Chapter 6
Molecular Approaches on Systematic Position, Genetic Polymorphism, and Classification of *Blastocystis* Isolates from Humans and Animals

Hisao Yoshikawa

Abstract *Blastocystis* was first reported as a harmless yeast found in human stool samples in 1912. This organism has been classified into the subphylum Sporozoa and then reclassified into the subphylum Sarcodina based on the morphological and physiological characteristics. Molecular sequencing study on the SSU rRNA gene of a human *Blastocystis* isolate showed that *Blastocystis* is not monophyletic with yeasts (*Saccharomyces*), fungi (*Neurospora*), sarcodines (*Naegleria*, *Acanthamoeba*, and *Dictyostelium*), or sporozoans (*Sarcocystis* and *Toxoplasma*) based on the partial sequence of the gene. Phylogenetic studies inferred with the entire sequence of the SSU rRNA gene of a human and a guinea pig isolates of *Blastocystis* revealed that the organism is a member of the stramenopiles. Furthermore, accumulation of the molecular data of human and animal *Blastocystis* isolates showed not only extensive genetic diversity among *Blastocystis* isolates from the same hosts but also genetic similarities were observed among the isolates from different hosts. Therefore, a consensus terminology of *Blastocystis* sp. is recently proposed for the isolates from mammalian and avian hosts including humans and it classifies all isolates into nine subtypes. Recently, however, several additional novel subtypes have been reported based on the phylogenetic studies of the partial sequences of the SSU rRNA gene. Therefore, the genus *Blastocystis* may be the most genetically polymorphic organism that parasitizes a wide range of animals.

6.1 Introduction

Blastocystis hominis was originally isolated from humans and reported as a harmless yeast by Brumpt (1912). The taxonomic classification was first challenged by morphological and physiological characteristics in cultures and *Blastocystis*

H. Yoshikawa (✉)
Department of Biological Sciences, Nara Women's University, Nara, Japan
e-mail: h.yoshikawa@cc.nara-wu.ac.jp

H. Mehlhorn et al. (eds.), *Blastocystis: Pathogen or Passenger?*,
Parasitology Research Monographs 4,
DOI 10.1007/978-3-642-32738-4_6,

showed many protozoan characteristics rather than yeast (Zierdt et al. 1967). Physiologically, *Blastocystis* is strictly anaerobic and growth of the organisms is supported in some intestinal protozoan media, while no growth in fungal media. The optimal pH and temperature for growth are 7.0–8.0 and 37 °C, while no growth under than pH 6.0 and 33 °C, respectively. Morphologically, *Blastocystis* lacks a cell wall, contains nucleus, smooth and rough endoplasmic reticulum, Golgi complex, and mitochondria-like organelle. Subsequently, this organism was proposed as a sporozoan and then reclassified as a sarcodine by Zierdt and colleagues based on the more detail morphological characteristics in in vitro cultures (Zierdt and Tan 1976; Zierdt 1991). However, morphological features of this parasite do not fit to any other protozoa. Therefore, molecular studies are essential to estimate or classify systematic position among a wide range of the phylum protozoa.

6.2 Molecular Approach on Phylogenetic Affinity

The fist molecular systematic approach was undertaken by using the parasite's small subunit rRNA (SSU rRNA) gene sequences with those from different eukaryotes (Johnson et al. 1989). Based on the partial sequences, it was shown that *Blastocystis* is not monophyletic with the yeasts (*Sacchromyces*), sarcodines (*Naegleria, Acanthamoeba*, and *Dictyostelium*) or sporozoan (*Sarcocystis* and *Toxoplasma*). It was concluded that close relationships to the yeasts or the apicomplexas/sporozoans are not supported. A later study (Silberman et al., 1996) revealed entire sequence of the SSU rRNA gene of two *Blastocystis* isolates from a human and a guinea pig and it showed that *Blastocystis* could be a member of the heterogeneous group of stramenopiles. Stramenopiles are a diverse heterogeneous group of unicellular and multicellular algae and protists, including heterotropic and photosynthetic representatives (Patterson 1989). This diverse group includes brown algae, diatoms, oomycetes, and several other lineages. The closest relative of *Blastocystis* in the stramenopiles is *Proteromonas lacertae*, a commensal flagellate in the hindgut of lizards and amphibia (Leipe et al. 1996). However, unlike other stramenopiles, no flagella and tubular hairs have ever been observed in *Blastocystis*.

