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Use of ELISAs in field studies of rabbit haemorrhagic disease (RHD) in Australia

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SUMMARY

ELISA techniques developed for the veterinary diagnosis of Rabbit Haemorrhagic Disease (RHD) in domestic rabbits were used for studying the epidemiology of RHD in Australian wild rabbits. The combination of ELISA techniques that distinguished IgA, IgG and IgM antibody responses and a longitudinal data set, mainly based on capture-mark-recapture of rabbits, provided a reliable basis for interpreting serology and set the criteria used to classify rabbits' immunological status. Importantly, young with maternal antibodies, immune rabbits and rabbits apparently re-exposed to RHD were readily separated. Three outbreaks of RHD occurred in 1996–7. The timing of RHD outbreaks was mainly driven by recruitment of young rabbits that generally contracted RHD after they lost their maternally derived immunity. Young that lost maternal antibodies in summer were not immediately infected, apparently because transmission of RHDV slows at that time, but contracted RHD in the autumn when conditions were again suitable for disease spread.

INTRODUCTION

Rabbit Haemorrhagic Disease (RHD), also known as Viral Haemorrhagic Disease or Rabbit Calicivirus Disease was first described in domestic rabbits in China [1]. It subsequently spread, primarily by trade in contaminated rabbit products, to other countries in Asia and then into Europe, eventually reaching Mexico and Cuba and African countries including Morocco. The causative agent, Rabbit Haemorrhagic Disease Virus (RHDV), was found to be a calicivirus [2, 3] specific to the European rabbit *Oryctolagus cuniculus* (L.). Rabbits usually died 24–72 h after infection showing no outward signs of disease until a few hours before death. The virus caused necrosis of the liver followed by disseminated intravascular coagulation, with lungs, trachea and spleen showing congestion [4].

RHD spread into wild rabbits in Europe in 1988 causing heavy mortality [5, 6] and RHDV was subsequently imported into Australia in September 1991 to assess its potential as a biological control agent against wild rabbits that have been major environmental and agricultural pests since their introduction in 1859 [7]. After extensive testing in quarantine at the Australian Animal Health Laboratory [7] to confirm host specificity and efficacy, approval was given for tests to begin on Wardang Island, South Australia, again under quarantine, to evaluate the ability of RHD to spread and cause high mortality under Australian climatic conditions. It proved to be well suited to the Australian environment when it escaped from the island in October 1995 [8] and became established across southern Australia

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within 18 months [9]. It was subsequently unofficially introduced into New Zealand [10].

The initial spread of RHD in late 1995 reduced wild rabbit populations in inland South Australia by over 90% and was reliably recorded from reports of dead rabbits and confirmed using virus-capture ELISA on liver samples from cadavers [11, 12]. However, determining the persistence and recurrence of RHD in residual populations of rabbits was more difficult even though initial studies showed that serology gave some insight into its epidemiology [11].

A major breakthrough in field epidemiology was achieved with the introduction of enzyme-linked immunosorbent assays (ELISAs) developed in Italy for veterinary diagnosis of RHD in domestic rabbits [13, 14]. These ELISAs not only enabled the general detection of antibodies using competition-ELISA but also enabled the detection and titration of isotypes IgA, IgG and IgM. The isotype titres proved critical for the interpretation of field serology in four main areas:

Cross-reactive antibodies. Before RHD was introduced into Australia, sera from wild rabbits cross-reacted in some ELISA tests used for RHDV antibody detection. This background reactivity potentially confounded serological data.

Natural resilience of young rabbits. Young rabbits less than 3 weeks old do not develop disease when experimentally infected by RHDV but, from about 4 weeks of age, mortality progressively increases to reach adult rates at about 9 weeks [15]. As the immunological response of young rabbits also differs from that of adult rabbits, evidence of their infection required careful interpretation.

Maternal antibodies. Accurate discrimination between temporary maternal antibodies in the serum of young rabbits and longer-lasting antibodies in immune rabbits was essential.

Antibodies in previously infected rabbits. Little was known of the persistence of antibodies in previously infected wild rabbits. These might be maintained or slowly lost or rabbits could also be re-infected and their antibody titre enhanced as occurs with Norwalk human calicivirus [16].

In this paper, data from wild rabbits are reviewed in conjunction with relevant literature to develop criteria enabling accurate assessment of the immunological status of rabbits. These criteria are used in turn to help determine the occurrence of RHD in a natural rabbit population.

MATERIALS AND METHODS

Study area

The study centred on Gum Creek sheep station (31° 15′ S, 138° 45′ E) in the Flinders Ranges, South Australia – one of two inland sites where RHD was quickly discovered following its escape from Wardang Island. In November 1995 rabbits were sampled on the site by night shooting and sampling was subsequently extended well beyond the Flinders Ranges to confirm the apparent extent of spread of RHD. Depending on local abundance, small samples of rabbits were collected at intervals along broad transects extending 300 km east to Fowlers Gap, New South Wales, about 500 km south-east to Telopea Downs in western Victoria and Bordertown (South-east South Australia) and 400 km west to Ceduna (Far West Coast, South Australia).

Subsequently, attention was again directed to Gum Creek when a detailed study of the epidemiology of RHD in a free-living rabbit population began in February 1996. In this semi-arid rangeland, annual rainfall averages about 220 mm but is highly variable. Daily temperature ranges (minimum–maximum) average 3–14 °C in August and 18–34 °C in February. Rabbits live in large warrens, although their territories extend to include several adjacent warrens.

Capture of rabbits

Shot samples of rabbits were collected at night using a 0·22 calibre rifle from a vehicle equipped with a 100 W spotlight. Once confirmed dead, each rabbit was opened ventrally and a vacutainer was used to collect blood from the heart to obtain serum for ELISA. The spleen and approx. 1 g of liver were collected for detection of RHDV by virus-capture ELISA. The rabbit was sexed and its reproductive condition recorded. Body weight was recorded and an eye removed and preserved in 10% buffered formalin for age estimation based on dried eye lens weight [17]. At least five rabbits were collected from each point chosen for sampling but where rabbits were abundant samples of 30–50 rabbits were collected.

