Experimental Parasitology 191 (2018) 55–61

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Evaluating rodent experimental models for studies of *Blastocystis* ST1

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ARTICLE INFO

Keywords:
Blastocystis ST1
Experimental colonization
Rat
Susceptibility to colonization
Colonization longevity
In vivo model

ABSTRACT

Blastocystis is a common inhabitant of the human gut, colonizing at least one billion people at a prevalence ranging from < 10% to 100% in healthy human populations globally. The majority of carriers remain asymptomatic, suggesting that *Blastocystis* is largely a commensal, though *Blastocystis* has also been implicated in disease in some people. However, there are no *in vivo* model systems in which to experimentally test the impact of *Blastocystis* on mammalian hosts and the gut ecosystem and determine which factors underlie these variable clinical outcomes. We evaluated a rat model for sustaining of a human-derived *Blastocystis* ST1 and assess colonization success and longevity. Because of the broad host range of *Blastocystis*, we compared the rat with three other rodent species to establish the reproducibility of our method. *Blastocystis* was introduced by esophageal gavage and colonization success evaluated by *Blastocystis* culture. Culture was also used to determine that all animals were negative prior to colonization and negative controls remain *Blastocystis*-free. In this study, *Blastocystis* ST1 established in 100% of the outbred rats (*Rattus norvegicus*) and gerbils (*Meriones unguiculatus*) challenged. Rats were colonized asymptomatically for more than one year, but *Blastocystis* ST1 was not transmitted between rats. *Mus musculus* strain CD1 and *Mastomys coucha* were not susceptible to *Blastocystis* ST1. Thus, rats appear to be a suitable *in vivo* model for studies of *Blastocystis* ST1, as do gerbils though testing was less extensive. This work lays the foundation for experimental work on the role of *Blastocystis* in health and disease.

1. Introduction

Blastocystis is a common unicellular eukaryote that colonizes a wide range of non-mammalian and mammalian hosts (Alfellani et al., 2013a; Clark et al., 2013), including an estimated one billion humans (Scanlan and Stensvold, 2013). This protist belongs to the Stramenopiles, with the genetically most related organisms belonging to algae such as kelp and diatoms, although Stramenopiles encompass amphibian symbionts within Opalinata (Adl et al., 2012). The life cycle of *Blastocystis* involves direct transmission of environmentally resistant cysts between hosts by the fecal-oral route (Tan, 2008; Wawrzyniak et al., 2013). *Blastocystis* is most commonly found as a small sphere-like structure in stool and in culture (vacuolar form), though multiple forms have been described (Clark et al., 2013; Stenzel and Boreham, 1996). For a long time, morphological uniformity masked the true diversity within *Blastocystis*; but small subunit ribosomal DNA sequencing has demonstrated extensive genetic variability across the genus *Blastocystis* (Clark et al., 2013; Gentekaki et al., 2017). To date, at least 17 subtypes (ST1–ST17) have been identified based on small subunit (SSU) ribosomal DNA analysis in birds and mammals, nine of which have been found in humans (e.g., Alfellani et al., 2013b; Clark et al., 2013; Roberts et al., 2013). Many of the subtypes exhibit only moderate host specificity (Alfellani et al., 2013a). More than 90% of human *Blastocystis* carriage can be attributed to ST1–ST4 (Clark et al., 2013); ST4 is only common in Europe while ST1–ST3 appear to be common worldwide (Alfellani et al., 2013b). Apart from humans, ST1 has so far been found in stool from non-human primates, artiodactyls, perissodactyls, carnivores, and birds (Alfellani et al., 2013a), but within ST1, different SSU rDNA alleles are found in organisms isolated from humans and non-human primates (Alfellani et al., 2013c), indicating host specificity at this level.

The pathogenicity of *Blastocystis* is debated in the literature, and one of the current working hypotheses is that some STs might be pathogenic in some situations (Clark et al., 2013; Poirier et al., 2012; Tan, 2008).

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https://doi.org/10.1016/j.exppara.2018.06.009
Received 27 March 2018; Received in revised form 18 June 2018; Accepted 26 June 2018
Available online 28 June 2018
0014-4894/

Blastocystis has often been explored in relation to gut diseases such as Irritable Bowel Syndrome (IBS) or Inflammatory Bowel Disease (IBD) (e.g., Boorom et al., 2008; Poirier et al., 2012; Tan, 2008). However, accumulating evidence suggests that *Blastocystis* is by and large a commensal organism, as it is common in healthy human populations (Parfrey et al., 2014; Petersen et al., 2013; Scanlan et al., 2014) and is more common in healthy individuals than in for instance individuals with IBS (Krogsgaard et al., 2015) and IBD (Andersen et al., 2015; Rossen et al., 2015). Indeed, the presence of *Blastocystis* may even be indicative of healthy gut microbiota (Andersen et al., 2015; Andersen and Stensvold, 2016; Audebert et al., 2016; Nieves-Ramírez et al., 2018). Given the multiple impacts *Blastocystis* can have on mammalian hosts we refer to *Blastocystis* as a symbiont, a term that encompasses pathogenic, commensal, and beneficial relationships (Chabé et al., 2017; Leung and Poulin, 2008; Lukeš et al., 2015). Hence, *in vivo* experimental models for studying *Blastocystis* are critical for understanding the impact of this symbiont on host health, immunology and gut microbiota as well as deciphering the causes of variable clinical outcomes that are observed.

