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**Detection of virulent
strains of Newcastle
disease virus
in chickens previously infected
with Australian strains of the
virus**

**A report for the Rural Industries Research
and Development Corporation**

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'Detection of virulent strains of Newcastle disease virus in chickens previously infected with Australian strains of the virus'

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Foreword

Emergency animal disease outbreaks result from the incursion into Australia of dangerous animal disease from other countries, or from unusually severe diseases that occur in Australia from time-to-time. All types of farmed terrestrial and aquatic livestock as well as fauna can be affected by such incidents, so Australia has developed programs to combat and control such emergency animal disease outbreaks. Virulent forms of Newcastle disease (ND), infectious bursal disease and avian influenza (AI) are poultry diseases that have potential to cause these types of outbreaks; indeed emergency outbreaks of ND and AI have occurred in Australia in recent years. Diagnostic and disease surveillance tests able to be used for rapid detection of these diseases are obvious pre-requisites for preparedness to combat outbreaks of these diseases.

The situation in Australia with Newcastle disease virus (NDV) is epidemiologically complex, as there are benign and disease causing forms of the virus naturally circulating in the national poultry flock. In addition it is now known that there are Australian NDV strains that can change, for unknown reasons, from being able to cause only mild disease to being able to cause severe disease ie emergency animal disease outbreaks. The aim of the project described in this report was to develop tests able to readily detect the presence of virulent (emergency) strains of NDV, and to readily differentiate them from the less virulent and benign forms of the virus that circulate all the time in parts of the Australian poultry industry. A specific aim was to be able to do this in flocks that had been naturally infected with benign viruses, an infection that naturally immunised them against the virulent disease. This immunity confers protection against the development of the disease, but the immune chickens can still be infected with the virulent virus. They are thus covertly infected with virulent virus and are consequently able to be a silent source for spread of this virus. The ability to detect such covertly infected flocks would greatly assist efforts to control outbreaks of virulent ND. This report describes the development of tests for this purpose and the assessment of the specificity, sensitivity and usefulness of the tests for diagnosis and surveillance of virulent ND.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

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Executive Summary

A virus capture ELISA (vELISA) for Newcastle disease virus was developed and assessed for detecting the presence of virulent Newcastle disease virus (NDV) in the tissues of chickens previously infected with lentogenic (immunising) strains of the virus. The performance of the vELISA was compared with that of virus isolation (VI) in embryonating chicken eggs and to the polymerase chain reaction (PCR) system. The vELISA was demonstrated to be specific for NDV and, in albeit limited number of tests, able to detect virulent NDV, but not avirulent strains of the virus. The test also detected NDV in the tissues of immune and non-immune chickens that were infected with virulent virus, though certain tissues, bone marrow and spleen, were better targets for testing than others. However, the vELISA was not as sensitive at detecting the virus as VI or PCR. Indeed in terms of sensitivity PCR was better than VI, which was better than vELISA. The vELISA was only about 50% as sensitive as VI, and about 44% as sensitive as PCR. The vELISA, in fact, failed to detect NDV in some infected chickens and therefore was not considered to be suitable as an individual bird test.

Independent assessment of the value of the test as a virulent ND (vND) diagnostic test was undertaken by the Australian Animal Health Laboratory (AAHL) diagnostic team. They took advantage of specimens collected during the outbreaks of vND in NSW from 1998-2000 and their experience during that outbreak. Their assessment was that the test worked, albeit less sensitively than alternatives, but that it failed to provide critical information about the pathogenicity of the virus detected. Such information was considered to be vital given the very complex situation that existed with NDV in Australia. They therefore felt that the vELISA provided only marginal benefits, at best, and that their efforts were better directed at developing rapid tests based on the real-time PCR platform as this would provide information concerning both the detection of NDV plus vital data on its virulence, in a shorter timeframe than the vELISA could be performed.

Introduction

The Australian poultry industry is currently free of a number of contagious diseases that severely compromise chicken farming in many countries eg. Newcastle disease (ND), virulent avian influenza (AI), very virulent infectious bursal disease and avian pneumovirus. Formal plans for the control and eradication of exotic ND and avian influenza exist in Australia, under the auspices of the AustVetPlan banner, and there are cost sharing arrangements between the Australian and State Governments and the various livestock industries for the funding of these programs. The currently accepted strategy for control of ND and AI is eradication; indeed formal AI eradication programs have been undertaken four times in the last twenty years and there have been a number of outbreaks of virulent ND in the past five years. Review of the AustVetPlan plan for ND and AI, and the other plans, occurs regularly and technical and scientific problems that present difficulties to the goal of eradication are identified. These reviews also consider whether changes to disease control concepts and strategies are necessary on the basis of scientific developments, and/or resources available to disease control agencies, and/or the opinions of the relevant livestock industries.