In live-trapping studies, rabbits were caught in wire cage traps baited with chopped carrot and oats and set every 4–8 weeks from November 1995 to December 1997. Sixty traps were used on 15 warrens for 4 consecutive nights on each occasion. Visits were timed according to the dynamics of the rabbit population;

more visits were made when rabbits were breeding than during the dry summer when reproduction ceased. Traps were inspected each morning, and trapped rabbits were placed separately in clean hessian bags, sexed, and their reproductive status noted. A blood sample (2 ml) was taken from an ear vein and the rabbit was marked with a serially numbered metal ear tag if not previously captured. Body weight was recorded at each capture to estimate growth rate and calculate the approximate age of younger rabbits [18]. Rabbits were released into burrows near their point of capture. Traps were closed during the day to avoid exposing rabbits to heat stress. To reduce the risk of spreading RHD, the fresh bait used for re-setting traps each evening was prepared only after daily work with rabbits concluded and cleaning up had been completed. A serum sample was drawn off each blood sample and frozen for later ELISA analysis in the CSIRO Wildlife and Ecology laboratory in Canberra. A liver sample was taken from any freshly dead rabbit found on the area and examined by virus capture ELISA (see below) to determine whether or not RHDV was present.

ELISA methods

Competition ELISA (cELISA) for the detection of antibody in serum was performed as previously described [13, 14, 19] but with minor modifications. Maxisorp plates (Nunc) were coated overnight at 4 °C with 50 μl per well of a polyclonal anti RHDV rabbit serum diluted 1/10000 in carbonate buffer (pH 9·6). The plates were then washed with phosphate-buffered saline (PBS) containing 0·05% Tween 20 (PBS-T) initially for a few seconds and then three times for 1 min per wash accompanied by vigorous shaking. Excess liquid in the wells was removed by tapping the inverted plate sharply on paper towels. Plates were either used immediately or stored at −20 °C in plastic bags and used within 4 weeks. To each well of a plate 25 μl of PBS-T containing 1% yeast extract (Merck) (PBS-TY) was added as a blocking agent. All sera were tested in duplicate. Test sera were diluted directly on the plate by adding 7 μl of sample to each of the first wells of the titration. After shaking the plate, 7 μl of each diluted serum were transferred into the next well (approx. a fourfold dilution). This process was usually repeated to give, in total, six consecutive fourfold dilutions. Negative and positive reference sera (controls) were included on each plate usually at three dilutions. 25 μl of virus, used at a concentration previously determined by titration to give an optical density reading 1·1–1·3 at 492 nm (OD_{492}), was added to all wells and the mixture incubated at 37 °C for 1 h with shaking. Plates were washed and excess liquid removed as described above. Monoclonal antibody (Mab 1H8) conjugated to horseradish-peroxidase (1H8-HRP) and diluted in PBS-TY was then added to all wells to detect virus bound to the solid phase. After final washing, 50 μl of phosphate-citrate buffer (pH 5·0) containing *o*-pheylenediamine (OPD) (Sigma) at 0·5 mg/ml and H_2O_2 at 0·02% was added to all wells. The reaction was stopped after 5 min by the addition of 50 μl 1 M H_2SO_4 and the OD_{492} of the wells read in an automatic plate reader (Labsytems Multiscan MS). Sera were considered to be positive if the OD_{492} value of the first dilution was lower than the $OD_{492} \times 0{\cdot}75$ of the first dilution of the negative reference serum. The titre of each positive serum was taken as the dilution that reduced the OD_{492} by 50% compared with the initial wells of the negative reference serum.

Isotype ELISAs (isoELISAs) were performed essentially as previously described [19]. Briefly, to detect RHDV specific IgG, Mab 1H8, RHDV specific, was adsorbed to the Maxisorp plate at a concentration of 2 μg/ml by the method described above for the polyclonal serum in the cELISA. Virus was added to the plates at a concentration double that used in the cELISA and, after incubation and washing, sera were added and serially fourfold diluted starting from 1/40. A Mab anti rabbit IgG HRP conjugated was used to detect IgG bound to the virus. The final step for the isoELISAs for IgG, IgM and IgA was the addition of OPD and H_2SO_4 as for the competition ELISA. To detect IgM and IgA isotypes the phases of the ELISA reaction were inverted in order to avoid competition with IgG that is usually the predominant isotype. Mab anti rabbit IgM or anti rabbit IgA were adsorbed to the wells and then the sera were diluted as described above. Incubation with the antigen followed and then Mab 1H8-HRP was used to detect the RHDV bound to the plate. Sera were considered positive if the OD_{492} value at the 1/40 dilution was more than 0·2 OD units (two standard deviations) above the value of the negative serum used as a control. The titre of each serum was taken as the dilution that reduced the OD_{492} of the positive reference serum by 50%. Because isoELISA tests do not follow identical methodology, equivalent titres do not imply that isotypes are present in the same amounts.

For the detection of RHDV antigen, a virus capture

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ELISA (vcELISA) developed in the Australian Animal Health Laboratory was used [20].

Data analyses

As well as using direct inspection of the data to draw conclusions, data were analysed using generalized linear modelling (GLM) within Genstat 5, Version 4.1, Lawes Agricultural Trust (Rothamstead Experimental Station). Most analyses included cELISA, transformed to $\log_{10}$ (reciprocal cELISA titre + 1), as the response variate because cELISA is the major technique used in Australia for detection of RHD antibodies. The explanatory variables used were log-transformed IgA, IgG and IgM titres, body weight and eye-lens weight. Sex of each rabbit and month and location of sampling were also included as explanatory factors where appropriate. Stepwise regression was used to determine which variables and factors added significantly to the variance explained.

RESULTS

Initial spread of RHD

It was known from reports of dead rabbits and from livers handed in for vcELISA tests that RHD had spread rapidly in the area east the Flinders Ranges reaching Fowlers Gap in western NSW by late 1995. It had also spread westward to Ceduna in South Australia but, apparently, little further. Sampling of rabbits in these areas and subsequent ELISA testing confirmed that many of the surviving rabbits carried high titres of RHD antibodies. However, the virus had not spread southward with such rapidity and it was not until April 1996 that the first cases appeared along the Murray River in the farming area known as the Murray–Mallee.