A major obstacle to studying the host-*Blastocystis* relationship is the lack of a reliable animal experimental model (Ajjampur and Tan, 2016; Ehret et al., 2017). The most commonly used mouse model is not optimal because *Blastocystis* colonizations are quickly self-limiting and only young or immunosuppressed mice and susceptible (Ajjampur and Tan, 2016; Moe et al., 1997). In contrast, such susceptibility limitations appear not to pertain to rats, and rats appear to be more suitable for developing an animal experimental model (Chandramathi et al., 2010a; Hussein et al., 2008; Iguchi et al., 2007; Li et al., 2013; Yoshikawa et al., 2004) and has been already used for assessment of specific effect of *Blastocystis* spp. on the host organism (Chandramathi et al., 2010b, 2014; Iguchi et al., 2009). However, most studies in rats challenge young animals and have not confirmed the absence of *Blastocystis* prior to challenge (Ajjampur and Tan, 2016); detailed studies on the course of *Blastocystis* colonization in the rat model are lacking. Pigs also appear to be promising models for *Blastocystis* colonization (Fayer et al., 2014; Wang et al., 2014); however, the major limitation of this model are high costs for SPF breeding maintenance in contrast to rodent models. In general, rodents are the most preferred experimental models by broader scientific community due its cost, accessibility and controllable (Ehret et al., 2017).

In the present study we evaluated the rat model for human-derived *Blastocystis* ST1 to establish parameters of colonization and identify methodological pitfalls. Given the broad host ranges found in *Blastocystis* generally, we also compared the rat model to three other rodent species to establish its generality. This work lays the foundation for experimental work on the role of *Blastocystis* in health and disease.

2. Material and methods

2.1. Animals

Four rodent model systems were used: Specific Pathogen-Free (SPF) outbred Wistar rats (Envigo RMS B.V., Horst, Netherlands; the supplier Anlab s.r.o., Prague, Czech Republic), SPF CD1 white mice (bred in-house), SPF Southern multimammate mice (*Mastomys coucha*) (bred in-house), and SPF Mongolian gerbils (*Meriones unguiculatus*) (bred in-house). All animals were housed (rats) or breeding (CD1, *M. coucha*, *M. unguiculatus*) under SPF conditions in Individually Ventilated Cages systems (for small rodents and rats separately according to legislation requirements) and only sterilized chow (Complete feed mixture for SPF mice and rats, Velas a.s., Lysá n/L, Czech Republic), bedding and also tap water were used (all sterilized in autoclave always under specific conditions). Further, animals were housed under controlled temperature conditions (22 °C), photoperiod (12:12-h light-dark cycle), and were provided unlimited access to chow and water. Animal health status was visually inspected at regular 24-h intervals during daily routine. Animals were always acclimated to laboratory conditions prior to start of each experiment.

The study was carried out in the strict accordance with the recommendations in the Czech legislation (Act No. 166/1999 Coll., on veterinary care and on change of some related laws, and Act No. 246/1992 Coll., on the protection of animals against cruelty). The present experiments and protocols were approved by the Committee on the Ethics of Animals Experiments of the Biology Centre of the Czech Academy of Sciences (České Budějovice, Czech Republic) and by the Resort Committee of the Czech Academy of Sciences (Prague, Czech Republic).

2.2. Experimental set up

Experiments were carried out for two to four weeks, with the exception of the experiment establishing the longevity of *Blastocystis* ST1 colonization in rats, which was maintained for ca. one year. We used rats aged 16 weeks except for the experiment focused on the observation of age effect on *Blastocystis* ST1 colonization; here, we used rats aged 12, 16 and 20 weeks (this corresponds to three, four and five months respectively), and for the sensitivity experiment on other rodent models, we used younger than 8 and older than 8 week-old animals. Each experiment established the length of the prepatent period (the period from colonization to cyst shedding), and determined the start of the patent period (the initial day when shedding of cysts was observed). All animals were assessed for colonization with *Blastocystis* by xenic culture prior to the start of the experiment. All were negative.

