

Efficacy of the EG95 hydatid vaccine in a macropodid host, the tammar wallaby

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SUMMARY

In Australia, macropodids are common intermediate hosts for the cestode *Echinococcus granulosus*, and sylvatic transmission is maintained via wild dogs. The parasite causes mortality in a number of macropodid species and the sylvatic cycle provides a source of infection to domestic livestock and humans. We determined the efficacy of the hydatid vaccine, EG95 in the tammar wallaby, *Macropus eugenii*, challenging either 1 or 9 months post-vaccination. EG95 provides similar protection to that seen in sheep (96–100%). Control tammars were significantly more likely to become infected (odds ratio 29.44; CI 4.13, 209.97; $P=0.001$) and to develop more cysts (count ratio 26.69; CI 5.83, 122.19; $P<0.001$). The vaccination may be beneficial if administered pre-release in captive breeding programmes for endangered macropodids. Further work to develop oral delivery methods may enable vaccine administration of wild animals and thereby a reduction in sylvatic transmission.

Key words: *Echinococcus granulosus*, hydatid, marsupial, macropodid, EG95, vaccination.

INTRODUCTION

Echinococcus granulosus has a world-wide distribution and is an important zoonotic pathogen. A number of distinct genotypes of the parasite exist, possibly representing distinct species (Thompson and McManus, 2002). Parasites of the G1 genotype are the most widespread in their distribution and are responsible for the majority of human infections (McManus and Thompson, 2003). The G1 genotype of *E. granulosus* is promiscuous in its range of suitable intermediate host species. Domestic livestock are the most important hosts; however, numerous herbivorous wildlife species may also act as hosts (Eckert *et al.* 2001; Jenkins and Macpherson, 2003). In Australia, a number of species of macropodid marsupials have become infected with the G1 genotype of *E. granulosus*, maintaining sylvatic transmission of the parasite via wild dogs and dingoes (Jenkins and Morris, 1991; Bowles *et al.* 1992; Jenkins and Macpherson, 2003). The transmission dynamics of this cycle differ markedly from the domestic cycle in that wild dogs

(dingoes and dingo/domestic dog hybrids) typically have much heavier burdens of the adult parasite compared to domestic dogs (Jenkins and Morris, 1991; Lahmar *et al.* 2001) and the minimum time required for development in the macropodid intermediate host can be as little as 9 months compared to several years in sheep (Gemmell *et al.* 1986; Barnes *et al.* 2007a). This potentially very prolific cycle provides a source for the introduction of the parasite into domestic livestock (Grainger and Jenkins, 1996), is a potential direct source of infection for humans, and causes mortality, primarily due to pulmonary impairment, in a number of species of macropodid marsupial including several species listed on the IUCN's *Red List of Threatened Species* (Johnson *et al.* 1998; IUCN, 2006; Barnes *et al.* 2007a, 2008).

Control of transmission of *E. granulosus* is possible, particularly through measures to prevent infections in the definitive hosts, although eradication of the parasite has only been achieved in island settings (Craig *et al.* 2007). A vaccine has been developed for use in intermediate hosts of *E. granulosus* which greatly reduces susceptibility to infection (Lightowlers *et al.* 1996, 1999). Although vaccination of wildlife species presents many obstacles, this has been achieved in Europe and North America to protect wildlife from rabies using an oral