Pre-RHD serum reactivity

One hundred and sixty-nine sera were collected in late 1995 and early 1996 in South Australia's Murray–Mallee, Southeast and Far West Coast from sites ahead of the apparent spread of RHD. However, among these, using ELISA cut-offs determined for European domestic rabbits, 21% were negative, 79% were apparently positive on the basis of IgG isoELISA, with titres between 40 and 640 and 6% were positive to both IgG and cELISA. The cELISA titres were between 10 and 160. No serum had detectable IgA and IgM ELISA titres. The lack of IgA and IgM components and, above all, the presence of IgG reactivity in the absence of cELISA titre, indicated that these antibody responses were highly anomalous in comparison with the rabbits' normal antibody response to challenge with RHD. Consequently, they were considered to be similar, though not identical to the 'pre-existing' or 'cross-reactive' antibodies seen in rabbit sera in Europe prior to the spread of RHD [21].

Although this pre-existing serum reactivity was widespread, inspection of data from five nearby sites in the Murray–Mallee indicated that when RHD finally spread through the area antibodies resulting from RHDV infection were sufficiently distinct to distinguish areas where RHD was present or absent (Table 1). This was strongly reinforced by statistical analysis. A generalized linear model, fitted to these data after excluding non-significant explanatory factors and variables, explained 96% of the variance (Table 2). The presence of IgA isotype in sera, and high titres of IgG, indicated those rabbits that had been infected with RHDV.

It could also be concluded that pre-RHD serum antibodies did not protect rabbits from RHD. As RHD spread onto previously unaffected sites, heavy mortality occurred even though 70–80% of rabbits had been classified as weakly positive by IgG ELISA. However, it is possible that the serum reactivity modified the immune responses in some surviving rabbits.

Maternal antibodies

Although the ELISAs had previously been used to detect maternal antibodies in young domestic rabbits [19], the methodology was reconfirmed using sera from young wild rabbits whose mothers had survived the initial RHD outbreak at Gum Creek. The presence of IgA, indicating previous infection with RHDV, was used to sort these young wild-caught rabbits into two groups: (a) previously infected rabbits and (b) those that had not been infected. The cELISA titres of this latter group of rabbits, assumed to represent maternal antibodies, were analysed to confirm that they were exclusively IgG and were lost as young rabbits grew to maturity. Although the statistical analyses began with three sets of samples, from October, November and December 1996 respectively to enable consideration

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Table 1. *ELISA data from sera of rabbits shot at five nearby sites in the Murray–Mallee, South Australia, collected as RHD began to spread into the area in April 1996*

			ELISA titres			
Location	Sex	Weight (kg)	cELISA	IgG	IgM	IgA
Brookfield	F	1·500	0	40	0	0
Brookfield	M	1·500	0	160	0	0
Brookfield	M	1·650	0	40	0	0
Brookfield	M	1·425	0	160	0	0
Brookfield	M	1·525	0	0	0	0
Brookfield	M	1·525	0	40	0	0
Wunkar	F	1·500	15	40	0	0
Wunkar	M	1·300	0	0	0	0
Wunkar	F	1·775	0	40	0	0
Wunkar	F	1·350	0	40	0	0
Atze	M	1·375	0	160	0	0
Atze	F	1·300	0	80	0	0
Atze	F	1·350	0	160	0	0
Atze	F	1·250	0	0	0	0
Atze	M	1·325	0	0	0	0
Atze	F	1·550	0	0	0	0
Atze	F	1·275	0	40	0	0
Atze	F	1·600	0	160	0	0
Atze	M	1·675	0	40	0	0
Atze	M	1·550	0	40	0	0
Atze	M	1·425	0	40	0	0
California*	F	1·600	0	160	0	0
California	M	1·800	0	40	0	0
California	M	1·550	0	640	0	0
California	F	1·600	640	2560	40	40
California	F	1·650	320	1280	0	160
California	M	1·525	320	2560	40	640
California	M	1·725	5120	40960	640	640
California	F	1·500	10240	40960	1280	640
California	F	1·625	0	40	0	0
California	F	1·550	5120	40960	640	160
California	M	1·850	2560	40960	80	640
California	F	1·550	160	2560	0	160
California	M	1·475	2560	10240	40	2560
California	M	1·575	2560	10240	160	160
Kooloola	M	1·475	0	160	0	0
Kooloola	M	1·575	0	160	0	0
Kooloola	F	1·450	0	160	0	0
Kooloola	M	1·700	0	40	0	0
Kooloola	F	1·650	0	40	0	0
Kooloola	F	1·700	0	40	0	0
Kooloola	M	1·650	0	80	0	0
Kooloola	M	1·600	0	160	0	0
Kooloola	M	1·600	0	40	0	0
Kooloola	M	1·600	0	160	0	0

* RHD was confirmed to be present at 'California' on the basis of vcELISAs on liver samples from cadavers found on site. ELISA results are expressed as reciprocal dilutions.

of possible seasonal effects on titres, samples were pooled when initial analyses using GLM showed that the month of sampling was not a significant explanatory factor. Non-significant variables and factors were dropped from the final model, summarized in Table 3.

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Table 2. *Regression analysis of log-transformed cELISA titres from rabbits shot at five sites in the Murray–Mallee, South Australia*

Accumulated analysis of variance*					
Change	D.F.	Sum of squares	Mean square	Variance ratio	*F* probability
+ $\log_{10}$ (IgG titre)	1	75·50	75·50	1017·06	<0·001
+ $\log_{10}$ (IgA titre)	1	2·56	2·56	34·55	<0·001
Residual	42	3·12	0·07		
Total	44	81·19			
Estimates of parameters for fitted regression					
	Estimate	S.E.	t_{42}	*t* probability	
Constant	0·053	0·046	1·15	0·258	
$\log_{10}$ (IgA titre)	0·856	0·072	11·92	<0·001	
$\log_{10}$ (IgG titre)	0·512	0·087	5·88	<0·001	

* Explanatory variables initially fitted included log-transformed IgA and IgG titres and location.

