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ORIGINAL PAPER

Blastocystis tropism in the pig intestine

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Abstract *Blastocystis* has been reported in pig feces but the sites of development in the gastrointestinal tract are unknown. The present study was undertaken to determine predilection sites of *Blastocystis* in 11 naturally infected pigs examined at 20 weeks of age. At necropsy, feces and contents of the duodenum, jejunum, ileum, and cecum were examined by immunofluorescence (IFA) microscopy and PCR and tissues from these sites as well as the proximal and distal colon were processed for histology from pigs 1 to 5. Feces were examined by IFA microscopy, and segments from the jejunum and ileum were processed for histology from pigs 6 to 11. Multiple sections were cut from each tissue segment, and each was stained with the following: hematoxylin and eosin, polyclonal rabbit antibody to *Blastocystis*, and ParaFlor B™ monoclonal antibody to *Blastocystis*. *Blastocystis* was detected in feces of all 11 pigs by IFA microscopy and determined by PCR and gene sequencing to be subtype 5 for pigs 1–5. *Blastocystis* was also detected in the lumen contents removed from the cecum of pigs 1–5 examined by IFA microscopy and in the cecum of pigs 4 and 5 by PCR. *Blastocystis* was also observed in tissue sections from the jejunum of 7 of the 11 pigs, in the proximal and distal colon of pigs 1–5, and in the cecum of 4 of these 5 pigs but was not detected in the duodenum or ileum of any pigs. In tissue sections, *Blastocystis* was found primarily in the lumen usually associated with digested food debris, sometimes in close proximity or appearing to adhere to the epithelium, but no stages were found to penetrate the epithelium or the lamina propria.

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Introduction

Blastocystis is an intestinal parasite found worldwide in humans and animals and is often the most frequently detected parasite in epidemiological surveys (Tan 2008). Its higher prevalence in developing countries than in developed countries has been associated with poor hygiene, exposure to animals, and consumption of contaminated food or water (Tan 2008). Despite its high prevalence and ubiquitous presence, there are few studies that demonstrate the location of *Blastocystis* stages in the gastrointestinal tract of either humans or animals. Nearly all that report a location indicate the presence of *Blastocystis* in the colon, less often in the cecum, and rarely in other intestinal sites.

Of the reports of *Blastocystis* detected in feces of large animals, domesticated or wild (Abe et al., 2002; Stensvold et al. 2009; Santin et al. 2011; Fayer et al. 2012; Alfellani et al. 2013a, b; Tan et al. 2013), only one indicates a specific location within the gastrointestinal tract where *Blastocystis* was found (Pakandl et al. 1993). Of the reports involving laboratory rodents, only one study, involving immunosuppressed mice, detected *Blastocystis* throughout the intestine (Yao et al. 2005). Considering the high prevalence of *Blastocystis* infection in humans, reports are quite rare in which *Blastocystis* was found in locations other than the colon or of tissue invasion by *Blastocystis* (Stenzel and Boreham 1996).

Because pigs are infected with some *Blastocystis* subtypes found in humans and because so little is known of the locations within the gastrointestinal tract where *Blastocystis* is found, the present study was undertaken to examine naturally infected pigs from a research farm in Maryland. When 11 pigs with *Blastocystis* in their feces reached market weight as porkers and were killed, the subtype of *Blastocystis* was determined and portions of the intestinal tract below the stomach were examined to determine if *Blastocystis* exhibited tropism for any specific locations.

Materials and methods

Animal source and care

Thirty-two healthy 20-week-old crossbred pigs were born and raised at the Henry A. Wallace Agricultural Research Center in Beltsville, Maryland. They were naïve to vaccines but received iron and vitamin B complex injections at 1 week of age. They were raised in a closed building on a cement floor, each pig in a 3×3-m pen separated by metal fencing from pigs in adjacent pens, but still in close contact. The floor of each pen was rinsed daily to remove feces but water and feces flowed across the floor of adjacent pens before reaching a drain. Pigs were weighed weekly and were fed a vegetable-based ration daily, the quantity of which was based on the weight of each pig. Water was available ad libitum. Of 11 pigs naturally infected with *Blastocystis*, pigs 1–5 were selected based on having a high number of *Blastocystis* in their feces and pigs 6–11 were selected at random from the remaining group. All were euthanized in accordance with an approved protocol by the local animal care and use committee.

