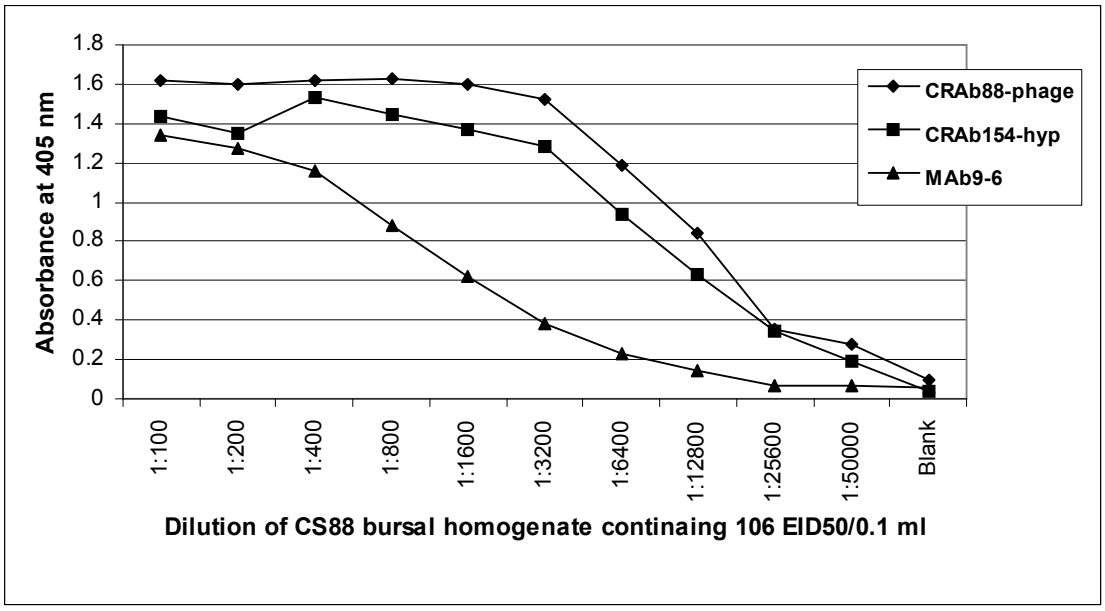


Figure 5. Comparison of CRAb88 phage & Mab 9-6 ability to detect CS88 antigen



Establish if there is interest in commercial kit development

IDEXX Laboratories, producers of a large number of diagnostic ELISA kits for poultry, were approached in September 2001 and offered CRA88. No response had been obtained. However, Synbiotics Corporation (San Diego, USA) expressed interest in CRAb88 late in 2002. They had evaluated & confirmed CRAb88 specificity and are currently looking at incorporating only CRAb88 into an ELISA that would be able to simultaneous differentiate all IBDV strains being either classical, variant or vvIBDV strains. In this case, the ELISA would consists of 3 – 4 monoclonal antibodies (for differentiation of USA variants) and CRAb88.

Replace E tag with B-tag and compare levels of expression

The system used to detect CRAb-SAb relies on a commercially available monoclonal antibody (anti E-tag). We originally intended to replace the E tag within CRAb88 with an alternative tag (B-tag) that is produced at AAHL in order to reduce the cost associated with the test. However, in the final test formulation CRAb88-phage was chosen over CRAb88-SAb. Thus this objective was modified as there was no need to replace the E tag contained within the soluble antibody with a B-tag.

Transfer of CRAb ELISA

All reagents, as well as the method for the CRAb ELISA, have been transferred to the Diagnostic Section of AAHL. The method has been evaluated and the specificity & sensitivity of the test were confirmed. All samples submitted to our Laboratory for IBDV diagnosis are now tested using the CRAb ELISA. Mab ELISA is also performed to determine whether IBDV detected is an Australian classical or variant strain.

Provisional patent for CRAb88

A full patent application that includes CRAb88 as well as other CRAbs was filed on June 6, 2002 (Australian Provisional Patent Application No PR5468).

