IN VITRO AND *IN VIVO* PHENOTYPIC STUDIES TO CHARACTERIZE *BLASTOCYSTIS* SP. SUBTYPE 3 (ST3) ISOLATED FROM ASYMPTOMATIC INDIVIDUALS, SYMPTOMATIC AND IRRITABLE BOWEL SYNDROME (IBS) PATIENTS.

NANTHINEY DEVI A/P RAGAVAN

FACULTY OF MEDICINE
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NANTHINEY DEVI A/P RAGAVAN

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Registration/Matric No: **MHA 120027**

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***IN VITRO* AND *IN VIVO* PHENOTYPIC STUDIES TO CHARACTERIZE *BLASTOCYSTIS* SP. SUBTYPE 3 (ST3) ISOLATED FROM ASYMPTOMATIC INDIVIDUALS, SYMPTOMATIC AND IRRITABLE BOWEL SYNDROME (IBS) PATIENTS.**

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ABSTRACT

The role of intestinal infection mediated-inflammation in the pathogenesis of irritable bowel syndrome (IBS) remains uncertain. *Blastocystis* sp. is the most common gut parasite found in the intestinal tract of humans. Its association with IBS is controversial, possibly as a result of irregular shedding of parasites in stool. The study aimed to screen for *Blastocystis* sp. in stool aspirate and stool samples in adult patients with and without IBS undergoing colonoscopy and measure the interleukin levels (IL-3, IL-5 and IL-8). This study suggests that stool aspirate sample may be a better method for sample collection for detecting *Blastocystis* sp. by PCR method during colonoscopy. Subtype 3 (ST3) was found to be the most predominant subtype. An increase in the interleukin levels demonstrates that *Blastocystis* sp. does have an effect in the immune system. The phenotypic characteristics of *Blastocystis* sp. ST3 isolated from asymptomatic individuals, symptomatic and irritable bowel syndrome (IBS) patients were analyzed. *Blastocystis* sp. ST3 isolated from IBS patients was shown to have a distinct growth profile, strong aggregation and clumping of parasites when stained with Modified Fields' stain with outer surface showing a greater binding affinity towards FITC Con A in symptomatic than asymptomatic isolates. Ultrastructural studies also showed that the parasite isolated from IBS patients possesses a rough, coarse surface with thicker surface coat and the presence of electron dense material. Ileum from the rabbit and Balb/C mice showed the highest number of muscle twitching when introduced with *Blastocystis* sp. ST3 antigen (Blasto-Ag ST3) derived from *Blastocystis* sp. infected IBS patient. The present study is the first to demonstrate the phenomenon of gut environment facilitating adaptation of parasites possibly for survival leading to phenotypic differences for *Blastocystis* sp. within a particular subtype. An *in vitro* model to study the pathogenicity of *Blastocystis* sp. ST3 was designed by comparing the

degree of mucosal sloughing, inflammation and necrosis of tissue of the *Wistar* rat's ileum, caecum, colon and rectum orally inoculated with *Blastocystis* sp. ST3 cysts isolated from asymptomatic individuals, symptomatic and IBS patients. This is the first study to provide evidence that occurrence of mucosal sloughing, inflammation and necrosis was seen to be higher in IBS group compared to symptomatic and asymptomatic group. This study supports the earlier findings that intestinal inflammation does play an important role in the clinical manifestation of IBS patients. With this, the association between *Blastocystis* sp. infection and inflammation was investigated. Wnt signaling pathway had been linked with inflammation and colorectal cancer. In the present study, we attempted to assess the effects Blasto-Ag derived from non-IBS and IBS patients respectively on the growth and gene expression of cancer cells, HCT116 *in vitro* compared with normal cells, CCD-18Co. The results revealed that the proliferation rate and migration of cells for both normal and cancer cells were significantly higher when induced with IBS Blasto-Ag ST3 compared to non-IBS Blasto-Ag ST3 implying that it may play a role in advancing colorectal tumor progression in IBS patients. Wnt analysis showed that a large number of canonical Wnts involved in the canonical pathway for both normal and cancer cells upon exposure of both the non-IBS and IBS Blasto-Ag ST3. However, IBS Blasto-Ag ST3 showed a greater effect on both cells compared to non-IBS Blasto-Ag ST3. The present study also conclusively provides evidence for the first time the factors such as the frequency of toilet visit in a day, the timing of toilet visit, the stool forms, and patient's moods as well as emotions can influence the shedding pattern of *Blastocystis* sp. cysts in an IBS patient. Stools should be collected in the morning; especially samples should be taken from the semi-solid form every time one visits the toilet even within a day for diagnostic purposes.

ABSTRAK

Peranan jangkitan usus yang menyebabkan keradangan dalam '*irritable bowel syndrom'* ataupun lebih dikenali sebagai IBS masih menjadi tanya soal. *Blastocystis* sp. adalah salah satu parasit usus yang hidup di dalam usus manusia dan haiwan. Parasit ini sukar ditemui di dalam najis pesakit IBS semasa ujian najis dijalankan di makmal, kemungkinan besar akibat fenomena kehadiran parasit yang tidak menentu di dalam najis. Oleh itu, pengajian ini bertujuan untuk mengesan *Blastocystis* sp. menggunakan sampel aspirasi najis dari kolon pesakit IBS yang menjalani pemeriksaan kolonoskopi dan dibandingkan dengan pesakit tanpa IBS. Selain itu, sampel najis juga dianalisis dan tahap interleukin (IL-3, IL-5 dan IL-8) pesakit tanpa-IBS dan IBS diukur. Keputusan penyelidikan menunjukkan kaedah PCR adalah terbaik untuk mengesan kehadiran parasit dengan menggunakan sampel aspirasi najis dari kolon semasa kolonoskopi manakala subjenis 3 (ST3) merupakan subjenis yang paling utama dijumpai dalam pengajian ini. Pesakit IBS yang ditambah pula dengan jangkitan parasit mempunyai peningkatan dalam tahap interleukin menunjukkan bahawa *Blastocystis* sp. mempunyai kesan terhadap sistem imun mereka berbanding pesakit tanpa-IBS. Ciri fenotip *Blastocystis* sp. ST3 daripada individu asimptomatik, pesakit simptomatik dan IBS juga dianalisis. Sehingga kini, tiada sebarang kajian dilakukan untuk menunjukkan perbezaan dari segi fenotip dalam sub-jenis yang sama iaitu ST3. Ini merupakan kajian pertama membuktikan *Blastocystis* sp. daripada pesakit IBS adalah berbeza berbanding parasit yang diasingkan daripada pesakit asimptomatik dan simptomatik. *Blastocystis* sp. daripada pesakit IBS mempunyai kadar pembiakaan yang berbeza manakala pengagregatan kuat dan pengumpalan parasit adalah lebih ketara apabila diwarnakan dengan '*Modified Fields' stain*'. Parasit daripada pesakit IBS juga berupaya menyerap pewarna FITC Con A pada kadar yang lebih tinggi menyebabkan permukaannya

berkilau dengan terang disebaliknya permukaan parasit daripada asimptomatik dan simptomatik adalah pudar dan tidak begitu terang. Lebih-lebih lagi, pengajian ultrastructural menunjukkan parasite daripada pesakit IBS mempunyai permukaan yang lebih kasar, tebal dan kehadiran elektron yang padat kelihatan di vakuol. Antigen daripada parasit IBS (IBS Blasto-Ag ST3) didedahkan pada usus kecil (ileum) arnab dan tikus Balb/C tikus menghasilkan kadar denyutan otot usus yang lebih tinggi berbanding antigen parasit daripada asimptomatik dan simptomatik. Kajian ini adalah yang pertama untuk menunjukkan fenomena persekitaran usus memudahkan penyesuaian parasit mungkin untuk hidup yang membawa kepada perbezaan fenotip untuk *Blastocystis* sp. ST3. Kajian ini adalah kajian pertama menunjukkan fenomena persekitaran usus boleh menyebabkan perubahan fenotip parasit berlaku agar ia dapat meruskan kehidupanya. Model *in vitro* untuk mengkaji patogenesiti *Blastocystis* sp. ST3 telah direka untuk membandingkan tahap kehakisan mukosa, radang dan nekrosis tisu daripada ileum, kolon, usus buntu dan rektum tikus *Wistar* yang disuntik melalui mulut dengan *Blastocystis* sp. sista ST3 yang diasingkan daripada individu asimptomatik, pesakit simptomatik dan IBS. Ini adalah kajian pertama memberi bukti di mana kehakisan mukosa, radang dan nekrosis tisu daripada kumpulan IBS adalah lebih tinggi berbanding kumpulan asimptomatik dan simptomatik. Kajian ini menyokong penemuan sebelum ini di mana keradangan usus memainkan peranan yang penting dalam manifestasi simptom klinikal pesakit IBS. Kajian lanjut untuk menyiasat hubungan antara usus pesakit dan parasit dijalankan. Keradangan juga dikenali sebagai faktor pencetus awal untuk perkembangan kanser. Selain itu, mekanisma Wnt telah dikaitkan dengan kanser kolorektal. Dalam kajian ini, penilaian terhadap kesan *Blastocystis* sp. ST3 antigen (Blasto-Ag ST3) yang diperolehi daripada pesakit tanpa-IBS dan pesakit IBS masing-masing dikaji dari segi pembiakan sel dan expresi gen dalam sel-sel kanser, HCT116 *in vitro* (dibandingkan dengan sel-sel normal, CCD-

18Co). Percambahan sel assay telah dijalankan untuk mengkaji kesan antigen pada pertumbuhan sel *in vitro* dalam sel-sel kanser dibandingkan dengan sel-sel normal. Keupayaan IBS Blasto-ag ST3 daripada kumpulan IBS menyebabkan percambahan sel yang lebih tinggi dan penghijrahan *in vitro* menunjukkan bahawa parasit ini boleh memainkan peranan penting dalam perkembangan tumor kolorektal pesakit IBS. Dengan ini, hubungan antara *Blastocystis* sp. ST3 dan keradangan dikaji. Dalam kajian ini, kami cuba untuk menilai kesan *Blastocystis* sp. ST3 antigen (Blasto-Ag ST3) yang diperolehi daripada pesakit bukan-IBS dan IBS dan dedahkannya kepada sel-sel normal dan kanser secara *in vitro.* Keputusan menunjukkan bahawa kadar percambahan dan pemindahan sel untuk kedua-dua sel normal dan kanser adalah lebih tinggi apabila didorong dengan IBS Blasto-Ag ST3 berbanding bukan IBS Blasto-Ag ST3 membayangkan bahawa ia boleh memainkan peranan dalam memajukan perkembangan tumor kolorektal di pesakit IBS. Analisis Wnt menunjukkan bahawa sebilangan besar Wnt gene terlibat dalam laluan *'canonical'* (perencatan β catenin pemfosforilan) untuk kedua-dua sel normal dan kanser apabila didehkan kepada Blasto-Ag bukan-IBS dan IBS. Walau bagaimanapun, IBS Blasto-Ag mempunyai kesan yang lebih tinggi pada kedua-dua sel berbanding bukan IBS Blasto-Ag. Expresi Wnt3 dan Wnt7a gen adalah tinggi di kedua-dua sel-sel apabila didedahkan kepada IBS Blasto-Ag ST3 mencadangkan bahawa gen ini boleh menjadi penanda biologi radang. Kajian ini buat kali pertama membuktikan bahawa faktor-faktor seperti kekerapan lawatan tandas dalam sehari, masa lawatan tandas, bentuk-bentuk najis dan perasaan serta emosi boleh mempengaruhi corak tidak menentu *Blastocystis* sp. sista dalam pesakit IBS. Najis perlu diambil pada waktu pagi, bentuk separuh pepejal dan setiap kali ke tandas untuk sepanjang hari. Cadangan ini boleh digunakan untuk tujuan diagnostic.

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Nanthiney Devi Ragavan

March 2016

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°	Degree
µl	Microliter
µm	Micrometer
AES	Amino-Terminal Enhancer of Split
AFU	Affinity fluorescence unit
AIDS	Acquired Immune Deficiency Syndrome
APC	Adenomatous Polyposis Coli
AP-PCR	Arbitrarily-primed PCR
ATCC	American Type Culture Collection
AXIN1	Axin 1
Balb/C	Albino, laboratory-bred strain of the house mouse
BCL9	B-Cell CLL/Lymphoma 9
bp	Base pair
BTRC	Beta-Transducin Repeat Containing E3 Ubiquitin
CCD18-Co	Normal colon fibroblast
CCND	Cyclin D
CER1	Cerberus 1
CHD	Chromodomain Helicase DNA Binding Protein
c-MYC	c-Myelocytomatosis
CRC	Colorectal cancer
CREBBP	CREB Binding Protein
CTNNB1	Catenin (Cadherin-Associated Protein) Beta 1

DNA	Deoxyribonucleic acid
DVL	Dishevelled
EDTA	Ethylenediaminetatraacetic acid
EF-1α	Elongation factor 1α
EP300	E1A Binding Protein P300
FECT	Formal ether concentration technique
FITC	Fluorescein isothiocyanate
FOSL-1	FOS-Like Antigen 1
FRAT	Frequently Rearranged In Advances T-Cell Lymphomas
FZD	Frizzled
g	Gram
GSK3-β	Glycogen Synthase Kinase 3 Beta
h	Hour
HCT116	Human colorectal carcinoma cells
IBS	Irritable Bowel Syndrome
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilo Dalton
LEF	Lymphoid enhancer-binding factor
LRP	Low Density Lipoprotein Receptor Related Protein1
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
NK	Natural killer

NKD	Naked Cuticle Homolog
nm	Nanometer
NRPS	Non-ribosomal polyketide synthase
PAR	Protease-activated receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Power of hydrogen
PKS	Polyketide synthase
qPCR	Quantitative PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcription
sec	Second
SEM	Scanning electron microscopy
SFRP	Secreted Frizzled Receptor Protein
sIgA	Secretory immunoglobulin A
SNPs	Single nucleotide polymorphisms
sp.	Species
SPSS	Statistical Package for the Social Science
SSU_rRNA	Small sub-unit ribosomal ribonucleic acid
ST	Subtype
STS	Sequence-tagged site
Taq	*Thermus aquaticus*
TCF	Transcription factor 7

TEM	Transmission electron microscopy
Th	T helper
TJ	Tight junction
TMP-SMX	Trimethoprim/sulfamethoxazole
TNF-α	Tumor necrosis factor-alpha

LIST OF APPENDICES

Chapter 1: Introduction

1.1 Research background

Blastocystis sp. is a protozoan parasite which belongs to the Phylum Stramenophile (Ciferri et al., 1938; Arisue et al., 2002). This parasite is known to be polymorphic and exists in different forms such as, amoeboid, avacuolar, cyst, granular, multivacuolar and vacuolar (Stenzel & Boreham, 1996; Suresh & Smith, 2004).

Blastocystis sp. has a worldwide distribution and is found to have a high prevalence in developing countries especially in the tropics and subtropics (Ashford & Atkinson, 1992; Zierdt et al., 1995) especially among children and adults. Its prevalence has been attributed to improper sanitation systems, poor personal hygiene, consumption of contaminated water and food as well as close proximity with animals (Stenzel & Boreham, 1996; Abdulsalam et al., 2012). The clinical symptoms in a symptomatic patient are non-specific including abdominal pain, diarrhea, nausea, vomiting abdominal pain, anorexia, weight loss and flatulence (Stenzel & Boreham, 1996; El-Shazly et al., 2005; Abdulsalam et al., 2012).

Diagnosis is by direct smear, light microscopy, *in vitro* cultivation and polymerase chain reaction (PCR) (Stenzel & Boreham, 1996). The epidemiological studies carried out previously have used a variety of these methods in detecting *Blastocystis* sp., the most sensitive being quantitative PCR (Poirier et al., 2012).

Morphologically the organism looks the same under the microscope but extensive genetic studies have revealed many subtypes. Stensvold et al., (2013) had

reported that there are 17 STs (ST1-ST17), in which ST1-ST9 were found in humans. Several studies have reported that genotypes of the parasite especially *Blastocystis* sp. ST3, may influence the pathogenicity in patients in Malaysia (Tan et al., 2008), Singapore (Wong et al., 2008) and the United States (Jones et al., 2009). All these studies showed that ST3 is the most predominant subtype

The pathogenicity of *Blastocystis* sp. is still controversial as this parasite can be isolated from asymptomatic individuals and patients with gastrointestinal symptoms (Tan et al., 2002; Stensvold et al., 2007). Patients infected with *Blastocystis* sp. did not exhibit any symptoms and were categorized as asymptomatic carrier. Meanwhile, patients infected with *Blastocystis* sp. and suffering from gastrointestinal symptoms such as diarrhea, abdominal pain, nausea and flatulence were categorized as symptomatic patient (Dagci et al., 2014). A recent study showed that *Blastocystis* sp. isolated from asymptomatic individuals and symptomatic patients are phenotypically different but belong to the same subtype (Tan et al., 2008). The pathogenicity of *Blastocystis* sp. still remains uncertain with conflicting reports on the pathogenic status of the organism (Levy et al., 1996; Carrascosa et al., 1996; Leelayoova et al., 2004; Andiran et al., 2006). However, there is much confusion on the role of this parasite in human disease (Tungtrongchitr et al., 2004; Leder et al., 2005).

Blastocystis sp. can also be an opportunistic parasite when it is present in immunocompromised individuals, especially in AIDS and cancer patients (Horiki et al., 1999; Kurniawan et al., 2009; Chandramathi et al., 2012). *Blastocystis* sp. had been known to have a potential association with irritable bowel syndrome (IBS) (Carrascosa et al., 1996; Giacometti et al., 1999; Yakoob et al., 2004; Boorom et al., 2008; Yakoob et al., 2010a; Jimenez et al., 2012). Both IBS and *Blastocystis* sp. infection share the

same symptoms such as constipation, abdominal pain, diarrhea, cramps, nausea and fatigue (Qadri et al., 1989; Graczyk et al., 2005; Boorom et al., 2008). It is possible that *Blastocystis* sp. infection in an IBS patient could further exacerbate the existing gastrointestinal symptoms. These patients were categorized as *Blastocystis* sp. infected IBS patients. IBS is also known functional gastrointestinal disorder based on the symptoms (Yakoob et al., 2010a). The symptoms are characterized by chronic diarrhea, excessive intestinal gas, abdominal pain, bloating and alteration of bowel habits which is based on Rome III criteria (Hussain et al., 1997). Rome III criteria had been used to categorize the functional gastrointestinal disorders based on the patient's clinical symptoms (Longstreth et al., 2006). Rome III criteria for IBS are usually when patients had recurrent abdominal pain or discomfort and a marked change in bowel habit, with symptoms experienced on at least three days or at least 3 months with two or more of the following

a) pain is relieved by a bowel movement;
b) onset related to a change in frequency of stool;
c) onset of pain is related to a change in the appearance of stool (Drossman & Dumitrascu, 2006).

The difference between *Blastocystis* sp. infected IBS patients and *Blastocystis* infected symptomatic patients is the timescale of suffering of the gastrointestinal symptoms. *Blastocystis* sp. infected IBS patients are usually confirmed by clinician based on Rome III criteria. A study had reported that at least 60% of people with IBS tend to have psychological disorder especially anxiety and depression (Whitehead et al., 2002). All these could contribute to work absenteeism (Maxion-Bergemann et al., 2006; Paré et al., 2006) which results in a poor quality of life as well as become major

contributors to economic burden (Longstreth et al., 2003; Hulisz, 2004; Wilson et al., 2004; Schmulson et al., 2006).

The pathophysiology of IBS remains obscure and there is no single contributory cause identified for the distinct clinical presentations seen in this heterogeneous gut disorder (Sainsbury & Ford, 2011). Other factors that may contribute to the symptoms of IBS include altered gut motility in response to the enhanced modulation of visceral perception, psychosocial factors or visceral hypersensitivity (Thompson et al., 2000).

Apart from that, the onset of symptoms is more likely to happen after an ongoing gut infection accompanied by diarrhea and vomiting (Spiller & Garsed, 2009). This could be due to bacterial or parasitic infection which trigger the gut and further exacerbate the recurring symptoms of IBS. Gut infection could result in abnormalities of gut flora, inflammation of intestinal tissues and altered bowel function.

Gwee (2005) postulated that inflammatory changes in adult with post-infectious IBS can be seen after a gut infection resulting in intestinal infection mediated-inflammation. A few studies also revealed that parasites such as *Blastocystis* sp. (Hussain et al., 1999; Giacometti et al., 1999), *Dientamoeba fragilis* (Windsor & Johnson, 1999), *Giardia lamblia* (D'Anchino et al., 2002) and *Entamoeba histolytica* (Sinha et al., 1997) can contribute to the symptoms of IBS.

Mediators of inflammation can result in gene modification which triggers mutation and alter the expression of genes, which eventually can lead to cancer (Hussain & Harris, 2007). There are many signaling pathways involved in cancer progression. Vascular endothelial growth factor (VEGF signaling pathway) (McMahon,

2000), cyclic adenosine monophosphate (cAMP) pathway (Huang et al., 2005), p53 pathway (Vazquez et al., 2008), TGF-β pathway (Derynck et al., 2001) and Wnt signaling pathway (Giles et al., 2003) show that gene mutation can lead to cancer progression. Out of the many signaling pathways, Wnt signaling pathway is very much associated with colon cancer. The Wnt signaling pathway regulates the gene transcription in the nuclear level and play a major role in the pathophysiology of inflammation especially colorectal cancer (Bienz & Clevers, 2000; Giles et al., 2003). This pathway also influence the proliferation and migration of cells (Toyama et al., 2010).

1.2 Justification of the study

Detection methods used in epidemiological studies influence the accuracy of prevalence data of *Blastocystis* sp. However the poor sensitivity of detection methods often leads to generating false negative results. This definitely makes the comparison of epidemiological data generated from different countries a challenge. Moreover, the phenomenon of irregular shedding of this parasite reported previously (Vennila et al., 1999) complicates the matter further. Therefore, stool aspirate samples from non-IBS and IBS patients who are undergoing colonoscopy have been used in the present study to assess the prevalence of *Blastocystis* sp. in IBS patients. Direct microscopy, *in vitro* cultivation, formalin ether concentration technique (FECT) and polymerase chain reaction (PCR) methods have been used previously to detect the presence of the parasite. There are only two studies carried out in Thailand and both showed a negative association between *Blastocystis* sp. and IBS patients (Tungtrongchitr et al., 2004; Surangsrirat et al., 2010). Thus far, there have been no studies carried out in Southeast Asian countries to show a positive association between both despite reports from other countries (Giacometti et al., 1999; Yakoob et al., 2004; Yakoob et al., 2010a, Yaakob et al., 2010b; Dogruman-Al et al., 2010; Jimenez et al., 2012) claiming that there is. Thus far, only stool samples and no other source of samples have been used to assess the prevalence of *Blastocystis* sp. from IBS patients. In this study, the interleukin levels including interleukin 3, 5 and 8, were measured to compare the interleukin levels in four different groups a) non-IBS group, IBS group, non-IBS patients infected with *Blastocystis* sp. (non-IBS Blasto) group and IBS patients infected with *Blastocystis* sp. (IBS Blasto) group.

The cyst form is resistant to unfavorable conditions of the gut and it is known to be the infective stage. Once, the cyst form reaches a favorable condition which could help in its growth and survival, excystation will occur. The parasite reproduces through binary fission in the host (Lee et al., 1985). Parasites such as *Entamoeba histolytica*, *Giardia intestinalis*, *Dientamoeba fragilis* and *Blastocystis* sp. are also vulnerable to unfavorable conditions at different stages in their life cycle (Baron & Yaeger, 1996). It is pertinent to assess if the gut condition of the host can influence the phenotypic characteristics of the parasites. Tan et al. (2008) showed that *Blastocystis* sp. derived from asymptomatic individuals and symptomatic patients were phenotypically and genotypically different.

