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Research paper

Detection of *Cryptosporidium hominis* and novel *Cryptosporidium* bat genotypes in wild and captive *Pteropus* hosts in Australia

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ABSTRACT

Spillover of zoonotic pathogens from wildlife to humans has been identified as a primary threat to global health. In contrast, the process of reverse pathogen transmission (zooanthroponosis), whereby pathogens move from humans into wildlife species is still largely unexplored. Globally, increasing urbanisation and habitat loss are driving many wildlife species into urban and regional centres. In Australia, large numbers of flying foxes now live in close proximity to humans, increasing the risk of zooanthroponosis. The protozoan parasite *Cryptosporidium* is an emerging zoonotic parasite that infects a wide range of vertebrates yet there are limited studies on transmission potential of *Cryptosporidium* between humans and urban wildlife. To explore the presence of zooanthroponosis in flying foxes in Australia the occurrence and diversity of *Cryptosporidium* was investigated in urbanised wild and captive flying foxes. PCR screening of faecal samples ($n = 281$) from seven wild sites and two captive facilities identified the presence of *Cryptosporidium* in 3.2% (95% CI 1.5% to 6.0%) of faecal samples. In faecal samples from wild sites *Cryptosporidium* occurrence was 1.7% (95% CI 0.3% to 4.8%) versus 5.9% (95% CI 2.2% to 12.4%) in samples from captive individuals, with no significant difference between captive and wild sites ($p = 0.077$). Multilocus sequencing using three loci, 18s rDNA, actin and *gp60* was used to identify *Cryptosporidium* in flying fox species. The host specific *Cryptosporidium hominis* was identified in faecal samples from two captive flying foxes, and one of these samples was confirmed as *C. hominis* at both actin and *gp60*. Sequencing of the 18s rDNA also revealed four novel *Cryptosporidium* genotypes in wild and captive individuals, actin and *gp60* amplification and sequencing were unreliable for all four novel genotypes. These novel genotypes have been designated *Cryptosporidium* bat genotypes VIII–XI. This first report of *Cryptosporidium* spp. in Australian flying foxes indicates zooanthroponotic transmission of *Cryptosporidium* from humans to flying foxes within a captive environment and extends the diversity of this globally important parasite.

1. Introduction

Zoonotic pathogens account for approximately 75% of emerging infectious diseases (EID) in humans (Taylor et al., 2001), with spillover from wildlife to humans having been identified as one of the primary threats to global health (Jones et al., 2008; Epstein and Price, 2009). As a result, emerging zoonoses are increasingly being recognised as playing a fundamental role in determining community health (John, 2013). The intricate balance between human, animal and ecosystem health has led to a systems approach under the banner of 'One Health' (John, 2013).

One still largely unexplored aspect of global heath is the potential transmission of human-borne pathogens to wildlife and domestic animals (zooanthroponosis) (Epstein and Price, 2009). Infections with zoonotic protozoan parasites, such as *Cryptosporidium*, *Giardia* and *Toxoplasma* (Current and Garcia, 1991; Dubey, 2009; Feng and Xiao, 2011) are known to be responsible for high levels of disease and morbidity in humans, but these zoonotic pathogens also infect wild and domestic animals (Taylor et al., 2001). Spillover of anthroponotic pathogens into wildlife therefore has the potential to cause significant and widespread disease burden, which may subsequently threaten Australia's unique ecosystem and perpetuate disease emergence (Ryan and Power, 2012).

The protozoan parasite, *Cryptosporidium*, is a primary cause of diarrhoeal disease worldwide (Fayer et al., 1997). Contamination of recreational water and drinking water with *Cryptosporidium* oocysts is a major source of cryptosporidiosis, and transmission can also occur via direct contact with faeces from infected hosts (Current and Garcia, 1991; Rose et al., 1991; DuPont et al., 1995).

