REVIEW

Recent advances in *Blastocystis* sp. research

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Blastocystis sp. is a eukaryotic protozoan symbiont commonly found in the gut of humans and animals. Its role in the gut remains controversial due to conflicting reports of its pathogenicity. This paper provides a review of its morphology, life cycle, potential pathogenicity, subtype diversity, treatment, and detection based on latest research. Studies on *Blastocystis* sp. in the Philippines are also summarized with recommended future directions.

KEYWORDS

Blastocystis, detection, pathogenicity, subtype, Philippines

INTRODUCTION

Blastocystis sp. is the most commonly-encountered eukaryotic gastrointestinal symbiont in humans and animals. Its life cycle and role in the gastrointestinal tract, whether it be as a commensal or as a parasite, remain unclear despite the application of recent advances in molecular and immunological methods to *Blastocystis* sp. research. Much of the current knowledge on *Blastocystis* sp. comes from examination of its SSU rRNA gene and culture of pathogenic isolates. There are currently 17 known subtypes (STs) of *Blastocystis* sp. in mammals (including humans) and birds (Alfellani et al. 2013a; Alfellani et al. 2013b; Alfellani et al. 2013c; Stensvold et al. 2009a) and 8 non-mammalian and avian STs (NMASTs) (Cian et al. 2017; Yoshikawa et al. 2016a) based on SSU rRNA gene sequences. Certain isolates, particularly isolate B from a diarrheic patient and WR-1 from a laboratory rat, can cause apoptosis of cell lines, induce a pro-inflammatory response, and penetrate intestinal tissues.

These are all signs of potential pathogenicity that may be associated with its proteinase activity. Most of the mammalian and avian STs have low host-specificity but some STs can also be found in water samples leading to possibilities of zoonotic, anthroponotic, and waterborne transmission. Certain STs have also been linked to pathogenicity but this still remains unclear since different isolates of the same ST demonstrate varying pathogenic activity. This paper reviews recent advances in *Blastocystis* sp. morphology, diversity, pathogenicity, life cycle, treatment, and detection. Moreover, studies of *Blastocystis* sp. in the Philippines are summarized with recommended future directions.

MORPHOLOGY AND LIFE CYCLE

The life cycle of *Blastocystis* sp. has yet to be fully understood. Nonetheless, recent studies have shed light on how it is transmitted and how it changes from one morphological form to another. The symbiont exhibits the following morphologic forms: vacuolar, granular, amoeboid, cyst (Stenzel and Boreham 1996) and a proposed precyst form (Suresh et al. 2009). Results of a detailed study of how *Blastocystis* sp. changes between these morphologic forms in an infected patient were presented by Suresh et al. (2009). They showed that the spherical vacuolar and granular forms are the most distinct and commonly-encountered form of *Blastocystis* sp. in fecal smears and *in vitro* cultures. These forms have a size range of 2-200 µm in diameter with an average of 4-15 µm. (Tan 2008). The vacuolar form is comprised of a large central vacuole with clumps of homogeneous material inside and a cytoplasm with organelles concentrated at its peripheral rim (Suresh et al. 2009). The

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central vacuole accumulates electron dense material during the stationary phase of growth suggesting accumulation of lipids and carbohydrates for cell growth (Yoshikawa and Hayakawa 1996). Cysteine proteases that may be related to inducing host inflammatory response are also localized in this organelle (Puthia et al. 2008). Other organelles include one or more nuclei, Golgi apparatus, endosome-like vacuoles, microtubules, and mitochondrion-like organelles (MLOs) (Tan 2008). *Blastocystis* sp. axenic cultures require anaerobic conditions to grow (Ho et al. 1993) but the MLOs have enzymes and features of both mitochondria of aerobic eukaryotes and hydrogenosomes of anaerobic eukaryotes (Lantsman et al. 2008). These include succinyl-CoA synthetase that is compatible with both GTP and ATP activity characteristic of hydrogenosomes (Hamblin et al. 2008). Features similar to mitochondria include mitochondrial DNA, pathways for amino acid metabolism, iron-sulfur cluster biogenesis, and translocase of outer membrane (Tom70) (Stechmann et al. 2008; Tsaousis et al. 2012; Tsaousis et al. 2011). These enzymes and pathways were determined from MLO genome analysis of *Blastocystis* sp. Nand II (ST1) (Stechmann et al. 2008; Tsaousis et al. 2012; Tsaousis et al. 2011). MLOs possess cristae visible under an electron microscope (Suresh et al. 2009). Raman et al. (2016) also noted that isolates from patients with gastrointestinal symptoms tend to have more abundant MLOs compared to isolates from asymptomatic patients. An outer surface coat or slime layer is also observed in cultures of vacuolar/granular forms (Zaman et al. 1998) which is hypothesized to be responsible for trapping bacteria for nutrition, protection against osmotic shock, and/or protection against host innate immune response (Stenzel and Boreham 1996; Tan 2008; Tan et al. 2002; Zaman et al. 1999). Granular forms are similar in size and shape as the vacuolar forms except for dense granules within the central vacuole. Homogeneous material within the central vacuole of the vacuolar form becomes more compact and dense in the granular form (Suresh et al. 2009). The latter form is also possibly an intermediate form for producing more viable cells as a survival reaction to stress (Dhurga et al. 2016). Non-viable granular forms tend to show higher granularity compared to viable granular forms (Yason and Tan 2015). The vacuolar and granular forms of *Blastocystis* sp. in pigs have been observed to occupy the jejunum and cecum but not the ileum and duodenum (Fayer et al. 2014). These occupy the lumen of the gut or attach to the intestinal epithelia but no signs of penetration of tissues have been observed so far in pigs (Fayer et al. 2014; Wang et al. 14a). In contrast, *in vivo* pathogenicity studies in immunocompromised mice and rats showed vacuolar forms penetrating the intestinal submucosa with sloughing of the epithelial layer as well as attachment to intestinal epithelia (Abdel-Hafeez et al. 2016b; Elwakil and Hewedi 2010; Hussein et al. 2008).

The vacuolar/granular forms of *Blastocystis* sp. soon adopt a more irregular shape as an amoeboid form. This morphologic form lacks certain organelles such as Golgi complex, surface coat, coated pits, and mitochondria (Suresh et al. 2009). Pseudopodia appear as either sharp projections or large round projections (Tan and Suresh 2006a) even though this morphologic form is non-motile (Tan 2008). Amoeboid forms are observed in xenic cultures (Lanuza et al. 1996) after at most 4 days in liquid media (Tan and Suresh 2006b) suggesting a role in phagocytosis of bacteria for additional nutrition necessary for encystation (Suresh et al. 2009). These forms can also be observed in axenic cultures forming colonies in soft agar media (Ng and Tan 1999; Tan et al. 1996; Valido and Rivera 2007). The amoeboid form is the proposed pathogenic form of *Blastocystis* sp. although different studies show varying associations with symptoms of pathogenicity in infected patients. For example, Tan and Suresh (2006b) observed the presence of this morphologic form in 10 symptomatic patients and noted its absence in 10 asymptomatic patients while Katsarou-Katsari et al. (2008) found the amoeboid form in a patient with urticaria. In contrast, Souppart et al. (2009) reported its presence in a single symptomatic patient and in two asymptomatic patients. As a consequence, further investigation is needed to ascertain the role of the amoeboid form in pathogenicity.

The precystic form has been reported in earlier studies as a highly refractile cyst-like stage with a thick, fuzzy coat surrounding the organism and with a vacuole formed within the protozoan (Singh et al. 1995; Suresh et al. 1994). This precystic stage was induced *in vitro* by incubating *Blastocystis* sp. vacuolar/granular forms in encystation medium, consisting of saline solution with yeast extract and 50% horse serum, with bacterial soluble products of *Proteus vulgaris* (Suresh et al. 1993). Tan (2004) added this 'intermediate cyst' stage to a proposed life cycle of *Blastocystis* sp. Its presence was confirmed by a detailed electron microscope study of the morphology of an oval precystic stage (Suresh et al. 2009). It was characterized by the formation of ribosome-like particles resulting in a dense cytoplasm and of a homogeneous electron-dense fuzzy coat, and disappearance of the central vacuole. This description was similar to that of the precystic stage induced by *in vitro* encystation of Suresh et al. (1993) and Singh et al. (1995) except for the absence of a central vacuole. Further study is required to confirm whether these are indeed the same morphological forms.

The cyst form is the transmissible form as demonstrated in *in vivo* experiments by oral inoculation of Wistar rats (Iguchi et al. 2007; Yoshikawa et al. 2004d), chicken (Iguchi et al. 2007; Tanizaki et al. 2005), quails, geese (Tanizaki et al. 2005), and BALB/c mice (Moe et al. 1997). The *in vitro*-induced cyst-like form of Suresh et al. (1993) can also infect Wistar rats via oral inoculation. The cyst stage is relatively small at 3-5 µm in diameter distinguished by a spherical to oval dense body bound by a three-layered cyst wall with a loose outer surface and containing 1-4 nuclei, glycogen deposits, and small vacuoles (Parija and Jeremiah 2013; Suresh et al. 1993; Tan 2008; Zaman 1994). Likewise, present is a pore probably for excystation (Suresh et al. 2009). Cyst forms are also 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 (Parija 2013; Tan 2008). *Blastocystis* sp. has been observed to adopt the cyst form in BALB/c mice to avoid host immune response (Zhou et al. 2010). Bacteria are suspected of playing a role in inducing encystment (Suresh et al. 1993; Suresh et al. 2009).

