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Molecular characterization of *Blastocystis* isolates from zoo animals and their animal-keepers

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Abstract

Blastocystis is an enteric protist and one of the most frequently reported parasitic infections in humans and a variety of animal hosts. It has also been reported in numerous parasite surveys of animals in zoological gardens and in particular in non-human primate species. PCR-based methods capable of the direct detection of *Blastocystis* in faeces were used to detect *Blastocystis* from various hosts, including nonhuman primates, Australian native fauna, elephants and giraffes, as well as their keepers from a Western Australian zoo. Additional faecal samples were also collected from elephants and giraffes from four other zoos in Amsterdam (The Netherlands), Antwerp (Belgium), Melbourne and Werribee (Australia). Information regarding the general health and lifestyle of the human volunteers were obtained by questionnaire. Overall, 42% and 63% of animals and zoo-keepers sampled from the Western Australian zoo were positive for *Blastocystis*, respectively. The occurrence of *Blastocystis* in elephants and giraffes from other cities was similar. This is the first report of *Blastocystis* found in the elephant, giraffe, quokka, southern hairy nosed wombat and western grey kangaroo. Three novel and what appear to be highly host-specific subtypes (STs) of *Blastocystis* in the elephant, giraffe and quokka are also described. These findings indicate that further exploration of the genetic diversity of *Blastocystis* is crucial. Most zoo-keepers at the Perth Zoo were harbouring *Blastocystis*. Four of these zoo-keeper isolates were identical to the isolates from the southern hairy nosed wombat and five primate species.

Keywords: *Blastocystis*, characterization, elephant, giraffe, zoo

1. Introduction

Blastocystis is an enteric protist and one of the most frequently reported parasitic infections in humans and a variety of animal hosts (Abe, 2004; Amin, 2002; Windsor et al., 2002). It has also been reported in numerous parasite surveys of animals in zoological gardens and in particular in non-human primate species (Abe et al., 2002; Lim et al., 2008; Pérez Cordón et al., 2008; Stensvold et al., 2009a).

Previous studies have shown that *Blastocystis* is common among animal handlers, namely zoo-keepers and abattoir workers, indicating that animals may pose a significant zoonotic source of *Blastocystis* for humans (Salim et al., 1999). This, however, is difficult to determine considering that few of these isolates from such environments have been characterized using molecular tools (Parkar et al., 2007; Stensvold et al., 2009a).

Blastocystis displays considerable degree of genetic heterogeneity and there are currently ten subtypes (ST1-10) that have been isolated from mammalian, avian, reptilian and amphibian hosts (Belova and Krylov, 1998; Noël et al., 2005; Noël et al., 2003; Stensvold et al., 2007a; Yoshikawa et al., 2004b). Some subtypes (STs), for example ST5, appears to be highly host specific for pigs and cattle, while other STs display moderate or low host specificity (Noël et al., 2005; Stensvold et al., 2009a) and may be considered zoonotic. However, to date, a limited variety of mammalian species have been screened for *Blastocystis*, making

it likely that further yet undiscovered STs may exist. Exploration of the genetic diversity of *Blastocystis* is crucial. The taxonomy of *Blastocystis* is still a controversial area and it has been proposed that each ST may correspond to different species of *Blastocystis* (Noël et al., 2005). Each species may possess unique phenotypic characteristics that may well dictate its zoonotic potential.

The aim of the present study was to determine the occurrence of *Blastocystis* among animals and their handlers at an urban Australian zoo and from animals from four additional locations, and to genetically characterize positive samples in order to understand the epidemiology and zoonotic potential of this parasite.

2. Materials and Methods

2.1 Study sites and collection of animal samples

2.1.1. Animal samples

The Perth Zoological Gardens (Perth Zoo), in South Perth, Western Australia, houses approximately 1300 animals from 200 different species. A total of 76 fresh faecal samples were collected from a variety of animals. Some animals had previously been screened for *Blastocystis*. Animals had variable levels of contact with zoo-keepers. Several Australian native fauna species were included in the study (Table I). Samples were collected from animals in eight of the 15 general exhibits at the Perth Zoo between April and August 2007.