Examination for *Blastocystis*

Feces from pigs were examined by immunofluorescence (IFA) microscopy using ParaFlor B™ antibody (Boulder Diagnostics, Boulder, CO) as described (Fayer et al. 2012) and by PCR as described (Santin et al. 2011). At the time of necropsy, segments of duodenum, jejunum, ileum, and cecum between 1.5 and 3.0 cm long were taken from five pigs (pigs 1–5), tied off with string, excised, placed on ice, and taken to the laboratory for testing. Adjacent pieces of tissue as well as proximal and distal colon approximately 1.25 cm long were placed in 10 % neutral buffered formalin (NBF) with the exception of the cecum which was frozen; all were later processed for histology. Segments of jejunum and ileum were taken from six additional pigs (pigs 6–11), placed in 10 % NBF, and processed for histology.

In addition to examination of feces, lumen contents from each segment of intestine (duodenum, jejunum, ileum, and cecum) from pigs 1–5 were examined by IFA microscopy using ParaFlor B™ antibody as described (Fayer et al. 2012) and by PCR as described (Santin et al. 2011). Tissues fixed in formalin and the frozen tissues were sent to a commercial histology laboratory for sectioning and mounting on glass microscope slides. One slide from each segment from each pig was stained with hematoxylin and eosin. Multiple additional sections from each location from each pig were left unstained. Glass microscope slides with unstained tissue sections affixed were processed as described (Elsasser et al. 2004). Briefly, slides were deparaffinized in three successive xylene baths followed by hydration to aqueous buffer through a decreasing gradient of ethanol:water concentrations. To maximize antigen detection, slides were incubated in hot citrate (pH 6.0) for 30 min followed by a 10-min wash in 0.1 % Triton X-100 in PBS. For fluorescence detection using ParaFlor B™ antibody, sections were incubated in a PBS-blocking buffer containing 5 % normal mouse serum; primary antibody was applied undiluted for 2 h at room temperature in a humidified chamber, and then, nuclei were stained with DAPI. Slides were rinsed with water and coverslips were mounted with antifade reagent (ProLong Gold™, Molecular Probes, Eugene, OR). For antigen imaging by horseradish peroxidase (HRP), hydrated specimens were additionally treated to inhibit pseudoperoxidase reactivity (Bloxall™, Vecta Laboratories, Burlingame, CA), blocked with 5 % normal rabbit serum in PBS, and incubated overnight at 4 °C with HRP-conjugated rabbit anti-*Blastocystis* IgG (1.3 μg/ml final dilution) (Gould and Boorom 2013). Slides were incubated subsequently in 3,3′-diaminobenzidine hydrochloride (0.75 mg/ml in 0.05 M TRIS buffer pH 7.5) for 4 min. Nuclei were counterstained with Carazzi's hematoxylin for 1 min. Slides were dehydrated through 100 % ethanol, immersed in xylene, and coverslips mounted. Color images were captured using an Olympus DP70 CCD camera attached to an Olympus BX40 light microscope and color balanced to neutrality with regard to redshift to account for bulb element illumination intensity. Black and white images were captured using a Zeiss AxioCam MRm camera attached to a Zeiss Axioskop brightfield microscope.