REPRODUCTION

Binary fission is the most commonly-observed form of reproduction of *Blastocystis* sp. Other forms of reproduction, such as budding, plasmotomy and schizogony, have been proposed by Govind et al. (2002) based on light microscopy and DNA staining. However, *Blastocystis* reproduction has become a subject of debate due to lack of electron microscopy results and use of stress-induced conditions, such as metronidazole treatment, (Govind et al. 2003, Tan and Stenzel 2003, Windsor et al. 2003). Plasmotomy and budding have since been observed with scanning electron microscopy and DNA staining in *Blastocystis* sp. vacuolar forms under xenic conditions (Zhang et al. 2012). Additionally, Tan and Suresh (2007) have also observed plasmotomy in amoeboid forms using transmission electron microscopy. Thus, binary fission, budding, and plasmotomy are the only forms of reproduction observed in *Blastocystis* sp. with sufficient evidence from light and electron microscopy and DNA staining (Yamada and Yoshikawa 2012).

Growth in xenic culture may have an influence on viability and reproduction of other non-vacuolar forms of *Blastocystis* sp. Amoeboid forms were the most commonly-observed morphologic forms in a xenic culture from a symptomatic patient (Tan and Suresh 2006b) with plasmotomy as a form of reproduction (Tan and Suresh 2007). Yamada and Yoshikawa (2012) also observed plasmotomy and budding in xenic *Blastocystis* sp. cultures. In contrast, Yason and Tan (2015) have shown that amoebic and granular forms of axenic ST1 *Blastocystis* sp. are non-viable and can be artifacts. The same study also showed that granular forms of axenic ST1, ST4, and ST7 cultures have lower viability with increasing granularity. A variety of forms of reproduction have been proposed for *Blastocystis* sp. but only a few have been confirmed. Caution should be practiced in identifying forms of reproduction since microscopy results may be misinterpreted. It is best that conclusions are made after confirming results using various methods, such as light and electron microscopy and nuclear DNA staining. Moreover, it is recommended that the ST of the culture used and conditions, whether xenic or axenic, are indicated. Other microorganisms may have a role in inducing or maintaining the viability of the other morphological forms. Observations on xenic culture may provide an insight into the viability of *Blastocystis* sp. in the gut microbiota.

TAXONOMY AND DIVERSITY

Examination of the full SSU rRNA gene sequence of *Blastocystis* sp. revealed much about the organism in terms of phylogenetics. It was previously considered a yeast but after a phylogenetic analysis of the gene, it was classified as a Protozoa and grouped with other Stramenopiles, particularly in the Heterokonta with the commensal *Proteromonas lacertae* (Noël et al. 2003; Tan et al. 2002). Intra-species classification, previously based on several criteria such as electrophoretic karyotypes, random fragment length polymorphism (RFLP) patterns of SSU rRNA gene, and protein profiles (Tan et al. 2002), has led to several types of intra-species groupings (e.g., groups, ribodemes, clades) that made comparison of results difficult. Consequently, Stensvold et al. (2007b) suggested the use of a consensus terminology based on STs from full SSU rRNA gene sequences of isolates from mammals and birds. Examination of this gene revealed the existence of 17 STs that can infect various species of mammals and birds leading to possibilities of zoonotic and anthroponotic transmission (Alfellani et al. 2013a; Alfellani et al. 2013b; Alfellani et al. 2013c; Noël et al. 2005; Rivera 2008; Stensvold et al. 2009a; Yoshikawa et al. 2004b). Previously, *Blastocystis* sp. isolates were assigned species epithets based on their host, e.g., *Blastocystis hominis* in humans, *Blastocystis ratti* in rodents. However, surveys from various hosts have shown low host-specificity of the various STs (Abe 2004; Noël et al. 2005; Noël et al. 2003; Rivera 2008). Thus, mammalian and avian samples belonging to the 17 STs are now referred to as '*Blastocystis* sp.' and sometimes indicating its known ST (e.g. *Blastocystis* sp. ST1). Sequencing of partial sequences and the use of ST-specific primers have also been used for ST identification. The most widely-used barcoding sequence is the 600 bp sequence of the 5'-end of the SSU rRNA gene amplified by the primers RD5 and BhRDr (Scicluna et al. 2006). The sequence-tagged site (STS) primers (Yoshikawa et al. 2004c) are ST-specific although these are limited only to identification of ST1-ST9 but have the advantage of identifying individual STs in mixed ST infections (Stensvold 2013b; Yoshikawa and Iwamasa 2016).

A series of studies has recently analyzed all available data on STs present in both humans and animals worldwide at the time based on both SSU rRNA gene sequences and STS primers (Alfellani et al. 2013a; Alfellani et al. 2013b; Alfellani et al. 2013c). The results showed that ST1 to ST9 have been detected in humans with ST1 to ST4 as the most commonly encountered while ST9 so far has been identified only in humans (Alfellani et al. 2013b). Moreover, ST prevalence patterns were also found on different animal groups (Alfellani et al. 2013a; Alfellani et al. 2013c). New data on human *Blastocystis* sp. ST prevalence have become available after studies compiled by Alfellani et al. (2013b) showed the predominance of ST1-ST4 in both symptomatic and asymptomatic patients from Brazil (David et al. 2015), Colombia (Ramirez et al. 2014; Sánchez et al. 2017), Ecuador (Helenbrook et al. 2015), Egypt (El-Fetouh et al. 2015), France (El Safadi et al. 2016), India (Das et al. 2016; Pandey et al. 2015), Indonesia (Yoshikawa et al. 2016b), Iran (Alinaghizade et al. 2017; Badparva et al. 2016; Khademvatan et al. 2017; Khoshnood et al. 2015), Ireland (Scanlan et al. 2014), Italy (Mattiucci et al. 2015), Lao People's Democratic Republic (Sanpool et al. 2017), Lebanon (El Safadi et al. 2013), Libya (Abdulsalam et al. 2013), Malaysia (Nithyamathi et al. 2016), Mexico (Villegas-Gómez et al. 2016), Netherlands (Bart et al. 2013), North Cyprus (Seyer et al. 2017), the Philippines (Adao et al. 2016a; Belleza et al. 2016), Qatar (Abu-Madi et al. 2015), Senegal (El Safadi et al. 2014), Tanzania (Forsell et al. 2016), Thailand (Jantermtor et al. 2013; Palasuwan et al. 2016; Popruk et al. 2015; Sanpool et al. 2015; Thathaisong et al. 2013), Tunisia (Abda et al. 2017), Turkey (Dogan et al. 2017), and the United States of America (Scanlan et al. 2016). Among the four most common STs, ST3 was the most commonly-identified ST in humans worldwide (Alfellani et al. 2013b), a trend that was evident in most of the more recent surveys in asymptomatic populations and routine outpatient clinics in Africa (Abda et al. 2017), Europe (Bart et al. 2013; Scanlan et al. 2014), Southeast Asia (Adao et al. 2016a; Jantermtor et al. 2013; Nithyamathi et al. 2016; Palasuwan et al. 2016; Popruk et al. 2015), West Asia (Abu-Madi et al. 2015; Alinaghizade et al. 2017; Dogan et al. 2017; Khademvatan et al. 2017), and the Indian subcontinent (Pandey et al. 2015). Moreover, it is also the most commonly-encountered ST in patients with irritable bowel syndrome (IBS) (Das et al. 2016; Ramirez et al. 2014) as well as in patients showing symptoms of gastrointestinal diseases such as diarrhea and flatulence (El Safadi et al. 2014; Jones et al. 2009; Mattiucci et al. 2015; Sanpool et al. 2015; Souppart et al. 2010; Wong et al. 2008). However, this does not imply that ST3 is the only pathogenic ST or that all ST3 isolates are pathogenic. The other three most common STs in humans, ST1, ST2, and ST4, have also been associated with gastrointestinal symptoms (El-Fetouh et al. 2015; Eroglu et al. 2009; Jimenez-Gonzalez et al. 2012; Moosavi et al. 2012; Ramirez et al. 2014; Souppart et al. 2009; Stensvold et al. 2011; Whipps et al. 2010). Since ST1 to ST4 are the most commonly-found STs in both symptomatic and asymptomatic patients, more studies are necessary to determine their association with pathogenicity.

