

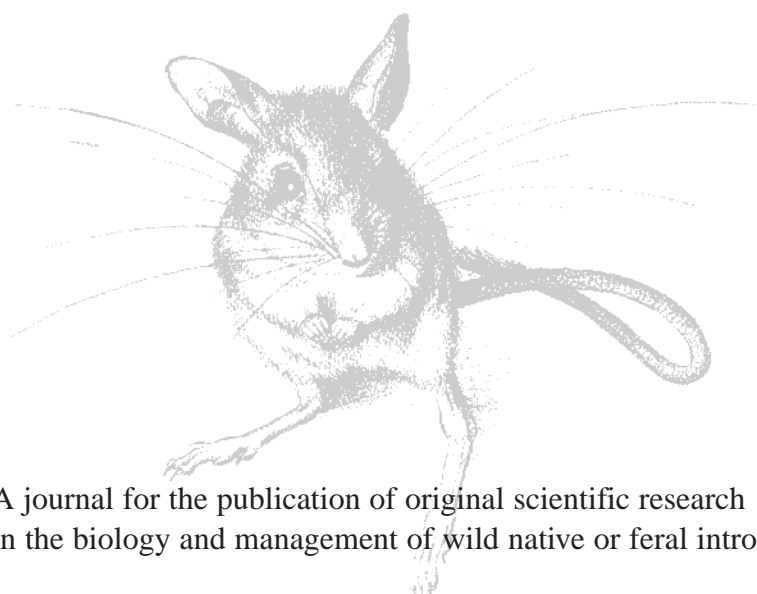
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## **An ELISA for Epidemiological Studies of Myxomatosis: Persistence of Antibodies to Myxoma Virus in European Rabbits (*Oryctolagus cuniculus*)**

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### *Abstract*

An ELISA (enzyme-linked immunosorbent assay) for detecting antibodies to myxoma virus was characterised in wild rabbits for use in epidemiological studies. Virus neutralisation assays and virus challenge were used to define sera from rabbits as positive or negative for myxoma-virus antibodies. In a group of naturally infected wild rabbits, antibodies to myxoma virus were readily detectable by ELISA each month for at least 12 months in all rabbits, including those where neutralising antibodies could no longer be detected. Maternally transferred antibodies could be detected in kittens born to immune does for approximately six weeks after birth. IgM antibodies to myxoma virus were detected by ELISA only during the active disease and recovery phase of myxomatosis. The ratio of IgM:IgG at a standard serum dilution provided an index of time since infection and a confirmatory assay for early myxomatosis, because the detection of IgM corresponded approximately with the onset of clinical signs. Rabbit antibodies to the orthopoxvirus, vaccinia, did not cross-react in the ELISA.

### **Introduction**

Myxoma virus (MV) is a member of the poxvirus family, in the genus *Leporipoxvirus* (Francki *et al.* 1991). It is the aetiological agent of myxomatosis in European rabbits (*Oryctolagus cuniculus*) and was released in Australia during 1950 as a biological control for wild rabbits. The virus is now endemic in the Australian rabbit population. Although myxomatosis was initially highly successful in controlling rabbits, there was rapid selection for attenuated virus strains, which were more efficiently transmitted, and genetically resistant rabbits (Fenner 1983). The proportion of rabbits, in field populations, that survives infection cannot be determined clinically (Fenner *et al.* 1953) and so serological assays to determine the proportions of immune and susceptible animals in a population have been used in many epidemiological studies of myxomatosis. The main assays used have been virus neutralisation (Fenner *et al.* 1953), complement fixation (Fenner *et al.* 1953) and immunodiffusion (Sobey *et al.* 1966, 1970). In addition, there has been one report of an enzyme-linked immunosorbent assay (ELISA) (Wetherall *et al.* 1983).

Rabbits that have recovered from myxomatosis appear to have lifelong immunity to the disease, although occasional increases in antibody titres in monitored field rabbits suggest that re-exposure to MV with transient virus replication may occur in some rabbits (Fenner *et al.* 1953; Fenner and Ross 1994). In studies on wild rabbits that have recovered from myxomatosis, antibodies to MV, measured by virus-neutralisation tests, persisted at high levels for at least 20 months. In the same rabbits, antibodies to MV measured by complement-fixation tests had initial high titres that declined 8–32-fold after approximately six months and then remained at moderate titres for the remainder of the study (Fenner *et al.* 1953).

In contrast, Williams *et al.* (1973) report that, in wild rabbits in the field, antibody titres, measured by double immunodiffusion to soluble MV antigens (Sobey *et al.* 1966, 1970), fluctuated over short time intervals. In more than 50% of immune rabbits, antibodies to MV

became undetectable at some stage during the sampling only to reappear at later sampling. These fluctuations were unrelated to clinical outbreaks of myxomatosis. The immunodiffusion assay has been widely used in Australia for epidemiological studies of myxomatosis (Williams *et al.* 1973; Sobey and Conolly 1975; Shepherd *et al.* 1978; Edmonds *et al.* 1978); however, its reliability for accurate studies must be questioned.

More recently, Wetherall *et al.* (1983) used an ELISA to follow the early immune response to infection of laboratory rabbits with an attenuated strain of MV. These authors also examined the proportion of seropositive rabbits at a field study site both immediately after an epidemic of myxomatosis and in a population where myxomatosis had not been seen for some years. However, they did not use the ELISA to measure either the duration of the humoral response to MV infection in wild rabbits recovered from natural infection or to assess whether antibody titres fluctuate in the field as described by Williams *et al.* (1973). No correlation of ELISA with a standard assay such as virus neutralisation has been reported.