Thus far, there have been no studies carried out to elucidate phenotypic characteristics of *Blastocystis* sp. isolated from asymptomatic, symptomatic and IBS patients having the same subtype. This study therefore would enable us to assess if gut environment conditions from these patients can influence phenotypic characteristics of *Blastocystis* sp. To achieve this, *Blastocystis* sp. ST3 was isolated from three different groups, namely asymptomatic individuals, symptomatic and IBS patients through a clinical and field survey.

Blastocystis sp. could also cause gut mucosal inflammation in IBS patients (Gwee, 2005). Several studies have been carried out to study the role of proteases from *Blastocystis* sp., which are associated with the pathogenicity of the parasites especially *Blastocystis* sp. ST3 (Sio et al., 2006; Abdel-Hameed & Hassanin, 2011). Hence, the present study aimed to elucidate the pathogenic effects of *Blastocystis* sp. ST3 in experimentally infected *Wistar* rats inoculated with cysts from three different groups' i.e. asymptomatic individuals, symptomatic and IBS patients by comparing the degree

of mucosal sloughing, inflammation, necrosis of tissue in the ileum, colon, caecum and rectum.

A study done by Ustün and Turgay (2006) postulated that the occurrence of mucosal inflammation contributing to gastrointestinal symptoms could be due to *Blastocystis* sp. infection. IBS patients having intestinal mucosal inflammation for a long term could possible develop colorectal cancer (CRC) but the underlying mechanism remains unclear. Kumarasamy et al. (2013) demonstrated that there was a positive association between *Blastocystis* sp. and colorectal cancer (CRC). In the present study, there is a need to evaluate the association between inflammation and IBS condition. Previous studies carried out provide evidence that solubilized antigen of *Blastocystis* sp. (Blasto-Ag) may result in *in vitro* proliferation of human colorectal carcinoma cells, HCT116 (Chandramathi et al., 2010b; Chan et al., 2012; Kumarasamy et al., 2013). Kumarasamy et al. (2013) showed that Blasto-Ag ST3 triggered a higher proliferation rate compared to Blasto-Ag ST1, ST2, ST4 and ST5 when introduced into the colorectal cancer cell lines (HCT116).

There is a need to study the effects of Blasto-Ag ST3 derived from two different groups, non-IBS and IBS patients on the cell proliferation rate of in vitro cancer cells, HCT116 in comparison with normal cells, CCD-18Co. In this study, asymptomatic and symptomatic isolates were grouped together and categorized as non-IBS. In addition to that, cell migration assay was carried out to measure the number of cells traversing a porous membrane, in response to the Blasto-Ag ST3 derived from non-IBS and IBS patients. A gene expression study was carried out to further study the association between inflammation and IBS in colorectal cancer patient. The expression of 75 genes

in both cell lines (normal cells and cancer cells) introduced with Blasto-Ag from non-IBS and IBS was also evaluated.

Factors influencing the shedding pattern of cysts were seen in a case study involving an IBS patient over a period of 30 days. A questionnaire to be filled concerning the time of the day and consistency of stool, and the emotional status and foods consumed and not the quantity of food consumed. The factors assessed were a) the frequency visiting the toilet in a day, b) the timing of visiting the toilet, c) the stool forms, d) the type of mood the patient was in whilst frequenting the toilet and e) food intake. This is also the first study to associate IBS patients infected with *Blastocystis* sp. ST3 with moods and emotions.

This pertinent study especially highlighting the influence of stress on shedding patterns of cysts can provide greater insights on the transmission patterns. The knowledge on the factors influencing the shedding pattern can definitely assist in the detection of the parasite, as well as influence treatment of *Blastocystis* sp. infected IBS patients.

1.3 Objectives of the study

1) To assess the prevalence of *Blastocystis* sp. in irritable bowel syndrome (IBS) patients by:
    a) Comparing the prevalence of *Blastocystis* sp. from stool and stool aspirate samples obtained from IBS and non-IBS patients.
    b) subtyping to assess the predominant subtype of *Blastocystis* sp. associated with IBS
    c) To assess the interleukin level (IL-3, IL-5 and IL-8) in serum samples from four different groups; non-IBS group, IBS group, non-IBS patients infected with *Blastocystis* sp. (non-IBS Blasto) group and IBS patients infected with *Blastocystis* sp. (IBS Blasto) group.

2) To establish phenotypic characteristics of *Blastocystis* sp. ST3 isolated from asymptomatic individuals, symptomatic and irritable bowel syndrome (IBS) patients i.e. :
    a) Growth profile, generation time and average size of *Blastocystis* sp. ST3 isolates from three different groups (asymptomatic, symptomatic and IBS).
    b) Staining characteristics of *Blastocystis* sp. ST3 isolates using Modified Fields' stain
    c) Cytochemical staining of *Blastocystis* sp. ST3 isolates using Fluorescein isothiocyanate (FITC)-labelled Con A (*Canavalia ensiformis*)
    d) Surface characteristics of *Blastocystis* sp. ST3 from the three different groups using scanning electron microscopy.
    e) Ultrastructural of *Blastocystis* sp. ST3 isolates from the three different groups using transmission electron microscopy.

f) Muscle twitching differences assessed on the ileum of Balb/C mice and rabbits.

3) To assess the histopathological changes during *in vivo* experimental infection with *Blastocystis* sp. ST3 isolated from asymptomatic individual, symptomatic and IBS patients in the intestinal tract of *Wistar* rats.

4) Molecular studies to assess the effects *Blastocystis* sp. ST3 antigens derived from non-IBS and IBS patients respectively on the growth of colon cancer cells (in comparison with normal cells) by :
   a) assessing the degree of cell proliferation caused by the antigens on *in vitro* cancer cells compared to normal cells
   b) measuring the number of cells traversing a porous membrane, in response to the Blasto-Ag ST3 using the cell migration assay
   c) Elucidating the role of Wnt signaling pathway in non-IBS and IBS patients

5) To study the factors that influence the shedding of *Blastocystis* sp. cysts in an IBS patient – An evidence based case study.

Chapter 2: Literature Review

2.1 Parasitic infection

Parasitic infections can cause serious risk of morbidity and death worldwide. At least 10.9 million deaths are reported worldwide due to certain types of parasitic diseases (WHO, 2005) particularly in people from developing and third world countries. Meanwhile in industrialized countries, the incidence of parasitic infection among immigrants and travellers who return from endemic countries have been quite high (Jelinek et al., 1997). Intestinal parasites can be categorized into two groups namely protozoa and the helminths. Protozoa are single celled organisms and the mode of reproduction is by binary fission; the hosts include both humans and animals (Lee et al., 1985). Intestinal parasites can be transmitted to humans or animals via contaminated water or food through the fecal oral route. *Blastocystis* sp., *Dientamoeba fragilis* and *Giardia lamblia* are examples of protozoan parasites. Meanwhile, helminths at the adult stage are relatively larger in size and can be seen with naked eyes (Barrett, 1981). Helminths are further categorized into nematodes, trematodes and cestodes which include hookworms, ascaris, tapeworms and filarial worms.

2.2 *Blastocystis* sp.

Blastocystis sp. is a common intestinal parasite which can be found in the intestinal tract of humans and animals (Boreham & Stenzel, 1993). This parasite is anaerobic and is a genetically heterogeneous protist (Arisue et al., 2002). It is a polymorphic protozoan which exists in various forms such as amoeboid, avacuolar, cyst, multivacuolar, granular, and vacuolar (Stenzel & Boreham, 1996; Suresh & Smith,

2004). The vacuolar life cycle stage ranges from 4 to 15μm in size and is the most common form seen in stool samples. The clinical symptoms of *Blastocystis* sp. seen in infected patients are mainly diarrhea, abdominal pain and other non-specific gastrointestinal symptoms such as nausea, anorexia, vomiting, weight loss, lassitude, dizziness, and flatulence (Ustün & Turgay, 2006). Previous studies have emphasized the morphological aspects of *Blastocystis* sp., while studies on the taxonomy, life cycle, mode of reproduction and pathogenicity of this parasite are still lacking. Several *in vivo, in vitro* and also epidemiological studies postulate that *Blastocystis* sp. could be a pathogenic parasite (Windsor et al., 2002). However, the pathogenicity of this parasite remains controversial (Zierdt, 1991) due to its occurrence in both asymptomatic individuals and symptomatic patients (Tan et al., 2008).

2.2.1 Taxonomy

The classification of *Blastocystis* sp. was found to be challenging. In the year 1911, the organism was proposed to be a harmless saprophytic yeast of the intestinal tract (Alexeieff, 1911). Alexeieff contrived the genus name, "*Blastocystis*", meanwhile Brumpt gave the species name, "*hominis*" (Alexeieff, 1911; Brumpt, 1912). *Blastocystis* was then assigned to the yeast of the genus *Schizosaccharomyces* based on the characteristics of this organism (Knowles & Gupta, 1924).

Swellengrebel (1917) suggested that this parasite could be a degenerating cell. Zierdt and Tan (1976) then categorized this organism to the subphylum Sporozoa and later Zierdt (1988) assigned it in the phylum Sarcomastigophora according to the parasite's reproductive methods. Ultrastructural studies carried out by (Dunn et al., 1989) revealed that *Blastocystis* sp. was shown to have a similar structure to other

organisms with few other unique characteristics. Johnson et al. (1989) suggested that *Blastocystis* sp. could be either protozoan or yeast according to ribosomal RNA sequencing and phylogenetic analysis. *Blastocystis* was then categorized into a different genus by itself in which, it is known as *Blastocystis hominis (*Jiang & He, 1993).

PHYLUM	Protista
SUBPHYLUM	Blastocysta
CLASS	Blastocystea
ORDER	Blastocystidea
GENUS	*Blastocystis*
SPECIES	*hominis*

There are many questions regarding the taxonomy and phylogenetic analysis based on the elongation factor 1α (EF-1α) gene sequences and the molecular analysis of *Blastocystis* small sub-unit ribosomal RNA (SSU rRNA). Silberman et al. (1996) placed the organism within the stramenopiles and closely associated to *Proteromonas.* The taxonomic status of *Blastocystis* was previously considered controversial due to its resemblance to Protista.

The stramenopiles comprising of a complex heterogeneous group consist of both unicellular and multicellular protists with flagella surrounded by lateral hair which is absent in *Blastocystis* (Patterson, 1989). A proposal had been made to revise the five kingdom classification in order to place this organism in another kingdom called "Chromista".

This group includes both heterotrophs and autotrophs consisting of brown algae, golden-brown algae, diatoms, slime nets, and water molds. The stramenopiles have been placed within the terminal 'crown' groups of the SSU rRNA tree with the green plants, animals, red algae and fungi. The species name for *Blastocystis* sp. was previously derived based on the host such as *Blastocystis hominis* from humans (Carbajal et al., 1997) and *Blastocystis ratti* form rats (Chen et al., 1997). However it has been shown that *Blastocystis* sp. being ubiquitous can be isolated from a variety of animal hosts and with increasing evidence pointing to animal-human transmission (Noël et al., 2005) confusion could set in with regard to the naming of species. Therefore, we just refer to as *Blastocystis* sp.

Hence the host specificity and the pathogenic potential of various isolates are linked with the sequence variations in the SSU rRNA. Members of the genus are further categorized into several subtypes (ST) which then can be termed as species (Noel et al., 2005). Better correlation was seen between the small sub-unit rDNA (SSU-rDNA) and ST and thus far 17 STs have been reported (Stensvold, 2013). In the present study the nomenclature will be standardized by using STs.

2.3 Prevalence

Blastocystis sp. is one of the most common parasites seen in humans and animals and is shown to have a worldwide distribution. This parasite can be isolated from fecal samples by culturing in 3ml Jones' medium supplemented with 10% horse serum. This culture method has been used to detect *Blastocystis* sp. in both adult patients (Cavalier, 1998) and pediatric (Cirioni et al., 1999). Children from different geographical areas were reported to have various gastrointestinal symptoms due to *Blastocystis* sp. infection in which higher detection of parasites were seen in diarrhoeic stools compared to stools from other symptomatic patients (Graczyk et al., 2005). A recent survey in children from Senegal, Africa showed 100% prevalence and was considered to be the highest prevalence rate worldwide thus far recorded (El Safadi et al., 2014).

Among adults, *Blastocystis* sp. was found to be higher in immunocompetent individuals compared to asymptomatic individuals (Nimri & Meqdam, 2004; Yakoob et al., 2004) and also in immunocompromised patients (Mohandas et al., 2002; Zali et al., 2004). *Blastocystis* sp. infection in human immunodeficiency/AIDS patients however showed no correlation (Albrecht et al., 1995) although other studies (Cirioni et al., 1999; Prasad et al., 2000; Rivero-Rodríguez et al., 2013; Paboriboune et al., 2014) showed a higher incidence of *Blastocystis* sp. infection in HIV patients. Hematological malignant patients who went through chemotherapy-induced neutropenia were also found to have a higher incidence of *Blastocystis* sp. infection accompanied with other gastrointestinal problems such as abdominal pain, flatulence and diarrhea (Taşova et al., 2000).

The prevalence rate of *Blastocystis* sp. differs within various communities, regions and also from country to country (Stenzel & Boreham, 1996). The prevalence rate of this parasite was found to be higher in developing countries compared to developed countries. In developed countries, the prevalence rate of *Blastocystis* sp. ranged from 0.5% in Japan (Horiki et al., 1997) to 23% in the United States (Amin, 2002). Meanwhile, in developing countries like Argentina (Basualdo et al., 2007) and Brazil prevalence rates of 27.2% and 40.9% respectively were recorded.

Prevalence was also influenced within the same country depending on which social strata people came from. The lower social strata class showed a higher prevalence rate compared to the higher social strata people, which probably is due to poor hygiene practices and poor water quality (Borda, et al., 1996; Wilairatana et al., 1996; Cirioni et al., 1999; Guignard et al., 2000; Taamasri et al., 2000).

Presence of *Blastocystis* sp. was also seen in other animals based on molecular studies, which have implication on the zoonotic transmission to humans (Thathaisong et al., 2003; Abe, 2004) and phylogenetic analyses done previously (Arisue et al., 2003; Noel et al., 2003). Invertebrates (Alexeieff, 1911; Belova & Krylov, 1997), monkeys (McClure et al., 1980), amphibian (Yoshikawa et al., 2004), pigs (Budéjovioe, 1991; Thathaisong et al., 2003), reptiles (Teow et al., 1992), cockroaches (Zaman et al., Ng et al., 1993), birds (Stenzel et al., 1994), rats (Chen et al., 1997), lizards (Suresh, et al., 1997b), cats (Duda et al., 1998), dogs (Duda et al., 1998; Daryani et al., 2008) and chickens (Lee & Stenzel, 1999) have been found to be infected with *Blastocystis* sp.

2.4 Biology

2.4.1 Morphology

Blastocystis sp. is a polymorphic organism which exists in vacuolar, granular, amoeboid and cyst forms (Figure 2.1) (Stenzel & Boreham, 1996; Tan et al., 2002).. The sizes and shapes of these forms may differ due to the surrounding gut conditions of the host which can be influenced by drugs, osmotic differences, and the metabolic status of the host (Dunn et al., 1989; Stenzel et al., 1991; Boreham & Stenzel, 1993). The different morphology of this parasite can have important diagnostic implications.

2.4.1.1 Vacuolar form

The vacuolar forms are spherical in shape with a large central vacuole occupying 90% of the cell's volume with a thin peripheral band of cytoplasm surrounding it. The vacuolar forms are largely found in *in vitro* cultures and fresh fecal samples. Stool sample is one of the most common sources for the detection of this parasite. The size of the vacuolar form ranges from 2 to 200 μm in diameter (Stenzel & Boreham, 1996). *Blastocystis* sp. cells in culture are surrounded by a slimy layer or capsule which is known as the surface coat (Zierdt & Tan, 1976). Coated pits are also seen on the cell membrane which plays a role in the endocytosis process (Dunn et al., 1989). Organelles like golgi apparatus, mitochondria-like-organelles and endoplasmic reticulum have been seen in the central vacuole. The central vacuole consist of tiny fine granular or flocculent material scattered unevenly, and is known to have a storage function for some metabolic products (Boreham & Stenzel, 1993). Rounded or elongated shapes of cytoplasmic materials have been seen in the large central vacuole (Suresh et al., 1995)

and it was postulated that aggregation of this material could be associated with programmed cell death (Nasirudeen et al., 2001).

2.4.1.2 Granular form

Granular forms are similar to vacuolar forms of *Blastocystis* sp. but relatively larger in size ranging from 15 to 200 μm with an average diameter between 4 and 15μm (Stenzel & Boreham, 1996). Granules can only be seen in the central vacuole of the granular form but not present in the vacuolar form. It is reported that the granules arise from the vacuolar form as an effect of increased serum concentration in the culture media, antibiotics and when axenisation attempts are being carried out (Stenzel & Boreham, 1996). Central vacuole and cytoplasm of granular forms of *Blastocystis* sp. may have lipid granules of various morphological types (Zierdt, 1973). Dunn et al. (1989) suggested that the granules are myelin-like inclusions with small vesicles, crystalline granules and lipid droplets. Thus far there are three different granules reported, the metabolic, reproductive and lipid granules. The reproductive granules have been found to play an important role in schizogony-like division to produce viable progenies of *Blastocystis* sp. (Zierdt et al., 1967; Zierdt, 1991; Suresh et al., 1994). However, the role of granular forms in helping the parasite to reproduce is yet to be accepted for more strong evidence (Boreham & Stenzel, 1993; Stenzel & Boreham, 1996; Tan & Stenzel, 2003).

2.4.1.3 Amoeboid form

Studies carried out by Suresh et al. (1994) and Singh et al. (1995) suggested that amoeboid forms of *Blastocystsis* sp. could be the intermediate form between the cyst and vacuolar form. Amoeboid forms are irregular in shape and usually have one or two large pseudopods (Zierdt & Tan, 1976) with a few Golgi bodies, mitochondria and endoplasmic reticulum (Tan et al., 2001). The size of amoeboid forms range from 10-15μm in diameter (Tan & Zierdt, 1973). A few studies suggested that this form is associated with protease activities which possess a higher pathogenic potential by further exacerbating the gastrointestinal symptoms in the host (Tan & Suresh, 2006; Zhang et al., 2012; Rajamanikam & Govind, 2013).

2.4.1.4 Cyst form

The cyst form described earlier has been isolated from fecal material (Stenzel, Boreham, & McDougall, 1991). The cysts are spherical in shape and generally smaller compared to both vacuolar and granular forms. Cysts from animal hosts are relatively larger in size compared to cysts isolated from humans which range from 3-6μm in diameter (Zaman et al., 1995; Stenzel et al., 1997). The cysts from humans are bi-nucleated and the nuclei can vary from one up to four (Stenzel et al., 1991). The cystic form consists of dense cytoplasm consisting of many mitochondria and small vacuoles made of glycogen or lipids. The cysts have a multi-layered cyst wall which can withstand unfavorable conditions. As such, cysts are thought to be the infective stage which can excyst in the host's intestine (Moe et al., 1997).

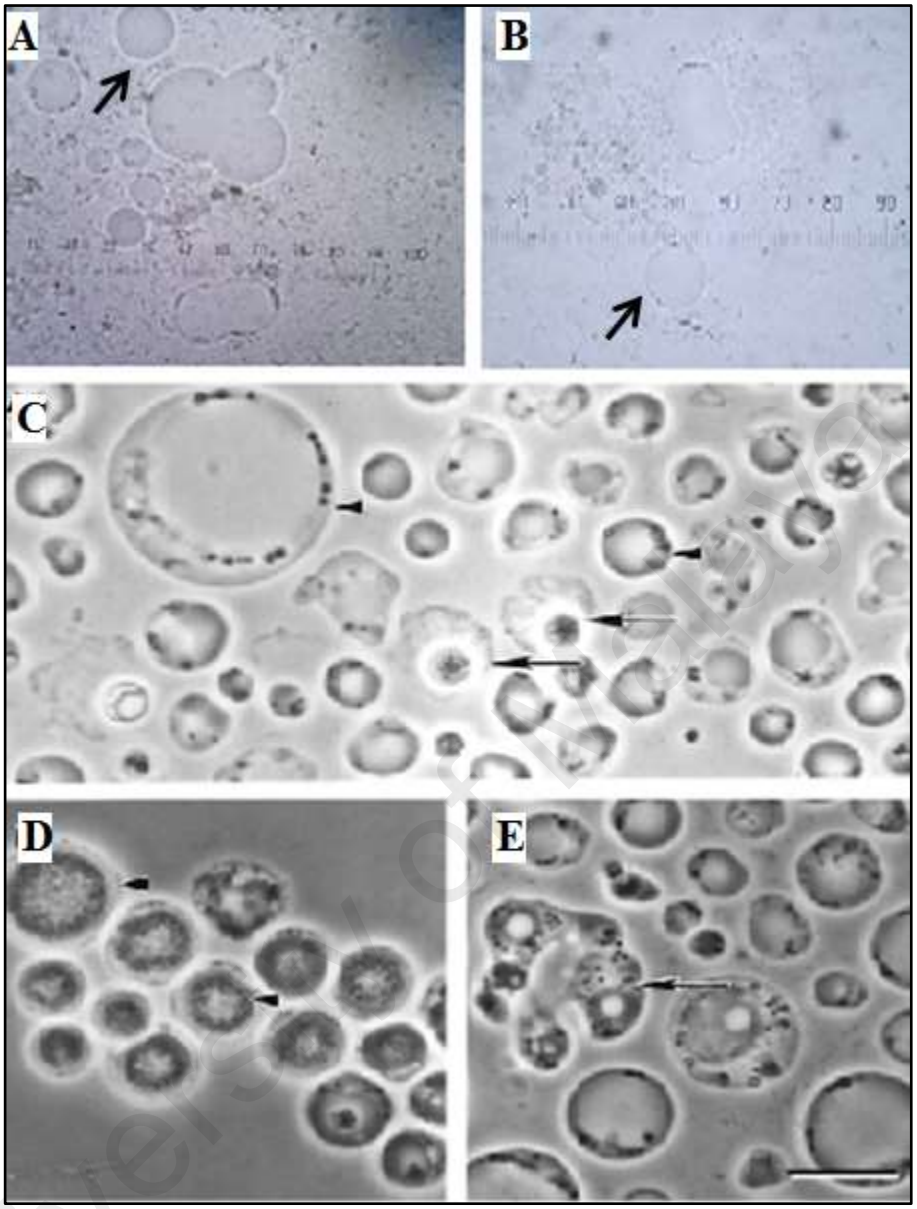

Figure 2.1: Morphological forms of *Blastocystis* sp. (A) & (B) vacuolar forms (arrows), (C) vacuolar and cysts form (arrows) (D) granular forms (E) amoeboid form [Figure 2.1 A & B: The photograph originally generated from this project; Figure 2.1 C, D & E adapted from Tan et al., 2008].

2.4.2 Life cycle

There are many conflicting reports on the life cycle of *Blastocystis* sp. (Tan et al., 2002). Many modes of reproduction have also been suggested (Singh et al., 1995). The life cycle of *Blastocystis* sp. incorporates all four forms of this parasite which include amoeboid, cysts, granular and vacuole. Various life cycles have also been proposed by other researchers (Zierdt, 1973; Boreham & Stenzel, 1993; Singh et al., 1995).