Certain STs are commonly encountered in various animal groups. Alfellani et al. (2013c) compiled most of the available ST data at the time and found that ST6 and ST7 are most commonly identified in birds, ST4 in rodents and marsupials, and ST5 and ST10 in Artiodactyls (e.g., pigs, sheep, deer, cattle) (Alfellani et al. 2013c; Stensvold et al. 2009a). Moreover, ST1 was the most commonly identified ST in non-human primates (Alfellani et al. 2013a). Subsequently, first reports of *Blastocystis* sp. infection in snow leopard, grey kangaroo, red kangaroo, and ostrich have appeared (Roberts et al. 2013). Novel ST-host associations have also been reported including ST1 and ST3 infections in cattle and ST8 in marsupials (Ramirez et al. 2014), ST11 in elephants and ST12 in giraffes (Roberts et al. 2013), ST7 in pigs and ST14 in goat (Adao et al. 2016b), and ST6 in cattle (Badparva et al. 2015). Reptiles, insects particularly cockroaches, amphibians, and bivalves also host

Blastocystis sp. Reptilian and insect isolates are referred to as different species compared to the 17 STs in mammalian and avian samples (e.g., *Blastocysis cycluri*, *Blastocystis pythoni*) due to differences in SSU rRNA gene sequences and in optimal growth temperature requirements. The SSU rRNA gene sequences of poikilothermic animal isolates formed separate clusters from those of the 17 mammalian and avian STs (Alfellani et al. 2013c). However, a recent phylogenetic analysis of Yoshikawa et al. (2016a) after addition of 12 new full SSU rRNA gene sequences of poikilothermic animal isolates showed formation of three reptilian clusters. Additionally, their study also indicated possible grouping of cockroach samples with ST17 and reptilian and amphibian isolates with ST15. More sequences from poikilothermic animal isolates may reveal new separate clusters that may be closely related to ST15 and ST17. Moreover, reptile, amphibian, and cockroach isolates do not grow optimally at 37°C. Reptilian isolates showed optimum growth at a room temperature of 24°C (Singh et al. 1996; Teow et al. 1992) but *Blastocystis cycluri* from iguana can tolerate incubation at 37°C (Yoshikawa et al. 2004b). Amphibian isolates also grew best at room temperature (Yoshikawa et al. 2004a) although an amphibian isolate (AFJ96-H1) showed optimum growth at 31 and 34°C (Yoshikawa et al. 2004b). On the other hand, cockroach isolates had optimum growth at both 25 and 37°C (Zaman et al. 1993). *Blastocystis* sp. has also been identified in mollusks particularly the marine bivalve *Donax* sp. (Pérez-Cordón et al. 2007) and the freshwater bivalve *Anodonta anatina* (Słodkowicz-Kowalska et al. 2015). However, both studies only reported microscopic identification of *Blastocystis* sp. in the gills and/or feces of these bivalves without any PCR verification or ST identification.

The abovementioned studies showed that *Blastocystis* sp. as a species is genetically diverse with a wide host range. Certain patterns of ST host-specificity are being discovered as more subtyping studies are done. Moreover, host range is definitely not limited to vertebrates, especially mammals and birds, as new evidence reveals more new subtypes in non-mammalian and non-avian hosts.

ZOONOTIC AND WATERBORNE TRANSMISSION

The low host-specificity and presence of cysts in water indicate possible zoonotic and waterborne transmission of *Blastocystis* sp. Animal handlers, pet owners, and people who have access to contaminated water are at risk of possible infections. Lee et al. (2012) found *Blastocystis* sp. ST4 in humans, pigs and buffaloes, and in a nearby river water in a village in Nepal indicating possible zoonotic and waterborne transmission. Salim et al. (1999) noted a high degree of association between *Blastocystis* sp. infection and animal handlers as compared to asymptomatic individuals residing in high-rise flats in Malaysia with little to no contact with animals. Li et al. (2007b) also reported association between pig ownership and *Blastocytis* sp. infection among villagers in Yunnan province in China. Identification of similar *Blastocystis* sp. STs in both animals and their respective animal handlers provided further evidence of zoonotic transmissions. Alfellani et al. (2013a) reported the presence of ST8 in zookeepers handling zoo primates commonly infected by this ST. Zookeepers in Perth zoos in Australia were also found to have *Blastocystis* sp. STs similar to those found in wombats and primates under their care (Parkar et al. 2007; Parkar et al. 2010). Yoshikawa et al. (2009) identified *Blastocystis* sp. ST1 and ST2 in children and rhesus monkeys living in the same area in Kathmandu, Nepal. Moreover, Rivera (2008) also found similar *Blastocystis* sp. STs in pigs and monkeys and their respective human handlers indicating possible zoonotic or even anthroponotic transmission. Yan et al. (2007) identified *Blastocystis* sp. ST5 in pigs and their owners/handlers in several Chinese provinces. Companion animals, e.g., pets are also possible sources of *Blastocystis* sp. zoonotic transmission. Belleza et al. (2015) noted that dog ownership is a risk factor in the acquisition of *Blastocystis* sp. Several recent studies on ST identification of *Blastocystis* sp. in dogs (Osman et al. 2015; Wang et al. 2013) showed that domestic dogs also carry *Blastocystis* sp. particularly ST1, ST2, ST4, ST5, ST6, and ST10. Only ST10 is not known to infect humans. Parkar et al. (2007) identified ST5 in a villager and a dog both residing in a village in Thailand indicating the possibility of dogs as sources of zoonotic transmission of *Blastocystis* sp.

Waterborne transmission of *Blastocystis* sp. has been associated with consumption of untreated water (Abdulsalam et al. 2012; Anuar et al. 2013; Helenbrook et al. 2015; Lee et al. 2012; Leelayoova et al. 2004; Li et al. 2007b; Nimri and Batchoun 1994; Nimri 1993; Taamasri et al. 2002). Viable cysts have been reported in wastewater (Banaticla and Rivera 2011; Suresh et al. 2005), river water (Ithoi et al. 2011; Lee et al. 2012) and drinking water (Leelayoova et al. 2008) and identified as ST1 (Banaticla and Rivera 2011; Lee et al. 2012; Leelayoova et al. 2008), ST2 (Banaticla and Rivera 2011), and ST4 (Lee et al. 2012). In some of these studies, the presence of similar *Blastocystis* sp. STs in both water sample and people utilizing the water source was noted adding evidence to waterborne transmission (Lee et al. 2012; Leelayoova et al. 2008). In particular, Leelayoova et al. (2008) identified *Blastocystis* sp. ST1 in 77.9% of schoolchildren attending a primary school in central Thailand as well as in the water supply of the school. Moreover, the presence of viable *Blastocystis* sp. in both influent and effluent wastewater may indicate an inadequate treatment process (Banaticla and Rivera 2011). Thus, wastewater leaks are possible sources of waterborne transmission.

Identification of similar *Blastocystis* sp. STs between two hosts, or sources in the case of water samples, can imply possible transmission of the protozoan between these hosts or sources. However, this may not always be the case since *Blastocystis* sp. also exhibits intra-ST diversity. For instance, *Blastocystis* sp. ST3 found in a certain population of people may be genetically distinct from those found in another population. Genetic diversity analyses of the SSU rRNA gene region of *Blastocystis* sp. have been recently used to analyze cryptic host diversity and specificity (Vargas-Sanchez et al. 2015; Villegas-Gomes et al. 2016; Villanueva-Garcia et al. 2017). Tests, such as nucleotide diversity (π), haplotype polymorphism (θ), gene flow (*Nm*), genetic differentiation index (F_{ST}), and Tajima's D test, have elucidated intra-ST diversity of *Blastocystis* sp. between populations of hosts carrying the protozoan. For example, the same ST1 population was found and transmitted between populations of howler monkeys but the identified ST2 populations were genetically distinct in these same populations of howler monkeys (Villanueva-Garcia et al. 2017). Likewise, *Blastocystis* sp. in children in Mexico were more genetically distinct than those in adults implying that adults are more likely to transfer *Blastocystis* sp. to other adults compared to children (Villegas-Gomes et al. 2016). Another study showed that *Blastocystis* sp. from IBS patients are less genetically diverse compared to those in asymptomatic populations implying that only certain populations are found in IBS patients (Vargas-Sanchez et al. 2015).

The host range of *Blastocystis* sp. could be related to the pathogen's genetic diversity. Certain patterns of host-specificity that have been uncovered are reported in the previous section. In this section elucidated on certain STs, particularly ST1, that are considered generalists and possibly are transmitted among different hosts through waterborne transmission. Genetic diversity studies can reveal not only patterns of host range but

also possible routes of transmission. This kind of information is particularly useful in assessing risks of infection.

