MORPHOLOGY DESCRIPTION, SUBTYPING, DISTRIBUTION AND PHYLOGENY OF *Blastocystis* sp. IN LIVESTOCK ANIMALS OF TWO WEST COAST STATES IN MALAYSIA

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

~	Approximately
*	Asterisk
χ^2	Chi-squared test
o	Degrees
=	Equal to
>	Greater than
km	Kilometre
km^2	Kilometre squared
<	Less than
≤	Less than or equal to
µl	Microlitre
µm	Micrometre
mg	Milligrams
mA	Milli Amperes
mM	Milli Molar
-	Minus
M	Molar
nm	Nanometre
%	Percentage
±	Plus, or minus
P	Probability
√	Tick

LIST OF ABBREVIATIONS

ACTG	Adenine, Cytosine, Thymine, Guanine
ANOVA	Analysis of Variance
BIC	Bayesian Information Criterion
BLAST	Basic Local Search Alignment Tool
C	Celsius
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
DVS	Department of Veterinary Services
EDTA	Ethylenediaminetetraacetic Acid
GIS	Geographic Information System
G+I	Gamma distribution and evolutionarily invariable
HDMS	Hexamethyldisilazane
HKY	Hasegawa-Kishino-Yano
IACUC	Institutional Animal Care and Use Committee
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IL	Illinois
Inc.	Incorporated
$MgCl_2$	Magnesium Chloride
ML	Maximum Likelihood
MLST	Multilocus Sequence Typing
NCBI	National Centre for Biotechnology Information
NGS	Next Generation Sequencing
NHP	Non-Human Primate
NJ	Neighbor Joining
OR	Odds Ratio
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
qPCR	Real-time Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA

rpm	Revolutions Per Minute
RFLP	Restriction Fragment Length Polymorphism
SAR	Stramenopiles, Alveolata, and Rhizaria
SEM	Scanning Electron Microscopy
sp.	Species
SPSS	Statistical Package for Social Science
SSU rDNA	Small Sub-Unit Ribosomal Deoxyribonucleic Acid
SSU rRNA	Small Sub-Unit Ribosomal Ribonucleic Acid
ST	Subtype
STS	Sequence Tagged Site
T92	Tamura 3-Parameter
Taq	*Thermus aquaticus*
TEM	Transmission Electron Microscopy

LIST OF APPENDICES

DESKRIPSI MORFOLOGI, SUBJENIS, TABURAN, DAN FILOGENI *Blastocystis* sp. DALAM HAIWAN TERNAKAN DARI DUA NEGERI DI PANTAI BARAT MALAYSIA

ABSTRAK

Blastocystis sp. merupakan protista unisel, anaerobik yang menghuni usus bagi pelbagai perumah haiwan, termasuk manusia. Ia merupakan organisma menarik dari pelbagai aspek biologi, epidemiologi, walaubagaimanapun patogeninya masih tidak diketahui. Di Malaysia, industri penternakan merupakan sektor yang sedang giat berkembang, dengan menyediakan sumber protein yang baik, peluang pekerjaan kepada populasi umum, dan merupakan suatu jaringan bagi kontak manusia-haiwan. Kajian ini adalah bertujuan untuk menyelidik deskripsi morfologikal dan epidemiologikal bagi *Blastocystis* dalam haiwan ternakan dari Pulau Pinang dan Perak, Malaysia serta peranan penting haiwan ternakan dalam transmisi organisma ini kepada manusia. Sejumlah 701 haiwan ternakan telah disaring bagi jangkitan *Blastocystis* di Pulau Pinang dan Perak, Malaysia. Di Pulau Pinang, sampel tinja segar daripada 127 lembu, 149 kambing, 100 biri-biri, dan kandungan seka daripada 174 burung puyuh telah menjalani pengkulturan *in-vitro* menggunakan medium Jones terubahsuai. Bentuk dan dimensi bagi sel *Blastocystis* daripada perumah tersebut telah dinilai menggunakan pemerhatian mikroskopik cahaya bagi slaid terwarna-Giemsa, manakala elektron mikroskop telah digunakan untuk menghuraikan struktur permukaan bagi pencilan yang terpilih. Kaedah kod bar DNA telah digunakan untuk pengecaman subjenis; setelah itu, analisis filogenetik bagi jujukan-jujukan yang diperoleh dilakukan. Di Perak, sampel tinja daripada 51 lembu, 50 kambing, dan 50

biri-biri telah disimpan pada -20ºC, sebelum menjalani pemeriksaan melalui kaedah kod bar DNA, dan seterusnya analisis filogenetik bagi jujukan-jujukan yang diperoleh. Keseluruhannya, di Pulau Pinang sebanyak 21.3% (117/550) haiwan yang disaring adalah positif *Blastocystis* sp. Prevalens jangkitan adalah lebih signifikan tinggi dalam kambing (35.6%) berbanding dalam lembu (15.7%), biri-biri (14%) dan burung puyuh (17.2%) (x^2 = 25.349, $p < 0.001$). Jantina, sistem penternakan, dan daerah dari mana haiwan tersebut di sampel telah dikenalpasti sebagai faktor risiko potensi yang berhubung kait dengan jangkitan *Blastocystis* sp. dalam haiwan ternakan ruminan di Pulau Pinang. Kebanyakan bentuk vakuolar membulat dan beberapa bentuk granular bagi *Blastocystis* diperhatikan dalam haiwan ternakan dari Pulau Pinang menggunakan mikroskop cahaya. Pencilan *Blastocystis* daripada burung puyuh menunjukkan saiz yang lebih besar (9.09 to 33.33 μm) daripada yang lain, manakala pencilan kambing adalah diameter paling kecil (1.40 to 4.40 μm). Min diameter sel bagi pencilan daripada ruminan kecil (kambing dan biri-biri) adalah berbeza secara signifikan daripada lembu dan burung puyuh ($p \leq 0.001$). Seperti yang ditunjukkan melalui kajian ultrastruktur, pencilan *Blastocystis* daripada haiwan ruminan adalah kasar, nipis dan kot permukaan tebal, manakala pencilan daripada burung puyuh mempunyai kot permukaan tebal dengan tekstur serat berspan dan berlekuk-lekak. *Blastocystis* sp. ST4, ST5, ST10, ST13, ST14, ST15, dan ST25 telah dikenalpasti dalam haiwan ternakan ruminan dari Pulau Pinang; ST5 and ST14 adalah yang paling kerap kali dikenalpasti. *Blastocystis* sp. ST6 merupakan satu-satunya ST yang diperhatikan dalam burung puyuh. Tiga subjenis iaitu, ST5, ST10, dan ST14 dikesan daripada haiwan ternakan ruminan dari Perak yang mana ST10 adalah yang paling lazim. Analisis filogenetik menyokong penempatan subjenis dengan sokongan 'bootstrap' yang tinggi, kecuali dalam kes pencilan ST5 daripada lembu di Perak di mana ia

