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First molecular characterisation of *Cryptosporidium* and *Giardia* from *Bubalus bubalis* (water buffalo) in Victoria, Australia

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ABSTRACT

We conducted a molecular epidemiological survey of *Cryptosporidium* and *Giardia* from *Bubalus bubalis* (water buffalo) on two extensive farms (450 km apart) in Victoria, Australia. Faecal samples ($n = 476$) were collected from different age groups of water buffalo at two time points (six months apart) and tested using a PCR-based mutation scanning-targeted sequencing-phylogenetic approach, employing markers within the small subunit of ribosomal RNA (designated p*SSU*) and triose phosphate isomerase (p*tpi*) genes. Based on p*SSU* data, *Cryptosporidium parvum*, *Cryptosporidium bovis* and *Cryptosporidium* genotypes 1, 2 (each 99% similar genetically to *Cryptosporidium ryanae*) and 3 (99% similar to *Cryptosporidium suis*) were detected in two (0.4%), one (0.2%), 38 (8.0%), 16 (3.4%) and one (0.2%) of the 476 samples tested, respectively. Using p*tpi*, *Giardia duodenalis* assemblages A and E were detected in totals of 56 (11.8%) and six (1.3%) of these samples, respectively. *Cryptosporidium* was detected on both farms, whereas *Giardia* was detected only on farm B, and both genera were detected in 1.5% of all samples tested. The study showed that water buffaloes on these farms excreted *C. parvum* and/or *G. duodenalis* assemblage A, which are consistent with those found in humans, inferring that these particular pathogens are of zoonotic significance. Future work should focus on investigating, in a temporal and spatial manner, the prevalence and intensity of such infections in water buffaloes in various geographical regions in Australia and in other countries.

1. Introduction

Cryptosporidium and *Giardia* are common aetiological agents of protozoal enteritis of humans and animals, including livestock, companion animals and wildlife (Fayer, 2004; Thompson and Monis, 2004; Thompson et al., 2008; Jex et al., 2011). Species of both genera are faecal-orally transmitted through resilient, infective stages (oocysts or cysts) (Korich et al., 1990; Carpenter et al., 1999; Betancourt and Rose, 2004), usually *via* water, food or direct contact (Cacciò et al., 2005; Smith et al., 2007). Clinical signs of disease can range from self-limiting diarrhoea in immune-competent individuals (O'Donoghue, 1995; Homan and Mank, 2001) to chronic and life-threatening infection in immune-compromised or -suppressed individuals (Hunter and Nichols, 2002; Petri et al., 2008; Stark et al., 2009). Humans have been reported to become infected with a range of species and genotypes of *Cryptosporidium* (Smith et al., 2006) or *Giardia* (Foronda et al., 2008). However, based on current molecular data (Cacciò et al., 2005; Xiao and Fayer, 2008), *Cryptosporidium hominis, Cryptosporidium parvum* and *Giardia duodenalis* assemblages A and B are responsible for the majority of known human disease cases. Of these species, *C. hominis* is recognised to be transmitted from human to human (Xiao et al., 2004), whereas *C. parvum* and *G. duodenalis* infections may be acquired through human–human or animal–human transmission (Xiao and Ryan, 2004; Xiao and Feng, 2008).

Livestock animals have been implicated as a source of human cryptosporidiosis and giardiasis, based on the molecular epidemiological studies conducted in various countries (Hunter and Thompson, 2005; Budu-Amoako et al., 2011). There is a considerable amount of data demonstrating that dairy and beef cattle harbour zoonotic species and genotypes of *Cryptosporidium* and *Giardia*, representing a potentially significant reservoir of infection to humans (e.g., Santín et al., 2004; Geurden et al., 2008; Xiao and Feng, 2008; Feng and Xiao, 2011). Interestingly, there is an age-specific stratification for *Cryptosporidium* in cattle. Usually, *C. parvum* is found principally in pre-weaned cattle, *Cryptosporidium bovis* and *Cryptosporidium ryanae* are mostly found in weaned calves and yearlings, and *Cryptosporidium andersoni* is most common in adult cattle (Santín et al., 2004). In contrast to the situation in cattle, very

