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Source: Journal of Wildlife Diseases, 44(4) : 1036-1040

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-44.4.1036

Journal of Wildlife Diseases, 44(4), 2008, pp. 1036–1040

Development and Evaluation of Immunoblot-based Serodiagnostic Tests for Hydatid Infection in Macropodids

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ABSTRACT: This study examined the suitability of immunoblot assays using antigen B from hydatid cyst fluid (HCF) and the recombinant protein EpC1 to estimate the prevalence of hydatid infection in threatened small macropodid populations. When HCF antigen B was used on sera from 29 wild eastern grey kangaroos (*Macropus giganteus*) examined for the presence of hydatid cysts at necropsy (17 positive and 12 negative), and from five captive-bred macropodids, the sensitivity and specificity were 88% and 41%, respectively. Following optimization, the use of rEpC1 on sera from 51 wild eastern grey kangaroos (48 positive and 3 negative at necropsy), and from 9 captive bred macropodids, resulted in sensitivity and specificity of 48% and 92% respectively.

Key words: *Echinococcus granulosus*, hydatid, immunoblot, immunodiagnosis, macropodid.

Cystic hydatid disease is caused by the larval stage of the taeniid cestode, *Echinococcus granulosus*. In Australia, a sylvatic cycle of the parasite exists in which macropodids are common intermediate hosts (Jenkins and Macpherson, 2003). Prevalence varies across the country and between different species of macropodid, but up to 57% of a population may be infected (Jenkins and Morris, 2003). Infection can be fatal in some of the smaller macropodid species, many of which are endangered (Johnson et al., 1998; Barnes et al., 2008), but the impact of infection at a population level is unknown (Eldridge, 1997). Cystic hydatid disease is an important zoonosis worldwide, and sylvatic transmission has been associated with human cases in Australia (Jenkins and Macpherson, 2003).

Radiography is the only technique validated for the diagnosis of hydatid infections in living macropodids (Barnes et al., 2007a), but it requires some degree of anesthesia and is unreliable for the detection of pulmonary cysts <1 cm in diameter and of cysts in other organs. Immunoblotting is considered a sensitive immunodiagnostic technique for the detection of anti-*E. granulosus* antibodies in human sera (Zhang et al., 2003). Two antigens, native antigen B and the recombinant fusion protein EpC1-GST, have reasonable sensitivity and specificity in humans (Li et al., 2003; Zhang et al., 2003). However, reliable methods for immunodiagnosis in other species, such as sheep, have not been developed (Moro et al., 1999).

If the promising results seen in human immunodiagnosis could be replicated in macropodids, serologic analyses could yield valuable information on the prevalence of anti-*E. granulosus* antibodies in wild macropodid populations. This would provide a useful tool in the investigation of the epidemiology of hydatid infections in these hosts. In this study, the immunodiagnostic tests reported to be useful in human hydatid infections were modified and evaluated to assess their suitability for use in macropodids.

Positive serum samples were collected from eastern grey kangaroos (*Macropus giganteus*) shot between June 2004 and May 2005 as part of the government's harvest policy on properties within a 160-km radius of Roma, southern Queensland (26.57°S, 148.78°E). These samples were taken from a subset of animals in which post-mortem findings (cyst number, size, location, fertility, and degenerative status) were described as part of a separate study (Barnes et al., 2007b). The overall prevalence of *E. granulosus* infection in this area was 3%. The property on which each

TABLE 1. Classification of sera used in the pilot and main tests based on presence–absence of hydatid cysts at postmortem, wild–captive bred status, and species of macropodid.

Post-mortem status	Origin	Species	Pilot testing	Main testing
Positive	Wild	Eastern grey kangaroo	17	48
Negative	Wild	Eastern grey kangaroo	12	3
	Captive-bred	Eastern grey kangaroo		9
		Tammar wallaby	5	

animal was shot and the organ(s) in which cysts were found were recorded.

Negative sera were classified as either "wild post-mortem-negative" or "captive-bred." Negative wild post-mortem-negative sera were collected from eastern grey kangaroos in which no evidence of hydatid infection could be found during careful post-mortem examination; these kangaroos came from the same locations where positive animal sera were obtained. Negative captive-bred sera were collected from captive-bred eastern grey kangaroos from CSIRO Sustainable Ecosystems, Canberra ($n=2$) and the Taronga Zoo, Sydney ($n=7$). The latter samples were not available at the time of the pilot testing; therefore, five samples from tammar wallabies (*M. eugenii*), captive-bred at CSIRO Sustainable Ecosystems, were used in that portion of the study. There was no history of hydatid infection among animals bred at either of these locations. The details of sera used in both the pilot and main tests are summarized in Table 1.