Lentogenic (non-pathogenic and low virulence) strains of NDV have been endemic in Australian poultry flocks since the late 1960s. Much is now known about these strains, particularly ways of differentiating such lentogenic strains from exotic types. It is also known that these endemic strains confer at least short term immunity in chickens to challenge with the most virulent exotic strains of NDV, and indeed one - the V4 strain or derivatives of it - is used internationally as a vaccine. However, the presence of natural infections with these strains in Australian poultry flocks creates potential difficulties to any exotic NDV control program. One of these problems - the fastest possible differentiation of lentogenic and virulent strains of the virus - has been intensively studied, as this was identified as a key element in the earliest possible detection of the presence of a virulent strain of the virus. Another problem is the identification of the presence of exotic strains of the virus in flocks previously infected with endemic strains of the virus. This aspect has not been examined in detail. Flocks infected with these local strains are partially or effectively immunised against development of clinical Newcastle disease (ND) following challenge with virulent strains, though they are not immune to infection with these virulent strains. Thus they can be 'silently' or covertly infected with the virulent virus, and consequently act as a potential source for multiplication and spread of this virus during a control program. Such an event needs to be detected quickly and efficiently so that appropriate disease control measures can be introduced. Consequently, there is a need to monitor such flocks during a control program and to identify, as early as possible, the presence of virulent strains. Currently this would be done by long established serological (hemagglutination inhibition) and/or virus isolation (in fertile eggs) testing, though each of these strategies has inefficiencies and difficulties, and certainly do not use some of the laboratory technologies (eg. ELISA, PCR) now routinely used in diagnostic and surveillance testing for other diseases. These newer technologies have considerable potential advantages in sensitivity, specificity and efficiency and in scope, and clearly have considerable relevance to the earliest possible detection of exotic NDV in flocks previously infected with local strains of the virus.

This clearly happened during the outbreaks of virulent ND that occurred in NSW during 1998, 1999 and 2000. Indeed, PCR testing to determine the virulence of NDV strains was used as a frontline diagnostic test during the outbreaks, with great benefit to disease control implementation. The use of PCR and nucleotide sequence testing in this way revealed hitherto undetected and unexpected information, and yielded dramatic results. It demonstrated (a) that the NSW outbreaks were caused by changed or mutated Australian lentogenic strains of the virus, and (b) a totally unexpected complexity or mixture of NDV strains within infected flocks (Westbury 2001). A number of flocks were found to be simultaneously infected with lentogenic and virulent virus, and with so-called transitional strains, strains that on nucleotide sequencing were intermediate between lentogenic and virulent viruses (Westbury 2001). This was absolutely new information about the disease and the virus that had never been described before, anywhere in the world. This has implications for concepts of how virulent ND would be controlled in Australia in the future, and on the laboratory

technology used to diagnose the disease and for methods of surveillance; simply, the 'goal posts moved'. A new phase in virulent ND virus control emerged and this centred on the need, the absolute need, to have nucleotide sequence information for virus characterisation, pathotyping, molecular epidemiological purposes and for virus surveillance. This development largely rendered superfluous the use of a virus capture ELISA for NDV as described and envisaged in the original proposal behind this project. The NSW outbreaks of virulent ND also saw an acceleration, for immediate disease control purposes, of the development and use of PCR and nucleotide sequence testing of ND viruses using virus grown in allantoic fluid, in paraffin embedded blocks and in fresh, chilled tissue samples. Thus the original concept of this project was overtaken by the NSW outbreaks of virulent ND.

Nevertheless, development and assessment of the virus capture ELISA (vELISA) occurred, with the specimens collected during outbreaks giving a unique opportunity to test and validate the technique under real outbreak conditions. Regrettably the vELISA test, when used under these circumstances, failed to detect virulent ND virus in some tissue samples of naturally infected chickens even though the virus could be isolated using chicken embryo inoculation, and detected PCR testing ie. it was of lower sensitivity. This lower sensitivity and the change in emphasis in the data required for ND diagnosis impinged upon the real usefulness of the vELISA for diagnosis and surveillance.