Fresh faecal samples were also obtained from elephants and giraffes at the Melbourne Zoo and giraffes from the Werribee Open Range Zoo in Victoria, Australia (Table II). Faecal samples from elephants and giraffes were collected from Amsterdam (The Netherlands) and Antwerp (Belgium) zoos (Table II) and fixed in 70% ethanol.

2.1.2 Questionnaire and collection of human samples

Zoo-keepers working with animals in the study groups were approached to voluntarily participate in the study by providing a faecal sample and completing a questionnaire. Questionnaires were designed to provide information regarding the participant's general health and lifestyle, occupational experience and current contact with zoo animals. Questionnaire data and single faecal samples from 19 zoo-keepers (40% response rate) were collected between April and August during two consecutive years (2007 and 2008). Faecal samples and questionnaire data from 22 individuals who did not have close interactions with exotic animals were also collected as part of the control population in the same time period in 2007. Permission was obtained from both the Murdoch Human Ethics Committee and the Zoo's Research and Ethics Committees for the collection of faecal samples and questionnaire data from the respondents.

2.2 Microscopy screening

All human samples collected were screened for gastrointestinal parasites using the zinc sulphate flotation method followed by microscopy (Weller et al., 1945).

2.4 DNA extraction

DNA was extracted from fresh faeces using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocol, with the modifications previously specified (Parkar et al., 2007). DNA from samples fixed in 70% ethanol were washed with milliQ water prior to the extraction method for fresh faeces.

2.5 Polymerase Chain Reaction (PCR)

At the beginning of our study, the PCR protocol from a previous study (Parkar et al., 2007) was used to amplify ~1100bp region of *Blastocystis* SSUrDNA. The primary PCR utilized previously published forward and reverse primers (RD3, 5`-GGG ATC CTG ATC CTT CCG CAG GTT CAC CTA C-3`; RD5, 5`-GGA AGC TTA TCT GGT TGA TCC TGC CAG TA-3`) for PCR amplification under the conditions previously described by Clark (1997). The secondary PCR utilized previously published forward and reverse primers (F1, 5`-GGA GGT AGT GAC AAT AAA TC-3`; R1, 5`-CGT TCA TGA TGA ACA ATT AC-3`) for PCR amplification under the conditions described by Böhm-Gloning et al (1997).

However, it was later discovered that the secondary PCR primers described by Böhm-Gloning et al (1997) do not amplify all *Blastocystis* isolates (Wong et al., 2008), in particular ST3. Consequently, negative samples were screened again using previously published forward and reverse primers in the secondary PCR

(bl1400ForC, 5`- GGA ATC CTC TTA GAG GGA CAC TAT ACA T-3`; bl1710RevC, 5`- TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3`) under conditions previously described (Stensvold et al., 2006). As bl1400ForC and bl1710RevC amplify ~310bp region of *Blastocystis* SSUrDNA, another secondary PCR using previously published primers (F1, 5`-GGA GGT AGT GAC AAT AAA TC-3`; R2, 5`- ACT AGG AAT TCC TCG TTC ATG-3`) were used to reamplify positive isolates under previously described conditions (Wong et al., 2008) in order to obtain larger fragments (~1100 bp) for phylogenetic analysis.

2.6 Sequencing and phylogenetic analysis

Bands representing the 1100 bp amplified PCR products were excised from a gel and purified using the UltraClean GelSpin DNA Purification Kit (MO BIO Laboratories, Inc.). Manufacturer's kit protocols were followed, except that DNA was eluted using 30µl of ultrapure PCR water and incubated at room temperature for 10 min prior to centrifugation at 10,000g for 30 s. PCR products were also purified from reactions using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation) according to the manufacturer's kit protocol. The PCR products were sequenced in both directions using an ABI 3730 capillary sequencer. Sequences were analysed using FinchTV and compared with previously published sequences from GenBank using the BLAST 2.2.9 program (http://www.ncbi.nlm.nih.gov/blast). *Blastocystis* STs 11, 12 and 13, and the western grey kangaroo sequences were deposited into GenBank (GU256899-GU256937).

