Direct characterization of *Blastocystis* from faeces by PCR and evidence of zoonotic potential

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SUMMARY

In vitro propagation followed by PCR, and a PCR-based method capable of the direct detection of *Blastocystis* in faeces were utilized to detect *Blastocystis* from various hosts in Australia, including primates and their handlers from the Perth Zoo. In addition, *Blastocystis* isolates from dogs and humans living in a localized endemic community in Thailand were also characterized genetically. PCR-based detection directly from faeces was shown to be more sensitive compared with *in vitro* culture for the detection of *Blastocystis*. Moreover, phylogenetic analysis of *Blastocystis* isolates amplified utilizing *in vitro* techniques prior to PCR revealed that this method favoured the preferential amplification of *Blastocystis* subtype 5 over subtype 1. This study is the first to provide molecular-based evidence supporting the zoonotic potential of *Blastocystis* in dogs, possums and primates in a natural setting.

Key words: *Blastocystis*, *in vitro* amplification, zoonoses, molecular characterization.

INTRODUCTION

Blastocystis is one of the most frequently encountered protozoan parasites reported in humans (Amin, 2002; Windsor *et al*. 2002). Although *Blastocystis* has previously been detected in various hosts from Australia, including humans, cats, dogs and chickens (Duda *et al*. 1998; Lee and Stenzel, 1999; Hellard *et al*. 2000), they were not genetically characterized. Moreover, Australian native fauna had not previously been screened for *Blastocystis*.

There are several methods used to detect *Blastocystis* infections, which include wet smears, concentration methods and *in vitro* amplification which was recently shown to be the most sensitive method to detect *Blastocystis* from faecal samples (Zaman and Khan, 1994; Leelayoova *et al*. 2002; Suresh and Smith, 2004; Termmathurapoj *et al*. 2004). However, due to the variable morphological forms that *Blastocystis* exhibits, it is impossible to distinguish among different species and subspecies of *Blastocystis* solely based on morphology without the use of molecular techniques (Yoshikawa *et al*. 2004*b*). It has been reported that the PCR detection of *Blastocystis* directly from faecal specimens is rather insensitive (Termmathurapoj *et al*. 2004). Hence, *in vitro* propagation is still used widely to facilitate molecular characterization.

Recent molecular studies have shown that *Blastocystis* displays considerable genetic heterogeneity in conserved genes, such as the small subunit of nuclear ribosomal RNA gene (SSU) and the elongation factor-1α gene (Ho *et al*. 2000; Arisue *et al*. 2003; Yoshikawa *et al*. 2003, 2004*a*, *c*; Abe *et al*. 2003*a*, *b*; Noel *et al*. 2003, 2005; Abe, 2004). Studies based on the SSU rRNA gene have revealed the presence of 7 groups or subtypes of *Blastocystis* displaying low host specificity. It is possible that these groups or subtypes correspond to different species of *Blastocystis* (Noel *et al*. 2005).

The aims of the present study were to develop a reliable PCR-based method for the detection and characterization of *Blastocystis* directly from faeces and to compare this method to *in vitro* culture for the diagnosis of *Blastocystis* infections. Moreover, we aimed to genetically characterize isolates of *Blastocystis* from a variety of animal hosts in Australia and Thailand, including isolates from animals and humans living in a localized endemic

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focus of parasitic infection, in order to explore the zoonotic potential of this parasite.

MATERIALS AND METHODS

Sampling strategy

Fresh faecal samples were obtained from these species of Australian native small marsupials in Julimar (100 km from Perth, Western Australia): chuditch (*Dasyurus geoffroii*), woylie (*Bettongia penicillata*), quenda (*Isoodon obesulus*) and brush-tailed possums (*Trichosurus vulpecula*). Samples were also collected from the following captive primates and their human handlers (zoo-keepers) from the Perth Zoo, Western Australia: Hamadryas baboon (*Papio hamadryas hamadryas*), black and white ruffed lemur (*Varecia variegatus*), crested macaque (*Macaca nigra nigra*), colobus (*Colobus guereza*), orang utan (*Pongo pygmaeus abelii*), ring tailed lemur (*Lemur catta*), spider monkey (*Ateles geoffroyi*), vervet (*Cercopithecus aethiops*) and white cheeked gibbon (*Nomascus leucogenys*). The samples were screened for *Blastocystis* using both PCR directly from faecal samples, as well as PCR following *in vitro* propagation (see Table 1) and formed the basis for the comparative study.