bergabung dengan jujukan-jujukan bagi rujukan ST21 dengan sokongan bootstrap yang sederhana melalui kaedah hubung kait jiran dan persamaan maksimum, masing-masing. Kajian ini telah menunjukkan jangkitan *Blastocystis* zoonotik dan enzootik dalam haiwan ternakan di kawasan kajian. Ia juga mendedahkan bahawa tekstur berserat pada permukaan sel *Blastocystis* berbeza antara perumah haiwan.

MORPHOLOGY DESCRIPTION, SUBTYPING, DISTRIBUTION AND PHYLOGENY OF *Blastocystis* sp. IN LIVESTOCK ANIMALS OF TWO WEST COAST STATES IN MALAYSIA

ABSTRACT

Blastocystis sp. is a unicellular, anaerobic protist inhabiting the intestinal tract of diverse animal hosts, including humans. It is a fascinating organism with various aspects of its biology, and epidemiology, even though the pathogenicity is still unknown. In Malaysia, the livestock industry is a continuously growing sector, providing a good source of protein, employment to the general population, and an avenue for constant human-animal contact. This study was aimed at investigating the morphological description, subtypes, distribution, and phylogeny of *Blastocystis* in livestock animals from Penang and Perak, Malaysia, and the possible role of livestock animals in the transmission of this organism to humans. A total of 701 livestock animals were examined for *Blastocystis* infection in Penang and Perak, Malaysia. In Penang, fresh faecal samples from 127 cattle, 149 goats, 100 sheep, and caecal contents from 174 quails were subjected to *in-vitro* cultivation using a modified Jones medium. Forms and dimensions of *Blastocystis* cells from these hosts were assessed using light microscopic observations of Giemsa-stained slides, while electron microscopy was used to describe the surface structure of selected isolates. DNA barcoding method was used for subtype identification; thereafter, phylogenetic analyses of sequences obtained were carried out. In Perak, faecal samples from 51 cattle, 50 goats, and 50 sheep were preserved at -20ºC, before undergoing screening by DNA barcoding method, and subsequent phylogenetic analyses of sequences

obtained. Overall, in Penang, a total of 21.3% (117/550) of animals screened were positive for *Blastocystis* sp. The prevalence of infection was significantly higher in goats (35.6%) than in cattle (15.7%), sheep (14%) and quail (17.2%) ($x^2 = 25.349$, $p < 0.001$). Gender, farming system, and district from which the animals were sampled were identified as potential risk factors associated with *Blastocystis* sp. infection among ruminant livestock animals in Penang. Numerous round-shaped vacuolar forms and a few granular forms of *Blastocystis* were observed in livestock animals from Penang using light microscopy. *Blastocystis* isolates from quails appeared larger in size (9.09 to 33.33 μm) than others, while isolates from goats were the smallest in diameter (1.40 to 4.40 μm). The mean cell diameter of isolates from small ruminants (goat and sheep) differed from those of cattle and quail significantly ($p \leq 0.001$). As revealed by ultrastructural studies, *Blastocystis* isolates from ruminant animals had a rough, thin and dense surface coat, while isolates from quails had a thick surface coat with fibrous spongy texture and indentations. *Blastocystis* sp. ST4, ST5, ST10, ST13, ST14, ST15, and ST25 were identified in ruminant livestock animals from Penang; ST5 and ST14 were the most frequently identified. *Blastocystis* sp. ST6 was the only ST observed in quail. Three subtypes namely, ST5, ST10, and ST14 were detected from ruminant livestock animals from Perak in which ST10 was the most common. Phylogenetic analyses supported the subtypes allocated to isolates with high bootstrap support, except in the case of ST5 isolates from cattle from Perak which clustered with ST21 reference sequences with moderate bootstrap supports by the Neighbour-joining and Maximum likelihood methods, respectively. This study has demonstrated zoonotic and enzootic *Blastocystis* infection in livestock animals in the study area. It has also revealed that the fibrous texture of *Blastocystis* cell surface varies between the host animals.

CHAPTER 1

INTRODUCTION

1.1 Research background

Blastocystis sp. is a single-celled, anaerobic protist that resides in the intestinal tract of various animals, including humans, all over the world (Skotarczak, 2018). It is an enteric eukaryotic symbiont that is frequently found in mammals and birds (Tan, 2008; Adao and Rivera, 2018). *Blastocystis* was first identified very early in the 20th century; however, substantial advancements in comprehending its biology did not occur until the late 1900s (Tan *et al.*, 2008). *Blastocystis* sp. belongs to the Stramenopiles, a complex assemblage of "botanical protists" consisting of heterotrophic and photosynthetic members (Silberman *et al.*, 1996; Tan, 2008; Ahmed and Karanis, 2018). It is often referred to as an unusual Stramenopile due to its anaerobic nature and lack of flagella or tubular hairs (Clark *et al.*, 2013; Ajjampur and Tan, 2016; Stensvold and Clark, 2016a). It is a fascinating organism from numerous perspectives (Arisue *et al.*, 2002).

Blastocystis sp. is extremely polymorphic; six morphological forms have been identified (Lepczyńska *et al.*, 2017), with each form showing extensive variations in size (Parija and Jeremiah, 2013). Four forms are most typically described in the literature - granular, vacuolar, cyst, and amoeboid forms (Tan *et al.*, 2002; Ajjampur and Tan, 2016); other less frequently encountered forms are the multivacoular and avacuolar (Stenzel and Boreham, 1996; Ahmed and Karanis, 2018). Although some degree of ultrastructural variation has been discussed (Stenzel *et al.*, 1997; Lee and Stenzel, 1999; Yoshikawa *et al.*, 2007), *Blastocystis* sp. isolates from different hosts have been described to have very similar morphology (Beghini *et al.*, 2017).