The first life cycle of *Blastocystis* sp. as proposed by Alexeieff (1911) demonstrated that the parasites undergo binary fission and autogamy which is a sexual reproductive process (Figure 2.2). The proposed life cycle was however refuted by other researchers. (Zierdt, 1973) proposed another new life cycle by light microscopy studies where the vacuolar form was seen to differentiate into the granular form which released the daughter vacuolar cells from the central body by plasmotomy process (Figure 2.3). Another life cycle proposed by Singh et al. (1995) was based on ultrastructural changes seen in the parasites during an *in vivo* encystment. Thick and thin walled cysts were introduced for the first time and they were suggested to be responsible for external and internal transmission respectively (Singh et al., 1995) (Figure 2.4).

The life cycle proposed by Stenzel & Boreham, (1996) showed that the most predominant form was small avacuolar cells which had a surface coat (Figure 2.5). The avacuolar form is suggested to pass through the intestine and differentiate to form a multi-vacuolar form with thick surface coat. Upon forming into a cyst wall, the thick surface coat will then detach. The cyst is known to be the infective stage of the life cycle and was found to be able to resist unfavorable condition in the intestine of the host.

Based on the life cycle proposed by Stenzel & Boreham, (1996) the amoeboid form arises from the avacuolar life cycle stage whilst the vacuolar form arises from the multi-vacuolar stage. Previous studies also revealed that *Blastocystis* sp. display distinct modes of reproduction such as binary fission, schizogony, budding, and formation of sac-like pouches which induce progenies (Govind et al., 2002). The process of schizogony has been suggested to take place within the thin-walled cyst which then ruptures to release daughter vacuolar forms.

A revised life cycle proposed by Tan (2004) incorporates the involvement of animals (Moe et al., 1997) which revealed that *Blastocystis* sp. could be a potential zoonotic organism (Figure 2.6). The mode of transmission of cysts in human and animals is via fecal-oral route. The cysts will then excyst into vacuolar form and reproduce through binary fission (Tan, 2004). Tan (2008) also included the zoonotic genotypes of this parasite which are from subtype one to seven with other host specificities (Figure 2.7). The life cycle proposed that seven or more species (or STs) of *Blastocystis* sp. from animals can infect humans who can be possible hosts to various zoonotic subtypes. Contaminated water and food can be one of the sources of transmission of this parasite. Viable cysts of *Blastocystis* sp. have been found in the drinking water which can infect humans and animals (Leelayoova et al., 2008). Several studies have reported that ST1 and ST4 are one of the common subtypes to be found in humans (Leelayoova et al., 2008; Domínguez-Márquez, et al., 2009). Meanwhile ST6 and ST7 have also been found in mammals and birds. Studies in Malaysia (Tan et al., 2008), Singapore (Wong et al., 2008) and United States (Jones et al., 2009) have shown that ST3 to be one of the most predominant subtypes seen. *Blastocystis* sp. ST3 was also found in animal samples (Souppart et al., 2009; Meloni et al., 2011). Animal to human transmission cannot be ruled out. Certain subtypes can only infect animals and are not

found in humans. The prevalence rate was found to be higher in humans who have contact with animals, providing evidence for the existence of zoonotic transmission (Requena et al., Devera, 2003; Khan & Alkhalife, 2005).

Another life cycle proposed included the encystation and excystation of *Blastocystis* sp. (Figure 2.8) (Parija & Jeremiah, 2013). In the colon, the cyst will excyst into vacuolar forms (Moe et al., 1997) which will then develop into amoeboid, avacuolar, granular and multivacuolar forms. All these forms do play an important role in the pathogenicity of this parasite (Zhang et al., 2012). The vacuolar form of this parasite then encysts into cysts prior to expulsion through fecal matter which then can be further transmitted to other humans and animals.

There are many ongoing studies carried out to elucidate the role of amoeboid, avacuolar, granular and multi-vacuolar forms of *Blastocystis* sp. which include molecular studies to elucidate the life cycle of *Blastocystis* sp. as well as to establish the possibility of zoonotic transmission in humans and animals.

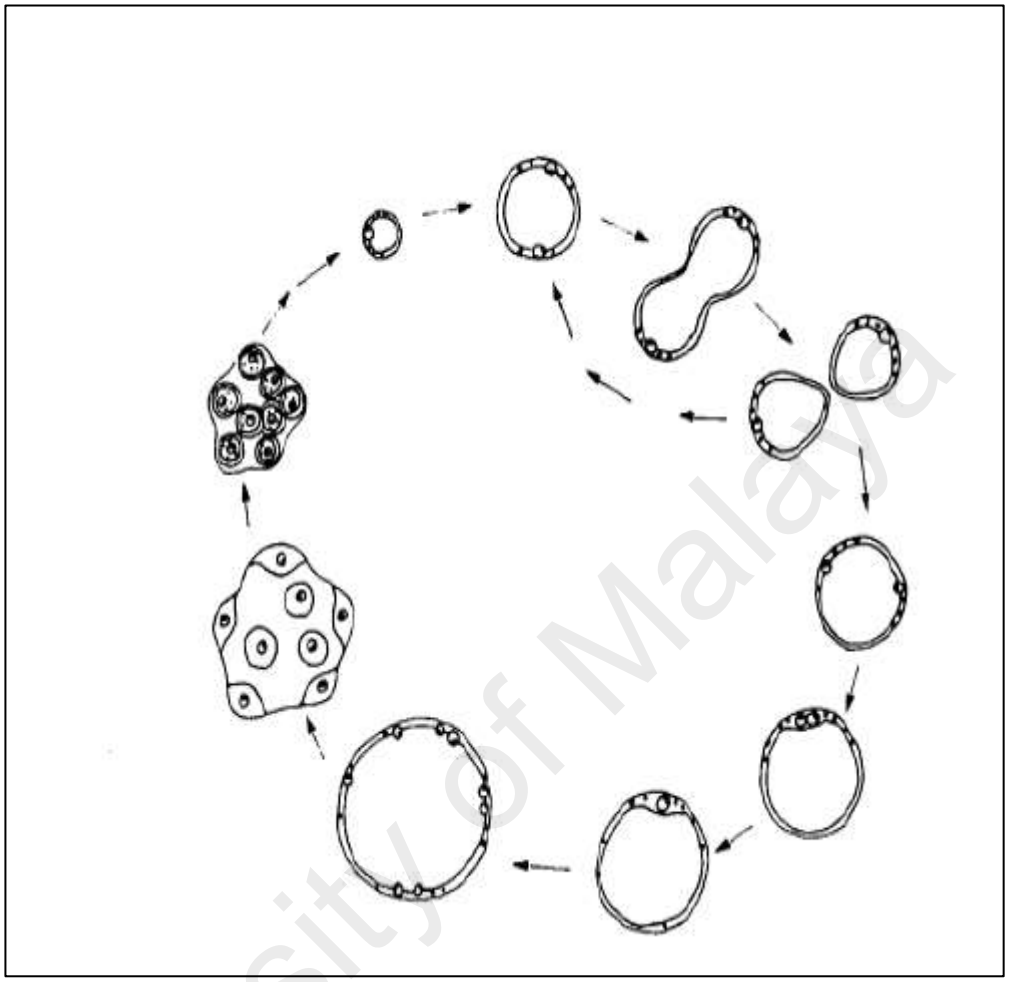

Figure 2.2: Life cycle of *Blastocystis* sp. proposed by Alexeiff (1911)

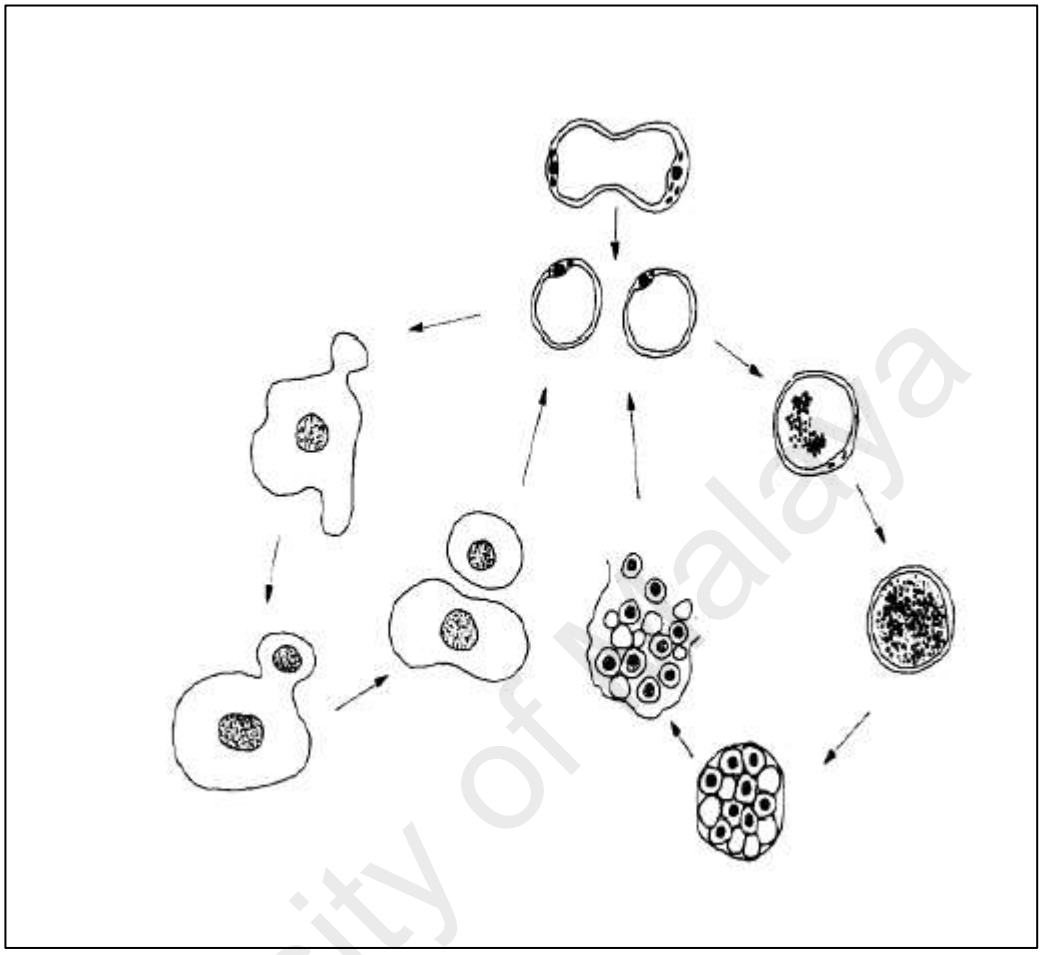

Figure 2.3: Life cycle of *Blastocystis* sp. proposed by Zierdt (1973)

[adapted from Boreham and Stenzel, 1993]

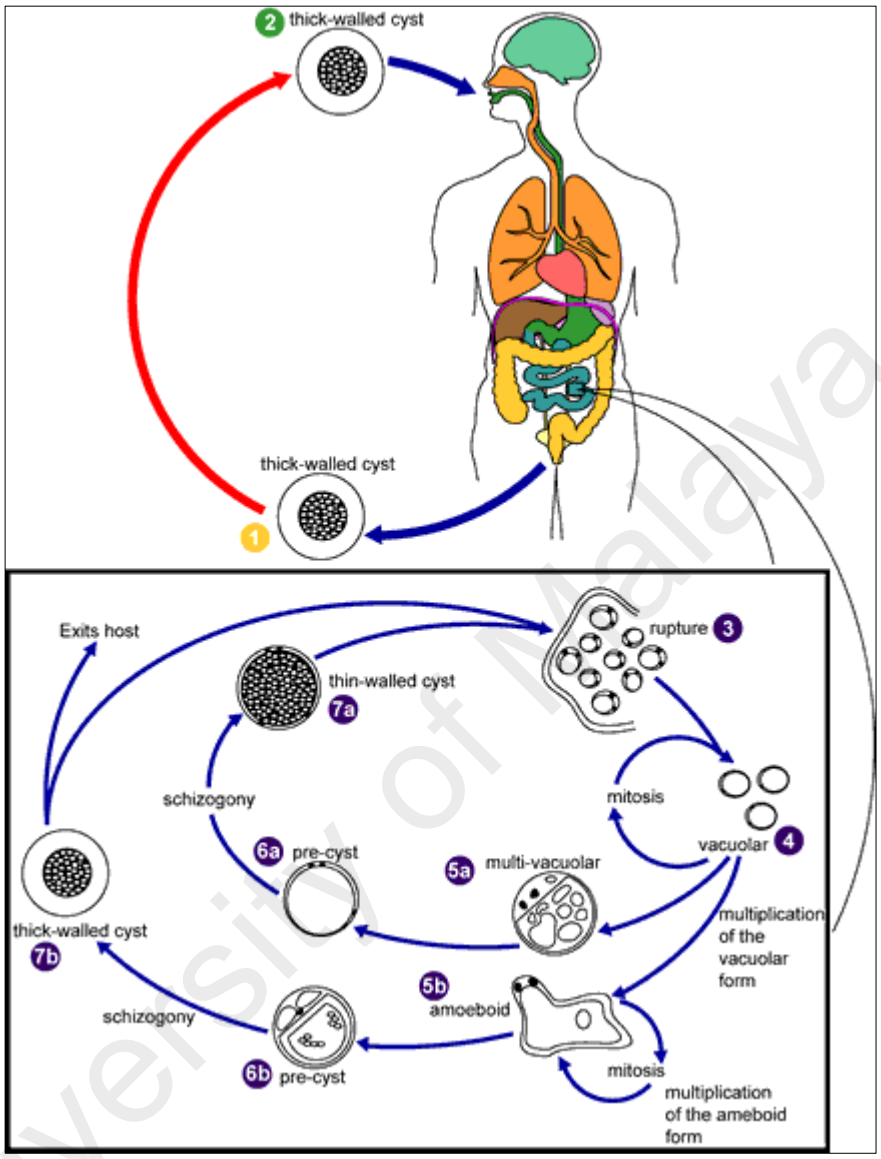

Figure 2.4: Life cycle of *Blastocystis* sp. proposed by Singh (1995)

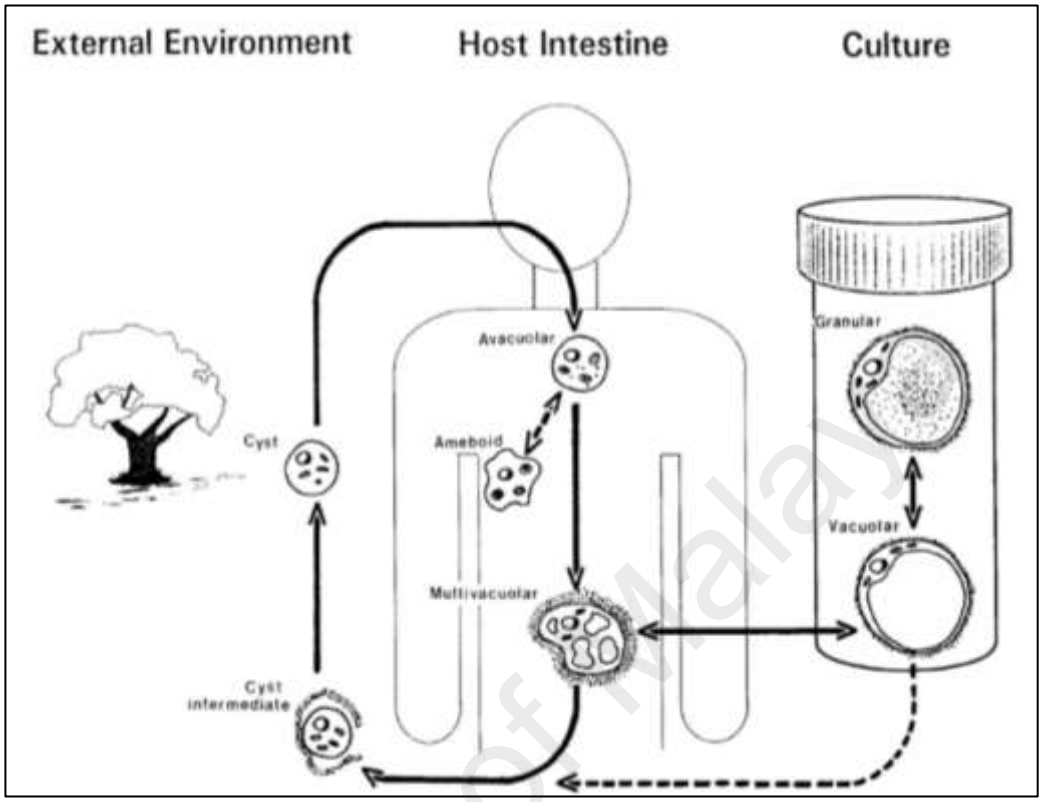

Figure 2.5: Life cycle of *Blastocystis* sp. proposed by Stenzel and Boreham (1996)

[adapted from Stenzel and Boreham, 1996].

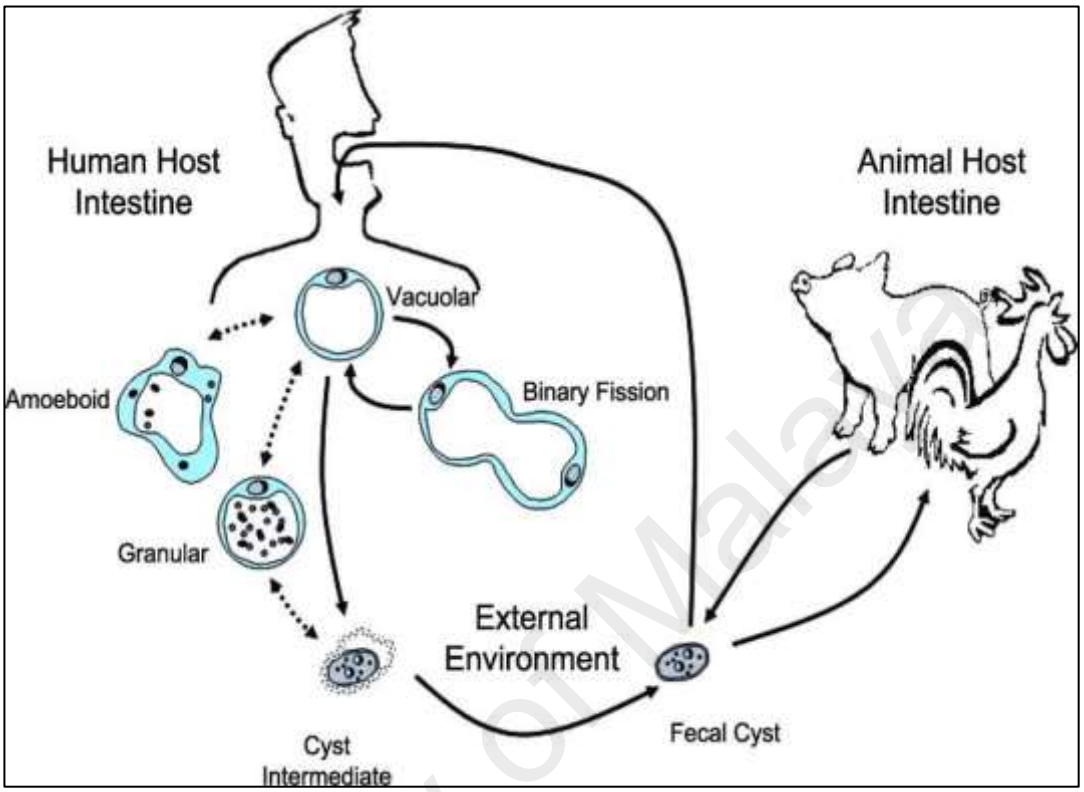

Figure 2.6: Life cycle of *Blastocystis* sp. proposed by Tan (2004)

[adapted from Tan et al., 2004]

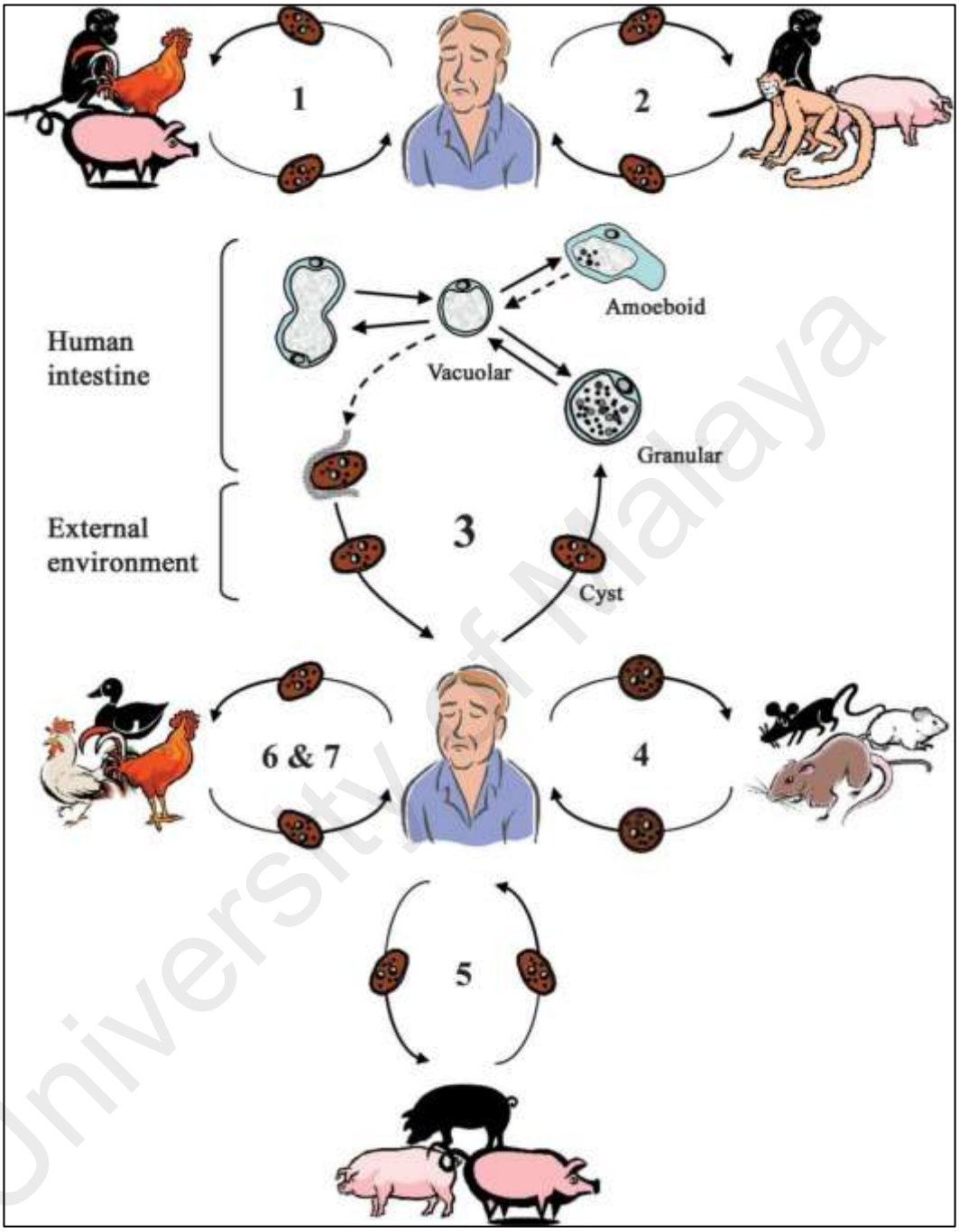

Figure 2.7: Life cycle of *Blastocystis* sp. as proposed by Tan (2008)

[adapted from Tan et al., 2008].

