Giardia and *Cryptosporidium* in Pristine Protected Catchments in Central Eastern Australia

We report the first confirmed records of the pathogenic protozoa *Giardia lamblia* and *Cryptosporidium parvum* from small remote streams in pristine protected areas in Australia, beyond the reach of urban or agricultural contamination.

INTRODUCTION

Giardia lamblia and *Cryptosporidium parvum* are unicellular protozoa which can survive for an extended period outside their animal hosts, as cysts or oocysts, respectively, in soils or water (1). They are a significant cause of severe gastrointestinal disease; are widespread in developing nations where watercourses commonly suffer human faecal contamination; and are a major cause of travellers' diarrhoea (2). They are also widespread in the developed nations of Europe, North America and South Africa, even in small streams in protected catchments (3–10).

Their dispersal mechanism has been controversial. In North America it was ascribed initially to native mammals; later, to humans (11–13). It remains unresolved, because the organisms were already widespread in that continent long before techniques were developed to detect their (oo)cysts in waterbodies, at concentrations around 10^{-2} g^{-1} , rather than in animal faeces at around 10^8 g^{-1} (1, 3–10).

Australia provides a unique opportunity to examine their distribution and dispersal. It is remote from other continents, and has a relatively high standard of public health and effective protection of drinking-water catchments. In Australia, *Giardia* and *Cryptosporidium* are widespread in watercourses subject to urban or agricultural contamination (14). Clinical cases of giardiasis or cryptosporidiosis have been recorded for many decades (1), but generally in individuals who have travelled overseas. There have been very few outbreaks of giardiasis or cryptosporidiosis in Australia, all associated with young children and their parents and carers, and all ascribed to direct faecal-oral or localized groundwater transmission (15–17). During 1998, the municipal water supply for Sydney, Australia's largest city, was threatened by *Cryptosporidium* contamination, forcing over 3 million people, a sixth of the nation's population, to boil drinking water for several weeks (18).

Unlike in North America and New Zealand, where remote areas have been screened for the presence of (oo)cysts (12, 19) the distribution of *Giardia* and *Cryptosporidium* in undisturbed water catchments in Australia remains unknown. Whilst human-infective *Giardia* cysts have been isolated from native fauna in Australia (20), there have been no rigorous analyses for presence or absence of *Giardia* or *Cryptosporidium* (oo)cysts in pristine watercourses. Relatively few Australian watercourses are completely free from human contamination (21). Here, we identified a set of rivers whose entire catchments are included in longstanding national parks upstream of all human infrastructure and with no feral stock; and sampled a series of tributary streams, successively smaller and closer to springs and sources,

and with fewer opportunities for potential contamination even by occasional backcountry hikers.

MATERIALS AND METHODS

We sampled 10 rivers in 7 national parks in the Central Eastern Rainforest Reserve Australia World Heritage Area (CERRA WHA), between 28° 17' 00" and 29° 31' 17" S and 152° 18' 42" and 153° 43' 36" E. Samples were taken between January and May 1998. Stream characteristics were as follows: flow velocity 0.5–2.3 m s^{-1} , turbidity 1–2 NTU, mean temperature 18.5°C, pH 5.5–8.0, mean PO_4^{3-} 0.034 mg L^{-1} , $\text{NO}_2^- + \text{NO}_3^-$ 0.12 mg L^{-1} , NH_4^+ 0.005 mg L^{-1} .

Each watercourse was sampled by direct micropore filtration of 100–140 L on site, using specially-designed portable battery-powered apparatus carried on foot. We used 2 different filtration techniques. In the first, we pumped 100–140 L samples, in 20-L subsamples, through separate flat circular Sartorius® nitrocellulose membrane filters of 3 μm pore size and 142 mm diameter; a total filter area of $100 \times 10^3 \text{ mm}^2$. The filters were preceded by 10 μm Gelman® prefilters. In the second, we filtered 140 L samples through Gelman® Envirocheck® folded-cartridge polyethersulfone membranes of 1 μm pore size, with an effective filtration area of $130 \times 10^3 \text{ mm}^2$. Up to 3 cartridges were needed for each sample depending on stream turbidity. All filters and prefilters were kept on ice after use, and treated within 72 hrs. *Giardia* cysts are oval, 8–12 μm long and 7–10 μm wide. *Cryptosporidium* oocysts are spherical, 4–6 μm in diameter. The 1 μm Envirocheck® cartridges trap both. The 3 μm flat filters trap *Giardia* cysts and also trap most *Cryptosporidium* oocysts. A few of the smaller *Cryptosporidium* oocysts may pass through the 3 μm flat filter. All sites were therefore also sampled using the Envirocheck® cartridges. Positive records of *Cryptosporidium* oocysts are of course equally valid for both filtration types.

