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First report of *Cryptosporidium* species in farmed and wild buffalo from the Northern Territory, Australia

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Abstract

A molecular epidemiological survey of *Cryptosporidium* from water buffalo (*Bubalus bubalis*) in the Northern Territory in Australia was conducted. Fecal samples were collected from adult farmed (*n* = 50) and wild buffalo (*n* = 50) and screened using an 18S quantitative PCR (qPCR). Positives were typed by sequence analysis of 18S nested PCR products. The qPCR prevalence of *Cryptosporidium* species in farmed and wild buffalo was 30 and 12 %, respectively. Sequence analysis identified two species: *C. parvum* and *C. bovis*, with *C. parvum* accounting for ~80 % of positives typed from the farmed buffalo fecal samples compared to 50 % for wild buffalo. Subtyping at the 60 kDa glycoprotein (gp60) locus identified *C. parvum* subtypes IIdA19G1 (*n* = 4) and IIdA15G1 (*n* = 1) in the farmed buffalo and IIdA18G3R1 (*n* = 2) in the wild buffalo. The presence of *C. parvum*, which commonly infects humans, suggests that water buffaloes may contribute to contamination of rivers and
waterways with human infectious Cryptosporidium oocysts, and further research on the epidemiology of Cryptosporidium in buffalo populations in Australia is required.

Keywords

Cryptosporidium; Buffalo18S; C. parvum; C. bovis gp60

Introduction

Cryptosporidium is an important protozoan parasite that infects a wide range of animals including humans (Xiao 2010). The parasite is fecal orally transmitted via water, food, or direct contact (Burnet et al. 2014). Clinical symptoms in immune-competent individuals can include self-limiting watery diarrhea, abdominal pain, nausea, and vomiting but in immune-compromised individuals, infections can result in chronic or protracted diarrhea (Chalmers and Davies 2010). The environmental stage of the parasite (oocyst) is resistant to inactivation by commonly used drinking water disinfectants (Baldursson and Karanis 2011; Burnet et al. 2014). As a result of this, contamination of water supplies is a major mode of transmission, and Cryptosporidium was the etiological agent in 60.3 % (120) of the waterborne protozoan parasitic outbreaks reported worldwide between 2004 and 2010 (Baldursson and Karanis 2011). Of the twenty-nine recognized Cryptosporidium species (Ryan and Hijjawi 2015; Zahedi et al. 2015), C. parvum and C. hominis have been responsible for the majority of infections in humans (Xiao 2010).

Livestock animals have been implicated as a source of human cryptosporidiosis based on molecular epidemiological studies conducted in various countries (Xiao 2010; Santín 2013; Abeywardena et al. 2014; Abeywardena et al. 2015). However, relatively little is known about the range of species and genotypes of Cryptosporidium in other members of the family
Bovidae, including water buffalo (*Bubalus bubalis*), but to date, *C. parvum*, *C. ryanae*, *C. bovis*, *C. ubiquitum*, a “*C. suis*-like” genotype, and *C. ryanae* variants have been reported (Gómez-Couso et al. 2005; Cacciò et al. 2007; Feng et al. 2012; Venu et al. 2012; Abeywardena et al. 2013a; Abu Samra et al. 2013; Amer et al. 2013; Helmy et al. 2013; Inpankaew et al. 2014; Mahfouz et al. 2014; Ma et al. 2015; Abeywardena et al. 2015; Aquino et al. 2015; Helmy et al. 2015).

Water buffalo are one of the important livestock animals in many regions of the developing world. The world’s water buffalo population was estimated as 195 million in 2011, being mostly distributed in Asia, some Mediterranean regions and Latin American countries (Robertson et al. 2014). Water buffalo were brought to Australia between 1824 and 1886 from South-East Asia to provide working animals and meat for the remote northern settlements (Letts 1964). When the early settlements were abandoned, the buffaloes were released, where they became feral populations. Currently, both farmed and feral buffalo herds exist in Australia, with the majority of feral buffaloes in the Northern Territory (in Arnhem Land) with a minimum estimated population of wild buffalo of approximately 98,000 buffalo across 92,000 km$^2$ of country (Saalfeld 2014).