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recombinant vaccinia virus (Blancou *et al.* 1986; Rupprecht *et al.* 2004). In addition, Haydon *et al.* (2006) have demonstrated that low-coverage vaccination strategies can be successful in reducing the reproductive number of an infectious agent below 1 thereby reducing the extent of rabies in the endangered Ethiopian wolf. Wildlife vaccination is usually considered for 3 reasons: when the disease is a public health risk, when there is an economic impact on livestock production or when there is a threat to an endangered species (Cross *et al.* 2007). All 3 are relevant to *E. granulosus* transmission and impacts in Australia. Vaccination may be particularly applicable to the control of hydatid infection in closely managed populations of endangered species and could be used pre-release in captive breeding and re-introduction programmes that form part of recovery plans for such species (Nolan and Johnson, 2001; Lundie-Jenkins and Lowry, 2005; Menkhorst and Jarman, 2005, 2007a). In order to assess the potential for the use of vaccination as a control measure for hydatid infection in macropodid marsupials, we undertook an experimental study of the efficacy of vaccination in reducing or preventing *E. granulosus* infection in the tammar wallaby, *Macropus eugenii*. This species is maintained in captive breeding colonies and has been shown to be highly susceptible to infection with the parasite (Barnes *et al.* 2007a).

MATERIALS AND METHODS

Animals

Tammar wallabies were obtained from a breeding colony maintained at CSIRO Sustainable Ecosystems, Canberra. All tammars were born in captivity and had no previous exposure to *E. granulosus*. They were kept in open grassy yards (30 × 50 m) at CSIRO Sustainable Ecosystems, and supplemented with a diet of ewe and lamb pellets (15% protein; Young Stockfeeds, Young, NSW); water was available *ad libitum*.

All experimental procedures were approved by the CSIRO Sustainable Ecosystems Animal Ethics Committee (Application Number 05/06-07).

Study design

In a pilot study, 4 juvenile tammar wallabies (7–8 months of age, not yet fully independent of their mother) were vaccinated with 2 × 1 ml doses of EG95 (50 µg plus either 0.1 mg or 1.0 mg Quil A (Brentag Biosector, Denmark)) 5 weeks apart. Six weeks after the booster vaccination, they were challenged with 8000 *E. granulosus* eggs (see Barnes *et al.* 2007a). Six non-vaccinated tammars of the similar age (i.e. now all 10–11 months of age) were challenged concurrently with the same dose of eggs. One vaccinated tammar in this group died during the trial from

causes unrelated to the trial. These animals were monitored until necropsy at 9–16 months post-infection (p.i.).

For the main trial, 40 wallabies (10–11 months of age) were allocated to 4 experimental groups of 10 animals. Twenty tammars were vaccinated with EG95 (50 µg plus 1.0 mg Quil A in a volume of 1 ml) at time 0, and then received an identical booster injection 4 weeks later. Ten of these tammars (4 females and 6 males) and 10 control, non-vaccinated tammars (all females) were challenged 1 month later with 8000 *E. granulosus* eggs (immediate challenge group). One vaccinated tammar in this group died during the trial from causes unrelated to the trial. These animals were necropsied at 12 months post-immediate challenge.

The remaining 2 groups of animals (10 vaccinated – 6 females and 4 males; and 10 non-vaccinated – 8 females and 2 males) were challenged with the 8000 *E. granulosus* eggs 9 months after the second vaccination (delayed challenge group). These animals were necropsied 8–12 months post-delayed challenge.

Vaccine preparation

The EG95 vaccine was prepared as a glutathione S-transferase fusion protein and purified. Each dose was prepared as a sterile solution containing 50 µg protein plus Quil A adjuvant as described by Lightowlers *et al.* (1996).

Collection of eggs for infection of experimental tammars

For each experimental infection, fresh mature, gravid *E. granulosus* were collected from the intestine of a naturally-infected wild dog shot by an Officer of the Rural Lands Protection Board, Yass, New South Wales, during the normal course of his duties. The eggs were recovered as described by Barnes *et al.* (2007a). When required, the eggs were re-suspended and counted using a modified Fuch's Rosenthal chamber and diluted in distilled water to 4000 eggs/ml.

