FINAL REPORT TO
BUREAU OF RURAL SCIENCES

1997/98 RESEARCH PROJECT

THE DEVELOPMENT OF A RAPID FIELD TEST FOR MEASURING THE SEROLOGICAL STATUS OF WILD RABBIT TO THE RABBIT CALICIVIRUS

Victorian Institute of Animal Science
Principal Investigator: John Humphrey
Report prepared by Penny Fisher, John Waddington and Steve McPhee

Project Objectives and Methodology:

To produce a sensitive, reliable and robust test that will require minimal expertise to perform, but will give an immediate and unequivocal result concerning the serum antibody status of any wild rabbit. This rapid test will be a major determinant in the release or non-release of the rabbit Calicivirus in the field, for the biological control of wild rabbits.

The test is designed to detect the presence of antibodies to rabbit Calicivirus (RCV) by binding RCV or the dominant capsid protein of RCV to polymer (latex) beads. The matrix will then react with several drops of whole rabbit blood – the presence of antibodies to RCV is then detected as a visible agglutination of the white beads on to a black background. If the presence of antibodies can been correlated with the ability of the rabbit to resist a challenge of RCV, a working test will give an unequivocal result concerning the antibody status of a wild rabbit within a few minutes. In the developmental stages of the rapid field test, duplicate samples will be tested in the rapid field test and the Capucci RCV ELISA to ascertain the sensitivity and specificity of the new field test.

Expected outcomes and outputs:

The expected outcome of the test is an immediate assessment of the serological status of a wild rabbit population at the time of bleeding rather than waiting for a laboratory result.

The results from the laboratory-based test represents the serological status of the wild rabbit population of at least one week prior to the results being received by the RCD coordinators.

The rapid field test, in contrast, will give the current serological status of the wild rabbit population in the area. An immediate decision relating to the release of the Calicivirus can be made, or an alternative strategy implemented. Simple design and ease of interpretation means that field based staff can test rabbits, with reduced costs from preparation, shipping and laboratory analysis of samples for results.

Summary of results against expected outcomes:

This is the final report for the project commenced in 1997/98, and originally planned to take a year to complete. Significant delays in the delivery of this project have been due to technical problems. The initial and central issue related to the inability to purify sufficient quantities of pure virus as the reactive coating for a polymer matrix (beads) to be used in a field-based rapid test. Subsequent milestones of testing and development of the field test have not been completed due to the lack of a cost-effective method for generating sufficient quantities of pure RCV virus proteins. This was despite extensive consultation and attempting a range of laboratory methods for growing and extracting the protein. Potential alternative methods of achieving this have been identified, but would require further scoping as new research in conjunction with progress in the ongoing validation of the c-ELISA for monitoring RCV in field populations of rabbits.

Further delays in completion of this report have been due to the departure of Dr. John Humphrey from the Victorian Institute of Animal Science.

Progress against milestones and achievement criteria:

1) **Purification of the Calicivirus from field-infected rabbits**

Purification of rabbit Calicivirus virus for the initial coating of the latex beads was completed in November 1997. The virus was purified from the livers of field caught Victorian rabbits. The rabbits were inoculated via the oronasal route and maintained in captivity at VIAS. The livers of the rabbits that died from infection with the Calicivirus were stored at –80C prior to the processing procedures. Briefly the livers were thawed then homogenised on ice, in a 1:5 ratio weight for volume (w/v) of sterile calcium and magnesium free Dulbecco's phosphate buffered saline (PBS) pH 7.2 using a Pro 200 homogeniser. The homogenate was then centrifuged at 2000g on a Beckman bench top centrifuge to remove removed most of the gross cell debris. The virus particles were then concentrated by precipitation with 8% Polyethylene glycol 6000. The precipitated virus preparation was then ultracentrifuged through a 20% w/v sucrose cushion at 100,000g for 90 minutes but unfortunately yielded only small quantities of pure virus.

The low recovery of virus may have been due to the rabbits dying more than 40 hours after inoculation via the oronasal route. This has previously been reported by Lorenzo Capucci (1999).

Binding of this purified virus to the latex beads obtained from the liver homogenates gave good results when tested against a small number of rabbit serum (n=10). The test was evaluated by its ability to predict which of the pre-bleed rabbit serum results correlated with the results of the challenge experiment (see attachment 1 Animal Ethics Committee protocol) ie. agglutination $\Rightarrow$ presence of antibodies $\Rightarrow$ resistant to oral RCV challenge for 7/10 rabbits. However, larger scale testing against rabbit validation trial serum from various Victorian field sites (n=94) did not give good predictions of antibody presence (The agglutination test agreed with results of the Capucci ELISA in less than 50% of the samples tested.). This disappointing result may have been due to the presence of antibodies present in the serum reacting with other proteins copurifying with the Calicivirus from the liver homogenates.

Following this result, the possibility of using an 'indirect' test was investigated. This involved the purification of polyclonal rabbit IgG purified from rabbit serum using protein Sepharose 4B. The purified IgG was coated onto the latex beads. The rabbit serum was then reacted with an equal volume of liver homogenate from a Calicivirus infected rabbit. A 50ul volume of this mixture was then reacted with an equal volume of the antibody coated beads to detect the presence of free virus. If no antibodies were present in the serum sample the virus in the liver homogenate would be free to react with the antibody coated beads and cause agglutination. Agglutination in this test indicated the absence of antibody in the serum.

