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Molecular characterization of *Cryptosporidium* spp. from wild rats and mice from rural communities in the Philippines

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ABSTRACT

In order to examine the prevalence of *Cryptosporidium* in wild rodents in the Philippines and understand the role wild rodents play in the transmission of this parasite to humans and livestock, 194 fecal samples from wild rats and mice from Luzon and Mindoro islands were examined. Molecular screening at the 18S and actin gene loci identified an overall prevalence of 25.8% (95%CI: 19.8, 32.5). Sequence and phylogenetic analysis of both loci identified *C. parvum*, *C. muris*, *C. scrofarum*, rat genotypes I-IV and a *C. suis*-like genotype in the rat-derived isolates and is the first report of *C. suis*-like and *C. scrofarum* in rats. Mixed infections were identified in 24% of the *Cryptosporidium* positive isolates. Rat genotypes II, III and IV showed high intragenotypic variation at the 18S gene locus compared to the actin locus.

1. Introduction

Cryptosporidium is a ubiquitous protozoan parasite capable of infecting humans and a wide variety of animals. The disease, cryptosporidiosis, usually manifests as self-limiting watery diarrhea, with symptoms ranging in severity and chronicity depending on the age and immunological status of the host. With a low infectious dose, infection with *Cryptosporidium* usually results from ingestion of food or water contaminated with the oocyst stage of the life cycle (Xiao and Ryan, 2004). Currently 26 valid species of *Cryptosporidium* and >50 different genotypes are recognized (Elwin et al., 2012; Kváč et al., 2013; Ren et al., 2012; Xiao, 2010). Molecular data indicates that eight *Cryptosporidium* species/genotypes are responsible for most human cryptosporidiosis cases, including *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. ubiquitum*, *C. cuniculus* and *C. viatorum* (Chalmers et al., 2011; Elwin et al., 2012; Xiao, 2010; Xiao and Feng, 2008) with *C. parvum* and *C. hominis* by far, the most common species in humans worldwide (Xiao, 2010).

Earlier studies into the epidemiology of *Cryptosporidium* spp. in rats and mice suggested that rodents may be important reservoir hosts for the parasite (Chalmers et al., 1997; Quy et al., 1999; Torres et al., 2000). Studies of *Cryptosporidium* in rats conducted in Australia, China, Japan, the United Kingdom (UK) and New Zealand (NZ), have reported a prevalence ranging from 2% to 49%, whereas studies in mice conducted in Australia, China Poland, Spain, the UK, and the United States (US) have reported a prevalence ranging from 1% to 62% (Chalmers et al., 1997; Chilvers et al., 1998; Foo et al., 2007; Iseki, 1986; Kimura et al., 2007; Klesius et al., 1986; Lv et al., 2009; Miyaji et al., 1989; Paparini et al., 2012; Sinski et al., 1993; Yamura et al., 1990). Most of the earlier epidemiological studies however, were based on morphological identification of *Cryptosporidium* sp. with no molecular data to support the identification. Recent genotyping studies carried out in rats and mice, have identified the zoonotic *C. parvum*, *C. meleagridis*, *C. muris*, potentially zoonotic *C. tyzzeri*, and host adapted species such as mouse genotype II and rat genotypes I, II, III and IV (Feng et al., 2009; Foo et al., 2007; Kimura et al., 2007; Lv et al., 2009; Paparini et al., 2012).

In the Philippines, rodents cause important agricultural problems in rural communities with damage to rice crops (both pre- and post harvest) causing significant economic losses to the farmers (Singleton et al., 2010). The widespread presence and close proximity of rodents to humans and domestic animals within the rural rice growing communities poses a public and veterinary health risk as these rodents are capable of contaminating large areas including food storage, water sources and domestic and peri-domestic habitats with their fecal droppings, facilitating the dissemination and transmission of *Cryptosporidium* (Meerburg et al., 2009; Singleton, 2003; Singleton et al., 2010). The purpose of the present study is to examine the prevalence of *Cryptosporidi-*

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um amongst species of wild rats and mice common in rural communities in two of the main islands of the Philippines and the potential of these rodents as reservoirs for human-infectious *Cryptosporidium*.

2. Materials and Methods

2.1. Sample sites and species trapped

Trapping of wild rats (*Rattus* sp.) and mice (*Mus* sp.) was conducted in two different municipalities (Calauan and Los Baños) located approximately 10 km from each other in Laguna, on the island of Luzon, and in the municipality of San Jose on the island of Mindoro (Fig. 1). In Calauan (14°08′N,121°25′E), trapping was carried out in small rice fields (1–3 hectares per household), which were interspersed with houses, shops and fish pens and located along an access road. In Los Baños, traps were set in a local fresh produce market and on the research farm of the International Rice Research Institute (IRRI) (14°18′N, 121°22′E). In San Jose (12°21′N, 121°05′E), rice farming is more intense and rice fields where trapping was conducted spanned approximately 7 hectares with sparse distribution of houses between each rice field. Morphological measurements were used to confirm the identity of the species as described in Htwe et al. (2012).

There were five species of rodents trapped: the rice-field rat (*Rattues argentiventer*), the pacific rat (*Rattus exulans*), the brown rat (*Rattus norvegicus*), the Asian house rat (*Rattus tanezumi*), and house mouse (*Mus musculus*). All rats and mice were dissected and faecal samples from the rectum or the large intestinal section closest to the rectum were collected and placed in individual 10 mL polyethylene bottles containing 2.5% potassium dichromate and sent to Murdoch University, Western Australia for processing and analysis. A total of 194 faecal samples were collected from wild house mice ($n = 16$), rice-field rats ($n = 24$), a pacific rat ($n = 1$), brown rats ($n = 70$) and Asian house rats ($n = 83$) (Table 1).