Blastocystis also exhibits genetic polymorphism based on nucleotide differences of its small subunit ribosomal RNA (SSU rRNA) gene (Clark, 1997; Stensvold *et al.*, 2007; Alfellani *et al.*, 2013a; Yoshikawa *et al.*, 2016b; Liu *et al.*, 2022). While *Blastocystis* organisms of significant genetic variability have been isolated from the same host species, genetically similar isolates have also been identified from different host species (Yoshikawa *et al.*, 2016b). Assigning species epithet to *Blastocystis* isolates according to host origin is, consequently, impractical and misleading (Stensvold *et al.*, 2007; Tan, 2008; Clark *et al.*, 2013). Stensvold *et al.* (2007) proposed a naming system by which genetic variants were identified as *Blastocystis* sp. subtypes, and each subtype of *Blastocystis* was assigned a number. Hence an isolate is labeled *Blastocystis* sp. subtype *n*, where *n* is the number allocated to the subtype to which it belongs.

Based on phylogenetic analysis of the small subunit ribosomal RNA (SSU rRNA) gene of *Blastocystis* sp., about 32 subtypes (STs 1 - 32) of *Blastocystis* have been proposed in a wide range of hosts such as humans, other mammals, and birds (Stensvold *et al.*, 2012b; Alfellani *et al.*, 2013b; Lepczyńska *et al.*, 2017; Stensvold and Clark, 2020; Higuera *et al.*, 2021; Maloney *et al.*, 2021a; Maloney *et al.*, 2021b). Twenty eight of these subtypes have been acknowledged while four are deemed invalid and are awaiting either confirmation or rejection by further data (Stensvold and Clark, 2020). In the literature, ten *Blastocystis* subtypes (STs 1 - 9, and ST12) have been very commonly reported as human subtypes, with ST9 typically considered to be an exclusively humans subtype (Clark *et al.*, 2013; Ahmed and Karanis, 2018; Lhotská *et al.*, 2020; Stensvold *et al.*, 2020); while ST10, ST11, and ST13 to ST32 have also been typically regarded as non-human subtypes only (Stensvold *et al.*, 2009; Parkar *et al.*, 2010; Higuera *et al.*, 2021). Nevertheless, *Blastocystis* sp. ST9 has been identified in

chickens from Malaysia (Noradilah *et al.*, 2017a) and in ring-tailed lemurs from China (Ma *et al.*, 2020a); also very recently, the isolation of ST10 and ST14 from healthy Senegalese school children (Khaled *et al.*, 2020), ST10 from a Syrian refugee in Lebanon (Khaled *et al.*, 2021) and ST16 from Colombian children (Osorio-Pulgarin *et al.*, 2021) were reported.

The genetic diversity exhibited by *Blastocystis* sp. is one of the main factors contributing to its ubiquitous nature (Ramírez *et al.*, 2017). It is estimated that over one billion individuals are infected with *Blastocystis* sp. worldwide. Generally, a higher prevalence has been reported in humans in developing countries than in developed countries, and this has been related to exposure to animals, waste disposal, standards of hygiene, and consumption of contaminated food or water (Tan *et al.*, 2008). An estimated prevalence of *Blastocystis* sp. in animals (in general) is not available, nonetheless, there is the likelihood of infection rate being higher in animals than in humans based on information from various studies on different animal hosts (Betts *et al.*, 2020). From several parts of the globe, mammals, aves, reptiles, amphibians, fishes, and insects have been found to carry *Blastocystis* sp.; and in Southeast Asia, Malaysia serves as one of countries in which the most investigations on *Blastocystis* sp. in animals have been conducted. Wild and companion animals, livestock animals, birds, reptiles, and arthropods from Malaysia have been reported as hosts to several *Blastocystis* sp. STs (Chuong *et al.*, 1996; Suresh *et al.*, 1997; Tan *et al.*, 2013; Chandrasekaran *et al.*, 2014; Farah Haziqah *et al.*, 2018a; Farah Haziqah *et al.*,2018b; Farah Haziqah *et al.*,2018c; Adrus *et al.*, 2019).

Transmission of *Blastocystis* sp. is believed to be by faecal-oral routes (Clark *et al.*, 2013), primarily by consuming food or drink contaminated with cysts (Tan *et*

al., 2008) or through contact with infected humans and animals (Maloney *et al.*, 2021b). The role of animals as reservoirs of *Blastocystis* strains colonizing humans is, up till now, vague (Valença-Barbosa *et al.*, 2019). The low host-specificity of *Blastocystis* sp. subtypes indicates possible zoonotic transmission (Adao and Rivera, 2018) and suggests that animals could be a significant potential reservoir for spreading the infection to humans (Ahmed and Karanis, 2018). Infection of rats and chickens with *Blastocystis* sp. isolates from humans under experimental conditions have been successful; this achievement establishes the possibility that *Blastocystis* sp. could be transmitted between human and animal (Iguchi *et al.*, 2007; Ajjampur and Tan, 2016; Růžková *et al.*, 2018). Besides, higher risk and frequency of *Blastocystis* sp. infection have been described in animal handlers and feeders for example those working in zoos, slaughterhouses, and research facilities, and those involved in intensive animal farming or industrial livestock production; this category of people have continuous interactions with animals (Rajah Salim *et al.*, 1999; Abe *et al.*, 2002; Tan, 2008; Stensvold *et al.*, 2009; Parkar *et al.*, 2010; Ahmed and Karanis, 2018). The above-mentioned reports immensely emphasize that *Blastocystis* sp. has zoonotic capabilities. Nonetheless, additional proof is still needed to conclusively establish that zoonotic transmission of *Blastocystis* sp. occurs (Parkar *et al.*, 2010; Lee *et al.*, 2012b; Mohammadpour *et al.*, 2020).

Further epidemiological information is fundamental to identify potential human infection reservoirs in animals and assess the risk of zoonotic transmission by different animal groups, especially those commonly in contact with humans such as poultry and livestock. Molecular epidemiological studies will not only aid in the demonstration of transmission patterns of *Blastocystis* sp. but will also illustrate the differences in subtype distribution among host groups and within geographical regions

(Stensvold and Clark, 2016b). It is of importance that epidemiological studies of *Blastocystis* sp. be designed to examine large numbers of different animal types and at numerous sites, not minding whether such sites are located in close proximity to one another (Alfellani *et al.*, 2013c). This appears essential as studies on *Blastocystis* sp. infection in goats (Tan *et al.*, 2013) and camels (Alfellani *et al.*, 2013c) have shown that subtype distribution can vary among sampling locations even within the same geographical region.