Methodology and Results

Virus capture ELISA (vELISA) for Newcastle disease virus

The vELISA was developed and assessed during this project used polyclonal NDV specific, egg yolk extracted antibody coated to the solid phase of the ELISA plate as the virus trapping or capture method. The antigen was either allantoic fluid containing ND virus, or tissue extracts or homogenates from NDV infected chickens. Capture of ND virus was detected by a panel of three monoclonal antibodies (mabs) to NDV. The three mabs were selected from a large number of NDV mabs held at AAHL. This specific panel of three mabs was chosen because at least one of the three reacted with all the ND virus strains with which they were tested. The range of ND virus strains tested in this way covered the spectrum of avirulent, lentogenic, mesogenic and velogenic, viscerotropic, neurotrophic and pneumotropic strains of NDV held at AAHL. Testing various times and incubation temperatures for each step optimised the test, by testing various blocking strategies to reduce background, and different methods for demonstrating binding of the detection mabs. Different enzyme substrates were also examined. Optimisation and cut-off values for positive and negative were determined using pure allantoic fluid harvested from 9-14 day old chicken embryos as well as similar allantoic fluid known to contain NDV. Positive:negative values were then determined using various tissue homogenates from SPF chickens, and from SPF chickens infected with either lentogenic or virulent strains of the virus.

Once the basic operational and computational values of the test were established, the test was used in chicken infection studies. It was compared with virus isolation as the 'gold' standard technique for detecting ND virus in tissue samples, and in some instances with PCR. Separate trials were conducted with three virulent virus strains, namely Herts 33/56, Texas GB and Australian virulent virus (vNSW) and with the Australian strain V4. These trials were conducted to demonstrate that the vELISA was able to detect ND virus in tissues of infected chickens, and the tissues of these chickens most likely to contain vELISA detectable virus. The results of these trials are presented in Tables 1 and 2.

Table 1. Number of chickens in three experimental groups in which virus was detected in the specified tissues using virus capture ELISA

	Herts 33/56	Texas GB	vNSW	Total
Brain	-	-	-	
Trachea	-	+(1/5)	-	1/15
Lung	+(2/5)	+(3/5)	+(2/5)	7/15
Blood-wbc	+(3/5)	+(2/5)	+(2/5)	7/15
Liver	-	-	-	
Pancreas	+(1/5)	-	-	1/15
Spleen	+(5/5)	+(5/5)	+(4/5)	14/15
Kidney	+(3/5)	+(3/5)	+(2/5)	8/15
Galt	+(1/5)	-	+(2/5)	3/15
Caecal tonsil	-	+(1/5)	-	1/15
Bone Marrow	+(5/5)	+(4/5)	+(5/5)	14/15

Notes:

- 5 chickens per virus group
- x/y = number of specimens in which virus was detected using vELISA/number of specimens tested
- control group infected with strain V4 ~ virus was not detected in any tissues of chickens killed five days after experimental infection
- chickens infected with virulent NDV tested when found dead or following euthanasia when affected clinically
- galt = gut associated lymphoid tissue.

This trial demonstrated that the vELISA failed to detect NDV in the group infected with strain V4 when these chickens were tested five days after experimental infection. The vELISA detected virulent virus in experimentally infected chickens, though the virus was detected more frequently in some tissues than others. The trial also demonstrated that bone marrow and splenic tissue samples appeared to be the tissues of choice, for each of the virulent virus strains used. However, kidney, lung and buffy coat cells were also potentially valuable samples

Table 2. Comparison of the results obtained using vELISA and virus isolation in chicken embryos

	Herts		Texas GB		vNSW		V4	
	Elisa	VI	Elisa	VI	Elisa	VI	Elisa	VI
Brain	-	-	-	3/5	-	3/5	-	-
Trachea	-	4/5	1/5	3/5	-	3/5	-	3/5
Lung	2/5	4/5	3/5	5/5	2/5	4/5	-	2/5
Blood	3/5	4/5	2/5	4/5	2/5	3/5	-	-
Pancreas	1/5	1/5	-	-	-	-	-	-
Spleen	5/5	5/5	5/5	5/5	4/5	5/5	-	2/5
Kidney	3/5	4/5	-	-	2/5	4/5	-	-
Galt	1/5	3/5	1/5	4/5	-	-	-	-
Caecal tonsil	-	2/5	1/5	4/5	5/5	5/5	-	2/5
Bone Marrow	5/5	5/5	4/5	5/5	5/5	5/5	-	2/5
Total +/50	20	32	19	34	17	31	0	10
% detection	40%	64%	38%	68%	34%	62%	0%	20%

Notes:

- 5 chickens per group
- VI = virus isolation
- x/y = number of chickens detected to be infected using either vELISA or virus isolation
- galt = gut associated lymphoid tissue
- % detection = percentage of chicken samples detected to be infected using either vELISA or virus isolation

This comparison demonstrated that the vELISA was not as sensitive as virus isolation in chicken embryos at detecting NDV when assessed using all tissue types. This was not an unexpected result. However, in selected tissues such as spleen and bone marrow the detection rates using vELISA and virus isolation were comparable – for spleen (vELISA 14/14, vi 15/15) and bone marrow (vELISA 14/15,vi 15/15). The V4 group of chickens were infected as judged by virus isolation, though this was not detected by vELISA.