PATHOGENICITY

Blastocystis sp. has been associated with a variety of diseases over the years. It is prominently associated with gastrointestinal disorders, such as diarrhea and abdominal pain (Andiran et al. 2006; Cabrine-Santos et al. 2015; Cirioni et al. 1999; Garavelli et al. 1991; Ghosh et al. 1998; Gil et al. 2016; Jerez Puebla et al. 2014; Karasartova et al. 2015; Kaya et al. 2007; Rajič et al. 2015; Rao et al. 2003; Tan and Suresh 2006b; Taşova et al. 2000; Vogelberg et al. 2010), IBS (Azizian et al. 2016; Fouad et al. 2011; Lepczyńska et al. 2016; Nourrisson et al. 2014; Yakoob et al. 2010a, b; Yakoob et al. 2004), and with appendicular peritonitis (Fréalle et al. 2015). Immuno-compromised patients, such as cancer and AIDS patients (Cirioni et al. 1999; Tan et al. 2009), iron deficiency anemia patients (El Deeb and Khodeer 2013), and those recovering from surgery or treatment (e.g., chemotherapy, renal transplant) (Ghosh et al. 1998; Gil et al. 2016; Karasartova et al. 2015; Rao et al. 2003; Taşova et al. 2000) are commonly reported to be at risk of pathogenic *Blastocystis* sp. infection. *Blastocystis* sp. has also been associated with presence of non-gastrointestinal related disorders. Most prominent of these are skin lesions and urticaria (Bálint et al. 2014; Casero et al. 2015; Katsarou-Katsari et al. 2008; Pasqui et al. 2004; Rajič et al. 2015; Valsecchi et al. 2004; Vogelberg et al. 2010) as well as Hashimoto's thyroiditis (Rajič et al. 2015), and formation of splenic cysts (Santos et al. 2014). However, other studies found no correlation between *Blastocystis* sp. infection and gastrointestinal disorders (Alinaghizade et al. 2017; Chen et al. 2003; Coskun et al. 2016; Dogan et al. 2017; Khademvatan et al. 2017; Rossen et al. 2015; Surangsrirat et al. 2010; Thamrongwittawatpong and Surangsrirat 2006). In fact, *Blastocystis* sp. is also commonly encountered in asymptomatic patients (Anuar et al. 2013; Pegelow et al. 1997; Santos and Rivera 2013; Velasco et al. 2011; Yaicharoen et al. 2005). Thus, this gastrointestinal protozoan is equally common in both symptomatic and asymptomatic patients. Most notably, El Safadi et al. (2014) reported *Blastocystis* sp. infection in all 93 children sampled with or without gastrointestinal disorders in the Senegal River basin area. *Blastocystis* sp. may even be a normal part of a healthy human gut flora (Lukeš et al. 2015). These discrepancies in associations make it difficult to assess the pathogenicity of *Blastocystis* sp. based on simple correlations with disease presence. Nonetheless, pathogenic isolates of *Blastocystis* sp. have been identified. The two most extensively studied pathogenic isolates are isolate B (ST7) from a symptomatic patient from Singapore and WR-1 (ST4) from a laboratory rat. *In vitro* and *in vivo* experiments on these two isolates have shown possible mechanisms and pathogenic effects of *Blastocystis* sp. on host intestines. The more prominent signs of pathogenicity of the organism are induction of apoptosis (Puthia et al. 2006; Walderich et al. 1998) and infiltration of host intestinal tissues (Abdel-Hafeez et al. 2016b; Elwakil and Hewedi 2010; Iguchi et al. 2009; Moe et al. 1997). In addition, Chandramathi et al. (2010c) also observed high levels of hyaluronidase, which is produced by nematodes and *Entamoeba histolytica* for infiltration of host skin and gut in rats. In contrast to these apoptotic effects, proliferation of human colonic cancer cell line HCT116 when incubated with *Blastocystis* sp. have been observed (Chan et al. 2012; Chandramathi et al. 2010a; Kumarasamy et al. 2013) leading to suspicions of *Blastocystis* sp. as a cause of colon cancer. However, this remains under investigation.

Vacuolar cells of *Blastocystis* sp. have been observed in *in vitro* studies to cause disruption of intestinal epithelial monolayer and of occluding tight junctions (ZO-1), and increased membrane permeability (Mirza et al. 2012; Wu et al. 2014a; Wu et al. 2014b). *Blastocystis* sp. can induce apoptosis of human adenocarcinoma HT-29 and Chinese hamster ovary cell lines (Walderich et al. 1998), IEC-6 rat intestinal epithelial cell lines (Puthia et al. 2006), and Caco-2 human colonic cancer cell lines (Wu et al. 2014b). *Blastocystis* sp. isolate B induces apoptosis of cell lines through activation of caspase-3 and caspase-9 (Wu et al. 2014b) and rho-kinase and HMG-CoA of host cells (Mirza et al. 2012). Moreover, this isolate can also adhere to Caco-2 cells leading to epithelial barrier dysfunction of the host cells (Wu et al. 2014a). In contrast, Puthia et al. (2006) observed contact-independent mechanism of inducing apoptosis to IEC-6 rat cell lines of *Blastocystis* sp. WR-1.

In vivo infection experiments showed that *Blastocystis* sp. can cause mucosal sloughing and inflammatory response in mice and rats through infiltration of the lamina propria (Elwakil and Hewedi 2010; Hussein et al. 2008; Li et al. 2013; Pavanelli et al. 2015). In particular, *Blastocystis* sp. isolate B and isolate H (ST7) have been observed to attach to and degrade mucin leading to disruption of crypt architecture and goblet cell loss in infected C57/BL6 mice previously treated with 2% DSS (Dextran sodium sulfate) (Ajjampur et al. 2016). Rats, instead of mice, are recommended as animal models since they are natural hosts of *Blastocystis* sp. but screening for current infection prior to start of experiments is required (Ajjampur and Tan 2016). Older mice are refractory to *Blastocystis* sp. (Ajjampur and Tan 2016) requiring induction of mild colitis using 2% DSS prior to infection (Ajjampur et al. 2016). They can also be utilized as immunosuppressed individuals in pathogenicity experiments (Abdel-Hafeez et al. 2016b). Nonetheless, *Blastocystis* sp. from symptomatic patients have been observed infiltrating the lamina propria of immunocompetent mice in *in vivo* pathogenicity assays (Elwakil and Hewedi 2010; Pavanelli et al. 2015). Further, mice may also be more suitable for immunology studies as the possibility of rats exhibiting prior immunity to *Blastocystis* sp. can lead to errors (Ajjampur and Tan 2016). Pigs have also been used for observation of pathogenicity. Similar to rats, healthy pigs are naturally infected with *Blastocystis* sp. with no signs of damage to their intestines (Fayer et al. 2014; Wang et al. 2014a). Anti-*Blastocystis* sp. IgA have also been observed more often in stool samples of immunosuppressed swine than in healthy swine (Wang et al. 2014b). Iguchi et al. (2007) also observed that not all human isolates of *Blastocystis* sp. of similar ST can infect chickens and rats. In their study, rats and chickens were inoculated with two cysts each of human isolates belonging to ST2, ST3, ST4, and ST7. Only one isolate each of ST4 and ST7 successfully infected both chickens and rats while the other two isolates of same STs and both ST2 isolates infected only chickens. Both ST3 isolates failed to infect both chickens and rats. Thus, caution should be observed when choosing a suitable animal model.

Pathogenicity may also vary among isolates of the same ST. Isolates from symptomatic patients have been observed to cause mucosal sloughing, inflammatory cell infiltration, and infiltration of lamina propria in mice (Abdel-Hafeez et al. 2016b; Elwakil and Hewedi 2010; Moe et al. 1997; Pavanelli et al. 2015) and rats (Chandramathi et al. 2014; Hussein et al. 2008; Iguchi et al. 2009). Specifically, Ajjampur et al. (2016) demonstrated that *Blastocystis* sp. isolates B and H from symptomatic patients are capable of infiltrating the intestinal tissues of C57/BL6 mice after inducing colitis with 2% DSS leading to death. Isolates from stool samples of infected mice in this study were also capable of infecting and causing tissue damage in other mice. *Blastocystis* sp. ST1 and ST3 from

symptomatic individuals have also been observed to cause tissue damage in Wistar rats (Hussein et al. 2008; Li et al. 2013). In contrast, isolates from asymptomatic individuals are weakly pathogenic or show no signs of pathogenicity in *in vitro* and *in vivo* assays. For instance, Hussein et al. (2008) observed that ST3 isolates from asymptomatic individuals were weakly pathogenic to Wistar rats while isolates from symptomatic individuals of the same ST caused tissue damage. Different ST7 isolates also exhibited different degrees of epithelial permeability in Caco-2 cells (Wu et al. 2014a) and tissue damage in C57/BL6 mice (Ajjampur et al. 2016). Moreover, *Blastocystis* sp. RN94-9 (ST4) did not cause mucosal sloughing in laboratory rats in inoculation experiments (Iguchi et al. 2009) and *Blastocystis* sp. Nand II (ST1) also did not exhibit cytopathic effects when incubated with colonic epithelial cell lines HT-29 and T-84 (Long et al. 2001). In addition, pathogenic effects may be host-specific. Wu et al. (2014b) observed epithelial layer disruption and apoptosis of human colonic cancer cell line Caco-2 cells by isolate B isolated from a human host but not by WR-1 isolated from rat.

Proteases both excreted or on the cell surface of *Blastocystis* sp. are suspected virulence factors and their activity may determine pathogenicity of an isolate. Cysteine proteases localized in the central vacuole (Puthia et al. 2008) are alleged to cause apoptosis and membrane permeability of host cells (Mirza et al. 2012). Serine, cysteine, and/or metalloproteases of *Blastocystis* sp. are possibly linked to increased paracellular permeability and perturbation of tight junctions of gut epithelial cells observed in biopsies of IBS patients (Poirier et al. 2012). High protease activity of *Blastocystis* sp. is suspected of causing IBS (Lepczyńska et al. 2016). Results of studies on cysteine protease activity of pathogenic strains correlated well with those from separate studies on *in vitro* and *in vivo* pathogenicity. Azocasein assays showed higher cysteine protease activities in *Blastocystis* sp. isolate B and ST3 isolates from symptomatic patients than in WR-1 and ST3 isolates from asymptomatic patients (Abdel-Hameed and Hassanin 2011; Mirza and Tan 2009; Rajamanikam and Govind 2013; Sio et al. 2006). Moreover, a 32 kDa protease has been implicated as a possible virulence factor (Abdel-Hameed and Hassanin 2011). Higher protease activities have also been observed in relation to neutral pH (Sio et al. 2006) and presence of amoebic forms of *Blastocystis* sp. (Rajamanikam and Govind 2013). Cysteine proteases, such as legumain and cathepsin B, are suspected virulence factors that may have immunomodulatory effects on human hosts (Ajjampur and Tan 2016). Variations in cysteine protease activity and protein sequence may be related to pathogenicity of *Blastocystis* sp. although further study is needed.