4. Development of RFLP

4.1 Background

Using RFLP, Jackwood & Sommer (1999) showed that vaccine strains used in and outside of the USA belonged to one of 6 different RFLP groups. Analysis of strains obtained outside of the USA revealed that many IBDV strains fell into in RFLP group 6 including vvIBDV, however vvIBDV were found to contain a specific *Ssp*I cleavage site absent in classical or variant strains. Four Australian strains included in this study were found to produce a "new" RFLP pattern and possessed the *Ssp*I sight characteristic of vvIBDV (Table 2). As evident from Table 2, the four Australian IBDV strains produced an RFLP pattern identical to that of IBDV strains isolated from Korea. The aim was to determine the RFLP pattern of all Australian IBDV strains isolated to date using the method of Jackwood and Sommer (1999), and ato test if an *SspI* cleavage site was present in these strains. Comprehensive results of these studies have been published (Sapats & Ignjatovic, 2002).

4.2 Method

IBDV strains used

Australian IBDV strains used for RFLP analysis were: vaccine strains 002/73 and V877, classical field strains 06/95, 01/96, N1/99, N2/99, K-3, M-4, R-1, T-4, A-1, SS-1, and Y5-3 and variant field strains 01/94, 02/95, 03/95, 04/95, 08/95, C-1, H-1, and 05-5. Included also were IBDV samples used in the study of Jackwood & Sommer (1999) and designated Aus1 and Aus2 (Table 2). These were obtained from the same source (Fort Dodge, Castle Hill, Australia), and are designated B72270 in this study being Websters Infectious bursal Disease Vaccine (V877, live virus). Additionally, samples of another two batches of the same Australian vaccine were included (B07140 and B91080). Other strains used were overseas classical strain 52/70, vvIBDV strain CS88 and variant E.

Preparation of Viral RNA, cDNA synthesis and RFLP

RNA was extracted from IBDV positive bursal homogenate using RNeasy mini kits (Qiagen, CA, USA) and cDNA produced using a cDNA synthesis kit (Boehringer Mannheim, GmbH, Germany**).** Jackwood and co-workers (1997, 1998, 1999) did not publish the sequence of the primers used but they did provide the exact position of the of the viral genome they amplify in PCR and then use for RFLP analysis. Using this information along with an alignment of 162 IBDV sequences deposited in GenBank, , we selected two primers J1 & J2 (Sapats & Ignjatovic 2002) which where conserved in most IBDV strains, including Australian strain 002/73. These two primer sequences were expected to amplify a 743 base pairs (bp) fragment using the PCR. PCR products were purified using a PCR purification kit (Qiagen) and digested with three restriction enzymes *Bst*N1, *Mbo*I or *Ssp*I that were used in RFLP analysis by Jackwood *et al.,* (Table 2). Digested DNA was visualized on agarose gels, stained and sizes of the RFLP bands estimated by comparing them to DNA standards.

4.3 Results & Discussion

Primers J1 & J2 generated a PCR fragment of 743 base pairs t with all overseas strains available at AAHL (variants E and GLS, classical strains 52/70, 1/68 and APHIS and vvIBDV strain CS88) as well as with all Australian IBDV strains isolated to date including vaccine strains (result not shown). This PCR product was then digested with three restriction enzymes, *Bst*N1, *Mbo*I and *SspI*. Comparison of the RFLP patterns obtained for reference strains VarE, 52/70 & CS88 with those generated by Jackwood (Table 2) showed that the sizes of fragments generated by each enzyme were exactly the same as those of Jackwood.