Blastocystis sp. is a cosmopolitan enteric protist whose role in the gut, either as a pathogen, a commensal, or even a beneficial member, remains controversial (Adao and Rivera, 2018). It has been recorded present in the intestines of both healthy and symptomatic humans and animals (Tan, 2008). Infection with *Blastocystis* has been associated with non-specific gastrointestinal symptoms such as diarrhoea, abdominal pain, nausea, and vomiting; intestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS); and urticaria in some patient populations (Scanlan, 2012; Ajjampur and Tan, 2016). Also, outcomes from *in vitro* and *in vivo* studies have revealed the pathogenic potential of *Blastocystis* sp., leading to the submission of likely models of pathogenesis (Betts *et al.*, 2020). However, a confounding factor in establishing the pathogenic ability of *Blastocystis* is its high degree of genetic diversity coupled with the asymptomatic status of most carriers (Gentekaki *et al.*, 2017); thus, the pathogenic role of this organism as the primary cause of enteric symptoms remains undecided (Piubelli *et al.*, 2019). Extensive understanding or knowledge of the different genetic variants of *Blastocystis* sp. could hold the key to harmonizing the contrasting opinions on its pathogenicity (Scicluna *et al.*, 2006).

1.2 Justification of study

The Malaysian livestock industry does not only supply beneficial animal protein but also offers paid employment to the populace, thus making this industry a fundamental part of the agricultural sector (Loh, 2002). This industry is continually expanding especially as ruminant livestock production in Malaysia is inadequate to meet consumer demands (International Trade Administration, 2021). Gastrointestinal infections have been known to cause health problems in livestock, thereby, resulting in economic losses for the farmers (Ifqiyyah *et al.*, 2021). Also, infectious diseases associated with livestock have become a major threat to human health with veterinarians, farmers, and slaughterhouse workers at higher risk of infection (Klous *et al.*, 2016). Irrespective of the farming system employed in livestock production, livestock animals worldwide have been reported as hosts to *Blastocystis* (Hublin *et al.*, 2020). Transmission of *Blastocystis* is majorly through the oral-faecal route; animal-to-human and human-to-animal transmission can occur (Hublin *et al.*, 2020). The presence of potentially zoonotic STs of *Blastocystis* in livestock animals has been frequently reported, and animal handlers such as animal farmers and slaughterhouse workers have been observed to be at risk of *Blastocystis* infection (Tan, 2008). There is, however, a paucity of substantial data on the prevalence, distribution, and predictors of *Blastocystis* sp. infection and its subtypes in livestock animals in Malaysia. The prevalence of *Blastocystis* infection in ruminant livestock from Perak has been reported only by Hemalatha *et al*. (2014), and there has not been any report of such on Penang livestock animals. Reports on the morphological characteristics of *Blastocystis* have reduced lately. Previous ultrastructural studies of *Blastocystis* have been mainly on isolates from humans and birds, and one study on *Blastocystis* isolate of cattle by Widisuputri *et al.* (2021). Although isolates from different host are morphologically

identical, ultrastructural studies may reveal subtle host related surface characteristics of *Blastocystis.* Thus, information on the morphology, distribution and determinants of *Blastocystis* infection is important, more so as *Blastocystis* is a prevalent protist and its role in its human and non-human host gut is still uncertain (Andersen and Stensvold, 2016).

1.3 Research objectives

This study embarks on the following objectives:

1. To determine the prevalence and the potential risk factors of *Blastocystis* sp. infection in the following livestock animals from Penang, Malaysia:
    a) Ruminant livestock animals consisting of farm-reared cattle, goats, and sheep
    b) Poultry animals consisting of farm-reared quail birds

2. To examine the phenotypic characteristics of *Blastocystis* sp. in livestock animals from Penang, Malaysia such as:
    a) General morphology based on staining characteristics using Giemsa stain
    b) Ultrastructural description of surface coat using scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

3. To determine the following genotypic characteristics and the evolutionary relationships of *Blastocystis* sp. from livestock animals in Penang and Perak, Malaysia using the DNA barcoding method:
    a) Subtype diversity and frequency of *Blastocystis* sp.

b) Subtype distribution of *Blastocystis* sp. using Geographic Information System (GIS)

CHAPTER 2

LITERATURE REVIEW

2.1 History and taxonomy of *Blastocystis* sp.

The journey to the taxonomic classification of *Blastocystis* sp. has been a complicated one (Stensvold and Clark, 2016a; Ahmed and Karanis, 2018). *Blastocystis* was first observed by Brittan (1849) and Swayne (1849) while investigating London's historic cholera epidemic and was tagged as cholera bodies along with other parasitic ova and cysts that were unknown at that time (Parija and Jeremiah, 2013). Its definition as a distinct organism was by Alexeieff at the beginning of the 20th century and for a long time afterward, its classification remained controversial (Denoeud *et al.*, 2011). At different times in its taxonomic history, *Blastocystis* has been (mis)classified as the cyst form of a flagellate, an amoeba, yeast, and a sporozoan (Silberman *et al.*, 1996; Stensvold and Clark, 2016a).

Alexeieff, in 1911, was the first to give a taxonomically significant description of this organism; he portrayed it as a harmless gastrointestinal saprophytic yeast and named it *Blastocystis enterocola* (Alexeieff, 1911). The specific epithet "*hominis*" was proposed by Brumpt in 1912 for *Blastocystis* isolates from humans, *B. hominis* then became the name recognized worldwide (Brumpt, 1912; Zierdt, 1991; Stenzel and Boreham, 1996; Parija and Jeremiah, 2013). Much later, Zierdt *et al*. (1967) described this organism as more of a protozoan than a yeast-based on failure to grow on fungal media, resistance to antifungal agents, susceptibility to antiprotozoal drugs, optimal growth at 37°C and neutral pH; and the presence of more than one nucleus, mitochondrion-like organelles, Golgi apparatus, and endoplasmic reticulum as revealed by physiological and electron microscopy studies. *Blastocystis* was thereafter

classified under the subphylum Sporozoa and a separate suborder Blastocystina, in 1978 by Zierdt, based on proposed methods of division (Zierdt, 1978). In 1988, however, Zierdt reclassified the organism under the subphylum Sarcodina (Stenzel and Boreham, 1996). Phylogenetic analysis of small subunit ribosomal DNA (SSU rDNA) helped in the most recent classification of *Blastocystis* under the phylum Heterokontophyta by Silberman *et al.* (1996). This phylogenetic position has subsequently been confirmed by the analysis of other genes by Arisue *et al.* (2002).