Radiological and clinical examinations and blood sampling

Each tammar was anaesthetized using 2–3% isoflurane (Attane, Pharmtech) using the methods described by Barnes *et al.* (2007a). Chest radiographs were taken of each tammar at 0, 4, 8, 12 p.i. as described by Barnes *et al.* (2007a). On these occasions each tammar was given a brief clinical examination. The tammars in the pilot study were also radiographed and examined at 9, 14 and 16 months p.i. Tammars were monitored visually every 2–3 days

throughout the trial for signs of respiratory distress. All tammars were euthanased and necropsied at the end of each trial or earlier if radiographic changes in the lungs raised concerns that the welfare of the animals was likely to be compromised before the next radiographic examination. Blood samples (3–5 ml) were taken from the lateral coccygeal vein of tammars in the main trial at 0, 1, 2, 6, 8, 10 and 14 months after the initial vaccination.

Infection of experimental tammars with E. granulosus eggs

Infection of tammars was undertaken when the animals were lightly anaesthetized as described by Barnes *et al.* (2007a). Two ml of distilled water containing approximately 8000 *E. granulosus* eggs were administered using soft plastic tubing inserted into the oesophagus.

Post-mortem examinations

Tammars were euthanased with 140 mg/kg sodium pentobarbitone (Lethobarb, Virbac, Peakhurst, Australia). At necropsy the presence or absence of hydatid cysts was initially assessed by gross visual inspection and palpation. Lung, liver, spleen, kidney, heart and brain were then sectioned at 3–5 mm intervals. The location of each cyst was recorded. The presence or absence of degenerative changes (caseation or calcification) was noted. Cysts were defined as non-viable if they contained only caseous or calcified material and no cyst fluid could be aspirated. When present, fluid was removed from cysts by sectioning or aspiration and examined with a stereomicroscope for the presence or absence of protoscoleces. Any lesions of uncertain aetiology were fixed in 10% buffered formalin. These were later processed using standard histological methods (Lillie, 1954) with sections cut at 4 μm and stained with H & E. Sections were examined to determine the presence or absence of the laminated layer that is diagnostic for cysts of *Echinococcus* spp (Kilejian *et al.* 1961). Sections from cysts of uncertain degenerative status were examined for signs of involution of the laminated layer, degeneration of the germinal membrane and presence of caseous, necrotic or calcified material within the cyst.

ELISA assessment of anti-EG95 antibody titres

Anti-EG95 antibodies in wallaby sera were detected by ELISA. Reagent volumes were 100 μl unless otherwise stated. An optimal concentration of the antigen, EG 95 recombinant protein, (1 $\mu\text{g}/\text{ml}$ in 50 mM carbonate buffer, pH 9.6) was incubated in ELISA plates (Microton, Greiner, Germany) overnight at 4 °C. Antigen was discarded and the plate

washed 3 times with phosphate-buffered saline and 0.05% Tween20 (Sigma) (PBS-T). Non-specific binding sites were blocked with 200 μl of 5% skim milk powder in PBS (PBS-SM) for 1 h at 37 °C. Wallaby sera diluted in PBS-SM was added to the plates and serially diluted from 1/1000. Controls (no antigen present) were included for each serum and a positive control serum was tested on each plate. Plates containing diluted sera were incubated for 1 h at 37 °C, and then washed as above. An optimal concentration (1/1000 in PBS-SM) of rabbit anti-kangaroo IgG H&L antiserum (Bethyl Laboratories, USA, A140-104) was added to each well. After incubation at 37 °C for 1 h, the antiserum was discarded, the plates washed and goat anti-rabbit HRP conjugate IgG (DAKO, Denmark) was added at 1/1000 in PBS-SM. Following further incubation at 37 °C for 1 h, the conjugate was discarded, the plates washed and activated TMB substrate (3,3',5,5'-tetramethylbenzidine, Sigma) was added to each well. Plates were incubated at room temperature for 15 min; the reactions stopped by addition of 0.5 M H_2SO_4 , and the optical density (OD) read at 490 nm. Acceptance of the data from each plate was determined provided the OD of the positive control sera at 2 dilution points (1/32 000 and 1/512 000) fell within 2SD of levels previously calculated from 20 replicate titrations of the positive control. Antibody titres were calculated from the projected titre at which the sera had an OD value of 1.6. Samples having antibody levels which failed to reach this OD at the highest dilution were recorded as being negative.