Thus, the two reasons for undertaking this project were as follows: first, to provide a convenient, well-characterised assay, correlated with virus neutralisation as a standard assay, for use in epidemiological studies of myxomatosis; and, second, to re-examine the persistence of detectable antibodies to MV in wild rabbits under field conditions. The work reported here describes the use of an ELISA to detect antibodies to MV, including isotype-specific IgG and IgM detection, and follows the antibody titre to MV in naturally infected wild rabbits for up to two years. In addition, the persistence of maternally transferred antibodies detected by ELISA in kittens has been determined.

## Materials and Methods

### *Viruses and Cells*

The Lausanne (LU) and Glenfield (GV) strains of MV used in these studies were derived from the virus stocks plaque-purified and described by Russell and Robbins (1989). Field strains of MV (Gungahlin/1-91 and Hall/11-90) were isolated from rabbit tissues by culture in Sirc cells. Vaccinia virus (WR strain) was obtained from Mr Harold Bults, CSIRO Division of Wildlife and Ecology. Virus stocks were cultured in RK13 or Sirc cells grown in minimal essential medium (MEM) supplemented with 10% newborn bovine serum, 200 units  $\text{mL}^{-1}$  penicillin and 100  $\mu\text{g mL}^{-1}$  streptomycin. Plaque assays and plaque reduction neutralisation assays were performed on Vero cell monolayers in MEM supplemented as above. Virus titres are expressed as plaque-forming units (pfu) per millilitre.

### *Antigen Preparation*

Antigen for ELISA was prepared from intracellular MV concentrated essentially as described by Russell and Robbins (1989), with the exception that the trypsin digestion was omitted and Dounce homogenisation was replaced by sonication. Briefly, virus was grown in Sirc or RK13 cells in 180- $\text{cm}^2$  tissue-culture flasks until uniform cytopathic effect was observed; 6–12 flasks were used for each preparation. The infected cell monolayers were washed twice with phosphate-buffered saline (PBS) (pH 7.2) and the cells were scraped from the flask and pelleted by centrifugation (800 g, 10 min). The pellets were resuspended in 5 mL of PBS and sonicated to release intracellular MV. This suspension was digested with DNaseI (25  $\mu\text{g mL}^{-1}$ ) and RNaseA (50  $\mu\text{g mL}^{-1}$ ) at 37°C for 30 min with frequent agitation. Virions were pelleted by centrifugation (250 000 g, 20 min in a Beckman TLS-55 rotor) through a step gradient formed by overlaying 0.54 mL 10% dextran T10 with the same volume of 36% sucrose, both solutions in 10 mM Tris HCl at pH 8.0 and (1 mM ethylenediamine tetra-acetic acid (EDTA)). The pellet was resuspended in PBS and the step-gradient centrifugation repeated. The final pellet was resuspended in 0.5–1.0 mL of PBS, aliquoted and stored frozen at –20°C. This produced a high-titred (approximately  $10^9$  pfu  $\text{mL}^{-1}$ ) antigen stock. The LU strain was chosen as the standard antigen because it grew rapidly to high titre in cell culture.

### *ELISA*

Antigen (50  $\mu\text{L}$  in PBS at pH 7.2) was added to each well of 96 well plates (Nunc) at a dilution determined by titration against a standard reference serum to produce an optical density (OD) at 405 nm of 1.0 at a serum dilution of 1/100. Absorption extended for 2 h at 37°C followed by three washes with 5% (w/v) skim milk powder in PBS (milk/PBS). Plates were then blocked with milk/PBS overnight at 4°C. Sera

were centrifuged (5 min at 13000 *g* in a microfuge) and then diluted in milk/PBS: 50  $\mu$ L per well at a minimum dilution of 1:50 (concentrations higher than 1:50 produced high background). Plates were incubated at 37°C for 2 h, washed three times with PBS and 0.05% Tween 20 (PBS/Tween), and 50  $\mu$ L of an anti-rabbit-Ig horseradish-peroxidase conjugate (Silenus) diluted 1:1000 in milk/PBS was added. Incubation extended for 30 min at 37°C, and was followed by six washes with PBS/Tween and the addition of 100  $\mu$ L of substrate [ABTS (2,2-azino bis 3-ethylbenzthiazoline sulphonate) at 1 mg mL<sup>-1</sup> plus 0.06% hydrogen peroxide, in 0.1 M citrate phosphate buffer at pH 4.0]. After incubation of the plates for 20 min at room temperature, absorbance at 405 nm was read on a Biorad model 3350 microplate reader. For each plate, the zero absorbance reading was set on wells containing antigen alone plus substrate. Standard positive and negative serum controls were included in each assay. Results are reported as either titres (reciprocal of the last dilution producing a positive assay) or as optical density readings at 405 nm (OD 405 nm) for a standard serum dilution of 1:100 (unless otherwise stated).

Assays for IgM or IgG followed the same format with the substitution of the appropriate anti-isotype conjugate (Southern Biotechnology). Conjugate concentrations for anti-rabbit-IgM (1:1000) and anti-rabbit-IgG (1:4000) were determined by titration against the standard anti-rabbit-Ig conjugate such that the anti-rabbit-IgG conjugate was diluted to give the same OD 405 nm with serially diluted test sera as the standard anti-rabbit-Ig conjugate. In contrast, anti-rabbit-IgM conjugate was used at a dilution that produced the maximum positive OD 405 nm for sera from rabbits early in the course of myxomatosis, and a minimal background. Thus, there is no direct relationship between IgM and IgG OD 405 nm values. There was very little cross-reactivity between the anti-rabbit-Ig conjugate and the anti-rabbit-IgM conjugate at the dilutions used, indicating that the anti-Ig conjugate was not detecting significant amounts of IgM. Background in IgM assays was higher and more variable than that in Ig or IgG assays.