In addition, faecal samples were collected from a tiger (*Panthera tigris sumatrae*), rhinoceros (*Ceratotherium simum simum*), otter and fishing cat (*Prionailurus viverrinus*) from the Perth Zoo. Faecal samples from domestic cats and dogs were collected from veterinary clinics and animal refuges and from Aboriginal communities in the central desert in Western Australia. *Blastocystis*-positive human faecal samples were obtained from a medical diagnostic laboratory in Perth, Western Australia. Two human and 3 dog faecal samples for *Blastocystis* were also collected from a localized parasite endemic community 150 km east of Bangkok, Thailand. The aforementioned samples were fixed in 20% dimethylsulfoxide (DMSO) and subjected to PCR only.

In vitro *cultivation*

Fifty mg of fresh faecal material was inoculated into a sterile screw-top tube containing 3 ml of Jones' medium supplemented with 10% horse serum (Jones, 1946; Suresh and Smith, 2004). Each sample was cultured in duplicate and incubated at 37 °C for 24–48 h. The cultures were examined for the presence of various morphological forms of *Blastocystis* using light microscopy at 400× magnification.

Prior to DNA extraction, 1 of the duplicate cultures was pelleted via centrifugation at 400 ***g*** for 10 min and the supernatant discarded. The resultant pellet was stored at −20 °C until required.

Blastocystis purification

The remaining duplicate culture was purified using the *Blastocystis* purification method as previously described (Hoevers *et al*. 2000; Snowden *et al*. 2000) in order to reduce bacterial contamination and to concentrate *Blastocystis* organisms. The other duplicate culture was pelleted via centrifugation at 400 ***g*** for 10 min at 22 °C. The supernatant was discarded and the pellet was resuspended in 3 ml of phosphate-buffered saline (PBS, pH 7·4). This suspension was overlaid on to a Ficoll-Paque column and centrifuged at 2000 ***g*** for 20 min at 22 °C (Suresh and Smith, 2004). *Blastocystis* separated into a band approximately 1 cm from the surface. This layer was collected and resuspended in 8 ml of PBS and centrifuged at 500 ***g*** for 5 min, which was repeated 6 times. The resultant pellet was resuspended in 1 ml of PBS and centrifuged at 500 ***g*** for 5 min. The resultant pellet was stored at −20 °C until required.

DNA extraction

DNA was extracted from faeces fixed in 20% DMSO, as well as from pelleted and purified cultures of *Blastocystis* using QIAamp DNA Stool Mini Kit (Qiagen, Germany). The manufacturer's protocol was used, with the following exceptions. (i) Faecal material fixed in 20% DMSO was washed 3 times with distilled water prior to extraction. (ii) The faecal material was suspended in 1·4 ml of ATL lysis buffer as specified by the manufacturer's protocol (Qiagen, Germany) and ruptured by 3–5 freeze-thaw cycles in liquid nitrogen and 95 °C water bath, respectively. (iii) The DNA was eluted from the matrix with 50 or 100 μl of AE elution buffer (Qiagen, Germany).

PCR amplification

A nested PCR reaction was used to amplify an approximately 1100 bp region of the SSU rDNA. 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 described by Clark (1997). The secondary PCR utilized previously published forward and reverse primers (forward, 5′-GGA GGT AGT GAC AAT AAA TC-3′; reverse, 5′-CGT TCA TGA TGA ACA ATT AC-3′) under the conditions described by Bohm-Gloning *et al*. (1997). The PCR was carried out in 0·2 ml tubes in a Perkin Elmer GeneAmp 2400 thermocycler.