The phylum Heterokontophyta, also known as Stramenopila, is a complex and heterogeneous evolutionary assemblage that comprises brown algae, golden-brown algae, diatoms, slime nets, water moulds, and oomycetes (Silberman *et al.*, 1996; Arisue *et al.*, 2002; Ahmed and Karanis, 2018). Stramenopiles, along with the Alveolata and Rhizaria, are members of the eukaryotic supergroup known as SAR - Stramenopiles, Alveolata and Rhizaria (Gentekaki *et al.*, 2017). Stramenopila is an extremely diverse eukaryotic group that includes unicellular and multicellular protists. Members comprise heterotrophs such as free-living flagellates, parasites of plants (for example *Peronospora*), parasites of animals (for example *Phythium insidiosum*), and organisms resembling fungi in terms of cytology and ecology; and numerous autotrophs, mostly algae (Derelle *et al.*, 2016). The stramenopiles are characterized by mitochondria with tubular cristae and, unlike all other eukaryotes, have tripartite tubular hairs either on their cell surface or, more commonly, on their long anterior flagellum.

Blastocystis is a very unusual stramenopile. Although it has mitochondria with tubular cristae, it is anaerobic, it lacks flagella and flagellar hairs, and its most characteristic feature is a crescent cap of heterochromatin in the nucleus (Silberman *et al.*, 1996; Arisue *et al.*, 2002; Denoeud *et al.*, 2011). The delay in the appropriate

classification of *Blastocystis* could, among other reasons, be attributed to the presence of multiple morphological forms in its host (Silberman *et al.*, 1996) and its lack of regular stramenopile features (Stensvold and Clark, 2016a). Stramenopiles, to date, contain only a single other human-infective eukaryote, *Pythium* (Stensvold and Clark, 2016a). The most recent taxonomy and classification of *Blastocystis*, according to Stensvold *et al.*, (2020), are as follows:

Kingdom: Sar

Phylum: Stramenopiles

Class: Bigyra

Order: Opalinata

Family: Blastocystidae

Genus: Blastocystis

Species: Currently not applicable

Taxonomy within the genus *Blastocystis* has remained unresolved. Initially, *Blastocystis* isolates from humans were referred to as *Blastocystis hominis*; while isolates from non-human hosts were either described as *Blastocystis* sp. or in a few cases named after their hosts for example *Blastocystis galli* from chicken, *Blastocystis lapemi* from sea-snake, *Blastocystis pythoni* from reticulated python, and *Blastocystis ratti* from (Belova and Kostenko, 1990; Teow *et al.*, 1991; Singh *et al.*, 1996; Chen *et al.*, 1997; Noel *et al.*, 2005; Stensvold *et al.*, 2007). However, most *Blastocystis* sp. isolates from its wide range of hosts have been found rather indistinguishable/identical by light and electron microscopy (Noel *et al.*, 2005). Hence, differentiating one isolate

from another based on morphological criteria alone has proven challenging (Tan, 2008). Besides, *Blastocystis* sp. isolates from one host have shown broad genetic diversity while genetic homology has been observed in isolates from different host species. Thus, species designation of *Blastocystis* has become difficult (Arisue *et al.*, 2003) and the host-specific naming of species is considered inaccurate (Stensvold *et al.*, 2007).

A *Blastocystis* subtype terminology was proposed when it became apparent that the earlier mentioned specific epithets were illogical and stood for several quite different entities (Stensvold *et al.*, 2020). This subtype nomenclature was introduced by Stensvold *et al.* (2007), this involved isolates being described as *Blastocystis* sp. and assigned to subtypes based on polymorphism of SSU rRNA gene. To date, about 32 *Blastocystis* sp. subtypes have been proposed (Higuera *et al.*, 2021).

2.2 Morphology of *Blastocystis* sp.

The morphology of *Blastocystis* has been described by several authors based on light and electron microscopy. The organism has been reported to exist in different morphological forms with each form showing considerable variations in size and shape (Parija and Jeremiah, 2013).

In early reports, *Blastocystis* was mostly described as spherical cells of diameter ranging from 5 - 20 µm, with a large central body (also called a central vacuole, internal body, or reserve body), a thin peripheral rim of cytoplasm, multiple nuclei each with a cap of condensed chromatin, cytochrome-free mitochondria and a thick, mucilaginous coat surrounding the organism. Separate smaller cells with glycogen and lipid inclusions, thick walls, no vacuole and, thought to be to be a resistant stage were also described (Boreham and Stenzel, 1993a; Stenzel and Boreham, 1996).

Currently, *Blastocystis* has been documented in at least six distinct morphological forms namely: vacuolar, granular, amoeboid, avacuolar, multivacuolar, and cyst; the cyst is believed to be the infective stage while the amoeboid form is supposedly more actively involved in the emergence of clinical symptoms (Tan and Suresh, 2006). The cyst, amoeboid, granular, and vacuolar forms of *Blastocystis* are very commonly observed, whereas the avacuolar and multivacuolar forms are less frequently encountered cell forms.

2.2.1 Vacuolar form

This is the most commonly observed morphological form in both *in-vitro* cultures and faecal/stool samples (Stenzel and Boreham, 1996; Tan, 2008; Poirier *et*

al., 2011). It is also the most easily recognizable of *Blastocystis* sp. forms (Wawrzyniak *et al.*, 2015). Vacuolar forms (Figure 2.1a) are mostly seen as spherical cells, although irregularly shaped cells may be present in cultures (Stenzel and Boreham, 1996). When stained, it has a large membrane-bound central vacuole of unknown function occupying more than 90% of the cell volume; the cytoplasm with all the organelles is visible as a thin peripheral layer between the vacuole and the cell membrane (Ajjampur and Tan, 2016; Stensvold and Clark, 2016a). These organelles include multiple nuclei, Golgi apparatus, endosome-like vacuoles, microtubules, and cytochrome-free mitochondria-like organelles (Arisue *et al.*, 2002; Tan, 2008). Vacuolar forms vary greatly in size, ranging from 2 µm to more than 200 µm in diameter with the average diameter of cells usually being between 4 and 15 µm (Stenzel and Boreham, 1996).