Another molecular approach has also been employed with the amino acid sequence of a highly conserved gene, elongation factor 1α (EF-1α). By using a maximum likelihood method of protein phylogeny, it was concluded that *Blastocystis* is not close to fungi and the divergence of the parasite is earlier than those of *Trypanosoma*, *Euglena*, *Dictyostelium*, and other higher eukaryotes (Nakamura et al. 1996). However, it was not possible to resolve the positions of *Blastocystis, Entamoeba, Plasmodium*, and *Tetrahymena* due to the low bootstrap probabilities. The subsequent study showed that, based on the genetic distance between homologous genes, *Blastocystis* from humans and animals diverged within the same group and the isolates all belonged to the same genus (Ho et al. 2000). The data also suggested an earlier emergence of *Blastocystis* among eukaryotes and a close relative of *Entamoeba histolytica*. Interestingly, these molecular phylogenetic studies of *Blastocystis* SSU rRNA and EF-1 gene sequences are apparently disparate conclusions on taxonomic or phylogenetic affiliations. Therefore, molecular approaches on systematic position of *Blastocystis* was conducted with multiple conserved genes, SSU rRNA, cytosolic-type 70-kDa heat shock protein, traslation elongation factor 2, and the non-catalytic 'B' subunit of vacuolar ATPase of *Blastocystis* with other stramenopiles (Arisue et al. 2002). This study clearly demonstrated that *Blastocystis* is a stramenopile and also alveolates are the closest relatives of *Blastocystis*/stramenopiles. More recently, phylogenetic analysis based on the conserved 5.8S rRNA gene with 12 stramenopiles, 2 fungi, 3 algae, and 3 alveolates showed *Blastocystis* positioned within stranenopiles, with *Proteromonas lacertae* as its closest relative (Hoevers and Snowden 2005).

6.3 Genetic Polymorphism and Molecular Classification

A large number of molecular studies have revealed that *Blastocystis* isolates from humans and animals appear to be extensively polymorphic in genetics (Yoshikawa et al. 1996, 1998, 2000, 2004a, b, c; Böhm-Gloning et al. 1997; Clark 1997; Hoevers et al. 2000; Snowden et al. 2000; Kaneda et al. 2001; Abe et al. 2003a, b, c; Abe 2004; Noël et al. 2003, 2005). In the earlier studies, random amplified polymorphic DNA (RAPD) analysis and restriction fragment length polymorphism (RFLP) analysis of the small subunit ribosomal RNA (SSU rRNA) genes have been applied to reveal genetic diversity among *Blastocystis* populations from humans to animals (Yoshikawa et al. 1996, 1998; Böhm-Gloning et al. 1997; Clark 1997; Hoevers et al. 2000; Snowden et al., 2000; Kaneda et al. 2001; Abe et al. 2003a, b). Namely, genetic variations within human and animal *Blastocystis* isolates were observed by RAPD analysis (Yoshikawa et al. 1996, 1998). An isolate from a chicken showed similar RAPD profiles with human isolates from USA and Japan, but not with five isolates from Singapore. This result indicates the genomic polymorphism among *Blastocystis* isolates from humans and animals and also a possibility of zoonotic potential of the parasite. In addition, subtype classification has been proposed for different genotypes among human *Blastocystis* isolates revealed by RAPD profiles and then developed subtype-specific PCR primers from the unique sequences of the RAPD products (Yoshikawa et al. 1998, 2000, 2003). Subsequently, subtype classification is generally accepted for consensus terminology for the genus *Blastocystis* (Stensvold et al. 2007). Conversely, Clark (1997) showed the sequence diversity among 30 human *Blastocystis* isolates using RFLP analysis of the amplified SSU rRNA genes and proposed seven ribodeme classification (riboprinting) among the isolates. This study showed extensive genetic diversity, over 7 % divergence between ribodemes 1 and 2 of human *Blastocystis* isolates. Surprisingly, this is approximately four times the genetic distance between the homologous genes of *Entamoeba histolytica* and *E. disper*. However, recent accumulation of RFLP analysis and sequencing of the SSU rRNA gene of human and animal isolates, it became apparent that RFLP approach is limited for use for classification of *Blastocystis* isolates. Therefore, genetic polymorphism among various human and animal *Blastocystis* isolates has been investigated based on the sequencing of the SSU rRNA gene.