Within each experiment we used three groups of animals (Fig. 1): an experimental group (n = 4 or 6), a positive control group (n = 4), and negative control group (n = 2) (for details see Fig. 1 and Table 1). Animals in the experimental group were challenged with *Blastocystis* from the fecal samples of donor (*Blastocystis*-positive human or positive rats). The positive control group involved the animals continuously colonized with *Blastocystis* cysts obtained from a donor human fecal sample, because such challenged animals exhibited a 100% colonization rate. This means that if some recipient animals (those animals

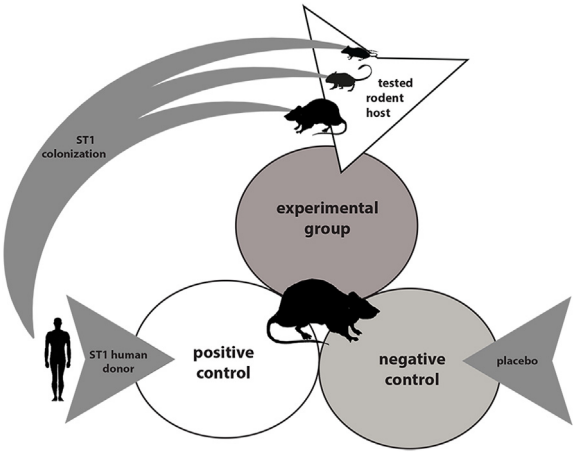

Fig. 1. Graphical visualization of the general experimental design. Within each experiment, we used three groups of animals: an experimental group (n = 4 or 6), a positive control group (n = 4), and negative control group (n = 2). Animals in the experimental group were challenged with *Blastocystis* ST1 from fecal material of the donor (*Blastocystis*-positive human, rats or gerbils). The positive control group involved animals always colonized with *Blastocystis* cysts obtained from a human fecal sample, because such challenged animals exhibited a 100% colonization rate – this means that if some recipient animals (those in experimental group) remained negative after *Blastocystis* colonization from non-human donors, we had a control group, which confirmed correct processing of doses with cysts using sucrose gradient. The negative control group included negative animals for *Blastocystis* ST1 and they were challenged with placebo (i.e., sterile saline).

Table 1
Summary of all performed experiments within the study focused on an evaluation of *Blastocystis* ST1 in the rat model system. The list of experiments is sorted according to the results section. Used animals: RN – *Rattus novergicus*, MC – *Mastomys coucha*, MM – *Mus musculus*, MU – *Meriones unguiculatus*; Type group: A-experimental group, B-positive control, C-negative control (for details see Fig. 1); Repetition – means number of performed experiments; *p.c.* – post colonization.

Experiment	Animals	Type group	Repetition	Age (weeks/ months)	Colonization length
Human-to-rat colonization	*RN*	**A** (n = 6) **C** (n = 2)	3 ×	16/4	12 colonized rats observed for ca. 2 months *p.c.*, 6 rats observed in experiment below
Longevity of colonization in rats	*RN*	**A** (n = 6)	1 ×	16/4	2 × 11th mnts, 3 × 12th mnts, 1 × 13th mnts
Age effect on colonization in rats	*RN*	**A** (n = 4) **C** (n = 2)	3 ×	12/3, 16/4, 20/5	long-term colonizations
Rat-to-rat colonization	*RN*	**A** (n = 4) **B** (n = 4) **C** (n = 2)	3 ×	16/4	–
Susceptibility of other rodent models	*MC* *MM* *MU*	**A** (n = 4) - 2 groups (< 8 weeks, > 8 weeks) **C** (n = 2)	1 ×	< 8 weeks > 8 weeks	observed for 4 months *p.c.* *MU* – colonizations observed for 15 mnts *p.c.*
Gerbil-to-rat colonization	*RN*	**A** (n = 4) **B** (n = 4) **C** (n = 2)	3 ×	16/4	–

supposed to acquire colonization) remained negative after *Blastocystis* inoculation from non-human donors, we had a control group, confirming correct processing of doses with *Blastocystis* ST1 cysts (Fig. 1). The negative control group included negative animals for *Blastocystis* ST1 and they were inoculated with placebo (sterile saline).

To ascertain that all animals were *Blastocystis*-free prior to each experiment, we examined them (three samples per animal collected on three consecutive days) using xenic *in vitro* cultivation (details below) prior to *Blastocystis* challenge. As a rule, all animals arriving in our animal facility are screened in order to ensure that they are free of any intestinal parasitic colonizations and, further, housed under SPF conditions to prevent colonization (for details, see above). We used animals of different ages in order to identify any age-related differences in susceptibility (for details, see summary of experiments in the Results section).

