LP130100035 Final Report

Innovative approaches to limiting the public health risks of *Cryptosporidium* and *Giardia* in Australian Catchments.

Investigators: Prof. Una Ryan, Dr. Lihua Xiao, Dr. Charlotte Oskam, Dr. Andrew Ball, Dr. Andrew Bath and Claire McInnes.

The project commenced in March 2014 and the aims of the project were:

1. Identify and enumerate *Cryptosporidium* and *Giardia* (C&G) in faecal samples from marsupials, sheep, cattle, rabbits and sewage treatment plants (STP) across various drinking water catchments in two states using digital PCR (dPCR) (which provide more accurate enumeration of C&G).
2. Use Next Generation sequencing to identify the species and subtypes of C&G present in marsupials, cattle, sheep, rabbits and STP sites in these catchments.
3. Determine the viability of *Cryptosporidium* oocysts detected using cell culture.
4. Conduct an analysis of farming and land management practices to determine if particular management practices are associated with a higher or lower prevalence of zoonotic genotypes in cattle and sheep.
5. Conduct modelling and quantitative microbial risk assessments (QMRA) for the various catchments based on the data generated under aims 1-4.
6. Develop targeted control strategies on the basis of the information gained from QMRA modeling.

Background:

Waterborne parasitic protozoan diseases have a worldwide distribution (Cotruva et al., 2004), result in four billion cases of diarrhea, 1.6 million deaths annually (www.who.int) and 62.5 million Disability Adjusted Life Years (DALYs) worldwide (Wright and Gundry, 2009). Diarrhea belongs to the five most common disease-related causes of death (www.who.int) and is responsible for 21% of deaths of children younger than five years of age (Kosek et al., 2003).

Cryptosporidium and *Giardia* are the most prevalent waterborne parasitic infections and represent the major public health concern of water utilities in developed nations, as the oo/cyst stage are highly resistant to environmental stresses and disinfection treatments (including chlorine treatment of community water supplies) (Baldursson and Karanis, 2011).

Both parasites are responsible for enteric disease in a wide range of hosts, may be chronic and life-threatening in immunocompromised individuals (Hunter et al., 2007) and there is currently no effective drug to treat cryptosporidiosis in Australia (Rossignol, 2010). Between 2004 and 2010, *Cryptosporidium* and *Giardia* were responsible for 95.5% of the waterborne protozoan parasitic outbreaks that have been reported worldwide and 46.7% of these occurred on the Australian continent (Baldursson and Karanis, 2011). Oo/cyst transport to surface water can occur by deposition of manure directly in the water or by wash off in surface runoff. Humans, wildlife and domestic livestock all potentially contribute *Cryptosporidium* and *Giardia* to surface waters. Identification of the sources/carriers of human pathogenic strains is therefore essential for accurate risk assessment and catchment management.

The zoonotic *C. parvum* and the anthroponotic *C. hominis* are the most common species in humans worldwide (Xiao, 2010), but more than eight other *Cryptosporidium* species/genotypes can be responsible for human cryptosporidiosis cases. Few genotyping studies have been

conducted in Australia, but to date, *C. hominis, C. parvum, C. meleagridis* (from birds and humans), *C. fayeri* (from marsupials), *C. andersoni* and *C. bovis* (from cattle), have been reported in humans in Australia (cf. Ryan and Power, 2012). In 2009, *C. fayeri* (from marsupials), was identified in a woman in Sydney and identical subtypes were found in marsupials in the area and there have also been several reports of *C. parvum* and *C. hominis* in marsupials (Ryan and Power, 2012).

Conclusive molecular evidence, linking contamination of water supplies by animals in catchments with outbreaks of cryptosporidiosis in human populations, is scant; however, several studies have strongly linked outbreaks of cryptosporidiosis with sheep and cattle grazing near the implicated reservoir, catchment or river (eg. Yang et al. 2008; Ruecker et al. 2007) and one recent waterborne outbreak in the UK was caused by *C. cuniculus* (from rabbits) (Chalmers et al., 2009; 2011). *Cryptosporidium cuniculus, C. fayeri, C. bovis* and *C. parvum* among others have been detected in Australian water supplies (unpublished data). Zoonotic *Giardia duodenalis* genotypes have been identified in cattle and sheep (Caccio and Ryan, 2008) and a high prevalence of zoonotic *Giardia* genotypes have also been identified in marsupials (Thompson et al., 2008; Thompson et al., 2010), which warrants further investigation.