Crude sheep hydatid cyst fluid (HCF) and the recombinant rEpC1 glutathione S-transferase (GST) fusion protein (rEpC1-GST) were prepared as previously described (Li et al., 2003). Because rEpC1-GST is expressed as a recombinant fusion protein in *E. coli* attached to GST, it may include *E. coli* and GST antigens; thus sera that contain anti-*E. coli*, anti-GST antibodies, or both, may give a false positive result. To minimize this possibility, diluted sera were presorbed with anti-*E. coli* and anti-GST antibody-absorbing nitrocellulose beads, as previously described (Li et al., 2003).

Antigens were electrophoresed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 12% (w/v) gels and transferred onto nitrocellulose membrane (Li et al., 2003). The membrane was blocked with 5% (w/v) skim milk in PBS for 1 hr at 37 C and cut into 3-mm-wide strips and stored at 4 C until used. Serum samples were diluted in phosphate buffered saline (PBS) Tween-20 (PBS containing 0.05% or 0.1% [v/v] Tween-20) and then incubated on a rocking platform with anti-*E. coli* and anti-GST antibody-absorbing nitrocellulose beads for 2 hr at room temperature or overnight at 4 C. The immunoblot protocol that followed was similar to that outlined by Li et al. (2003). Following development with 4-chloro-1-naphthol, a dark purple color, at the position of the rEpC1-GST band or the antigen B band on the HCF strip at 8/12 kDa, represented a positive reaction.

In the pilot testing, sera from 17 post-mortem-positive eastern grey kangaroos, 12 wild postmortem-negative eastern greys, and five captive-bred negative tammar wallabies were assessed by immunoblotting using serum diluted 1:50, followed by sheep anti-wallaby IgG (Marsupial Research Laboratory, Newcastle University, New South Wales) diluted 1:5,000, and then by conjugated donkey anti-sheep IgG diluted 1:1,000 followed by development. Both rEpC1 and HCF test strips were evaluated.

A strong positive, a weak positive, and a negative sample (captive-bred) were selected for use in further optimization of the technique. At this stage, the alternative second antibody, rabbit anti-kangaroo IgG (Bethyl Laboratories, Montgomery, Texas, USA) became available and was

TABLE 2. Diagnostic performance of antigen B and the recombinant EpC1-glutathione S-transferase (rEpC1-GST) fusion protein for hydatid infection in macropodids using immunoblotting. All sera were from eastern grey kangaroos, unless otherwise stated.

Antigen testing	Test positive/cysts detected	Sensitivity % (CI)	Test negative/cysts not detected		Specificity % (CI)
			Captive-bred	Wild	
Pilot testing[a]					
Antigen B	15/17	88.2 (72.7, 100)	4/5[b]	3/12	41.2 (5.0, 77.3)
rEpC1-GST	7/17	41.2 (5.5, 76.8)	5/5[b]	10/12	88.2 (63.7, 100)
Main testing[c]					
rEpC1-GST	23/48	47.9 (31.1, 64.7)	9/9	2/3	91.7 (47.4, 100)

[a] Serum dilution 1:50; sheep anti-wallaby IgG dilution 1:5,000; conjugated donkey anti-sheep IgG dilution 1:1,000.

[b] Tammar wallaby sera.

[c] Serum dilution 1:25; rabbit anti-kangaroo IgG dilution 1:1,000; conjugated goat anti-rabbit IgG dilution 1:1,000.

also evaluated in combination with conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, California, USA).

In the main testing, sera from 48 post-mortem-positive eastern grey kangaroos, three wild post-mortem-negative eastern grey kangaroos, and nine captive-bred negative eastern grey kangaroos were tested using serum diluted 1:25, rabbit anti-kangaroo IgG diluted 1:1,000, and conjugated goat anti-rabbit IgG diluted 1:1,000. Only rEpC1-GST test strips were evaluated. Results from captive-bred and wild post-mortem-negative animals were pooled to form the "cysts not detected" group because the goal was to develop a test capable of distinguishing between sera from animals with and without cysts (i.e., clinical infection). Sensitivity and specificity were calculated for all tests. To account for clustering by place of origin, the 95% confidence intervals were calculated using the Huber–White estimate of variance (Rogers, 1993) with Stata Version 9 (StataCorp, College Station, Texas, USA, 2006) software.