This indirect test required an additional incubation step and necessitated the use for extra equipment. In addition, because of the possibility of cross-contamination of critical reagents and because agglutination indicated the absence of antibody rather than its presence it was decided that this increased level of complexity would be too difficult for field staff to use. Although a major advantage of this test would have been an almost immediate result in a field situation at the time of bleeding, further development of this test method was not continued.

2) Attachment of the purified virus to the polymer matrix – determine that coating has occurred by electron microscopic examination

The very small quantities of purified virus obtained from the liver homogenates were attached to the latex beads by incubating at room temperature in 0.1M glycine–HCl pH 8.2. A number of experiments using the Electron microscope were performed to detect attachment of rabbit Calicivirus to the polystyrene beads. When viewed under the electron microscope the latex beads appears as black smooth surfaced particles, however it was not possible to detect virus particles attached to the surface of the beads.

Discussions with Dr. Brian Cooke (CSIRO) revealed that AAHL Geelong had cloned the major capsid protein of RCV, the VP60 molecule. Lorenzo Capucci had produced monoclonal antibodies to this same protein for use in his ELISA test. The cost of obtaining even small amounts of this protein from Spain was too expensive, and the Spanish were reluctant to supply more than a few milligrams of this protein, which was insufficient to coat the latex beads.

Efforts were made to generate more workable quantities of pure viral coat proteins (VP60) in *E.coli* by using the *E.coli* plasmid vectors pGex and pET30b.

The gene that codes for the VP60 capsid protein of RCV was cloned into both the pGex and pET30b vectors. These were transformed into the appropriate *E. coli* hosts (JM109), however no aqueous soluble VP60 protein was produced following induction by IPTG when the *E. coli* were incubated at 37^{o}C. We have therefore tried to grow the *E. coli* containing the pGex vector at lower temperatures and also tried to maximise the aeration of the broth during incubation. None of these procedures produced any material in the aqueous phase therefore the VP60 molecule produced using the pGex vector could not be purified on the glutathione column (GST affinity chromatography). The pET30b vector, containing the 1.8kb cDNA fragment cloning for the VP60 molecule, produced the VP60 molecule with the histidine tags when induced with IPTG. The presence of the histidine tags was confirmed by the anti-his-peroxidase tag in the western blot. This molecule was also not soluble in the aqueous phase but was soluble in the 8M Urea. The Urea solubilised molecule however did not bind to the Nickel Column because the histidine tags were "buried" inside the molecule and were not accessible for binding. We have therefore taken a different tack and tried to purify the VP60 molecules from the inclusion bodies.

Overexpression of many recombinant proteins in *E. coli* often results in the formation of insoluble and inactive material known as inclusion bodies. The recombinant VP60 capsid protein of RCV also forms inclusion bodies when both these vectors were grown in *E. coli*. Purification of the recombinant proteins in relatively pure form from *E. coli* is often difficult due to the amount of contaminating proteins binding to the inclusion bodies. A number of different procedures were available, including washing and centrifugation using sucrose gradients, to remove unwanted proteins from the *E. coli* cells prior to sonication (which results in the release of the inclusion bodies from the *E. coli* cells).

A simple and effective measure was simply;

- Washing the *E. coli* cells 5 times with cold Phosphate Buffered saline with 100 ml volumes. The cells were resuspended after each wash by vigorous pipetting and

centrifuged at 10,000 rpm for 10 minutes on a Sorvall centrifuge with a SS34 rotor between washes.

- Following the final washing step the *E. coli* cells were resuspended in PBS then sonicated using the Brandon Sonifier 20 seconds on, 40 seconds off, at setting 7 on ice. This was repeated 5 times.
- The sonicated *E. coli* cells were then washed 5 times with PBS to remove other soluble proteins released following sonication.
- Following sonication the inclusion bodies were further purified using both aqueous two phase systems and sucrose density gradients with the latter proving the more effective.

The inclusion bodies were then pelleted in Eppendorf tubes and resuspended in varying concentrations of SDS and dithiothrieitol and incubated at 37^{o}C to determine the optimal concentrations for solubilising the sucrose purified inclusion bodies. The inclusion bodies of the pET30b vector were found to be soluble in relatively low concentrations of SDS if sufficient DTT was present. When the solubilised protein was run on an acrylamide gel the dominant band visualised was the VP60 and it was about 60-70 % pure. However, this process has only produced relatively small quantities of the 60-70% pure virus, insufficient for coating the latex beads. Large amounts of inclusion bodies with extractable VP60 protein could possibly be generated by using fermenters, however these products may not be as pure, due to the possible presence of other proteins with similar isoelectric points to the VP60 protein.

