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Diversity and host specificity of *Blastocystis* in syntopic primates on Rubondo Island, Tanzania ☆

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ABSTRACT

The isolated ecosystem of Rubondo Island National Park, Tanzania is an interesting model site, inhabited by an assembly of primate species with various histories: two introduced primate species, *Pan troglodytes* (chimpanzee) and *Colobus guereza* (colobus), and a single indigenous species *Chlorocebus aethiops pygerythrus* (vervet monkey). Apart from important lessons for future introduction/re-introduction projects, Rubondo National Park offers a unique place to study the patterns of transmission of primate parasites and their host specificity. *Blastocystis* was detected using standard microscopy, together with PCR-based determination and the prevalence and subtype identification of *Blastocystis* was determined in each primate species. Subtype (ST) 1 was detected in all three Rubondo primate populations; ST2, ST3 and ST5 were found in colobus and vervet monkeys. All chimpanzee isolates of *Blastocystis* belonged exclusively to ST1, which formed a discrete group, suggesting that Rubondo chimpanzees are colonized by a single, host-specific *Blastocystis* strain that circulates among the members of the group. Phylogenetic analyzes indicated that transmission of *Blastocystis* did not occur between Rubondo primate populations. Observed host specificity of *Blastocystis* provides a new understanding of the transmission and distribution of *Blastocystis* among sympatric hosts under natural conditions.

1. Introduction

The genus *Blastocystis* is an assemblage of protists commonly occurring in humans and various animals including pigs, monkeys, guinea pigs, rodents, birds and reptiles (Abe et al., 2002; Tan, 2004). Although historically classified among the yeasts, fungi or sporozoans (Zierdt, 1991), *Blastocystis* is currently referred to as a member of the Stramenopiles (a diverse group that includes brown algae, diatoms, slime nets and water moulds) (Silberman et al., 1996). Among the six life stages that have been described, the cyst represents an infectious stage responsible for fecal-oral transmission between animals and/or humans (Stenzel and Boreham, 1991; Salim et al., 1999; Tan, 2004; Suresh et al., 2005).

Several molecular studies have addressed the genetic diversity of *Blastocystis* isolates. Up to 13 distinct subtypes (ST1–ST13) in different host species have been distinguished by phylogenic analyzes or with other methods (RFLP, riboprinting) using the ssrDNA as a marker (Clark, 1997; Yoshikawa et al., 1998, 2004a; Abe et al., 2003a; Stensvold et al., 2009a; Parkar et al., 2010). High similarity or even identity of sequences of *Blastocystis* suggests the transmission of some isolates between humans and a wide range of domestic or zoo animals living in close contact (Parkar et al., 2007, 2010; Rivera, 2008; Stensvold et al., 2009a). Weak host specificity of *Blastocystis* from humans was furthermore demonstrated by successful experimental infection of mice and rats with human isolates (Moe et al., 1997; Hussein et al., 2008). However, several STs show various levels of host specificity (Stensvold et al., 2009a).

A few studies have focused on primates as hosts of *Blastocystis* and seven STs have been recorded from non-human primates in captivity (Abe et al., 2003b; Rivera, 2008; Stensvold et al., 2009a;

☆ Note: Nucleotide sequence data reported in this paper are available in the GenBank database under the accession nos. HQ286904 to HQ286916 and JF792494 to JF792497.

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Teichroeb et al., 2009), as well as among free ranging rhesus monkeys (Yoshikawa et al., 2009).

Pathogens with a broad host spectrum may be a serious risk factor for endangered species, mainly in translocation programs when released animals interact widely, not only with the indigenous fauna but also with a plethora of local parasites and other pathogens (Cunningham, 1996; Pedersen et al., 2005). However, studies of the gastrointestinal parasites of primates released into the wild are scarce. The isolated ecosystem of Rubondo Island National Park, Tanzania, is inhabited by two introduced primate species, namely *Pan troglodytes* (common chimpanzee) and *Colobus guereza* (colobus monkey) and a single indigenous species *Chlorocebus aethiops pygerythrus* (vervet monkey). In addition to lessons for future introduction/re-introduction projects, Rubondo Island National Park offers a unique place to study the patterns of transmission of primate parasites and their host specificity.

Since 2006 we have been monitoring the parasites in populations of all four primate species (including humans) on Rubondo Island. Herein, we present the results of the detection of *Blastocystis* by standard microscopy, together with a PCR-based determination of prevalence and ST identification of *Blastocystis* in each primate species. Genetic identification of *Blastocystis* STs sheds new light on the transmission and distribution of *Blastocystis* among various sympatric hosts under natural conditions.