Sequencing and cloning

Positive PCR products, approximately 500 bp in length, purified using Exonuclease I/Shrimp Alkaline Phosphatase (ExoSAP-IT™) (USB Corporation, Cleveland, OH), were sequenced in both directions using the same PCR primers in 10 μl reactions, BigDye™ chemistries, and an ABI3130 sequencer analyzer (Applied Biosystems, Foster City, CA). Sequence chromatograms of each strand were aligned and examined with Lasergene software (DNASTAR, Inc., Madison, WI). When mixed infection within a specimen was suspected from the sequence traces, the PCR products of small subunit ribosomal DNA (SSU rDNA) were cloned using the

TOPO TA Cloning kit (Invitrogen Corp., Carlsbad, CA) and transformants were selected from each specimen and screened by PCR and sequenced in both directions using M13 forward and reverse primers. Up to eight clones from each specimen were sequenced.

Negative PCR results were tested for inhibition. Known DNA was added to those samples found negative followed by retesting that confirmed that inhibition was not present.

The nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers KF410594-KF410611.

Results

Blastocystis was detected in feces of all 11 pigs by IFA microscopy (Table 1). Pigs 1–5 were the most heavily infected. Lumen contents were examined from pigs 1–5 but not from pigs 6–11. Contents from the duodenum, jejunum, and ileum from pigs 1–5 were examined by ParaFlor B™ and by PCR, but all were found negative for *Blastocystis*. Lumen contents from the cecum were found positive for *Blastocystis* by IFA microscopy using ParaFlor B in pigs 1–5 and by PCR in pigs 4 and 5. Feces from pig 2 contained *Blastocystis* subtype 5d. For both PCR-positive cecum contents (pigs 4 and 5) as well as feces from pigs 1, 3, 4, and 5, multiple subtypes within each specimen were suspected based on the sequence traces, so those feces were subjected to cloning. Heterogeneity in nucleotide sequences was observed among clones (Table 1). Nucleotide sequences obtained from direct sequencing and cloning showed a total of 13 different nucleotide sequences (identified as a–m), all belonging to subtype 5 (Table 1).

Findings from histologic sections are also shown in Table 1. The proximal and distal colons from pigs 1–5 stained with polyclonal rabbit anti-*Blastocystis* antibody and with ParaFlor B™ were found positive for *Blastocystis* and were the most heavily infected portions of the gastrointestinal tract. Numerous organisms were observed in the lumen of a single ×400 field in some sections of the colon. ParaFlor B™-stained sections are shown in Fig. 1a, b. Small numbers of *Blastocystis* were observed in tissue sections of the cecum from pigs 1, 2, 4, and 5 (Figs. 1c; 2a, b) and in the jejunum of pigs 1–5 (Fig. 1d), but none were observed in the duodenum or ileum. As many as two forms were observed per field in the sections of the cecum, but in the sections in which the jejunum was positive, only one to three *Blastocystis* were found in each section. *Blastocystis* was found primarily in the feces that remained at the periphery of the lumen after tissues were processed. However, little or no feces were present in sections of the duodenum, jejunum, and ileum. Some organisms were in proximity to but not touching epithelial cells in both colon and cecum; others appeared within mucus near the epithelium (Fig. 2a). A few organisms appeared closely adherent to the epithelium (Figs. 1c; 2b–d). These appeared nearly round or triangular with no clear internal features such as nuclei,

Table 1 Feces, lumen contents, and intestinal tissues examined for *Blastocystis* by molecular and microscopic methods

Pig no.	Feces PCR	Feces IFA[a]	Lumen contents			Tissue sections[b]				
			Duodenum, jejunum, and ileum IFA /PCR	Cecum PCR	Cecum IFA[a]	Duodenum	Jejunum	Ileum	Cecum	Proximal and distal colon
1	ST5a and ST5b	+	−/−	−	+	−	+	−	+	+
2	ST5d	+	−/−	−	+	−	+	−	+	+
3	ST5c, ST5d, ST5e, and ST5f	+	−/−	−	+	−	+	−	−	+
4	ST5c, ST5i, and ST5j	+	−/−	ST5c, ST5g, and ST5k	+	−	+	−	+	+
5	ST5g and ST5h	+	−/−	ST5c, ST5l, and ST5m	+	−	+	−	+	+
6	ND	+	ND	ND	ND	ND	−	−	ND	ND
7	ND	+	ND	ND	ND	ND	−	−	ND	ND
8	ND	+	ND	ND	ND	ND	−	−	ND	ND
9	ND	+	ND	ND	ND	ND	+	−	ND	ND
10	ND	+	ND	ND	ND	ND	−	−	ND	ND
11	ND	+	ND	ND	ND	ND	+	−	ND	ND