In Australia, marsupials, cattle, sheep and rabbits are the dominant animals inhabiting water catchment areas with densities of 400-2000 km^2 (Ryan and Power, 2012) and contribute a large volume of manure to catchments. Therefore, it is important to determine the potential role that these host species play in the dissemination of *Cryptosporidium* and *Giardia* to drinking water sources and the associated human health risks.

This project aimed to conduct for the first time in Australia, a comprehensive quantitative analysis of genotypes of *Cryptosporidium* and *Giardia* in marsupials, rabbits, cattle, sheep and humans via sewage treatment plants (STP) across different catchments and states, over a three-year period to determine if these animals were the source of *Cryptosporidium* and *Giardia* infection in humans. We also aimed to enumerate the numbers of *Cryptosporidium* and *Giardia* (oo)cysts present in samples and based on the data generated, develop targeted control strategies on the basis of the information obtained for improving catchment management.

Outcomes:
The start of the project was delayed due to the addition of a fourth Industry Partner (SeqWater from Queensland) subsequent to the awarding of the grant in 2013. The large number of additional samples from Queensland (653 faecal samples and 470 Waste Water Treatment Plant - WWTP samples) meant that not all aims of the project could be completed in the required timeframe and therefore a decision was made not to conduct viability analysis using cell culture but to conduct viability analysis using vital dyes where appropriate. It also greatly limited QMRA analysis.

We first developed and validated a digital PCR assay (see Yang et al., 2014) and also developed a next generation sequencing (NGS) assay for *Cryptosporidium* (Paparini et al., 2015).

This project then conducted long-term monitoring of *Cryptosporidium* and *Giardia* in water catchments serving Western Australia (WA), New South Wales (NSW) and Queensland (Qld), Australia.

In total 5,774 faecal samples from 17 known host species and 7 unknown bird samples, in 11 water catchment areas were screened for *Cryptosporidium* over a period of 30 months (July

2013 to December 2015) and the results are summarised below (see Zahedi et al., 2016a and Zahedi et al., 2018a for more details).

The overall prevalence of *Cryptosporidium* across the various hosts sampled was 18.3% (1,054/5,774; 95% CI, 17.3-19.3). Of these, 873 samples produced clean Sanger sequencing chromatograms, and the remaining 181 samples, which initially produced mixed chromatograms suggesting the presence of multiple different sequences, were reanalysed by Next- Generation Sequencing (NGS) to resolve the presence of *Cryptosporidium* and the species composition of potential mixed infections.

The overall prevalence of confirmed mixed infections was 1.7% (98/5,774), and in the remaining 83 samples, NGS only detected one species of *Cryptosporidium*. Of the 17 *Cryptosporidium* species and four genotypes detected (Sanger sequencing combined with NGS), 13 are capable of infecting humans; *C. parvum, C. hominis, C. ubiquitum, C. cuniculus*, *C. meleagridis, C. canis, C. felis, C. muris, C. suis, C. scrofarum, C. bovis, C. erinacei* and *C. fayeri*.

Oocyst numbers per gram of faeces (g^{-1}) were also determined using qPCR (with ddPCR calibrated standards), with medians varying from 6,021 - 61,064 across the three states. The detection of human-infectious *C. hominis* in cattle and kangaroo faeces and the high prevalence of the zoonotic *C. parvum* in cattle are significant findings. In addition, two novel *C. fayeri* subtypes and one novel *C. meleagridis* subtype were identified. This is also the first report of *C. erinacei* in Australia. Future work to monitor the prevalence of *Cryptosporidium* species and subtypes in animals in these catchments is warranted.