As shown in Table 3, Australian IBDV strains could be classified into 12 distinct molecular groups. Group A1 contained vaccine viruses, groups A2 – A9 classical field strains and groups A10 - A12 variant field strains. None of the 12 groups identified were identical to any of the molecular groups identified previously. Jackwood and Sommer (1997; 1998; 1999) have shown that by using RT/PCR-RFLP vaccine strains prevalent in the USA could be placed into one of the six molecular groups (Table 2). Furthermore over 184 field isolates from 17 different countries belonged to an additional 41 molecular groups (Jackwood & Sommer, 1997; Jackwood & Sommer, 1998; Ture et al., 1998; Jackwood & Sommer 1999; Meir et al., 2001). Considering that Australian IBDV strains form an additional 12 molecular groups, clearly the existence of a such large number of molecular groups (in total at least 59) argues against the effective use of this method for IBDV strain differentiation. Our results showed that even minor differences between strains are detected by this method, which in reality are not related to any other antigenic, pathogenic or protective property of the IBDV strain analysed. For example, 11 field strains from NSW, WA and Qld which are very similar to vaccine viruses antigenically and at the nucleotide level (Sapats & Ignjatovic, 2000; Ignjatovic *et al*., 2002) could be classified into 9 distinct molecular groups.

Our results also showed that a specific *Ssp*I site that is used to predict a very virulent IBDV phenotype was absent from all Australian strains, contrary to the published finding of Jackwood & Summers, (1999). We obtained three virus samples from the same source as did Jackwood & Summers (1999); those samples being vaccine batches, with one sample being identical for both studies (B72270). In addition, the *Bst*N1 and *Mbo*I RFLP patterns obtained by us and by Jackwood for samples B72270 differed, indicating that the samples identified as Aus1 & Aus2 were in effect not Australian IBDV strains. One Australian strain (N1/99) contained an *Ssp*I site; this site was however located at a different position than that found in vvIBDV strains. It is known that cross-contamination of samples prior to PCR can occur necessitating the use of specialised PCR suites. To our knowledge, such stringent conditions were not applied in the study of Jackwood & Summers (1999).

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Table 2. Jackwood's IBDV molecular groups identified by RT/PCR-RFLP

Mol Group	Strain	Country	*SspI*	*BstNI* 424	350	209	172	154	139	119	*MboI* 480	403	362	234	229	112
1	DelA	USA	-	■			■			■				■	■	
1	1084A		-													
2	Del-E	USA	-	■			■			■		■			■	
2	MD		-													
2	80/03		-													
3	STC	USA	-				■	■	■	■			■		■	
3	VarVax	Vaccine	-													
4	228E	Vaccine	-			■	■	■		■			■		■	
4	D78		-													
4	D278		-													
4	Bur706		-													
4	Baxendal		-													
4	BurVac		-													
4	TAD C		-													
4	TAD F		-													
4	Gum		-													
4	NZ-A	NewZeal	-			■	■	■		■			■		■	
4	NZ-B	NewZeal	-													
5	Lukert	Vaccine	-	■			■			■	■				■	
5	Edgar		-													
5	Bursine		-													
5	BioBur W		-													
5	Avimune		-													
6	AK2	France	+	■			■			■			■		■	
6	FD1	Sth Africa	+													
6	FD2	Sth Africa	-													
6	S1	Spain	+													
6	N5	Netherland	+													
6	S293	Mexico	-													
6	S599	PortoRico	-													
New	FD9110	Korea	+	■			■			■			■		■	
New	FD9696	Korea	+													
New	Aus1	Australia	+													
New	Aus2	Australia	+													
New	Aus3	Australia	+													
New	Aus4	Australia	+													