[a] Feces and lumen contents stained with ParaFlor B™ were examined by immunofluorescence (IFA) microscopy

[b] Tissue sections were considered positive for *Blastocystis* only after both polyclonal anti-*Blastocystis*-stained sections and ParaFlor B™-stained sections were found positive

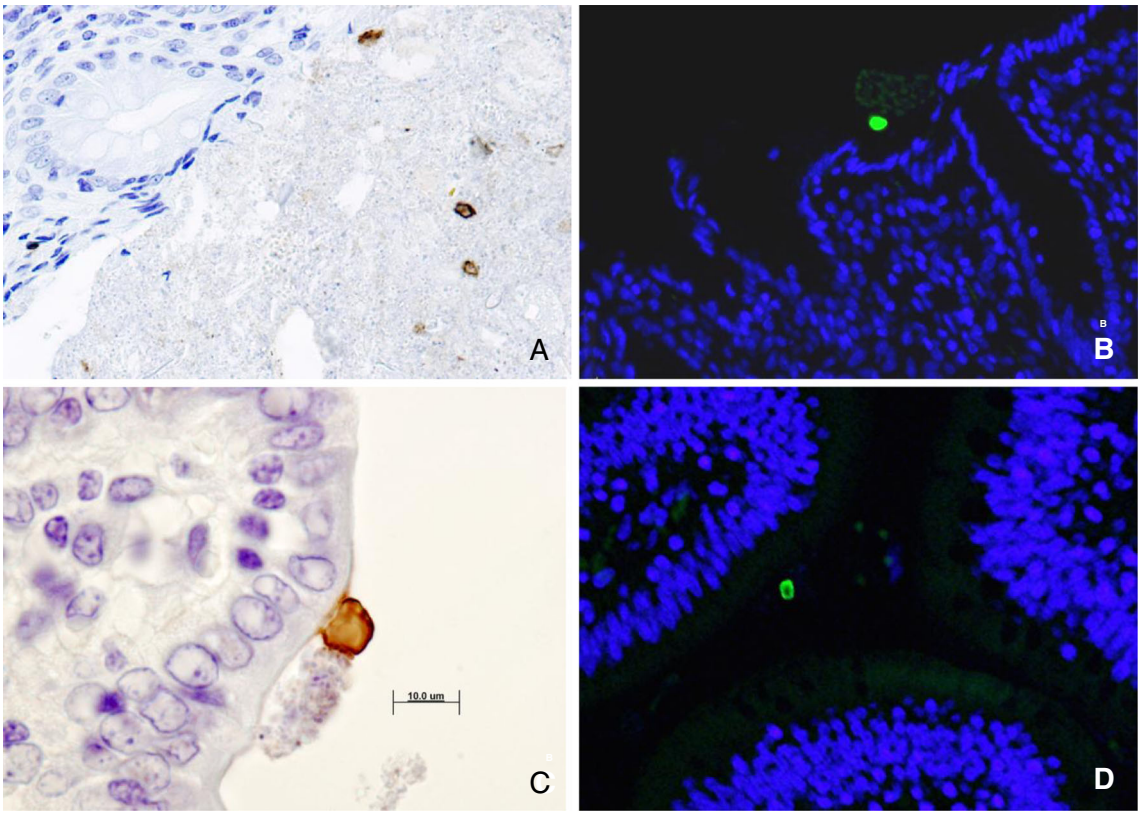

Fig. 1 Histologic sections of colon (**a**, **b**), cecum (**c**), and jejunum (**d**). In **a**, **c**, stained with horseradish peroxidase and hematoxylin, *Blastocystis* is stained *brown*. In **b**, **d**, stained with ParaFlor B™ and DAPI, *Blastocystis* is stained *green*. All *Blastocystis* are within or attached to feces within the lumen. In **c**, *Blastocystis* is closely adhered to the surface of the epithelium and to feces