Table 3. *Estimates of parameters for the regression fitted to log-transformed cELISA titres from young rabbits with maternal antibodies (i.e. seropositive but lacking IgA) live-caught at Gum Creek in October, November and December 1996*

	Estimate	S.E.	t_{106}	*t* probability
Constant	0·643	0·145	4·45	< 0·001
$\log_{10}$ (IgG titre)	0·4902	0·0414	11·84	< 0·001
Weight	−0·635	0·114	−5·56	< 0·001

Explanatory variables initially fitted included log-transformed IgG titres, body weight and month.

The fitted model,

$$\log_{10}(\text{cELISA}+1) = 0{\cdot}643 + 0{\cdot}4902 \times \log_{10}(\text{IgG}+1) - 0{\cdot}635 \times \text{weight (kg)},$$

explained 82% of the variance. Log-transformed cELISA titres were clearly explained in terms of log-transformed IgG titre and the negative body weight parameter showed that maternal antibodies declined in larger, older rabbits.

In this analysis, data from some rabbits had high leverage in the fitted regression. These were generally large rabbits, 1·3 kg or above, that had lost all trace of maternal antibodies. Similar animals could be justifiably excluded from future analyses because their inclusion would undoubtedly distort fitted regressions.

Antibody persistence and change in previously infected rabbits

Among rabbits that had been infected with RHDV but had survived and formed antibodies, it was anticipated that titres would remain constant or slowly decline with time after infection. However, GLM analyses (not shown) indicated no clear decline in antibody cELISA titres with body weight (as an indicator of age). The sera of previously infected rabbits showed only a decline in IgM and, to a lesser extent, IgA titres with body weight. This could be explained in terms of isotypes IgM and IgA being less persistent than IgG following initial infection. Interestingly, month of sampling was also a significant explanatory variable of cELISA titres suggesting a seasonal pattern in antibody titres rather than a simple diminution of titre with time since infection.

The failure of analyses using GLM to provide a clear picture of antibody persistence in young rabbits probably relates to the complexity of responses of rabbits of different ages and individual variation between rabbits. However, the absence of a long-term decline in the antibodies of older rabbits was not surprising in view of subsequent investigations. Table 4 provides examples of five individual rabbits for which records of cELISA and isotype titres were

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Table 4. *Examples of rabbits that recovered from RHD and subsequently showed variation in titres (expressed as reciprocals) of cELISA, IgG and IgA*

		Titres*				
Date	Weight (kg)	cELISA	IgG	IgM	IgA	Notes
Rabbit no. 1383 (male)						
28 Feb 96	1·500	> 640*	2560	N	320	
8 May 96	1·575	1280	5120	N	< 160	
18 Nov 96	1·650	< 2560	10240	N	2560	
17 Dec 96	1·550	ns†	10240	N	40	
16 Apr 97	1·750	< 640	5120	N	40	
14 Aug 97	1·700	> 640	10240	N	N	
18 Dec 97	1·725	1280	10240	N	1280	
Rabbit no. 1386 (female)						
28 Feb 96	1·550	640	20480	40	N	
18 Nov 96	1·775	< 2560	10240	< 40	2560	
19 Feb 97	1·675	< 640	40960	N	40	
15 Apr 97	1·775	1280	20480	N	< 40	
21 Jun 97	1·850	1280	20480	N	160	
12 Aug 97	2·000	< 640	40960	N	40	15 days pregnant
3 Oct 97	2·000	1280	10240	N	160	Lactating
11 Nov 97	1·800	1280	> 10240	N	160	
17 Dec 97	1·825	2560	< 40960	N	1280	Lactating, not pregnant
Rabbit no. 1387 (male)						
28 Feb 96	1·450	> 640	2560	N	< 40	
10 Oct 96	1·575	< 2560	< 40960	< 40	640	
19 Nov 96	1·650	1280	40960	N	40	
18 Dec 96	1·600	1280	20480	< 40	< 40	
18 Feb 97	1·550	> 640	10240	N	< 40	
20 Jun 97	1·725	1280	2560	N	2560	
12 Aug 97	1·650	1280	< 40960	N	< 40	
Rabbit no. 2268 (male)						
1 Aug 96	1·625	320	320	N	N	
4 Oct 96	1·725	ns	ns	ns	ns	
18 Nov 96	1·650	> 640	2560	N	160	
16 Dec 96	1·575	> 640	20480	N	N	
18 Feb 97	1·550	640	10240	N	N	
15 Apr 97	1·675	640	< 10240	N	N	
20 Jun 97	1·625	320	> 2560	N	N	
13 Aug 97	1·675	640	1280	N	N	
17 Dec 97	1·675	640	5120	N	N	
Rabbit no. 2612 (female)						
4 Oct 96	1·025	> 160	640	N	2560	
20 Nov 96	1·425	> 40	320	N	< 40	
18 Dec 96	1·525	< 40	160	N	N	
21 Jun 97	1·850	320	320	N	5120	18 days pregnant
13 Aug 97	1·800	80	160	N	640	Lactating, not pregnant
11 Nov 97	1·875	80	> 160	N	640	
17 Dec 97	1·775	80	160	N	2560	

* > indicates slightly more than, < slightly less than.
† ns, no sample.

obtained on frequent occasions. In these rabbits, IgM isotype antibodies were not generally detectable except in association with the initial infection with RHD. However, IgA antibodies were more persistent though highly variable. IgA often fell to very low levels then reappeared again at moderate titres (IgA > 160). Re-exposure to RHDV seems the most likely explanation for these serological patterns and

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Table 5. *Summary of different immunological classes developed on the basis of cELISA, isotypes titre and body weight*

	Titre*				
Class	cELISA	IgG	IgM	IgA	Notes
Negative	−	−	−	−	
Pre-existing antibodies	± (rare)	+	−	−	
Maternal antibodies	+	+	−	−	Rabbits < 1300 g
Previously infected rabbits					
Recent infection	**+**	**+**	**+**	**+**	IgM > 640
Past infection	+	+	±	±	
Re-infected rabbits	**+**	**+**	−	+	IgA > 160

* **+**, high titre; +, low titre; −, no antibodies.

the boosting of antibody titres would certainly explain why cELISA and IgG titres persist and remain relatively high over long periods.