Sequencing and phylogenetic analysis

Bands representing amplified PCR products were excised from a gel and purified using the

Table 1. Numbers of samples positive for *Blastocystis* when subjected to *in vitro* amplification and PCR

Source and/or geographical location	Host	Number of samples (*n*)	Positive by culture†	Positive by PCR
Julimar	Chuditch	29	0	4
	Possum	5	0	2
	Quenda	3	0	2
	Woylie	3	0	1
Perth Zoo	Baboon	2	2	2
	Black and white ruffed lemur	2	2	2
	Crested macaque	2	0	2
	Colobus	1	N/A	1
	Fishing cat	1	N/A	0
	Human	4	0	4
	Orang utan	2	0	2
	Otter	1	N/A	0
	Rhinoceros	1	N/A	0
	Ringtailed lemur	3	0	1
	Spider monkey	1	N/A	1
	Tiger	2	N/A	0
	Vervet	2	2	2
	White cheeked gibbon	2	2	2
Australian diagnostic laboratory	Human	6	N/A	6
Australian central desert	Dog	10	N/A	2
Australian veterinary clinics	Cat	5	N/A	0
	Dog	5	N/A	0
Australian animal refuges	Cat	5	N/A	0
	Dog	5	N/A	0
Thailand	Dog	3	3	N/A*
	Human	2	1	1
Total		107	12	37

† N/A, not subjected to *in vitro* amplification; N/A*, not subjected to PCR as faecal samples were not available.

UltraClean™ GelSpin DNA Purification Kit (MO BIO Laboratories, Inc.). Manufacturer's kit protocols were followed, except that DNA was eluted using 30 μl of ultrapure PCR water and incubated at room temperature for 10 min prior to centrifugation at 10 000 ***g*** for 30 s. The PCR products were sequenced in both directions using an ABI 3730 capillary sequencer. Sequences were analysed using SeqEd v 1.03 (Applied Biosystems) and compared with previously published sequences from GenBank™ using the BLAST 2.2.9 program (http://www.ncbi.nlm.nih.gov/blast).

Phylogenetic analysis was conducted using sequence data for a region of the SSU rDNA (~850 bp). Sequences were aligned using the program CLUSTAL W (Thompson *et al.* 1994) and then manually adjusted. Phylogenetic analysis of sequence data from *Blastocystis* isolates from this study as well as 26 other *Blastocystis* isolates retrieved from GenBank™ (Table 2) was carried out using MEGA v3.1 (Kumar *et al.* 2004). (http://www.megasoftware.net). Distance-based analysis was undertaken using Kimura-2-parameter and the tree was conducted using the UPGMA algorithm. Bootstrap values were calculated by the analysis of 1050 replicates from the UPGMA tree. *Proteromonas lacertae* was used as the outgroup.

RESULTS

Detection of Blastocystis *using* in vitro *amplification*

From a total number of 64 samples subjected to *in vitro* propagation, 12 (19%) were positive for *Blastocystis* (Table 1). The dominant morphological form in the cultures was the vacuolar form, with some cultures containing a few granular forms.

The use of the Blastocystis *purification method*

The *Blastocystis* purification method did not affect or improve the PCR amplification from DNA from *in vitro* cultures. The amplicons produced from both purified and unpurified cultures were of a similar abundance, yielding more than the required amount of DNA for sequencing purposes.

Detection of Blastocystis *directly from the faeces using PCR*

From a total number of 107 samples screened for *Blastocystis* using PCR directly from the faeces, 37 (35%) were positive for the organism. From the subset of 64 samples, which were also subjected to *in vitro* propagation, 27 (42%) samples were shown to be positive for *Blastocystis* (Table 1).