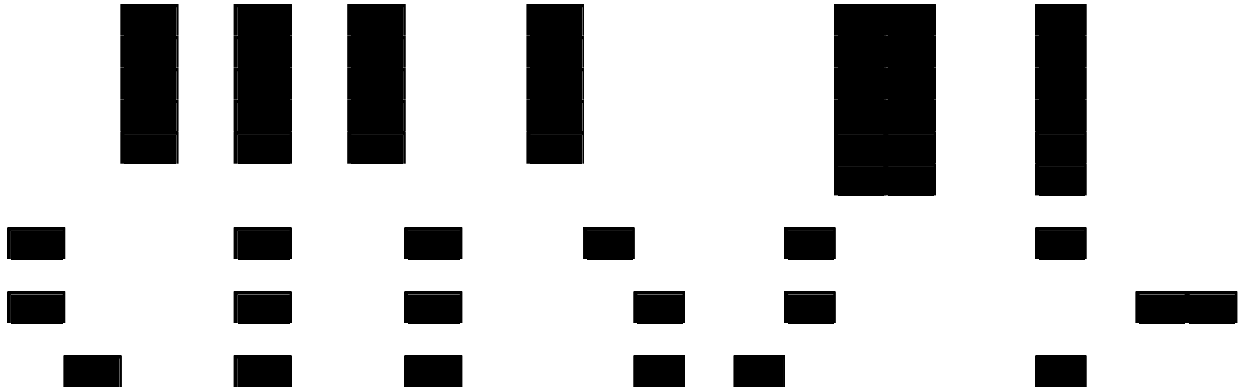
Adapted from Jackwood & Summers, (1999) Avian Diseases 43, 310 & web site: http://www.oardc.ohio-state.edu/ibdv,

SspI site is considered typical of vvIBDV strains

Table 3. RFLP pattern of Australian & representative overseas IBDV strains

Mol Group	Strain	Origin	*BstNI*								*MboII*										*SspI*				
			424	363	209	191	172	158	147	122	670	474	401	362	289	269	229	148	121	112	743	567	468	275	176
A1	002/73	Vaccine				■	■		■	■					■		■			■	■				
A1	V877	Vaccine				■	■		■	■					■		■			■	■				
A1	B07140	Vaccine				■	■		■	■					■		■			■	■				
A1	B72270	Vaccine				■	■		■	■					■		■			■	■				
A1	B91080	Vaccine				■	■		■	■					■		■			■	■				
A1	N2/99	NSW				■	■		■	■					■		■			■	■				
A2	06/95	NSW				■	■			■					■	■				■	■				
A3	M1	NSW				■	■		■						■		■			■	■				
A3	R-1	NSW				■	■		■						■		■			■	■				
A4	01/96	NSW				■	■			■					■		■	■			■				
A5	N1/99	NSW				■	■		■	■	■											■			■
A6	A-1	NSW				■	■			■					■		■			■	■				
A7	SS-1	NSW				■	■			■			■				■				■				
A8	K2	WA				■	■		■	■					■	■				■	■				
A8	T-4	WA				■	■		■	■					■	■				■	■				
A9	Y5-3	Qld				■	■		■	■			■			■					■				
A10	05-5	SA			■		■	■	■				■					■	■		■				
A11	C-1	Vic			■		■		■			■				■					■				

A12	**02/95**	**Vic**
A12	**01/94**	**Vic**
A12	**03/95**	**Vic**
A12	**04/95**	**Vic**
A12	**08/95**	**Vic**
A12	**H-1**	**Vic**
2	**Var E**	**USA**
6	**CS88**	**UK**
New	**52/70**	**UK**



5. Monitoring changes in local IBDV field isolates

5.1 Background

The aim was to monitor changes in local IBDV strains in order to detect any atypical changes, particularly those leading to an increase in virulence. For this reason, bursae were solicited from commercial flocks at different sites, including sites where IBDV had been previously isolated and two cases in which IBDV involvement was suspected.

5.2. Method

Field samples for IBDV isolation and their processing

Individual bursae collected from broilers were shipped on ice to our laboratory. Each bursa was weighed and a 10% w/v homogenate made in PBS. Homogenate was frozen and thawed 3 times, centrifuged at 3,000 rpm for 15 min and the aqueous phase collected. This supernatant was tested in an antigen ELISA using monoclonal antibody (Mab) 9-6 as a detecting antibody. If the sample was positive for IBDV antigen it was then re-tested in an antigen ELISA using a panel of four Mabs 9-6, 17-82, 44-18 and 39A.