Phylogenetic trees were constructed using sequence data from the amplified segments of the SSU rDNA (Figure 1: ~160bp; Figure 2: ~700bp; Figure 3: ~965bp). Sequences of isolates obtained from the present study were aligned with previously published sequences of *Blastocystis* obtained from GenBank (Table III) and isolates from the Perth Zoo (Parkar et al., 2007) using the program CLUSTAL W (Thompson et al., 1994) and then manually adjusted where necessary. Phylogenetic analysis was performed using MEGA v4.0.2 (Tamura et al., 2007). Distance-based analysis was undertaken using Kimura-2-parameter and the tree was constructed using the neighbour-joining algorithm. Bootstrap values were calculated by the analysis of 1400 replicates from the Neighbour-Joining tree. Analysis was also undertaken using the maximum parasimony algorithm. Bootstrap values were calculated by the analysis of 500 replicates from the maximum parasimony tree. *Proteromonas lacertae* was used as the outgroup.

3. Results

3.1 Occurrence of *Blastocystis* in animals

From the total number of 76 samples screened for *Blastocystis* from the Perth Zoo, 32 (42%) tested positive. Amongst the 40 primate samples collected, 50% were positive for *Blastocystis*. More than half of the primate species housed at the Western Australian zoo were infected with *Blastocystis*. In contrast, 33% of all non-primates sampled tested positive, with *Blastocystis* found in only five of the 16 species sampled.

188

189 The occurrence of *Blastocystis* in elephants and giraffes from Amsterdam,
190 Antwerp, Melbourne and Werribee was 57% (12/21) and 82% (19/23),
191 respectively.

192

193 3.2 Occurrence of *Blastocystis* in the zoo-keepers and control group

194 Questionnaire data revealed that all participants from the zoo had been working at
195 the zoo for at least a year, and that it was common for them to care for a variety of
196 different animal species from more than one exhibit. Only three zoo-keepers
197 cared for animals from one exhibit, with only one of them caring specifically for
198 elephants. Zoo-keepers were accordingly classified as primate keepers (working
199 exclusively with primates), non-primate keepers (working exclusively with non-
200 primates) or generalist keepers (working with both primate and non-primate
201 species).

202

203 In total, 12 out of 19 (63%) zoo keepers were infected with *Blastocystis*. Five
204 from nine of the non-primate zoo keepers and all five generalist zoo-keepers were
205 positive for *Blastocystis.* All of these individuals reported experiencing gastro-
206 intestinal related symptoms. Two of five of the primate zoo-keepers were also
207 positive for *Blastocystis*. No other parasites were detected using the zinc sulphate
208 floatation method and microscopy. Only two out of 22 (9%) individuals from the
209 control group were positive for *Blastocystis*, with one of these individuals

reporting gastro-intestinally related symptoms. Again, no other parasites were detected using the zinc floatation method and microscopy.

3.4 Phylogenetic analysis

DNA sequences obtained from 34 *Blastocystis* isolates on Genbank were included in the phylogenetic analysis (Table III). The rooted neighbour-joining tree identified 13 clades (Figures 1, 2 and 3). Ten of these clades correspond to STs identified in the consensus based on previous molecular studies (Figure 1). There was strong support in placing all elephant, giraffe and quokka isolates within separate novel host-specific clades or STs that we assign as ST11, ST12 and ST13, respectively. The other isolates from this study clustered within ST1, ST2, ST3 and ST4.

All isolates of *Blastocystis* isolated from the non-human primates at the Perth Zoo belonged to ST1 and ST2. Three isolates from primate zoo-keepers also belong to ST1 and were similar to the primate isolates, as well as previously characterized isolates from primate zoo-keepers. The only isolates from this study in ST1 that did not belong to the primates or primate zoo-keepers was isolated from the southern hairy nosed wombat (from the Australian bushwalk exhibit) and zoo-keeper 14, who cared for animals in the Australian bushwalk exhibit.