Table 2. *Blastocystis* isolates obtained from GenBank™ for phylogenetic analysis

Host	Country of origin	Reference	Accession number
Cattle	Japan	Abe (2004)	AB107964
Cattle	Japan	Abe (2004)	AB107965
Duck	France	Noel *et al*. (2003)	AY135412
Human	France	Noel *et al*. (2003)	AY135402
Human	Japan	Arisue *et al*. (2002)	AB023578
Human	Japan	Yoshikawa *et al*. (2004*c*)	AF408426
Human	Japan	Yoshikawa *et al*. (2004*c*)	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	Singapore	Arisue *et al*. (2003); Noel *et al*. (2005)	AF408427
Human	Thailand	Arisue *et al*. (2003)	AB070992
Human	Thailand	Thathaisong *et al*. (2003)	AF439782
Human	Thailand	Noel *et al*. (2005)	AY618266
Lizard	Singapore	Noel *et al*. (2005)	AY590116
Monkey	Japan	Arisue *et al*. (2003)	AB070997
Monkey	Japan	Abe (2004)	AB107969
Monkey	Japan	Abe (2004)	AB107970
Monkey	Japan	Abe (2004)	AB107967
Monkey	Japan	Abe (2004)	AB107968
Partridge	Japan	Abe (2004)	AB107972
Python	Singapore	Noel *et al*. (2005)	AY590112
Pig	Japan	Arisue *et al*. (2003)	AB091248
Rat	Japan	Arisue *et al*. (2003)	AB091251
Rat	Singapore	Noel *et al*. (2005)	AY590114

Phylogenetic analysis

DNA sequences obtained from 22 *Blastocystis* isolates (Table 3) were included in the phylogenetic analysis. The rooted UPGMA tree identified 7 clades, which corresponded to the 7 known groups/subtypes identified in previous molecular studies (Arisue *et al*. 2003; Abe *et al*. 2003*a*, *b*; Noel *et al*. 2003, 2005). There was strong bootstrap support for the clustering of subtypes 2 and 4 to the exclusion of subtypes 1, 3, 5, 6 and 7, but only moderate support for the delineation of subtypes 1, 5 and 6 from subtypes 3 and 7, as well as subtype 2 from 4 (see Fig. 1). With the exception of subtypes 1 and 5, which clustered together, the other subtypes were clearly separated from each other with strong bootstrap support.

All isolates of *Blastocystis* isolated from primates and their human handlers at the Perth Zoo were placed within subtypes 1 or 5, as were 3 dog and 1 human isolate from Thailand, and 1 dog isolate from Australia. The sequences of most primate isolates of *Blastocystis* were identical to each other and placed in subtype 1. However, isolates from the *in vitro* cultures (from baboon, vervet and black and white ruffed lemur) were not identical to those amplified directly from the faeces of the baboon, black and white ruffed lemur and vervet. Analyses of the sequence data representing the isolates amplified directly from faecal samples revealed the presence of mixed peaks in the sequencing chromatograms, whereas no mixed peaks were evident in the sequencing chromatograms from isolates obtained by *in vitro* cultivation. A *Blastocystis* isolate from 1 of the zoo-keepers (zoo-keeper (19) faeces) was 99·6% similar in sequence compared with those from the primates (baboon culture and white cheeked gibbon faeces (a)) at the Perth Zoo. A single *Blastocystis* isolate from a Thai human was shown to be 100% similar to an isolate from a dog living in the same community. Another human isolate from Thailand was placed within subtype 6. *Blastocystis* sequences from a possum and human in Australia were also shown to be 100% similar to each other and were placed in subtype 7.

DISCUSSION

Comparing PCR detection directly from the faeces to in vitro *propagation for the detection of* Blastocystis

Prior to this study, *in vitro* propagation was considered to be the most sensitive method for the detection of *Blastocystis* infections (Leelayoova *et al*.