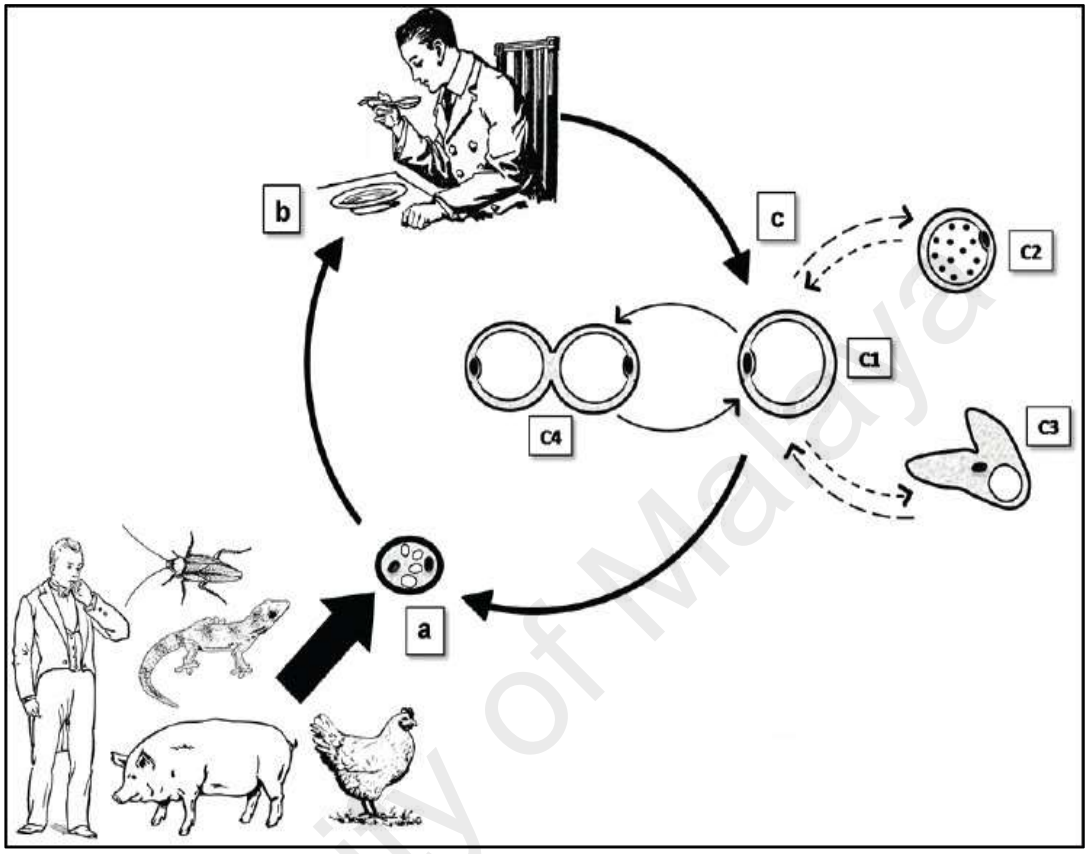

Figure 2.8: Life cycle of *Blastocystis* sp. as proposed by Parija and Jeremiah (2013)

[adapted from Parija and Jeremiah, 2013].

2.5 Pathogenicity and genotypes

There are many factors contributing to the pathogenicity of *Blastocystis* sp. Several studies have isolated parasites from asymptomatic individuals and symptomatic patients (Shlim et al.,1995; Böhm-Gloning et al., 1997; Svenungsson et al., 2000; Leder et al., 2005; Tan et al., 2008; Eroglu et al., 2009). The pathogenic role of *Blastocystis* sp. was questionable when it was seen in asymptomatic individuals who did not exhibit gastrointestinal symptoms (Udkow & Markell, 1993; Tan et al., 2002). The gastrointestinal symptoms such as anorexia, nausea, flatulence, diarrhea and abdominal pain are common symptoms attributed to *Blastocystis* sp. infection (Boreham & Stenzel, 1993).

Several other reports have suggested that *Blastocystis* sp. is associated with a variety of diseases which include colitis (Russo et al., 1988), terminal ileitis (Tsang, et al., 1989), tropical pulmonary eosinophilia (Enzenauer et al., 1990), ulcerative colitis (Jeddy & Farrington, 1991), reactive arthritis (Lakhanpal et al., 1991), chromic urticarial (Pasqui et al., 2004) and hypoalbuminemia (Nassir et al., 2004). Studies have also shown that this parasite can be opportunistic and pathogenic in immunocompromised populations, especially in AIDS patients (Ok et al., 1997; Cirioni et al., 1999; Taşova et al., 2000; Lebbad et al., 2001; Rivero-Rodríguez, et al., 2013; Paboriboune et al., 2014). *Blastocystis* sp. infection coupled with other intestinal disorders such as irritable bowel syndrome (IBS) and colorectal cancer (CRC) can further exacerbate gastrointestinal symptoms (Boorom et al., 2008; Chandramathi et al., 2012).

An *in vitro* model was established using *Blastocystis* sp. culture filtrate and this was used to study the capability of transcription factors in human colorectal cells, inflammatory responses and the release of cytokines (Long et al., 2001; Puthia et al., 2008). Inflammatory cytokines have the ability to affect human epithelial cells due to *Blastocystis* sp. infection and can further down regulate the immune system (Long et al., 2001). Besides that, *Blastocystis* sp. was also shown to trigger the cytopathic and cellular immune responses which also cause downregulation of tumor necrosis factor-alpha (TNF- α) and upregulation of interleukin-6 (IL-6) and interleukin-8 (IL-8). This was observed after peripheral blood mononuclear cell (PBMC) were exposed to solubilized antigens of *Blastocystis* sp. culture filtrate (Blasto-Ag) (Chandramathi et al., 2010b).

Blasto-Ag has been used to study the proliferation rate of human colorectal carcinoma cells (HCT116) which increased when exposed to Blasto-Ag (Chandramathi et al., 2012). Another study showed that Blasto-Ag derived from symptomatic isolates demonstrated a higher cell proliferation rate compared to asymptomatic isolates (Chan et al., 2012). Vinoth et al. (2013) revealed that Blasto-Ag from ST3 isolates triggered a higher proliferation rate of HCT116 cells compared to five other subtypes. Blasto-Ag from ST3 isolates have the shown the ability to weaken the immune system and assist in the growth of colorectal cancer cells. This study demonstrated that subtype especially ST3 does have an influence in the pathogenicity of the parasites.

Genotype of the parasite has also been associated with pathogenicity of the parasite (Souppart et al., 2009). Kaneda et al. (2001) highlighted that ST1, ST2 and ST4 were associated with the gastrointestinal symptoms but no such association was shown between such symptoms and ST3. In contrast, 100% of the urticarial patients were

shown to be infected with *Blastocystis* sp. ST3 (Hameed et al., 2011). Other studies have shown that ST1 is more prevalent after ST3 (Kaneda et al., 2001; Li et al., 2007; Dogruman-Al et al., 2008; Souppart et al., 2009) however the role of subtypes and their respective pathogenic effects remain un-elucidated. The correlation between asymptomatic and symptomatic groups with genotypes is still lacking (Böhm-Gloning et al., 1997; Yoshikawa, et al., 2004a).

Blastocystis sp. ST3 was also found to have a higher protease activity at 32kDa which could play a role in the pathogenicity of the parasite (Abdel-Hameed & Hassanin, 2011). Studies showed that proteases of *Blastocystis* sp. could degrade immunoglobulin A (IgA) (Sio et al., 2006; Abdel-Hameed & Hassanin, 2011) whilst others demonstrated greater virulence in which immunoglobulin A (Ig A) can disrupt the barrier of intestinal gut and trigger the immune response (Puthia et al., 2005, Mirza & Tan, 2009; Puthia et al., 2008).

Various studies have been carried out to eliminate this parasite or to reduce the symptoms the parasite causes. Drugs such as metronidazole, nitazoxanide, and TMP-SMX have been used to eradicate this parasite (Sekar & Shanthi, 2013). However, there are challenges in eradicating this parasite as certain patients have been found to be resistant to the medication prescribed (Tan et al., 2008). Previously, many researchers had used microscopy and *in vitro* cultivation as detection methods but not molecular methods (Stenzel et al., 1994; Singh et al., 1996; Stenzel et al., 1997; Yoshikawa et al., 2007).

Comprehensive studies have been carried out to study the molecular aspects of *Blastocystis* sp. which resulted in the categorization based on subtype establishing ST1 to ST13 (Yoshikawa et al., 2003; Yoshikawa et al., 2004a). These subtypes vary

according to hosts and different geographical locations. Seven major subtypes have been identified by PCR-based molecular methods which belong to human and other mammals (Yoshikawa et al., 2004b; Noel et al., 2005). Further studies carried out revealed that there are a total of 17 STs thus far reported (Alfellani et al., 2013b). Out of 17 STs, 90% of human isolates are infected with *Blastocystis* sp. ST1-ST4 (Malheiros et al., 2011; Alfellani et al., 2013a), meanwhile others were infected with *Blastocystis* sp. ST5-ST9. Eight subtypes (ST10-ST17) had been found to be in non-human hosts too (Alfellani et al., 2013b).

Recently, a rapid method in *Blastocystis* sp. subtyping had been developed which is similar to DNA barcoding in animals (Scicluna et al., 2006) by PCR method (Yoshikawa et al., 2004a). The arbitrarily-primed PCR (AP-PCR) analysis has been used to develop the sequence-tagged site (STS) primers from of known strains of *Blastocystis* sp. Subtypes were then amplified to tally the phylogenetically distinct clades implied from the SSU rRNA sequences (Arisue et al., 2003; Yoshikawa et al., 2004a). This method uses independent primers set for each subtype for subtyping the parasite. However, DNA barcoding method only requires a single set of primers to amplify in which sequencing is carried out at 600bp region of the SSU rRNA of *Blastocystis* sp. to detect the established subtypes (Scicluna et al., 2006).

Poirier et al. (2011) established a highly sensitive method by using real-time quantitative PCR (qPCR) to detect *Blastocystis* sp. in human feces. Partial sequence of the *Blastocystis* sp. SSU rRNA gene was targeted by direct sequencing of the qPCR products. This method had not only the ability to detect various forms of *Blastocystis* sp. but was able to quantify the parasite at the same time. These new methods if

standardized could facilitate epidemiological studies and improve categorization of parasites according to subtypes.

2.6 Irritable bowel syndrome (IBS)

2.6.1 Epidemiology

Several reports have suggested an association between *Blastocystis* sp. and irritable bowel syndrome (IBS) which is known to be a functional gastrointestinal disorder (Hussain et al., 1997; Giacometti et al., 1999; Yakoob et al., 2004; Karanis et al., 2007). The prevalence of this intestinal disorder worldwide is reported to be 2-22% in the West (Rey & Talley, 2009) and 15% in Malaysia (Rajendra & Alahuddin, 2004; Tan et al., 2003). Meanwhile in industrialized countries, at least 5%–24% of people are known to be affected (Longstreth et al., 2006). The symptoms of IBS are not persistent and often accompany other functional disorders (Whitehead et al., 2002).

IBS although not a life threatening disease, can cause the patient to suffer in silence (Wilson et al., 2004). IBS also results in a major economic burden for healthcare providers with a significant impact on the quality of life of patients (Hulisz, 2004; Longstreth et al., 2003). The Rome III criteria have been used to categorize the functional gastrointestinal disorders according to the patient's clinical symptoms. Rome III criteria for IBS is usually recurrent abdominal pain or discomfort and a marked change in bowel habit for at least six months, with symptoms experienced on at least three days or at least 3 months with two or more of the following: pain is relieved by a bowel movement; onset related to a change in frequency of stool; onset of pain is related to a change in the appearance of stool (Drossman & Dumitrascu, 2006).

2.6.2 Causative agent and IBS

The etiopathogenesis and pathophysiology of IBS is still not clearly understood whereby it could be because of gastrointestinal motility, visceral hypersensitivity or biopsychosocial factors which involve the biological, psychological and social aspects of the patients (Camilleri &Choi, 1997). These authors also suggested that altered gut motility and food intake could contribute to biopsychosocial factors. Emotional states such as anxiety disorder, depression, fear or patients traumatized as a result of their early life stress have been shown to influence gut motility and exacerbate IBS symptoms (Mayer et al., 2001).

Brain gut axis mechanism associated with IBS have been attributed to a factor contributing to the dysfunctional interaction between the brain and the gut (O'Mahony et al., 2011). The bi-directional communication between the central nervous system and cardiovascular, immune and other symptoms through neural and endocrine mechanism has been shown to be different among individuals (McEwen & Gianaros, 2010). Long term emotional instability, mood swings and stress can create imbalance in the body systems resulting in the passing of loose and soft forms of stools with accompanying gastrointestinal symptoms.

Psychosocial factors, altered motility and hypersensitivity of the GI tract are known to be causative agents for the occurrence of IBS, however the exact cause is still unknown. Inflammatory changes of the gut in the adults with post-infectious IBS (PI-IBS) have been shown to be quite common especially for patients diagnosed with IBS. Often an acute enteric infection will result in creating an additional mechanism for intestinal infection mediated-inflammation (Gwee, 2005).

2.6.3 *Blastocystis* sp. and IBS

Invading pathogens which include bacteria or parasites can also be a causative agent in causing IBS. Studies carried out to establish the diversity of bacteria seen in the intestinal gut of IBS patients provided information on the role of gut bacteria (Rajilić–Stojanović et al., 2011) and the type of gut microflora (Simrén et al., 2012). Several studies carried out showed that parasitic infection could contribute to the development of IBS (Hussain et al., 1997; Giacometti et al., 1999). Parasites such as *Blastocystis* sp. (Hussain et al., 1997; Giacometti et al., 1999), *Dientamoeba fragilis* (Windsor & Johnson, 1999), *Entamoeba histolytica* (Sinha et al., 1997) and *Giardia lamblia* (D'Anchino et al., 2002) have been associated with IBS. The challenge in ascribing *Blastocystis* sp. to be a contributory factor is that the symptoms caused by the parasite and IBS are similar. Many studies carried out showed that this parasite had been associated with the gastrointestinal disorder, IBS (Markell & Udkow, 1986; Hussain et al., 1997; Giacometti et al., 1999; Yakoob et al., 2004; Boorom et al., 2008). The common symptoms shared by both *Blastocystis* sp. and IBS are constipation, diarrhea, abdominal pain, nausea, fatigue and cramps (Graczyk et al., 2005). Hussain et al. (1997) showed that the sera collected from *Blastocystis* sp. infected IBS patients have a higher level of IgG antibody levels compared to a healthy group. However the first epidemiological study carried out two years later showed a statistically significant association between *Blastocystis* sp. infected IBS patients and the control group (Giacometti et al., 1999). Three other studies however carried out showed no association between *Blastocystis* sp. One was carried out in Mexico (Ramirez-Miranda et al., 2010) and two others from Thailand (Tungtrongchitr et al., 2004; Surangsrirat et al., 2010). This could be due to the irregular shedding of *Blastocystis sp.* is known to have and variation in stool detection methods. Other parasites such as *Giardia*

intestinalis and *Dientamoeba fragilis* were also reported to have intermittent shedding, which demonstrates that single stool sample collection may give false results (Van Gool, et al., 2003).

An interplay of various factors have been shown such as food intake (Öhman & Simrén, 2010), intestinal microbiota, impairment epithelial barrier and immunological response. This could influence and alter structural tight junction proteins in the ileum and large intestine which increases the intestinal permeability of the gut (Barbara et al., 2012).

The gut of IBS patients possesses intestinal microbiota which can differ biologically and clinically from that of normal individuals. Intestinal microbiota in IBS patients could be a contributory cause to the symptoms of the host. IBS patients have been shown to be more prone to develop low grade inflammation, increase in immunological response, formation of abnormal gases, acidic fecal microbiota and poor metabolism of protein and carbohydrate (Bonfrate et al., 2013).

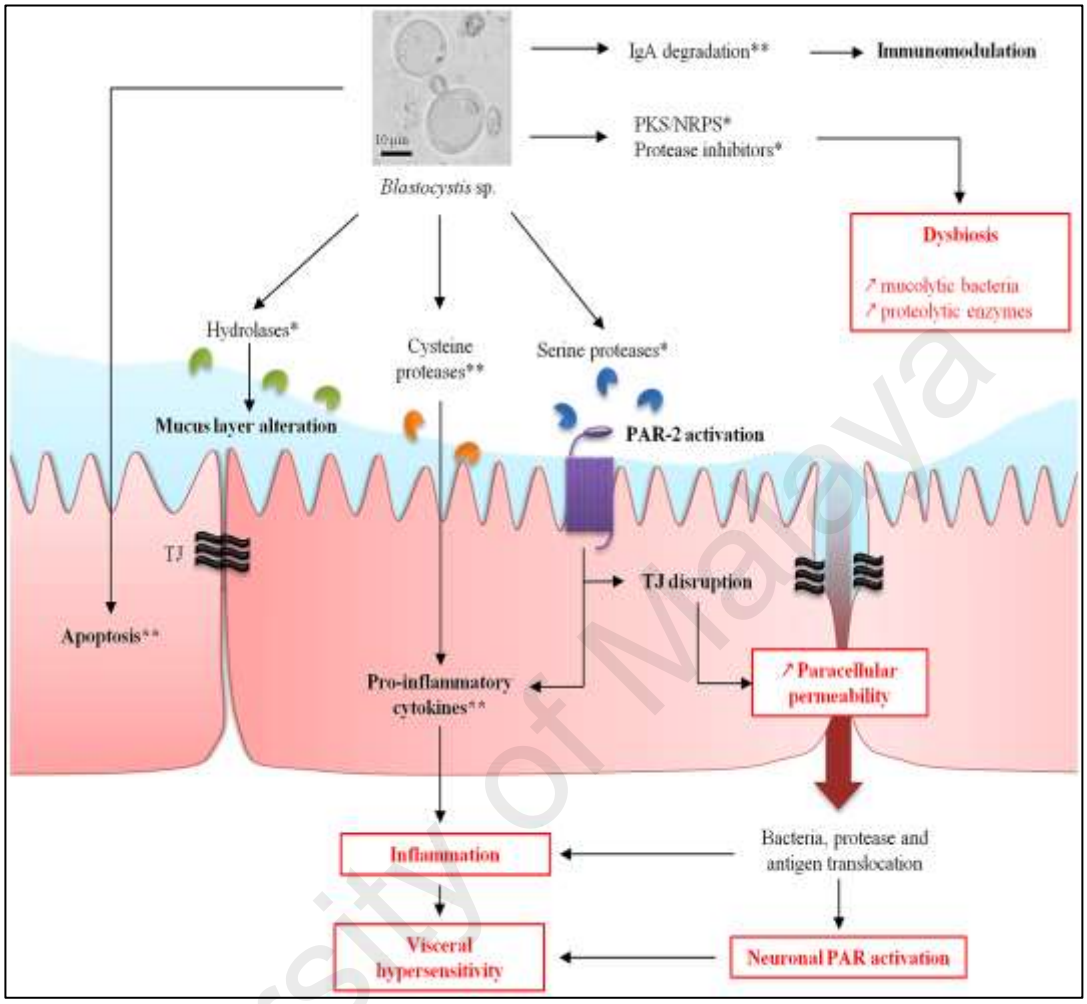

Figure 2.9: Schematic representation of pathogenicity for *Blastocystis* sp.

Derived from genomic (*) and experimental (**) data of the study showed a possible link with IBS pathophysiology mechanisms (in red).

IgA, immunoglobulin A; NRPS, non-ribosomal polyketide synthase; PAR, protease-activated receptor; PKS, polyketide synthase.

[Adapted from (P. Poirier, Wawrzyniak, Vivares, Delbac, & El Alaoui, 2012)]

Several studies showed that impairment of epithelial barrier is due to visceral pain associated with IBS and this can cause intestinal motility with increased sensitivity of the gut (Figure 2.9). An *in vitro* study on colonic biopsies obtained from IBS patients demonstrated a heightened level of para-cellular permeability which was associated with the destruction of tight junctions (TJs) (Piche et al., 2009). Low grade inflammation was seen in the intestine of IBS patients (Chadwick et al., 2002). Protease-activated type 2 (PAR-2) was then postulated to take part in low grade inflammation and increased level of gut permeability (Bueno, 2008). Activation of PAR-2 occurs when the cleavage of the N-terminal domain of the receptor was initiated by serine-proteases. Peptide ligands were released which bind to receptors which enhanced the opening of TJs causing inflammation to be promoted. Diffusion of pathogens such as bacteria and foreign antigens to sub-mucosa occur due to increased intestinal permeability which will then trigger inflammation. Activating a few members of the PAR family have also been suggested to generate abdominal pain (Steck et al., 2013).

Gecse et al. (2008) revealed that stools collected from IBS patients had a higher proteolytic activity compared to healthy individuals. Protease activity has been demonstrated in supernatants from *Blastocystis* sp. ST4 and ST7 axenic isolates (Tan, 2008). Other studies have also shown the association between proteases and the pathogenicity of *Blastocystis* sp. (Sio et al., 2006; Abdel-Hameed et al., 2011). Protease from this parasite was shown to break down immunoglobulin A (IgA) and subsequently lead to the destruction of intestinal barrier which can trigger an immune response (Puthia et al., 2005; Puthia et al., 2008; Mirza & Tan, 2009). Hence, proteases such as cysteine, metalloproteases or serine derived from bacteria or *Blastocystis* sp. have been shown to contribute to the development of IBS symptoms and mechanisms (Denoeud et

al., 2011). This destruction could lead to the imbalance of gut microbiota in IBS patients compared to healthy individuals (Lee & Bak, 2011) and may trigger low grade inflammation of the intestinal mucosa which further exacerbates IBS symptoms. Innate immunity activated due to gut infection results in the inflammation of the gut.

Long term inflammation can influence gene expression and may cause gene modification which can lead to colorectal cancer (Hussain & Harris, 2007). These involve the signaling transduction pathway responsible for regulating the expression of different genes, and in the process, eliminating parasites and regulating inflammation. However, abnormal activation of the signaling transduction pathway plays a role in the etiology of cancer. Wnt signaling pathway is one of the signaling transduction pathways activated and it is very much associated with colon cancer.

Chapter 3: Studies to assess the prevalence of *Blastocystis* sp. in stool aspirate samples collected from irritable bowel syndrome patients.

3.1 Introduction

The prevalence data of *Blastocystis* sp. in irritable bowel syndrome (IBS) patients has still not been established which pose a challenge when it comes to associate the pathogenicity of this parasite to IBS conditions. Moreover, no association was found between the eradication of this parasite and the clinical response to treatment for IBS patients infected with *Blastocystis* sp. symptoms of IBS patients. *Blastocystis* sp. is one of the most common gut parasites found in the intestinal tract of humans and animals (Windsor, 2007). The organism exists in various morphological forms such as vacuolar, granular, amoeboid, cyst, avacuolar and multi-vacuolar forms and is transmitted through the fecal-oral route (Tan & Suresh, 2007). The usual mode of diagnosis of *Blastocystis* sp. in stool samples is via direct microscopy, *in vitro* culture technique and by polymerase chain reaction (PCR) method.

Epidemiological studies on *Blastocystis* sp. depend entirely on various detection methods. The first epidemiological study to report on the potential link between *Blastocystis* sp. and IBS was done by Giacometti et al. (1999). *Blastocystis* sp. was found to be significantly present in IBS patients compared to patients with gastrointestinal symptoms. Several other studies investigating the association between *Blastocystis* sp. and IBS saw a higher prevalence of *Blastocystis* sp. in IBS patients compared to asymptomatic or symptomatic patients (Yakoob et al., 2004; Yakoob et al., 2010a; Yaakob et al., 2010b; Dogruman-Al et al., 2010; Jimenez-Gonzalez et al., 2012). However, three studies carried out previously showed no association between

Blastocystis sp. and IBS in which one was from Mexico (Ramirez-Miranda et al., 2010) and the other two, from Thailand (Tungtrongchitr et al., 2004) and (Surangsrirat et al., 2010).