Attempts to purify the VP60 molecule from the other contaminating proteins using the Biorad Rotofor did not eventuate. Although it was possible to solubilise the recombinant proteins using low concentrations of dithiothreitol and sodium dodecyl sulphate anew and unexpected problem arose. We believed that the next step following solubilising was to isoelectrically focus the recombinant proteins using the Rotofor. This procedure would not only have purified the VP60 molecule but the recombinant protein would have been at the correct pH for binding to the latex beads. However when the ampholytes (the small molecules that generate the pH gradients for the focusing to occur) were added to the solubilised proteins they immediately came out of solution.! This was an unexpected and disappointing result. Proteins bind optimally to latex beads

at or near their iso-electric point. We had expected that this was our final step prior to binding the protein to the latex beads so you can imagine our frustration at this point. Biorad ,the company supplying the ampholytes, stated that other researchers had encountered similar problems when purifying recombinant proteins from inclusion bodies

3) Test the polymer matrix with rabbit serum from various geographical areas and compare the results with the Capucci ELISA

Due to the inability to generate sufficient quantities of high purity protein for coating latex beads, comparative tests of the bead matrix against rabbit serum also tested through the Capucci ELISA have not been possible. Ongoing work towards the validation of the c-ELISA has to date, delivered some conflicting results, so that current use of the c-ELISA as a standard for comparison with a rapid field may not be appropriate (see attachment 2 Interim Report , Validation of the "Capucci" Competitive Enzyme Linked Immuno Sorbant Assay (ELISA) to measure rabbit Calicivirus immunity in populations of Wild European Rabbits prepared by S. McPhee and D. Berman.)

4) Test the coated beads in a rabbit slide test with whole blood in the field

Not completed – see above

5) Field test verified prior to use and registration for use in the field

Not completed – see above

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Organisations / Individuals consulted or collaborating

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Expenditure of BRS funds for project to June 1999

ITEM	AMOUNT
Total BRS contribution (1997/98)	**$46 720.00**
Salary (includes on-costs)	18 300
Overheads	6 150
Vehicle mileage	1 200
Laboratory consumables	18 500
Animal facilities/technician	7 660
TOTAL	**$51 810***

* all BRS funds expended (overexpenditure against project)

Justification for expenditure against project proposal:
Salary plus on-costs: More staff time of the VPS-3 scientist than originally planned was required due to the problems and extended work devoted to the initial laboratory stages of purifying RCV proteins.
Overheads: increased concomitant with salary expenditure
Vehicle mileage: Less than anticipated expenditure as project did not progress to field testing stage where most travel would have been required.
Laboratory consumables: Expenditure less than anticipated as project did not progress to 'mass production' stage of reagents and biological components of field test
Animal facilities/technician: Expenditure greater than anticipated due to extended use of sera from captive rabbits for initial testing.

Attachment 1

Animal Ethics approval details (not available in electronic copy – hard copy supplied)

Attachment 2

Appendix 1.

Rabbit Calicivirus Disease Program

Biological Control Initiative Against the European Wild Rabbit

Interim Report *Jun 99*

Prepared by Steve McPhee, Agricultural Technical Services Pty. Ltd.

AGRICULTURE VICTORIA

A business of the Department of Natural Resources and Environment

1. Project:

Validation of the "Capucci" Competitive Enzyme Linked Immuno Sorbant Assay (ELISA) to measure rabbit calicivirus immunity in populations of Wild European Rabbits.

2. Principal Investigators:

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3. Background:

To investigate the epidemiology of Rabbit Calicivirus (RCV) under different climatic conditions, monitoring sites have been established across the nation to measure the impact of RCV on rabbit abundance and to also measure the seasonal variation in the levels of immunity in rabbit populations.

Critical measurements in understanding the epidemiology of this disease are the prevalence of RCV immunity in rabbit populations. Blood samples from 20 to 30 shot rabbits are collected on a regular basis from the monitoring sites. The presence of serum antibodies to RCV is measured by the Capucci competitive ELISA. This is a relatively new assay that requires validation to confirm that interpretations of results are consistently reliable. If the degree of susceptibility to RCV can be reliably defined within populations of rabbits then management decisions for the control of rabbits can be implemented more effectively, and the indiscriminate release of virus into non-susceptible populations can be avoided.

A critical evaluation of the Capucci ELISA using reference rabbit sera is essential in determining the sensitivity and specificity of the test and for facilitating the predictions of mortality in exposed rabbits. To date no systematic attempt has been made to validate the Capucci ELISA therefore interpretation of field serology is difficult.

4. Project Objectives

4.1 To validate the accuracy of the Capucci Competitive ELISA, for the detection of antibodies to RCV in wild European rabbits, based on the experimental challenge of rabbits with RCV, and using death or survival as a measure of assay sensitivity and specificity.

4.2 Use serological data obtained to accurately predict the survival or death of individuals to Rabbit Calicivirus so the assay can be used to define the susceptibility of populations of wild rabbits.

4.3 To implement a standard procedure to be adopted by all laboratories for the Capucci competitive ELISA. Note a standard method has been circulated by ANQAP.

4.4 To enable RCV serology to be accurately interpreted and therefore optimise the impact of RCV integrated with sustained strategic management of rabbits.

5. Methods:

To validate the Capucci competitive ELISA, rabbits were captured from selected monitoring sites and bled to determine immune status prior to being challenged with a lethal oral dose of RCV. Rabbits were then monitored over a 10 day post challenge period and surviving rabbits were euthanased at the end of the program. Survival or death of challenged individuals was correlated with pathological data and serological results of the Capucci cELISA.