To date, only one study of *Cryptosporidium* in farmed water buffalo in Victoria, Australia, has been conducted (Abeywardena et al. 2013a), and nothing is known about the prevalence and species of *Cryptosporidium* infecting water buffalo in Northern Australia. Therefore, the aim of the present study was to use molecular tools to better understand the public health risks of *Cryptosporidium* sp. in both wild and farmed buffalo herds to drinking water supplies in the Northern Territory (NT) of Australia.
Materials and Methods

Sample collection and processing

Buffalo fecal samples were collected in August 2015 from (1) wild buffalo \( n = 50 \) located near the Maude Creek cattle station, 30-km south of Katherine, NT (the herd was originally sourced from the Phelp River, South-Eastern Arnhem Land) and (2) farmed buffalo \( n = 50 \) from the Northern Territory Government, Department of Primary Industry and Fisheries (DPIF) buffalo farm (Beatrice Hill Farm), near the Adelaide River, NT by Tropical Water Solution staff. Samples were collected from freshly deposited fecal samples into individual 75-ml fecal collection pots using a scrapper to expose and scoop from the center of the scat pile. All samples were stored at 4 °C, shipped to Murdoch University, and stored at 4 °C until analyzed. All fecal samples were collected from adult animals; the wild buffalo were between 2–5 years of age, and the farmed buffalo were between 3 and 4 years of age. Approval for faecal collection was obtained from the Northern Territory Buffalo Industry Council and individual property owners directly by phone. As the samples were collected directly from the ground and not per rectum, specific animal ethics approval was not required.

DNA isolation

Genomic DNA was extracted from 250 mg of each fecal sample using a Power Soil DNA Kit (MolBio, Carlsbad, California). A negative control (no fecal sample) was used in each extraction group.

PCR amplification of the 18S rRNA gene

All samples were screened for the presence of Cryptosporidium at the 18S rRNA locus using a quantitative PCR (qPCR) previously described (King et al. 2005; Yang et al. 2014). Each 10-μl PCR mixture contained 1× Go Taq PCR buffer (KAPA Biosystems), 3.75 mM MgCl₂,
400 μM of each dNTPs, 0.5-μM 18SiF primer, 0.5-μM 18SiR primer, 0.2-μM probe, and 1U/reaction Kapa DNA polymerase (MolBio, Carlsbad, California). The PCR cycling conditions consisted of one pre-melt cycle at 95 °C for 6 min and then 50 cycles of 94 °C for 20 s and 60 °C for 90 s.

Samples that were positive by qPCR were amplified at the 18S locus using primers which produced a 611-bp product as previously described (Silva et al. 2013) with minor modifications; the annealing temperature used in the present study was 57 °C for 30 s, and the number of cycles was increased from 39 to 47 cycles for both primary and secondary reactions. PCR contamination controls were used including negative controls and separation of preparation and amplification areas.

**PCR amplification of the gp60 gene**

Samples that were typed as *C. parvum* at the 18S locus were subtyped at the 60 kDa glycoprotein (gp60) locus using a nested PCR as previously described (Zhou et al. 2003).

**Sequence analysis**

The amplified DNA from secondary PCR products was separated by gel electrophoresis and purified for sequencing using an in-house filter tip method (Yang et al. 2013). Purified PCR products from both loci were sequenced independently using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer’s instructions at 57 and 54 °C annealing temperature for the 18S rRNA and gp60 loci, respectively. Sanger sequencing chromatogram files were imported in Geneious Pro 8.1.6 (Kearse et al. 2012), edited, analyzed, and aligned with reference sequences from GenBank using Clustal W (<http://www.clustalw.genome.jp>).
Statistical analysis

Statistical analyses (Fisher’s exact test) were performed using SPSS 22 for Windows (SPSS Inc. Chicago, USA), to determine if there was a statistical difference in the prevalence of Cryptosporidium in farmed versus wild buffalo.

Results

Prevalence of Cryptosporidium in farmed and wild buffalo

The qPCR prevalence of Cryptosporidium species in the farmed buffalo was 30 % (15/50) (95 % CI, 17.9–44.6) and was 12 % (6/50) (95 % CI, 4.5–24.3) in the wild buffalo. This difference was significant ($P = 0.048, P < 0.05$).

Cryptosporidium species detected in farmed and wild buffalo

Sequence analysis at the 18S locus was only successful for six of the fifteen farmed buffalo positives and four of the six wild buffalo samples. Two Cryptosporidium species, C. parvum and C. bovis, were identified in both types of the samples. In the farmed buffalo samples, five were C. parvum and one was C. bovis, whereas for the wild buffalo, two were identified as C. parvum and two were C. bovis.