Statistical analyses

Protection against infection was calculated as the percentage reduction in the mean number of viable hydatid cysts in vaccinated animals in comparison to the mean number of viable cysts in control animals. Other analyses were undertaken using Stata Version 9 (StataCorp, College Station, TX, USA, 2006). To assess associations between the trial (pilot/immediate challenge/delayed challenge), vaccination status (control/vaccinated) and sex on the risk of development of hydatid infection, logistic regression was undertaken with each variable fitted separately and assessed in comparison with the null model using the likelihood ratio test. The odds ratio (OR) and 95% confidence interval (CI) were also calculated for each of these variables, other than the reference group, using the corresponding beta-coefficients and associated standard errors. As the distribution of observed proportions of tammars with various numbers of cysts was closer to that expected with a negative binomial, rather than a Poisson distribution, negative binomial regression was undertaken to assess associations between the trial, vaccination status and sex on the number of cysts that developed. Each variable was fitted separately and assessed in comparison with

Table 1. Numbers of hydatid cysts and levels of protection achieved in tammam vaccination trials using the EG95 recombinant vaccine

(Immediate and delayed challenge vaccinated tammams were challenged at 1 and 9 months after their last vaccination, respectively.)

Experimental group	No. cysts found in each tammam (no. showing evidence of degeneration, if any)										Mean no. viable cysts	Protection ^a (%)		
	0	1	1	2	8	10	(1)							
Pilot, control	0	1	1	2	8	10	(1)				3.50	100.00		
Pilot, vaccinated	0	0	0								0.00			
Immediate challenge, control	0	0	0	0	0	0	0	1	1	3	0.50	100.00		
Immediate challenge, vaccinated	0	0	0	0	0	0	0	0	0	0	0.00			
Delayed challenge, control	0	0	1	1	2	(1)	2	2	3	(1)	6	40	5.50	96.36
Delayed challenge, vaccinated	0	0	0	0	0	0	0	0	0	1	2	(1)	0.20	

^a Calculated as the percentage reduction in the mean number of viable cysts in vaccinated tammams compared with the mean number of viable cysts in control tammams.

Table 2. Associations between trial, vaccination status and sex of the tammam wallaby on the presence (odds ratio) and number of hydatid cysts (count ratio) found at necropsy

Variable	Odds ratio (CI)	<i>P</i> -value	Count ratio ^a (CI)	<i>P</i> -value
<i>Trial</i> ^b		0.027		0.002
Pilot	Reference group		Reference group	
Immediate challenge	0.11 (0.01, 1.02)		0.14 (0.03, 0.70)	
Delayed challenge	1.53 (0.18, 13.35)		1.89 (0.49, 7.35)	
<i>Vaccination</i> ^c		0.001		<0.001
Vaccinated	Reference group		Reference group	
Control	29.44 (4.13, 209.97)		26.69 (5.83, 122.19)	
<i>Sex</i> ^d		0.333		0.569
Female	Reference group		Reference group	
Male	3.32 (0.29, 37.69)		1.56 (0.32, 7.95)	

^a Estimated ratio of mean number of cysts for animals in the exposed group relative to the reference group.

^b Adjusted for vaccination status.

^c Adjusted for trial.

^d Adjusted for trial and vaccination status.

the null model using the likelihood ratio test. The count ratio (CR, estimated ratio of mean number of cysts for animals in the exposed group relative to the reference group) and 95% CIs were calculated for each variable other than the reference group using the corresponding beta-coefficients and associated standard errors. For both logistic and negative binomial regression, variables with a univariable $P < 0.25$ were progressively included in multivariable models in a forward selection process whereby variables were retained if the associated P -value from the likelihood ratio test was less than 0.05. Interaction terms between 2 significant variables were also tested in the models.