5.1 Schedule of Events:

Rabbits were bled at the time of capture (day –7), prior to RCV challenged (day 0) and at days 7 and 10 post challenge. Rabbits were monitored over a 10 day, post challenge period and surviving rabbits euthanased at the end of the program. Survival or death of individuals was correlated with pathological data and serological results of the Capucci c-ELISA

Figure 1

5.2 Capture

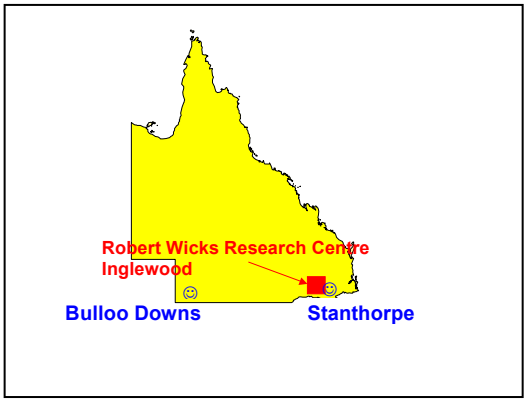
Within Victoria rabbits were captured from the Bendigo, Beechworth, Ballan and Hattah areas and animals were housed at the NRE, Bendigo Laboratories. The Queensland program was conducted at the Robert Wicks Research Centre, Inglewood, and rabbits were collected from Bulloo Downs and the Satnthorpe area. (Fig. 2.)

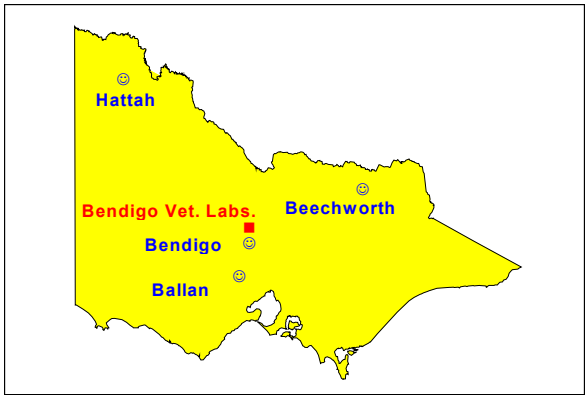
Rabbits were captured using ferrets to flush rabbits from warrens into nets, spotlighting and live capture using landing nets, and baited cage traps. Capture methods were approved by the VIAS animal ethics committee.

Figure 2 Capture sites and location of animal Laboratories

Queensland Capture Sites

Victorian Capture Sites





5.3 Housing

At the time of capture rabbits were tagged, bled from ear vein and physical measurements such as sex, weight, condition score, reproductive status, and flea burden were recorded. Animals were transported to enviromentaly controlled animals houses, individually caged and held over a 7 day pre-challenge period to ensure no wild virus was active amounst the captured rabbits.

5.4 Allocation, Date Challenged and Number Treated

Rabbits were randomly allocated into two groups on the basis of liveweight. A control group of 4 to 10 animals from each of the capture areas were not challenged with RCV. In Victoria control rabbits were housed in separate rooms to the RCV challenged animals where as in Queensland non-challenged rabbits were individually caged in the same areas as the challenged rabbits.

Table 1 Date and number of rabbits treated

Location	Date of Challenge	Number of Rabbits	
		Control	Challenged
Victoria			
Bendigo	23 Oct 98	10	51
Beechworth	13 Nov 98	6	49
Ballan	29 Jan 99	8	52
Hattah	4 Mar 99	5	40
Queensland			
Bulloo Downs	4 Mar 99	4	103
Stanthorpe	29 Apr 99	4	52

5.5 Dosing

Rabbits challenged with RCV received an oral dose of 0.5ml (1500xLD50) of rabbit calicivirus suspension (Batch Number: RCD 1A). This inoculum was stored at -80^{o} C since time of manufacture. Rabbits allocated to the control group received an oral dose of 0.5 ml of a saline solution.

5.6 Monitoring and Sampling Procedures

Rabbits were fed an ad-lib diet of fresh carrot and manufacture pellets and/or oats and were monitored 2 to 3 time's daily, food and water intake, clinical and behavioural observations were recorded. Time to death was also recorded and cause of death determined by pathological and serological examination. Clinically sick animals as determined by convulsions, or an inability to move to food or water, were euthanased and processed to collect blood, organs and tissue.

Surviving rabbits were euthanased on day 10 and necropsied, with the following blood and pathology samples collected.

1) Blood samples were collected into 10-ml blood clotting tubes and held overnight to coagulate. The serum was decanted into 2-ml vials and stored in a freezer at -20^{o}C.
2) Liver and spleen samples (frozen), kidney, liver, and lung sections as well as eye preserved in 10% buffered formalin.

Sera from 30 rabbits captured in the Ballan area were collected at 24 hours and immediately prior to RCV challenge. These samples were utilised to evaluate the reproducibility of the c-ELISA. by measuring the variation within animals and within samples.

5.7 Measurements

5.7.1. Physical Sex, weight, condition score, reproductive status, flea burden and time to death

5.7.2. Estimated Age of Rabbits. Collected eyeballs were stored in 10% buffered, neutral formalin for at least 30 days, processed, dried and weighed at days 4 and 7. Predicted age was determined as described by Myers and Gilbert (1968).