granules, or vacuoles, except for a light central area surrounded by a peripheral layer with a distinctly stained outer wall. Adherent organisms, some nonadherent organisms in close proximity to the epithelium, and a few in the lumen appeared similar to those shown in Figs. 1c and 2b–d, whereas many organisms in the lumen and feces varied greatly in shape from nearly round to elongate to those with an irregular periphery and from 6 to 20 μm in diameter or long axis, most had little or no internal staining, and staining with IFA and HRP varied from pale to dense. There were no discernible differences within or between locations in the intestinal tract that were helpful in determining a life cycle pattern. Invasion, disruption, or other microscopic evidence of pathology in the epithelium and the lamina propria were not observed. *Blastocystis* was not found in histologic sections of the duodenum or ileum.

Discussion

Three major forms of *Blastocystis*, described as vacuolar, granular, and amoeboid, have been photographed and described from in vitro samples (Stenzel and Boreham 1996; Tan 2008). However, a baffling range of morphologic forms has been identified including the medusa head and chestnut burr cell forms (Zierdt 1991; Parija and Jeremiah 2013). Furthermore, a great deal of variability has been noted in size and shape of the organism, possibly altered by environmental conditions and treatment of specimens prior to examination or the influence of osmotic conditions, but the influence of preservatives, diluents, and staining solutions on *Blastocystis* morphology has not been adequately assessed (Stenzel and Boreham, 1996). Because great morphological differences were seen in the present study and because the immunologically based staining methods did not reveal internal structures, comparison and naming of the forms observed in the present study were difficult and could not be compared with previous descriptions that have been based primarily on phase-contrast microscopy and transmission electron microscopy of cultured specimens. Therefore, to prevent misidentification of forms seen in the present study, they were not described as comparable to culture-derived forms.

Blastocystis subtype 5 was identified in feces from pigs 1–5 and in the lumen contents from pigs 4 and 5. In six of those seven PCR-positive specimens, cloning was necessary because mixed infection was suspected based on the nucleotide sequence traces. Sequence heterogeneity was found in all those samples with up to four different nucleotide sequences identified in the fecal specimen from pig 3. It is possible that the heterogeneity is due to the presence of multi-copy genes of