A negative association between *Blastocystis* sp. and IBS could be the result of poor detection methods which hampers the sensitivity of detection and thereby generates false negative results. IBS patients who are infected with *Blastocystis* sp. seek other medical treatment without knowing that this parasite could be the main causative agent causing the gastrointestinal symptoms. Obtaining false negative results could also result in patients missing the opportunity for treatment which could have relieved the symptoms.

Detecting this parasite in fecal samples and by *in vitro* cultivation has been a challenge due to the irregular shedding of this parasite reported previously (Vennila et al., 1999). Intermittent shedding has been seen not only for *Blastocystis* sp. but other parasites as well, such as *Giardia intestinalis* and *Dientamoeba fragilis*. Vennila et al., (1999) had also suggested collecting stool samples for the three consecutive days in order to improve the chances of identifying positive stools.

Colonoscopy, whilst not indicated in all patients with IBS, is occasionally utilized to exclude organic causes of symptoms. During colonoscopy, a long, flexible thin tube was inserted into the rectum. A small video camera, attached to the tip of the tube was used routinely to screen for the presence of ulcers, polyps and inflammation. At the same time, aspirates of fluid (stool aspirates) in the colon were flushed out through another tube and collected in 1500cc CRD Liners. Stool aspirates, collected directly during colonoscopy may circumvent many of the limitations of standard stool

collection techniques for *Blastocystis* sp. that has been described. In the present study, the presence of *Blastocystis* sp. among consecutive adult Malaysian patients undergoing colonoscopy was assessed. The prevalence of *Blastocystis* sp. was compared between patients with and without IBS, according to Rome III criteria.

In this study, the interleukin (IL) levels of IL-3, IL-5 and IL-8 were also measured to compare the interleukin levels between four groups, non-IBS patients group, IBS patients group, non-IBS patients infected with *Blastocystis* sp. (non-IBS Blasto) group and IBS patients infected with *Blastocystis* sp. (IBS Blasto) group. Interleukin levels were measured to further confirm the pathogenic potential of *Blastocystis* sp. isolated from IBS patients.

3.2 Materials and methods

3.2.1 Sample Collection

A hospitalized-based cross sectional study was performed between May 2010 and May 2011, where adult patients attending the weekly colonoscopy at University Malaya Medical Centre (UMMC) for an index examination for various indications were invited to participate in the study. Patients were recommended to undergo colonoscopy for normal screening and also for the prevention of colorectal cancer (Figure 3.1). IBS patient with persistent diarrhea often choose colonoscopy when prescribed medication has failed to reduce their gastrointestinal symptoms. A single investigator interviewed all patients prior to colonoscopy, and IBS was defined according to the Rome III criteria (Longstreth et al., 2006) together with a normal or insignificant colonoscopy finding. Patients with a recent use of antibiotics, particularly Metronidazole, were excluded. A

total of 123 patients were briefed about this study but only 109 patients participated. They were divided into two different cohorts; 74 (67.90%) and 35 (32.1%) were non-IBS and IBS respectively. Inclusion and exclusion criteria used for the selection of the patients are provided in Appendix 1. A standardized bowel preparation regime consisting of bisacodyl and low-residue diet for two days followed by a 2-liter polyethylene glycol and electrolyte lavage solution (PEG-ELS) was used for all patients undergoing colonoscopy at this institution. Colonoscopy was performed using standard video-endoscopes with variable stiffness (CF 160AL, Olympus, Tokyo, Japan). All patients received a combination of Midazolam 2.5 mg to 5 mg and Pethidine 25 mg to 50 mg as sedation prior to colonoscopy. Stool samples were collected after two weeks from the patients. The protocol for this study was approved by Medical Ethics Committee, University Malaya Medical Centre (MEC Ref No. 793.13).

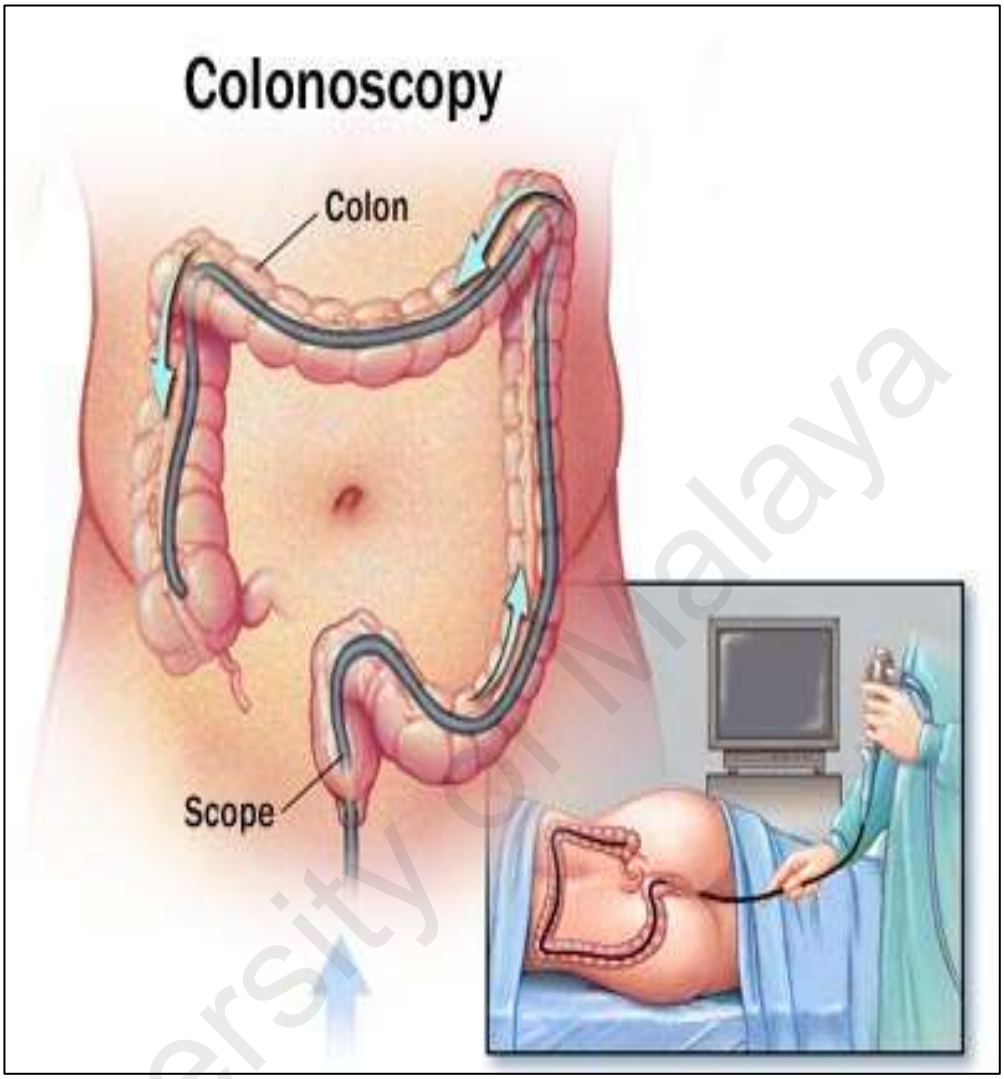

Figure 3.1: **An illustration of the colonoscopy procedure performed.**

(Source: http://greatlakesgastroenterology.com/features/colonoscopy/)

3.2.2 Questionnaire

The patients were required to fill in the patient's consent form (Appendix 2). A standard questionnaire (Appendix 4) was used in this study. The patients were asked to report on their gastrointestinal symptoms, and the lifestyles of the individuals were assessed before the collection of stool aspirate samples. A medical doctor confirmed the IBS patients based on Rome III criteria (Appendix 3). Other medical histories of the patients were extracted from their medical records in the hospital data system. Patients were also contacted via telephone for an interview based on the questionnaire filled out by them.

3.2.3 Stool processing

Stool aspirate samples were collected in 1500cc CRD Liners following direct aspiration during colonoscopy from the non-IBS and IBS patients (Figure 3.2). The samples were then transported to the Department of Parasitology and stool aspirate samples were then spun in 50ml Falcon tubes at 3000rpm for 10 min and the sediment assessed for the parasite using direct microscopy. In addition, 50mg of the sediment was then cultured in 3ml Jones' medium supplemented with 10% horse serum and kept in incubator at 37°C. The cultures were screened for *Blastocystis* sp. after 24, 48 and 72 hours using light microscopy. Formal ether concentration techniques (FECT) were also done to screen for the presence of other intestinal parasites. Various staining techniques were carried out, such as Ziehl- Neelsen, modified trichrome and trichrome staining methods to detect for *Cryptosporidium*, microsporidia and other parasites as well. Stool aspirate samples were kept at 4°C for deoxyribonucleic acid (DNA) extraction using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany). Stool samples collected after

two weeks were cultured in 3ml Jones' medium supplemented with 10% horse serum and screened for *Blastocystis* sp. after 24, 48 and 72 hours using light microscopy.

Figure 3.2: Stool aspirate samples received from the Endoscopy Unit, 4th Floor, University Malaya Medical Centre (UMMC).

3.2.4 Subtyping of *Blastocystis* sp.

The genomic DNA of *Blastocystis* sp. for all stool aspirate samples collected was extracted using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) based on the manufacturers' protocol. All the samples were subjected to sequence tagged site (STS) primer-polymerase chain reaction (PCR) using the seven sets of primers previously described by (Yoshikawa et al., 2004b). Two to five microliters of DNA preparations were used to amplify the genomic sequences in a 20µl reaction containing 1x PCR buffer (Fermentas, USA). PCR conditions consisted of 1 cycle of initial denaturing at 95^0C for 5 mins, followed by 40 cycles of denaturing at 95^0C for 1 min, annealing at 56.3^0C for 1 min 30 seconds and extending at 72^0C for 1 min, and an additional cycle of elongation at 72^0C for 10 min using Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, USA). The amplified products were then electrophoresed in 1.5% agarose gels (Promega, USA) in Tris–borate-EDTA buffer. Gels were stained with ethidium bromide and photographed using an ultraviolet gel documentation system (UVitec Ltd., Cambridge, United Kingdom). PCR amplification for each primer pair were done in triplicate.

3.2.5 Blood sample collection

Blood samples were collected from all the patients who came to the colonoscopy unit. 3cc blood samples were collected in plain EDTA tubes. Blood was allowed to coagulate and was centrifuge at 2000rpm for 10 mins to obtain the serum samples. The serum samples were kept at -20ºC until inflammatory cytokine measurements were done.

3.2.6 Inflammatory cytokines

Human IL-8, IL-3 and IL-5 ELISA (Enzyme-Linked Immunosorbent Assay) purchased from Bio-Rad (Raybiotech, Norcross, Georgia, USA) were used in this study. This assay uses an antibody which is specific to IL-8, IL-3 and IL-5 coated on a 96-well plate. All reagents, samples and standards were prepared as instructed. 100µl of sample were added into each well and incubated for 2.5 h at room temperature. The specific interleukins (IL-8, IL-3 and IL-5) which present in the serum samples will bind to the wells by the immobilized antibody. The wells were washed and 100µl of Biotin antibody were added and incubated again for one hour at room temperature. After washing away unbound biotinylated antibody, 100µl of horseradish-peroxidase (HRP) conjugated streptavidin was added into the wells. The wells were again washed and 100µl tetramethylbenzidine (TMB) substrate solution was added to the wells and colour develops in proportion to the amount of respective interleukin binding. The Stop Solution changed the colour from blue to yellow, and the intensity of the colour was measured at 450 nm using a microplate reader.

3.2.7 Statistical analysis

Double data entry was performed using Microsoft Excel 2010 and statistical analyses conducted with IBM Statistical package for Social Sciences for Window SPSS (version 21). Categories such as gender, age group, food intake were treated as categorical variables and expressed as percentages. Pearson chi-square analysis test was used to examine the differences in prevalence of *Blastocystis* sp. in non-IBS and IBS patients. Odds ratios (OR) and 95% confidence intervals (CI) were tabulated. For the purposes of analysis for the inflammatory cytokines, patients' diagnoses were classified

as IBS, non-IBS, IBS Blasto and non-IBS Blasto. The latter consisted of any clinically significant colonoscopy findings. The inflammatory cytokines measurements were analyzed using Student's t-test. Statistical significance was defined as p value of <0.05.

3.3 Results

323 patients were screened during the study period and 109 (88.6 %) patients agreed to participate in the study. Among the 109 patients, 74(67.90%) and 35(32.1%) were non-IBS and IBS respectively. In this present study, with regards to age groups, a total of 10(9.2%) participants were aged <50 years and 99(90.8%) were aged ≥ 50. The age range of participants was ranging from 18 to 96 years, with a median age of 65 years. This study consisted of 49(45%) males and 60(55%) females and the ethnicity was: Malay, 19.3%; Chinese, 67%; Indian, 10.1%; and Sikh, 3.7% (Table 3.1).

The overall prevalence for *Blastocystis* sp. among 109 patients was 9.17% (Table 3.2). All stool aspirate samples were initially found to be negative for *Blastocystis* sp. and all other parasites when examined by direct microscopy, formal ether concentration techniques (FECT) as well as the *in vitro* culture method. However, when a PCR amplification method was utilized for the stool aspirate samples, we were able to identify *Blastocystis* sp. in 6 (17.1%) IBS and 4 (5.5%) non-IBS patients (p=0.047) (Table 3.3). Subtyping of *Blastocystis* sp. was as follows: non-IBS (ST2; n=1, ST3; n=2 and ST5 n=1) and IBS (ST3; n=3, ST4; n=2 and ST5 n=1). Fresh stool samples were collected from all 109 patients two weeks after performing colonoscopy. The direct microscopy, *in vitro* cultivation and FECT methods were all negative for *Blastocystis* sp. for all the 109 stool samples.

The association of *Blastocystis* sp. infection based on the analysis of demographic data showed that there was a significant association between the presence of *Blastocystis* sp. and the food intake. The results showed that *Blastocystis* sp. prevalence was significantly higher in the IBS group compared to non-IBS group. Patients with high fiber diet had a lower risk of being infected with *Blastocystis* sp. (Table 3.4).

In this study, non-IBS persons were normal and healthy individuals, meanwhile non-IBS Blasto were asymptomatic individuals infected with *Blastocystis* sp., and IBS Blasto group were *Blastocystis* sp. infected IBS patients. A comparison of serum cytokines IL-3, IL-5 and IL-8 between non-IBS, IBS, non-IBS Blasto and IBS Blasto groups was demonstrated (Figure 3.1). IL-8 levels were significantly elevated in the IBS Blasto group and IBS group ($p<0.05$) compared to non-IBS and non-IBS Blasto group. The levels of IL-3 were only seen to be significantly higher in IBS Blasto group and IBS group ($p<0.05$) compared to non-IBS. Meanwhile, the IL-5 levels were significantly higher in IBS Blasto group ($p<0.05$) compared to non-IBS and non-IBS Blasto group.

Table 3.1: General characteristics of the patients (n = 109)

Characteristics	Frequency (%)	
Groups:		
non-IBS	74	(67.9)
IBS	35	(32.1)
Gender:		
Male	45	(41.3)
Female	64	(58.7)
Age group:		
< 50	10	(9.2)
≥ 50	99	(90.8)
Ethnicity:		
Malay	21	(19.3)
Chinese	73	(67.0)
Indian	11	(10.1)
Sikh	4	(3.7)

Table 3.2: Analysis of four methods used to detect *Blastocystis* sp. in stool aspirate and stool samples.

Techniques	N	Samples, positive (%)	
		Stool aspirate	Stool
Direct microscopy	109	0 (0%)	0 (0%)
In vitro cultivation	109	0 (0%)	0 (0%)
Formal ether concentration technique (FECT)	109	0 (0%)	0 (0%)
Polymerase chain reaction (PCR)	109	10 (9.17%)	Nil

Table 3.3: Percentage of *Blastocystis* sp. infection detected in non-IBS and IBS patients

Groups	Techniques			
	Direct microscopy	***In vitro* cultivation**	**FECT**	**PCR**
non-IBS (n= 74)	negative	negative	negative	4 (5.5%)
IBS (n= 35)	Negative	Negative	negative	6 (17.1%)*

*$p < 0.05$ is the comparison done between non-IBS and IBS and patient

Table 3.4: Subtypes of *Blastocystis* sp. found in stool aspirate samples via PCR method

Technique	non-IBS, n=74	IBS, n=35
PCR	4 (5.5%)	6 (17.1%)
Subtypes		
ST1	Nil	Nil
ST2	1 (25%)	Nil
ST3	2 (50%)	3 (50%)
ST4	Nil	2 (33.3%)
ST5	1 (25%)	1 (16.7%)
ST6	Nil	Nil
ST7	Nil	Nil
ST8	Nil	Nil
ST9	Nil	Nil

Table 3.5: Analysis of risk factors for *Blastocystis* sp. infection

Variables		N	*Blastocystis* sp. infection		OR (95% CI)	p-value
			no	%		
Gender						
	Male	45	3	6.7	1.719 (0.420,7.042)	0.447
	Female	64	7	10.9		
Age group (years)						
	< 50	10	0	0	1.112 (1.041, 1.188)	0.292
	≥ 50	89	10	10.1		
Group						
	non-IBS	74	4	5.4	3.621 (0.951,17.788)	0.047[a]*
	IBS	35	6	17.1		
Ethnicity						
	Malay	21	3	14.3	0.519 (0.122, 2.201)	0.366
	Others	88	7	8		
Food intake						
	Low fibre	66	9	13.6	0.151 (0.018, 1.236)	0.046[a]*
	High fibre	43	1	2.3		

N: Number examined; no: Number positive.

Reference group marked as OR = 1; CI: Confidence interval.

[a]Significant association, *p < 0.05

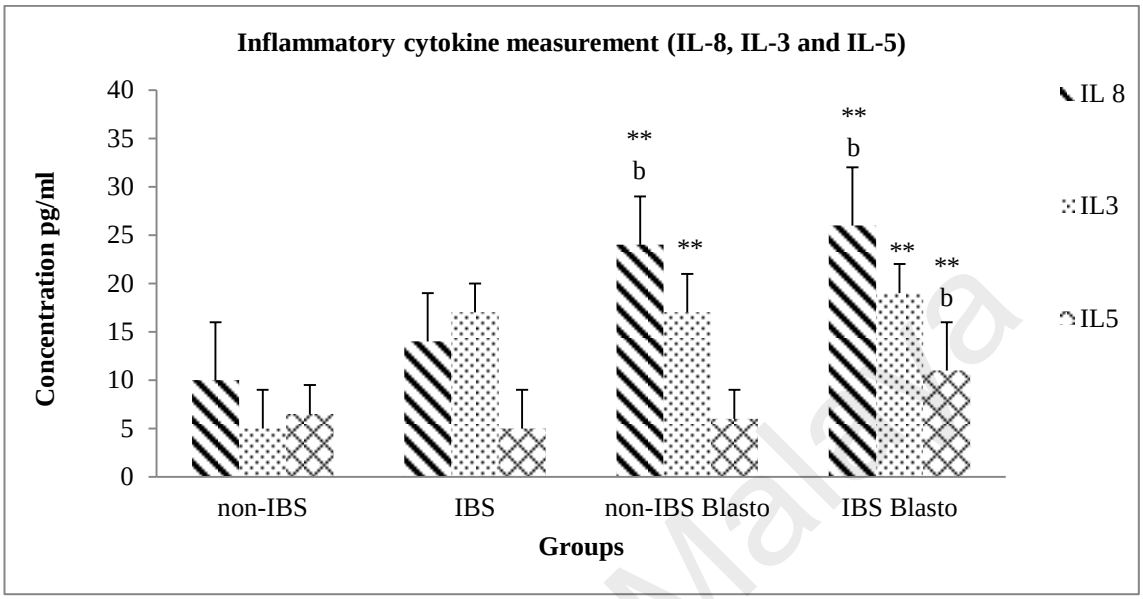

Figure 3.3: Levels of serum for IL-3, IL-5 and IL-8.

Data is given in mean ± SD. **P < 0.05 is the comparison done against non-IBS group. ^{b}P < 0.05 is the comparison done against IBS group.

3.4 Discussion

The pathogenicity of *Blastocystis* sp. is still disputable as adults with an acute infection are known to develop gastro-intestinal symptoms similar to IBS, usually following travel to an endemic geographical location. However, its' association with IBS, a chronic gastrointestinal disorder, is less certain. The irregular shedding of *Blastocystis* sp. in stools as well as the intermittent shedding of other protozoa such *Giardia intestinalis* and *Dientamoeba fragilis* does imply that single stool examination has a low sensitivity for detecting (Vennila et al., 1999). In patients with *Entamoeba histolytica*, up to four to nine separate fecal examinations were required to make the diagnosis (Sinha et al., 1997). With additional stool examinations, the diagnostic yield has been shown to increase by 22.7% for *Entamoeba histolytica*, 11.3% for *Giardia intestinalis* and 31.1% for *Dientamoeba fragilis* (Van Gool et al., 2003).

A prevalence study done in Thailand showed that 13.6% of IBS patients were infected with *Blastocystis* sp. compared to 20% of normal patients (Tungtrongchitr et al., 2004). These results were not significantly different. Another study done by Surangsrirat et al., (2010) on IBS patients infected with *Blastocystis* sp. was also found to be not statistically significant; where 10.0% and 16.7% were positive in control and IBS patients respectively. However, in the present study it was found that the results were statistically significant, 17% (6/35) of IBS patients were infected with *Blastocystis* sp. compared to 5.5% (4/74) control patients. This present study is the first in Southeast Asia to show that the results of associating *Blastocystis* sp. to IBS were statistically significantly positive. However, the prevalence of *Blastocystis* sp. among IBS patients in the present study was somewhat lower than previous published reports (Giacometti et al., 1999; Dogruman-Al et al., 2010; Jimenez-Gonzalez et al., 2012). This may be due to

patient selection in our study, as all patients were undergoing colonoscopy, which is not commonly indicated in patients with IBS. The present study also showed that PCR method used in stool aspirate samples was more sensitive than direct microscopy, *in vitro* culture method and trichrome stain. Purgatives and rapid colonic transit prior to colonoscopy may have flushed all the parasites out providing an initial negative result for direct microscopy, *in vitro* culture technique and trichrome staining technique. Traces of DNA of *Blastocystis* sp. were able to be detected by the PCR technique demonstrating that PCR could be a better method in detecting this parasite in IBS patients who were undergoing colonoscopy. This study suggests that IBS patients who are undergoing colonoscopy should be screened for this parasite by using PCR method in order for them to eradicate this parasite immediately if they are found to be positive for *Blastocystis* sp. This present study supports the earlier research done by Kumarasamy et al. (2014) suggesting that stool aspirate samples can be an advantage to detect the parasite as the samples are collected directly from the washout during colonoscopy. This may then address the issue of irregular shedding of *Blastocystis* sp.

Blastocystis sp. does trigger the immune system of the patients and many studies have been done to study the pathogenicity of this parasite. The increase of oxidative damage and pro-inflammatory cytokines by *Blastocystis* sp. infection in animal models had been reported (Chandramathi et al., 2010c). Apart from that, solubilized antigen from *Blastocystis* sp. (Blasto-Ag) has the ability to downregulate peripheral blood mononuclear cells (PBMC) while facilitates the growth of human colorectal cells (Chandramathi et al., 2010). The role of the immune system in the human body is to fight against any invading antigens or parasites by innate and acquired immunity. Phagocytosis occurs in innate immunity in which phagocytes such as monocytes, neutrophil and natural killer (NK) cells will eliminate the invading antigens or parasites.