RESULTS

No adverse local or systemic reactions were noted following the administration of EG95. In the pilot study, tammams were euthanased from 9 to 16 months p.i. Hydatid cysts developed in 5/6 control and 0/3 vaccinated tammams.

In the main study immediate challenge group, all tammams were euthanased at 12 months p.i. Three of 10 control tammams and 0/9 vaccinated tammams became infected. In the delayed challenge group, tammams were euthanased from 8 to 12 months p.i. Eight of 10 control tammams and 2/10 vaccinated tammams developed hydatid cysts (Table 1). Overall infection rates were 61.5% (16/26) control tammams and 9.1% (2/22) vaccinated tammams. Control tammams were significantly more likely to become infected (OR 29.44; CI 4.13, 209.97; $P = 0.001$, Table 2). There was also a significant association between the trial and likelihood of infection, with tammams in the immediate challenge group less likely (OR 0.11; CI 0.01, 1.02) and those in the delayed challenge group more likely (OR 1.53; CI 0.18, 13.35) to become infected compared to the reference group, the pilot study ($P = 0.027$, Table 2). There was no association between sex of the tammam and the likelihood of becoming infected ($P = 0.333$, Table 2). It was not possible to fit the interaction term between trial and vaccination status to the model.

Table 3. Distribution of hydatid cysts in various organs for each of the experimental groups in which tammars became infected

(Cysts were not found in tammars from the pilot study vaccinated group or the immediate challenge vaccinated group.)

Experimental group	No. infected tammars in group/No. in group	Location and number of cysts (no. showing signs of degeneration)						Total
		Lung	Pleural cavity	Cardiac muscle	Skeletal muscle	Kidney	Root of mesentery	
Pilot, control	5/6	21 (1)	1	0	0	0	0	22 (1)
Immediate challenge, control	3/10	3	0	0	0	2	0	5
Delayed challenge, control	8/10	48	1	3	3 (1)	1 (1)	1	57 (2)
Delayed challenge, vaccinated	2/10	0	0	0	1	0	2 (1)	3 (1)
Total		72	2	3	4	3	3	87

The numbers of hydatid cysts found in each tamar at post-mortem are shown in Table 1. In control tammars, cyst number ranged from 0 to 40, whereas, among the vaccinated tammars the range was 0–2 cysts with vaccination conferring 96.36–100% protection across the trials. Control tammars were more likely to develop more cysts (CR 26.69, CI 5.83, 122.19, $P < 0.001$, Table 2). There was also a significant association between the trial and the number of cysts developing, with tammars in the immediate challenge group developing fewer cysts (CR 0.14; CI 0.03, 0.70) and those in the delayed challenge group developing more cysts (CR 1.89; CI 0.49, 7.35) compared to the reference group, the pilot study ($P = 0.002$, Table 2). There was no association between the sex of the tamar and the number of cysts developing ($P = 0.569$, Table 2). It was not possible to fit the interaction term between trial and vaccination status to the model.

Seventy-two of the 87 (82.8%) hydatid cysts recovered from the 18 infected tamar wallabies were in lung tissue (Table 3). Cysts were also found free in the pleural cavity, in the cardiac and skeletal muscle, in the kidney and at the root of the mesentery (Table 3). Four cysts were classified as non-viable; 3/84 cysts (in lung tissue, skeletal muscle and kidney) in control tammars and 1/3 cysts (in the root of the mesentery) in vaccinated tammars (Tables 1 and 3). Protoscoleces were seen in some cysts from tammars necropsied from 8 months onwards. Due to variation in time of necropsies relative to infection it was not possible to compare the likelihood of cysts becoming fertile between control and vaccinated animals.