Other isolates from this study belonged to ST3 and ST4. Three isolates belonging to generalist zoo-keepers were characterised as ST3, and were similar to

previously characterized human isolates. An isolate from the control group belonged to ST4, and was identical to previously characterized isolates from a human and a rat.

4. Discussion

4.1 *Blastocystis* in zoo animals

Previous studies of *Blastocystis* in zoo environments have found high prevalences of the organism amongst primate and avian hosts (Abe et al., 2002; Pérez Cordón et al., 2008). The present study has also shown that many of the primates at the Western Australian zoo are infected with *Blastocystis*, and that these isolates are similar to previously characterized isolates (Figure 2). This confirms previous studies showing that ST1 and ST2 appear to be zoonotic and primarily consisting of isolates from human and non-human primates. However, few studies have screened other zoo animals for *Blastocystis* (Abe et al., 2002; Lim et al., 2008; Pérez Cordón et al., 2008). Our study is the first to report the isolation of *Blastocystis* from the following hosts: Asian elephant, giraffe, quokka, southern hairy nosed wombat and the western grey kangaroo.

Elephants and giraffes have been screened previously at Osaka Zoo but were negative for *Blastocystis* (Abe et al., 2002). However, it is possible that these animals may harbour the parasite as *in vitro* cultivation was used as the method for detection, which is known to preferentially amplify some STs over others (Parkar et al., 2007), and to lack sensitivity due to several factors which may

affect the viability and reproduction of *Blastocystis* (Leelayoova et al., 2002; Zaman and Khan, 1994).

The significance of *Blastocystis* infections and these novel STs in elephants, giraffes and Australian native fauna is not clear. The limited parasitological data recorded for elephants and giraffes in the past 50 years have largely been confined to helminths and *Sarcocystis* (Bengis et al., 1998; Garijo et al., 2004; Goossens et al., 2005; Vidya and Sukumar, 2002). However, our study has demonstrated that the newly assigned ST11 seems to be host-specific, as it was not detected in any other species at the study site, and was found in elephants from four different geographical locations (Figure 3). Further studies are required to determine whether *Blastocystis* occurs in African elephants, and if so, whether these isolates are genetically similar or distinct from those in ST11.

This is the second study to report *Blastocystis* infection in Australian native fauna, although novel STs were identified in the present study. The western grey kangaroo isolate, like the giraffe isolates, belongs to the newly assigned ST12 (Figure 3). The quokka isolates (ST13), and the western grey kangaroo isolate, are distinct from the brushtailed possum isolate previously characterized (Parkar et al., 2007). Some studies had recorded novel and/or zoonotic genotypes of *Cryptosporidium* and *Giardia* in various species of Australian native fauna (Adams et al., 2004; McCarthy et al., 2008; Thompson et al., 2008), and the significance of these findings are not fully understood. Further studies are

279 required in order to determine the incidence in other populations, host-specificity
280 and zoonotic significance. These findings also stress the importance of screening
281 other hosts for *Blastocystis* in order to determine the genetic diversity and
282 taxonomy of this parasite.
283
284 4.2 Zoonotic potential
285 High prevalence of *Blastocystis* infections has previously been reported amongst
286 zoo-keepers (Salim et al., 1999). In the present study, 12 out of 19 zoo-keepers
287 were positive, compared to two out of 22 from the control group. Seven of these
288 isolates were sequenced and characterized successfully. Two isolates were found
289 to be similar to some isolates from the primates in ST1 and ST2, and one isolate
290 was identical to primates and the southern hairy nosed wombat in ST1 (Figure 2).
291 These findings indicate that the high prevalence amongst zoo-keepers may be due
292 to the close contact between the animals and zoo-keepers, which may facilitate the
293 transmission of *Blastocystis*. Thus, there is epidemiological evidence to support
294 the zoonotic potential of *Blastocystis.*
295
296 The potential for waterborne transmission of *Blastocystis* to occur in the zoo
297 environment should also be taken into account as Leelayoova et al (2008)
298 demonstrated that the high prevalence of *Blastocystis* ST1 amongst Thai
299 schoolchildren may be due to consuming *Blastocystis* ST1 contaminated drinking
300 water from a rainwater tank on school grounds. One of the tasks routinely
301 performed by zoo-keepers is the cleaning of animal enclosures. The cleaning of

