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Diversity of *Cryptosporidium* in brush-tailed rock-wallabies (*Petrogale penicillata*) managed within a species recovery programme

Elke T. Vermeulen [a,*], Deborah L. Ashworth [b], Mark D.B. Eldridge [a,c], Michelle L. Power [a]

[a] Department of Biological Sciences, Macquarie University, North Ryde, NSW, 2109, Australia
[b] Office of Environment and Heritage, PO Box 1967, Hurstville, NSW 2220, Australia
[c] Australian Museum Research Institute, Australian Museum, 6 College Street, Sydney, NSW 2010, Australia

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ABSTRACT

Host–parasite relationships are likely to be impacted by conservation management practices, potentially increasing the susceptibility of wildlife to emerging disease. *Cryptosporidium*, a parasitic protozoan genus comprising host-adapted and host-specific species, was used as an indicator of parasite movement between populations of a threatened marsupial, the brush-tailed rock-wallaby (*Petrogale penicillata*). PCR screening of faecal samples (n = 324) from seven wallaby populations across New South Wales, identified *Cryptosporidium* in 7.1% of samples. The sampled populations were characterised as captive, supplemented and wild populations. No significant difference was found in *Cryptosporidium* detection between each of the three population categories. The positive samples, detected using 18S rRNA screening, were amplified using the actin and *gp60* loci. Multi-locus sequence analysis revealed the presence of *Cryptosporidium fayeri*, a marsupial-specific species, and *C. meleagridis*, which has a broad host range, in samples from the three population categories. *Cryptosporidium meleagridis* has not been previously reported in marsupials and hence the pathogenicity of this species to brush-tailed rock-wallabies is unknown. Based on these findings, we recommend further study into *Cryptosporidium* in animals undergoing conservation management, as well as surveying wild animals in release areas, to further understand the diversity and epidemiology of this parasite in threatened wildlife.

1. Introduction

Disease emergence presents a significant risk to the conservation of endangered wildlife. The risks of disease are leading to growing concern of the cost–benefit efficiency of the supplementation strategy (Kock et al., 2010). Species recovery actions such as the supplementation of dwindling populations with captive bred animals may introduce parasites atypical to the recovery species or exacerbate prevalence of existing pathogens due to stress and immune status of captive bred individuals, which may spread these pathogens into its new environment (Moberg, 1985; Cunningham, 1996). Control of disease risks requires a sound understanding of host–parasite interactions, both in threatened species and of hosts that may contribute to disease emergence. Further, parasites specific to the target species may not survive translocation or other conservation processes, thereby unbalancing the natural host–parasite relationship (Moir et al., 2012).

Cryptosporidium, a protozoan parasite with a broad vertebrate host range and variable host specificity, represents a potential indicator of disease risks associated with conservation management. This research strategy is particularly applicable to threatened Australian marsupials where the occurrence of human derived *Cryptosporidium* species has not been conclusively determined (Hill et al., 2008; Ng et al., 2011; Dowle et al., 2013).

Of the 26 described *Cryptosporidium* species (reviewed in Ryan et al., 2014), twelve have been reported in both humans and other hosts: *C. parvum, C. hominis, C. ubiquitum, C. andersoni, C. bovis, C. cuniculus, C. muris, C. canis, C. felis, C. meleagridis, C. suis* and *C. fayeri* (Xiao et al., 2001; Gatei et al., 2002; Xiao, 2002; Leoni et al., 2006; Robinson et al., 2010; Waldron et al., 2010). Each of the *Cryptosporidium* species reported in humans have been found in the Australian environment (Ryan and Power, 2012; Abeywardena et al., 2013; Nolan et al., 2013), though human infections in Australia are predominantly *C. parvum* and *C. hominis* (Waldron et al., 2011).

Despite *Cryptosporidium* being identified in 16 marsupial species from 7 families (reviewed in O'Donoghue, 1995 and Power, 2010), identifications of *Cryptosporidium* to species level is limited to recent studies employing molecular tools (Warren et al., 2003; Hill et al., 2008; Power and Ryan, 2008; Ryan et al., 2008; Yang et al., 2011).

* Corresponding author. Department of Biological Sciences, Macquarie University, North Ryde, NSW 2109, Australia. Tel.: +61 2 9850 9259; fax: +61 2 9850 8245.
E-mail address: elke.vermeulen@students.mq.edu.au (E.T. Vermeulen).

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Following molecular identification, marsupials were found to be susceptible to two host-adapted *Cryptosporidium* species, *C. fayeri* and *C. macropodum* (Power and Ryan, 2008; Ryan et al., 2008). Several other host-specific genotypes have also been described in marsupials including brushtail possum genotype I from brushtail possums (*Trichosurus vulpecula*) (Hill et al., 2008) and kangaroo genotype I from western grey kangaroos (*Macropus fuliginosus)* (Yang et al., 2011).