2.2. Sample processing and DNA extraction

Approximately 400 mg of faecal matter from intestinal sections or faeces in 2.5% potassium dichromate were transferred into a 2.0 mL centrifuge tube. All faecal samples were then rinsed with distilled water and centrifuged at 10,000×*g* and the supernatant removed. This was repeated twice to wash off any residual potassium dichromate. Total DNA was extracted from the faecal samples using a Powersoil DNA Kit (MOBIO, Carlsbad, California, USA), according to the manufacturer's protocol with minor modifications. Briefly, approximately 250 mg of faecal sample was measured into the 2.0 mL tube containing beads provided by the manufacturer and subjected to 5 freeze-thaw cycles (liquid nitrogen/80 °C), followed by 10 min of boiling to ensure lysis of the thick-walled *Cryptosporidium* oocysts and release of DNA. The final elution volume was adjusted to 50 μL of Buffer 6 from the kit manufacturer's recommended volume of 200 μL of Buffer 6 in order to increase DNA concentration. DNA was stored at −20 °C until required.

2.3. PCR amplification and sequencing

All 194 faecal DNA samples were screened for the presence of *Cryptosporidium* at the 18S rRNA locus and at the actin gene locus using a two-step nested PCR as previously described (Ng et al., 2006; Ryan et al., 2003), with the annealing temperature for the actin locus lowered to 55 °C. All PCR amplification was carried out with positive controls (*C. hominis* DNA) and negative controls which contained no DNA. Secondary PCR products were separated by gel electrophoresis and fragments corresponding to the expected length were excised and purified using an MOBIO UltraClean 15 DNA purification kit (MOBIO, Carlsbad, California, USA). Purified PCR products were then sequenced using an ABI Prism Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to manufacturer's protocol with the annealing temperature raised to the respective temperatures of the secondary PCR primers used.

2.4. Sequence and phylogenetic analysis

Nucleotide sequences were analyzed using ChromasPro version v2.3 (http://www.technelysium.com.au) and aligned with reference sequences of *Cryptosporidium* species and genotypes from GenBank using ClustalW (http://www.genome.jp/tools/clustalw). Neighbor joining analysis with evolutionary distances calculated using Tamura-Nei parameters and maximum likelihood analysis were conducted using MEGA version 5.05 (Tamura et al., 2011). A sequence of *Monocystis agilis* (GenBank accession no. AF457127) was used as an outgroup for the 18S rRNA analysis, whereas a *Plasmodium falciparum* (GenBank accession no. M19146) sequence was used as an outgroup in the analysis of the actin gene. Bootstraping using 1000 replicates was carried out to assess the reliability of inferred tree topologies. Sequences from a recent rat study in Australia (Paparini et al., 2012) were obtained from GenBank under accession numbers JX294358-JX294376. Representative sequences for each *Cryptosporidium* species/genotype generated from this study have been deposited in GenBank under accession numbers JX485388-JX485418.

3. Results

3.1. Prevalence of Cryptosporidium in rats and mice

PCR screening at the 18S rRNA gene locus and actin locus detected 50 *Cryptosporidium* positives from the 194 faecal DNA samples; a prevalence of 25.8% (95% CI: 19.8, 32.5). No *Cryptosporidium* was detected in the house mice, rice-field rat and the pacific rat. The prevalence in rats was 28.1% (95% CI: 26.1, 35.3) (Table 1). The highest proportion of positive isolates were identified from the Asian house rat (37/50) followed by the brown rat (13/50). The prevalence of *Cryptosporidium* was highest in the municipality of Calauan at 63% (95% CI: 48.7, 75.7) followed by Los Baños at 31.8% (95% CI: 18.6, 47.6). San Jose had the lowest prevalence of *Cryptosporidium* at 2.1% (95% CI: 0.3, 7.3) (Table 1).

3.2. Sequence and phylogenetic analysis of the 18S rRNA gene locus

Of the 50 positives identified at the 18S rRNA gene locus, sequence analysis was successful for 44 of these and 7 different *Cryptosporidium* species and genotypes were identified; *C. muris* ($n = 3$), *C. scrofarum* ($n = 4$), rat genotype I ($n = 1$), rat genotype II ($n = 6$), rat genotype III ($n = 19$), rat genotype IV ($n = 6$) and *C. suis*-like genotype ($n = 5$) (Table 2). Phylogenetic analysis revealed that rat genotypes I, II and III formed a separate clade from the other intestinal *Cryptosporidium* with rat genotypes II and III grouping closely, exhibiting a genetic similarity of 98.6% and 99.0% respectively with rat genotype I and 99.5% with each other (7 SNPs over 450 bp of sequence) (Fig. 2). Intra-genotypic variation was observed within the rat genotype isolates identified in the present study at the 18S locus (Fig. 2). Isolates clustering with rat genotype III exhibited genetic similarities ranging between 99.3% and 100%. Rat genotype IV isolates from the present study along with W19 genotype variants from a previous study (Jiang et al., 2005) formed a cluster, with genetic similarities ranging between 99.0% and 99.8% (Fig. 2). The genetic similarity between rat genotype IV and its clos-