5.7.3. Antigen Capture ELISA. The livers of all rabbits were processed and assayed using an antigen capture ELISA to determine the presence of virus in liver. (Collins et al, 1996)

5.7.4. Capucci Competative ELISA. Antibodies to RCV were detected using the c-ELISA, as described by Capucci et al (199?). The series of samples collected from individual rabbits were assayed on the same plate at a 1:10 serum dilution and each sample was assayed in triplicate. Results are expressed as a percentage inhibition against a set of standardised negative control sera from the same plate.

5.7.5. Detection of antibody immunoglobins IgG, IgM and IgA. Improved methods for measuring antibody isotopes have been developed in Italy by Cappuci. The CSIRO laboratory, Wildlife and Ecology at Canberra under the direction of Dr Brian Cooke have developed and implement a series of isotyping ELISA,s to detect antibody isotypes and measure their serum titres. These more sensitive tests enable the detection of rabbits with maternal antibodies, re-infected rabbits and rabbits that have recovered from infection. Rabbits collected from the Beechworth site in Victoria have been assayed for antibody isotypes and serum titres.

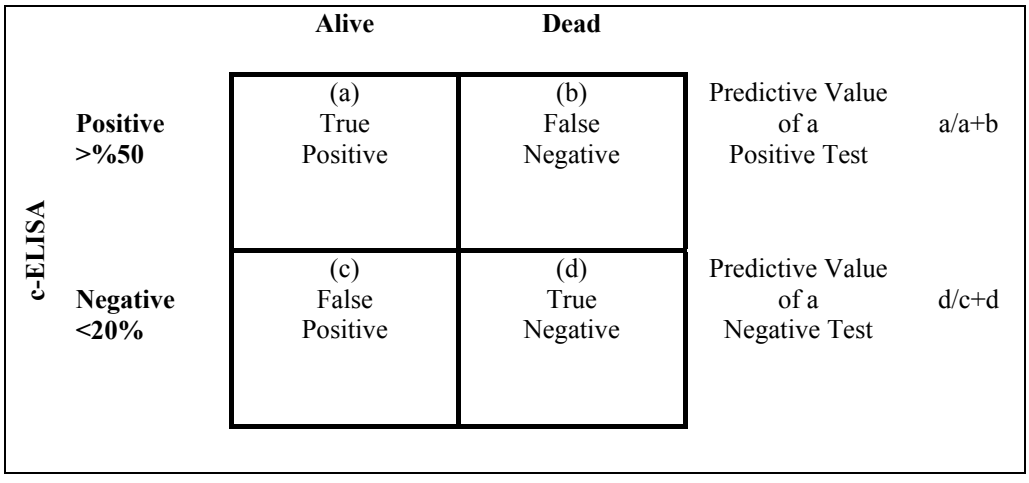
5.8 Analysis

5.8.1 Validity

All diagnostic tests should be backed by sound data comparing their accuracy with an appropriate standard. The standard of validity is termed the "gold standard", this refers to the method of determining whether a disease is truly present or not. The gold standard for evaluating the c-ELISA was determined by the survival or death of rabbits post RCV challenge

The performance characteristics of a diagnostic test can be evaluated by using the 2 X 2 table (fig. 3). Results are used to estimate test sensitivity, specificity, predictive value, accuracy and the prevalence of disease in the population.

Figure 3 Diagnostic test (2 x 2 table) outcomes and definitions.



Sensitivity = a/(a+c) **Accuracy** = (a+d)/(a+b+c+d)

Specificity = d/(b+d) **Prevalence** = (a+c)/(a+b+c+d)

Definitions:

(i) Sensitivity and Specificity

a) Test Sensitivity Is defined as the proportion of infected or diseased individuals that test positive. Sometimes referred to as the *true-positive rate.*

b) Test Specificity Is defined as the proportion of disease free individuals with a negative test. Sometimes referred to as the *true-negative rate*

c) False Positive Rate Likelihood of a positive result in animals known to be free of the disease and equals (1-Specificity)

d) False Negative Rate Likelihood of a negative result in animals known to have the disease and equals (1-Sensitivity)

In summary sensitivity and the false negative rate describe how the test performs in animals with the disease, whereas specificity and the false-positive rate describe how the test performs in animals without the disease

(ii) Predictive Values (Probability that a test result reflects the true disease status)

a) Positive Predictive Value.(PPV) Is the probability of disease in an animal with a positive (abnormal) test result

b) Negative Predictive Value (NPV) Is the probability of that an animal does not have the disease when the test result is negative

Where as sensitivity and specificity are absolute properties of a test and do not change for any given cutoff value, predictive values are relative, varying with the prevalence of the disease in the population from which the animals came.

(iii) Effect of Prevalence on Predictive Values

Diagnostic tests are used in populations with widely varying disease frequencies. This has no effect on Sensitivity or specificity, but predictive values may vary considerably. As the prevalence of infection decreases the positive predictive value also decreases but the negative predictive value increases.

(iv) Accuracy

Is the proportion of all tests, both positive and negative, that are correct. It is often used to express the overall performance of the test. However its value is subject to the same constraints as predictive value and is correct only for the population used to standardise the test.