NGS was also conducted on *Cryptosporidium* glycoprotein 60 (*gp60*) subtypes to identify the within-host diversity of *Cryptosporidium* as this has implications for has implications for our understanding of the epidemiology, disease severity and evolution of *Cryptosporidium* virulence (see Zahedi et al., 2017a for more details). Briefly, Sanger and NGS sequencing of *gp60* amplicons from *Cryptosporidium hominis* (n=11), *Cryptosporidium parvum* (n=22) and *Cryptosporidium cuniculus* (n=8) DNA samples from Australia and China were compared. Sanger sequencing identified only one *gp60* subtype in each DNA sample: one *C. hominis* subtype (IbA10G2) (n=11), four *C. parvum* subtypes belonging to IIa (n=3) and IId (n=19) and one *C. cuniculus* subtype (VbA23) (n=8). NGS identified the same subtypes initially identified by Sanger sequencing, but also identified additional *gp60* subtypes in *C. parvum* and *C. cuniculus* but not in *C. hominis*, DNA samples. The number of *C. parvum* and *C. cuniculus* subtypes identified by NGS within individual DNA samples ranged from two to four, and both *C. parvum* IIa and IId subtype families were identified within the one host in two samples.

These findings also have implications for our understanding of the epidemiology and transmission dynamics of *Cryptosporidium*, as previous studies have relied on Sanger sequencing, which may not reflect the extent of within-host diversity and result in incorrect assumptions regarding transmission of the parasite. More extensive studies employing NGS approaches on a wider range of samples are important to determine the extent of *Cryptosporidium* within-host genetic diversity and should be an essential prerequisite for vaccine, drug and epidemiological studies.

The diversity of *Cryptosporidium* species in sewage was analysed using NGS analysis of 730 raw influent samples from 25 Australian wastewater treatment plants (WWTPs) across NSW,

QLD and WA, between 2014 and 2015 and the data generated is summarised below (see Zahedi et al., 2018b for more details).

The overall *Cryptosporidium* prevalence was 11.4% (83/730): 14.3% (3/21) in NSW; 10.8% (51/470) in QLD; and 12.1% (29/239) in WA. A total of 17 *Cryptosporidium* species and six genotypes were detected by NGS. In NSW, *C. hominis* and *Cryptosporidium* rat genotype III were the most prevalent species (9.5% each). In QLD, *C. galli, C. muris* and *C. parvum* were the three most prevalent species (7.7%, 5.7%, and 4.5%, respectively), while in WA, *C. meleagridis* was the most prevalent species (6.3%). The oocyst load/litre ranged from 70 to 18,055 oocysts/L (overall mean of 3,426 oocysts/l: 4,746 oocysts/l in NSW; 3,578 oocysts/L in QLD; and 3,292 oocysts/l in WA).

The data generated demonstrated that *Cryptosporidium* is prevalent in the raw influent of wastewater treatment facilities across Australia. NGS was central to unravelling the large diversity of *Cryptosporidium* species and genotypes in these samples and revealed the potential contribution of livestock, wildlife and birds (in addition to humans), to wastewater contamination. While human waste is a major contributor to WWTPs, the data from the present study suggests that abattoirs and poultry processing plants etc., could also be major contributors to wastewater treatment facilities. NGS analysis of the vertebrate species contributing to the wastewater will also help with determining the origin of the *Cryptosporidium* species detected in wastewater samples, and clearly further research is required to better understand the sources of *Cryptosporidium* in Australian wastewater.

Due to time constraints, *Giardia* was only analysed in QLD and NSW samples (Zahedi et al., 2019 – in preparation). The overall *Giardia* prevalence in QLD samples was 24.3% (159/653, 95%CI; 21.1-27.8). This comprised Assemblage A 36/653 (5.5%, 95%CI; 3.9-7.6), Assemblage B 35/653 (5.4%, 95%CI; 3.8-7.4) and Assemblage E 80/653 (12.2%, 95%CI; 9.8-15). In NSW, the prevalence was 11.9% (181/1521, 95%CI; 10.3-13.6). This comprised Assemblage A 66/1521 (4.3%, 95% CI; 3.4-5.5), Assemblage B 32/1521 (2.1%, 95%CI; 1.4-3.0) and Assemblage E 83/1521 (5.5%, 95%CI; 4.4-6.7). In addition, 88 microscopically *Giardia*-positive isolates from QLD were typed and in addition to *G. duodenalis* assemblage A (50% (44/88) and assemblage B (38·6% (34/88), assemblage E was identified in 6·8% (6/88) of samples. This is the first report of assemblage E in humans in Australia, indicating that in certain settings, this assemblage may be zoonotic (see Zahedi et al., 2017b for more details).