Criteria for determining immunity classes

Using antibody isotypes, rabbits could be placed in six serological categories. These were: (i) *Negative*, no antibodies to RHD detected; (ii) *Pre-RHD reactivity*, mainly detected at low titre by IgG isoELISA, uncommonly detected by cELISA as well, not reactive in IgA and IgM isoELISAs; (iii) *Maternal antibodies*, detected by cELISA, antibodies exclusively IgG, (no IgM or IgA), restricted to rabbits less than 11 weeks old, or less than 1·3 kg; (iv) *Rabbits with antibodies following recent infection* cELISA includes both IgG and IgA components and high levels of IgM (> 640); (v) *Rabbits with antibodies from past infection*, cELISA normally includes components of IgG and IgA isotypes. IgA must be present in rabbits < 1·3 kg to distinguish them from rabbits carrying maternal antibodies; (vi) *Re-infected rabbits*, restricted to older, mature rabbits, cELISA components IgG and IgA with the latter having a titre > 160, no IgM.

To facilitate comparisons, these criteria are also given in Table 5. In using these criteria, there was usually little difficulty in deciding the status of rabbits. Even when antibody titres in immune adult rabbits occasionally fell to such low levels that IgA became undetectable (e.g. rabbit no. 2268, Table 4) and rabbits then had antibody patterns similar to those of young with maternal antibodies, body weight (⩾ 1·3 kg) provided a distinguishing measure. Furthermore, the immunological history of older rabbits was generally so well known that there was no confusion over their correct immunological category.

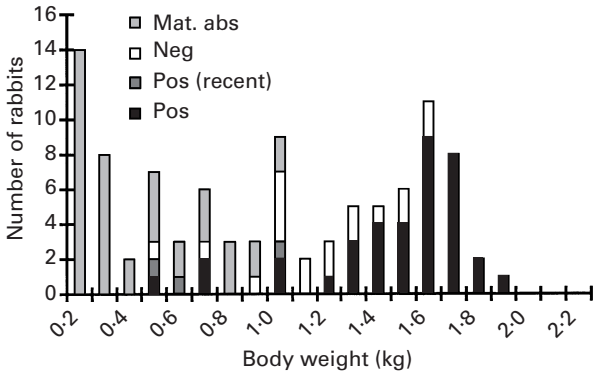

Fig. 1. The serological status of rabbits in different weight classes at Gum Creek in November 1996. Young rabbits below 0·5 kg had maternal antibodies, but as these declined many young became infected with RHD. All breeding adult rabbits (> 1·4 kg) had high titres of antibodies to RHD.

Very young rabbits that formed few antibodies or lost them soon after infection presented a potentially greater problem for classification. Some may have lost antibodies to the extent that they were misclassified as being seronegative or still retaining maternal antibodies. However, such problems probably occurred infrequently. Fewer than 3% of young had equivocal IgA titres and could not be readily categorized. In addition, less than 10% of seronegative young survived later RHD outbreaks relative to seropositive rabbits of the same age (B. D. Cooke, unpublished data). This confirmed that the most of seronegative rabbits were indeed susceptible and that no major classification errors were made.

Field application

The criteria on serological status were applied to all field samples collected from Gum Creek during 1996 and 1997. Figure 1 provides an example drawn from

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Table 6. *Summary of data from Gum Creek showing indices of population size and broad changes in immune status of population with recruitment of young and passage of RHD*

	1995	1996						1997						
	29–30	28 Feb –	8–11	31 Jul–2	1–10	18–21	15–18	17–20	15–18	20–23	12–23	2–5	11–14	17–20
Sample date	Nov	1 Mar	May	Aug	Oct	Nov	Dec	Feb	Apr	Jun	Aug	Oct	Nov	Dec
Rabbits caught (*n*)	3	22	26	14	72	115	65	66	59	40	37	22	68	76
Known alive*	–	–	30	30	87	122	80	70	79	54	49	52	92	85
% Pre-existing abs.	25	4	0	0	1	2	0	0	3	4	0	0	0	0
% Negative	9	0	0	0	10	18	14	41	23	19	11	0	32	44
% Previously infected	66	96	100	27	26	41	60	57	51	77	86	25	19	36
% Maternal abs.	0	0	0	73	63	39	26	2	24	0	3	75	49	20
Young recruited														
Recently infected†	0	1	2	0	4	3	1	0	0	1	0	2	2	0
Re-infected, IgA ⩾ 160‡	–	–	–	0/16	3/7	7/18	0/9	0/9	0/8	3/8	0/10	0/1	1/3	4/6
RHD present											?			

* Number of rabbits known to be alive was calculated by keeping a tally of marked rabbits not caught during one visit but recaptured on later visits.

† Recently infected rabbits (IgM > 1/640).

‡ Old previously infected rabbits with high IgA titres (⩾ 160) were considered re-infected; data show number of rabbits with high IgA/number of old rabbits caught. Data from the first three samples were not included to avoid confusion with recently infected rabbits.

§ Only 3 rabbits were live-trapped on the initial trip to Gum Creek, so data are based on a sample of 21 rabbits shot on the area.

rabbits collected on 18–21 November 1996. The serological status of the rabbits is given in relation to body weight to provide an indication of age-related changes. Adult rabbits (1·4 kg or above) were mostly immune survivors of the initial RHD epizootic in 1995.

Most young below 0·5 kg had maternal antibodies (IgG isotype only) but, by the time rabbits reached 0·8 kg only half had maternal antibodies and few rabbits retained them beyond 1·0 kg. The loss of maternal antibodies explained why the number of seronegative rabbits increased among the older sub-adult rabbits. However, some young rabbits had clearly been infected with RHDV and had survived.

Rabbits classed as 'recently infected' weighed between 0·5 and 1·0 kg. From their growth rates it was estimated that rabbits of 0·5 kg were approx. 37 days old (5 weeks) and weights of 0·8 kg and 1·0 kg corresponded to 56 and 70 days (8 weeks and 10 weeks) respectively. This implied that some young rabbits may have become infected as they lost their maternal antibodies at about 8 weeks (range 5–11 weeks).

Figure 1 also shows that a number of adult rabbits were seronegative. These had been born early in the 1996-breeding season, and had presumably lost their maternal antibodies without subsequently contracting RHD. However, all 18 of the older rabbits that had survived the first outbreak of RHD had high IgG antibody titres and 7 had IgA titres above 160 (see above) suggesting that they had been re-infected with RHDV.