5.8.2 Evaluating Cutoffs

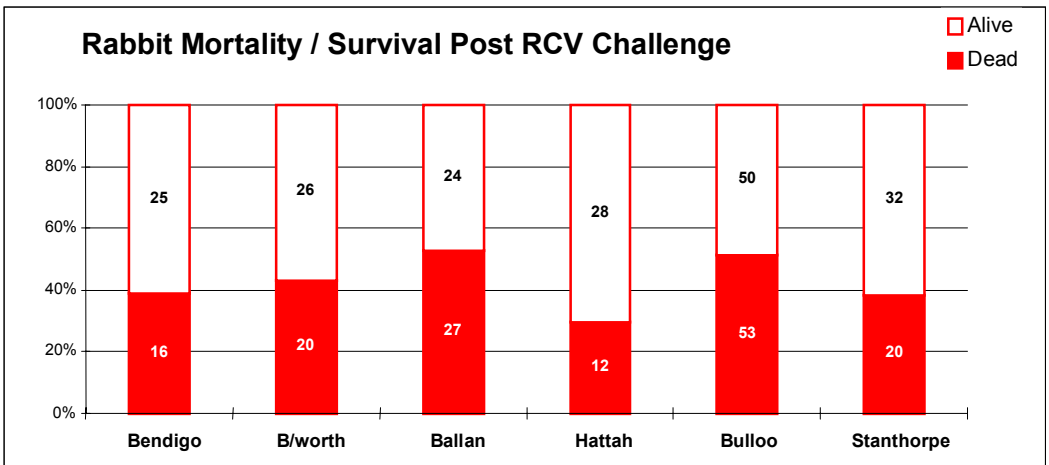
For test results that fall along a continuum, e.g., ELISA cutoffs, test performance can be depicted graphically by plotting a response-operating characteristic (ROC) curve, which compares the true-positive rate (sensitivity) on the vertical axis, with the false-positive rate (1-specificity) on the horizontal axis. The ROC curve provides a simple method for evaluating a test's ability to discriminate between health and disease over a complete spectrum of operating conditions, and it can be used to select cutoffs or to compare diagnostic tests.

6. Interim Results

6.1 Mortality / Survival Post RCV Challenge

For all sites 64% (range 70% to 48%) of RCV challenged rabbits survived lethal oral dose of RCV (Fig 4

Figure 4



6.2 Analysis of Rabbit Sera using the c-ELISA

Rabbit sera from all sites have been screened using the c–ELISA at a 1:10 dilution and serum profiles have been plotted against mortality or survival for individual rabbits.). More detailed analysis of sera for antibody isotypes and serum titres and further analysis of interactions between site of capture, age structure are still to be performed.

6.3 Confirmation of Death Due to RCV Challenge.

Livers from all rabbits have been assayed using the antigen capture ELISA. Death by RCV was confirmed by the presence of antigens in liver from all rabbits dying with in 7 days post RCV challenge. No antigens were found in the livers of rabbits surviving the challenge.

6.4 Immune Status and Rabbit Age

Rabbit lenses have been processed and correlated with immune status (Fig. 4). Preliminary analysis of immune status plotted against lens weight demonstrates that there are very few susceptible rabbits (<20% inhibition) greater than 12 months of age. (Lens weight of 200mg approximately equals 12 months old.). A more detailed correlation between sex, age, structure, site of capture, survival/mortality and immune status is to be performed.

6.4 Detection of RCV Antibody Immunoglobins

Sera samples from the Beechworth site have been sent to Brian Cooke's laboratory to determine serum titres for antibody isotypes. Interpretation of serum profiles related to the amplitude and sequence's of occurrence of the antibody isotypes IgG, IgA and IgM indicate that a relatively high proportion (8/18) of surviving rabbits may have been subjected to a primary infection at the time of RCV challenge. The results for animals dying from RCV challenge are still to be interpreted. Consideration is being given to test for antibody immunoglobins for the remaining five sites to compare serum profiles with mortality or survival.

6.5 Evaluation of the Optimum Cutoff for the c-ELISA

Evaluation of and comparison of selected cutoffs for each capture site using response-operating characteristic curve analysis and likelihood ratio analysis is still to be performed

6.6 Validity of the C-ELISA

A preliminary analysis of the sensitivity, specificity, and the positive and negative predictive values has been tabulated for selected cutoffs used in Victoria (20%, and 50%) and Queensland (15%, and 25%)

6.6.1 Sensitivity (the proportion of RCV immune individuals surviving, that test positive.)

There is an apparent variation in the sensitivity of the test between sites for the all selected cutoffs. (Fig 6a) E.g., at the Hattah site the sensitivity remains high (100%) with all the immune rabbits being above the 50 % cutoff. Whereas, for the Beechworth site the number of immune rabbits testing positive declines from 76% to 36% across the respective cutoff points. The sensitivity of the test is relatively poor at the 25% cutoff, for 4 of the 6 sites, 18% to 40% of surviving rabbits tested negative (Fig 6a & 5). With the exception of Hattah the sensitivity declines more rapidly after the 25% cutoff point indicating that an appropriate cutoff for sensitivity should be < 25% inhibition