#### *Plaque Reduction Neutralisation Assays*

Myxoma-virus neutralisation assays were performed as plaque reduction neutralisation assays (PRNAs) on Vero cell monolayers in six-well dishes (Nunc) with constant virus concentration (approximately 100 pfu of LU) and two-fold serum dilutions from 1:10 to 1:320. Virus and serum dilutions were mixed and incubated at 37°C for 60 min. Duplicate aliquots from each tube were then plated onto Vero monolayers for plaque development. Titres were expressed as the reciprocal of the last dilution of serum producing more than 50% reduction in plaque numbers compared with normal serum. Undiluted sera were heated to 56°C for 30 min prior to testing to inactivate complement.

#### *Positive and Negative Sera*

Standard positive-control serum was provided by Mr Harold Bults (CSIRO Division of Wildlife and Ecology) from a rabbit immunised with Shope fibroma virus and then hyperimmunised with the LU strain of MV. This serum neutralised MV in PRNAs with a titre of more than 1000. It was aliquoted to provide a standard control. Negative sera were obtained from 18 laboratory rabbits maintained free of MV. After testing each individual serum with ELISA, these sera were pooled and the pool aliquoted to provide standard negative serum. Sera from wild rabbits observed with clinical symptoms of myxomatosis were confirmed as positive for specific MV antibodies by plaque reduction neutralisation. These sera were used to determine high and low positive values in the ELISA.

Additional sera were available from wild rabbits shot during a rabbit irruption in November 1990 on the New South Wales–South Australian border north-west of Broken Hill. Rabbits were sampled at eight widely separated locations over an area of approximately 400 km<sup>2</sup>. Animals were assessed for clinical myxomatosis and serum was collected.

For experimental determination of IgM levels, four domestic rabbits were infected with approximately 100 pfu, in a 0.1-mL volume of PBS, of a highly attenuated (grade 5; Fenner and Marshall 1957) strain of MV (Hall/11-90) by intradermal inoculation.

Antisera to vaccinia virus were prepared by inoculating two laboratory rabbits intradermally with  $5 \times 10^4$  pfu of vaccinia virus, WR strain, in 0.1 mL PBS.

#### *Rabbits*

Wild rabbits observed to have clinical myxomatosis during a natural epidemic in January 1991 were captured and released into an enclosed 0.15-ha paddock. Rabbits were ear-tagged for identification and allowed to recover from myxomatosis. Artificial warrens, shelter boxes and water were provided; pasture was supplemented with lucerne hay and oats. Approximately 50% of the rabbits recovered from myxomatosis and 20 rabbits were used for subsequent sampling. These rabbits were recaptured by hand-

netting and sampled for blood each month for the next 12 months. Some animals were sampled again two years after infection.

To determine the persistence of passively acquired maternal antibodies in kittens, litters born to six captive, myxoma-immune, wild rabbits, were held with their mothers from birth to nine weeks old, in small outside yards. Individual animals in the litter were identified by ear-notching. Lucerne hay, water and pasture were available *ad libitum*. Shelter boxes and shade were provided.

Laboratory rabbits were housed in individual cages in an environmentally controlled room. Water and pelleted food were available *ad libitum*; green vegetables were fed to the rabbits twice weekly.

All work involving animals was approved by the CSIRO Division of Wildlife and Ecology Gungahlin Animal Experimentation Ethics Committee.

## Results

### ELISA Development

Serum and antigen dilutions that were appropriate for routine use in the ELISA were determined by titrating sera from known-negative rabbits and positive sera defined by neutralisation assay. An example of a titration curve of standard positive control serum and serum from a known-negative rabbit (Fig. 1) shows that the linear range for the standard positive control extended from 1:100 to 1:10000 when the antigen concentration was adjusted to produce an OD 405 nm of about 1.0 at 1:100 dilutions of the serum. These concentrations of serum and antigen were originally determined by checkerboard titrations. Dilutions of negative sera below 1:50 produced high and inconsistent background (data not shown), and the minimum titres found for known-positive rabbits were 1:200. Therefore, dilutions of either 1:50 or 1:100 were used to maximise the difference between background and positive signal while still detecting low-titre positives. The end-point for titrations was empirically set at 0.1 OD units above the OD of the negative-control serum at a dilution of 1:100. Centrifuging the sera (13000 g, 5 min) after thawing and before use reduced non-specific background.

For epidemiological studies involving large numbers of samples, it is convenient to be able to assay sera at a single dilution, in this case, 1:100. Generally, it is sufficient to report results qualitatively as either positive or negative. Therefore, it was necessary to set cut-off values for positive and negative results. To determine cut-off points for positive and negative samples, 18 sera from known-negative rabbits and 21 positive sera were examined in duplicate at 1:100 dilution on duplicate 96-well plates. The positive sera were obtained from 19 wild rabbits that