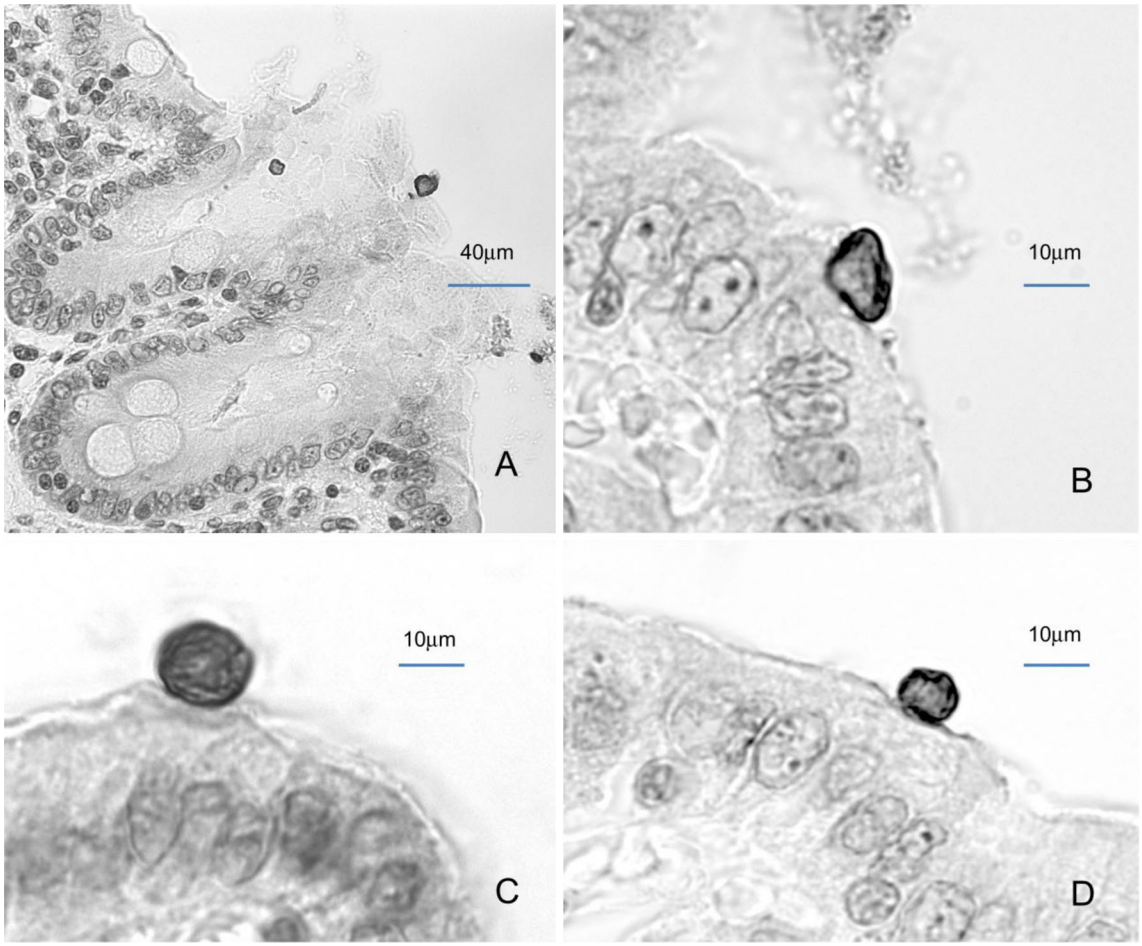

Fig. 2 Polyclonal rabbit serum conjugated with horseradish peroxidase- and hematoxylin-counterstained tissue sections of the cecum. **a** Two stages located in what appears to be mucus from goblet cells. **b–d** *Blastocystis* is closely adhered to epithelial cells

SSU rDNA in the *Blastocystis* genome. The 18S DNA gene is present in multiple copies and previous studies on *Blastocystis* have shown sequence heterogeneity (Santin et al. 2011). However, the possibility of infections with multiple subtypes in those samples could not be dismissed because in previous studies, more than one subtype was identified within a sample (Santin et al. 2011; Alfellani et al. 2013a, b).

Blastocystis was not found in the lumen contents of the duodenum, jejunum, or ileum of pigs 1–5 by molecular or microscopic methods, suggesting that either it was not there or was present in such small numbers that it was not detected. If *Blastocystis* was present, it seems unlikely that PCR failed to detect it because of inhibitory factors because it was detected both in cecum lumen contents and in feces using the same molecular methods. Small numbers were detected by IFA microscopy in cecum lumen contents in pigs 1–3 although cecum contents from these pigs were negative by PCR. A few *Blastocystis* were observed in tissue sections of jejunum from pigs 1–5 stained with ParaFlor B^{TM} and with polyclonal anti-*Blastocystis* antibody but not in jejunum lumen contents from these pigs by PCR or IFA. These apparent discrepancies in which extremely small numbers of *Blastocystis* were found in these areas and not in others or were detected by one method and not another may result from sample selection. The close association of *Blastocystis* with feces and the scarcity of feces in histological sections of the ileum may account for finding *Blastocystis* in tissue sections of the jejunum but not in the ileum of pigs 1–5. Likewise, feces were few or absent in tissue sections of the duodenum. Although very few *Blastocystis* were found in the jejunum, their presence indicates that *Blastocystis* is a parasite not only of the cecum and colon but also of the small intestine as well. Histological sections revealed *Blastocystis* adherent to the epithelium, but none were seen within the epithelium or lamina propria indicating that essential nutrients could be obtained from materials present in the intestinal lumen.