2.3. Doses of Blastocystis ST1 for colonization

Doses for colonization were prepared from donor human stool samples positive for *Blastocystis* ST1 using a sucrose gradient (Arrowood and Sterling, 1987): the principle of this gradient is based on the separation of particles of different density using two sucrose solutions (A, B) of different specific gravity (s. g.) prepared from Sheather's solution (s. g.- 1.27) and 20% TWEEN (Sigma–Aldrich, St. Louis, MI, USA) in PBS (Solution A: 100 mL Sheather's sucrose solution in 200 mL of 20% Tween/PBS, s. g. – 1.103; solution B: 50 mL of Sheather's sucrose solution in 200 mL of 20% Tween/PBS, s. g. – 1.064). In the first step, 30 mL of Solution A was added to the tubes, and then 30 mL of Solution B was layered on the top, followed by 15 mL of sample containing cysts. This suspension was centrifuged at 1870 × g/20 min/5 °C in a Hettich Universal 16R centrifuge (Hettich, Kirchlengern, Germany). Subsequently, the top 0.5 cm layer was removed, and the rest of the supernatant was transferred to clean tubes. Tubes were centrifuged again under the same conditions, then 3/4 of the resulted supernatant was removed and the sample was centrifuged again – this procedure was repeated three times. Eventually, a 3 cm-thick layer of sample remained, which was transferred to clean tubes and centrifuged again. The resulted sediment was used for preparation of doses of cysts.

The human donor was healthy and identified during a pilot study on the prevalence of *Blastocystis* in the gut-healthy human population in Czech Republic (unpublished data). One dose with *Blastocystis* ST1 cysts contained 10^3–10^4 cysts counted using a Bürker chamber (Fig. 2A).

For experiments designed to test the ability of rodent colonizations to be transmitted directly to rodents, we also prepared doses with cysts from fecal samples of rats and gerbils colonized with *Blastocystis* ST1 (Fig. 1). To increase the density of cysts in infectious doses from rat and gerbil feces, we collected fecal samples from *Blastocystis*-colonized animals over three consecutive days and stored the samples in PBS at room temperature. In these cases, one dose contained 10^2 cysts. All experimental animals used within this study were orally colonized using esophageal gavage.

2.4. Confirmation of Blastocystis ST1 colonization

Blastocystis colonization was confirmed following esophageal gavage with *Blastocystis* cysts using xenic *in vitro* cultivation of fresh stool with modified Jones' medium with 10% heat-inactivated horse serum (Leelayoova et al., 2002) as follows: A fecal sample about the size of a pea was transferred with a sterile swab to a glass cultivation tube (10 mL; Sigma–Aldrich) containing 4 mL of modified Jones' medium under anaerobic conditions (according to protocol described at http://www.blastocystis.net/p/lab-stuff.html).The culture was incubated at 37 °C for 72 h. Each culture was then subjected to sub-culturing by transferring 50–100 μL of the sediment using a sterile Pasteur pipette into 4 mL of fresh medium. Furthermore, each culture was examined with Lugol's Iodine by light microscopy at 400 × magnification (Olympus CX22LED) (Fig. 2B).

In order to identify the length of the *Blastocystis* ST1 prepatent period (i.e., the period before *Blastocystis* appeared in feces), the fecal samples were collected every day upon challenge of animals during the pilot study. Our results showed that *Blastocystis* ST1 cells start to appear from the 7th day post colonization (*dpc*) (Fig. 3). Based on this observation, we collected the fecal samples from challenged animals from the 7th *dpc* in order to evaluate whether animals were colonized or not (Fig. 3).

2.5. Evaluation of health status of rats colonized with Blastocystis ST1

In each experiment, we observed the health status of the animals challenged with *Blastocystis* ST1 using the following parameters: (i) development of unusual clinical signs, (ii) stool consistency, and (iii) body weight. We evaluated these parameters every week for each animal during each experiment.

2.6. Molecular diagnostics

Molecular detection and subtyping of *Blastocystis* relied on amplification of the SSU rRNA genes according to the method by Scicluna et al. (2006) and consensus terminology (Alfellani et al., 2013a; Stensvold et al., 2009). The cysts from the human sample were identified as *Blastocystis* ST1, and thus the same protocol was used for confirmation of the presence of *Blastocystis* ST1 in experimental