All vaccinated wallabies sero-converted, with specific IgG titres 1 month after the second vaccination ranging between 15 000 and 340 000, mean 135 000 (Fig. 1). At the time the delayed challenge group were infected, the antibody titres of that group of animals ranged from negative to 7500. Of the 2 wallabies that developed hydatid cysts, 1 had a negative titre and the other a titre of 12 000 at the time of infection. None of the control animals had a detectable level of specific antibody against the EG95

antigen at any time. Only 2 of the vaccinated and delayed challenge animals remained serologically positive 18 months after their initial immunization and the titres in these two animals were < 2000 . No boost in antibody titre was evident following the challenge infections.

DISCUSSION

These findings show that unvaccinated tammars are 29 times more likely to develop hydatid cysts when challenged with *E. granulosus* eggs compared to vaccinated animals. They are also likely to develop a significantly greater number of cysts. The 96–100% protection afforded by the course of 2 vaccinations is similar to the level of protection noted in the several vaccine trials that have been undertaken in sheep (Lightowlers *et al.* 1996, 1999; Lightowlers, 2006).

Both the likelihood of tammars developing cysts and the intensity of infection varied significantly between the trials. The trials varied both in methodology (relative timing of infection and challenge) and in *E. granulosus* eggs used for the challenge (each batch being from a different wild dog). Differences in infectivity of different batches of parasite eggs may have been a factor. Since the infectivity of the eggs used in the delayed challenge trial appeared to be greater than that used in the others it is not possible to make an objective assessment on whether the reduced protection (96% compared to 100%) afforded by the vaccine in this trial was due to a reduction in efficacy, differences in the susceptibility of the animals at the time of infection or a function of the greater challenge.

Hydatid cysts occur most commonly in the lungs of macropodid hosts; only 0.5% of cysts (1 liver cyst) were found outside of the thoracic cavity in 71 naturally infected eastern grey kangaroos and wallaroos and 5% of cysts (2 kidney cysts) in 11 experimentally infected tamar wallabies (Barnes *et al.* 2007a,b). A remarkable distribution in the location of hydatid cysts was found in the tammars involved in the delayed challenge (Table 2). In vaccinated tammars,