Relatively few studies have examined large domesticated animals for the presence of *Blastocystis. Blastocystis* sp. was found in feces on five pig farms in Southern Bohemia (Pakandl 1991). *Blastocystis* subtypes 1, 2, 3, and 5 have been detected in feces from 150, 7, 6, and 59 pigs, respectively, from Japan, Spain, France, Thailand, and Denmark (reviewed

by Stensvold et al. 2009); subtypes 1, 3, 5, and 10 were detected in feces from 2, 3, 16, and 22 cattle, respectively, from Japan and Denmark; ST-1 was found in 1 horse, ST10 was found in 1 deer, and ST3 and ST10 were found in 2 sheep. In peninsular Malaysia, *Blastocystis* ST1, ST3, ST6, and ST7 were detected in feces from 236 of 773 goats (Tan et al. 2013). In addition, ST5 was found in six pigs and ST10 was found in seven cattle in the USA (Santin et al. 2011). Nine other cattle were found infected with ST10 and ST14 in the USA (Fayer et al. 2012). None of these large animal studies examined the gastrointestinal tract to determine if *Blastocystis* demonstrated a tropism for any specific locations. In only one study, in which *Blastocystis* was experimentally transmitted from a human to gnotobiotic piglets, was *Blastocystis* detected in the intestinal tract of a large domesticated animal, and this was in the cecum of a piglet (Pakandl et al. 1993).

Most animal studies have been conducted with laboratory rodents. *Blastocystis* was found in the cecum and large intestine of rats fed culture-derived cyst stages (Suresh et al. 1993). An amoeboid form of *Blastocystis* with granular elements was observed in the cecum of Wistar rats orally inoculated with culture-derived cysts (Suresh et al. 1995). Immunocompetent BALB/c mice at 2, 4, and 6 weeks of age were orally inoculated with culture-derived cysts of *Blastocystis*, and most excreted cysts within a week, whereas those inoculated at 8 weeks of age did not become infected (Moe et al. 1996). *Blastocystis* was detected only in the cecum and colon, not in the small intestine. Histological examination revealed intense inflammatory cell infiltration, edema in the lamina propria, and mucosal swelling in the cecum and colon. Mostly, vacuolar and granular forms were found in the cecum, whereas only cystic forms were found in the colon. In the cecum, organisms were found mainly in the lumen contents but some were found at the edge of the mucosal epithelium. None were found within the lamina propria and no invasive lesions were observed. Cysts found in the colon were thought to have arisen from stages in the cecum. The only published report found in which *Blastocystis* was distributed throughout the gastrointestinal tract was in immunocompromised ICR mice inoculated with axenic culture forms and xenic cyst forms (Yao et al. 2005). Pathological findings were extreme, including severe edema, hyperemia, congestion, and exfoliation in the mucosa of the jejunum, ileum, cecum, and colon, but neither the presence of *Blastocystis* nor the absence of other pathogens could be confirmed in the photomicrographs. It is unclear whether these immunocompromised mice might have been infected with other pathogens. Heavily infected gnotobiotic guinea pigs infected with *Blastocystis* had gross cecal hyperemia and frequent penetration of the epithelium but not the lamina propria where a slight increase in cellularity was noticed (Zierdt 1991). A photomicrograph shows *Blastocystis* in the ileal mucosa of a guinea pig, but it is not clear if the organism is actually within or adherent to an epithelial cell. None of these aforementioned studies determined the subtype of *Blastocystis* involved in the infections. The tropism, ability to invade tissues, and the ability to initiate pathogenic change could vary with the subtype and result in the differences found among the studies.