Table 6 provides data on the changes in the percentage of rabbits of different antibody status in relation to the number of rabbits caught and known to be alive in the population at Gum Creek throughout 1996 and 1997. The percentage of the population classed as previously infected was reduced during each breeding season by the influx of young rabbits carrying maternal antibodies. However, many of these

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young lost their antibodies without becoming immediately infected with RHD and seronegative rabbits made up a significant part of the population in late spring and summer (November–March) each year.

The recurrence of RHD in late 1996, the first half of 1997 and again in late 1997 could be inferred from increases in the percentage of previously infected rabbits during October–November 1996, April–June 1997 and December 1997. These changes resulted mainly from the loss of seronegative rabbits as they became infected with RHDV and either died or added to the numbers of seropositive survivors. Individual rabbits that could be identified as having been recently infected or re-infected enabled an even more precise definition of the timing of disease outbreaks.

Rabbits with antibody patterns typical of pre-RHD reactivity were encountered relatively uncommonly once RHD became established. After the initial RHD outbreak finished in early 1996 they never amounted to more than about 4% of rabbits trapped and so did not cause a major problem with interpretation of field results. The decline in the proportion of rabbits showing background reactivity cannot be fully explained by arguing that RHD antibodies masked them. Seronegative rabbits made up about 40% of the population in some samples collected in 1996 and 1997 and persistent background reactivity should have been revealed under those circumstances. Some interaction between RHD and the factors causing background reactivity seems likely.

DISCUSSION

Pre-existing serum reactivity

Wild rabbits that were apparently RHD-positive on the basis of IgG isoELISA reactions were common in populations of wild rabbits across South Australia in advance of the spread of RHD in 1995. Similar evidence was obtained from sera collected in the Cooma district, New South Wales, prior to the escape of RHD from Wardang Island (T. Robinson, P. Kirkland, L. Capucci, unpublished data) and from a serological survey in New Zealand conducted before RHDV arrived [10]. Indeed, such observations are not new. RHD positive sera were found in Europe among rabbits not previously affected by RHD and in sera collected years before the sudden appearance of the disease [14, 21]. Further studies have demonstrated the existence in European domestic rabbits of a non-pathogenic virus genetically and antigenically closely related to RHDV [22]. However, the data obtained in Australia and Europe differ in at least two aspects. First, sera from rabbits in Europe were invariably positive to both cELISA and IgG ELISA, whereas in Australia positive results were obtained mainly with IgG ELISA and relatively few sera were weakly positive by cELISA as well. Second, serologically positive European rabbits were generally protected from RHD [14, 21] and this is clearly not the case for Australian or New Zealand rabbits [10]. So, if a RHDV related virus exists in Australasia, it is different from the non-pathogenic European virus and must be antigenically distant from RHDV.

From an epidemiological viewpoint, the pre-existing serum reactivity in the wild rabbit population did not confound serological data used to determine the initial spread of RHD through South Australia. Rabbits that survived infection with RHDV when it initially spread were consistently positive by cELISA. They also carried RHD-specific IgA, not present in sera prior to the spread of RHD, while IgG titres significantly higher than background levels enabled further discrimination.

Antibody levels in rabbits infected when young

RHDV has been detected by PCR in the livers of 4–10 day-old rabbits killed 12–72 h after infection, but viral replication is limited to small, scattered foci in the liver and disseminated intravascular coagulation is absent [7]. However, as RHD first spread across Australia it was not uncommon to find young rabbits weighing only 200–350 g (3–5 weeks old) that had died from RHD as indicated by high concentrations of RHDV in their livers. The absence of clinical RHDV infection in very young rabbits may initially result from the failure of liver cells or other tissues to support major virus replication but clearly this does not always apply after young wild rabbits come above ground at 3 weeks of age.

Nevertheless, rabbits less than 9 weeks old still show reduced mortality compared with adults [15] and this occurs at a time when the rabbit's immune system is still maturing. At birth, peripheral lymphoid tissues are undeveloped; Peyer's patches are not observed until 2–3 weeks of age and young rabbits less than 3 weeks old fail to make antibodies to a variety of common antigens. Circulating immunoglubulin (Ig) in newborn rabbits is primarily of the mother's allotype and the offspring's allotype Ig increases rapidly beginning at 4–6 weeks of age [23].

Serology of 5–8 week-old wild rabbits that survived experimental infection (1500 rabbit infectious doses, given intramuscularly) showed that their immune response was highly variable (B. Cooke, A. Robinson, unpublished data). Some young, even at 7 weeks of age, did not develop antibodies while others, only 5 weeks old, developed moderately high titres (IgM = 10240, IgA = 2560, IgG = 5120) within 2 weeks of inoculation. Nevertheless, the highest peak titres seen were in an 8-week-old rabbit (IgM = 40960, IgA = 5120, IgG = > 40960).

Despite the experimental evidence that rabbits infected at different ages show an increasing antibody response with age it was not possible to demonstrate a clear association between antibody patterns and body weight or age of previously infected rabbits in the field. However, this probably reflects the high variability of individual antibody responses, the wide range of ages when infection occurs and the added complication that rabbits are apparently re-infected on further exposure to RHDV over time.

Maternal antibodies

Analyses using GLM confirmed that rabbits with maternal antibodies could be readily identified because their antibodies were exclusively IgG isotype and characteristically declined with increasing body weight and age. Few rabbits carried maternal antibodies once they reached 1·2 kg in body weight.

Young rabbits acquire maternal antibodies across the placenta. This occurs in the last few days of pregnancy by active transport across the yolk sac. Although antibody isotypes IgG and IgM can both be transported in this way [24], only anti-RHDV IgG occurs in juvenile rabbits. Rabbits recovering from an initial RHDV infection are unlikely to rear young and thus RHDV specific IgM is unlikely to be found. The titre of antibody in late-stage embryos of wild rabbits, and presumably newborn young, approximates that of the mother (B. Cooke, unpublished data).