An analysis of farming and land management practices in WA, NSW and QLD was conducted via a questionnaire to determine if particular management practices were associated with a higher or lower prevalence of zoonotic genotypes in cattle and sheep. However, despite intensive efforts, the response rates from farmers to the questionnaire was very poor and therefore no statistically valid conclusions could be made.

Three reviews have also been completed (Ryan et al., 2014; Ryan and Hijjawi, 2015; Ryan et al., 2016 and Zahedi et al., 2016b).

Most significant outcomes:
The most significant findings were the detection of the human-infectious *C. hominis* in cattle and kangaroo faeces, the high prevalence of the zoonotic *C. parvum* in cattle, the high prevalence of the zoonotic *C. meleagridis* in wastewater and the identification for the first time of *Giardia duodenalis* assemblage E in humans in Australia.

Cryptosporidium hominis was identified by Sanger analysis and NGS in 5.2% (43/835; 3.8–6.9) of kangaroos screened in NSW and the median numbers of *C. hominis*/g^{-1} was 4831 with a range of 26–16,890 g^{-1} (Zahedi et al., 2016b), indicating that significant numbers of oocysts were present in some samples and DAPI staining indicated that they were viable. These kangaroo-derived *C. hominis* isolates were also analysed at the *gp60* locus using both Sanger and NGS (Zahedi et al., 2017a). In that study, unlike *C. parvum* isolates, in which additional within-host *gp60* subtype diversity was identified by NGS, only one *C. hominis* subtype was identified by both Sanger and NGS in the kangaroo-derived DNA samples, suggesting a single, recent introduction of *C. hominis* into kangaroos (Zahedi et al., 2017a). The *C. hominis* in the kangaroos may have come from spill-back from humans in the catchments, which may have also have spilled-over to infect cattle in the catchments. The lack of identification of *C. hominis* in kangaroos in NSW catchments prior to 2011 tends to support this. However, only a small fraction of samples were typed in those studies and it is not possible to determine if even the same kangaroo populations were analysed in the previous studies and therefore it is impossible to draw any real inferences. Collection site coordinates of *C. hominis* positive kangaroo and cattle samples in NSW indicated that there was a geographical overlap between areas from which six cattle and nine kangaroo *C. hominis* positives (including both subtypes IbA10G2 and IdA15G1) were collected (S-34.61278, E150.58498).

Cryptosporidium hominis was not detected in kangaroos from WA and kangaroo samples were not collected from QLD. Given the detection of *C. hominis* in kangaroos from NSW, the lack of data for QLD is an important knowledge gap and we recommend that future studies should be conducted in QLD to screen kangaroo faecal samples for *Cryptosporidium*.

Cryptosporidium hominis was detected in cattle faecal samples across all three states at a prevalence ranging from 4.5 to 14.1%. Although *C. hominis* predominately infects humans, it has been previously reported in cattle in Australia (Zahedi et al., 2016b), China (Chen and Huang, 2012, Zhang et al., 2018), Kenya (Kang'ethe et al., 2012), Korea (Park et al., 2006), Malawi (Banda et al., 2009), New Zealand (Abeywardena et al., 2012), and Scotland (Smith et al., 2005). However, there is no molecular evidence confirming transmission of *C. hominis* between cattle and humans, and therefore more studies should be conducted to fully elucidate the transmission dynamics of *C. hominis* in cattle. In the present study, two *C. hominis* subtypes were detected in cattle; IbA10G2 and IdA15G1. Subtype IbA10G2 is a dominant subtype responsible for *C. hominis*-associated outbreaks of cryptosporidiosis worldwide (Xiao, 2010). Subtype IdA15G1 was identified in three cattle isolates from WA and has been detected in humans from Victoria with a history of gastrointestinal disorders (Koehler et al., 2013). It is also the dominant subtype infecting Aboriginal people in WA (Ng-Hublin et al., 2017).