Although tissue invasion was not detected in the present study and no microscopic indications of tissue damage were observed, indirect evidence suggesting tissue pathology has been obtained in other studies. In animal studies, rats infected with *Blastocystis* had elevated levels of urinary hyaluronidase and an increase in the proinflammatory cytokines IL-6 and IL-8 in sera, which was interpreted as suggestive of mucosal invasion activity (Chandramathi et al. 2010) although microscopic evidence of *Blastocystis* in tissues was not presented to support this possibility. Likewise, in chronically infected rats with no histological evidence of invasion, mucosal sloughing, or cellular response, RT-PCR indicated upregulation of inflammatory cytokines (Iguchi et al. 2009). In another study, although invasion was not discussed, high levels of NO were found in tissues of the cecum and ileum of infected mice (Eida et al. 2008). Proteases secreted by *Blastocystis* might facilitate parasite adherence and initiate lesions by hydrolyzing intestinal mucosal connective tissue proteins (Sio et al. 2006). In human studies, *Blastocystis* proteases were detected in 17 of 18 symptomatic patients and protease activities at the 32-kDa band were detected in 11 of 18 symptomatic patients, suggesting a possible pathogenic role (Abdel-Hameed and Hassanin 2011). Puthia et al. (2005) explained that some parasite proteases evade or modulate the host immune response by degradation of host immune molecules, facilitate invasion by catalyzing degradation of connective tissues, and utilize specific host proteins for parasite metabolism and *Blastocystis* cysteine proteases mediate an IL-8 response (Puthia et al. 2008). In silico analysis of the *Blastocystis* subtype 7, genome sequence highlighted 22 genes coding for possible secretory proteases and 2 cysteine proteases, a cathepsin B and a legumain, were identified in the parasite culture supernatant (Wawrzyniak et al. 2012). These authors speculated that these proteases might act on intestinal cells and disturb gut function. These studies suggest that *Blastocystis* can elicit such inflammatory responses without actual invasion.

. In one study not involving rodents, microscopic examination of the lumen contents at death revealed *Blastocystis* in the cecum of monkeys and chickens (Yamada et al. 1987).

Considering the high prevalence of *Blastocystis* infection in humans, reports are quite rare of humans with either *Blastocystis* in locations other than the colon or with invasive *Blastocystis*. Duodenal secretions from the enteral string test were found positive for *Blastocystis. Blastocystis* was detected by electron microscopy in jejunal biopsies from three patients with AIDS, cryptosporidiosis, and diarrhea (Connolly et al. 1991). Aspirate of lumen fluids acquired by endoscopy from the lower ileum and cecum of a patient was positive for

Blastocystis (Matsumoto et al. 1987). Terminal ileitis secondary to infection with *Blastocystis* was reported in a 37-year-old male (Tsang et al. 1989). A 16-year-old hemophiliac with acquired immune deficiency-related complex presented with upper gastrointestinal symptoms in conjunction with high densities of *Blastocystis hominis* in duodenal secretions and in the stool (Narkewicz et al. 1989). A 46-year-old woman who presented with acute peritonitis was found to have cyst-like forms of *Blastocystis* in her peritoneal fluid (Patino et al. 2008). A laparotomy revealed perforation from an adenocarcinoma involving her bowel and peritoneum. In addition to the previous three reports, there are only two reported cases of invasive *Blastocystis. Blastocystis* was detected in mucosal ulcers in a 4-year-old girl with rectal bleeding (Al-Tawil et al. 1994), and colonoscopy of a 47-year-old male showed large ulcers in the cecum, hepatic flexure, and transverse colon with multiple small ulcers in the rectum (Janarthanan et al. 2011). Biopsies showed chronic inflammation plus multiple vacuolated and amoeboid structures confirmed by trichrome staining to be *Blastocystis*. A patient taking prednisone developed *Blastocystis*-associated diarrhea, and organisms were found in synovial fluid from the left knee (Lee et al. 1990). After metronidazole treatment, knee aspirate and stools were negative for *Blastocystis*. As in most of the animal studies, the subtypes involved in these infections were undetermined. Future reports of infection, whether of animals or humans, should indicate the subtype of *Blastocystis*. As data accumulates, the tropism pattern of each subtype might be identified and could provide guidance for diagnosis and treatment.

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