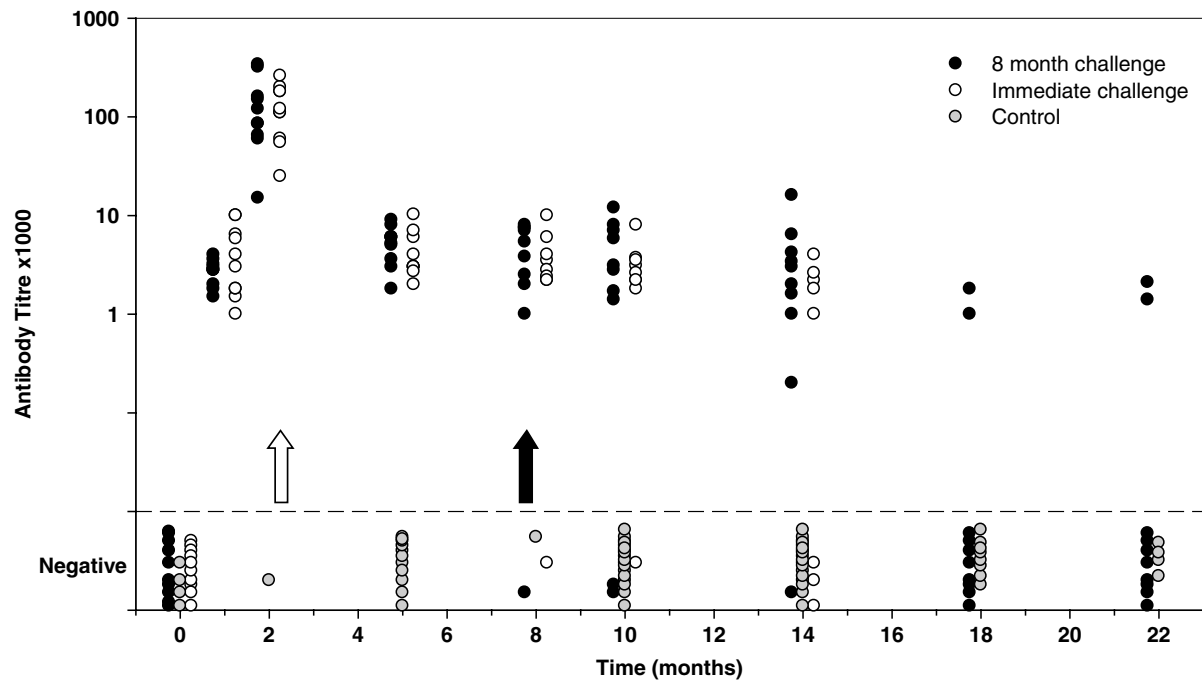


Fig. 1. Antibody titres of tamar wallaby sera after immunization with EG95-GST at 0 and 1 months. Sera from vaccinated animals that were challenged 4 weeks after the booster vaccination (immediate challenge, open arrow) are shown with open symbols and those in which the challenge infection was delayed until 9 months post-vaccination (black arrow) are shown with black symbols. Controls are shown with grey symbols.

the few cysts that were found were present in locations other than the lung, and in the control group, while the majority of the parasites were located in the lungs, cysts were identified also at a variety of other anatomical locations. There was insufficient data to conclude whether this unusual cyst distribution was a feature of the eggs used in the delayed trial or whether the vaccine may have influenced the location of hydatid cysts in vaccinated tammars. There has not been any reference made to vaccination affecting the location of hydatid cysts in sheep (Lightowlers *et al.* 1996, 1999; Heath *et al.* 2003).

Protoscoleces were identified from cysts as soon as 8 months p.i. This is sooner than the 9 months recorded in a previous experimental infection of tamar wallabies (Barnes *et al.* 2007*b*) and provides further evidence for the potentially more rapid intermediate phase of the parasite life cycle in the Australian sylvatic cycle compared to the domestic cycle. Overall, the proportion of cysts showing signs of degeneration (4.6%, 4/87) was similar to that recorded in previous experimental infection of tamar wallabies (7.5%, Barnes *et al.* 2007*a*) and is consistently lower than that recorded in sheep (Gemmell, 1966; Lightowlers *et al.* 1999). Data are insufficient to analyse differences in cyst degeneration between vaccinated tammars (1/3 cysts) and control tammars (3/84 cysts) but the higher proportion of degenerate cysts in the vaccinated animals suggests that vaccination may have some effect on developing cysts.

Specific antibody raised by the wallabies against the vaccine protein were similar to those seen in

EG95 vaccinated sheep (Woollard *et al.* 1998). The available evidence suggests that the vaccine has its effects through complement-mediated lysis of the invading oncosphere (Woollard *et al.* 2000; Lightowlers and Heath, 2004). The antibody titres seen in the 2 wallabies which did develop hydatid cysts when challenged 9 months after vaccination did not provide a clear association between protection and antibody titre at challenge. Data from the animals which were protected suggested that titres as low as 2000 were protective.

As multiple cysts in lungs and pleural cavity are known to have a rapid detrimental effect on the health of tammars (Barnes *et al.* 2007*a*) and endangered small macropodids (Johnson *et al.* 1998; Barnes *et al.* 2008), the EG95 vaccination in its current injectable form may be beneficial if administered pre-release in captive breeding programmes. The level of protection that has been found here, reducing both likelihood and intensity of infection, following experimental infection with *E. granulosus* in the tamar wallaby would suggest that the vaccine would also be a valuable tool for widespread control of hydatid transmission through wild animals such as, in Australia, macropodid marsupials. However, for this to be achievable, the challenge remains to develop a practical method for delivery of the vaccine to the numerous species of wild animal that play a role in sylvatic hydatid disease transmission.

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