Maternal antibodies specific for the non-pathogenic RHDV-related virus in Italian domestic rabbits lasted for 4–7 weeks depending on the titre of the mother [19]. Maternal antibodies against myxoma virus also persist for up to 8 weeks in the young of Australian wild rabbits [25, 26]. Results in the study at Gum Creek confirm this general figure and further suggest that some young rabbits retained anti-RHD maternal antibodies for up to 12 weeks (equivalent to 1·2 kg in Fig. 1). Such prolonged retention of maternal antibodies probably reflects the very high IgG antibody titres (> 40960) among some adult wild rabbits.

Laboratory observations show that maternal antibodies at low titres (c-ELISA titres ~10, IgG titre ~40) do not prevent contact infection with RHDV among caged wild rabbits. Rabbits with low titres also become infected if intramuscularly inoculated, but the presence of maternal antibodies strongly reduces mortality (B. Cooke, unpublished observations). Nevertheless, some uncertainty remains over the importance of maternal antibodies in natural populations. Studies on small round-structured human caliciviruses, such as the Norwalk, Mexico and Hawaii viruses, suggest that infections generally occur after infants lose their maternal antibodies at about 5 months of age [27]. A similar model may apply to rabbits and RHDV. Young rabbits retain maternal antibodies for an average of about 8 weeks in the field, much as they do in the laboratory. This indicates that antibodies must generally protect against infection. If young rabbits were infected well before they lost their maternal antibodies they would survive and the average age of rabbits with detectable maternal antibodies would fall markedly.

As Figure 1 shows, significant numbers of young, recently recovered rabbits were sometimes present in the population at Gum Creek despite generally high mortality caused by RHD. These survivors were between 5 and 10 weeks old making it likely that they had contracted RHD while 8 weeks old or less when their chances of survival would have been higher than those of older rabbits [15]. If they retained some maternal antibodies at that time they may have been further protected from RHD.

Antibody persistence and change in previously infected rabbits

After infection, if rabbits recover, IgM titres rapidly reach a peak within 2 weeks then decline quickly. IgA titres are more persistent but they also decline. IgG titres reach a peak more slowly and persist for many months. However, there is also good evidence from field observations that rabbits previously infected with RHDV may respond on re-exposure by showing significant rises in both IgA and IgG isotypes. This occurs despite the rabbits' relatively high titres of circulating antibodies. Wild rabbits showed similar increases in IgA and IgG antibodies when reinoculated orally or intramuscularly with 1500 RID RHDV

several months after recovery from an initial RHDV infection (B. Cooke, A. Robinson, J. Merchant, unpublished observations).

The strong rise in IgA titre suggests a mucosal response on re-exposure to the virus. Recent evidence that flies may transmit RHDV, either directly to mucosal surfaces or when rabbits ingest fly faeces on vegetation, increases the likelihood of this route of re-exposure [28]. A limited replication of RHDV in the mucosa of re-exposed rabbits would provide a simple explanation for the changes in IgA and IgG titres and this has a parallel in the enteric Norwalk virus affecting humans where repeated re-infection has been demonstrated clinically. With human calicivirus infections immunity appears to have two components comprising a local mucosal antibody resistance that prevents re-infection for 2–6 months and a longer-term resistance to infection that may be built up by repeated exposure [16].

Field epidemiology

The live-trapping study at Gum Creek began just as the initial outbreak of RHD was coming to an end. However, RHDV was persistent enough in the semi-arid environment for disease to break out whenever susceptible rabbits subsequently appeared in the population. Viable RHDV was shown to persist in a cadaver for 3 weeks [29]. It is also excreted in the urine of infected rabbits, potentially contaminating wide areas. As yet, chronically infected carrier rabbits have not been demonstrated. Although their existence has been suggested from a serological study in Spain [30] this needs to be re-evaluated in view of the evidence that rabbits may be reinfected with RHDV.

Each year new rabbits came into the population at Gum Creek as a result of breeding that occurred irregularly depending on rainfall. However, the timing of RHD outbreaks lagged behind, presumably because of the natural resilience of young below 8 weeks old and because young rabbits were protected by maternal antibodies for their first few weeks of life. RHD broke out at Gum Creek about 8 weeks after the first young rabbits were born in 1996, suggesting that it only recurred when increasing age and gradual loss of maternal antibodies provided a sufficient nucleus of fully susceptible young. RHD would probably have passed through the rabbit population in a single epidemic at that time except that many late-born young only lost their maternal antibodies and became susceptible after summer had begun and conditions became less suitable for spread of RHD [9, 31]. RHD returned to infect a growing nucleus of fully susceptible rabbits as the weather became cooler in autumn (April–June) 1997. At that time, susceptible rabbits included late-born young from 1996 and others born after early rains in March 1997. RHD again broke out in late spring (November) of 1997 as young born in late winter began to lose their maternal antibodies.

There is good evidence that rabbits with circulating antibodies to RHD may be readily re-infected. This was reflected in the maintenance of relatively high antibody titres during much of the year. Antibody titres in older rabbits increased whenever RHD passed through the population and confirmed the presence of disease even when very few young survived to show antibodies.

Apart from the initial outbreak, RHD has seldom caused sharp population reductions. Instead, it has tended to offset the extent of population increase expected with breeding. Effective recruitment into the immune breeding population was low and rabbit numbers at Gum Creek remained relatively stable over the 2-year study at levels generally 85–90% lower than those seen before RHD broke out [11].

Taken together the results clearly show that ELISA methods developed in Italy were appropriate for field studies of RHD in Australia. Inspection of field data, supported by statistical evaluation using GLM enabled the resolution of potential problems in interpreting serological data and ultimately provided a framework for classifying rabbits according to their immunological status. Applying these criteria to a wild rabbit population sampled over time provided an initial insight into the epidemiology of RHD in inland South Australia. It has enabled a start to be made on unravelling details of the epidemiology and effectiveness of RHD as a biological control in Australia and should assist with studies [5, 6, 30] seeking to understand the epidemiology and management of RHD for the conservation of wild rabbits in Europe.