2. Materials and methods

2.1. Study site

Rubondo Island (240 km^2) is situated at the southwestern corner of Lake Victoria (2°18′ S, 31°50′ E). The island became a forest reserve in 1928 and a half century later, in 1977, was formally declared a National Park. In the 1960s and 1970s, the Frankfurt Zoological Society (FZS) introduced groups of several mammalian species including 17 chimpanzees (*P. troglodytes*) and 20 colobus monkeys (*C. guereza*) onto the island (Grzimek, 1970; Borner, 1985). Detailed records of these introductions are not available, but it is known that the colobus monkeys originated from Mt. Meru, Tanzania. No systematic research has been conducted on colobus monkeys since their release, however it seems that they have adapted and their numbers have increased. The introduced chimpanzees were all wild born, originating from several West African countries, but they spent between 3.5 months to 9 years in captivity in Europe before their release. Most likely these chimpanzees were treated for gastrointestinal parasite infections using anthelminthic and anti-protozoal drugs in captivity and shortly prior to their release (Huffman et al., 2008). The chimpanzees survived and adapted to the new environment (Borner, 1985; Huffman et al., 2008) and their number has at least doubled (Moscovice et al., 2007). The only indigenous primate species is the vervet monkey (*C. a. pygerythrus*) occurring in several troops across the island. No population estimates are available; however, vervet monkeys are commonly seen in the forest and nearby human dwellings.

2.2. Sample collection, microscopic diagnostics and statistical analysis

We collected fecal samples of chimpanzees ($n = 206$), vervet monkeys ($n = 111$) and colobus monkeys ($n = 49$) from mid-July 2006 to mid-July 2008. Feces were collected when a group or individual was encountered, except in some instances for chimpanzees when we found the feces under night nests in the morning, in which case the samples were not older than 12 h. As the primate groups on Rubondo are not completely habituated to humans, the identification of individual animals was impossible. Eight stool samples of six researchers (two of them were sampled twice) working on Rubondo Island were also collected. We immediately divided 5 g (chimpanzees and humans) or 2 g (vervet and colobus monkeys) of samples into 20 ml vials in 10% formaldehyde and simultaneously 2 g (all primates) into 10 ml vials in 96% ethanol. Preserved feces were transported to the Department of Parasitology, Veterinary and Pharmaceutical University, Brno, Czech Republic. Before microscopic examination, each formaldehyde preserved sample was homogenized and strained through a sieve into a Falcon conical tube (P-Lab, Czech Republic), diluted with 0.025 M phosphate buffer solution and centrifuged for 10 min at 313*g* (MPV-340, swing up head). The remaining sediment was re-suspended in 5 ml of 10% formalin and examined microscopically using the merthiolate-iodine-formalin concentration (MIFC) method (Blagg et al., 1955) with the addition of Lugol's iodine solution. Cysts of *Blastocystis* were diagnosed using light microscopy based on their size and general appearance (Zaman, 1996; Ash and Orihel, 2007). The prevalence was set as the percentage of samples containing *Blastocystis* (Combes, 2001). The McNemar test was performed to compare the sensitivity of *Blastocystis* detection using either the MIFC sedimentation or PCR method.

2.3. DNA extraction, PCR amplification and sequencing

All samples preserved in ethanol were analyzed by PCR for the presence of *Blastocystis* DNA. Half of each sample preserved in 96% ethanol was washed five times with PBS followed by centrifugation (5 min at 19,722*g*). After centrifugation, the supernatant was removed and the sediment was dried at 40 °C on a dry-block. Total DNA was extracted using a QIAmp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocol with the following exceptions: (i) fecal material was re-suspended in 1.6 ml of ASL lysis buffer and (ii) DNA was eluted from the matrix with 100 µl of AE elution buffer (Qiagen, Germany).

The ssrDNA region was amplified by PCR using a Biometra thermocycler (BioTech, Czech Republic) and the following PCR mix: 12.5 µl of commercial Combi PPP (Taq-Purple DNA Polymerase PCR) Master Mix (Top-Bio, Czech Republic), 9.5 µl of PCR water, 1 µl of each primer mix (0.01 mM each) and 1 µl of template DNA. The following conditions were applied: 94 °C for 4 min (denaturation), 30 cycles at 94 °C for 1 min (denaturation), 56 °C for 1 min (annealing), 72 °C for 2 min 30 s (primer extension), followed by 10 min at 72 °C. DNA sequences of the *Blastocystis*-specific primer BhRDr (5′-GAGCTTTTAACTGCAACAACG-3′, Scicluna et al., 2006) and primer MedlinA (5′-CTGGTTGATCCTGCCAG-3′, Medlin et al., 1988) correspond to the ends of a 600 bp long region near the 5′ end of the *Blastocystis* ssrDNA (Scicluna et al., 2006). PCR product yield was mostly high and no additional bands were seen. For each set of PCRs, negative controls (samples without DNA) and positive controls (DNA from in vitro cultured *Blastocystis*) were used. The PCR products were checked on ethidium bromide-stained 1% Tris-acetate-EDTA electrophoresis buffer (TAE) agarose gels with a 100 bp ladder (Top-Bio, Czech Republic). In the case of samples that were positive for *Blastocystis* by microscopy but negative by PCR, inhibition was tested for. The isolated total DNA was mixed with DNA from a positive control at a ratio of 4:1 (Stensvold et al., 2006) and processed by PCR as described. If the PCR was still negative, the presence of inhibitors in samples was suspected. From each primate species, representative *Blastocystis*-positive samples from wet and dry seasons in each year were randomly selected for sequencing. Because samples could not be attributed to individuals and there was the possibility that the same individual was sampled more than once on a particular day, PCR products were selected from different sampling days (Petrželková et al., 2010). One representative sample from each researcher was chosen for sequencing. PCR products (~600 bp) were