Currently, 27 *Cryptosporidium* species are recognised and 20 have been reported in humans (Ryan et al., 2014). Two species, *Cryptosporidium hominis* and *Cryptosporidium parvum* cause over 90% of human cryptosporidiosis cases and are of public health significance (Morgan-Ryan et al., 2002; Jex et al., 2008). Although the majority of *Cryptosporidium* spp. appear to be highly host adapted, a number of species have been shown to infect a wide variety of vertebrate hosts

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(Thompson et al., 2005; Feng, 2010; Xiao, 2010; Waldron et al., 2011). The zoonotic *C. parvum* has been identified across a wide range of vertebrate hosts (Morgan et al., 1997; Xiao et al., 2004; Fayer, 2010), whereas the anthroponotic *C. hominis* has been found to be almost entirely host specific (Morgan-Ryan et al., 2002). Infections with *C. hominis* in non-human mammals has only been identified in cattle (Smith et al., 2005; Ng et al., 2011), kangaroos (Ng et al., 2011) and a dugong (Morgan et al., 2000) and experimental infections have been established in pigs, calves and mice (Akiyoshi et al., 2002; Guk et al., 2004). The host specificities of *Cryptosporidium* and low environmental transmission potential of *C. hominis* from non-human host sources therefore make this parasite an ideal candidate for an investigation into anthroponotic pathogen transmission to wildlife.

Approximately 25% of all living mammals are bats (Order Chiroptera), with over 1232 extant species spread across all continents, excluding the polar regions (Kasso and Balakrishnan, 2013; Ng and Baker, 2013). Globally, bats are of high economic value due to their role in ecosystem services where they act as pollinators, seed dispersers, insect predators and bioindicators (Fujita and Tuttle, 1991; Jones et al., 2009). Until recently the order Chiroptera was divided into the Mega- and Microchiroptera (Gray, 1821), commonly referred to as mega- and microbats. Now, bats are classified into the suborders, Yinpterochiroptera and Yangochiroptera with the former comprising all megabat species and three former microbat families Megadermatidae, Rhinolophidae and Rhinopomatidae (Tsagkogeorga et al., 2013). For ease we apply the terms megabat and microbat throughout this report and take 'megabats' to include the three microbat families above.

Australia hosts a total of 77 bat species, including eight species of megabats (Suborder: Yinpterochiroptera, family: Pteropodidae), commonly known as fruit bats or flying foxes (McKay et al., 1989). Flying foxes rely heavily on seasonal fruit and nectar food sources which are widely dispersed in native forests (Hall and Richards, 2000). The decline of these native food sources as a consequence of anthropogenic impacts has resulted in the movement of these animals into more highly populated areas where native gardens and parks provide reliable alternative food sources (Parry-Jones and Augee, 2001; Williams et al., 2006). These shifts into urbanised environments are placing mounting pressures on flying foxes, including Grey-headed flying foxes (*Pteropus poliocephalus*) which are currently classified as threatened under the IUCN guidelines (Markus and Hall, 2004; McDonald-Madden et al., 2005; IUCN, 2014).

The first case of *Cryptosporidium* in a bat was identified in a tissue sample of a microbat (Yangochiroptera: Vespertilionidae: *Eptesicus fuscus*) in 1998 (Dubey et al., 1998), followed by the identification of a *Cryptosporidium* "mouse" genotype in faecal samples of another microbat species (Yangochiroptera: Vespertilionidae: *Myotis adversus*) (Morgan et al., 1998; Morgan et al., 1999). Molecular typing of *Cryptosporidium* from bats in China found novel genotypes in four Yinpterochiroptera species. *Cryptosporidium* bat genotype I was identified in Chinese rufous horseshoe bat (Rhinolophdae: *Rhinolophus sinicus*) and Stoliczka's trident bat (Hipposideridae: *Aselliscus stoliczkanus*) and bat genotype II was identified in a Chinese rufous horseshoe bat, Fulvus roundleaf bat (Hipposideridae: *Hipposideros fulvus*) and Leschenault's rousette (Pteropidae: *Rousettus leschenaultia*) (Wang et al., 2013). Two further novel genotypes, bat genotype III and bat genotype IV were identified in Yangochiroptera from the United States of America (USA) and Czech Republic (Kváč et al., 2015). Bat genotype III was described from big brown bats (Vespertilionidae: *E. fuscus*) and bat genotype IV from the common pipistrelle (Vespertilionidae: *Pipistrellus pipistrellus*). Three further *Cryptosporidium* bat genotypes (V–VII) were described in Rhinolophdae: *Rhinolophus inops*, *Cynopterus brachyotis*, and *Eonycteris spelaea*, in the Philippines (Murakoshi et al., 2016). The zoonotic *C. parvum* was also identified in the common pipistrelle and western small-footed bat (*Myotis ciliolabrum*) in the Czech Republic and USA respectively (Kváč et al., 2015).