primate enclosures involve the use of water hoses, and it may be possible for zoo-keepers to acquire infection through contact with contaminated water. Previous studies found that *Blastocystis* cysts may be resistant to water treatment and clorination (Suresh et al., 2005; Zaki et al., 1996), and it is possible that cysts may also be resistant to various disinfectants, which may play a role in the transmission of *Blastocystis* in the zoo environment. Further studies are required to determine risk factors for zoonotic transmission of *Blastocystis*.

4.3 Host specificity occurring within the genus *Blastocystis*

Many of the *Blastocystis* STs display a broad host range, although they may not all be of zoonotic significance. Based on evidence from this study and previous studies (Abe, 2004; Abe et al., 2003; Noël et al., 2005; Parkar et al., 2007; Rivera, 2008; Stensvold et al., 2009a; Yan et al., 2007; Yoshikawa et al., 2003), it is likely that ST1, ST2, and ST4 are zoonotic as identical isolates of human and animal origin were identified and clustered within these STs. These STs also display a low host specificity (Noël et al., 2005), which is also likely for other STs containing isolates from different hosts. ST3 also contains isolates of animal and human origin. However, none of the human isolates are identical to the animal isolates and there is strong bootstrap support (Noël et al., 2005) to suggest that there may be high host specificity occurring within this ST. ST5 and ST10 also display a broad host range, containing isolates from primates, pigs (ST5 only), and livestock with low host specificity (Noël et al., 2005; Stensvold et al., 2009a). Whereas ST6 and ST7 seem to display degrees of host specificity, as human

isolates are distinctly different to avian isolates within these STs (Arisue et al., 2003; Noël et al., 2005; Yoshikawa et al., 2004a). ST8 contains isolates from primates, humans and a pheasant (Stensvold et al., 2009a) . ST9 currently consists of only two isolates, and these are of human origin (Noël et al., 2005). Although ST11, ST12 and ST13 seem to be host specific, further characterization studies of *Blastocystis* from elephant, giraffe and Australian native fauna species are required in order to determine whether these newly assigned STs are common amongst these animals, and whether they are geographic and/or host specific, with zoonotic potential.

Current phylogenetic analyses based on the SSU rRNA gene of *Blastocystis* isolates suggest that there is low host specificity occurring within the genus. The findings from this study along with an increasing number of epidemiological and subtyping studies, it is becoming increasingly evident that the frequency of STs differs significantly between hosts. Thus further studies focusing upon animal hosts not previously screened for *Blastocystis*, and the phylogenetic analysis of these isolates may help resolve the genetic diversity and taxonomic status of *Blastocystis*.

4.4 The limitations of current screening tools and its implications for the taxonomy of *Blastocystis*

A number of different methods to amplify *Blastocystis* SSU rRNA gene have been described previously (Böhm-Gloning et al., 1997; Clark, 1997; Jones II et al.,

2008; Jones et al., 2009; Menounos et al., 2008; Scicluna et al., 2006; Stensvold et al., 2007b; Stensvold et al., 2006; Termmathurapoj et al., 2004; Wong et al., 2008; Yoshikawa et al., 1998; Yoshikawa et al., 1996). Some methods primarily focus upon detection and subtyping of *Blastocystis* isolates using primers which amplify fragments smaller than 350bp (Menounos et al., 2008; Stensvold et al., 2007b; Stensvold et al., 2006) while others amplify products in excess of 600bp (Scicluna et al., 2006) and 1000bp (Termmathurapoj et al., 2004; Wong et al., 2008; Yoshikawa et al., 1998; Yoshikawa et al., 1996). As these primers are genus-specific, preferential amplification or no amplification of certain STs are possible. It has previously been reported that primers designed by Böhm-Gloning et al. (1997) do not amplify ST3 isolates (Wong et al., 2008), while ST3 is preferentially amplified by primers described in Stensvold et al. (2006). In order to account for possible sequence variation within primer sites, it is recommended to use multiple primer pairs (Stensvold et al., 2009b).