2.2.2 Granular form

The granular form of *Blastocystis* (Figure 2.1b) is also seen commonly in faecal samples and cultures (Ajjampur and Tan, 2016). It is similar in structure to the vacuolar form, except possessing morphologically and cytochemically different central vacuole contents (Stenzel and Boreham, 1996). The central vacuoles of the granular form have granules which are also sometimes observed in its cytoplasm (Velásquez *et al.*, 2021). Metabolic, lipid, and reproductive granules are three types of granules discovered by electron microscopic studies (Tan and Zierdt, 1973; Parija and Jeremiah, 2013). Granular forms often are slightly larger than the average vacuolar forms, and diameters of 10 to 60 µm, 15 to 25 µm, 3 to 80 µm, and 6.5 to 19.5 µm have been reported (Stenzel and Boreham, 1996).

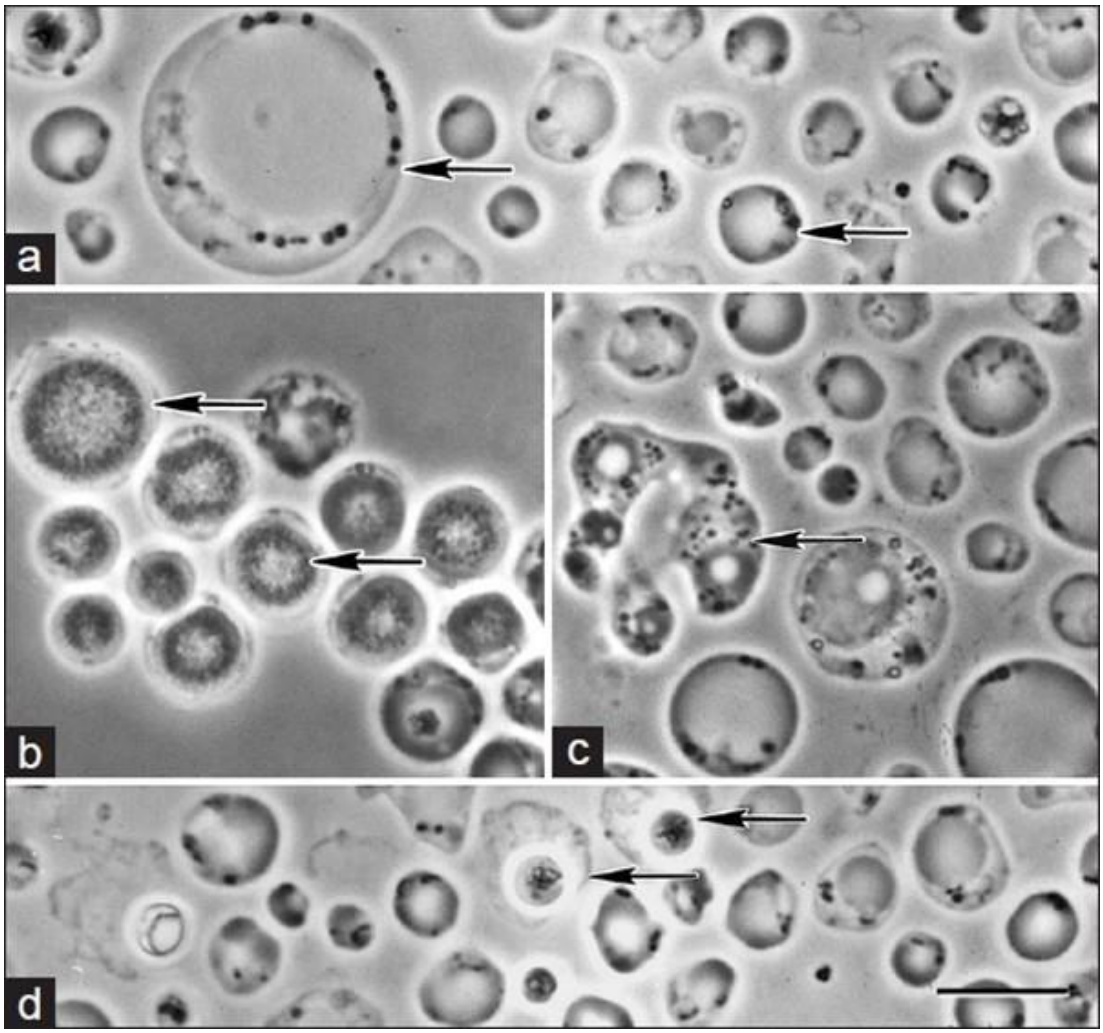

Figure 2.1 Morphological forms of *Blastocystis* (Parija and Jeremiah, 2013). (a) Vacuolar forms of *Blastocystis* having a large centrally placed vacuole showing extensive variation in size (arrows), (b) Granular forms with distinct granules filling the central body, (c) Amoeboid form with characteristic pseudopodia, (d) Cyst forms. Note the smaller size and the characteristic refractile cyst wall surrounded by loose irregular outer coat.

2.2.3 Amoeboid form

This form of *Blastocystis* is not often reported; and although the observation of non-locomotory pseudopod-like cytoplasmic extensions is commonly reported, the morphological descriptions available are somewhat contradictory (Tan, 2008; Velásquez *et al.*, 2021). The amoeboid form as shown in Figure 2.1c is suggested to be the pathogenic form of *Blastocystis* sp. Reports on the relationships with pathogenicity symptoms in infected individuals have varied (Adao and Rivera, 2018), but amoeboid forms of *Blastocystis* are more often seen in faeces of symptomatic patients (Parija and Jeremiah, 2013).

2.2.4 Cyst form

Cyst form is the smallest form of *Blastocystis* sp. measuring between 2 to 5 µm in diameter (Figure 2.1d). It is a resistant form and is believed to be how this organism is transmitted (Poirier *et al.*, 2011; Ahmed and Karanis, 2018). *Blastocystis* cysts are not frequently seen in laboratory cultures, they are variable in shape but are mostly ovoid or spherical (Tan, 2008). The cyst is protected by a multilayered cyst wall that appears to form beneath the surface coat (Stenzel and Boreham, 1996), and it has been found resistant to osmotic and temperature-related stress and can survive up to 19 days in water, a month at 25°C, and 2 months at 4°C (Tan, 2008; Adao and Rivera, 2018).