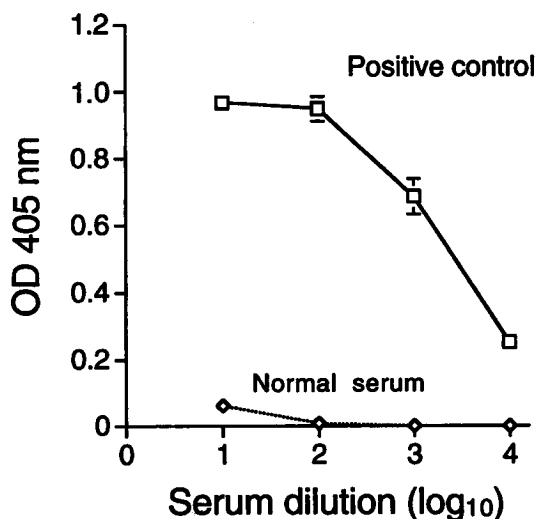


Fig. 1. Comparison of titrations of normal serum and positive-control serum in the myxoma virus ELISA (enzyme-linked immunosorbent assay). Sera were diluted 10-fold from 1:10 to 1:10000 and each dilution was measured in quadruplicate. Values are optical densities at 405 nm (OD 405 nm) (mean  $\pm$  s.d.; error bars were too small to plot on the negative serum).

had recovered from myxomatosis, and were confirmed by neutralisation assays and ELISA titration and two independent dilutions of the standard positive serum. The results are shown in Fig. 2. Little difference existed in OD values of the negative sera between the two plates: the mean OD 405 nm ( $\pm$  s.d.) of Plate 1 was  $0.03 \pm 0.02$  and of Plate 2 was  $0.03 \pm 0.03$  (range for both plates, 0–0.12). Between-plate differences were greater for some of the positive sera, but this did not alter the qualitative interpretation of the assay. The comparisons for standard positive-control serum were as follows: Plate 1,  $1.02 \pm 0.08$ ; Plate 2,  $0.89 \pm 0.02$ .

At least 0.25 OD units occurred between the lowest positive sample and the highest negative serum. The cut-off value for positive sera was therefore empirically set at 0.25 OD units above the mean background OD of the negative sera, which incorporated all the known-positive sera. The negative value was set at 0.1 OD unit above the mean background OD of the negative sera. This produced a higher cut-off value than that obtained by calculating three standard deviations from the mean, but included all the negative sera. Values between the positive and negative cut-offs were regarded as equivocal.

To further determine variation between plates, four negative samples were assayed in triplicate on each of three plates. Mean OD values ( $\pm$  s.d.) for the negative sera on each plate were as follows: Plate 1,  $0.03 \pm 0.01$ ; Plate 2,  $0.04 \pm 0.01$ ; and Plate 3,  $0.03 \pm 0.01$ . The standard positive-control serum was assayed in duplicate on each plate and mean values were 1.08, 1.18 and 1.03, respectively, for the three plates. These experiments, together with those described above, indicated that there was minimal variation between plates.

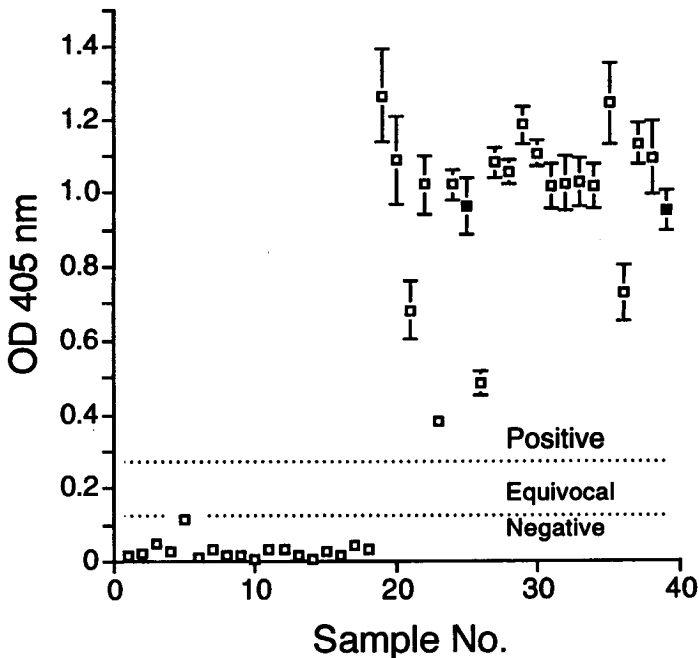


Fig. 2. Measurement of known positive and negative sera in myxoma virus (MV) ELISA (enzyme-linked immunosorbent assay) to determine cut-off values. Sera were diluted 1:100 and anti-MV antibodies measured at OD 405 nm (optical density at 405 nm; mean and range). Each serum was measured in duplicate on duplicate plates. The filled squares indicate the standard positive serum. Sera were regarded as positive (0.25 OD units above the negative-control sera mean), negative (below 0.1 OD units above the negative-control sera mean) or equivocal (between the negative and positive zones).

### *Sensitivity and Specificity of the ELISA*

Sera from wild rabbits that had recovered from myxomatosis were collected each month for 12 months and the antibody titres to MV determined by ELISA. Data are presented from 12 rabbits for which at least 10 monthly samples were available (Fig. 3). Neutralising titres were determined at the beginning and end of the sampling period for all 12 rabbits (Fig. 4) and monthly for three rabbits (Fig. 5). ELISA titres were strongly correlated to neutralisation titres in March 1991 samples ( $r = 0.95$ ,  $P < 0.001$ ; calculated with  $\ln$  titre) but less strongly correlated in February 1992 samples ( $r = 0.65$ ,  $P < 0.05$ ).

To confirm the specificity of the antibodies measured by both ELISA and neutralisation, four of the recovered wild rabbits, including two with low neutralising titres (Nos 4802 and 4825), were challenged at two years after infection with  $10^4$  pfu of the virulent LU strain of MV. None of the rabbits showed clinical signs of myxomatosis, although all four rapidly developed a circumscribed swelling at the site of injection. This had resolved by 10 days after inoculation. Two of the four rabbits had a significant increase in ELISA titre (Table 1). Rabbit 4802, which had low neutralising titres and low ELISA titres, showed a 32-fold rise in ELISA titre but did not develop clinical signs, indicating that the rabbit was immune to MV as had been shown by previous antibody results. Rabbit 4812, which had a prechallenge ELISA titre of 3200, had a four-fold increase in ELISA titre following challenge. The other two rabbits did not show significant changes in titres (as is common in clinical use, significance was regarded as a four-fold rise in titre).