Meanwhile, acquired immunity consisting of T helper (Th) lymphocytes can be further divided into cellular immunity, Th1 and acquired immunity, Th2 response. Th1 targets the intracellular pathogens while Th2 plays an important role in fighting against the extracellular pathogens by increasing the level of immunoglobulins (Ig) such as IgG, IgG and IgM. Inflammatory cytokines also play an important role in immune system. Cytokines are small proteins that are pertinent in cell signaling. Examples of cytokines are chemokines, interleukins (IL), interferon, lymphokines and tumor necrosis alpha (TNF-α). Interleukins, which are a group of cytokines, are categorized from interleukin 1 to 17. Each interleukin has their own role in the immune system. The present study involved the analysis of IL-3, IL-5 and IL-8.

IL-8, a chemoattractant for neutrophils and macrophages is also known to act as a pro-inflammatory mediator in cellular responses as seen previously when vaginal tract was exposed to *Trichomonas vaginalis* which indicate chronic inflammation (Oppenheim & Ruscetti, 2001). According to Kollmar et al., (2006), the expression of IL-8 is important in tumor progression via the alteration of the immune system and the regulation of tumor cell growth. The levels of interleukin were higher in IBS than non-IBS group implicating *Blastocystis* sp. infection to have an effect on inflammatory cytokines released by the cells. However, the level of interleukin varies between non-IBS Blasto and IBS Blasto group. A recent study done by Chandramathi et al., (2012) reported that *Blastocystis* sp. in patients possibly immunosuppressed due to chemotherapy could tend to become opportunistic and exploit the advantageous situation to multiply more rapidly causing symptoms to exacerbate. These authors further demonstrated *Blastocystis* sp. isolated from IBS patients showed various unique and different phenotypic characteristics compared to the same parasite isolated from symptomatic patients and asymptomatic individuals. This was attributed to the

adaptation of the parasite to the fluctuations of the gut environments especially in IBS patients (Yoshikawa et al., 2004b).

3.5 Conclusion

In conclusion, the study has shown PCR examination of stool aspirates during colonoscopy is a useful method of identifying the presence of *Blastocystis* sp. in adult patients with IBS. Whilst colonoscopy remains invasive and clearly not indicated in all symptomatic adults, it may be considered for patients negative for stool culture but with a high suspicion of *Blastocystis* sp. based on its characteristics symptoms. IBS patients with *Blastocystis* sp. showing elevated levels of interleukin demonstrate that *Blastocystis* sp. does have an effect on the immune system and can be a good indicator for *Blastocystis* sp. infection.

Chapter 4: Studies to establish phenotypic characteristics of *Blastocystis* sp. ST3 isolated from asymptomatic individuals, symptomatic and irritable bowel syndrome patients.

4.1 Introduction

Phenotypic differences between asymptomatic and symptomatic *Blastocystis* isolates have been shown through isoenzyme patterns (Mansour et al., 1995; Gericke et al., 1997), protein profiling (Kukoschke & Müller, 1991) sero-groups (Müller, 1994) and molecular characterization (Böhm-Gloning et al., 1997; Kaneda et al., 2001; Yoshikawa et al., 2004a; Yan et al., 2006; Stensvold et al., 2007; Tan et al., 2008). A recent study reported that solubilized antigen of *Blastocystis* sp. (Blasto-Ag) derived from symptomatic isolates was more pathogenic and possess the ability to weaken the cellular immune response compared to the asymptomatic isolates (Chan et al., 2012).

There have been 13 STs reported (Stensvold et al., 2007) with *Blastocystis* sp. ST3 shown to be the highest in Thailand with prevalence rates between 41.7-92.3% (Yoshikawa et al., 2004a); Egypt-54.55% (Hussein et al., 2008); Singapore- 78% (Wong et al., 2008); Turkey- 75.9% (Özyurt et al., 2008); Turkey-59.3% (Dogruman-Al et al., 2009); Germany- 21% (Böhm-Gloning et al., 1997) and France- 53.5% (Souppart et al., 2009). These studies demonstrate the importance of ST3 in terms of its prevalence and its pathogenic implications.

Blastocystis sp. has been associated with irritable bowel syndrome (IBS) (Boorom et al., 2008), and the consequences of gut conditions which obviously varies in asymptomatic individuals, symptomatic and IBS patients in terms of gut flora, pH, and

osmotic pressure and water potentials has never been investigated. It is obvious that the severity would be obviously seen more in IBS than in the gut conditions of symptomatic or asymptomatic condition (Malinen et al., 2005). Thus far, there have been no studies to demonstrate if there are any phenotypic changes by keeping the subtype constant and can environment play a part in changing the phenotypic characteristics. Chapter 3 had also showed a positive association between *Blastocystis* sp. ST3 and IBS.

The present study attempted to investigate if the three different gut conditions can influence phenotypic variation of the parasite by keeping the subtype constant. Hence, through field and other clinical surveys, *Blastocystis* sp. ST3 was isolated from three different groups, asymptomatic individuals, symptomatic and IBS patients. Ileum from the rabbit and Balb/C will be used to study the pathogenicity of *Blastocystis* sp. ST3 by exposing the ileum with *Blastocystis* sp. ST3 antigen (Blasto-Ag ST3) derived from the three different groups. This study aimed to observe the autonomic properties of ileum in response to the Blasto-Ag ST3.

4.2 Materials and Methods

4.2.1 Source of *Blastocystis* sp.

A total of 8 *Blastocystis* sp. isolates were obtained from four IBS patients (IBS1-4) and four symptomatic patients (S1-4) at a local gastroenterology clinic. Four asymptomatic isolates (A1-4) were obtained from a field survey at a local village located at Kuala Langat, Selangor. Irritable bowel syndrome was defined according to the Rome III criteria (Longstreth et al., 2006) whereas gastrointestinal symptoms varied from stomach bloating, diarrhea, abdominal cramp to excessive gas. Stool screening was also done for various intestinal parasites such as *Entamoeba histolytica*, *Giardia lamblia*, microsporidia, *Dientamoeba fragilis, Ascaris lumbricoides, Trichuris trichiura,* hookworm and *Taenia* sp. Patients who were positive for one or more of these parasites were excluded from the present study.

The parasites were isolated by culturing in 3ml Jones' medium supplemented with 10% horse serum (Gibco Laboratories, Life Technologies, Grand Island, New York) and maintained at 37^{o}C. The positive cultures were sub-cultured once every three days and also stored at -20^{o}C for subsequent subtyping. Consequently after isolation, the parasites were maintained in Jones' medium supplemented with 10% horse serum for at least one month prior to the elucidation of phenotypic characteristics.

4.2.2 Subtyping of *Blastocystis* sp.

The genomic DNA of *Blastocystis* sp. for all 12 isolates was extracted using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturers' protocol. All 12 *Blastocystis* sp. isolates were then subjected to sequence tagged site (STS) primer polymerase chain reaction (PCR) using the seven sets of primers previously described (Yoshikawa et al., 2004b). Two to five microliters of DNA preparations were used to amplify the genomic sequences in a 20µl reaction containing 1x PCR buffer (Fermentas, USA). PCR conditions consisted of 1 cycle of initial denaturing at 95^0C for 5 min, followed by 40 cycles of denaturing at 95^0C for 1 minute, annealing at 56.3^0C for 1 minute 30 seconds and extending at 72^0C for 1 minute, and an additional cycle of elongation at 72^0C for 10 minute using Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, USA).The amplified products were then electrophoresed in 1.5% agarose gels (Promega, USA) in Tris–borate-EDTA buffer. Gels were stained with ethidium bromide and photographed using an ultraviolet gel documentation system (UVitec Ltd., Cambridge, United Kingdom). PCR amplifications for each primer pair were done in triplicate.

4.2.3 Growth characteristics of *Blastocystis* sp.

The parasites of each isolate were pooled together from day 3 cultures to make a final concentration of $1x10^4$cells/ml in 3 ml screw-capped tubes containing Jones' medium supplemented with 10% horse serum. All cultures were kept in airtight screw-capped tubes and incubated at 37ºC for up to 10 days. All experiments were done in triplicate. The *Blastocystis* sp. count was carried out using a hemocytometer chamber (Improved Neubauer, Hausser Scientific Inc., Horsham, PA, USA) with 0.4 % trypan

blue dye exclusion (Sigma-Aldrich, Saint Louis, Missouri, USA) as the viability indicator. The parasite count was determined daily in cultures until all parasites became non-viable. Only viable cells that did not take up trypan blue stain were counted.

Fifty parasites were randomly chosen from every culture tube for size measurement every 2 days for the next 10 days. Statistical analysis was carried out using SPSS version 21. Generation time (GT) was calculated for the 24-h period during the most rapid growth based on following formula previously described (Chaudhari & Singh, 2011).

$$GT = \frac{t}{n} = \frac{t}{3.3 \log (b/B)}$$,where

B = number of cells at zero time

b = number of cells at end of time period

t = time period

GT = generation time

n = number of generations

log = logarithm to the base 10 (common log)

4.2.4 Modified Fields' Stain

The *Blastocystis* sp. isolates from day 3 culture were stained with Modified Fields' stain according to protocol previously described (Afzan et al., 2010). The slides were then viewed under 400X magnification. All experiments were done in triplicate.

4.2.5 Cytochemical staining

The parasites were then grown, pooled and centrifuged respectively to make smears on day 3 cultures with Fluorescein isothiocyanate (FITC)-labelled Con A (*Canavalia ensiformis*) (Tan et al., 2008) and examined with a fluorescence microscope (Wild-Leitz, LeitzWetzlar, Germany) using incident light transmission at 400X magnification. All experiments were done in duplicate. The results were then quantified by using percentage of fluorescent cells in 100 cells and affinity fluorescence unit (AFU) (scale of brightness of 1+, 2+, 3+ and 4+).

4.2.6 Scanning electron microscopy (SEM)

The parasites were washed three times with phosphate buffered saline (PBS) pH 7.4. The samples were centrifuged at 2000×g for 5 min. The pelleted cells were fixed with 4% glutaraldehyde and post-fixed with 1% osmium tetroxide**.** The specimens were then mounted on polycarbonate membrane (Nuclipore, Agar Scientific, USA) and dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90% and 100%). The specimens were critical-point dried with carbon dioxide coated with gold, and examined with a scanning electron microscope (FEI-Quanta 200 FESEM, USA).

4.2.7 Transmission electron microscopy (TEM)

Cultures containing *Blastocystis* sp. were collected from day 3 culture washed three times using PBS pH 7.4 and centrifuged at 2000× g, for 5 min. The pelleted cells were re-suspended overnight in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3 at 4^{o}C, washed thoroughly with cacodylate buffer and post-fixed for 30 min in 1% osmium tetroxide in cacodylate buffer. The fixed cells were dehydrated for 5 minutes in ascending series of ethanols (30%, 50%, 70%, 80%, 90% and 100%) and embedded in epoxy resin. Semithin sections were stained with toluidine blue. Ultrathin sections were cut using an ultramicrotome, contrasted with uranyl acetate and lead citrate and viewed using a transmission electron microscope (FEI-Quanta 200 FESEM, USA).

4.2.8 Physiology of rabbit and Balb/C mice's ileum

Clean *Blastocystis* sp. ST3 from three different isolates (A1, S1 and IBS1) via Ficoll-Paque density gradient centrifugation method as previously described previously (Chandramathi et al., 2010; Chan et al., 2012; Kumarasamy et al., 2013) were isolated. The lysates were sonicated at a frequency of 60hz and 0.5 amplitude for 10 cycles. The lysate was screened under a microscope to observe that the lysis had taken place. The sonicated samples were kept overnight at 4ºC and were centrifuged at 60,000xg for 15 minutes. The supernatants were then filter sterilized, and the protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, USA). Five concentration of *Blastocystis* sp. ST3 antigen (Blasto-Ag ST3) 0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml and 1.0mg/ml were prepared.

Three rabbits and three Balb/C mice were dissected. An abdominal midline incision was made and the intestine was lift out slowly and placed in a petri dish filled with Tyrode's solution. The ileum approximately 2-3cm was severed and the lumen was flushed gently with the Tyrode's solution by using a 5ml syringe. The ileum was then suspended in the organ bath. The organ bath connected to the Powerlab Programme is able to read the frequency and amplitude of ileum twitching (Figure 4.1). Five different concentrations of Blasto-Ag ST3 ranging from 0.2 to 1.0mg/ml were introduced directly to the ileum and then the ileum was soaked back into the Tyrode solution to obtain the frequency and amplitude of muscle twitching. The optimal muscle twitching could be determined based on the sensitivity of ileum in response to the Blasto-Ag ST3. Experiment was done in triplicate. This study has been approved by the Institutional Animal Care and Use Committee (IACUC), University Malaya. The ethics reference no: 2014-01-07/PARA/R/ADR.

Figure 4.1: Organ bath connected to the Powerlab Programme.

4.3 Results

4.3.1 Genotyping of *Blastocystis* sp.

Based on PCR amplification with the STS primers, all the 12 isolates from 3 different groups, asymptomatic, symptomatic and IBS amplified with the primer SB227 (~526 bp) (Yoshikawa et al., 2004b) (Figure 4.2). The PCR products for all three groups were determined as ST3. In this study, we found that ST3, which is known as a pathogenic subtype is also found in the asymptomatic isolates.

Figure 4.2: Gel image of *Blastocystis* sp. subtyping

Blastocystis sp. isolated from asymptomatic individuals L1-L4, symptomatic isolates L5-L8, and IBS isolates L9-12, ST3 (526bp), M = 100bp plus DNA marker.

4.3.2 Growth characteristics

A total of 12 *Blastocystis* sp. ST3 isolates from four asymptomatic individuals (A1-4), symptomatic patients (S1-4) and IBS patients (IBS1-4) respectively were used in the present study. The growth profile with an initial inoculation of $1.0X10^4$cells/ml showed three distinct and different growth profiles. A1-4 isolates showed the highest peak growth compared to the other two groups followed by S1-4 isolates and IBS1-4 isolates. The parasite peak count for A1-4 isolates ranged from $120X10^4$ cells/ml in isolate A1 to $496X10^4$ cells/ml in isolate A2 (Figure 4.3). The range of parasite count during peak growth in symptomatic isolates between $4.53X10^4$cells/ml to $9.33X10^4$cells/ml and in IBS isolates was between $33.1X10^4$cells/ml to $112.4X10^4$cells/ml (Figure 4.3). All IBS isolates peaked on day 5 including one and two of the symptomatic and asymptomatic isolates respectively. The remaining isolates peaked on day 4 with the exception of isolates S2 and A4 which peaked on day 6 (Figure 4.3). The asymptomatic isolates (average generation time: 5.97±1.52 h) grew faster than the symptomatic isolates (average generation time: 9.87±2.97 h) and IBS isolates (average generation time: 7.56±1.06 h) (Figure 4.4). Parasites from IBS isolates (IBS1-4) showed the largest diameter with a mean of 18.43$\pm$2.22µm compared to parasites of symptomatic isolates (isolates S1-4) 15.54$\pm$3.02µm and asymptomatic isolates (isolates A1-4) 11.76$\pm$0.82µm (Figure 4.5).

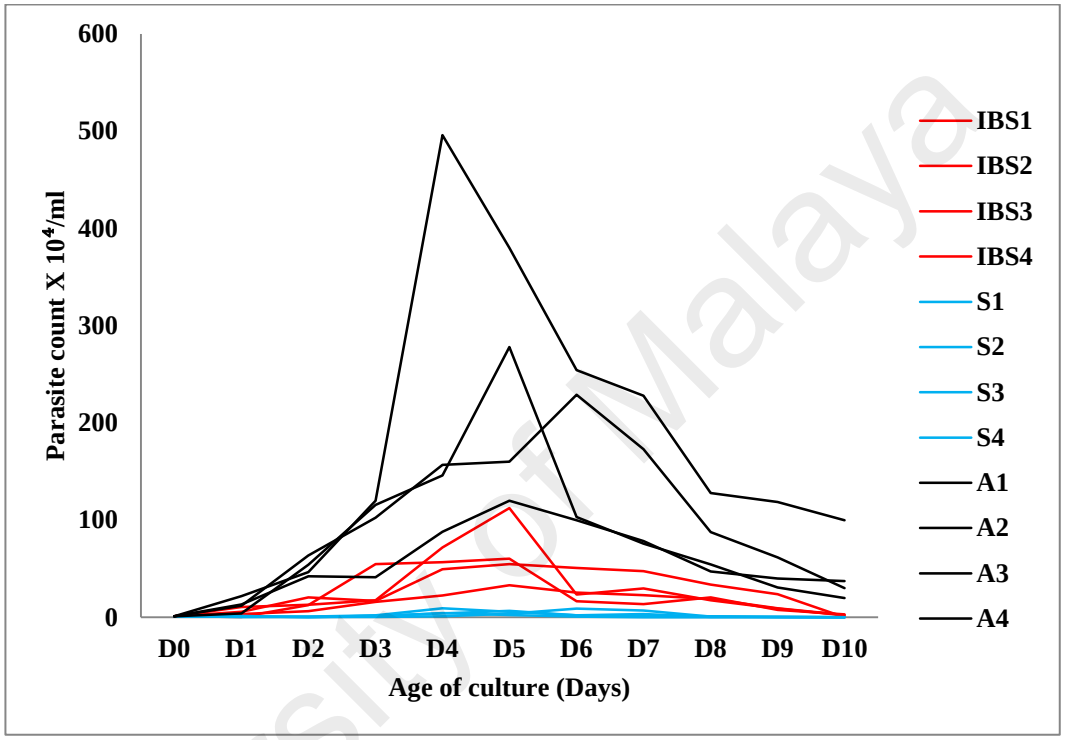

Figure 4.3: Growth profile of *Blastocystis* sp. ST3 isolates A1-4, S1-4 and IBS1-4 in culture

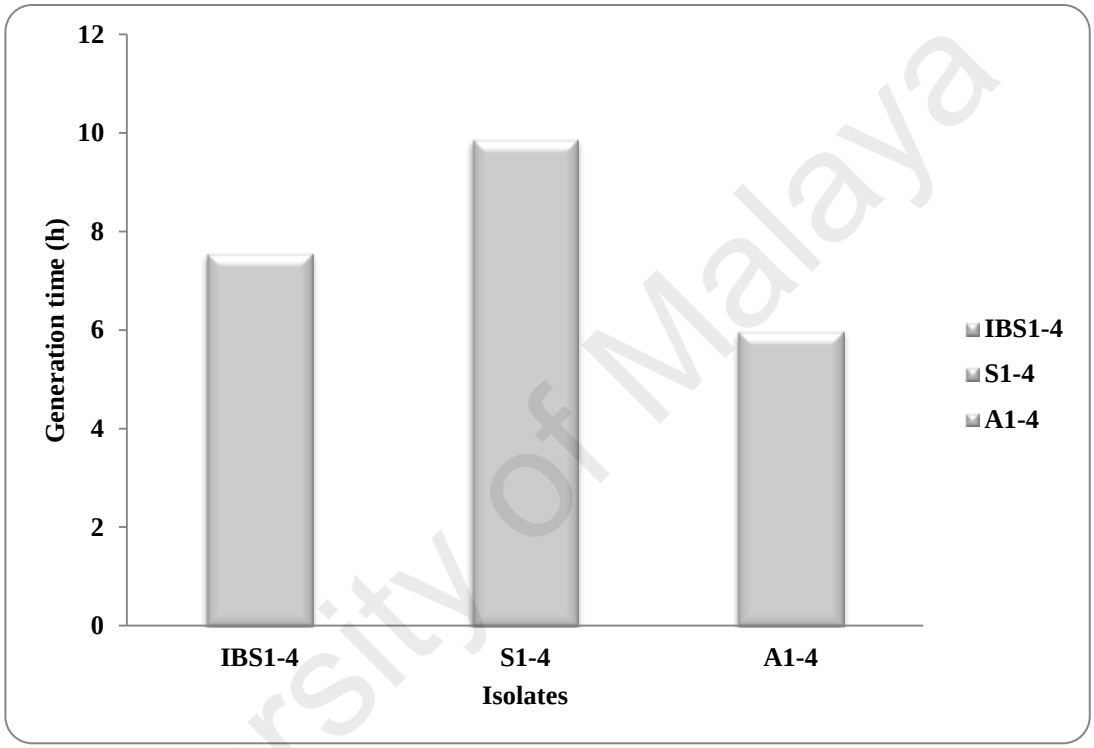

Figure 4.4: Generation time of *Blastocystis* sp. ST3 *for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4).

Comparison between generation time for asymptomatic isolates (A1-4) of *Blastocystis* sp. ST3 isolates, symptomatic isolates (S1-4) and IBS isolates (IBS1-4). Note that the asymptomatic isolates grew faster than both IBS and symptomatic isolates.

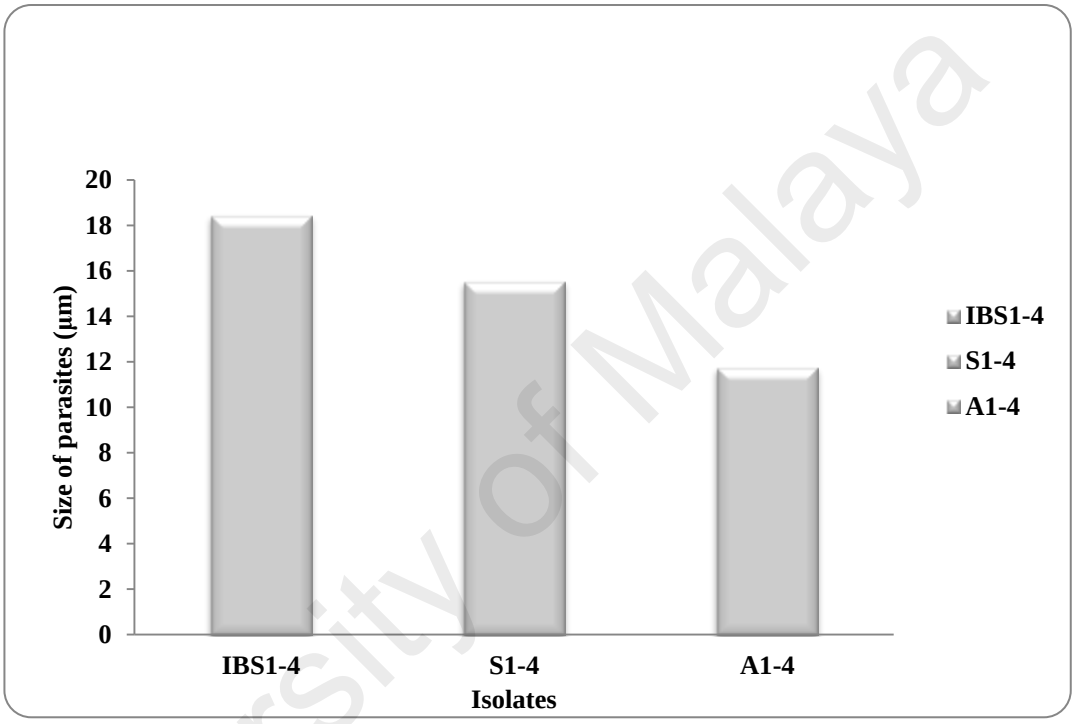

Figure 4.5: Average size of *Blastocystis* sp. ST3 *for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4); Comparison between average size of parasites for asymptomatic isolates (A1-4) of *Blastocystis* sp. ST3 isolates, symptomatic isolates (S1-4) and IBS isolates (IBS1-4).