Nucleotide sequencing

A hypervariable region (HVR) within the VP2 gene of IBDV has been previously identified between amino acids 211-340 and shown to contain conformational epitopes important for protection (Bayliss *et al.,* 1990; Heine *et al.,* 1991). Primers based upon highly conserved sequences located on either side of this region (B3 and B4) were synthesised and used in PCR to amplify the 211-340 region of field IBDV strains as previously described (Sapats &Ignjatovic, 2000). The cDNA obtained was sequenced and the translated amino acid sequences compared for all strains. Computer assisted programs (Clustal X) were used to align the amino acid sequences.

5.3 Results & Discussion

Characterisation of IBDV isolates from various commercial sites

Samples were obtained from19 broiler farms in the states of Victoria, NSW, SA & Qld and one layer farm in Qld (AH farm) (Table 4). Broilers were between 27 - 37 days of age, whereas layer the flock was 48 days-old. IBDV involvement was only suspected on two farms (GMF & AH farms). All field samples were tested initially in an antigen ELISA using Mab 9-6 and CRAb154 to determine if an IBDV antigen was present. Samples from 10 broiler farms were positive for IBDV. All samples positive for IBDV were also tested for cross-reaction with four Australian Mabs to determine if there was any antigenic change in these strains in comparison to vaccines. As shown in Table 4, IBDV strains from NSW (six strains designated 01/01, 03/01, 02/02, 03/02, 02/03 & 05/02) were all

classical like strains that were similar to vaccine strains 002/73 & V877. Two isolates from Qld (02/01 & 01/03) were also antigenically classical like strains. Two isolates from Vic (02/02 & 01/03) were variant like strains similar to IBDV variants isolated previously in Vic (02/95 & C-1 in Table 4).

One sample from each farm that contained the highest concentration of antigen by ELISA, was chosen as a representative isolate (Table 4) and used for sequencing. Included for sequencing was also the antigen negative sample from FF farm in SA. Sequencing confirmed the results obtained by Mabs and indicated that IBDV strains from NSW (01/01, 03/01, 02/02, 03/02, 02/03 & 05/02) and Old (02/01 & 01/03) were classical like strains that were similar to vaccine strains (Figure 6). The SA isolate (02/00) and two Vic (02/02 & 01/03) were variant like strains similar to IBDV variants isolated previously in Vic (Figure 6).

Overall the results have indicated that IBDV strains circulating on broiler farms in NSW, Qld and Vic have not changed antigenically or genetically in comparison to vaccine strains and strains isolated previously on broiler farms in these states. Therefore Australian strains remain a unique group of strains that can be differentiated genetically from IBDV strain of other countries. The results have also confirmed that variants are present in Vic and SA, and not in other States. In NSW and Qld only classical IBDV strains have been isolated thus far.