**Table 1.** ELISA (enzyme-linked immunosorbent assay) titres in wild rabbits challenged with  $10^4$  plate-forming units of virulent myxoma virus two years after recovery from myxomatosis

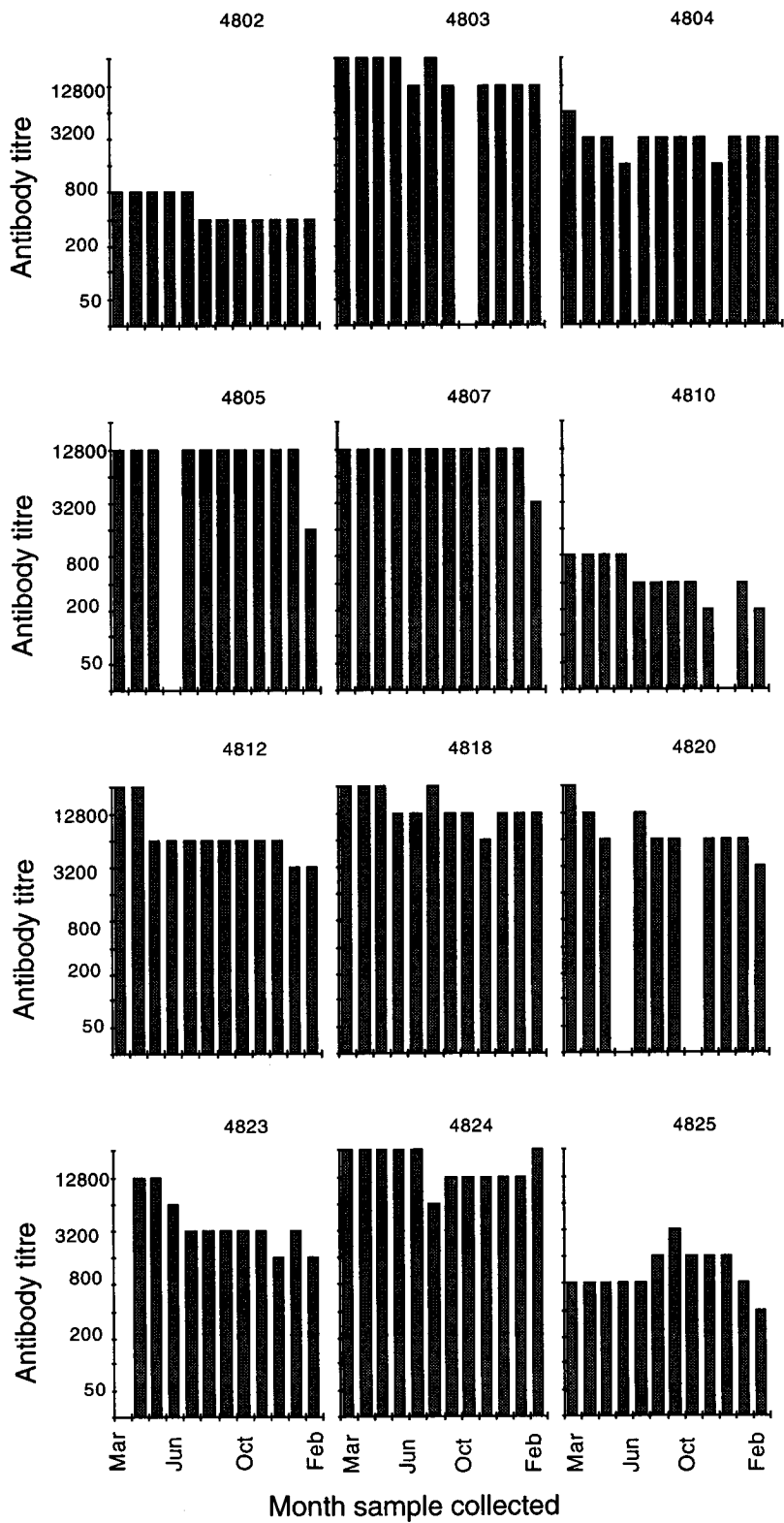
Postchallenge titre is that at 20 days after challenge. \*, significant alteration in titre (significance was defined as a rise of four-fold or greater)

Rabbit No.	Prechallenge titre	Postchallenge titre
4802	400	12800*
4812	3200	12800*
4823	3200	6400
4825	12800	6400

The specificity of the assay for MV was further determined by testing sera from two rabbits immunised with vaccinia virus. These sera specifically neutralised vaccinia virus (titres approximately 1000). Antisera to vaccinia virus did not bind to MV antigen in the ELISA, indicating that the common poxvirus NP antigen (Woodroffe and Fenner 1962) was not detected.

No differences in antibody titres occurred with LU or GV strains of MV as antigen in ELISA and no differences in neutralisation assays occurred between LU and Gungahlin/1-91, the field strain isolated from the wild rabbits used in these assays (data not shown).

**Fig. 3.** ELISA (enzyme-linked immunosorbent assay) titres shown for each captive wild rabbit recovered from myxomatosis for each month from March 1991 to February 1992. The identification number of each rabbit is indicated above each graph. Sera were serially diluted two-fold from 1:50 to 1:25600 and the titres are indicated as the reciprocal of the last positive dilution. Blank spaces for rabbits 4803, 4812, 4810, 4820, 4825 indicate that no sample was obtained for that rabbit in that month.