6.6.2 Specificity (the proportion non-immune individuals that die and have a negative test result).

Specificity is relatively high for the 25% to 50% cutoffs, with reduced (85% to 96%) variability between sites, indicating that the majority of the non-immune rabbits testing negative to the 25% cutoff ,died.(Fig. 6b)

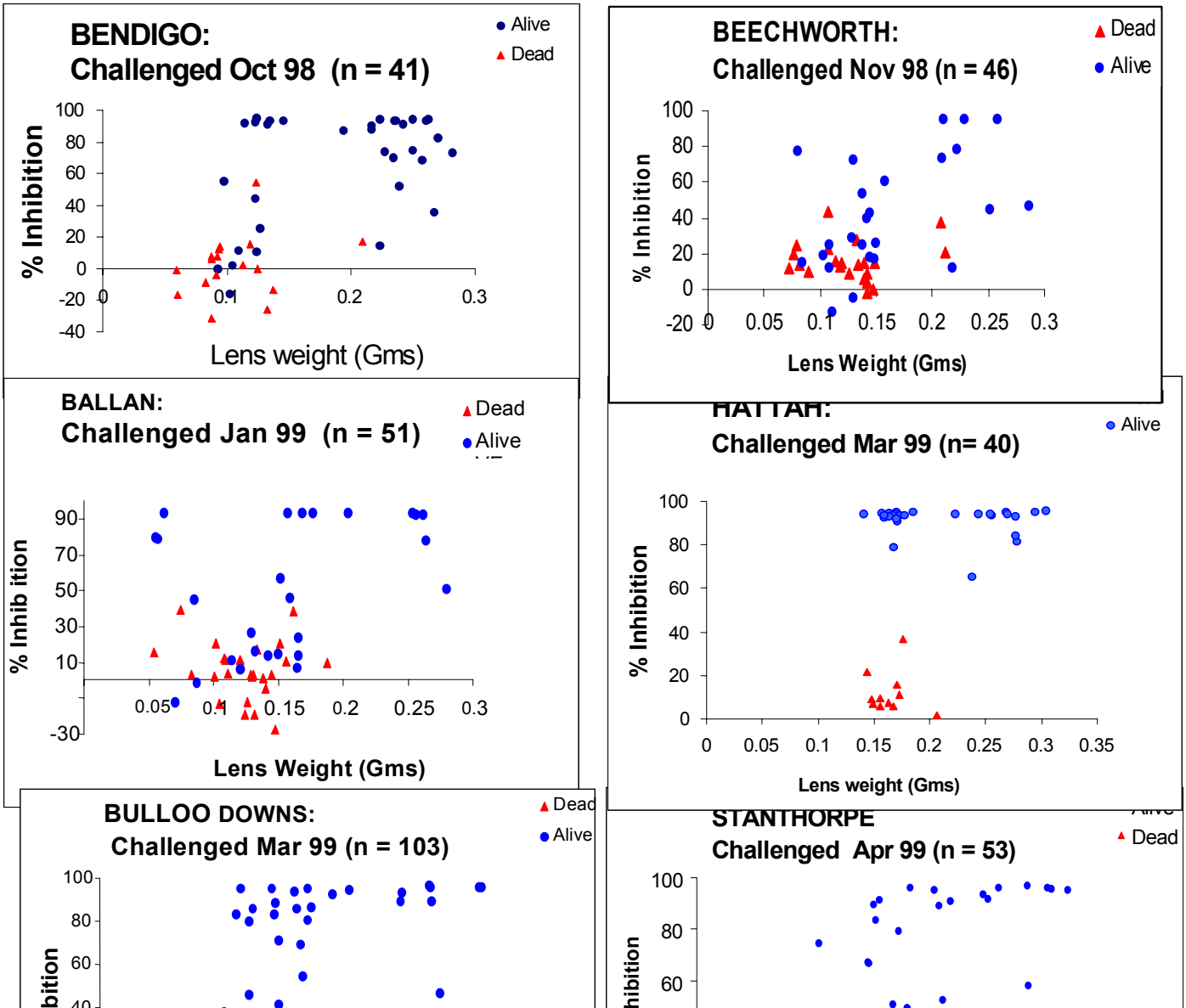
6.6.3 Positive and Negative Predictive Values (Probability that test results reflects the true disease status)

The NPV was variable between sites for all the selected cutoff points and with the exception of Hattah and Stanthorpe, the NPV was relatively poor due to the number of, surviving rabbits testing negative.(Fig 6d)

The PPV was least variable between sites for the 25% to 50% cutoffs and had a high, 83% to 96% probability of detecting true positive rabbits at the 25% cutoff point. (Fig. 6c)

These data indicate that rather than use two separate cutoffs to determine the proportion of sero-negative or sero-positive rabbits, a single cutoff point located between 20% and 25% may be adequate to measure the susceptibility of rabbit populations. A more detailed analysis of the existing data is underway to determine the influence of site location, time of sampling, and age structure on the test validity.

Figure 5 Rabbit mortality or survival after an oral dose (0.5ml) of RCV, comparing immune status and eye lens weight.