Although using different primer pairs may minimise the likelihood of preferential amplification, it can be difficult to perform accurate phylogenetic analyses if the primers amplify small products. The major limitation of performing phylogenetic analyses using small products is the inability to determine subgroupings and relationships within individual STs. Therefore, it is important that future research focusing upon the taxonomy and phylogenetic relationships among *Blastocystis* isolates amplify at least 1000bp of the SSU rRNA gene. Also, isolates belonging

to novel STs should be amplified using primer pairs with resulting products of at least 1000bp in order to create accurate phylogenetic trees.

In order to determine whether STs correspond to different species of *Blastocystis*, more discriminatory ST-specific genotyping tools are required to resolve host specificity and ST subgroupings. At present, phylogenetic analyses are derived from sequences of the SSU rRNA gene. Phylogenetic studies based on other loci, such as the elongation factor-1-alpha (EF-1α), intertranscriber region (ITS) may provide further insight into the taxonomic status of *Blastocystis*, including the subgroupings within individual STs.

In conclusion, the present study is the first to report *Blastocystis* isolated from elephants (ST11), giraffes (ST12), quokka (ST13) and western grey kangaroo (ST12). Further studies are required in order to determine the host specificity and zoonotic potential of these newly assigned STs, as well as their potential clinical impact. A high prevalence of *Blastocystis* was reported in animals and zoo-keepers from the Western Australian zoo. Some isolates from the zoo-keepers were similar or identical to isolates from the animals they work with, providing evidence to support the zoonotic potential of this parasite.

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566 **Tables**

567 Table I. Animals sampled from the Perth Zoo in this study.
568

General Exhibit	Host	Scientific Name	Samples (n)	Positive (n)
African savannah	African painted dog	*Lycaon pictus*	7	0
	Hamadryas baboon	*Papio hamadryas*	3	3
	Rothschild's giraffe	*Giraffa camelopardalis rothschildi*	4	4
	Southern white rhinoceros	*Ceratotherium simum simum*	2	0
Asian rainforest	Asian elephant	*Elephas maximus*	4	4
	Oriental small-clawed otter	*Aonyx cinereus*	2	0
	Silvery gibbon	*Hylobates moloch*	4	2
	Sulawesi crested macaque	*Macaca nigra nigra*	4	2
	Sumatran orang utan	*Pongo pygmaeus abelii*	4	4
	Sumatran tiger	*Panthera tigris sumatrae*	2	0
	Sun bear	*Helarctos malayanus*	2	0
	White cheeked gibbon	*Hylobates leucogenys*	4	2
Australian bushwalk	Quokka	*Setonix brachyurus*	2	2
	Southern hairy nosed wombat	*Lasiorhinus latifrons*	1	1
	Western grey kangaroo	*Macropus fuliginosus*	2	1
Cockatoo exhibit	Red tailed black cockatoo	*Calyptorhynchus banksii*	2	0
Lesser primates	Black-capped capuchin	*Cebus apella*	2	0
	Common marmoset	*Callithrix jacchus*	2	0
	Cotton-top tamarin	*Saguinus oedipus*	2	0
	Emperor tamarin	*Saguinus imperator*	1	0
	Pygmy marmoset	*Callithrix pygmaea*	2	0
	Ring-tailed lemur	*Lemur catta*	4	1
	White-fronted capuchin	*Cebus albifrons*	2	0
Main lake	Black and white ruffed lemur	*Varecia variegata variegata*	4	4
Reptile encounter	Woma	*Aspidites ramsayi*	1	0
Other animals	Fishing cat	*Prionailurus viverrinus*	2	0
	Muir's corella	*Cacatua pastinator pastinator*	2	0
	Tonkean macaque	*Macaca tonkeana*	2	2
	Sulphur crested cockatoo	*Cacatua galerita*	1	0
Total			**76**	**32**