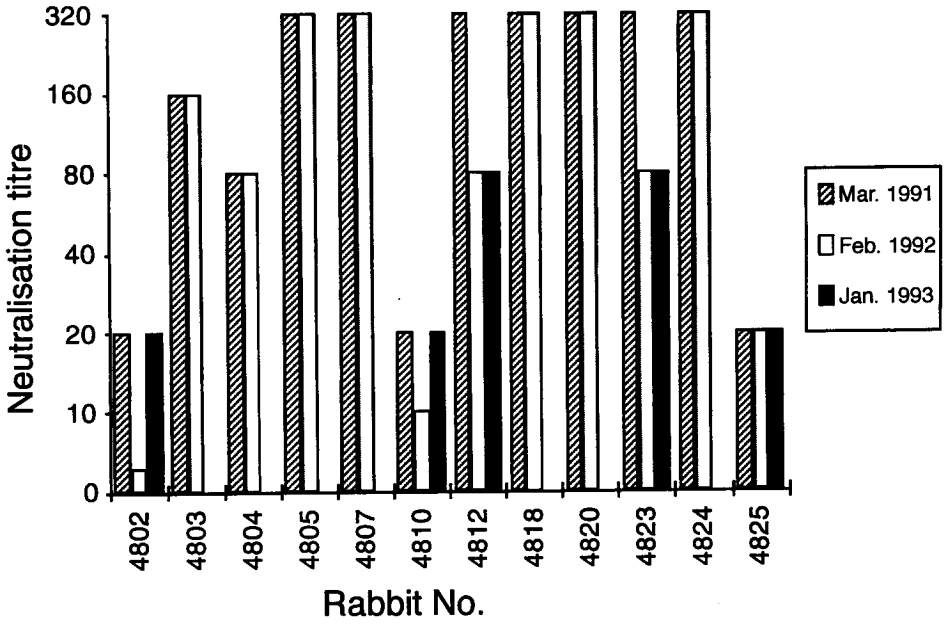


Fig. 4. Virus neutralisation titres of myxoma virus antibodies for 12 wild rabbits for March 1991 and February 1992. In addition titres for five rabbits in January 1993 are shown. Dilution intervals were two-fold from 1:10 to 1:320 and titres are indicated as the reciprocal of the highest dilution producing more than 50% reduction in plaque numbers.

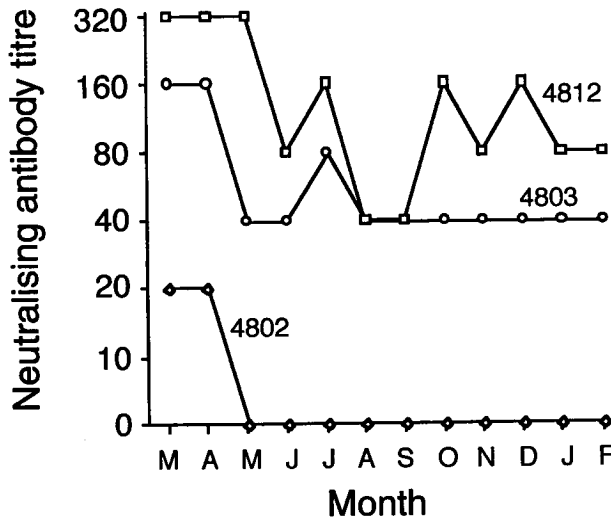


Fig. 5. Neutralising antibody titres for three wild rabbits recovered from myxomatosis for each month from March 1991 to February 1992.

*Persistence of Maternal Antibody in Wild Rabbits*

To examine the time for which placentally transferred antibodies to MV could be detected by ELISA, 24 wild-rabbit kittens born to six immune does were sampled from 9–10 days after birth onwards. Maternal ELISA titres ranged from 1600 to 6400. Antibody levels in kittens, as measured by ELISA OD 405 nm on 1:50 dilutions of sera, were very similar both within litters and between litters. By 6–9 weeks after birth, passively transferred antibodies could no longer be detected by ELISA (Fig. 6).

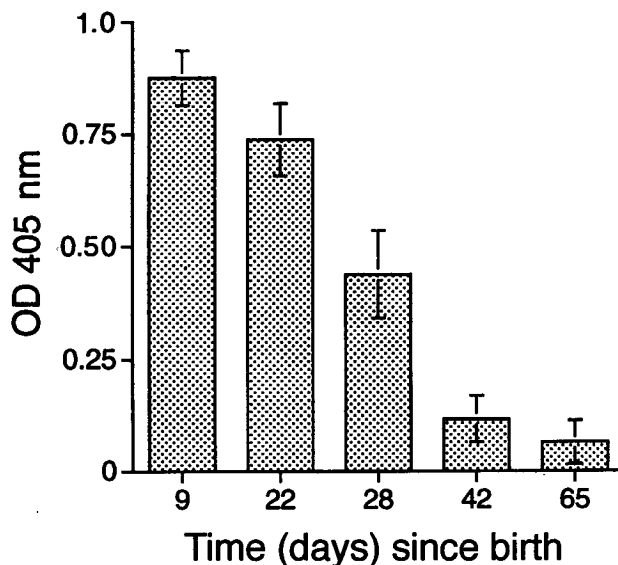


Fig. 6. Passively transferred maternal antibodies to myxoma virus in kittens born to six wild rabbits, measured at the indicated times after birth. OD 405 nm (optical density at 405 nm) was measured by ELISA (enzyme-linked immunosorbent assay) for duplicate 1:50 serum dilutions for each kitten. At each time, the values for all kittens were averaged. Error bars indicate one standard deviation from the mean.

