Impact of pH on the viability and morphology of *Blastocystis* isolates

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Abstract. *Blastocystis* sp. is ubiquitous in avian, mammalian and human hosts and propagates in either neutral or slightly alkaline conditions within the host’s gastro-intestinal tract. Of the few previous studies on this enteric protozoan parasite in feline and canine hosts, prevalence values have been shown to range between 0 to 70.8%. In view of the close association between humans, and canine and feline hosts as companion animals, faecal samples of 180 *Felis catus* and 82 *Canis lupus*, collected from Penang and Kuala Lumpur, Malaysia, were initially screened by in vitro cultivation followed by molecular characterization. No positive isolates were identified in culture but in 12 feline samples DNA barcoding detected a zoonotic subtype *Blastocystis* ST1 for the first time. Consequently, avian and human isolates, which had previously been successfully cultured, were used to investigate the impact of pH on the viability and morphology of *Blastocystis* sp. The use of Trypan blue showed that the number of viable cells increased when exposed to pH 4 and a significant increase in viability occurred in pH values of 5 to 7. Development of *Blastocystis* cells in both isolates was suppressed in media less than pH 5 followed by the disappearance of viable cells from avian isolates in more acidic media below pH 4. Morphologically at pH 4 cells from avian isolates were less rounded, and with wrinkled / shrunken surfaces, than the more normal rounded cells from human isolates. On the other hand, at values below pH 3, no viable cells in human isolates were visible. The present findings therefore confirm that gastro-intestinal pH is an important determinant of *Blastocystis* viability and consequently influences the epidemiology of infection within avian, mammalian and human hosts.

INTRODUCTION

The genus *Blastocystis* comprises a group of single-celled parasites belonging to the Stramenopiles which inhabit the gastro-intestinal tract of vertebrate and human hosts (Li et al., 2007; Leelayoova et al., 2008; Ithoi et al., 2011) and especially domesticated mammals (Parkar et al., 2010; Roberts et al., 2013; Hemalatha et al., 2014), rodents (Lavier, 1952; Chen et al., 1997), birds (Belova and Kostenko, 1990; Belova, 1991; Belova, 1992; Chandrasekaran et al., 2014), amphibians (Yoshikawa et al., 2004), and reptiles (Teow et al., 1991; Singh et al., 1996). Human and non-human *Blastocystis* isolates are morphologically similar and can be vacuolar (Stenzel and Boreham, 1996), granular (Tan, 2004), cyst (Tan, 2008) or amoeboid (Tan and Suresh, 2006). Human transmission involves the cyst form and occurs via the faecal-oral route. *Blastocystis*
species have low host specificity with possible zoonotic subtypes (Parkar et al., 2010).

Knowles and Das Gupta (1924) reported the presence of Blastocystis sp. for the first time in Australian domestic cats, with low prevalences of 11.7% recorded in cats from the USA (Ruaux and Stang, 2014) and 10.0% in Egypt (Maya, 2010) compared with a high prevalence of 67.3% in cats from Australia (Duda et al., 1998). Duda et al. (1998) also reported a high prevalence 0.8% in dogs using a culture-based study, whereas Wang et al. (2013) detected only 2.5% using molecular methods. Prevalences in dogs overall appear to be on low ranging from 1.3% in Cambodia (Wang et al., 2013), to 3.1% in Egypt (Awadallah and Salem, 2015), 3.4% in France (Osman et al., 2015), 9.7% in the USA (Ruaux and Stang, 2014), but with higher prevalences of 24.0% in India (Wang et al., 2013), 28.0% in Iran (Daryani et al., 2008) and 37.0% in Colombia (Ramírez et al., 2014).

These relatively low prevalences and in some instances the absence of infection in domesticated hosts from Germany (König and Müller, 1997), Malaysia (Chuong et al., 1996; Suresh et al., 1996) and Japan (Abe et al., 2002), suggested that companion animals are unnatural reservoir hosts. Sutton (2004) suggested that the low gastric pH in the alimentary tract, together with high concentrations of enzyme secretions, provides an unsuitable environment for Blastocystis to reproduce. Therefore, the objectives of the present study are to: (1) to review all reported cases of Blastocystis infections worldwide; (2) to study the prevalence of subtypes of Blastocystis in local stray populations of cats and dogs and (3) to determine the impact of low pH in the stomach of these hosts, on the morphology and viability of Blastocystis isolates.