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REFERENCES

1. Liu SJ, Xue XP, Pu BQ, Quian NH. A new viral disease in rabbits. Animal Husb Vet Med 1984; **16**: 253–5.
2. Ohlinger VF, Haas B, Meyers G, Weiland F, Thiel H-J. Identification and characterisation of the virus causing rabbit haemorrhagic disease. J Virol 1990; **64**: 3331–6.
3. Parra F, Prieto M. Purification of a calicivirus as the causative agent of a lethal haemorrhagic disease in rabbits. J Virol 1990; **64**: 4013–5.
4. Marcato PS, Benazzi C, Vecchi G, et al. Clinical and pathological features of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev Sci Tech Off Int Epizoot 1991; **10**: 371–92.
5. Villafuerte R, Calvete C, Blanco JC, Lucientes J. Incidence of viral haemorrhagic disease in wild rabbit populations in Spain. Mammalia 1995; **59**: 651–9.
6. Marchandeau S, Chantal J, Portejoie Y, Barraud S, Chaval Y. Impact of viral haemorrhagic disease on a wild population of European rabbits in France. J Wildl Dis 1998; **34**: 429–35.
7. Lenghaus C, Westbury H, Collins B, Ratnamohan N, Morrissy C. Overview of the RHD project in the Australian Animal Health Laboratory. In: Munro RK, Williams RT, eds. Rabbit haemorrhagic disease: issues in assessment for biological control. Aust. Gov. Printing Services, Canberra: Bureau of Resource Sciences, 1994: 104–129.
8. Fenner F, Fantini B. Biological control of vertebrate pests. The history of myxomatosis – an experiment in evolution. CABI, 1999.
9. Kovaliski J. Monitoring the spread of rabbit haemorrhagic disease virus as a new biological agent for the control of European rabbits in Australia. J Wildl Dis 1998; **34**: 421–8.
10. O'Keefe JS, Tempero JE, Motha MXJ, Hansen MF, Atkinson PH. Serology of rabbit haemorrhagic disease virus in wild rabbits before and after release of virus in New Zealand. Vet Microbiol 1999; **66**: 29–40.
11. Mutze G, Cooke B, Alexander P. Initial impact of rabbit haemorrhagic disease on European rabbit populations in South Australia. J Wildl Dis 1998; **34**: 221–7.
12. Cooke BD. Field epidemiology of rabbit calicivirus disease in Australia. In: Chasey D, Gaskell RM, Clarke IN, eds. Proceedings of the first international symposium on caliciviruses. European Society for Veterinary Virology, 1997: 151–5.
13. Capucci L, Scicluna MT, Lavazza A. Protocollo delle reazioni ELISA per la diagnosi virologica e sierologica della malattia emorragica virale del coniglio (RVHD) e virologica della European brown hare sindrome della lepre (EBHS). Selezione Vet 1991; **32**: 1381–97.
14. Capucci L, Scicluna MT, Lavazza A. Diagnosis of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev Sci Tech Off Int Epizoot 1991; **10**: 347–70.
15. Morrisse J-P, Le Gall G, Boilletot E. Hepatitis of viral origin in Leporidae: introduction and aetiological hypothesis. Rev Sci Tech Off Int Epizoot 1991; **10**: 283–95.
16. Johnson PC, Mathewson JJ, DuPont HL, Greenberg HB. Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults. J Infect Dis 1990; **161**: 18–21.
17. Dudzinski ML, Mykytowycz R. The eyelens as an indicator of age in the wild rabbit in Australia. CSIRO Wildl Res 1961; **6**: 156–9.
18. Dudzinski ML, Mykytowycz R. Analysis of weights and growth rates of an experimental colony of wild rabbits *Oryctolagus cuniculus* (L.). CSIRO Wildl Res 1960; **5**: 102–15.
19. Capucci L, Nardin A, Lavazza A. Sercoconversion in an industrial unit of rabbits infected with a non-pathogenic rabbit haemorrhagic disease-like virus. Vet Rec 1997; **140**: 647–50.
20. Collins BJ, White JR, Lenghaus C, Morrissy CJ, Westbury HA. Presence of rabbit haemorrhagic disease in rabbit tissues as revealed by a monoclonal antibody dependent capture ELISA. J Virol Meth 1996; **58**: 145–54.
21. Rodak L, Smid B, Valicek L. Identification of the viral haemorrhagic disease virus of rabbits as a calicivirus. Rev Sci Tech Off Int Epizoot 1991; **10**: 513–24.
22. Capucci L, Fusi P, Lavazza A, Pacciarini ML, Rossi C. Detection and preliminary characterisation of a new calicivirus related to rabbit haemorrhagic disease but non-pathogenic. J Virol 1996; **70**: 8614–23.
23. Knight KL, Crane MA. Generating the antibody repertoire in rabbit. Adv Immunol 1994; **56**: 179–218.
24. Merad Z, Wild AE. The route of maternal IgM transport to the rabbit fetus. Placenta 1992; **13**: 291–304.
25. Fenner F, Marshall ID. Passive immunity in myxomatosis of the European rabbit (*Oryctolagus cuniculus*): the protection conferred on kittens borne by immune does. J Hyg 1954; **52**: 321–36.
26. Kerr PJ. An ELISA for epidemiological studies of myxomatosis: persistence of antibodies to myxoma virus in European Rabbits (*Oryctolagus cuniculus*). Wildl Res 1997; **24**: 53–65.
27. Cubitt D, Parker S, Jiang X, Matson DO. A review of serological studies on human caliciviruses. In: Chasey D, Gaskell RM, Clarke I N, eds. Proceedings of the first international symposium on caliciviruses. European Society for Veterinary Virology, 1997: 10–6.
28. Asgari S, Hardy JRE, Sinclair RG, Cooke BD. Field

evidence of mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (DIPTERA: Calliphoridae) among wild rabbits in Australia. Virus Res 1998; **54**: 123–32.
29. Westbury H. Field evaluation of RCD under quarantine. Final report of Project CS 236 to the Meat Research Corporation.
30. Simon MC, Ortega C, Maynar P et al. Studies in wild rabbit (*Oryctolagus cuniculus*) populations in Navarra (Spain). I. Epidemiology of viral haemorrhagic disease. Gibier Faune Sauvage, Game Wildl. 1998; **15**: 47–64.
31. Smyth RE, Cooke BD, Newsome AE. The spread of rabbit calicivirus in relation to environmental factors. Canberra: CSIRO Wildlife and Ecology, 1997.