The pilot testing yielded reasonable sensitivity (88%), but low specificity (41%), using HCF strips for detection of antigen B, and a low sensitivity (41%) but greater specificity (88%) when the rEpC1-GST strips were used (Table 2). Among the negative animals, there was one false-positive result among the captive-bred tammar wallabies with antigen B, but none with rEpC1-GST. Optimization of rEpC1-GST suggested that a serum concentration of 1:25 was preferable to a 1:50 concentration. Rabbit anti-kangaroo IgG at a 1:1,000 dilution, combined with conjugated goat anti-rabbit IgG at 1:1,000 dilution, gave less nonspecific binding than the sheep anti-wallaby IgG–donkey anti-sheep IgG combination.

The main testing, which used rEpC1-GST strips, showed only a slight improvement in both sensitivity (48%) and specificity (98%, Table 2). Serum from the only animal with hepatic as well as pulmonary cysts produced a positive result. Of the 12 negative animals tested, the nine captive-bred animals were all negative.

Our attempts to develop a reliable serologic test, using both native antigen B in crude hydatid cyst fluid and the recombinant EpC1 protein for the diagnosis of hydatid infection in macropodids, thus proved unsuccessful. There are a variety of possible explanations for this, including production of low levels of specific antibodies, cross-reactions with antibodies produced in response to other parasites, and similar antibody responses of animals from endemic areas regardless of their visible infection status at post-mortem.

Hydatid infection in humans results in a

marked humoral response and a prolonged rise in specific antibody levels in most infected individuals (Zhang et al., 2003), but this does not appear to be the case in macropodid infections. The low sensitivities of the native antigen B (41%) and rEpC1-GST tests (41–48%) suggest that many infected macropodids do not produce detectable levels of specific anti-*E. granulosus* antibodies. Levels of antibodies directed against rEpC1-GST were low among most of the individuals that were seropositive, as demonstrated by weakly stained bands on the test strips compared to those from seropositive humans (Li et al., 2003).

In both humans and sheep, pulmonary cysts result in lower specific antibody levels than do hepatic cysts (Moro et al., 1999). This may provide further explanation for the low antibody titers detected in this study, given that only one of the 48 infected kangaroos had an extra-pulmonary cyst. Todorov et al. (1979) suggested that the presence of a thick, fibrous capsule in human pulmonary cysts restricted diffusion of antigens from the cyst, resulting in minimal antibody production. This is not supported in macropodid hydatid infections because the capsule is typically thin (Barnes et al., 2007a).

In this study, the use of antigen B resulted in low specificity. Some of the captive-bred animals in our study, animals that had never been knowingly exposed to *E. granulosus* eggs, reacted to antigen B but not to rEpC1. It is possible that false-positive results in unexposed animals result from cross-reactions of host antibodies, generated against other closely related parasites to the antigens used in these tests, as has been demonstrated in sheep (Yong et al., 1978). This suggestion is supported by the 100% specificity of the rEpC1-GST strips among sera from captive-bred macropodids because the recombinant protein EpC1 is specific to *E. granulosus*. False positives may also result from nonspecific binding, despite the measures we employed to remove antibodies to *E. coli* and GST.

Among the samples analyzed from wild kangaroos, it is possible that some of the false positives, judged by absence of visible hydatid cysts at post-mortem, in fact reflected previous exposure to *E. granulosus*. Many eggs fail to develop into cysts following ingestion, and evidence from experimental infections suggest that successful cyst development is 4–16 times less likely in macropodid species when compared to sheep (Barnes et al., 2007a). This may result in sera from exposed, but post-mortem-negative, macropodids giving positive test results. However, to be useful, a test must be able to distinguish between sera from animals with cysts, (i.e., clinical infection that may be detrimental to health) and sera from animals with no clinical evidence of infection.

Despite efforts to optimize the rEpC1 immunoblot, all permutations considered yielded an unacceptably low sensitivity or specificity, a result that precluded the practical application of immunodiagnosis with these protocols in macropodids. Further modifications to the techniques used, and further work exploring the immune response to infection of these hosts, may result in the development of a more-effective immunodiagnostic test.

We would like to thank D. Higgs, the Taronga Zoo, and CSIRO Sustainable Ecosystems for the provision of macropodid sera. This work was approved by the University of Queensland Animal Ethics Committee (Approval SVS/308/04/UQ). Tamsin Barnes received an Australian Government International Postgraduate Research Scholarship.

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Received for publication 26 December 2007.