Analytical protocols were based on an amalgam of methods from previous published studies, as below, and coincide closely with US EPA Standard Method 1622 (5, 22–26), which was adopted shortly after our samples were analyzed. Flat filters and prefilters were scraped and washed 3–4 times with 1% polyoxyethylenesorbitan monooleate (Sigma-Aldrich® Tween 80®). Cylindrical filters were shaken twice for 10 min in 240 ml Gelman® elution buffer (8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 , 2.9 g hydrated Na_2HPO_4 , 0.1 g sodium lauryl sulfate, 3 μl Tween 80, 150 μl Sigma® Antifoam B, 1.0 L deionized distilled water, adjusted to pH 7.4). All samples were centrifuged at 3000 x g for 10 min, resuspended and purified using a percoll-sucrose gradient.

For purification, samples were centrifuged in 250 ml tubes at 3000 x g for 10 min using a refrigerated fixed-angle centrifuge; resuspended in phosphate buffered saline (PBS) at pH 7.4 with 0.1% dodecyl sulfate and 0.1% Tween 80; centrifuged down to 10 ml; resuspended in 5 ml PBS underlayered with percoll-sucrose (SG 1.5); and centrifuged at 1050 x g for 10 min in a swinging-bucket rotor. The upper aqueous layer and percoll-su-

crose interface were centrifuged to a final pellet and stored at 4°C.

Purified pellets were stained with Meridian Diagnostics® Merifluor® fluorescein isothiocyanate (FITC)-labelled monoclonal antibodies specific to *Cryptosporidium* and *Giardia* epitopes, and examined by epifluorescent microscopy using a Zeiss® Axioscop®, with an excitation wavelength of 450–490 nm, a barrier filter of 510–520 nm, 400 x magnification and a Zeiss ZVS-47DEC digital camera. Bright green fluorescent bodies of appropriate dimensions were treated initially as presumptive (oo)cysts. All presumptive (oo)cysts were examined further using differential interference contrast (D.I.C.) microscopy. Any which demonstrated atypical external or internal morphological characteristics were removed from counts.

Bench-scale recovery rates were determined by analysing samples spiked with known concentrations of *Giardia* and *Cryptosporidium* (oo)cysts measured with a haemocytometer (26). Recovery rates were 53 ± 5.5% for the flat filter technique, similar to those for previous studies; and 64 ± 4% for Gelman® Envirocheck® cylindrical filters.

For each sample, successive 1.5 µg slide wells of the stained purified pellet were examined, until either *i*) both *Giardia* cysts and *Cryptosporidium* oocysts were detected; *ii*) 20 wells were counted; or *iii*) the entire pellet was used. Purified pellets ranged in wet-weight from 0.03–0.13 mg per 100 L sample. A total of 208 1.5 µg slide wells were counted (Table 1). On average, over 40% of each 100 L sample was examined completely.

RESULTS

Giardia cysts and/or *Cryptosporidium* oocysts were found in every stream, no matter how small, remote and pristine, and how close to the source (Table 1). Overall mean densities were 3.4 *Giardia* cysts and 1.8 *Cryptosporidium* oocysts per 100 L. Of the 208 slide wells counted, 2 contained 2 *Giardia* cysts, 15 contained 1, and 191 contained none (Table 1). Similarly, 10 contained 1 *Cryptosporidium* oocyst and 198 contained none. The frequencies of cysts and oocysts are slightly higher in the national parks of northernmost NSW (10/64, 4/64, respectively), than in those of southeast Qld (6/84, 4/84) and mid-north NSW (3/60, 1/60). The difference is significant at *p* < 0.05 for *Giardia* but not *Cryptosporidium*.

DISCUSSION

These are the first published records of *Giardia lamblia* and *Cryptosporidium parvum* from surface waters in pristine pro-

tected areas in Australia. Indeed, they are the first records from such small protected watercourses worldwide. The closest equivalent study in North America found that these protozoa were not present in the headwaters of protected montane catchments, though they were further downstream (3). Cyst concentrations detected in the CERRA WHA are as high as those in protected areas in North America (3–10).