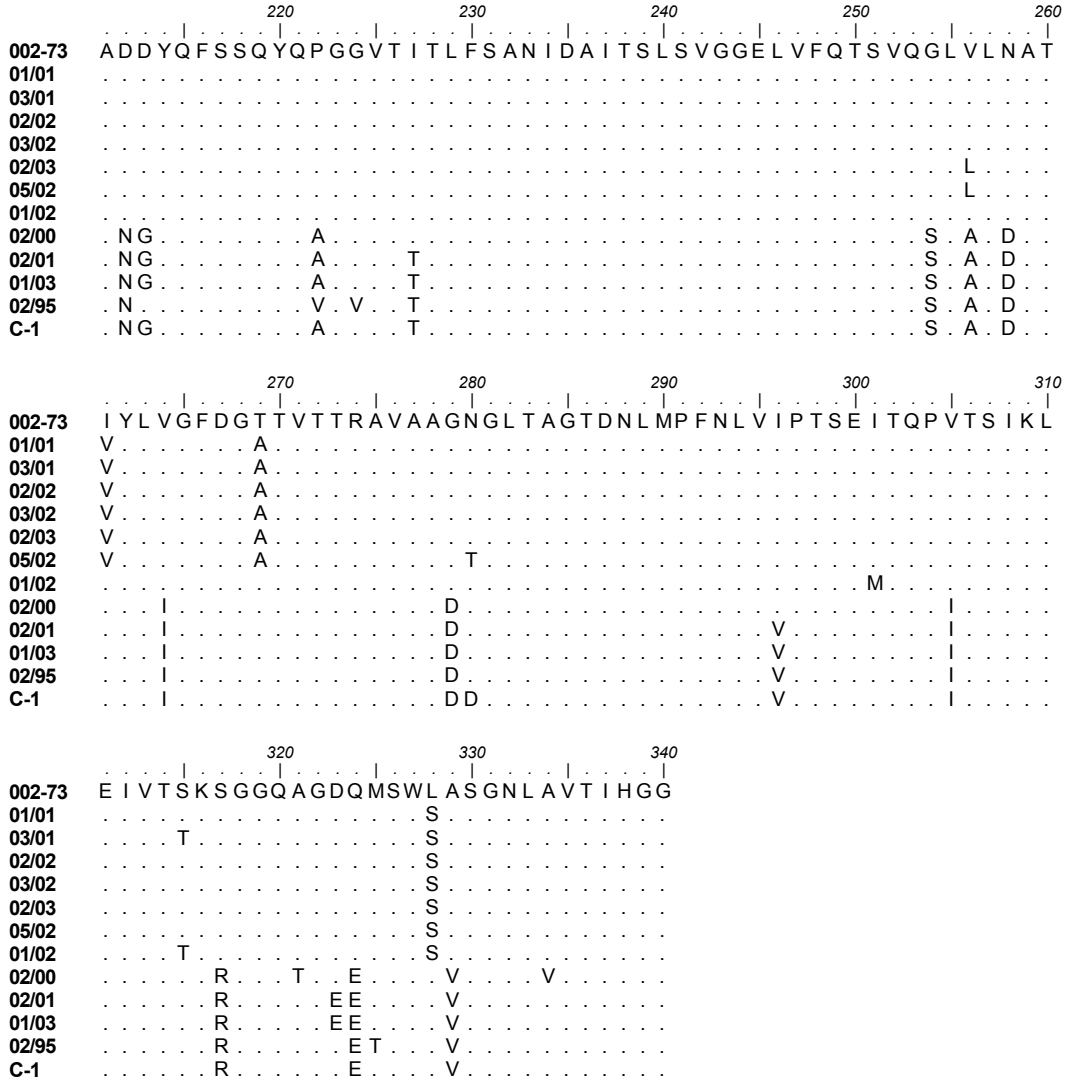
Table 4. Origin of samples collected for IBDV isolation during 2000/2003 and their reaction with Australian Mabs directed against the VP2 antigen

Origin of strains		Isolation			Strain	Reaction with Mab			
State	Farm	Date	Flock age (days)	No positive/ No tested		17-82	39A	44-18	9-6
Vic	R	07/01	30-34	0/2	NI				
	C	07/01	30-34	0/3	NI				
	L	07/01	30-34	0/4	NI				
	GFM*	10/01	35	1/1	02/01	+	-	-	+
	P	1011		0/3	NI				
	NNG	10/01		0/4	NI				
	L	02/03	30	2/2	01/03	+	-	-	+
SA	FF	28/6/00	28-33	2/2	02/00	-	-	-	-
NSW	D	03/01	30	1/1	01/01	+	+	+	+
	BA	5/01	41	1/1	03/01	+	+	+	+
	KL	09/02	34-37	2/2	02/02	+	+	+	+
	KPP	12/02	29	1/1	03/02	+	+	+	+
	AP	02/03	27-29	3/4	02/03	+	+	+	+
	M	12/02	30-32	0/9	NI				
	C	12/02	29	0/5	NI				
	G	12/02	33	6/6	05/02	+-	+-	+-	+-
Qld	B	09/02	33	4/6	01/02	+	+	+	+
	AH*	10/02	48L	0/5	NI				
	YF	02/03	33-34	7/14	04/02	+	+	+	+
Vaccine strains					002/73	+	+	+	+
					Bursavac live	+	+	+	+
					BursavacK 877	+	+	+	+
Australian					02/95	+	-	-	+

varinats	**C-1**	+	-	-	+

* IBDV involvement was suspected on the farm
+ or - = Reaction or no reaction with Mab in ELISA. NI = Not isolated. ND = Not done.