This trial provided very encouraging results, particularly that the vELISA could detect virulent virus in the tissues of chickens that died from the disease or were severely affected by it, though some tissues were better candidates for testing than others. Specifically, it demonstrated that spleen and bone marrow were potentially important targets for future testing. Importantly, it also demonstrated that the vELISA failed to detect strain V4 and thus it seemed likely on the basis of these early results that the concept that the vELISA would detect virulent virus and not avirulent or lentogenic virus could be valid. The trial also suggested that the vELISA could be a useful screening or group test for detecting virus, particularly when certain tissues were used, as the detection rate in these tissues using the vELISA was greatly inferior to that of virus isolation.

The next series of tests involved challenge of chickens immune to the development of clinical ND with virulent strains of the virus. The protocol for this work was as follows:

- 3-4 week old SPF chicken were immunised by the oro-nasal route with at least 10^6 EID₅₀ of V4 strain of the virus;
- the chickens were bled 14-21 days after immunisation to check for specific serum antibody to the virus;
- the chickens were challenged when all birds had a haemagglutination inhibition antibody titre of at least 2^3 ;

- each immune chicken was challenged by the oro-nasal route with a dose of virulent virus demonstrated to induce lethal disease in non-immune chickens;
- tissues of chickens were tested between 2 and 20 days after challenge with virulent virus; and
- the tissues collected from sampled chickens – blood, lung, spleen, kidney and bone marrow – were those found in earlier tests to be most likely to have detectable challenge virus.

The results of this trial are presented in Table 3.

Table 3. Detection rate of NDV in certain tissues by vELISA following challenge of immune chickens with virulent virus Herts 33/56

	Days after challenge with virulent virus					
	0	2	4	5	7	10
Blood - wbc	-	-	-	1/4	-	-
Lung	-	-	1/4	2/4	2/4	1/4
Spleen	-	-	2/4	4/4	¾	2/4
Kidney	-	-	-	2/4	2/4	1/4
Bone marrow	-	-	2/4	4/4	4/4	2/4

Notes:

- wbc = white blood cells
- x/y = number detected as infected with NDV/number tested

This study demonstrated that NDV virus could be detected using the vELISA in target tissues of immunised chickens when tested up to ten days after challenge. It also demonstrated that the virus detected, based on molecular testing, was the virulent virus Herts 33/56.

The results above prompted a series of tests to assess the performance of the test, though this time the sampling period was extended to 20 days. The results of these tests, using different challenge strains, are presented in Tables 4 to 7.

Table 4. Detection rate of NDV in certain tissues by vELISA following challenge of immune chickens with virulent virus Herts 33/56

	Days after challenge with Herts 33/56								Total
	4	5	7	10	12	14	16	19	
Wbc	1/4	1/4	-	-	-	-	-	-	2/32
Lung	-	1/4	-	2/4	-	-	-	-	3/32
Spleen	2/4	4/4	3/4	3/4	2/4	-	1/4	-	15/32
Kidney	-	1/4	2/4	2/4	-	-	-	-	5/32
Bone marrow	3/4	3/4	4/4	2/4	-	1/4	-	-	13/32
Total /20	6	10	9	9	2	1	1	-	

Notes:

- wbc = white blood cells
- x/y = number detected as infected with NDV/number tested

Table 5. Detection rate of NDV in certain tissues by vELISA following challenge of immune chickens with virulent virus Texas GB

	Days after challenge with Texas GB								Total
	4	5	7	10	12	14	16	19	
Wbc	1/4	-	1/4	-	-	-	-	-	2/32
Lung	1/4	2/4	1/4	1/4	-	-	-	-	5/32
Spleen	3/4	4/4	4/4	2/4	2/4	-	1/4	2/4	16/32
Kidney	1/4	2/4	-	-	-	-	-	-	3/32
Bone marrow	3/4	2/4	2/4	4/4	4/4	1/4	-	1/4	17/32
Total /20	9	10	8	7	6	1	1	3	

Notes:

- Wbc = white blood cells
- x/y = number detected as infected with NDV/number tested

Table 6. Detection rate of NDV in certain tissues by vELISA following challenge of immune chickens with virulent virus vNSW

	Days after challenge with vNSW								Total
	4	5	7	10	12	14	16	19	
Wbc	1/4	2/4	-	-	-	-	-	-	3/32
Lung	1/4	2/4	-	-	-	-	-	-	3/32
Spleen	-	1/4	3/4	-	-	-	1/4	1/4	8/32
Kidney	-	2/4	1/4	2/4	1/4	-	-	-	4/32
Bone marrow	3/4	2/4	3/4	-	3/4	-	-	2/4	13/32
Total /20	5	9	7	2	4	-	1	3	

Notes:

- Wbc = white blood cells
- x/y = number detected as infected with NDV/number tested