Certain isolates of *Blastocystis* sp. can cause host inflammatory response notably in cytokine production. Infection with the protozoa causes pro-inflammatory cytokine production (e.g., IL-8, IL-6) of colon cells (Abdel-Hafeez et al. 2016b; Chan et al. 2012; Chandramathi et al. 2010a; Chandramathi et al. 2010c; Iguchi et al. 2009; Kumarasamy et al. 2013; Puthia et al. 2008) through NF-κB (Chandramathi et al. 2010a; Puthia et al. 2008) and mitogen-activated protein (MAP) kinases (Lim et al. 2014). In contrast, Teo et al. (2014) reported NF-κB inhibition in monocytic cell line THP1-Blue. Moreover, Ragavan et al. (2015) reported that IBS patients infected with *Blastocystis* sp. also have elevated levels of IL-8 expression compared to IBS patients that are not infected with the protozoan. *Blastocystis* sp. infections also cause elevated productions of IgA in the intestinal lumen of pigs (Wang et al. 2014a) and BALB/c mice (Abdel-Hafeez et al. 2016b; Santos and Rivera 2009). In contrast, humans infected with *Blastocystis* sp. have low serum IgA (Nagel et al. 2015). Coincidentally, *Blastocystis* sp. WR-1 can also degrade human IgA (Puthia et al. 2005) but its relation to low serum IgA in patients infected with *Blastocystis* sp. remains a topic for further study. In addition, *Blastocystis* sp. infections are also known to cause oxidative damage to host gut (Chandramathi et al. 2010b), induce IgE production (Chandramathi et al. 2014), and activate Toll-like receptors, TLR-2 and TLR-4 (Teo et al. 2014).

Adding more controversy to the pathogenicity of *Blastocystis* sp. are more recent studies showing that this protozoan is part of normal gut microbiota and may even be beneficial to the host. It is already suggested that *Blastocystis* sp. along with other gut eukaryotes should be referred to as 'symbionts' rather than as 'parasites' due to their varying effects on the human gut as affected by factors such as gut microbiome composition, diet of host, and health of host (Lukeš et al. 2015). In this review, the definition of Leung and Polin (2008) was used for the term 'symbiosis' which is an intimate relationship between two organisms regardless of the outcome whether it would be beneficial, neutral, or detrimental. Thus, the term 'symbiont' encompasses all forms of symbiosis – mutualism, commensalism, and parasitism – and is not limited to a beneficial relationship to both host and microorganism. Identification of gut bacterial diversity and species richness using methods such as Next Generation Sequencing (NGS) has led to more information on association of certain bacteria with *Blastocystis* sp. However, results of such studies have again raised more questions rather than answers in determining the role of this eukaryote in the human gut. Nourrisson et al. (2014) reported association of *Blastocystis* sp. colonization with IBS class C that is associated with constipation. The use of quantitative PCR with specific primers in this study showed a decrease in abundance of the butyrate-producing bacteria *Faecalibacterium prausnitzii*. On the other hand, Audebert et al. (2016) found contrasting results. NGS results comparing gut bacterial diversity of 48 *Blastocystis*-colonized and 48 *Blastocystis*-free patients indicated an association of the gut eukaryote with higher gut bacterial diversity and species richness. Results of the study also showed more abundant *Faecalibacterium* and *Roseburia* as well as members of the Clostridia, Ruminococcaceae, and Prevotellaceae in *Blastocystis*-colonized individuals. Andersen et al. (2015) also observed similar associations of *Blastocystis* sp. carriage with dominance of *Prevotella* and *Ruminococcus* enterotypes. Their results showed that carriage of *Blastocystis* sp. may not be associated only with species richness but with the presence of certain *Prevotella* or *Ruminococcus* enterotypes as well (Andersen et al. 2015). All 9 patients positive for *Blastocystis* sp. in the study of Nourrisson et al. (2014) were colonized by ST4 with one patient having a ST4 and ST2 co-infection. Similarly, Andersen et al. (2015) noted that most of the *Blastocystis* sp. identified in their study was ST4 (21/58). The rest were ST3 (12/58), ST2 (11/58), ST1 (8/58), or mixed (6/58). ST identification was not performed in the study of Audebert et al. (2016). It is not yet determined whether *Blastocystis* sp. modulates the bacterial population of the host gut or tends to thrive more in certain populations. Based on the aforementioned studies, *Blastocystis* sp. is more likely associated with members of the Prevotellaceae and Ruminococcaceae or just with ST4. More studies are definitely needed to understand these associations and whether the changes that emerge are beneficial or not to the host.

The uncertain pathogenicity of *Blastocystis* sp. is a key issue on whether it should be included in disease control and surveillance or not. This is particularly important on account of its low host-specificity and the global shift of focus to 'One Health' or disease transmission between animals, humans, and the environment in tackling disease issues. As explained earlier, ST and intra-ST diversity are key aspects in determining zoonotic transmission. So far, there are no specific STs associated with disease in both humans and animals, but it is suspected that those

that are potentially pathogenic in humans may have zoonotic sources. Ajjampur et al. (2016) has come close to demonstrating the pathogenicity of *Blastocystis* sp. isolates B and H, both ST7 which is common in avian hosts. Swine are also possible sources of potentially pathogenic zoonotic transmission (Yan et al. 2007, Lee et al. 2012). However, tests for protease activity or *in vivo* or *in vitro* apoptotic tests were not carried out for these swine isolates. Despite uncertain pathogenicity, monitoring of the presence of *Blastocystis* sp. is still included in human health surveillance studies, particularly in international travelers to the United States (Harvey et al. 2013), in hospitalized diarrheal patients in India (Nair et al. 2010), and in Syrian refugees in Europe (Mockenhaupt et al. 2016) along with other parasites related to diarrhea (e.g., *Giardia lamblia*). In summary, *Blastocystis* sp. should not be left out in parasite surveys in both humans and animals. The inclusion of ST identification, if possible, is recommended most especially in diarrheic patients.

In vitro and *in vivo* studies all point to *Blastocystis* sp. as utilizing cysteine proteases to degrade the mucin layer and penetrate the gut epithelial membrane of the host. Vacuolar forms are usually observed in these studies even if amoebic forms are the suspected pathogenic forms. Again, genetic diversity may be related to varying degrees of pathogenicity of *Blastocystis* sp. However, this remains inconclusive as isolates of the same ST may have varying apoptotic activities or gut epithelia invasiveness. Moreover, results of pathogenic studies are mostly observations on one isolate, particularly *Blastocystis* sp. isolate B (ST7). These observations may not be the same with other isolates since other studies have shown that *Blastocystis* sp. is part of a healthy gut microflora. Further studies on other isolates are recommended as pathogenicity may be related to other factors.

PROGRAMMED CELL DEATH

Programmed cell death (PCD) is a characteristic of multicellular eukaryotic cells during development although it has also been observed in unicellular eukaryotes, particularly parasites such as *Giardia lamblia*, *Cryptosporidium parvum*, and *Entamoeba histolytica* (Nasirudeen 2005). PCD seems to be counterproductive to parasites residing within a host but it has its benefits. PCD may function to moderate parasite population to avoid death of the host, to prevent alerting host immune response, and to modulate apoptotic pathways of host cells particularly immune cells (Bruchhaus et al. 2007). PCD was first observed in *Blastocystis* sp. isolate B in the middle of colonies grown on agar medium (Tan et al. 2001a). Subsequent studies on this particular isolate soon determined that PCD in *Blastocystis* sp. can be triggered by the antiprotozoal drug metronidazole (Nasirudeen et al. 2004), the monoclonal antibody 1D5 which targets cell surface legumain (Tan et al. 2001b; Wu et al. 2010), and apoptosis-inducer staurosporine (STS) (Yin et al. 2010b). *Blastocystis* sp. cells undergoing PCD show characteristic features of multicellular cells undergoing apoptosis such as nuclear condensation, externalization of phosphatidylserine, maintenance of plasma membrane integrity with increasing permeability, cell shrinkage, and *in situ* DNA fragmentation (Nasirudeen et al. 2004; Nasirudeen et al. 2001; Wu et al. 2010; Yin et al. 2010b). Unlike apoptosis in multicellular eukaryotes, *Blastocystis* sp. PCD is caspase- and mitochondria-independent and may involve multiple mediators (Nasirudeen and Tan 2004, 2005; Tan and Nasirudeen 2005; Yin et al. 2010b). *In situ* DNA fragmentation, caused by exposure to monoclonal antibody 1D5, can only be partially inhibited by the mitochondrial transition blocker cyclosporine A and the pan-caspase inhibitor zVAD.fmk (Nasirudeen and Tan 2005) while the caspase-3-specific inhibitor Ac-DEVO-CHO has no effect (Nasirudeen and Tan 2004). However, these may be characteristic of monoclonal 1D5-induced PCD or of *Blastocystis* sp. isolate B or ST7 isolates. In contrast, Balakrishnan and Kumar (2014) observed high caspase-like activity in *Blastocystis* sp. ST3 isolates from symptomatic patients when PCD was induced by metronidazole treatment. PCD involves cell surface cysteine proteases since it can be triggered by monoclonal antibody 1D5 and inhibited by cysteine protease inhibitors such as the legumain-specific carbobenzoyl-Ala-Ala-AAsn-epoxycarboxylate ethyl esther (APE-RR) (Wu et al. 2010) and the broad-range cysteine protease inhibitor iodoacetamide (Yin et al. 2010b). *Blastocystis* sp. PCD in other STs requires further study as the mechanisms involved may differ depending on the ST or of the inducing agent used. The rate of apoptosis depends on the ST under study. For instance, ST3 exhibited the highest rate of apoptosis when compared to ST1, ST2, and ST5 after 72 hours of exposure to metronidazole (Dhurga et al. 2012). The relationship between PCD and pathogenicity remains a subject for future studies since both involve cysteine proteases. *Blastocystis* sp. also showed characteristics of autophagy induced by starvation and rapamycin treatment (Yin et al. 2010a). Its survival in the host gut is likewise a topic for further investigation.