The watercourses tested here are as well or better protected from contamination by *Giardia* and *Cryptosporidium* as any others worldwide, except perhaps for those in remote oceanic or subantarctic islands. The Australian continent has historically been free from many of the world’s otherwise widespread waterborne pathogens (20); and within Australia, there are very few water catchments which are equally undisturbed. Most protected areas either have roads and tracks, a history of logging, grazing or horse pasture, or feral dogs, cats, pigs, cattle, goats, horses, and donkeys, which routinely carry *Giardia* and *Cryptosporidium* (27–31). Hence, the results reported here indicate that *Giardia* and *Cryptosporidium* are probably present in every suitable habitat worldwide.

The mechanism by which they have reached these protected streams is likely to involve several factors. Whilst *Giardia* cysts and *Cryptosporidium* oocysts are robust and could potentially be carried as high-altitude atmospheric particles, the concentrations reported here are orders of magnitude too high for this to be the only source, and indicate a contaminated faecal input directly within the catchments concerned. Humans are an unlikely vector in this case, even though 1–3% of the human population in this region carries *Giardia* asymptotically (Wild, C. and Vogt, S., unpubl. data), because the sampling sites were specifically selected to exclude them. The most likely mechanism is that native marsupials or possibly feral cats, both of which can carry *Giardia* (11, 20), may have transmitted it from adjacent catchments with domestic stock, or from areas contaminated by humans further downstream in the same catchments. A wide variety of small- to medium-sized marsupials and other mammals are present in the study areas. Birds are another possible vector, but avian isolates may not be cross-infective to mammals (32, 33).

An expert inquiry into the 1998 Sydney water contamination event (18) identified several possible sources of *Cryptosporidium*, some pre- and some post-treatment. The results reported here indicate that most or all of the catchments for municipal water supply in Australia, and indeed worldwide, almost certainly contain *Giardia* cysts and *Cryptosporidium* oocysts at low concentrations. From a public health perspective the issue is now one of risk management. Broadly, treatment systems with

Table 1. Abundance of *Giardia* cysts and *Cryptosporidium* oocysts in water samples isolated from different watercourses at test sites.

National Park	River	Number of 1.5 µg wells counted for this sample	Total <i>Giardia</i> cysts in entire sample	Total <i>Cryptosporidium</i> oocysts in entire sample
Lamington	Canungra	8	1	2
Lamington	Canungra	16	1	0
Lamington	Canungra	16	1	0
Lamington	Canungra	12	1	0
Lamington	Purragulli	16	1	1
Lamington	Nixon	16	1	1
Border Ranges	Brindle	8	4	1
Toonumbah	Iron Pot	20	2	1
Mount Warning	Korrumbyn	16	3	1
Whian Whian	Minyon	20	1	1
Gibraltar Range	Surveyor	20	1	1
Gibraltar Range	Dandahra	20	2	0
Washpool	Coombadjha	20	0	1

flocculation or filtration steps remove protozoan cysts, whereas those with chlorination only do not (5–10).

Large holding dams with multi-day residence times may allow cysts to settle to bottom sediments; but these can be resuspended during storm floods, and there have been several major instances of cryptosporidiosis and associated gastrointestinal infection through ingesting water from lakes used for recreation (34, 35). The costs of retrofitting or replacing water treatment systems which do not currently remove protozoan cysts must therefore be balanced against the likely concentrations of cysts and oocysts in the catchment; the likelihood that they are viable and human-infective; and the likelihood that they will pass undamaged through existing treatment processes. Viability is testable using the 4,6-diamino-2-phenylindole (DAPI) method, and reverse-transcription PCR respectively as applied by Johnson et al., Mayer and Palmer, Stinear et al., and Kaucner and Stinear (36–39).

Giardia and *Cryptosporidium* are already significant threats to human health worldwide (40), and likely to become much more so in future. Without basic information on distribution and concentration of both protozoa, public health responses are likely to be hampered and ineffective. Now that field sampling techniques for remote areas are available, it is feasible to compile for empirical data for Australian water catchments. Whether this actually occurs in practice will depend on funding priorities by public health and water-supply authorities.

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