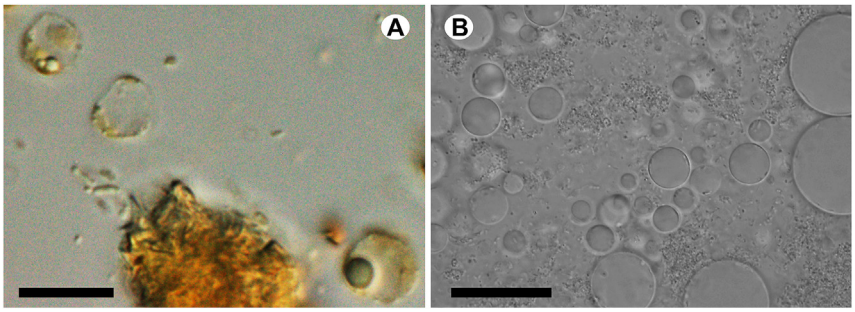

Fig. 2. *Blastocystis* ST1 life stages documentation. (A) Thick-walled cyst stage of *Blastocystis* ST1 with central vacuole and multiple nuclei on periphery, obtained from human donor detected using Merthiolate-Iodine-Formaldehyde (MIF) sedimentation stained with Lugole Iodine for better visualization and documented using microscope Olympus BX53/DP73; scale bar = 5 µm. (B) Vacuolar forms of *Blastocystis* ST1 from rat samples detected using xenic *in vitro* culture; scale bar = 50 µm.

animals. Total DNA was extracted using the PSP Spin Stool DNA Kit (Stratec Biomedical, Birkenfeld, Germany) according to the manufacturer's instructions. PCR amplicons were cloned using TOPO TA Cloning kit/PCR 2.1 – TOPO vector (ThermoFisher Scientific, Waltham, MA, USA). The obtained clones were cleaned using the High Pure Plasmid Isolation Kit (Roche Diagnostic, Basel, Switzerland), and then sequenced by GATC Biotech (Constance, Germany). As a rule, we take three clones per animal after confirmation of colonization using xenic *in vitro* cultivation. The sequences were compared with reference sequences in the *Blastocystis* subtype (SSU rRNA gene) and Sequence Typing (MLST) database available at http://pubmlst.org/blastocystis/.

3. Results

To identify the optimal animal experimental model for further studies of *Blastocystis* ST1, we carried out several experiments as summarized below (for details see Table 1).

3.1. Colonization of rats with Blastocystis ST1 cysts isolated from a healthy human donor

The aims of this experiment were to identify the susceptibility of outbred Wistar rats to colonization of *Blastocystis* ST1 isolated from human stool and to determine the length of the prepatent period. To confirm the results, we performed three independent experiments with two groups, an experimental group (n = 6) and a control group (n = 2) (Table 1). Fecal samples were collected every day following challenge with *Blastocystis* cysts from the human donor, and subject to culture, and examination. *Blastocystis* ST1 was detected in all animals between 7th and 12th *dpc*. Thus, Wistar rats are susceptible to *Blastocystis* ST1 colonization and colonization success rate was 100%. The prepatent period varied between seven and twelve days (Fig. 3), but generally lasted for 12 days, as *Blastocystis* ST1 was predominantly detected in rat stool by culture and/or observation only on the 12th *dpc* (Fig. 3).

3.2. Longevity of Blastocystis ST1 colonization in outbred rats

The aim of this experiment was to determine for how long *Blastocystis* ST1 colonization could persist in outbred rats. We performed long-term monitoring (ca. 14 months) of outbred rats colonized with *Blastocystis* ST1 chosen from the previous experiment with only one group (n = 6; see Table 1). Their fecal samples were collected and cultivated using xenic *in vitro* cultivation every week for assessment of *Blastocystis* ST1 colonization. This colonization could be detected for at least 11 months in all animals; however, spontaneous resolution was seen later on in all animals between the 11th and 13th month post colonization (*p.c.*) (Fig. 3 and Table 1). The rats were further monitored till the 14th month *p.c.*, but all remained negative.

3.3. Age effect on susceptibility to Blastocystis ST1 colonization in rats

The aim of the experiment was to reveal whether the age of outbred Wistar rats influences susceptibility to *Blastocystis* ST1 colonizations. Wistar rats were grouped according to age (12, 16 and 20 months old); hence, three groups (n = 4) of rats were included (for details, see Table 1). Colonization was established in all rats across all age categories in the same colonization system as described above (Fig. 3), indicating that age does not influence susceptibility to *Blastocystis* ST1 colonizations in outbred rats.