Table 7. Cumulative total of number of specimens in which NDV was detected by vELISA in the three challenge experiments above

	Days after challenge with virulent virus								Total/160
	4	5	7	10	12	14	16	19	
Wbc	3	3	1	-	-	-	-	-	7 (4%)
Lung	2	5	1	3	-	-	-	-	11 (7%)
Spleen	5	9	10	7	4	-	3	3	41(25%)
Kidney	1	5	3	2	1	-	-	-	12 (7%)
Bone marrow	9	7	9	6	7	2	-	3	43(27%)
Total/60	20	29	24	18	12	2	3	6	114
%detection	33	48	40	30	20	3	5	10	

The cumulative results indicated that the chances of detecting NDV using the vELISA were greatest between four and ten days after experimental infection. The tissues of choice were, at least with the virulent virus strains used in these tests, spleen and bone marrow.

The sensitivity of the test needed to be compared with results obtained using virus isolation attempts. The results of these studies are presented in Tables 8 to 11.

Table 8. Detection rate of NDV in certain tissues by virus isolation following challenge of immune chickens with virulent virus Herts 33/56

	Days after challenge with Herts 33/56								Total
	4	5	7	10	12	14	16	19	
Wbc	2/4	3/4	2/4	-	-	-	-	-	7/32
Lung	1/4	3/4	2/4	3/4	-	1/4	-	-	10/32
Spleen	3/4	4/4	4/4	4/4	3/4	1/4	2/4	-	21/32
Kidney	-1/4	2/4	4/4	4/4	-	-	-	-	11/32
Bone marrow	4/4	4/4	4/4	4/4	2/4	3/4	-1/4	-	22/32
Total/20	11	16	16	15	5	5	3	-	71

Notes:

- Wbc = white blood cells
- x/y = number detected as infected with NDV/number tested

Table 9. Detection rate of NDV in certain tissues by virus isolation following challenge of immune chickens with virulent virus Texas GB

	Days after challenge with Texas GB								Total
	4	5	7	10	12	14	16	19	
Wbc	1/4	2/4	3/4	1/4	-	-	-	-	7/32
Lung	2/4	4/4	3/4	2/4	-	1/4	-	-	12/32
Spleen	4/4	4/4	4/4	4/4	3/4	1/4	2/4	3/4	25/32
Kidney	3/4	4/4	2/4	-	1/4	-	-	-	10/32
Bone marrow	4/4	4/4	4/4	4/4	4/4	2/4	-	2/4	24/32
Total /20	14	18	16	11	8	4	2	5	78

Notes:

- Wbc = white blood cells
- x/y = number detected as infected with NDV/number tested

Table 10. Detection rate of NDV in certain tissues by virus isolation following challenge of immune chickens with virulent virus NSW

	Days after challenge with vNSW								Total
	4	5	7	10	12	14	16	19	
Wbc	2/4	3/4	1/4	2/4	-	-	-	-	8/32
Lung	3/4	4/4	3/4	1/4	-	-	-	-	11/32
Spleen	3/4	4/4	4/4	3/4	2/4	2/4	2/4	1/4	21/32
Kidney	2/4	4/4	2/4	1/4	2/4	-	1/4	-	12/32
Bone marrow	4/4	4/4	4/4	3/4	3/4	2/4	1/4	2/4	23/32
Total /20	14	19	14	10	7	4	4	3	75

Notes:

- Wbc = white blood cells
- x/y = number detected as infected with NDV/number tested

Table 11. Cumulative total of number of specimens in which NDV was detected by virus isolation in the three challenge experiments above

	Days after challenge with virulent virus								Total/160
	4	5	7	10	12	14	16	19	
Wbc	5	8	6	3	-	-	-	-	22(14%)
Lung	6	11	8	6	-	2	-	-	33(21%)
Spleen	10	12	12	11	8	5	6	4	68(42%)
Kidney	6	10	8	5	3	-	1	-	33(20%)
Bone marrow	12	12	12	11	9	7	2	4	69(43%)
Total/60	39	53	46	36	20	14	9	8	225
%detection	65	88	77	60	33	23	15	13	

These results demonstrated that the chances of detecting NDV by vELISA were greatest between days four and ten after experimental infection. The tissues of choice were spleen and bone marrow. Importantly, virus isolation was more sensitive than vELISA in detecting NDV in the specimens.

These results are summarised across all sample types in and across all sample types in Table 12.