MATERIALS AND METHODS

Collection of faecal samples and in vitro cultivation

Fresh faecal samples of 180 cats (Felis catus) and 82 dogs (Canis lupus) were collected from several animal shelters located in Perak, Penang Island, and in the Federal Territory of Kuala Lumpur in which the animals were individually housed in cages. Faecal samples were stored in stool containers and processed within 12 hours after collection. All feline and canine hosts were handled according to protocols approved by the Institutional Animal Care and Use Committee (IACUC), University Malaya (Case No.: ISB/31/01/2013/SMZ (R)).

Up to 2g of each faecal sample was inoculated into a sterile screw-top vial containing 3 ml of Jones medium supplemented with 10% heat-activated horse serum (Jones, 1946; Suresh and Smith, 2004) and incubated at 37°C for 48 to 72 hours.

DNA barcoding

Genomic DNA from Blastocystis was extracted using the Qiagen stool extraction kit according to the manufacturer's instructions. All isolates were subjected to DNA barcoding as described by Mohd Zain et al. (2017). From results of the Blast analysis, subtypes were assigned against databases at www.pubmlst.org/blastocystis and the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Blastocystis subtypes were reported in ST format which is the consensus terminology reported by Stensvold et al. (2007b).

Viability experiments

Avian Blastocystis isolates were obtained from a village chicken Gallus gallus (A3) and a peafowl Pavo cristatus (M2) whereas the three human Blastocystis isolates (H1, H2 and H3) were obtained from patients at the University of Malaya Medical Centre (UMMC). Human ethical approval was obtained in accordance with University Malaya Medical Centre research policy (Reference No.: 2054-12181). Six media (Jones medium supplemented with 10% horse serum) were prepared with descending values of from pH 6, 5, 4, 3, 2, and 1 and adjusted by adding 01M HCl. A control medium with pH 7 were also prepared.

Approximately 1 x 10^6 cells of day 3 cultures of avian isolates A3 and M2 and 1 x
$10^5$ cells of human isolates (H1, H2 and H3) were inoculated into 3 ml of each of the six media plus the control medium. Culture tubes were prepared in triplicate for each medium and subsequently incubated at 37°C. After 24 hours, cells were counted in 0.5% Tryphan blue solution using a haemocytometer chamber (Improved Neubauer, Hausser Scientific) and evaluated for indicators of viability such as distinct size, shape and staining properties.

**RESULTS**

**Prevalence of Blastocystis**

Of thirteen previous studies on Blastocystis infections in cats and dogs worldwide (Table 1) up to seven zoonotic genotypes from both feline and canine hosts were identified including ST1, ST2, ST3, ST4, ST5, ST6 and ST10 (Wang et al., 2013; Ruaux and Stang, 2014; Ramírez et al., 2014; Osman et al., 2015). More detailed morphological characteristics of Blastocystis sp. were described by Duda et al. (1998) in 70.8% infected dogs from Australia, whereby irregularly-shaped cells appeared to be vacuolar, with a thin outer rim of cytoplasm and barely discernable organelles surrounding a central vacuole. Duda et al. (1998) also showed by transmission electron microscopy that canine isolates of Blastocystis sp. appeared to be morphologically similar to cultured isolates of B. hominis. In short term cultures the cellular surface coat measured up to 3 µm thick and often with adhering bacteria. The nuclei were round or slightly elongate, measuring between 1-2 µm, with the majority of nuclei contained within a crescentric band of opaque material.

In the present study in vitro cultures of fresh faecal samples from 180 cats (Felis catus) and 82 dogs (Canis lupus) tested negative for Blastocystis sp. On the other hand, DNA barcoding successfully detected 20% (n=12/60) positivity for Blastocystis sp. in cat faecal samples with sequences assigned to ST1 (allele 4). There were additional five faint PCR bands might indicate an extremely low infection.

**Viability of Blastocystis**

Figure 1 showed that the number of viable cells of Blastocystis increase when cultured in a less acidic medium. Cultured isolates of avian and human origin in media ranging from pH 5 to 7 show cells which are round and vacuolar. When exposed to a medium of pH 4, avian isolates exhibit cells which become wrinkled and shrunken although cells from human Blastocystis isolates remain unaffected. In pH values of less than 4 both avian and human isolates become suppressed and no viable cells were observed (Figure 2).