4.3.3 Modified Fields' stain

The staining characteristics in parasites from all three groups were similar. However parasites from IBS isolates showed strong aggregation and clumping (Figure 4.6C), the intensity of which was markedly lowered in parasites of isolates S1-4 (Figure 4.6B). Parasites from A1-4 isolates were seen to be distinct with no clumping (Figure 4.6A).

4.3.4 Cytochemical staining of *Blastocystis* sp. ST3

The outer surface of parasites in IBS isolates (Figure 4.7C) showed greater binding affinities towards FITC-labelled Concanavalin A (Con A) than symptomatic isolates (Figure 4.7B) and asymptomatic isolates (Figure 4.7A). The fluorescence intensity and the percentage of the reactive forms of IBS isolates in FITC-labelled Con A stain range was (4+; 88-100%), symptomatic isolates, S1-4 (3+; 78-100%) and for asymptomatic isolates, A1-4 (1+; 78- 94%).

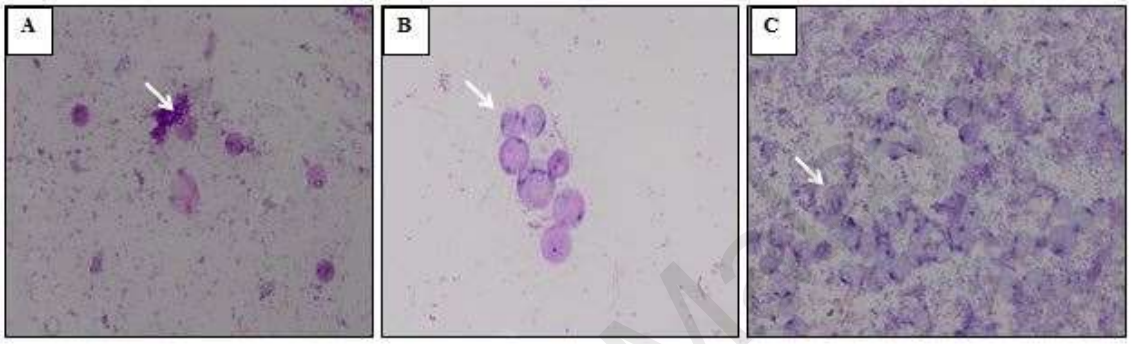

Figure 4.6: Comparison of clumping of *Blastocystis sp. ST3 stained with modified fields' stain for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4); (A): *Blastocystis* sp. A1 isolate were stained with modified fields' stain and the parasites were far apart; (B): Stained *Blastocystis* sp. S2 were attached together in a smaller number of parasite; (C): The IBS1 isolate seen to be clumped together in a large number.

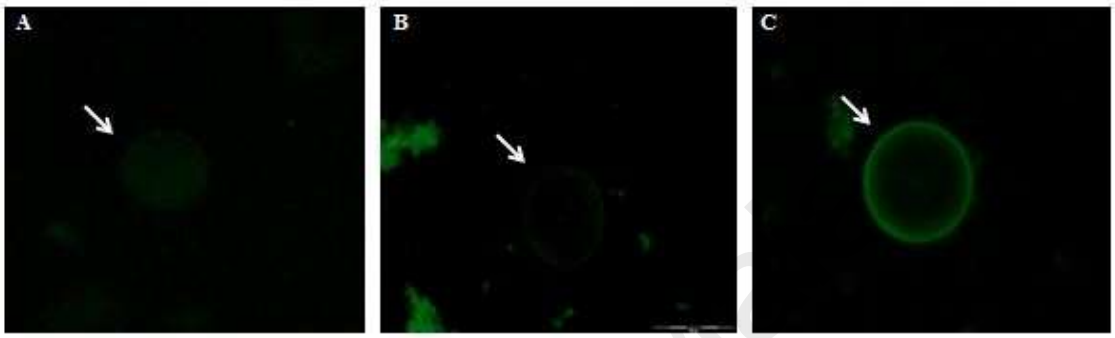

Figure 4.7: Comparison of binding affinities of *Blastocystis sp. ST3 stained with* FITC-labelled Con A ***(***A): asymptomatic isolates, isolates A1-4, AFU (1+), (B): symptomatic isolates, isolates S1-4, AFU (3+) and (C): IBS isolates (IBS1-4) which were stained with FITC-labelled Con A showed a greater binding affinities with scale brightness of AFU (4+)

The values are expressed as: AFU 1+ weak intensity, AFU 2+ medium intensity, AFU 3+ strong intensity (percentage of reactive forms).

4.3.5 Surface structure of *Blastocystis* sp. ST3

Blastocystis sp. ST3 forms in three different groups, asymptomatic isolates (Figure 4.8 A&B), symptomatic isolates (Figure 4.8 B&C) and IBS isolates (Figure 4.8 E&F) appeared to have different surface morphology respectively. Scanning electron microscopy (SEM) showed that *Blastocystis* sp. ST3 isolated from asymptomatic isolates possess a very smooth surface meanwhile the *Blastocystis* sp.ST3 isolated from symptomatic isolates showed slightly rough surface with tiny pores. In IBS isolates, the surface of *Blastocystis* sp. ST3 showed a very coarse and intensely folded surface.

4.3.6 Ultrastructure of *Blastocystis* sp. ST3

Blastocystis sp. ST3 in all three groups, asymptomatic isolates (Figure 4.9 A), symptomatic isolates (Figure 4.9 B) and IBS isolates (Figure 4.9 C-H) appeared to be rounded and oval shape. The IBS isolates also exhibited a dense material (Figure 4.9 C-H). In contrast, the dense material was not seen in any of the parasites from both the asymptomatic and symptomatic isolates.

Blastocystis sp. ST3 in IBS isolates showed a thicker layer of surface coat surrounding the parasites, 1032.76nm (Figure 4.10 C), 549.30nm (Figure 4.10 D), 700.55nm (Figure 4.10 E) and 792.26nm (Figure 4.10 F) compared to a relatively thinner layer seen in parasites from both asymptomatic isolate, 209.34nm (Figure 4.10 A) and symptomatic isolate, 343.08nm (Figure 4.10 B).

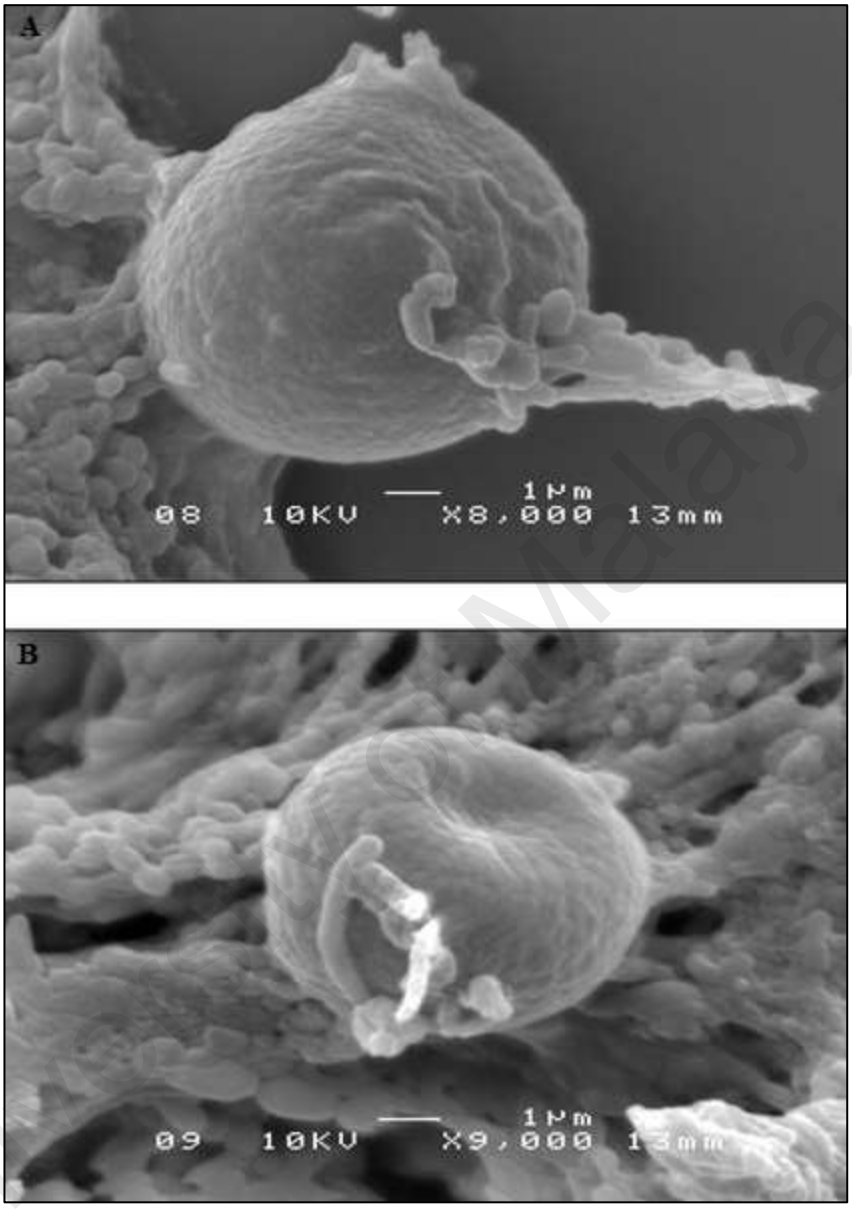

Figure 4.8: Comparison of scanning electron microscopy of *Blastocystis sp. ST3 for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4); Figure (A) & (B): Scanning electron microscopy showed that *Blastocystis* sp. ST3 isolated from A1 possess a smooth surface

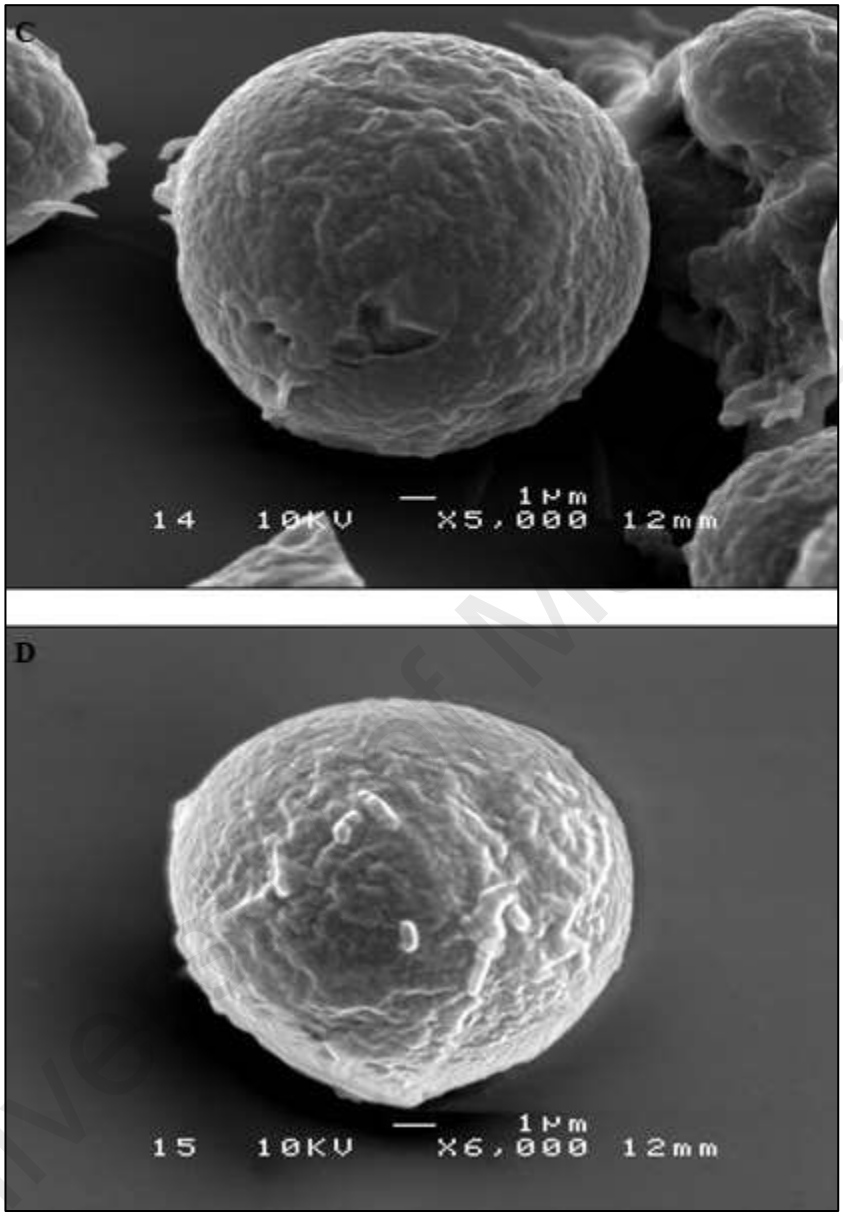

Figure 4.8, continued Comparison of scanning electron microscopy of *Blastocystis sp. ST3 for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4); Figure (C) & (D): Scanning electron microscopy showed that *Blastocystis* sp. ST3. isolated from symptomatic isolate, S1 showed slightly rough surface

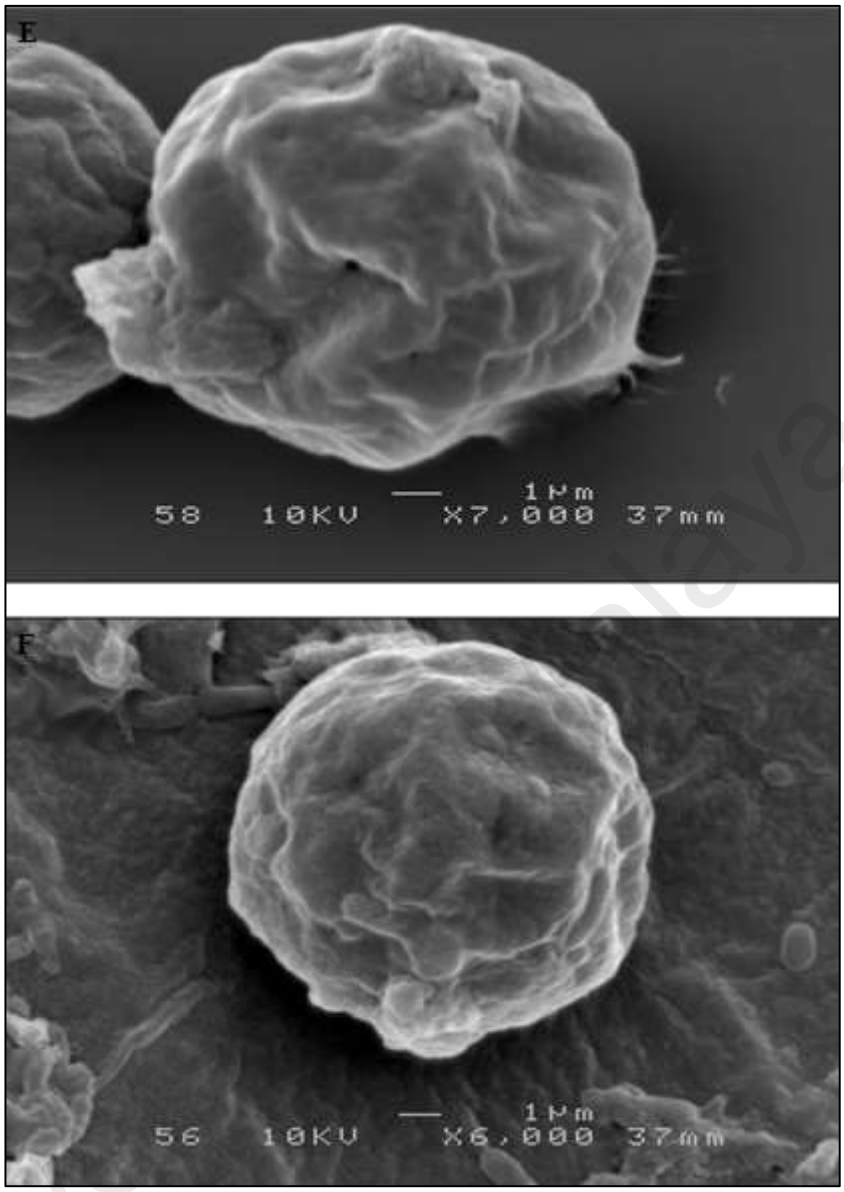

Figure 4.8, continued Comparison of scanning electron microscopy of *Blastocystis sp. ST3 for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4); Figure (D) & (E): Scanning electron microscopy showed that *Blastocystis* sp. ST3. isolated from IBS1 showed coarse and folded surface.

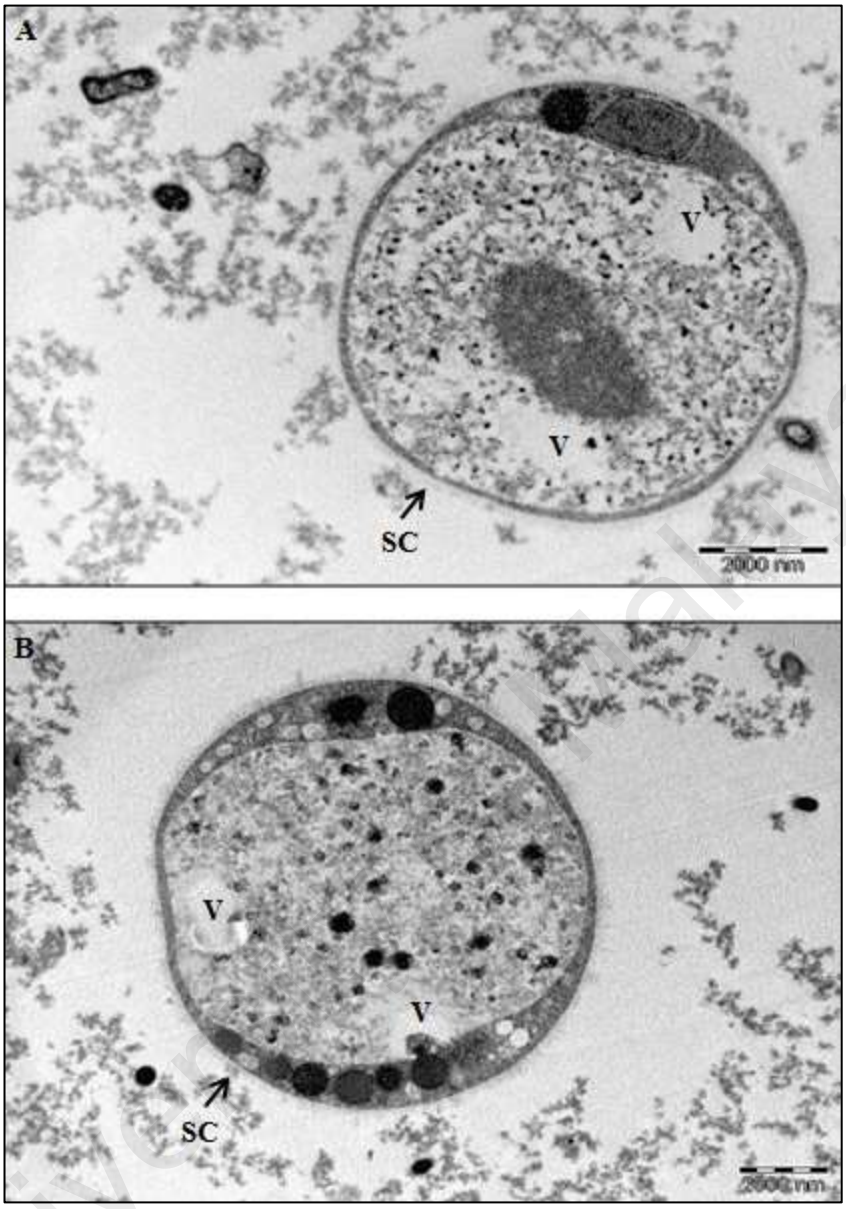

Figure 4.9: Comparison of transmission electron microscopy of *Blastocystis sp. ST3 for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4); Transmission electron micrographs (TEM) of asymptomatic isolates, isolate A1 (A) and *Blastocystis* sp. ST3 isolated from symptomatic isolates, S1. (B)

Electron dense material was not seen in parasites from both the asymptomatic and symptomatic isolates.

Note: (V) vacuole, (DM) dense material, (SC) surface coat

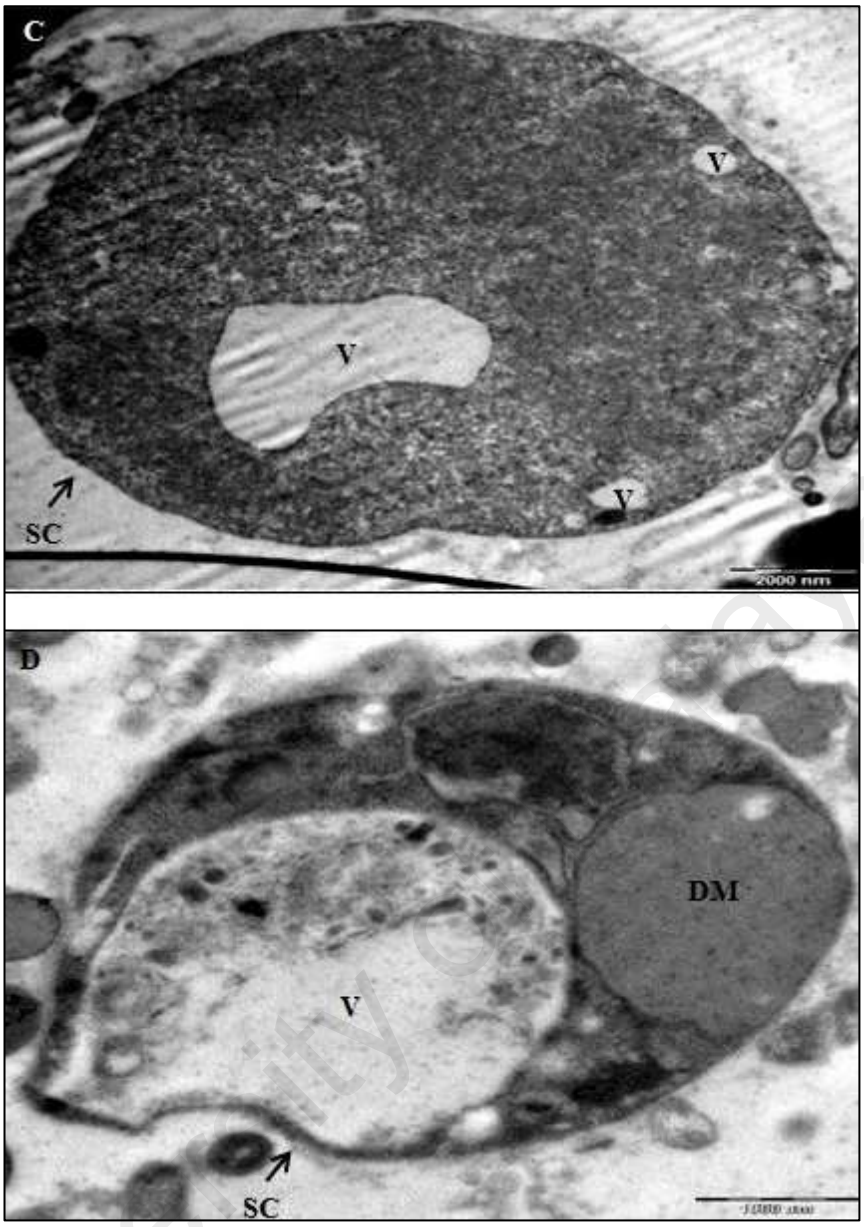

Figure 4.9, continued Comparison of transmission electron microscopy of *Blastocystis sp. ST3 for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4); Figure (C) & (D): Transmission electron microscopy showed Blastocystis sp. ST3 isolated from IBS showed to have electron dense material which can be seen within the central body of all IBS isolates.