Table 12. Detection rate (%) of NDV using vELISA and virus isolation on tissue specimens collected at specified times after experimental infection of ND immune chicken with virulent strains of the virus

a. Comparison of vELISA and virus isolation on samples collected on certain days after infection using all tissue samples

Days after infection:	4	5	7	10	12	14	16	19
vELISA	33%	48%	40%	30%	20%	3%	5%	10%
Virus isolation	65%	88%	77%	60%	33%	23%	15%	13%
Comparative sensitivity	51%	54%	52%	50%	60%	13%	33%	77%

b. Comparison of vELISA and virus isolation on defined tissues collected on all days specimens were collected

Tissue type:	WBC	Lung	Spleen	Kidney	Bone marrow
vELISA	4%	7%	25%	7%	27%
Virus isolation	14%	21%	42%	20%	43%
Comparative sensitivity	28%	33%	59%	35%	63%

Using virus isolation as the ‘gold’ standard method indicates that the vELISA is less sensitive in detecting NDV than virus isolation when compared on the basis of all samples collected on specified days after infection, and when the different tissues were compared. The vELISA never achieved greater than a 63% comparative sensitivity in these tests (excluding the specimens collected on day 19 on the basis of relatively small numbers). In addition, its detection rate obviously declined in comparison to virus isolation after day 12 (again excluding specimens collected on day 19), perhaps associated with a smaller quantum of virus in these samples as a result of an immune response in the chickens.

The virus detected using the vELISA in these tests was assumed to be virulent virus based on the earlier finding that the vELISA failed to detect strain V4 in the specified tissue samples in chickens experimentally infected with that virus. To confirm this, every third virus isolate in each of the challenge experiments described above was checked for virulence by molecular pathotyping using the technique described in the OIE Manual of Standards for Diagnostic Tests and Vaccines (2000). All these isolates were found to be of the virulent type.

Comparison of the relative sensitivity of the vELISA, virus isolation and the polymerase chain reaction (PCR) in detecting NDV in tissue samples was also undertaken. Specimens collected during the tests where Texas GB was used as the challenge virus were selected for this purpose, as testing all samples collected by PCR was considered to be impractical. This proved to be a more difficult task than originally envisaged because of what appeared to inherent error-prone nature of the test when

used directly on tissue samples. Nevertheless results were obtained and these are recorded below in Table 13.

Table 13. Detection rate of NDV in certain tissues by polymerase chain reaction (PCR) testing following challenge of immune chickens with virulent virus Texas GB

	Days after challenge with Texas GB								Total/160
	4	5	7	10	12	14	16	19	
Wbc	2/4	3/4	3/4	2/4	-	-	-	-	10/32
Lung	3/4	4/4	3/4	2/4	1/4	1/4	-	-	14/32
Spleen	4/4	4/4	12/4	4/4	3/4	2/4	3/4	3/4	27/32
Kidney	3/4	4/4	2/4	2/4	2/4	1/4	-	-	14/32
Bone marrow	4/4	4/4	4/4	4/4	4/4	3/4	2/4	2/4	27/32
Total/60	16	19	16	14	10	7	5	5	92
%detection	80	95	80	70	50	35	25	25	

Table 14. Comparison of detection rate (%) of NDV using vELISA, virus isolation and PCR on certain samples collected at specified intervals after infection of immune chickens with the virulent ND virus strain Texas GB

a. Comparison of three methods on certain days after infection using all tissue samples

	Days after infection	4	5	7	10	12	14	16	19
		All tissues	vElisa	33%	48%	40%	30%	20%	3%
	Virus isolation	65%	88%	77%	60%	33%	23%	15%	13%
	PCR	80%	95%	80%	70%	50%	35%	25%	25%
Comparative Sensitivity	Elisa vs Isolation	51%	54%	52%	50%	60%	13%	33%	77%
	Elisa vs PCR	42%	51%	50%	43%	33%	9%	20%	40%
	Isolation vs PCR	82%	93%	96%	86%	66%	66%	60%	52%

b. Comparison of three methods on defined tissues collected on all days specimens were collected

		WBC	Lung	Spleen	Kidney	Bone marrow
All days of tests	vELISA	4%	7%	25%	7%	27%
	Virus isolation	14%	21%	42%	20%	43%
	PCR	31%	44%	84%	44%	84%
Comparative sensitivity	vELISA vs isolation	28%	33%	59%	35%	63%
	Elisa vs PCR	13%	16%	30%	16%	32%
	Isolation vs PCR	45%	48%	50%	45%	51%

This data clearly indicated that the order of sensitivity in detecting NDV in samples, from most to least sensitive, was PCR > virus isolation > vELISA, a not unexpected result. During the period four to ten days after infection with virulent virus the detection rate by vELISA was 50% to 54% of the detection rate by isolation, and 42% to 51% of the detection rate by PCR. These sensitivity differences are also evident when detection rates in the tissues are compared. PCR testing is clearly the most sensitive way of detecting NDV in tissue samples, although it is a difficult and error prone technology, particularly when a nested PCR system has to be used. Indeed, it is difficult to contemplate using nested PCR testing of tissue samples as a routine diagnostic test for virulent ND because of the inherent difficulties in consistently obtaining valid results, and the ever-present possibility of contamination of the test, despite the best efforts of operatives to avoid such an event. New systems based on the real-time PCR process (so-called TaqMan assays) have overcome many of the difficulties of conventional PCR, particularly those associated with the use of nested systems, and these are the way of the future.