**DISCUSSION**

Global prevalences of Blastocystis infections in companion animals vary widely e.g. a high prevalence of Blastocystis was reported from an Australian culture-based study on dogs (Duda et al., 1998), whereas more recent studies from the same location reported a marked lower prevalence using molecular methods (Wang et al., 2013). Such variation appears to reflect the sensitivity of different screening methods (Stensvold et al., 2007a) e.g. light microscopy not only appears to have the lowest sensitivity but is also prone to false positives compared with PCR-based methodology (Roberts et al., 2011). This observation is largely based on Blastocystis sp. being pleomorphic with extensive variation in morphology (Wang et al., 2013). Xenic in vitro cultures appeared to be highly sensitive compared with concentration or direct smear/light microscopic techniques (Leelayoova et al., 2002). Blastocystis was also successfully cultured from two dog samples in an inspissated egg slant medium over a short growth period but these cultures did not materialize from cat faeces (Duda et al., 1998). In the present study, xenic cultures of Blastocystis took place in the Jones’ medium supplemented with 10% horse serum (Leelayoova et al., 2002; Suresh and Smith, 2004; Stensvold et al., 2007) but the cultivation of isolates did not occur in this medium. Instead a time-consuming DNA barcoding method, which sequenced a species-specific DNA fragment, successfully
Table 1. The prevalence (%) and zoonotic genotypes of *Blastocystis* sp. in canine and feline hosts worldwide

<table>
<thead>
<tr>
<th>Host</th>
<th>Reference/Origin</th>
<th>Prevalence (%)</th>
<th>Morphological characteristics</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Light microscope</td>
<td>SEM</td>
</tr>
<tr>
<td>Canine</td>
<td></td>
<td></td>
<td>–</td>
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<tr>
<td></td>
<td>Suresh <em>et al.</em>, 1996/Malaysia</td>
<td>– 0: Domestic dog</td>
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<td></td>
<td>Chuong <em>et al.</em> (1996)/Malaysia</td>
<td>– 0: Domestic dog</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>König and Müller (1997)/Germany</td>
<td>– 0: Domestic dog</td>
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<td>–</td>
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<tr>
<td></td>
<td>Duda <em>et al.</em> (1998)/Australia</td>
<td>– 51/72 (70.8): Domestic dog</td>
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<td></td>
<td></td>
<td>– The cells were irregular in shape.</td>
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<td></td>
<td></td>
<td>– Size: 3 to 10 µm in diameter (average 4.5 µm).</td>
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<td></td>
<td></td>
<td>– The cells appeared as the vacuolar form: a thin outer rim of cytoplasm, containing barely discernible organelles, surrounded a central vacuole of varying morphology.</td>
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<td></td>
<td></td>
<td>– <em>Blastocystis</em> sp. from canine isolates appeared morphologically similar to cultured isolates of <em>B. hominis</em>.</td>
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<tr>
<td></td>
<td></td>
<td>– In the shorter-term cultures, the cells had a thick surface coat up to 0.3 µm, often with adherent bacteria.</td>
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<tr>
<td></td>
<td></td>
<td>– The nuclei were rounded or slightly elongated and were approximately 1 to 2 µm in diameter.</td>
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<tr>
<td></td>
<td></td>
<td>– The nuclei commonly contained a crescentic band of electron opaque material and, sometimes, an additional ‘spot’ of electron opaque material.</td>
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<tr>
<td></td>
<td></td>
<td>– <em>ST1, ST2, ST3, ST4, ST5, ST6</em></td>
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<td>–</td>
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<tr>
<td></td>
<td>Abe <em>et al.</em> (2002)/Japan</td>
<td>– 0/54 (0): Pet dog</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Daryani <em>et al.</em> (2008)/Iran</td>
<td>– 14/50 (28.0): Domestic dog</td>
<td>–</td>
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<tr>
<td></td>
<td>Wang <em>et al.</em> (2013)/Australia</td>
<td>– 2/80 (2.5): Pet and pound dog (Australia)</td>
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<td></td>
<td></td>
<td>– 1/80 (1.3): Semi-domesticated dog (Cambodia)</td>
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<td></td>
<td>– 19/80 (24.0): Stray dog (India)</td>
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<td></td>
<td>Rüaux and Stang (2014)/USA</td>
<td>– 10/108 (9.7): Shelter-resident canines</td>
<td>–</td>
<td>–</td>
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<td></td>
<td>Ramirez <em>et al.</em> (2014)/Colombia</td>
<td>– 15/40 (37.0)</td>
<td>–</td>
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<td></td>
<td>Awadallah and Salem (2015)/Egypt</td>
<td>– 4/130 (3.07)</td>
<td>–</td>
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<td></td>
<td>Osman <em>et al.</em> (2015)/France</td>
<td>– 4/116 (3.4)</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Feline</td>
<td>Knowles and Gupta (1924)/India</td>
<td>Not mentioned</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>Duda <em>et al.</em> (1998)/Australia</td>
<td>– 35/52 (67.3)</td>
<td>–</td>
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</tr>
<tr>
<td></td>
<td>Maysa (2010)/Egypt</td>
<td>– 5/50 (10.0)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Rüaux and Stang (2014)/USA</td>
<td>– 12/103 (11.65): shelter-resident felines</td>
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</tr>
</tbody>
</table>
detected the presence of *Blastocystis* but only in cat samples. This method, which was the more sensitive than light microscopy and cultivation (Stensvold *et al.*, 2007a), proved to be a most useful proxy for intra-subtype diversity.