Figure 6. Alignment of deduced amino acid sequences of 10 IBDV strains isolated in this study. The complete deduced amino acid sequence of the hyper-variable region of VP2 of Australian vaccine strain 002/73 is shown from amino acid position 211 to 340. Below this sequence are aligned the deduced amino acid sequences for six IBDV isolates from NSW (01/01, 03/01, 02/02, 03/02, 02/03 &05/02), one (01/02) from Qld, two (02/01 & 01/03) from Victoria. & one (02/00) from SA. Included in the alignment are also the deduced amino acid sequences for two previously isolated variant strains 02/95 & C-1. For all strains only those amino acid residues that differ from 002/73 are shown.



6. OUTCOMES

:
(1) An ELISA for fast differentiation of vvIBDV strains that is available for use at AAHLs' Diagnostic Section.

(2) Conformation that no significant changes have occurred in circulating field strains and that Australian IBDV can still be differentiated from foreign IBDV strains.

7. References

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Plain English Compendium Summary

Project Title:	Diagnostic tools for differentiation of vvIBDV and characterisation of local IBDV strains
RIRDC Project No.:	CSA-15J
Researcher:	Jagoda Ignjatovic, Sandra Sapats and Gaylene Gould
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Objectives	(a) Develop ELISA & RFLP for differentiation of vvIBDV strains & (b) demonstrate that changes in local IBDV field isolates remain such that they can be clearly differentiated from all other overseas strains.
Background	Very virulent IBDV is exotic to Australia. A simple & rapid diagnostic test is needed in the case of an exotic incursion. In an earlier RIRDC project (CME97-06) a potential reagent specific for vvIBDV in ELISA was developed (CRAb88). Further evaluation of this reagent was needed. Restriction fragment length polymorphism (RFLP) was developed in the USA for the differentiation of IBDV strains & Australian strains were shown to be similar to vvIBDV strains. The usefulness of this method was also evaluated. It was also possible that vvIBDV like strains could emerge from local endemic strains & that monitoring of strains circulating in commercial poultry flocks would provide an early warning system.
Research	The specificity of CRAb88 was tested in two overseas laboratories against range of IBDV strains. RFLP method was introduced & tested with all Australian IBDV strains. IBDV field isolates were obtained from various commercial poultry sites and antigenic & genetic properties compared to other strains.
Outcomes	CRAb88 was shown to react only with vvIBDV detecting all vvIBDV regardless of their country of origin. An ELISA was subsequently developed & transferred to the diagnostic section of our laboratory. RFLP method was introduced, however by this method Australian IBDV strains belonged to 12 different groups which differed from other 47 groups identified among overseas strains. Thus RFLP is not suitable for simple & meaningful strain differentiation in Australia. No similarity between Australian & vvIBDV was detected by this method. Seven new IBDV isolates collected from broiler sites between 2001 & 2003 were similar antigenically &genetically to other Australian IBDV strains.
Implications	A simple and fast diagnostic test (ELISA) is now available in Australia for differential diagnosis of exotic vvIBDV incursion. Monitoring of local IBDV strains showed no major changes, in either their antigenicity or virulence.
Publications	Sapats S & Ignjatovic J. (2002). Restriction fragment length polymorphism analysis of the VP2 gene of Australian strains of infectious bursal disease virus. *Avian Pathology 31, 559-566* Ignjatovic J, Sapats S & Gould G. (2002). Characterisation of additional infectious bursal disease virus field isolates confirms existence of two distinct genetic groups in Australia. *Australian Veterinary Journal* 80, 689-694 Sapats SI, Heine HG, Trinidad L, Gould GJ, Foord AJ, Doolan SG, Prowse S & Ignjatovic J. (2003). Generation of chicken monoclonal antibody fragments that differentiate and neutralise infectious bursal disease virus (IBDV). *Archives of Virology* 148, 497-515