Table 3. *Blastocystis* isolates obtained in this study, labels for the isolates used for phylogenetic analysis and their subtypes

Host species	Isolates from cultures or faeces	Subtype
Dog (96)	Faeces	1
Human (1)	Faeces	
Human (2)	Faeces	
Human (3)	Faeces	7
Human (4)	Faeces	
Human (5)	Faeces	
Human (6)	Faeces	
Human, (zoo-keeper 19)	Faeces	1
Human, (zoo-keeper 44)	Faeces	
Human, (zoo-keeper 639)	Faeces	1
Human, (zoo-keeper Anon 02)	Faeces	
Baboon	Culture	1
Baboon	Faeces	1
Black and white ruffed lemur	Culture	5
Black and white ruffed lemur	Faeces	1
Colobus	Faeces	
Crested macaque	Faeces	1
Orang utan	Faeces	1
Brushtailed possum	Faeces	7
Brushtailed possum	Faeces	
Ring tailed lemur (a)	Faeces	1
Ring tailed lemur (b)	Faeces	1
Spider monkey	Faeces	
Thai dog (108)	Culture	5
Thai dog (428)	Culture	5
Thai dog (676)	Culture	5
Thai human	Culture	5
Thai human (H522H1)	Faeces	6
Thai human (H706H1)	Faeces	
Thai human (H1069H3)	Faeces	
Vervet	Culture	5
Vervet	Faeces	1
White cheeked gibbon (a)	Faeces	1
White cheeked gibbon (b)	Faeces	1

2002; Suresh and Smith, 2004). An advantage of the *in vitro* culture method is that low numbers of *Blastocystis* can be cultured for use in other (e.g., genotypic and/or phenotypic) tests (Suresh and Smith, 2004). A previous study comparing detection methods for the diagnosis of *Blastocystis* infections revealed that out of 23 *Blastocystis* positive samples, 98% and 52% were positive using *in vitro* culture and PCR detection directly from the faeces, respectively (Termmathurapoj *et al*. 2004). In the present study, 19% and 42% of samples were shown to be positive for *Blastocystis* using *in vitro* amplification and PCR detection directly from the faeces, respectively. This result suggests that the DNA extraction method used in the present study was more effective compared with the method used by Termmathurapoj *et al*. (2004), the PCR-based method used in this study for the detection of *Blastocystis* infections is more sensitive compared to *in vitro* amplification and that *in vitro* cultivation does not need to be performed to achieve increased sensitivity of molecular tests.

This is the first study to detect *Blastocystis* in Australian native marsupials. It should be noted that none of these samples were positive by culture, and some were positive by PCR. It may be possible that some isolates require longer than 48 h for the establishment of growth *in vitro*, as growth in some cultures may take up to 72 h of incubation before parasite stages can be detected, with subculturing into fresh medium every 48–72 h. In some studies, subculturing is performed up to 5 times before a sample is considered to be negative for the presence of *Blastocystis* (Yoshikawa *et al*. 2004*c*).

In previous studies using Jones' medium supplemented with 10% horse serum (Salim *et al*. 1999; Leelayoova *et al*. 2002; Suresh and Smith, 2004; Termmathurapoj *et al*. 2004; Yakoob *et al*. 2004; Yoshikawa *et al*. 2004*c*), only human faecal samples were screened for *Blastocystis*. However, several different types of media to cultivate *Blastocystis* have been reported, and it has been suggested that the usefulness of cultivation may depend on the reagents and protocols employed (Zaman and Khan, 1994; Leelayoova *et al*. 2002). Perhaps an alternative to Jones' medium should be evaluated for the detection of *Blastocystis* in non-human hosts, since *Blastocystis* is susceptible to changes in environmental conditions (Stenzel and Boreham, 1996; Leelayoova *et al*. 2002; Tan, 2004). Other types of medium, such as diphasic-agar slant medium have been effective for propagating *Blastocystis* from cattle, pigs and chickens (Yoshikawa *et al*. 1996, 1998; Abe *et al*. 2003*a*, *b*, *c*), and should be evaluated (along with other types of medium) in comparative diagnostic studies.

This study has shown that it is possible to detect *Blastocystis* directly from faeces solely using molecular tests with a greater sensitivity compared with *in vitro* propagation. A clear advantage of using molecular techniques to detect *Blastocystis* directly from the faeces for the diagnosis of *Blastocystis* infections is that the method is not dependent upon factors such as animal host species, viability of *Blastocystis* organisms, and the various reagents and protocols used in *in vitro* propagation. Another advantage of using molecular techniques compared with conventional methods is that it is less time consuming, and can provide more information in terms of genetic variability and determining zoonotic relationships (Traub *et al*. 2005).