Overall, the present results confirm the presence of low prevalences of *Blastocystis* especially in feline hosts. This suggests that both canine and feline hosts are not suitable reservoirs of infection for potentially zoonotic subtypes (ST1 – ST6 and ST10), which were
possibly transient or opportunistically infected through coprophagia of other host faeces or drinking contaminated water. Nevertheless, *Blastocystis* ST1 DNA was recovered from cat samples. The template for PCR amplification was likely to be DNA derived from non-viable *Blastocystis* cells damaged due to high gastric acid levels within the alimentary tract, as no positive isolates were successfully cultured in growth media.

Figure 2. *Blastocystis* cultures of A) avian and B) human isolates in a pH range of 1 - 4 with rounded or oval-shaped non-viable cells; C) avian isolates in pH4 with non-viable vacuolar and wrinkled/shrunken cells and D) human isolates in pH 4 with smaller viable vacuolar cells; typical rounded vacuolar cells in E) avian and F) human isolates within a pH range of 5 – 7.
However, ST1 is the most common human subtype in Thailand, Libya, Nigeria and Peninsular Malaysia (Alfellani et al., 2013; Leelayoova et al., 2008; Nithyamathi et al., 2016) and is associated with human irritable bowel symptoms (Alfellani et al., 2013). This subtype, which has been isolated from farm animals (Tan, 2008) predominantly occurs in dogs from Australia, Cambodia and India (Wang et al., 2013) also in a cat from USA (KJ872776) (Ruaux and Stang, 2014).

Physiologically, Blastocystis requires a neutral pH for optimum development and growth (Stenzel and Boreham, 1996) and the present study supports the view that pH values between 5 and 7 facilitate optimal growth of avian isolates. On the other hand, human isolates appear to survive over a wider pH range of 4 to 7 with smaller viable cells being observed at pH 4. This suggests that human isolates are more tolerant of less extreme acidic conditions especially under low pH levels in the human digestive tract (Kararli, 1995).

Growth of Blastocystis isolates from both feline and canine hosts was not observed in vitro using the Jones’ medium suggesting that viable cells or cysts were destroyed under the extreme acidic (< pH 4) conditions similar to those in the gastrointestinal tract. The low pH in the stomach in both hosts is attributable to gastric juice, produced by parietal/oxyntic cells, comprising concentrated hydrochloic acid up to 0.1 M, together with potassium and sodium chlorides. Gastric juice is involved in protein digestion and denaturation together with the activation of pepsin, but the inactivation of ingested microflora such as bacteria (Persson, 2008). On the other hand, gastric acid secretion in the avian gastrointestinal tract, unlike that in mammals, occurs only in the proventriculus where protein denaturation and activation of pepsinogen to pepsin takes place. According to Rynsburger (2009) in ageing broiler chickens, there is both a linear decline in pH from 5.20–3.37 in the proventriculus and also 3.49–3.27 in the gizzard, with a diet of oilseed, soybean and wheat also having an impact on pH.

CONCLUSION

Malaysian cats and dogs were clear of Blastocystis infections, despite molecular evidence to show that cats were opportunistically infected with the zoonotic subtype ST1. This could be attributed to the extreme acidity in the gastrointestinal tract that creates an unsuitable environment for the growth of Blastocystis. The viability of Blastocystis isolates in culture is pH-dependent with growth being suppressed at low pH whilst the yield of viable cells improves as pH rises to neutral.

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