3.4. Colonization of rats with Blastocystis ST1 cysts obtained from rats

The aim of this experiment was to find out whether transmission of *Blastocystis* ST1 originally isolated from human stool is possible between outbred Wistar rats. Hence, we prepared doses with cysts from rats colonized with *Blastocystis* ST1 isolated from human stool and shedding *Blastocystis* ST1 cysts (Table 1). We used two types of doses with cysts; the first type was prepared directly from fresh feces, while the second type of dose was prepared from feces collected over three consecutive days and stored in PBS at room temperature. However, in all experiments the rats remained negative upon challenge except

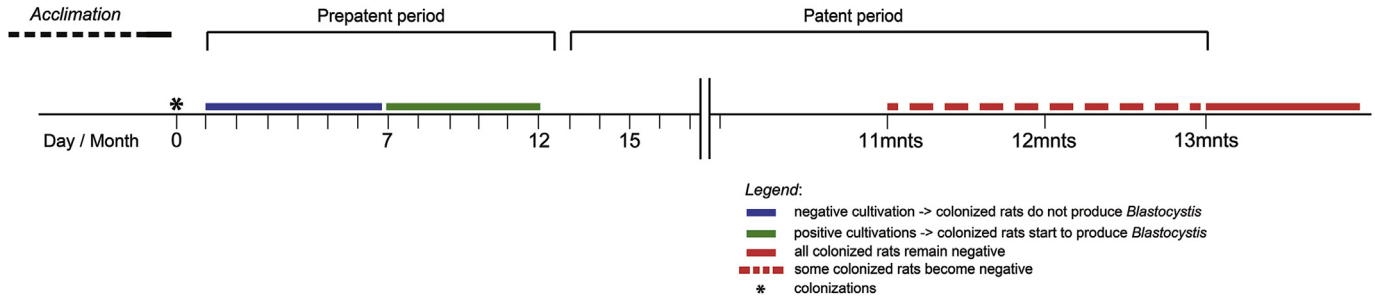

Fig. 3. Graphical visualization of *Blastocystis* ST1 colonizations from a human donor in the rat model system. Each experiment established the length of the prepatent period (the period from colonization to cyst shedding) and determined the beginning of the patent period (the initial day when shedding of cysts was observed). Fecal samples were collected every day upon challenge with *Blastocystis* cysts from the human donor, cultivated, and examined. *Blastocystis* ST1 was detected in all animals between 7th and 12th day post colonization (*dpc*). Thus, Wistar rats are susceptible to *Blastocystis* ST1 colonization from a human donor, and the colonization success rate was 100%. The prepatent period varies between 7th and 12th day *dpc*, but generally lasts 12 days.

positive control (Fig. 1).

3.5. Health status of rats colonized with Blastocystis ST1

We detected no detrimental effect on the health status of colonized rats; i.e., no clinical manifestations or changes in stool consistency were observed. Moreover, the body weight of all animals ranged between 190 and 230 grams, with no differences between colonized and non-colonized animals.

3.6. Susceptibility of other rodent models to Blastocystis ST1 colonizations

We performed experimental colonizations in other laboratory rodent models, namely CD1 white mice, multimammate mice (*Mastomys coucha*), and gerbils (*Meriones unguiculatus*). Mice were previously shown to be susceptible to *Blastocystis* only when colonized at an age younger than eight weeks (Moe et al., 1997). Therefore, we tested our ability to challenge these rodents with *Blastocystis* ST1 colonizations at two age levels: younger than eight weeks and older than eight weeks (Table 1). Interestingly, we detected *Blastocystis* ST1 colonizations in gerbils in both age categories, and the longevity of colonization was also long-term, with the colonization persisting until sacrifice of old animals at the age of ca. 15 months. Gerbils remained healthy as assessed by the observation of stable stool consistency and absence of clinical symptoms. All mice remained negative, and we continued examining stool samples for a month past the expected patent period to confirm this negative result.

3.7. Colonization of rats with biological material obtained from gerbils positive for Blastocystis ST1

The aim of this experiment was to investigate whether human-derived *Blastocystis* ST1 colonization could be transmitted directly from gerbils to rats. If so, this would remove the necessity of using human samples for experimental colonizations. The outbred rats were challenged with doses of cysts prepared from the *Blastocystis* ST1-colonized gerbils (Table 1). The rats remained negative except positive control (Fig. 1). These results were confirmed in a second experiment.

3.8. Molecular characterization of Blastocystis identified in the stool of experimental animals

All *Blastocystis* found in the stool of the experimental animals used in the present study were confirmed to be *Blastocystis* ST1 (sequence with accession no. MH021854 was deposit in GenBank).

4. Discussion

The question of whether *Blastocystis* (or any particular subtype of *Blastocystis*) is a pathogen or a commensal remains unanswered, due to conflicting results from epidemiological surveys and case reports (e.g., Gill et al., 2016; Krogsgaard et al., 2015; Parfrey et al., 2014; Petersen et al., 2013; Poirier et al., 2012; Rossen et al., 2015). To move investigations of the impact of *Blastocystis* on human health beyond correlations and delve into causative and mechanistic relationships with the host gut, immune system, and host-associated microbiota, it is essential to identify a suitable experimental animal. *Blastocystis* establishes long-term colonization of humans (Scanlan and Stensvold, 2013), and therefore relevant models should permit long-term and stable colonization.