Accumulated data of the SSU rRNA gene sequences of various *Blastocystis* isolates from humans and animals indicate that most of the phylogenetic clade show a mixed population of *Blastocystis* isolates from both humans and animals inferred with the SSU rRNA gene (Arisue et al. 2003; Noël et al. 2003; Yoshikawa et al. 2004c). These results strongly suggest that proposing of various new species names based mainly on host origins are problematic and cause confusion among *Blastocystis* isolates from various hosts (Yoshikawa et al. 2004c). Therefore, a consensus terminology of *Blastocystis* sp. is recently proposed for the isolates from mammalian and avian animal hosts including humans and it classifies all isolates into 9 subtypes (STs) based on the phylogeny inferred with SSU rRNA gene (Stensvold et al. 2007) (Table 6.1). The reason that *Blastocystis* sp. rather than *Blastocystis hominis* was proposed is because some reptilian and amphibian species seems to fall within the range of variation by the mammalian and avian clades (Yoshikawa et al. 2004c; Noël et al. 2005). If the reptilian or amphibian isolates are distinct species, all of the nine clades (nine subtypes) cannot be *Blastocystis hominis*. However, a most recent phylogenetic study inferred with the homologous gene including more wide range of isolates from vertebrate to invertebrate hosts has apparently shown that most isolates from poikilothermal animal hosts (i.e., reptiles, amphibians, and insects) are positioned into the basal clades among the genus of *Blastocystis*, suggesting that the isolates from poikilothermal animal hosts are different from homoiothermal animal hosts (i.e., mammals and birds) (Yoshikawa et al. 2007).

Based on the subtype classification, the majority of human *Blastocystis* belong to subtype 3, while *Blastocystis* isolated from several populations such as Iran, Malaysia, Pakistan, Philippines, and Thailand belong to subtype 1 (Table 6.1). However, the sample size of the latter group is relatively small in number (12–45 isolates) and discrepancy results were observed in the same countries in Pakistan and Thailand. Therefore more detail research should be accomplished in these countries to confirm which subtype is the dominant among the human *Blastocystis* populations. Moreover, recent findings of human infections with *Blastocystis* sp. STs 5, 8 and 9 (Yoshikawa et al. 2004a, b, c; Yan et al. 2007; Stensvold et al. 2008) clearly show that human is specific host capable of being infected by any *Blastocystis* subtypes. Recently, there have been reported additional novel STs from ST10 to ST13 based mainly on partial sequence (about 300–1,000 bp) of the SSU rRNA gene (Li et al., 2007a; Parkar et al. 2010; Stensvold et al. 2009b). However, there is no guarantee that phylogenetic tree inferred with partial sequence will fit to the tree inferred with the entire sequence of the same gene. Since subtype classification for *Blastocystis* sp. was originally inferred with the entire sequence of the SSU rRNA gene, full sequence of the SSU rRNA gene should deposit in GenBank as a reference sequence data for the novel subtypes.