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571 Table II. Samples collected from Melbourne, Werribee, Amsterdam and Antwerp
572 Zoos.
573

Zoo	Host	Scientific name	Samples (n)	Positive (n)
Melbourne	Asian elephant	*Elephas maximus*	5	5
	Giraffe	*Giraffa camelopardalis rothschildi*	1	1
Werribee	Giraffe	*Giraffa camelopardalis rothschildi*	2	1
Amsterdam	Asian elephant	*Elephas maximus*	14	6
	Giraffe	*Giraffa camelopardalis*	15	12
Antwerp	Asian elephant	*Elephas maximus*	2	1
	giraffe	*Giraffa camelopardalis*	5	5
Total			**44**	**31**

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576 Table III. *Blastocystis* isolates obtained from GenBank for phylogenetic analysis.

Host	Country of origin	Reference	Accession number
Cattle	Denmark	Stensvold et al. (2009a)	FM164412
Cattle	Japan	Abe et al. (2004)	AB107964
Duck	France	Noël et al. (2003)	AY135412
Human	France	Noël et al. (2003)	AY135402
Human	Japan	Arisue et al. (2002)	AB023578
Human	Japan	Yoshikawa et al. (2004c)	AF408426
Human	Japan	Yoshikawa et al. (2004c)	AY244621
Human	Japan	Arisue et al. (2003)	AB070987
Human	Japan	Arisue et al. (2003)	AB070988
Human	Japan	Arisue et al. (2003)	AB070989
Human	Japan	Arisue et al. (2003)	AB070990
Human	Japan	Arisue et al. (2003)	AB091238
Human	Japan	Arisue et al. (2003)	AB091239
Human	Japan	Arisue et al. (2003)	AB070986
Human	N/A	Arisue et al. (2002)	AB023499
Human	Japan	Yoshikawa et al. (2004c)	AF408425
Human	Singapore	Arisue et al. (2003) ; Noël et al. (2005)	AF408427
Human	Thailand	Thathaisong et al. (2003)	AF439782
Monkey	Japan	Arisue et al. (2003)	AB070997
Monkey	Japan	Abe et al. (2004)	AB107969
Monkey	Japan	Abe et al. (2004)	AB107970
Monkey	Japan	Abe et al. (2004)	AB107967
Monkey	Japan	Abe et al. (2004)	AB107968
Pheasant	Japan	Abe et al. (2004)	AB107971
Pig	Japan	Arisue et al. (2003)	AB091248
Rat	Japan	Arisue et al. (2003)	AB091251
Rat	Singapore	Noël et al. (2005)	AY590114

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Figure Legend

Figure 1. Neighbour-joining tree displaying the relationships among *Blastocystis* isolates, inferred by distance based analysis of SSU rDNA sequence data using Kimura's-2-parameter distance estimates. Some sequences used for comparison were from GenBank. Scale bar shows 0·5 substitutions (corrected) per base pair. Shaded squares indicate animal isolates from the Perth Zoo. Unshaded squares indicate zoo-keeper isolates, while the diamond indicates a human control isolate. The triangle indicates an isolate from the Perth Zoo from a previous study (Parkar et al., 2007).

Figure 2. Neighbour-joining tree displaying the relationships among *Blastocystis* isolates, inferred by distance based analysis of SSU rDNA sequence data using Kimura's-2-parameter distance estimates (bootstrap value on the left or only bootstrap value shown). Maximum parasimony estimates are also displayed (right). Some sequences used for comparison were from GenBank. Scale bar shows 0·2 substitutions (corrected) per base pair. Isolates marked with shaded squares and circles indicate animal and zoo-keeper isolates from our study the West Australian Zoo. Shaded triangle indicates a human control isolate. Isolates marked with unshaded symbols indicate previously characterized isolates from the Perth Zoo (Parkar et al, 2007).