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little is known about the range of species and genotypes of *Cryptosporidium* and *Giardia* in other members of the family Bovidae, including water buffalo (*Bubalus bubalis*). This species contributes substantially to the agricultural economies in many developing countries, and is widely used as a working animal (for transport and ploughing) and as a source of milk, meat and leather (Perera, 2011). Generally, bovids produce large amounts of faecal waste each day, and, if infected, can excrete substantial numbers of infective stages that contaminate the environment (Fayer et al., 2000; Budu-Amoako et al., 2011).

To date, molecular studies of *Cryptosporidium* of water buffaloes have been conducted in countries including Egypt (Amer et al., 2013), Italy (Cacciò et al., 2007), Nepal (Feng et al., 2012) and Spain (Gomez-Couso et al., 2005). Unlike cattle, a genotype with 99% similarity to *C. ryanae* has been characterised from water buffaloes more frequently than any other species of *Cryptosporidium* (Feng et al., 2012; Amer et al., 2013), while *C. andersoni* and *C. bovis* have not yet been reported. One molecular study of *Giardia* from water buffaloes conducted in Italy reported assemblages A and E (Cacciò et al., 2007, 2010). These studies suggest that water buffalo, like cattle, might also serve as an important reservoir of *Cryptosporidium* and *Giardia* for transmission to humans, which warrants investigations using molecular tools. In the present study, we genetically characterized *Cryptosporidium* and *Giardia* from water buffaloes in Victoria, Australia, and assessed their zoonotic potential. For this purpose, we utilized a PCR-based mutation scanning-targeted sequencing-phylogenetic approach, employing markers within the small subunit of ribosomal RNA (designated p*SSU*) and triose phosphate isomerase (p*tpi*) genes. As these two loci have been widely used for molecular characterisation of *Cryptosporidium* and *Giardia*, there is a substantial amount of sequence data available in public databases for comparative analysis.

2. Materials and methods

2.1. Sample collection, calves and management practices

Faecal samples (n = 476) were collected from water buffaloes (*Bubalus bubalis*; mixed sexes; various age groups), including 32, 232 and 212 samples from animals of <6 months, 6 months to 2 years, and >2 years, on extensive farms A and B, located 450 km apart in Victoria, Australia. Samples were collected from each farm, at two time points, six months apart. In total, 100 and 400 buffaloes were present on each of the two farms (Farm A and B, respectively) at each visit. Freshly deposited faecal samples were collected from paddocks or enclosures. On both farms, water buffaloes of >3 months of age were maintained together. Calves were born *in situ* on both farms and maintained with their dams up to 24 h to ensure that each calf had received colostrum. Then, calves were kept in pens, fed with whole milk, weaned at 3 months of age and released on to paddocks with cows. Farm A commenced farming swamp buffaloes in 1992, and introduced riverine buffaloes in 2002, whereas on farm B, all of the buffaloes were of the riverine type.