Preferential growth of Blastocystis in vitro

Previous molecular characterization studies of *Blastocystis* have only analysed *Blastocystis* isolates amplified *in vitro* (Ho *et al*. 2000; Arisue *et al*. 2003; Noel *et al*. 2003, 2005; Yoshikawa *et al*. 2004*c*). The present study was the first to compare isolates

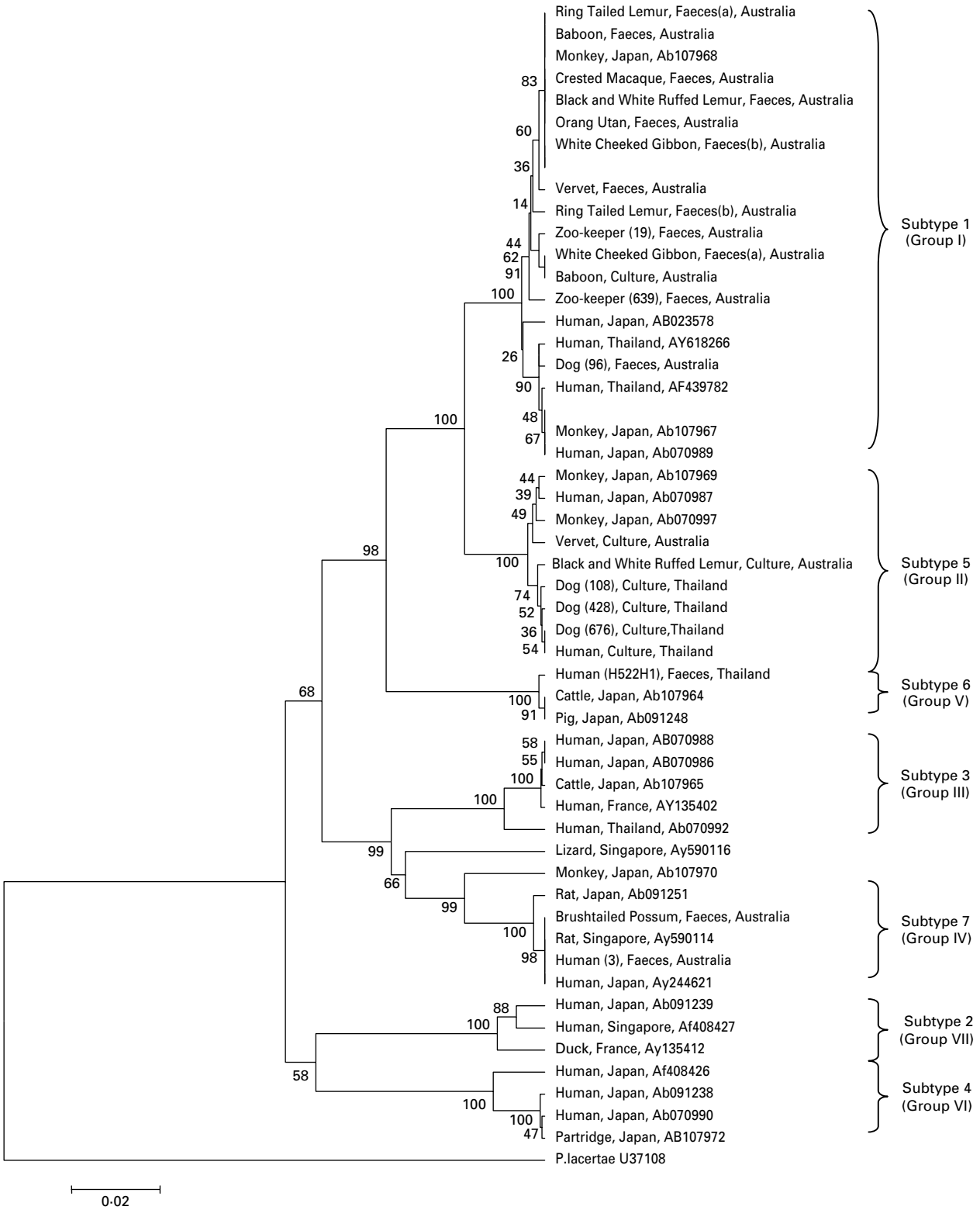

Fig. 1. UPGMA tree displaying the relationships among *Blastocystis* isolates, inferred by distance based analysis of SSU rDNA sequence data using Kimura's-2-parameter distance estimates. Some sequences used for comparison were from GenBank™ Scale bar shows 0·2 substitutions (corrected) per base pair.

obtained from *in vitro* cultures and those amplified directly from the faeces by the PCR. According to the phylogenetic tree constructed, *in vitro* isolates of vervet and black and white ruffed lemur origin belonged to subtype 5, whereas the isolates amplified directly from the faeces from these hosts belonged to subtype 1 (black and white ruffed lemur faeces and vervet faeces). The other *in vitro* isolate (from baboon) was identical to an isolate from a white cheeked gibbon (white cheeked gibbon faeces (a))

but was not identical to the baboon isolate amplified directly from the faeces (baboon faeces). These findings indicate that not only did these primates have mixed infections, which were evident due to the mixed peaks in the sequencing chromatograms for the isolates amplified directly from the faeces (there were no mixed peaks in the sequencing chromatograms from *in vitro* isolates), but also that preferential growth of a particular isolate can occur *in vitro* if the host is infected with more than one isolate. In the present study, *Blastocystis* subtype 5 overgrew subtype 1 *in vitro*.

Although *in vitro* propagation is considered one of the most sensitive methods currently available for the detection of *Blastocystis* infections, the organisms grown in culture may not accurately represent the parasite population sampled. In the early studies on genetic variability in *Giardia*, Assemblage A was considered to be the most commonly isolated genotype. However, it was later shown that the *in vitro* culturing methods used were preferentially selecting for Assemblage A genotypes (Thompson and Monis, 2004). As with more recent studies on *Giardia*, the ability to genetically characterize isolates of *Blastocystis* directly from faeces or environmental samples will provide a way of avoiding the potential selectivity imposed by laboratory amplification *in vitro*.