Figure 3. Neighbour-joining tree displaying the relationships among *Blastocystis* isolates, inferred by distance based analysis of SSU rDNA sequence data using Kimura's-2-parameter distance estimates. Some sequences used for comparison were from GenBank. Scale bar shows 0·2 substitutions (corrected) per base pair. Isolates indicated by circles are from elephants and those indicated by squares are from giraffes.

Table I. Animals sampled from the Perth Zoo in this study.

General Exhibit	Host	Scientific Name	Samples (n)	Positive (n)
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	Oriental small-clawed otter	*Aonyx cinereus*	2	0
	Silvery gibbon	*Hylobates moloch*	4	2
	Sulawesi crested macaque	*Macaca nigra nigra*	4	2
	Sumatran orang utan	*Pongo pygmaeus abelii*	4	4
	Sumatran tiger	*Panthera tigris sumatrae*	2	0
	Sun bear	*Helarctos malayanus*	2	0
	White cheeked gibbon	*Hylobates leucogenys*	4	2
Australian bushwalk	Quokka	*Setonix brachyurus*	2	2
	Southern hairy nosed wombat	*Lasiorhinus latifrons*	1	1
	Western grey kangaroo	*Macropus fuliginosus*	2	1
Cockatoo exhibit	Red tailed black cockatoo	*Calyptorhynchus banksii*	2	0
Lesser primates	Black-capped capuchin	*Cebus apella*	2	0
	Common marmoset	*Callithrix jacchus*	2	0
	Cotton-top tamarin	*Saguinus oedipus*	2	0
	Emperor tamarin	*Saguinus imperator*	1	0
	Pygmy marmoset	*Callithrix pygmaea*	2	0
	Ring-tailed lemur	*Lemur catta*	4	1
	White-fronted capuchin	*Cebus albifrons*	2	0
Main lake	Black and white ruffed lemur	*Varecia variegata variegata*	4	4
Reptile encounter	Woma	*Aspidites ramsayi*	1	0
Other animals	Fishing cat	*Prionailurus viverrinus*	2	0
	Muir's corella	*Cacatua pastinator pastinator*	2	0
	Tonkean macaque	*Macaca tonkeana*	2	2
	Sulphur crested cockatoo	*Cacatua galerita*	1	0
Total			**76**	**32**

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Zoo	Host	Scientific name	Samples (n)	Positive (n)
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Werribee	Giraffe	*Giraffa camelopardalis rothschildi*	2	1
Amsterdam	Asian elephant	*Elephas maximus*	14	6
	Giraffe	*Giraffa camelopardalis*	15	12
Antwerp	Asian elephant	*Elephas maximus*	2	1
	giraffe	*Giraffa camelopardalis*	5	5
Total			**44**	**31**

Table III. *Blastocystis* isolates obtained from GenBank for phylogenetic analysis.

Host	Country of origin	Reference	Accession number
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Human	Japan	Arisue et al. (2003)	AB070988
Human	Japan	Arisue et al. (2003)	AB070989
Human	Japan	Arisue et al. (2003)	AB070990
Human	Japan	Arisue et al. (2003)	AB091238
Human	Japan	Arisue et al. (2003)	AB091239
Human	Japan	Arisue et al. (2003)	AB070986
Human	N/A	Arisue et al. (2002)	AB023499
Human	Japan	Yoshikawa et al. (2004c)	AF408425
Human	Singapore	Arisue et al. (2003) ; Noël et al. (2005)	AF408427
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Monkey	Japan	Arisue et al. (2003)	AB070997
Monkey	Japan	Abe et al. (2004)	AB107969
Monkey	Japan	Abe et al. (2004)	AB107970
Monkey	Japan	Abe et al. (2004)	AB107967
Monkey	Japan	Abe et al. (2004)	AB107968
Pheasant	Japan	Abe et al. (2004)	AB107971
Pig	Japan	Arisue et al. (2003)	AB091248
Rat	Japan	Arisue et al. (2003)	AB091251
Rat	Singapore	Noël et al. (2005)	AY590114