DETECTION

Various methods of detection and laboratory diagnosis of *Blastocystis* sp. infections in humans have been continually used over the years. These methods include microscopy and xenic *in vitro* culture (XIVC), nucleic acid amplification-related methods (PCR, RT-PCR, pyrosequencing) and new methods such as matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) technology (Roberts et al. 2014b). An ELISA-based detection method for stool samples, the Copro-ELISA *Blastocystis* (Savyon Diagnostics) (Dogruman-Al et al. 2015) is likewise commercially-available. Microscopy and XIVC are the most commonly used methods of detection in clinical laboratories (Stensvold et al. 2009b) while nucleic acid amplification methods (PCR and RT-PCR) are generally employed in prevalence and subtyping studies (Roberts et al. 2014b; Stensvold 2013a). XIVC is more sensitive than microscopy (with or without trichrome stain) (Termmathurapoj et al. 2004) even when microscopy is coupled with a concentration technique such as formalin-ether concentration (FECT) (Stensvold et al. 2007a). It has been shown to be more sensitive than PCR (Santos and Rivera 2013). Live *Blastocystis* sp. enriched in culture are more easily identified compared to cells in preserved slides (Stensvold 2015). There are varieties of media used for XIVC detection in epidemiological and prevalence studies. These include Robinson's medium (Alfellani et al. 2013b; Yersal et al. 2016), Locke's egg (LE) medium (Santos and Rivera 2013; Yan et al. 2007), Ringer's solution (Li et al. 2007a; Li et al. 2007b), Tanabe-Chiba medium (Yoshikawa et al. 2016b), DMEM (Moosavi et al. 2012), Boeck-Drbohlav medium (Dominguez-Marquez et al. 2009), and a biphasic nutrient medium (Adao et al. 2016b; Banaticla and Rivera 2011; Belleza et al. 2015; Belleza et al. 2016; Dela Cruz et al. 2016; Rivera 2008). However, Jones' medium is most commonly used for XIVC (Abdulsalam et al. 2013; Alfellani et al. 2013b; Das et al. 2016; Jantermtor et al. 2013; Lee et al. 2012; Nagel et al. 2012; Nithyamathi et al. 2016; Rene et al. 2009; Sanpool et al. 2015; Souppart et al. 2010; Stensvold 2015; Stensvold et al. 2011; Tan et al. 2009; Thathaisong et al. 2013; Wong et al. 2008). Usually, XIVC is followed by PCR for subtyping, which can be achieved by either sequencing of the SSU rRNA gene or using subtype-specific (STS) primers. As XIVC enhances PCR amplification (Stensvold et al. 2007a; Termmathurapoj et al. 2004), certain reviews recommended ST

identification by PCR or RT-PCR from XIVC (Roberts et al. 2014b; Stensvold 2013a; Stensvold et al. 2007a). It has long been suspected that the choice of XIVC media may favor the growth of certain STs affecting the reported ST prevalence (Roberts et al. 2014b; Stensvold 2013a; Stensvold 2015) but evidence to prove this growth bias has yet to be found. Nonetheless, many researchers prefer direct stool DNA extracts over DNA extracts from XIVC (Abu-Madi et al. 2015; David et al. 2015; El Safadi et al. 2016; El Safadi et al. 2014; El Safadi et al. 2013; Eroglu et al. 2009; Forsell et al. 2016; Forsell et al. 2012; Helenbrook et al. 2015; Jones et al. 2009; Khoshnood et al. 2015; Malheiros et al. 2011; Mattiucci et al. 2015; Nagel et al. 2012; Pandey et al. 2015; Parkar et al. 2010; Popruk et al. 2015; Souppart et al. 2009; Villegas-Gómez et al. 2016). A variety of storage methods for stool samples before direct stool DNA extraction have also been employed including stool transport and recovery (S.T.A.R.) buffer (Bart et al. 2013; El Safadi et al. 2014), potassium dichromate solution (David et al. 2015), RNAlater (Helenbrook et al. 2015), freezing (Jones et al. 2009), phosphate buffered saline (PBS) (Malheiros et al. 2011), 70% ethanol (Forsell et al. 2012), or dried stool spots in filter paper (DSSFP) (Seyer et al. 2016). The barcoding primer pair RD5 and BhRDr (Scicluna et al. 2006) is the most commonly used and recommended for phylogenetic analyses and subtyping (Roberts et al. 2014b). These primers are best used with XIVC since they can also amplify non-specific DNA, particularly fungal DNA, when used with direct stool DNA extracts (Stensvold 2013a). In spite of this situation, the barcoding primers have been used on direct stool DNA extracts in several studies (Bart et al. 2013; David et al. 2015; El Safadi et al. 2014; El Safadi et al. 2013; Forsell et al. 2016; Forsell et al. 2012; Malheiros et al. 2011; Mattiucci et al. 2015; Pandey et al. 2015; Popruk et al. 2015). Other primers have also been used to amplify and/or sequence certain regions of the SSU rRNA gene. These include Blast 505-532/Blast 998-1017 (Santín et al. 2011), Blf/Blr (Grabensteiner and Hess 2006), F1/BHCRseq3 (Stensvold et al. 2007a), and BLF/BLR (Menounos et al. 2008). Other primers available for sequencing the entire SSU rRNA gene of *Blastocystis* sp. include RD3/RD5 and the primers of Böhm-Gloning et al. (1997) and Parkar et al. (2007), eukaryote-specific primers A and B of Medlin et al. (1988), and SR1F/SR1R with the inner primers F70, ABBH1, B6, and B71 (Abe 2004) or the inner primers 413-S, 827-S, 1256-S, and 1602-S (Rivera 2008). Full SSU rRNA gene sequencing is still useful in spite of the presence of barcode primers. Clark et al. (2013) recommended that new STs will only be declared if a full SSU rRNA gene sequence has a 5% or more difference with reference sequences of all the other STs. STS primers amplify specific STs without the need for sequencing. It also has the added advantage of identifying STs in mixed cultures. However, the STS primers still have some disadvantages. Some primers, particularly those used for identification of ST4, exhibit low sensitivity while STS primer regions exhibit sequence variations which may affect specificity (Stensvold 2013a). The STS primer set was once limited to the identification of ST1-ST7 but Yoshikawa and Iwamasa (2016) have since developed specific primer pairs for detection of ST1 to ST9, the *Blastocystis* sp. STs found in humans, in combination with XIVC. Scanlan et al. (2015) have also created ST-specific primers used in nested PCR with RD5/BhRDr with high specificity but these are limited to detection of ST1-ST4. There have been three RT-PCR methods developed for detecting *Blastocystis* sp. (Jones et al. 2009; Poirier et al. 2011; Stensvold et al. 2012). These primers have already been used on field samples for detection and subtyping of *Blastocystis* sp. from direct stool DNA extracts (El Safadi et al. 2016; El Safadi et al. 2014; Forsell et al. 2016; Jones et al. 2009). RT-PCR-detected STs that are different from those detected from barcoding PCR on the same sample have not been reported. The former, however, has the advantage of higher sensitivity compared to the latter. In fact, El Safadi et al. (2014) used RT-PCR to detect *Blastocystis* sp. on DNA extracts that were negative for barcoding PCR. This method showed 100% prevalence of the symbiont on the sample set. A new set of primers targeting the MLO gene has been developed for subtyping (Poirier et al. 2014). This study also reported on the utility of using the MLO gene, instead of the SSU rDNA gene for identifying intra-ST variation. In summary, a variety of methods are available for detection of *Blastocystis* sp. High sensitivity and specificity are possible with these methods along with the added advantage of identifying the ST of the isolate. The SSU rDNA is the usual target for detection and subtyping. However, the MLO gene and other genes may be useful in identifying intra-ST variation.