Zoonotic potential

In the present study, all 4 faecal samples collected from the zoo-keepers were positive for *Blastocystis*. Two of these samples were sequenced and characterized successfully. *Blastocystis* isolated from the zoo-keepers at the Perth Zoo were found to be similar to some isolates from the primates, which clustered into the same subtype (subtype 1). It has been reported that the close contact between animals in a zoo environment can facilitate the transmission of *Blastocystis*, and intimate association between humans and animals in such environments (Salim *et al*. 1999; Abe *et al*. 2002).

Similarly, a dog isolate (dog 676 culture) and a human isolate (human culture) from the same village in Thailand were identical genetically and belonged to subtype 5. The present study is the first to report a human isolate (human (Th522H1) faeces) belonging to subtype 6, which has been considered to be specific to pig and cattle isolates (Noel *et al*. 2005). These findings may be reflective of the interactions between humans and animals in rural Thailand, and provide evidence suggesting zoonotic transmission.

In addition, a human isolate (human (3) faeces) and a brushtailed possum isolate (brushtailed possum faeces) from the present study were genetically identical to each other, and to rat and human isolates from Singapore and Japan, respectively. The findings from the present study agree with previous studies, indicating the presence of 7 distinct subtypes which are not host specific, comprising isolates from humans and various other animals (Yoshikawa *et al*. 1996, 1998; Clark, 1997; Snowden *et al*. 2000; Arisue *et al*. 2003; Noel *et al*. 2005).

In conclusion, the present study demonstrated that PCR-based methods were more sensitive than *in vitro* propagation for the detection of *Blastocystis*. Comparisons between *Blastocystis* isolates from *in vitro* cultures and those amplified directly from faeces revealed that preferential amplification can occur *in vitro*. The present study also identified a number of isolates which may be of zoonotic significance.

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