Table 6.1 *Blastocystis* subtypes among human populations in various countries

		Subtype (ST)											
Country	No. of isolates	1	2	3	4	5	6	7	8	9	Unknown	Mixed ST	References
Bangladesh	26	2	0	24	0	0	0	0	0	0	0	0	Yoshikawa et al. (2004b)
China	35	13	2	14	0	0	0	0	0	0	1	5	Yan et al. (2006)
	78	16	1	55	1	0	0	0	0	0	3	2	Li et al. (2007b)
	192	47	9	116	1	0	1	0	0	0	8	10	Li et al. (2007a)
Denmark	99[a]	20	15	39	16	0	1	0	1	0	0	7	Rene et al. (2009)
	206	37	52	50	35	0	0	6	0	1	0	25	Stensvold et al. (2009a)
Egypt	44	8	4	24	8	0	0	0	0	0	0	0	Hussein et al. (2008)
	20	3	4	12	0	0	0	0	0	0	0	1	Souppart et al. (2010)
	100	15	0	39	0	0	23	13	0	0	0	10	Fouad et al. (2011)
Philippines	12	10[b]	0	0	0	0	0	0	0	0	2	0	Rivera and Tan (2005)
France	40	8	4	20	4	0	0	1	0	0	0	3	Souppart et al. (2009)
Germany	166	35	1	110	12	0	0	0	0	0	0	8	Böhm-Gloning et al. (1997)
	12	3	2	5	0	2	0	0	0	0	0	0	Yoshikawa et al. (2004b)
Greece	45	9	6	27	1	0	1	1	0	0	0	0	Menounos et al. (2008)
	51	7	5	32	1	0	1	5	0	0	0	0	Vassalos et al. (2010)
Iran	45	20[b]	4	16	0	0	2	3	0	0	0	0	Motazedian et al. (2008)
Italy	32	2	6	16	6	0	0	1	1	0	0	0	Meloni et al. (2011))
Japan	19	4	6	5	4	0	0	0	0	0	0	0	Kaneda et al. (2001)
	50	4	0	26	2	0	11	5	0	2	0	0	Yoshikawa et al. (2004b)
Malaysia	17	9[b]	1	7	0	0	0	0	0	0	0	0	Tan et al. (2008)
Nepal	20	4	4	12	0	0	0	0	0	0	0	0	Yoshikawa et al. (2009)
	63	2	0	0	12	0	0	0	0	0	8	41	Lee et al. (2012)
Pakistan	10	2	0	7	0	0	1	0	0	0	0	0	Yoshikawa et al. (2004b)
	179	87[b]	10	49	8	7	6	10	0	0	0	2	Yakoob et al. (2010)
Singapore	13	2	4	7	0	0	0	0	0	0	0	0	Wong et al. (2008)
Spain	51	1	2	0	48	0	0	0	0	0	0	0	Domínguez-Márquez et al. (2009)

(continued)

Table 6.1 (continued)

Country	No. of isolates	Subtype (ST)											References
		1	2	3	4	5	6	7	8	9	Unknown	Mixed ST	
Sweden	63	10	9	30	13	0	0	1	0	0	0	0	Forsell et al. (2012)
Thailand	153	7	0	138	2	0	0	0	0	0	0	6	Thathaisong et al. (2003)
	68	53[b]	15	0	0	0	0	0	0	0	0	0	Leelayoova et al. (2008)
Turkey	92	17	20	51	0	0	0	0	0	0	0	4	Dogruman-Al et al. (2008)
	87	8	12	66	1	0	0	0	0	0	0	0	Özyurt et al. (2008)
	32	0	3	29	0	0	0	0	0	0	0	0	Eroglu et al. (2009)
	19	0	8	10	0	0	0	0	0	0	0	1	Dogruman-Al et al. (2009a)
	66	10	9	38	0	0	0	0	0	0	0	9	Dogruman-Al et al. (2009b)
	50	8	5	32	0	0	0	0	0	0	0	5	Yoshikawa et al. (2011)
UK	30	3	1	22	4	0	0	0	0	0	0	0	Clark (1997)
	56	3	9	22	21	0	0	0	0	0	0	1	Scicluna et al. (2006)
US	7	1	0	6	0	0	0	0	0	0	0	0	Jones et al. (2009)
	5	1	1	1	1	0	0	0	1	0	0	0	Whipps et al. (2010)
Total	2297	487	225	1135	180	9	47	46	3	3	22	139	

[a] A total number of samples were obtained from 74 patients.

[b] The subtype 1 is the major subtype in these countries.

6.4 Conclusion

Ever since the first description of a harmless yeast *Blastocystis hominis* in humans in 1912, this eukaryotic microorganism has been classified into various subphylum based on morphological and physiological characteristics. However, recent accumulation of the molecular data of several conserved genes indicates that the genus *Blastocystis* is a member of the stramenopiles. Furthermore, an extensive genetic diversity has been recognized among *Blastocystis* isolates from humans and animals indicating that this parasite may be one of the most indistinguishable unicellular parasite composing from heterogeneous groups of different species. Because, it has been reported that the 629 positions from the university core region of the SSU rRNA gene, that should be very conservative, the maximum difference among the *Blastocystis* isolates was shown to be 3.6 %, which was comparable with the differences between *Saccharomyces cerevisiae* and *Pneumocystis carinii* (3.2 %) and between *Zea mays* and *Chlorella kessleri* (3.8 %) (Arisue et al. 2003). In addition, this protozoan parasite may not be host specific and many animal species are potential sources for reservoirs of zoonotic isolates. Further molecular research may facilitate species designation for this parasite and pathogenic potential of the specific genotype or species.

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