The role of *Pteropus* spp. (flying foxes) as vectors for viral pathogens, including Lyssavirus and Hendra virus within Australia, is well established, but studies investigating the role of bats as vectors for protozoan pathogens of human and/or veterinary importance are scarce (Ng and Baker, 2013). Although protozoan parasites belonging to the genera *Toxoplasma* (Sangster et al., 2012), *Hepatocystis* (Mackerras, 1958) and *Trypanosoma* (Mackerras, 1959; Austen et al., 2015) have been identified in flying foxes, *Cryptosporidium* diversity within these animals is currently unknown. The presence of infectious protozoan parasites in flying fox species may however have important implications for Australia's endemic fauna, livestock and human health, as host switching or spillover events could potentially lead to infections and ultimately disease outbreaks.

The aim of this study was to investigate the potential for zooanthroponotic pathogen transmission of *Cryptosporidium* spp. into Australian Flying foxes (*Pteropus* spp.). Samples from wild and captive sites in New South Wales (NSW) and Queensland (QLD) were collected and molecular methods were employed in order to explore *Cryptosporidium* presence and genetic diversity.

2. Materials and methods

2.1. Sampling sites and sample collection

Australian flying fox (*Pteropus* spp.) roosts are predominantly distributed along coastal regions and within tropical and subtropical zones (Hall and Hughes, 1987). Habitat loss has resulted in a shift of these animals into increasingly urbanised environments (Plowright et al., 2011). Consequently, most major east coast cities and towns are now continually occupied by flying fox roosts, facilitating easy access for sampling purposes (Plowright et al., 2011).

Faecal samples from nine flying fox sites along the east coast of Australia were collected for parasite analysis. Plastic sheets were placed below the roosting areas and discrete faecal samples were collected no >6 h after placement. The sample sites consisted of urban and rural locations in NSW (Centennial Park, North Avoca, Singleton, Tocal, Port Macquarie, Byron Bay, Gordon) and two captive facilities, one in NSW (Matcham) and one in QLD (Tolga) (Fig. 1). All wild sourced samples were opportunistically collected from Grey-headed flying fox (*P. poliocephalus*) camps in 2012 and 2013. Samples from bats in captivity in NSW were collected from *P. poliocephalus* held at the Wambina Flying Fox Sanctuary, Matcham (a re-release facility operated by the Wildlife Animal Rescue and Care Society Inc.), located 55 km north of Sydney in February and March 2015. Samples from captive bats in QLD were collected in April 2015 at the Tolga Bat Hospital located in the Atherton Table Lands near Cairns. The Tolga group consisted primarily of Spectacled and Little Red flying foxes (*Pteropus conspicillatus* and *Pteropus scapulatus* respectively) housed in one enclosure. Animals aggregated in single species groups and utilised specific areas of the enclosure and samples were collected from these areas. To minimise the risk of pseudoreplication within the captive groups, non-neighbouring samples were collected off the drop sheets. Faecal samples were irradiated by exposure to a ^{60}Cobalt source for 35 min to reduce pathogenic risk and stored at 4 °C until further processing.

2.2. DNA extraction and 18S rDNA PCR screening

DNA was extracted from faecal material (~150 mg) using the Isolate Faecal DNA kit (Bioline, London, UK) following the manufacturer's instructions. Extracted DNA was stored at −20 °C until further processing. Prior to PCR the DNA samples were combined with Gene Releaser (5 μl) (BioVentures, Inc., TN, USA) and overlaid with paraffin oil (Biotech Pharmaceuticals PTY LTD, Australia) before microwaving on high (7 min). Samples were initially screened for *Cryptosporidium* using a nested PCR protocol targeting a ~298 bp fragment of the 18s rDNA gene. Primary amplifications were performed using forward primer