In cattle, the prevalence of *Cryptosporidium* was high (22.3%–26.3%) across three states and *C. parvum* was the dominant species ranging from 39.1% to 50.7% of samples positive for *Cryptosporidium* in cattle in each state, followed by *C. bovis* (17.6%–28.1%), *C. muris* (8.1%–15.6%) *C. hominis* (4.7–14.1%), *C. ubiquitum* (2.7%) and *C. ryanae* (1.6%–19.7%). *Cryptosporidium andersoni* was not detected. Most of the cattle sampled were adult cattle and therefore the high prevalence of *C. parvum* is surprising, as other studies have suggested that *C. parvum* dominates in pre-weaned calves but that *C. bovis, C. ryanae* and *C. andersoni* dominate in older cattle (Santín et al., 2008). This highlights the importance of site-specific analysis for accurate QMRA analysis. The *C. parvum* gp60 subtypes identified (IIaA15G2R1, IIaA16G2R1, IIaA17G2R1, IIaA18G3R1, IIaA19G2R1, IIaA19G3R1 and IIaA13G1) are commonly identified subtypes in humans and animals worldwide (Xiao, 2010, Feng et al.,

2013), with the exception of subtype IIaA13G1, which has previously only been detected in a single human patient from WA (Ng-Hublin et al., 2013).

Cryptosporidium meleagridis is a common parasite of humans in Australia (Ryan and Power, 2012) and also infects a wide range of birds (Zahedi et al., 2016a), with many overlapping *C. meleagridis* subtypes found in both birds and humans; suggesting both anthroponotic and zoonotic transmission (Silverlas et al., 2012). This is evidenced by the fact that *C. meleagridis* is commonly reported in wastewater worldwide (Hashimoto et al., 2006; Feng et al., 2009; Li et al., 2012; Huang et al., 2017). In the present study, *C. meleagridis* was the most prevalent species detected in WWTP samples collected from WA and in many cases was the only species detected. However, it was not detected in NSW or QLD. Although a variety of bird species are commonly seen at WWTPs in Australia, particularly around lagoons and clarifiers (secondary and tertiary treatment), the raw sewage entries to most WWTPs are covered, and not exposed and accessible to birds and animals. Some of the *C. meleagridis* detected in WWTPs in WA could have been originated from humans, however, further investigation revealed that the raw influent samples were taken directly from the distribution chamber located just before the primary ponds, which was only covered with a layer of mesh, providing easy access to bird contamination. Alternatively, industrial sources of wastewater from poultry farms could also be a major contributor. The predominance of the bird-specific *C. galli* in WWTP samples from QLD also confirms the potential role birds may play in contamination of wastewater by *Cryptosporidium*, but currently data on the contribution of poultry farms to WWTP in both WA and QLD is lacking and is an important knowledge gap. To date, there has only been one report of *C. galli* in wastewater (Ramo et al., 2017), however, *C. baileyi*, another avian *Cryptosporidiu*m species, has been reported in several studies from China (Feng et al., 2009; Li et al., 2012; Huang et al., 2017). It is possible that the high levels of *C. meleagridis* and *C. galli* detected in WA and QLD respectively, were due to contamination in our laboratory. However, this is unlikely as neither species were included as controls on the same Illumina MiSeq run and quality filtering removed all reads <100. The high number of *C. meleagridis* reads in WA (107 to 58,246 reads/sample) and *C. galli* reads in QLD (129 to 32,164 reads/sample) supports their validity. In addition, if it was due to gross contamination, then both species would be randomly distributed across all samples, with mixtures of both species in some samples and this was not the case.

Analysis of *Giardia* in sporadic human cases of giardiasis in QLD identified *G. duodenalis* assemblage E (previous though to be specific to livestock) in six out of 88 microscopically *Giardia*-positive isolates (Zahedi et al., 2017b). At the *gdh* locus, all six were 100% identical to a WA cattle-derived assemblage E isolate (GenBank accession number HQ398327) and at the *tpi* locus, were 100% identical to a Victorian sheep isolate (GenBank accession number GQ444454). This is the first report of assemblage E in humans in Australia.