*Detection of IgM to MV*

To differentiate active or recent infection from low or maternal antibody titres, production and detection of IgM to MV was investigated by ELISA. Four domestic rabbits were infected with a highly attenuated strain of MV that did not cause mortality, and serum IgM or IgG levels were determined with isotype-specific conjugates.

IgM levels were high nine days after infection, about the time that clinical signs were becoming apparent, and declined to background 30–37 days after infection. In contrast, IgG peaked 30 days after infection and remained at high levels over the next month (Fig. 7). Clinical signs were most prominent 12–25 days after inoculation, followed by rapid recovery. The results with ELISA for IgM and IgG suggest that IgM:IgG OD ratios had some predictive value for the stage of infection. High IgM:IgG OD ratios indicated recent infection, ratios around unity were found during the main clinical phase of the infection and low IgM:IgG ratios were present in the recovery phase.

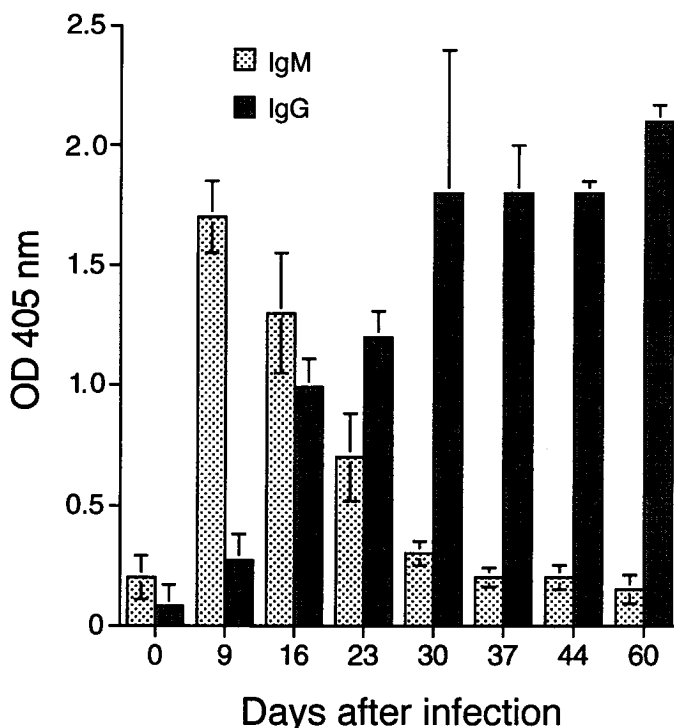


Fig. 7. IgG and IgM levels determined by ELISA (enzyme-linked immunosorbent assay) using 1:100 dilutions of serum from four rabbits at the indicated times after infection with a very attenuated strain of myxoma virus. Antibody levels shown are means ( $\pm$  s.d.) of duplicate determinations from all four rabbits.

To examine the persistence of IgM to MV in wild rabbits, sera from the 12 rabbits recovered from myxomatosis for March and April 1991, that is, at least 30–60 days after infection, were tested for IgM and IgG to MV. All 12 rabbits were strongly positive for IgG (mean OD 405 nm for March was  $1.26 \pm 0.06$  and for April was  $1.28 \pm 0.07$ ) but were the same as background (i.e. negative) for IgM (mean value for March was  $0.19 \pm 0.02$  and for April was  $0.21 \pm 0.02$ ). This result indicates that in wild rabbits naturally infected with MV, IgM levels did not persist.

#### *Epidemiological Use of the ELISA*

The predictive value of the IgM and IgG ELISA was investigated with sera collected from 34 wild rabbits during a myxomatosis epidemic in western New South Wales. These sera were analysed for IgM and IgG levels to MV. The ELISA results were compared with the clinical assessment of the rabbits made at the time of sampling. Twenty of the rabbits were scored as clinically positive. Of these, 19 had levels of IgM to MV consistent with the diagnosis. One rabbit had undetectable IgM and IgG and a re-examination of the field notes indicated it had been scored clinically positive on the basis of scabby eyelids. These are observed in rabbits recovering from myxomatosis but are not diagnostic in the absence of other signs. The antibody results suggested that this rabbit had not been infected with MV. The remaining 14 rabbits were scored as clinically negative. Of these, seven were negative for IgM and IgG; two had high IgM:IgG ratios, indicating very recent infection; and two had low IgM but high IgG, indicating recovery from infection and subsequent immunity. The remaining three rabbits had IgM levels of about 0.5 and no IgG, which may indicate a very early stage of infection.

## Discussion

The ELISA results indicate that this assay is suitable for field surveys of antibodies to MV. First, the ELISA detected specific antibodies to MV as confirmed by both neutralisation assays and challenge infection of rabbits. No cross-reaction with rabbit antibodies to vaccinia virus (an orthopoxvirus) occurred, although sera from only two rabbits were tested. Antibody detection by ELISA was more sensitive than that by neutralisation assays. For example, Rabbit 4802, which had neutralising titres too low to calculate a 50% endpoint for much of the sampling period, but was protected from challenge, was clearly positive for MV antibodies when measured by ELISA. Second, antibody titres to MV, measured by ELISA, in naturally infected wild rabbits remained positive for at least two years and did not fluctuate from positive to negative. Thus, the assay has both high sensitivity (i.e. the proportion of animals that are truly positive and are detected as positive) and specificity (i.e. the proportion of animals that are truly negative and are detected as such) for MV antibodies in rabbits.