TREATMENT

The earlier sections explained the role of *Blastocystis* sp. in the human intestinal flora, which is still controversial due to reports of both its pathogenicity and benign/beneficial role to the host. This has led to referral of detection of *Blastocystis* sp. in stool samples as 'carriage' rather than 'infection'. This issue has already been tackled in reviews along with the necessity of drug treatment against *Blastocystis* sp. in human intestinal flora (Kurt et al. 2016; Stensvold et al. 2010). Treatment of *Blastocystis* sp. infection is reserved for patients with unexplained gastrointestinal symptoms. Research is still ongoing on the susceptibility of *Blastocystis* sp. to drugs despite the looming controversy on the necessity of treatment. Metronidazole is the first drug of choice for treatment but has not always been effective (Kurt et al. 2016; Roberts et al. 2014b; Stensvold et al. 2010). This drug can induce PCD in *Blastocystis* sp. (Nasirudeen et al. 2004). Several studies have shown the efficacy of this drug in eradicating the protozoa in infected patients albeit not in all cases (Haresh et al. 1999; Moghaddam et al. 2005; Nigro et al. 2003; Roberts et al. 2014a). Mirza et al. (2011) even demonstrated ST-related variations in metronidazole susceptibility of this gut protozoan. In this study, ST4 isolates were found more susceptible to metronidazole than ST7 isolates. Moreover, quick re-infection of the patient and the lack of the *ntr* and/or *nim* gene that induce/s production of the toxic form of metronidazole in *Blastocystis* sp. unlike in *Entamoeba* sp. and *Giardia* sp. indicated poor drug efficacy rather than resistance (Stensvold et al. 2010). However, there are metronidazole-resistant WR-1 ST4 isolates developed for study in various drug treatment experiments (Dunn et al. 2012). Other drugs that are effective in treatment of *Blastocystis* sp. infection include paromomycin and cotrimoxazole (Kurt et al. 2016; Stensvold et al. 2010). On the other hand, Roberts et al. (2015) suggested trimetoprim-sulfamethoxazole (TMP-SMX) as a first choice for treatment of *Blastocystis* sp. infection after it proved more effective than metronidazole in an *in vitro* study. TMP-SMX can also eradicate *Blastocystis* sp. infection in some although not all cases much like metronidazole. Moghaddam et al. (2005) reported eradication of *Blastocystis* sp. in only two out of 9 patients while Nagel et al. (2014) reported eradication in only six out of 10 *Blastocystis* sp.-positive IBS patients even when TMP-SMX was used in combination with secnidazole and diloxanide furoate. There are a number of compounds and plant extracts that have been tested for cytotoxic activity against *Blastocystis* sp. infections. The protozoan was found to be susceptible to the protease inhibitor E-64 (Al-Mohammed et al. 2013), monolaurine (lauric acid) and lactoferine (*Lactobacillus acidophilus*) in both *in vitro* and *in vivo* studies (Ismail et al. 2016). Moreover, the antimicrobial peptide LL37 exerted a cytotoxic effect on *Blastocystis* sp. except for isolate B, which showed resistance to the effects of the peptide (Yason et al. 2016). A number of plant extracts are effective against *Blastocystis* sp. in both *in vivo* and *in vitro* studies (Table 1).

Table 1: Plant extracts and other natural products with cytotoxic activity against *Blastocystis* sp.

Natural product	Solvent	Concentration	Type of study	Reference
Brucea javanica	Water	500 µg/ml	*in vitro*	Yang et al. (1996)
Coptis chinensis	Water	100 µg/ml	*in vitro*	Yang et al. (1996)
Brucea javanica	Dichloromethane	2 mg/ml	*in vitro*	Sawangjaroen and Sawangjaroen (2005)
Quercus infectoria	Methanol	2 mg/ml	*in vitro*	Sawangjaroen and Sawangjaroen (2005)
Nigella sativa	Water	100 and 500 µg/ml	*in vitro*	El Wakil (2007)
Serenoa repens	Ethanol	5 mg/ml	*in vitro*	Grabensteiner et al. (2008)
Thymus vulgaris	Ethanol	5 mg/ml	*in vitro*	Grabensteiner et al. (2008)
Vitis vinifera (seed)	Ethanol	5 mg/ml	*in vitro*	Grabensteiner et al. (2008)
Cucurbita pepo (fruit)	Ethanol	5 mg/ml	*in vitro*	Grabensteiner et al. (2008)
Allium sativum	Water	0.01 and 0.1 mg/ml	*in vitro*	Yakoob et al. (2011)
Ferula asafoetida (oil)	Ethanol	40 mg/ml	*in vitro*	El Deeb et al. (2012)
Ferula asafoetida (powder)	Ethanol	116 mg/ml	*in vitro*	El Deeb et al. (2012)
Green tea extract	Water	20-35 mg/ml	*in vitro* and *in vivo*	Al-Mohammed et al. (2013)
Achillea millefolium	Methanol	198.8 µg/ml	*in vitro*	Özbilgin et al. (2013)
Zingiber officinale	Water	20 mg/kg/day	*in vivo*	Abdel-Hafeez et al. (2015)
Allium sativum	Water	20 mg/kg/day	*in vivo*	Abdel-Hafeez et al. (2015)
Mallotus oppositifolius	Ethanol	27.8 µg/ml	*in vitro*	Christensen et al. (2015)
Vemonia colorata	Ethanol	117.9 µg/ml	*in vitro*	Christensen et al. (2015)
Zanthoxylum zanthoxyloides, cortex	Ethanol	255.6 µg/ml	*in vitro*	Christensen et al. (2015)
Z. zanthoxyloides, radix	Ethanol	335.7 µg/ml	*in vitro*	Christensen et al. (2015)
Eythrina senegalensis	Ethanol	527.6 µg/ml	*in vitro*	Christensen et al. (2015)
Clausena anisata	Ethanol	3114 µg/ml	*in vitro*	Christensen et al. (2015)
Eurycoma longifolia	Ethyl acetate	1.0 mg/ml	*in vitro*	Girish et al. (2015)
Purica granatum, peel	Water	3 g/kg	*in vivo*	Abdel-Hafeez et al. (2016a)

GENOMIC STUDIES

Whole genome sequences are available for the most extensively-studied *Blastocystis* sp. isolates WR-1 (ST4) (Wawrzyniak et al. 2015), isolate B (ST7) (Denoeud et al. 2011), and NandII (ST1) (Eme et al. 2017). MLO genomes for *Blastocystis* sp. Nand II (ST1) and DMP/02-328 (ST4), which have been used to study proteins and metabolic pathways present in the organism (Pérez-Brocal and Clark 2008; Stechmann et al. 2008; Tsaousis et al. 2012; Tsaousis et al. 2011), likewise, are accessible. In the same manner, whole genome sequences may also provide clues to pathogenicity of *Blastocystis* sp. either through production of proteases that affect integrity of host gut epithelium or of products that affect host gut microbiota. Whole genome analysis has already identified 20 secreted proteases that may be involved in the possible virulence of *Blastocystis* sp. with seven of these being specific to WR-1 (Wawrzyniak et al. 2015). Moreover, up to 2.5% of *Blastocystis* sp. NandII (ST1) genome consist of genes, acquired through lateral gene transfer, that may facilitate survival of the organism in the host gut (Eme et al. 2017). These include genes that influence metabolism, oxygen-stress response, host immune response evasion, and pathogenicity. Anaerobic metabolism of *Blastocystis* sp. may have been developed through lateral gene transfer (Eme et al. 2017). Notably, a tryptophanase gene has been identified in *Blastocystis* sp. Nand II. This gene produces indole which influences biofilm formation, cell-to-cell signaling of microorganism and establishment of host gut epithelial barriers that prevent intestinal inflammation (Lee et al. 2015). Indole production and the effect of the compound on host gut microbiota may be the link between *Blastocystis* sp. and intestinal disorders related to gut epithelia dysbiosis such as IBS and inflammatory bowel disease (IBD). IBS is a gastrointestinal disorder with typical symptoms such as abdominal pain and altered bowel movement but with no sign of disease that may cause such symptoms (Madden and Hunter 2002, Chey et al. 2015). It is divided based on three criteria as follows: IBS with diarrhea (IBS-D), IBS with constipation (IBS-C), and IBS with mixed bowel pattern (IBS-M) (Chey et al. 2015). IBD, on the other hand, is a chronic gastrointestinal disorder characterized by inflammation of the gut with presence of lesions. Ulcerative colitis and Crohn's disease are the two forms of IBD (Reiff and Kelly 2010). Dysbiosis or imbalance of gut microbiota is the suspected cause of both IBS and IBD (Carding et al. 2015). Several studies

Table 2: Subtypes and species of *Blastocystis* sp. identified in various sources using both barcode sequences and STS primers.

Source/host	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST14	*B. pythoni*	Mixed	Total
Human	55	6	107	30	8	0	0	0	0	1	207
Pig	14	1	0	0	18	0	2	0	0	0	35
Chicken	0	0	0	0	0	1	4	0	0	1	6
Duck	0	0	0	0	0	0	2	0	1	0	3
Goat	0	0	0	0	0	0	0	1	0	0	1
Wastewater	7	2	0	0	0	0	0	0	0	0	9
Philippine macaque	2	1	1	0	0	0	0	0	0	0	4
Box turtle	0	0	0	0	0	0	0	0	1	0	1
Dog	1	2	4	3	3	0	0	0	0	0	13
Total	79	12	112	33	29	1	8	1	2	2	279

examined the association among IBS, IBD, and *Blastocystis* sp. As with the other gastrointestinal disorders, association between IBS and *Blastocystis* sp. remains unclear with several studies both supporting (Yakoob et al. 2006, Yakoob et al. 2010, Yakoob et al. 2010b, Dogruman-Al et al. 2010, Fouad et al. 2011, Azizian et al. 2016) and contradicting (Thamrongwittawatpong and Surangsrirat 2006, Tungtrongchitr et al. 2006, Surangsrirat et al. 2010, Dogruman-Al et al. 2009) it. In particular, IBS-D has been found to be associated with either *Blastocystis* sp. ST1 (Yakoob et al. 2010b, Fouad et al. 2011) or ST3 (Azizian et al. 2016). Other studies did not identify the ST of *Blastocystis* sp. or the type of IBS. Unlike IBS, the association between *Blastocystis* sp. and IBD has not been extensively-studied. Boorom et al. (2007) hypothesized an association between IBD and *Blastocystis* sp. However, recent studies showed contradictory results (Dogruman-Al et al. 2009, Rossen et al. 2015, Coskun et al. 2016). Studies on direct effects of *Blastocystis* sp. and production of indole on gut microbiota that causes gastrointestinal disorders are still lacking.