Here, we test the hypothesis that the rat is a suitable model for experimental colonization with *Blastocystis* ST1 colonizations derived from humans. Rats are becoming more commonly used to test effects of *Blastocystis* spp. on the host organism (summarized in Ajjampur and Tan, 2016). Previous work demonstrated only short-term colonization of mice with human-derived *Blastocystis*, and only in a limited developmental window (Ajjampur and Tan, 2016; Moe et al., 1997). In contrast, several studies suggest that rats are more permissive, with even small doses of cysts sometimes leading to long-term colonizations (when observed) in Wistar and Sprague–Dawley rats (Chandramathi et al., 2014; Chen et al., 1997; Iguchi et al., 2009; Hussein et al., 2008; Li et al., 2013; Yoshikawa et al., 2004). In the present study, we established stable reservoir colonizations of *Blastocystis* ST1 originally isolated from a healthy human male in outbred SPF Wistar rats and observed no detrimental effects on the rat in terms of behavior, weight status, or fecal consistency. We extend previous work by establishing (i) the length of the pre-patent period, (ii) the longevity of colonization, and (iii) assessing the possibility of transmission by cysts isolated form rat and gerbil feces.

To be of greatest research value, the animal model should mimic human *Blastocystis* colonization, thus enabling downstream experiments comparing the impact of potentially pathogenic versus commensal *Blastocystis* subtypes. Importantly, rats appear to reflect the pathogenicity characteristics of human *Blastocystis* carriage, opening the possibility of studying the interaction of this common symbiont across the span of clinical outcomes observed in human populations. Li et al. used *Blastocystis* ST1 isolated from a human donor with diarrhea and showed that the rats also showed symptoms upon colonization, predominantly lethargy and infiltration of intestinal mucosa by *Blastocystis* ST1 (Li et al., 2013). Other studies also suggest correlations between human and rat symptoms following colonization with *Blastocystis* derived from human donors (Chandramathi et al., 2014; Hussein et al., 2008; Kumarasamy et al., 2017), though these studies have limitations. A study by Iguchi et al. demonstrated a pathogenicity of *Blastocystis* ST4 on rats experimentally colonized by *Blastocystis* from reservoir rats (Iguchi et al., 2009). Interestingly, *Blastocystis* ST4 was frequently detected in human patients with acute diarrhea (Clark et al., 2013; Stensvold et al., 2011). The human donor colonized by *Blastocystis* ST1 used in the present study showed no clinical symptoms within several years of observation, and we did not detect overt pathohistology in the colonized rats. Similarly, a few other studies observed no clinical symptoms in experimental rats colonized by *Blastocystis* from human donors (Chandramathi et al., 2010a; Iguchi et al., 2007; Yoshikawa et al., 2004). A key future direction for this model with rats will be assessing the impact on the rat host of *Blastocystis* isolated from human donors with diarrhea and other clinical symptoms to determine whether the pathogenicity is transferred to the rat model. The diverse effects of *Blastocystis* ST1 on mammalian hosts might be caused (i) by intra-subtype genetic variability in pathogenicity traits (Clark et al., 2013), (ii) by different intensity of colonization that might have some effect (Pavanelli et al., 2015) or (iii) by accompanying microorganisms. Several studies have shown that *Blastocystis* is associated with higher bacterial diversity in fecal microbiota than *Blastocystis*-free subjects (Andersen et al., 2015, 2016; Audebert et al., 2016; Nieves-Ramírez et al., 2018). As a result, Ajjampur and Tan (2016) argue that future studies with *in vivo* models that use xenic *Blastocystis* for colonization need to disentangle the impacts of gut microbiota and *Blastocystis*, During our experiments, we observed that the prepatent period of *Blastocystis* ST1 colonization ranged from seven to twelve days, but most animals started shedding cysts on the 12th *dpc*. We found similar results by culture; vacuolar forms appeared in xenic cultures of rat stool between the 7th and the 12th *dpc*. These results are consistent with other rat experimental studies on *Blastocystis* using *per os* administration of cysts, which found that the length of the prepatent period ranged from six to 15 days post colonization (Iguchi et al., 2007, 2009; Li et al., 2013; Yoshikawa et al., 2004).