Sequences at the gp60 locus were obtained for five of the farmed buffalo C. parvum positives, and C. parvum subtypes IIdA19G1 ($n = 4$) and IIdA15G1 ($n = 1$) were identified. In the wild buffalo, gp60 sequences were obtained for both C. parvum positives, and both were identified as IlaA18G3R1.
Discussion

The present study described the prevalence and molecular characterization of Cryptosporidium species in the farmed and wild buffalo from the Northern Territory in Australia. The prevalence of Cryptosporidium species in the farmed and wild buffalo fecal samples was 30.0 and 12.0 % respectively. A previous study of Cryptosporidium in farmed water buffalo in Victoria reported a prevalence of 13.0 % (62/476) (Abeywardena et al. 2013a). Other studies have reported prevalences in buffalo ranging from 5.7 to 62.1 % (Amer et al. 2013; Inpankaew et al. 2014; Abeywardena et al. 2014; Aquino et al. 2015; Ma et al. 2015).

Cryptosporidium parvum is the most commonly reported zoonotic species of Cryptosporidium infecting humans and was the most common species detected in farmed buffalo (~80 % of positives typed) and in wild buffalo accounted for 50 % of the positives typed. This species has been detected previously in buffaloes in many countries including Spain, Italy, Australia, Egypt, India, and Thailand (Amer et al. 2013; Cacciò et al. 2007; Gómez-Couso et al. 2005; Maurya et al. 2013; Abeywardena et al. 2013a; Inpankaew et al. 2014; Mahfouz et al. 2014; Aquino et al. 015). Previous studies have reported that C. parvum was the predominant species in young buffaloes (Cacciò et al. 2007; Maurya et al. 2013; Inpankaew et al. 2014), and in the previous study in Australia, C. parvum was only detected in buffalo <6 months of age (Abeywardena et al. 2013a). In the present study, however, all buffalo samples were adults (2–5 years), indicating that this species can also commonly infect adult buffaloes.

Cryptosporidium bovis was detected in ~20 and 50 % of the farmed and wild buffalo isolates-typed, respectively. This species has previously been reported in buffalo in Egypt (Helmy et al. 2013), South Africa (Abu Samra et al. 2013), Australia (Abeywardena et al. 2013a), and
China (Ma et al. 2015). In the previous study in Australia, *C. ryanae* variants (reported as genotypes 1 and 2) and a *C. suis*-like genotype (reported as genotype 3) were also reported in buffaloes (Abeywardena et al. 2013a). *Cryptosporidium bovis* is predominantly a parasite of livestock and has only been reported in humans on a few occasions (Khan et al. 2010; Ng et al. 2012).

The *C. parvum* subtype IIA18G3R1 was identified in the two wild buffalo samples. IIA18G3R1 is a common subtype and has been reported widely in both cattle and humans worldwide including Australia (Plutzer and Karanis 2009; Ryan and Power 2012). The *C. parvum* subtypes IIdA19G1 and IIdA15G1 were identified in the farmed buffalo in the present study. Both are considered zoonotic subtypes (Wang et al. 2014). The *C. parvum* IId subtype family has been reported mainly from sheep and goats but has also been reported in humans and cattle (Plutzer and Karanis 2009; Xiao 2010; Wang et al. 2014). The *C. parvum* IIdA15G1 subtype has been detected in livestock and humans (Plutzer and Karanis 2009; Xiao 2010; Wang et al. 2014), including a human in Australia (Ng et al. 2010). The IIdA19G1 subtype is less common but has been reported in humans and animals (Xiao 2010; Wang et al. 2014). In China, IId is the predominant *C. parvum* subtype (Wang et al. 2014), and both of these IId subtypes are commonly found in bovine animals in China including yaks (Wang et al. 2014; Qi et al. 2015). However, this subtype is not common in dairy cattle in Australia (Ng et al. 2012; Abeywardena et al. 2013b). Previous analysis has indicated that *C. parvum* IId subtypes were probably dispersed from Western Asia to other geographical regions (Wang et al. 2014). As water buffalo in Australia came from Asia (Letts 1964), it is likely that the IId subtype family was introduced into Australia with the introduction of buffalo. In the previous study in Australia (Abeywardena et al. 2013a), gp60 subtyping was not conducted.
Conclusion

The preliminary data from the present study indicates that water buffaloes have the potential to contribute to the zoonotic transmission of *C. parvum* via contamination of water, as buffaloes usually wallow in rivers, streams, and other water sources (Abeywardena et al. 2014). These findings indicate the need (1) for further characterization of the prevalence, intensity of infection, and species of *Cryptosporidium* in buffalo populations across Australia and (2) to determine the levels of oocysts in rivers and waterways flowing from buffalo farms and wild buffalo locations, particularly those that flow into water reservoirs used for drinking water.

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References