Understanding the whole genome of *Blastocystis* sp. has shed new light on its physiology, potential pathogenicity, and potential in influencing the gut microbiota. These studies showed that *Blastocystis* sp. may have an indirect effect on causing gastrointestinal disorders through introducing changes in its neighboring gut microflora rather than an active role in causing disease. Information obtained from whole genome analysis may provide clues on possible mechanisms of the effect of this gut protozoan on the health of the host gut.

STUDIES IN THE PHILIPPINES

Researches on *Blastocystis* sp. in the Philippines are mostly on molecular diversity although there are also studies on comparison of detection methods (Santos and Rivera 2013), colony growth (Valido and Rivera 2007), and immune response (Santos and Rivera 2009). Results of studies on the molecular diversity of Philippine *Blastocystis* sp. isolates have already been published at the time riboprinting was still used before the advent of full SSU rRNA gene sequences (Rivera and Tan 2005; Tan and Rivera 2009). Surveys on ST diversity on various sources based on SSU rRNA gene sequences soon followed but these were mostly limited to the island of Luzon, particularly Metro Manila (Adao et al. 2016a; Adao et al. 2016b; Banaticla and Rivera 2011; Belleza et al. 2015; Belleza et al. 2016; Evidor and Rivera 2016; Rivera 2008). ST surveys in humans and animals in other parts of the country are therefore encouraged. Most of the abovementioned studies involved culture of the *Blastocystis* sp. first on biphasic medium supplemented with 10% horse serum and antibiotics (Rivera 2008) before DNA extraction and PCR. ST1 to ST7 have been grown on this medium as well as the rare STs such as ST14 and *Blastocystis pythoni* (Adao et al. 2016b). The study of Rivera (2008) was among the first to determine the lack of host-specificity of certain *Blastocystis* sp. STs. Other publications on ST diversity showed similarities in ST distribution with the findings of other surveys conducted in other parts of the world. For instance, ST3 is the most commonly detected ST in Filipino patients (Adao et al. 2016a; Belleza et al. 2016) while ST5 is the most frequently isolated ST in pigs in the Philippines (Adao et al. 2016b; Evidor and Rivera 2016). In addition, certain STs, particularly ST1, are common in humans, animals, and water samples (Adao et al. 2016a; Adao et al. 2016b; Banaticla and Rivera 2011; Belleza et al. 2016; Rivera 2008). The distribution of various *Blastocystis* sp. STs identified from various sources in the Philippines is summarized in Table 2. Studies on potential pathogenicity and cross-infection are crucial under Philippine conditions since *Blastocystis* sp. is highly prevalent in both humans and animals reaching as high as 40.7% in asymptomatic children (Baldo et al. 2004) and 36.8% in symptomatic patients (Tan and Rivera 2009) as observed in Metro Manila. Similarly, *Blastocystis* sp. carriage has been reported in as high as 38.5% of 122 pigs in Bay, Laguna (Dela Cruz et al. 2016). The prevalence of *Blastocystis* sp. in various hosts in the Philippines is presented in Table 3. Collating ST diversity and distribution in various sources can show which STs are most likely distributed by zoonotic, anthroponotic, or waterborne transmission. Moreover, additional studies on pathogenic potential of such isolates as well as immune response to such infections in both *in vitro* and *in vivo* assays can determine if *Blastocystis* sp. carriage in Filipinos poses a pathogenic risk or is a normal or even beneficial part of a healthy gut microbiome.

CONCLUSION

Blastocystis sp. has high molecular diversity which may or may not be related to host-specificity or influence on host gut microbiota. Future studies should definitely address these relationships whether or not they pertain to SSU rRNA gene diversity, MLO gene diversity, or protease activity diversity. Molecular diversity even within the same STs of *Blastocystis* sp. also implies diversity in physiology and more importantly potential pathogenicity, which greatly affects the significance of further research in a clinical aspect. It is this uncertainty that needs to be addressed before research in treatment and clinical detection can truly progress. Addressing results of *in vivo* and *in vitro* pathogenicity experiments on *Blastocystis* sp. isolates WR-1 (ST4), isolate B (ST7), and NandII (ST1) as characteristics of

Table 3: Prevalence of *Blastocystis* sp. in various sources from published studies in the Philippines.

Source	Prevalence	Location	Study
Humans	70/127 (40.7%)	Metro Manila	Baldo et al. (2004)
Humans	50/136 (36.8%)	Philippine General Hospital and College of Public Health (Manila)	Rivera and Tan (2005)
Guinea pig	2/4 (50%)	Arranque market, Metro Manila	Rivera and Tan (2005)
Pig	7/22 (31.8%)	Bustos, Bulacan	Rivera and Tan (2005)
Squirrel	5/5 (100%)	Arranque market, Metro Manila	Rivera and Tan (2005)
Chicken	6/19 (31.6%)	Bustos, Bulacan	Rivera and Tan (2005)
Chinese chicken	6/8 (75%)	Antipolo, Rizal	Rivera and Tan (2005)
Duck	8/16 (50%)	Antipolo, Rizal	Rivera and Tan (2005)
Lovebirds	9/18 (50%)	Arranque market, Metro Manila	Rivera and Tan (2005)
Quail	3/4 (75%)	Arranque market, Metro Manila	Rivera and Tan (2005)
Turkey	3/4 (75%)	Antipolo, Rizal	Rivera and Tan (2005)
Iguana	1/1 (100%)	Arranque market, Metro Manila	Rivera and Tan (2005)
Influent wastewater	7/31 (23%)	Various locations	Banaticla and Rivera (2011)
Effluent wastewater	2/31 (7%)	Various locations	Banaticla and Rivera (2011)
Humans	28/110 (25%)	San Isidro, Rodriguez, Rizal	Santos and Rivera (2013)
Humans	165/1271 (12.98%)	Pateros, Metro Manila	Belleza et al. (2015)
Philippine macaques	5/50 (10%)	Parks and Wildlife Bureau, Quezon City, Metro Manila	Casim et al. (2015)
Pigs	16/71 (22.5%)	Victoria, Laguna	Adao et al. (2016b)
Chicken	3/14 (21.4%)	Victoria, Laguna	Adao et al. (2016b)
Pigs	4/28 (14.3%)	Pila, Laguna	Adao et al. (2016b)
Goat	1/6 (16.7%)	Pila, Laguna	Adao et al. (2016b)
Chicken	2/20 (10%)	Pila, Laguna	Adao et al. (2016b)
Duck	3/14 (21.4%)	Pila, Laguna	Adao et al. (2016b)
Humans	29/35 (82.86%)	Pateros, Metro Manila	Adao et al. (2016a)
Dogs	21/145 (14.5%)	Pateros, Metro Manila	Belleza et al. (2016)
Pigs	47/122 (38.5%)	Bay, Laguna	Dela Cruz et al. (2016)
Pigs	14/100 (14%)	Metro Manila	Evidor and Rivera (2016)

Blastocystis sp. in general may be misleading. For instance, tissue damage in Wistar rats caused by isolate B (ST7) could be a characteristic of this particular isolate or a gene unique to this isolate. The discovery of lateral gene transfer in *Blastocystis* sp., an important research breakthrough, may indicate the possibility of genes found only in certain STs or even isolates.

Future studies should not be limited to potential pathogenicity and molecular diversity since the life cycle and influence on the gut microbiome are important aspects of *Blastocytis* sp. research as well. The cyst form is poorly studied as well as its induction and mechanism of transfer. Non-vacuolar forms, such as the amoebic and granular forms, may be dying cells due to low viability rather than actual viable morphological forms. The case of the amoebic form is a debatable issue since this morphological form has been implicated as the pathogenic form of *Blastocystis* sp. It may be important to indicate the predominant morphological form during pathogenicity and protease activity assays since this may also have an influence on the results. Metagenomic studies also imply that *Blastocystis* sp. has an indirect effect on host gut health through dysbiosis. Other isolates may lack capabilities to infiltrate or disintegrate tight junctions of cells in the gut epithelia, but they may have an influence on gut microbiota diversity that can have detrimental effects on the host gut.

Although its prevalence and diversity in the Philippines are high, *Blastocytis* sp. is mostly neglected due to its inconclusive

pathogenicity. More studies on the protozoan's life cycle, potential pathogenicity, molecular diversity, and its influence on host gut microbiota are needed. Identification of potentially pathogenic isolates remains important but other research tools should be explored. As aforementioned elsewhere, *Blastocystis* sp. can indirectly affect host gut health through its influence on gut microbiota diversity. Whole genome and protein analyses are important approaches that may elucidate on these complexities.

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