Although there are reports of *C. parvum* and *C. hominis* in marsupials, these are based only upon a molecular signature from a faecal DNA sample, and an infection has never been confirmed using other methods such as parasite isolation (Hill et al., 2008; Ng et al., 2011; Dowle et al., 2013). The molecular detection of *C. parvum* and *C. hominis* in marsupial hosts has also been associated with an inability to confirm at greater than a single locus, namely the 18S rRNA. Passage of *C. parvum* of *C. hominis* oocysts through the marsupial gut is the likely reason for identifications of these *Cryptosporidium* species in marsupials (Dowle et al., 2013). The only confirmed case of *Cryptosporidium* infection in a marsupial that was not host specific was an infection of *C. muris* in captive greater bilbies (*Macrotis lagotis*) being bred for release into natural habitat (Warren et al., 2003).

Here we use molecular methods to detect and identify *Cryptosporidium* in the brush-tailed rock-wallaby (BTRW), *Petrogale penicillata*. This species is listed as 'endangered' in New South Wales, Australia (NSW *Threatened Species Conservation Act* 1995) and 'near threatened' on the IUCN Red List of Threatened Species across eastern Australia (IUCN, 2013). There is an approved NSW Recovery Plan for the species (DECC, 2008), as well as an approved National Recovery Plan (Menkhorst and Hynes, 2010). These plans identify supplementation of small colonies with captive bred individuals as an important recovery strategy and over the last few years several translocations of individuals between captive breeding facilities and wild populations have occurred (Menkhorst and Hynes, 2010). As rock-wallaby populations have experienced variable levels of human intervention, studying their parasites provides a platform to examine the effect of conservation management on the host–parasite relationship. Hence, our aim was to detect and identify *Cryptosporidium* species infecting wild, captive bred, and supplemented brush-tailed rock-wallaby populations.

2. Methods

2.1. Sample collection and sites

Brush-tailed rock-wallabies were once abundant in south-eastern Australia but are now reduced to fragmented populations in New South Wales and Victoria (Eldridge and Close, 2005). Dispersal between populations, which are located in steep, rocky habitats, is rare (Browning et al., 2001). For this study, seven BTRW sites were sampled between March 2010 and July 2013 (Table 1). Sample collection dates were spread evenly across three seasons (Autumn, Summer and Winter), with ~10 samples collected in Spring (2010 and 2012), spread evenly across the four years. The origin of each population varied and included three categories: one site with a BTRW population kept in a captive breeding facility (captive bred), sites where free-ranging populations had been supplemented with captive bred individuals (supplemented) and two pristine sites with only free-ranging animals (wild). Fresh faecal samples were collected in vials containing silicon beads from each site opportunistically from unknown individuals during routine colony management by the Office of Environment and Heritage staff and were then stored at 4 °C until further processing. The highest number of samples was obtained from Square Top in Warrumbungle National Park since this was a major release site.

2.2. DNA extraction and PCR screening

Genomic DNA was extracted from faecal material (~150 mg) using the ISOLATE Fecal DNA kit (Bioline, London, UK) following manufacturer's instructions. The extracted DNA was stored at –20 °C until further analysis. Directly prior to each PCR, the DNA samples were treated with GeneReleaser (BioVentures, Inc., TN, USA) by combining equal volumes of DNA and GeneReleaser, and subjecting the mixture to 7 min in a 500 W microwave.

2.3. PCR screening at the 18S rRNA locus

DNA samples were initially screened for *Cryptosporidium* using nested PCR to amplify a partial fragment of the 18S rRNA. The primary reaction followed the methodology of Xiao et al. (1999) but with a lower $MgCl_2$ concentration (2 mM). The secondary reaction comprised the primers 18S IF and 18S IR and followed the method of Morgan et al. (1997). PCRs were performed using Red Hot Taq DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA) as previously described (Hill et al., 2008). Both reactions were modified to increase specificity for *Cryptosporidium* by lowering the concentration of dNTPs to 50 μM.

Longer 18S rRNA fragments were generated for samples testing positive for *Cryptosporidium* using the 18S IF and 18s IR primer set. The longer fragments were amplified using the primers of Xiao et al. (1999) for both primary and secondary reactions, following conditions as previously described by Waldron et al. (2011), inclusive of dNTPs and $MgCl_2$ concentrations as described above.

2.4. PCR amplification at confirmatory loci

To confirm 18S rRNA positives, DNA samples were screened at two additional loci, actin and glycoprotein 60 (*gp60*). For the actin locus, a nested protocol (Sulaiman et al., 2002) was performed with minor modifications. To improve specificity for *Cryptosporidium,* the concentration of $MgCl_2$ was lowered to 2 mM, dNTPS to 50 μM, and the annealing temperature raised to 54 °C in the secondary reaction.

Table 1
The rate of *Cryptosporidium* detected at the different loci per screened site and site category. All sites are in New South Wales; the precise location is withheld for some sites for the safety of the animals. KV means Kangaroo Valley. Samples at the loci (18S rRNA, actin and *gp60*) were deemed as positive after DNA sequencing.

Site	Population category	No. of samples	18S rRNA (298 bp)	18S rRNA (825 bp)	Actin	*gp60*
KV Mountain	Wild	55	7	7	2	3
KV River	Supplemented	43	2	1	0	1
KV Creek	Supplemented	10	4	3	0	0
Nattai	Wild	30	3	3	1	1
Square Top	Supplemented	123	5	4	0	0
Waterfall Springs	Captive bred[a]	39	2	2	1	2
Jenolan Caves	Supplemented	24	0	0	0	0

[a] Wallabies in a captive breeding facility.