Figures 6 a,b,c,& d A preliminary analysis of the sensitivity, specificity, and the positive and negative predictive values has been tabulated for selected cutoffs used in Victoria (20%, and 50%) and Queensland (15%, and 25%)

Figure 6a

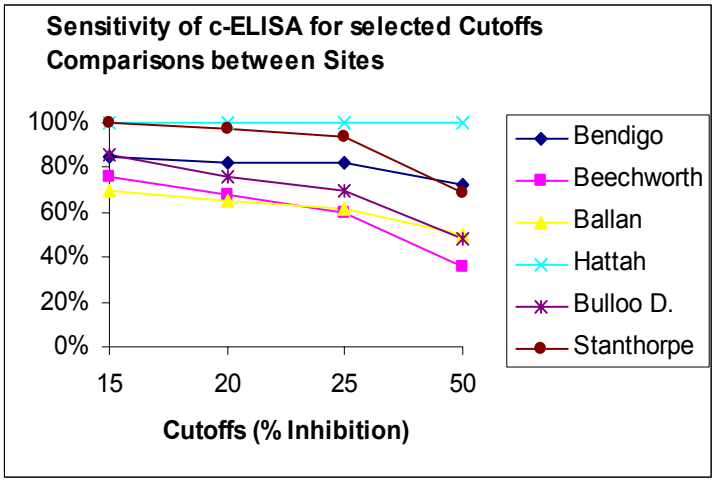


Figure 6b

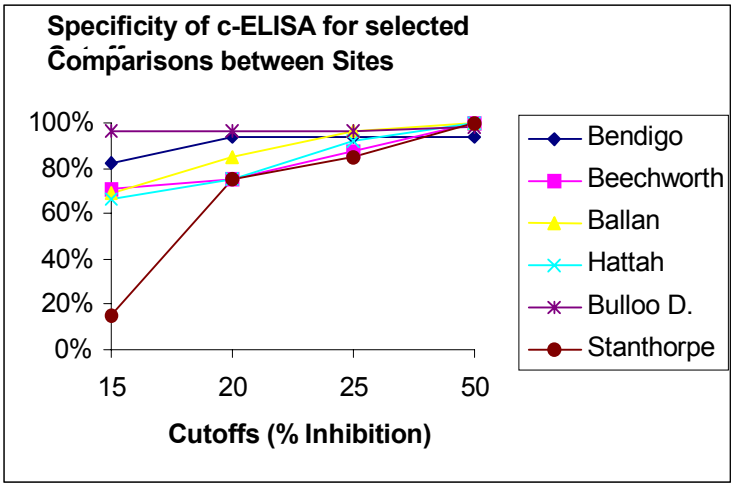


Figure 6c

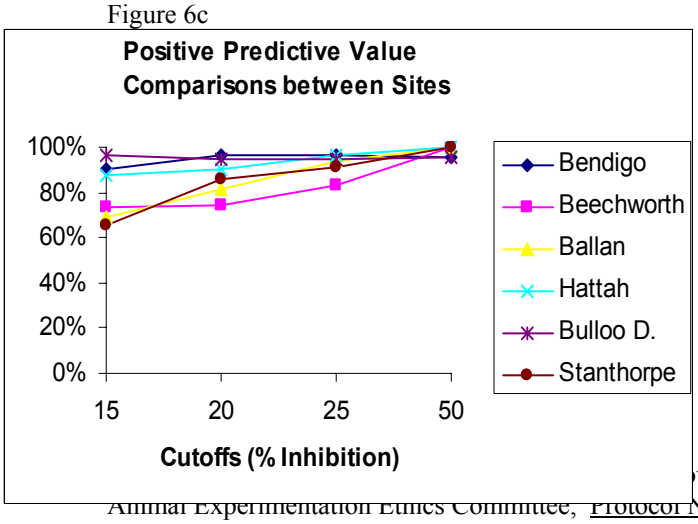
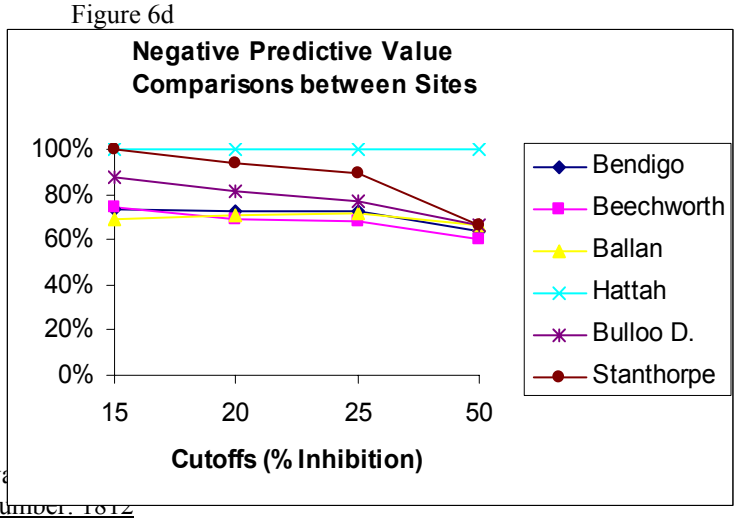


Figure 6d



Animal Experimentation Ethics Committee, Protocol Number: 1812

9. Acknowledgments

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10. References

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