Transfer of vELISA for detection of NDV to AAHL's diagnostic group

The results obtained using the vELISA to detect NDV in the tissues of chickens, either immune or non-immune, prompted transfer of the test to the AAHL's diagnostic group. The group was in the process of testing samples collected from chickens during the outbreaks of virulent ND and had samples stored from earlier outbreaks in NSW in 1998, 1999 and 2000. These samples were tested using the vELISA and the results compared to those obtained using virus isolation and PCR. An immediate problem was encountered because of higher than expected background readings in tissues collected from commercial chickens known not to be infected with NDV. The parameters of the experimental test had been calculated on results obtained with SPF chickens. This required re-calculation of these positive/negative threshold parameters using commercial chickens. The protocol of the test eg. blocking steps and reagents, also had to be adjusted to diminish the background levels when tissues from commercial chickens were used in the test. Following these modifications, the vELISA was used on field samples that had already been tested for the presence of NDV by other means. The results obtained were in line with the results obtained during the experimental studies ie. the sensitivity results were of the same order in that vELISA was generally about 50% as sensitive as virus isolation in detecting the presence of virulent ND virus in samples of clinically affected chickens. However, it became evident during this testing that mere detection of NDV was the beginning rather than the end of ND diagnosis. The presence of low virulence, and virulent viruses in a number of flocks during the NSW outbreak, and the additional complications of the so-called intermediate or transitional strains of the virus in some of these same flocks, meant that molecular characterisation of the virus had become relatively more important than hitherto. Indeed it could be said to have become essential.

Thus the emphasis in NDV diagnosis switched to the most rapid possible demonstration of the pathogenicity of the virus. Molecular characterisation is the best tool for this task so diagnostic efforts are now heavily directed at molecular detection and characterisation of the virus, with other tests providing a supportive role. Thus the diagnostic group found the results obtained using the vELISA to be merely a signpost that NDV was present but not providing critical information about the virus on which diagnostic decisions could be based, despite the data that indicated that the vELISA only detected virulent virus. Doubts remains about the overall validity of this concept as the basis of the claim rests on data obtained with one avirulent strain and three virulent strains of the virus. The diagnostic requirement is that pathotyping must be carried out. It would be very difficult to obtain sufficient data using a large number of strains to validate the concept that the vELISA definitively only detected virulent virus. New developments in molecular pathotyping using real-time PCR have the potential to enable acquisition of this information within two to three hours of the arrival of a specimen in the laboratory. For this reason, diagnostic practitioners think that the need for a vELISA of the type examined in this project has past. The sensitivity is too low, and results providing the critical information about the pathogenicity of the virus as well as its presence in the sample can be obtained using new technology in the same timeframe as that required to undertake an ELISA test.

Discussion

A virus capture ELISA (vELISA) able to detect NDV in tissue samples of chickens infected with the virus was developed. The ELISA used a standard virus capture format of virus capture with a polyclonal chicken, yolk derived antibody, detection of virus using a pool of three monoclonal antibodies to NDV and an anti-mouse antibody (mab) conjugate. The monoclonal antibodies were chosen for their ability to bind to a range of ND virus strains held in the NDV repository of AAHL. They were chosen because they were the mabs that provided the best possible reactivity with the ND virus strains tested in the laboratory screening process. Background levels in chickens not infected with ND virus were determined, though it was necessary during the project to recalculate the figure determined using SPF chickens as it proved to be unworkable when tissues from commercial chickens were used in the test.

The test was found to be useable and to provide specific results. An important early finding was that the vELISA failed to detect NDV in chickens known to be infected with strain V4 of the virus. This suggested that only virulent virus was detected, presumably because virulent virus was present in higher quantities in the tissues of infected chickens. This observation needs to be extended, if further development of the test is to be undertaken, by testing chickens infected with lentogenic strains of the virus, and the so-called transitory strains of the virus ie. the strains that only require one amino-acid change at the cleavage site of the fusion protein to become fully virulent.

The vELISA was found able to detect virulent ND virus in the tissue samples of chickens clinically affected by virulent ND virus strains and in the tissues of ND immune chickens challenged with virulent virus, even though these chickens were sub-clinically infected. The virus was most readily detected in the immune chickens between 4 and 12 days after challenge with virulent virus, though this occurred in some chickens up to 19 days after challenge. Bone marrow and the spleen of challenged chickens were the tissues from which the virus was most likely to be detected.