2.2.5 Avacuolar form

This *Blastocystis* form lacks a central vacuole and measures approximately 5 µm in diameter (Stenzel and Boreham, 1996). Avacuolar cells are usually uninucleate but are sometimes observed to be binucleate, the nuclei are bigger than those of other

morphological forms (Parija and Jeremiah, 2013). This form is frequently found in fresh faeces of symptomatic patients.

2.2.6 Multivacuolar form

This form multiple possesses small vacuoles of different sizes interconnected to each other or lying discrete in the cytoplasm, and measures within 5-8 µm in diameter (Stenzel and Boreham, 1996). This form has one to two nuclei and is also frequently found in fresh faeces of symptomatic patients (Tan, 2008).

2.3 Transmission and life cycle of *Blastocystis* sp.

Recent studies have shed light on the transmission of *Blastocystis* (Adao and Rivera, 2018). The cyst form of *Blastocystis* is responsible for its dissemination in the environment (Poirier *et al.*, 2011). Transmission is principally achieved via faecal-oral routes (Ahmed and Karanis, 2018) and a host can become infected through direct transmission routes (animal-to-animal, human-to-human, animal-to-human, and human-to-animal) or indirect transmission routes (foodborne and waterborne) (Ahmed and Karanis, 2018; Hublin *et al.*, 2020).

Information on the life cycle of *Blastocystis* sp. is inadequate (Poirier *et al.*, 2011), and poorly documented (Wawrzyniak *et al.*, 2015), thus the life cycle of *Blastocystis* sp. is still not completely comprehended (Adao and Rivera, 2018). The suggested life cycle of *Blastocystis* (Figure 2.2) commences when a susceptible host (man or any animal) ingests the cyst form. On reaching the large intestine, the organism excysts and develops into the vacuolar form which then undergoes binary fission (Tan, 2008). Vacuolar forms may develop into granular or amoeboid forms, but it is unclear how the transition from one form to another happens (Hublin *et al.*, 2020).

Different modes of reproduction such as binary division, budding, endodyogeny, multiple fission, plasmotomy, and schizogony have been reported (Carneiro Santos *et al.*, 2017); nonetheless, binary fission of the vacuolar forms is the most commonly observed and well-established mode of reproduction (Parija and Jeremiah, 2013; Ahmed and Karanis, 2018). Cysts are formed as the organism passes along the large intestine; the cysts are shed into faeces, thereby, continuing the chain of transmission (Hublin *et al.*, 2020).

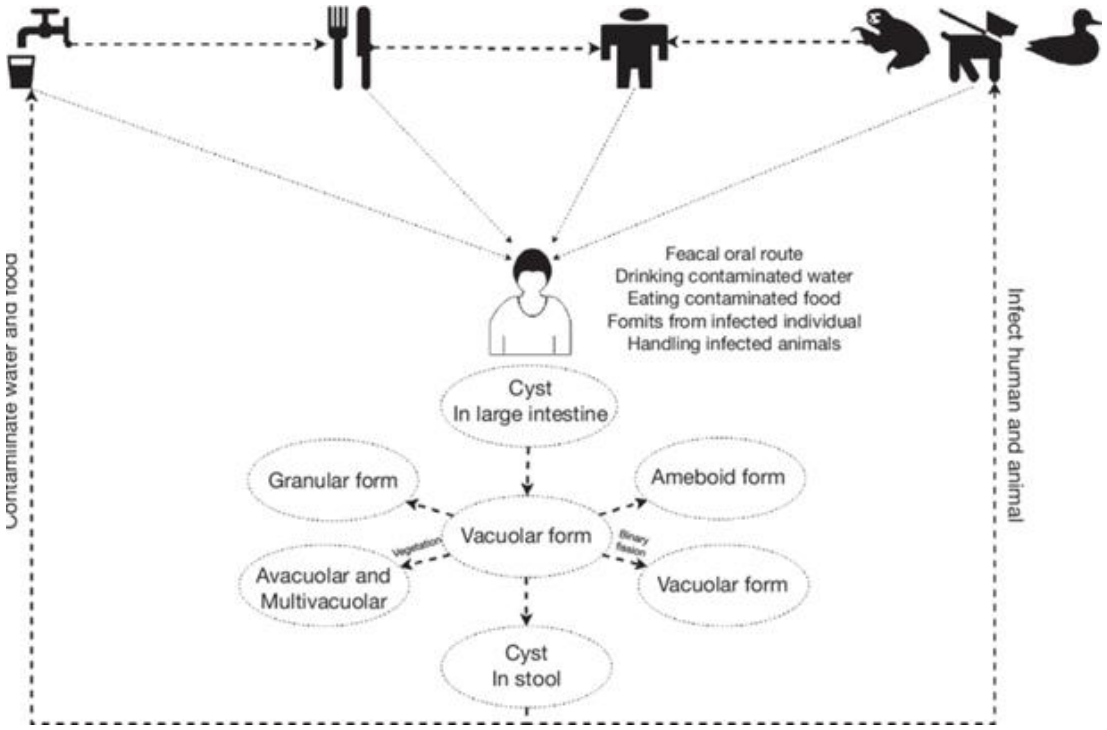

Figure 2.2 Transmission and life cycle of *Blastocystis* agreed by consensus (Ahmed and Karanis, 2018).

2.4 Detection of *Blastocystis* sp.

Over the years, *Blastocystis* sp. detection has relied mostly on the screening of host feacal samples by light microscopy (direct microscopy and concentration techniques), *in-vitro* cultivation, and PCR-based molecular methods.

Diagnosis of *Blastocystis* sp. by light microscopy is fast and often aided using several staining methods, such as Trichome, Giemsa, iron hematoxylin, Gram, and Fields staining, to accentuate its morphological features. This morphology-based method is, however, difficult and requires expertise because of the varying sizes and polymorphic nature of *Blastocystis* sp. isolates (Stensvold *et al.*, 2006). Culturing involves the cultivation of *Blastocystis* in xenic and axenic culture media such has Locke-egg medium, Robinson's medium, and Jones' medium. Although *in-vitro* cultivation is a more sensitive method for *Blastocystis* sp. detection than light microscopy (Suresh and Smith, 2004), it is a time-consuming method, and both methods are incapable of differentiating between isolates at any level.