Results from ELISA are available in a few hours, whereas PRNAs take 6–7 days and require facilities for tissue culture and the maintenance of cell lines. Complement-fixation assays, while reasonably rapid, are not as accurate as neutralisation assays and can not be used with haemolysed sera (Fenner *et al.* 1953). The ELISA also has the advantages that very small volumes (<5  $\mu$ L) of serum are required and haemolysed sera can be used without any problems.

An ELISA for MV has been described previously (Wetherall *et al.* 1983). However, there was no assessment of the persistence of antibodies in wild rabbits. Nor was there any attempt to assess the sensitivity and specificity of the assay by using an alternative serological test as a 'gold standard'. Factors such as maternal antibodies or the use of ELISA to measure IgM were discussed but not measured. Importantly, the background ODs of normal sera in the ELISA reported by Wetherall *et al.* (1983) were much higher (more than 10-fold) than was acceptable for the assay described here, and could have led to a number of positive samples being judged as equivocal or negative. The reason for this high background is not known but it may have been related to the use of low dilutions (1:50) of a commercial MV suspension leading to high levels of non-viral material on the plates. In the assay reported here, antigen dilutions of 1:250–1:1200 were routinely used, depending on the antigen preparation, and the use of a reference serum enabled standardisation of each batch of antigen to produce consistent results.

The main serological assay used for epidemiological studies of MV in Australia over the last 25 years has been the double-immunodiffusion assay described by Sobey *et al.* (1966). This assay has been criticised for its low sensitivity compared with neutralisation assays (Vaughan and Vaughan 1969). Its suitability for field use is also questionable since in particular locations more than 50% of 'immune' rabbits were reported as seronegative during late winter or early spring (Williams *et al.* 1973). Such decline and disappearance of MV antibody titres occurred even in small groups of rabbits in captivity. In one example, 13 of 16 immune rabbits were seronegative in midwinter (Williams *et al.* 1973). In the absence of detailed local knowledge of the epidemiology of MV, the immunodiffusion assay would have limited predictive value.

The low sensitivity of the immunodiffusion assay has led to some speculative interpretations of fluctuating antibody levels in wild rabbits that have recovered from myxomatosis. For example, it was suggested that the fluctuations in titre could be explained by latent infection of rabbits with MV. Williams *et al.* (1972, 1973) proposed that viral recrudescence occurred as antibody titres declined, followed by stimulation of antibody production and control of infection. There is very little epidemiological or experimental support for this proposition (Fenner and Ross 1994).

The work reported in this paper was undertaken, in part, to determine whether such fluctuations in antibody titres were a significant feature of immunity to MV. When using the ELISA, all rabbits that were initially seropositive were clearly positive on resampling each month for 12 months (see Fig. 3). Even rabbits with low neutralisation titres were obviously positive by ELISA. These results confirmed earlier work (Fenner *et al.* 1953) showing that neutralisation titres to MV remained elevated for at least 20 months after infection.

All Australian strains of MV must be derived from either LU or the GV progenitor, the standard laboratory strain, as these are the only strains that have been released in Australia (Fenner and Ratcliffe 1965). Previous work has indicated that all strains of myxoma virus tested cross-react antigenically, although some field strains have been shown to have a difference in soluble antigens (Fenner and Woodroffe 1965; Fenner 1965; Woodroffe and Fenner 1965). To ensure that the ELISA would recognise antibodies to all field strains of MV, it was tested on antigen prepared from both LU and GV strains of MV. No difference existed between the two antigens by ELISA. As a further check, the MV strain isolated from the naturally infected wild rabbits was compared with LU in neutralisation assays, by using sera from the wild rabbits; again, there were no differences in titres.

The time for which maternal antibodies to MV in kittens could be detected by ELISA was similar to those in other reports. Fenner and Marshall (1954) showed that, at birth, kittens born to immune does had passively transferred antibodies to MV that were equivalent to serum titres in the doe. The titres of these antibodies had fallen to low levels by seven weeks after birth, when measured by either complement-fixation or neutralisation tests.

The use of ELISA to detect IgM to MV in recently infected rabbits indicated that this assay could be useful in epidemiological studies, to determine whether active infections are occurring. This is important in field infections of genetically resistant wild rabbits with attenuated strains of MV, as clinical symptoms may be minimal. IgM to MV was present in laboratory rabbits, infected with attenuated MV, only during the early and active stages of the clinical disease (see Fig. 6), and it was at very low levels 30 days after infection. This result was confirmed by examining sera from a group of wild rabbits known to have recently recovered from myxomatosis.

The variable background associated with the IgM assay and the low levels of IgM to MV in the serum made IgM values alone difficult to interpret. The most useful comparison was the ratio of IgM and IgG OD values at a serum dilution of 1:100. This ratio was evaluated in rabbits collected during an epidemic of myxomatosis and proved useful for understanding the disease and immune state in individual rabbits. This would be especially valuable where samples are being collected from study sites at widely separated times where the onset of epidemic myxomatosis or low levels of endemic myxomatosis might not be detected clinically or by conventional serology.

The ratio of IgM and IgG values also provides a tool for examining serum antibody results in the equivocal zone, the area between known positives and known negatives for which there were no data during the development of the assay. True positive results in this zone could be due to either persistent maternal antibodies, indicated by low body weight and the absence of IgM or subclinical infection with MV, in which case IgM should be present. Rabbits that have been infected with MV but had very low antibody responses could also fall in this zone. As the assay is used in further and more extensive field studies, it should be possible to evaluate the settings for positive and negative cut-offs more rigorously.

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