However, the vELISA was demonstrated to be significantly less sensitive in detecting virus in controlled tests than virus isolation and PCR testing. This was not an unexpected result as virus isolation and PCR involve virus or, virus genetic material, amplification. The vELISA was only about 50% as sensitive at detecting ND virus as virus isolation in the period 4 to 12 days after infection, and, on average, about 44% as sensitive as PCR in detecting virus in this time period. There was a trend for it to become even less sensitive after this time period, probably because of diminishing quantities of the tissues caused by the immune response of the chicken to the virus and the lack of an amplification cycle in the test. The test would therefore certainly not detect all NDV infected chickens, be they clinically or sub-clinically infected with the virus. It could therefore not be considered an individual bird test, but, rather, a flock-screening test.

Independent testing of the vELISA by the diagnostic team at AAHL revealed some generic problems with the test. The paradigm of ND diagnosis in Australia has shifted as a result of the outbreaks of virulent ND in NSW from 1998. An unexpected complexity developed as a result of this outbreak. We now have the concept of virulent ND caused by an exotic virulent virus introduced from another country, and virulent virus caused by Australian strains that have become virulent by some means. We also have molecular complexity amongst ND virus strains within Australia – we have avirulent, lentogenic, virulent strains and strains that on molecular characterisation are almost virulent. This complexity and the need to differentiate Australian strains from others, has put almost total emphasis in diagnosis and surveillance on molecular characterisation of ND virus strains. Molecular characterisation needs to be undertaken as quickly as possible and this is best done, currently, using virus strains propagated in chicken embryos ie. following virus isolation. Use of a real-time PCR platform offers the opportunity to do this directly from tissues within 2-3 hours of receiving the strain at a laboratory. This is where the emphasis in ND diagnosis in Australia is now heading. These requirements have made the use of the vELISA somewhat redundant as the results obtained from it indicate only the presence of NDV, until more substantial information can be obtained about the

specificity of the results ie. whether the vELISA definitively only detects virulent virus. This would require a very substantial amount of additional work. Furthermore, virus isolation is currently necessary for conventional PCR testing to proceed as such testing of chicken tissues is problematic because of non-specific inhibition of the PCR testing when tissues are used, and the likelihood of laboratory cross contamination when nested PCR systems are used on tissue samples. This becomes more likely when workloads in PCR laboratories become high and intensive. Thus surveillance is probably currently better done using virus isolation in chicken embryos, virus detection by hemagglutination and haemagglutination inhibition tests, followed by PCR and nucleotide sequencing testing of ND virus in the allantoic fluid of the embryo. In the near future it likely that this system will be replaced by real-time PCR testing of tissue samples, as is now done with testing for Australian bat lyssavirus.

vELISA and allantoic fluid

The early development stage of the vELISA involved testing normal allantoic fluid of 9-14 day old chicken embryos, and similar allantoic fluid containing NDV. These tests provided the first indication that NDV could in fact be detected using the developmental vELISA. However this was not the purpose for which the test was envisaged. Nevertheless, there has been interest in the use of the test for this purpose and reagents used in the test have been transferred to the virology group at the NSW Elisabeth Macarthur Agricultural Institute (EMAI) as scientists at that Institute expressed interest in using the procedure to test for the presence of NDV in allantoic fluid. AAHL has not contemplated using the test in this way, being satisfied with currently available tests for this purpose – conventional haemagglutination (HA) test in microtitre plates or the rapid HA plate test using concentrated chicken red blood cells, a test that can detect the presence of HA activity in allantoic fluid within 10 to 15 seconds. Nevertheless the vELISA may offer some advantages for large scale testing of chicken embryos for NDV and work at EMAI may explore this possibility.

Implications

A vELISA for NDV was developed and it was able to detect NDV in the tissues of infected chickens, though not as effectively as techniques such as virus isolation and PCR. The complexity of the NDV situation in Australia is such that the paradigm of ND diagnosis in Australia has changed and this change has made the approach taken in the development of the vELISA during this project somewhat obsolete as there is now a need for vital and rapid virulence testing of the virus, and for additional molecular epidemiological information to help determine the source of the virus. This type of information cannot be effectively be provided by ELISA tests. Technology development and the natural history of NDV in Australia has caused the value and relevance of ELISA testing, in the way originally envisaged in the project, to be diminished. In plain language, the technique has been superseded.

Recommendation

In light of the above, it is recommended that no further development of the vELISA occur as tests for the same purpose (detection of NDV) but with greater sensitivity and utility and usefulness are now available or under development and being assessed.

Reference

Westbury, H.A. (2001) Newcastle disease virus: an evolving pathogen? *Avian Pathol.* **30**: 5-11.