A previous study in Egypt reported a high prevalence of assemblage E – 62·5% (25/40) in children living in agricultural areas in Egypt (Abdel-Moein and Saeed, 2016). In that study, assemblage E was detected in 42.1% of *Giardia* positive diarrheic and 81% of non-diarrheic children suggesting that assemblage E may cause clinical giardiasis (Abdel-Moein and Saeed, 2016). The high prevalence of assemblage E was attributed to the fact that the children lived in rural villages with large cattle populations (Abdel-Moein and Saeed, 2016). Other studies in Egypt have reported assemblage E in 15 and 11.1%, respectively, of *Giardia* positive human samples (Foronda et al. 2008; Helmy et al. 2014). In Brazil, assemblage E was identified in 34% (15/44) of *Giardia*-positive samples amongst preschoolers (aged between 10 months and 4 years) in a community of Rio de Janeiro (Fantinatti et al., 2016). In that study, all samples

were collected from children attending a day-care unit located in the slum with no sewerage network coverage, and stray animals including pigs and cattle moving throughout the location (Fantinatti et al. 2016).

Assemblages A and E are common amongst sheep and cattle in Australia with assemblage E the most dominant assemblage in hoofed animals (Nolan et al., 2010; Ng et al., 2011; Abeywardena et al., 2013; Yang et al., 2014; Asher et al., 2016). In the present study, the individuals that were positive for Assemblage E were experiencing diarrhoea, came from both rural and urban areas and shed variable levels of cysts in their feces. All were identical to assemblage E from Australian cattle and sheep, suggesting possible zoonotic transmission. The data generated demonstrates that zoonotic transmission from cattle and sheep may be occurring and warrants further investigation.

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2. Zahedi, A, Monis, P, Gofton, AW, Oskam, CL, Ball, A, Bath, A, Bartkow, M, Robertson, I, Ryan, U., 2018a. *Cryptosporidium* species and subtypes in animals inhabiting drinking water catchments in three states across Australia. Water Res. 134, 327-340.
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1. Zahedi, A., Gofton, A., Greay, T., Monis, P., Oskam, C., Ball, A., Bath, A., Watkinson, A., Robertson, I., Ryan, U., 2018. Profiling the diversity of *Cryptosporidium* species and genotypes in wastewater treatment plants in Australia using next generation sequencing. Global Microbiome 2018 Symposium. August 17th. (Invited Speaker)
2. Zahedi, A., Monis, P., Gofton, A.W., Oskam, C.L., Ball, A., Bath A, Bartkow, M., Robertson, I., Ryan, U., 2018. Metagenomic analysis of *Cryptosporidium* species and genotypes in wastewater treatment plants. 14th International Congress of Parasitology (ICOPA). 19th-24th of August, Daegu, South Korea. p16.
3. Zahedi, A., Gofton, A., Jian, F., Paparini, A., Oskam, C., Ball, A., Robertson, I., Ryan, U., 2017. Next Generation Sequencing uncovers within host genetic diversity of *Cryptosporidium gp60* subtypes. Australian Society for Parasitology conference, Blue Mountains, Australia. 26th - 29th June, p41.
4. Zahedi, A., Paparini, A., Watkinson, A., Oskam, C., Robertson, I., Ryan, U., 2016. Molecular characterization of species and genotypes of *Cryptosporidium* in animals inhabiting 3 main water catchments (Lake Baroon, Logan River, North-Pine River) in South-East Queensland (QLD). International Congress of Tropical Medicine and Malaria, Brisbane, Australia. Australia. September 18th - 22nd, p178.
5. Zahedi, A., Phasey, J., Boland, T., Ryan, U., 2016. First report of *Cryptosporidium* species in farmed and wild buffalo from the Northern Territory, Australia. International Congress of Tropical Medicine and Malaria, Brisbane, Australia. Australia. September 18th - 22nd, p74.

6. Zahedi, A., Paparini, A., Jian, F., King, B., Monis, P., Ball, A., Robertson, I., Ryan, U., 2015. Prevalence and molecular characterization of *Cryptosporidium* species in animals inhabiting Sydney water catchments. Australian Society for Parasitology conference, Auckland, New Zealand. June 29th - July 2nd, p44.
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