We found that human-derived *Blastocystis* ST1 establishes long-term colonizations in rats, mimicking characteristics of human *Blastocystis* colonization, and we also found that host age does not influence susceptibility. *Blastocystis* establishes long-term colonizations in humans; therefore, an animal model should also ideally permit long-term colonizations. In experiments assessing the longevity of *Blastocystis* ST1

colonizations, we found that these were maintained for 11–13 months *p.c.*. No previous studies of *Blastocystis* colonization in rat models have included such information. Moe et al. included data on the maximal duration of *Blastocystis* sp. colonization in a mouse model, which ranged from two to three weeks depending on the age of the animals (Moe et al., 1997). Another important aspect monitored in our study was the effect of age on the susceptibility to *Blastocystis* colonizations in rats, as humans of all ages and with robust immune systems can be colonized with *Blastocystis*. In contrast to humans, only young mice (3–8 weeks of age) are susceptible to *Blastocystis* observed colonization (Moe et al., 1997), but were not susceptible at any age in our study. In contrast, in the present study, rats of all ages could be consistently colonized with *Blastocystis* ST1 cysts derived from human stool. We successfully colonized rats aged three, four, and five months. Other studies demonstrated the susceptibility of rats to *Blastocystis* spp. in animals aged three to six weeks (Iguchi et al., 2007; Hussein et al., 2008; Li et al., 2013; Yoshikawa et al., 2004), demonstrating that both developing and mature rats can readily be colonized.

Together, these observations suggest that the rat model is amenable to research on interactions between *Blastocystis* subtypes and the host. One important limitation is the inability to transfer human-derived *Blastocystis* ST1 colonization between rats. We performed several experiments with doses containing *Blastocystis* ST1 cysts prepared from the feces of *Blastocystis*-positive rats, but all recipient animals remained negative. In contrast, controls in these experiments and throughout using *Blastocystis* ST1 cysts isolated directly form a human donor worked 100% of the time. One of the explanations could be that *Blastocystis* ST1 does not properly encyst in the rat gut due to different physiological factors or gut microbiome composition (Skotarczak, 1984). Iguchi et al. showed that different *Blastocystis* subtypes (ST2, ST3, ST4 and ST7) may differ in their ability to encyst in various host (chicken, rats and humans) (Iguchi et al., 2007). Interestingly, the gut ciliate, *Balantioides coli*, encysts only in its reservoir hosts (suids), whereas in other hosts, such as humans and great apes, *B. coli* does not encyst and only trophozoite stages are shed in feces (Pomajbíková et al., 2010). *Blastocystis* forms two types of cysts – thick-walled ones responsible for transmission between hosts, and thin-walled one responsible for autoinfection within host gut (Singh et al., 1995). Our observations might suggest that the cysts observed in *Blastocystis*-positive rats contain only thin-walled cysts that are unable to survive in the environment or colonize other individuals.

We tested other rodent models for their ability to produce infective cysts of human-derived *Blastocystis* ST1, which would enable research without the need to continually access and manipulate human donor stool to prepare doses of cysts. We colonized CD1 mice, multimammate mice, and gerbils with *Blastocystis* ST1 isolated from a human donor, and only gerbils proved susceptible to colonization regardless of age. While others have successfully colonized mice younger than eight weeks (Ajjampur and Tan, 2016; Moe et al., 1997), we did not succeed in doing so (Table 1). Several explanations for this discrepancy are possible, including: (i) use of different mouse genotypes – we use CD1 while others used BALB/c, albino mice and others, which may be more sensitive to *Blastocystis* colonization, and (ii) use of different *Blastocystis* subtypes – most previous *in vivo* mouse models did not determine subtype (summarized in Ajjampur and Tan, 2016).

The prepatent period in experimentally colonized gerbils ranged between five and seven *dpc*, with colonization lasting for 15 months. Interestingly, gerbils are often used for experimental encystation of *Giardia duodenalis* trophozoites obtained from *in vitro* culture because of their higher pH of stomach and hindgut (Beasley et al., 2015). Such colonized gerbils are able to produce *G. intestinalis* cysts, which can be used for subsequent colonization of other experimental animals (D. Květoňová *personal communication*). However, in case of *Blastocystis* ST1, the rats remained repeatedly negative after inoculation of *Blastocystis* ST1 cysts isolated material from gerbils. Thus, gerbils do not produce infective *Blastocystis* ST1 cysts.

5. Conclusion

Based on our results, the rat model of *Blastocystis* ST1 colonization appears to be a highly promising experimental model for testing of the impact of this important and but poorly understood symbiont on mammalian hosts (including humans) and determination of the factors that underlie the variable clinical outcomes of *Blastocystis* carriage. The future use of this *in vivo* model may lay the very foundation for experimental work leading towards finally pinpointing the role of *Blastocystis* in health and disease.

Declarations of interest

None.

Financial support

This work is financially supported by Young Investigators grant from the Human Science Frontiers Program (RGY0078/2015) to KJP and LWP, and by European projects (KJP): Modbiolin (7th Framework programme, no. 316304) and European project for the promotion and popularization of research, development and innovation (Operational Programme).

Acknowledgements

This work could not have be done without a great support of our colleagues, Martin Kváč and Bohumil Sak, from the Laboratory of Veterinary and Medical Protistology (Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exppara.2018.06.009.

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