Molecular techniques have been employed in the diagnosis of *Blastocystis* sp. These methods are more sensitive than microscopy and culture, and reveal the genetic diversity of *Blastocystis* sp. subtypes; but they cost more and are primarily used in research laboratories (Hublin *et al.*, 2020). The commonly used molecular techniques are PCR-based and they include PCR with subtype-specific sequence-tagged site (STS) primers, and PCR amplification using genus-specific primers followed by Sanger sequencing of the SSU rRNA gene (Stensvold, 2013a). Sequencing is not needed for PCR using STS primers, it can detect infection by mixed subtypes but the detectable number of subtypes is limited (ST1-ST9), thus infections by other subtypes may go undetected (Stensvold, 2013b). Although this method is incapable of precise

classification of mixed subtype infections, Sanger sequencing of fragments of the SSU rRNA gene is more frequently used in molecular studies of *Blastocystis* sp. (Maloney *et al.*, 2019b).

PCR amplification of the SSU rRNA gene has been by either real-time PCR (qPCR), conventional PCR, or nested PCR; and several PCR protocols have been developed and applied by different authors for the diagnosis and subtype (ST) classification of *Blastocystis* sp. as shown in Table 2.1. So far, published real-time PCR assays for *Blastocystis* have had their limitations for *Blastocystis* screening. The assay of Jones *et al.* (2008) was shown to support DNA amplification from ST1, ST3, and ST4 isolates; however, it is theoretically difficult to estimate specificity and sensitivity based on gene copy numbers since the gene targeted in this report is undefined. While the assay described by Poirier *et al.* (2011) was designed as a genus-specific PCR that targeted the SSU rRNA gene, the amplicon was 339 base pairs long. In diagnostic PCRs, considerably shorter amplicons are generally desired to boost sensitivity(Stensvold *et al.*, 2012a). Additionally, this assay and that of Stensvold *et al.* (2012a) have enabled the amplification of DNAs from *Blastocystis* strains that belong to all subtypes previously identified in humans only making them unsuitable for the study of *Blastocystis* in animal hosts

A drawback of nested PCR is the potential for increased contamination despite proven sensitivity. Likewise, conventional PCR methods amplifying very short amplicons have the drawback of limited subtyping accuracy; although this would not impede diagnostic uses (Santín *et al.*, 2011).

Table 2.1 PCR amplification of SSU rRNA gene for screening and subtype identification of *Blastocystis* sp.

Type of PCR	Fragment length (SSU-rDNA Region)	Primers	References	Hosts	Subtypes identified
qPCR	152 base pairs	prMSJ2 (Forward) CACACTGTGATTCTCGGG prMSJ2 (Reverse) GAAATGGAAGATGGAATTGATGAC	Jones *et al.* (2008)	Human	STs 1, 3, 4
	320 to 342 base pairs, depending on the subtype	qPCR (Forward) BL18SPPF1 AGTAGTCATACGCTCGTCTCAAA BL18SR2PP (Reverse) TCTTCGT TACCCGTTACTGC	Poirier *et al.* (2011)	Human	STs 1-9
	190 base pairs	qPCR Blasto F5 (Forward) GGTCCGGTGAACACTTTGGATTT Blasto F2 (Reverse) CCTACGGAAACCTTGTTACGACTTCA	Stensvold *et al.* (2012a)	Human	STs 1-9
Nested PCR	~1100 base pairs	RD3 (Forward) GGGATCCTGATCCTTCCGCAGGTTCACCTAC RD5 (Reverse) GGAAGCTTATCTGGTTGATCCTGCCAGTA	Parkar *et al.* (2007)	Human, diverse animals	STs 1, 5, 6, 7
		Forward GGAGGTAGTGACAATAAATC Reverse CGTTCATGATGAACAATTAC	Wang *et al.* (2013)	Dog	STs 1, 5

Type of PCR	Fragment length (SSU-rDNA Region)	Primers	References	Hosts	Subtypes identified
Conventional PCR	~600 base pairs (5' one-third of the SSU rDNA - barcode region)	RD5 (Forward) ATCTGGTTGATCCTGCCAGT BhRDr (Reverse) GAGCTTTTTAACTGCAACAACG	Scicluna *et al.* (2006)	Human, Primate, Pig	STs 1-5
			Stensvold (2013b)	Human, Cattle, Camel	STs 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14
			Wang *et al.* (2013)	Dog	STs 1, 4, 5, 6
			Noradilah *et al.* (2017b)	Human	STs 1, 2, 3, 4
			Noradilah *et al.* (2017a)	Domestic animals	STs 1, 2, 3, 4, 5, 6, 7, 8, 9, 10
			Lee *et al.* (2018)	Cattle	STs 1, 5, 10, 14
			AbuOdeh *et al.* (2019)	Diverse animals	STs 4, 10, 14, 17
			Chang *et al.* (2021)	Goats	STs 1, 4, 5, 6, 10, 14
			Song *et al.* (2021)	Chinese Bamboo rats	STs 4, 5
			Mohammad Rahimi *et al.* (2021)	Cattle, Sheep, Chickens	STs 1, 5, 7, 10, 14
	310 base pairs (3' end of the SSU rDNA)	bl1400 (Forward) GGAATCCTCTTAGAGGGACACTATACAT bl1710 (Reverse) TTACTAAAATCCAAAGTGTTCATCGGAC	Stensvold *et al.* (2006)	Human	NA

Type of PCR	Fragment length (SSU-rDNA Region)	Primers	References	Hosts	Subtypes identified
Conventional PCR	~260 base pairs	BLF (Forward) CGAATGGCTCATTATATCAGTT BLR (Reverse) TCTTCGTTACCCGTTACTGC	Menounos *et al.* (2008)	Human	STs 1, 2, 3, 4, 6, 7
	~500 (479) base pairs (Santin region)	Blast 505–532 (Forward) GGAGGTAGTGACAATAAATC Blast 998–1017 (Reverse) TGCTTTCGCACTTGTTCATC	Santín *et al.* (2011)	Human, Cattle, Swine, Primate, Chicken	STs 1, 2, 3, 4, 5, 6, 7, 10