2.2. Isolation of genomic DNA from faecal samples, and PCR amplification

Genomic DNA was extracted from individual faecal samples using the PowerSoil DNA isolation kit (MoBIO, USA) (*cf.* Abeywardena et al., 2013), and then frozen at −20 °C until molecular testing. Each genomic DNA sample was tested specifically for the presence of *Cryptosporidium* and *Giardia* DNA, employing genetic markers (designated p*SSU* and p*tpi*) in the small subunit (*SSU*) nuclear ribosomal RNA gene and triose phosphate isomerase (*tpi*) genes, respectively. For the amplification of p*SSU*, nested PCR was performed in a 50 μl volume containing 2.0 mM of $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each oligonucleotide primer and 1.25 U of Mango*Taq* polymerase in a standard PCR buffer (Bioline, USA). In the primary reaction of *SSU* for *Cryptosporidium*, primers XF2 (forward: 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′) and XR2 (reverse: 5′-AAGGAGTAAGGAACAACCTCCA-3′) (Xiao et al., 1999) were employed, and primers pssu-f (forward: 5′-AAAGCTCGTAGTTGGATTTCTGTT-3′) and pssu-r (reverse: 5′- ACCTCTGACTGTTAAATACRAATGC-3′) (*cf.* Nolan et al., 2010a) were utilised for the secondary reaction to amplify p*SSU* (~240 bp). The cycling protocol for the primary amplification included an initial cycle of 94 °C/5 min (initial denaturation), followed by 30 cycles of 94 °C/45 s (denaturation), 45 °C/2 min (annealing), and 72 °C/1.5 min (extension) and a final extension of 72 °C/10 min. From 1 μl of primary amplicon, the nested amplification was performed using a cycling protocol of 94 °C/5 min (initial denaturation), followed by 35 cycles of 94 °C/30 s (denaturation), 55 °C/30 s (annealing) and 72 °C/30 s (extension), followed by a final extension of 72 °C/10 min.

For the amplification of p*tpi*, nested PCR was performed in a 50 μl volume containing 3.0 mM of $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each oligonucleotide primer and 1.25 U of Go*Taq* polymerase in standard PCR buffer (Promega, USA). In the primary reaction for *tpi* of *Giardia*, primers AL3543 (forward: 5′-AAATTATGCCTGCTCGTCG-3′) and AL3546 (reverse: 5′-CAAACCTTTTCCGCAAACC-3′) were employed, whereas primers AL3544 (forward: 5′-CCCTTCATCGGIGGTAACTT-3′) and AL3545 (reverse: 5′-GTGGCCACCACTCCCGTGCC-3′) were utilised in the secondary reaction to amplify p*tpi* (530 bp) (*cf.* Nolan et al., 2010b). The cycling protocol for primary amplification included a cycle of 94 °C/5 min (initial denaturation), followed by 35 cycles of 94 °C/45 s (denaturation), 50 °C/45 s (annealing) and 72 °C/60 s (extension), followed by a final extension of 72 °C/10 min. The secondary PCR was identical, except that the annealing temperature was 60 °C.

2.3. Mutation scanning, sequencing and phylogenetic analyses

For p*SSU* amplicons, single-strand conformation polymorphism (SSCP) analysis (Gasser et al., 2006) was carried out as described previously (Jex et al., 2007). For p*tpi* amplicons, restriction endonuclease fingerprinting (Orita et al., 1989; Zhu and Gasser, 1998) was employed, using the enzyme *Rsa*I (Promega) (*cf.* Nolan et al., 2010b). Amplicons representing each banding profile were selected and treated with exonuclease I and shrimp alkaline phosphatase (Fermentas), according to the manufacturer's instructions, and then sequenced in both directions by direct, automated sequencing (BigDye Terminator v.3.1 chemistry, Applied Biosystems, USA), using the same primers employed in secondary PCR. The quality of each sequence was assessed based on the corresponding electropherogram using the program BioEdit (Hall, 1999), and the sequences determined were compared with known reference sequences using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST).

Some p*SSU* amplicons were cloned prior to sequencing. In brief, amplicons were purified using minicolumns (Wizard PCR Preps DNA Purification System, Promega) and ligated into the pGEM-T-Easy vector (Promega) and *Escherichia coli* (α-select chemically competent cells, Bioline) as described previously (Abeywardena et al., 2013). Then, the cells were plated on to agar plates and incubated overnight at 37 °C. Ten colonies were picked from each plate and grown at 37 °C overnight for plasmid purification (using Wizard Plus SV Minipreps DNA Purification System, Promega). Thereafter, inserts were PCR-amplified using the primers pssu-f and pssu-r (Section 2.2); following the SSCP analysis of all amplicons, some