Note: (V) vacuole, (DM) dense material, (SC) surface coat

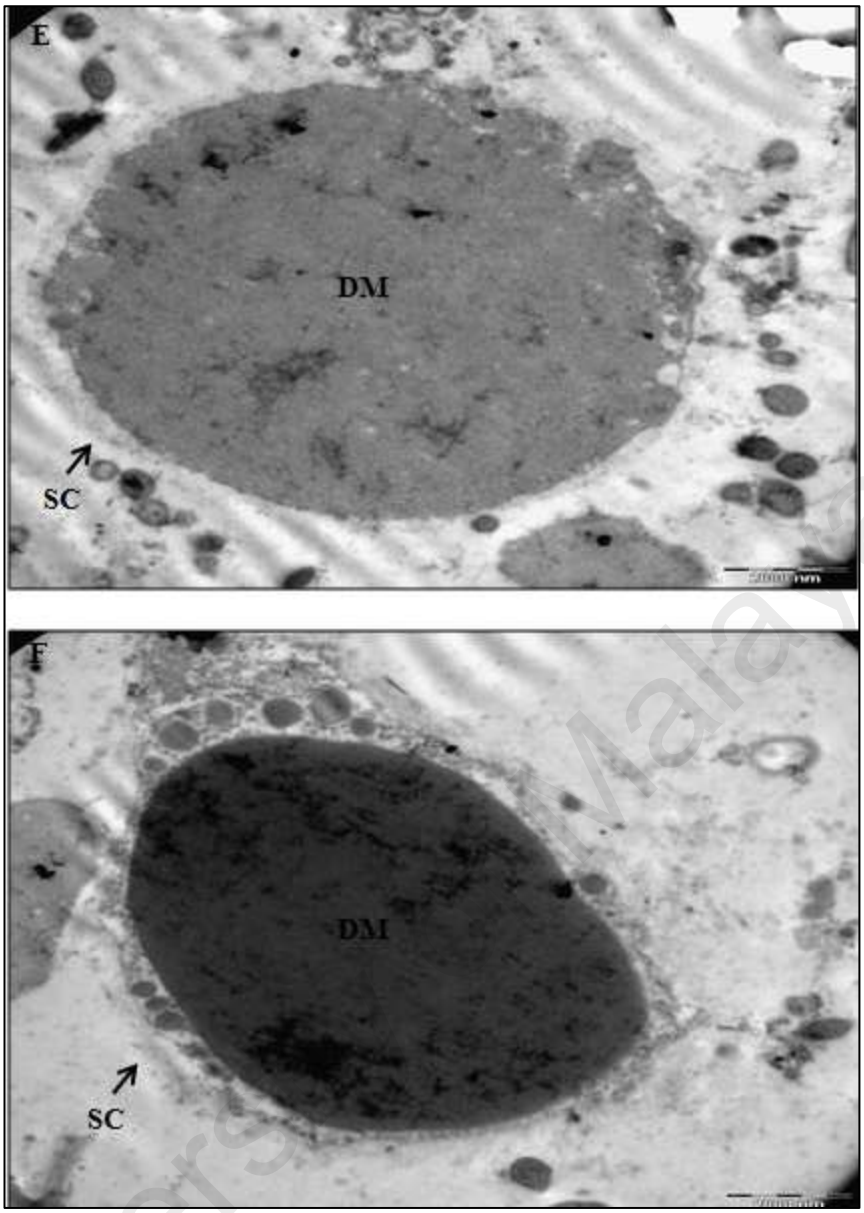

Figure 4.9, continued Comparison of transmission electron microscopy of *Blastocystis sp. ST3 for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4); Figure (E) & (F): Transmission electron microscopy showed *Blastocystis* sp. ST3. isolated from IBS patients showed to have electron dense material which can be seen within the central body of all IBS isolates.

Note: (V) vacuole, (DM) dense material, (SC) surface coat

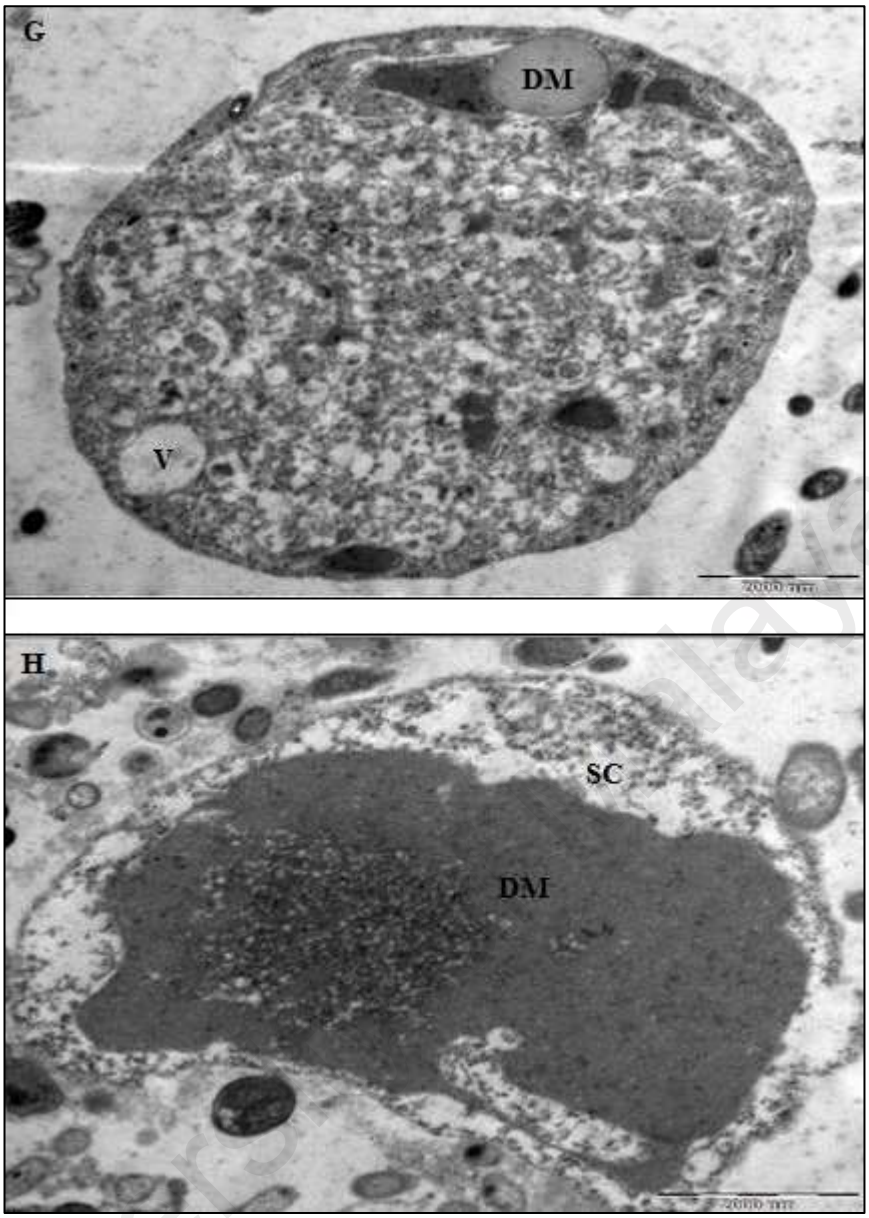

Figure 4.9, continued Comparison of transmission electron microscopy of *Blastocystis sp. ST3 for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4); Figure (G) & (H): Transmission electron microscopy showed *Blastocystis* sp. ST3. isolated from IBS showed to have electron dense material which can be seen within the central body of all IBS isolates.

Note: (V) vacuole, (DM) dense material, (SC) surface coat

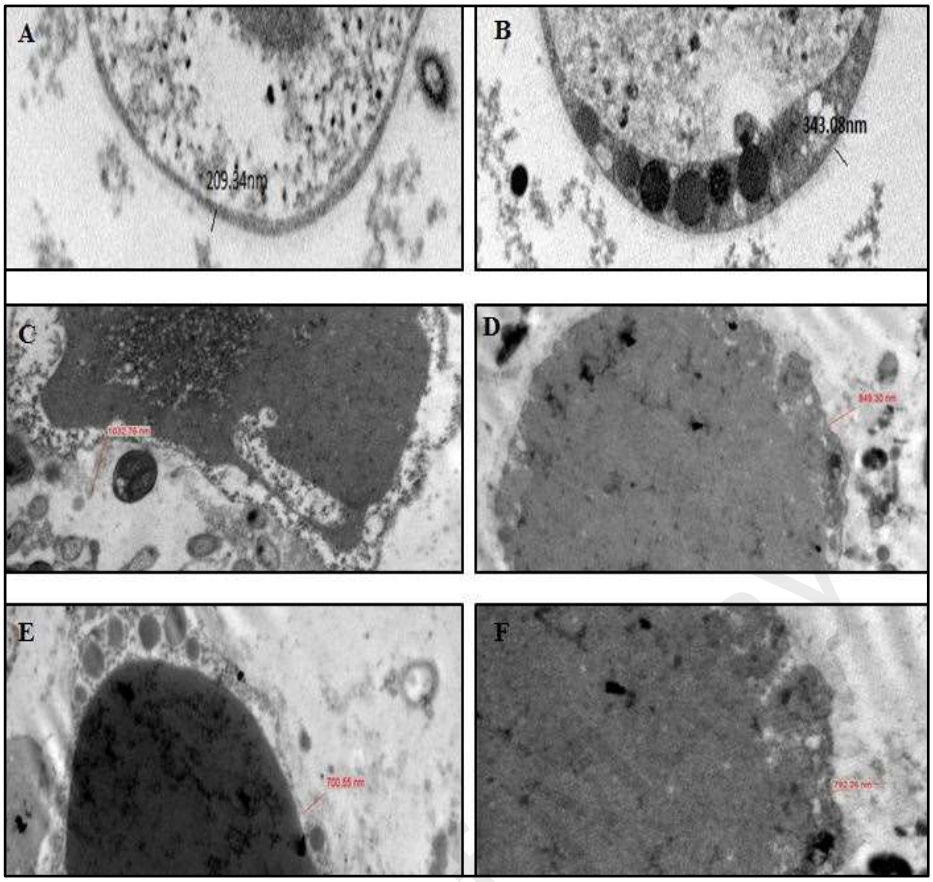

Figure 4.10: Comparison of thickness of surface coat of *Blastocystis sp. ST3 for* asymptomatic isolates (A1-4), symptomatic isolates (S1-4) and IBS isolates (IBS1-4), and; *Blastocystis* in IBS isolates (C-F) revealed to have a thicker layer of membrane surrounding the parasites which is also known as a surface coat compared to a relatively thin layer of membrane in both asymptomatic isolates and symptomatic isolates (A) & (B) respectively.

4.3.7 Physiology of rabbit and Balb/C mice's ileum

An average of twitching per minute of both the rabbit and Balb/C mice's ileum was recorded. 1ml of Jones' medium was introduced directly on the ileum in the organ bath as the control showed an average of 12 and five twitching per minute for the rabbit's and mice's ileum respectively. Rabbit's ileum showed no effect on muscle twitching upon exposure of 0.2, 0.4, 0.6 and 0.8mg/ml for the Blasto-Ag ST3 derived from three different groups (asymptomatic, symptomatic and IBS). However, 1mg/ml Blasto-Ag ST3 was found to have an effect on the rabbit's ileum. Rabbit's ileum exposed to IBS Blasto-Ag ST3 showed to have the highest number of muscle twitching, 15 twitching per minute compared to 13 per minute for symptomatic Blasto-Ag ST3 and 12 twitching per minute for asymptomatic Blasto-Ag ST3. The experiment was then conducted on Balb/C mice's ileum. All five concentrations of Blasto-Ag ST3 were found to have an influence on the muscle twitching of the mice's ileum. IBS isolates showed the highest twitching of ileum per minute when 1.0mg/ml of Blasto-Ag was introduced (12 twitching/minute) compared to Blasto-Ag derived from asymptomatic and symptomatic isolates (Table 4.1).

Table 4.1: Twitching of Balb/C mice's ileum per minute upon exposure of Blasto-Ag ST3 derived from asymptomatic, symptomatic and IBS isolates

Groups	Concentration (mg/ml)	Twitching/minute
Control (Jones' medium)		5
Asymptomatic	0.2	3
	0.4	4
	0.6	4
	0.8	5
	1.0	5
Symptomatic	0.2	6
	0.4	5
	0.6	6
	0.8	7
	1.0	7
IBS	0.2	10
	0.4	10
	0.6	10
	0.8	11
	1.0	12

4.4 Discussion

The pathogenicity of *Blastocystis* sp. is still disputed because of its presence in both asymptomatic individuals and symptomatic patients (Al & Hökelek, 2006; Yan et al., 2006). Out of 15 subtypes (ST1-ST15) based on the small subunit rRNA, nine have been found in human (ST1-ST9) (Stensvold et al., 2009). Previous studies have demonstrated that parasites from asymptomatic and symptomatic isolates have shown phenotypic differences (Tan et al., 2008; Chan et al., 2012) but these differences could have been largely due to subtype differences. In the present study, the fortuitous discovery of *Blastocystis* sp. ST3 from three different groups namely asymptomatic individuals, symptomatic and IBS patients have been studied to assess if differing gut conditions can influence phenotypic differences.

Growth profiles have been used previously to demonstrate phenotypic differences (Tan et al., 2008) and in the present study three distinct growth profiles and generation times were seen for asymptomatic, symptomatic and IBS isolates, providing evidence that the rate and possibly the biology of reproduction could be influenced by gut conditions. The generation time shows that the asymptomatic isolates grew faster than symptomatic and IBS isolates which was similar with a previous study by Tan et al., (2008). Furthermore the IBS isolates are larger in diameter compared to parasites from symptomatic and asymptomatic. It is interesting to note that the size variation in IBS isolates is the highest compared to the other two groups implying that the growth conditions in IBS gut would have conferred this diversity in size, growth rate and generation time. There was a notable clumping of parasites seen in all isolates from IBS which was absent totally in all asymptomatic isolates and partially seen in symptomatic isolates. The surface coat of parasites from IBS isolates has been shown to be folded

and coarse as seen by SEM and its surface coat seen more prominently thicker by TEM studies. It is likely that this surface could be sticky which has resulted in the clumping of the parasites which is seen in the modified fields' stain. FITC Con A was used to identify the carbohydrate surface of *Blastocystis* sp. The surface coat also showed a high FITC Con A binding with 88-100% of cells in all IBS isolates showing affinity fluorescence unit (AFU) of 4+ compared to symptomatic and asymptomatic isolates which showed only 3+ and 1+ respectively. The higher FITC Con A binding may imply that the surface texture of the cell wall could cause the adherence of bacteria to the parasite surface coat, thereby influencing the pathogenicity of the parasite.

The digestive system's purpose is to facilitate the breakdown and absorption of nutrients into the bloodstream. The environment has to be supplemented with the right gut flora (Giannella et al., 1972), pH (Guyton, 2006) and the right amount of secretion of acid, bile and pancreatic juices (Bengmark, 1998). The pH of gastric acid and small intestine is between pH 1.5 to 3.5 and pH 7.0 to 9.0 respectively. Recent studies have dispelled the belief that IBS is a purely psychological disorder. There have been clear demonstrations of distinct abnormalities of the gut mucosa including immune activation, increased release of inflammatory mediators and impaired gut barrier function. It is obvious that the microbiota and the gut conditions vary between asymptomatic to symptomatic conditions with prominent differences such as low grade inflammation, abnormal gases, immunological response, low fecal microbiota acid level with poorer protein and carbohydrate metabolism in IBS patients (Bonfrate et al., 2013). Furthermore the gut diversity with increased bacteria has been reported to be seen within the mucus layer sometimes in the deeper portions previously reported in IBS patients using the fluorescent in situ hybridization method possibly due to a common barrier defect within mucosal layers (Noor et al., 2010; Joossens et al., 2011).

Significant differences in CD3+, CD4+, CD8+, lamina propia leukocytes (LPL) and intraepithelial lymphocytes (IEL) between post infectious-IBS (PI-IBS) patients and healthy controls have been shown providing evidence that there has been aberrant mucosal immune response to the luminal environment in PI-IBS (Sundin et al., 2012). The severity in IBS conditions and the influence of these adverse changes in gut condition obviously seem to have a pronounced effect on the phenotypic expression of *Blastocystis* sp. seen in the present study.

Studies have implicated that genotypes of *Blastocystis* sp. are the influencing factors for the parasite's pathogenicity especially subtype 3, where in Malaysia (Tan et al., 2008), Singapore (Wong et al., 2008) and USA (Jones et al., 2009), evidence for its pathogenicity was clearly demonstrated. *Blastocystis* sp. ST3 was also found to be the only subtype in 100% of patients suffering from urticaria in Egypt (Hameed et al., 2011). Blasto-Ag of ST3 showed the most predominant subtype compared to five other subtypes in triggering a higher proliferation rate in colorectal cancer cells (Kumarasamy et al., 2013). Tan et al. (2008) also demonstrated ST3 to be pathogenic when comparing the phenotypic characteristics between asymptomatic and symptomatic isolates of *Blastocystis* sp.

The present study however is the first to demonstrate phenotypic variation within ST3 isolates from three environments namely asymptomatic, symptomatic and IBS. Hence environmental adaptability for survival purposes could be a possible reason for the phenotypic variation to exist. The thicker surface coat shown by the ultrastructural study in IBS isolates could influence cytopathic effect of *Blastocystis* sp. towards the intestinal lining of the gut. *Blastocystis* sp. lysate and live parasites have been shown to trigger cytopathic effects on Chinese Hamster Ovary cells (Thompson et al., 1993; Walderich et

al., 1998). The coarse and uneven surface with folding seen in parasites of IBS isolates showing high Con A binding as well the aggregation and clumping seen in stained smears provide evidence that the surface is sticky. This becomes ideal to facilitate adherence to bacteria (Suresh et al., 1997a) as well as to the intestinal lining which can exacerbate inflammation.

In IBS condition inflammation of the intestinal could cause leaky gut syndrome which facilitates undigested food substances to pass between the cells of the intestinal epithelial layer to the bloodstream causing damage on the intestinal wall (Ma, 1997), which in turn will reduce the efficiency of nutrient absorption. Previous studies have shown that the interplay between luminal factors such as food, living bacteria in the intestine, epithelial barrier and the mucosal immune system can alter structural rearrangement of tight junction proteins in the small intestine and colon which can result in the increment of intestinal permeability especially in PI-IBS and in IBS with diarrhea (Barbara et al., 2012). Dietary intake could also influence the intestinal gut flora causing a digestive problem which then can lower the immune system (Öhman & Simrén, 2010). Other than that, a studies done to evaluate the role of single nucleotide polymorphisms (SNPs) for interleukin (IL)-8 and IL-10, comparing between IBS patients infected with *Blastocystis* sp. and asymptomatic patients found out that it can actually alter individual sensitivity increasing the relative risk in the development of *Blastocystis sp. infected IBS patients* (Olivo-Diaz et al., 2012).

Verne et al. (2003) also reported that IBS can be classified by altered visceral perception. Visceral perception of the gut is very much associated with the activation of serotonin, 5-HT3 receptor. Numerous studies have linked up with the visceral sensitivity with IBS patients (Chang et al., 2003; Naliboff et al., 2006). Studies showed

that serotonin plays an important role in causing the excitatory and inhibitory gut neurotransmitters which trigger in the smooth muscle contraction or relaxation (Singh et al., 2003). Serotonin also known as neurotransmitter can be found in gastrointestinal tract responsible in regulating the intestinal movement which causes the bowel irritation and pathophysiologic disturbances in IBS patients (Crowell, 2004). Serotonin and proteases influence the gut motility and both have been known to be secreted by *Blastocystis* sp. (Puthia et al., 2008). Miwa et al., (2001) showed that intestinal biopsies from patients with constipation with predominant IBS secreted higher levels of serotonin *in vitro.* Other than that, serotonin is also secreted by some gastrointestinal protozoa which cause diarrhea and elevated serum serotonin levels in humans (Banu et al., 2005). Lucas et al., (2010) reported that motility and visceral hypersensitivity play an important role in contributing to the IBS condition in patients and this has been attributed to the low grade intestinal inflammation.

Moreover, IBS patients who are suffering from chronic fatigue and fibromyalgia could be due to the dysfunction of autonomic nervous system representing the physiological pathway (Tougas, 2000). Autonomic properties of ileum in response to the Blasto-Ag ST3 were observed based on the twitching of the ileum. The present study is the first to assess the effect of Blasto-Ag ST3 on the ileum twitching and the potential effect on gastrointestinal tract of the infected host. The study clearly demonstrated that Blasto-Ag ST3 from IBS isolates has a higher effect on the twitching rate seen in the ileum of both rabbit and mice compared to Blasto-Ag ST3 from asymptomatic and symptomatic isolates. Therefore, it is possible that a similar a scenario can be seen in IBS patients infected with *Blastocystis* sp. which could have contributed to the manifestation of clinical symptoms. The differing gut motility upon exposure of Blasto-Ag ST from three different groups (asymptotic, symptomatic and

IBS isolates) could be associated with the phenotypic differences of this parasite described earlier in this chapter (Figure 4.3-4.10). This study also suggests that Balb/C mice can be a better and cheaper animal model to study the twitching of ileum upon exposure of Blasto-Ag ST3 compared to rabbits. Balb/C mice showed an optimal response when introduced to the lowest concentration of Blasto-Ag ST3, 0.2mg/ml.

The present finding has very important implications. Previously all evidence has been pointing to subtype 3 to be the pathogenic one. However the present study cautions on forming such a conclusion and suggests that ascribing subtype to pathogenicity could be an over generalization. It is evident that gut environment can influence phenotypic expression of even the same subtype. A similar study had been done on assessing the phenotypic characterization of *Entamoeba histolytica* whereby the interaction between the parasites and host component such as bacterial flora and mucins can trigger the parasites to be more pathogenic by exhibiting their virulence factor (Padilla-Vaca & Anaya-Velázquez, 2010).

It is beyond the scope of the present study to postulate the details of the gut environment although others have suggested that an unhealthy gut has a complex open ended ecosystem which can be a host for various microorganisms (Rajilić-Stojanović et al., 2007; Ley et al., 2008; Scanlan & Marchesi, 2008). This study has diagnostic implications and it is important to ensure that a proper and a detailed study be undertaken before forming any definite conclusion as gut environment does play a part in expressing and influencing phenotypic.

4.5 Conclusion

There have been no studies thus far providing evidence for phenotypic variation within a particular subtype. The present study is the first to demonstrate the phenomenon of gut environment facilitating adaptation of parasites possibly for survival leading to phenotypic differences for *Blastocystis* sp. ST3.

Chapter 5: *In vivo* experimental infection studies with *Blastocystis* sp. ST3.

5.1 Introduction

The worldwide distribution of *Blastocystis* sp. and increase in the infection rate demonstrate the zoonotic potential and the parasite's low host-specificity (Poirier et al., 2012). The infective stage of this parasite is the cyst form and it can be transmitted through the fecal-oral route (Yoshikawa et al., 2000). Several studies showed phenotypic differences in *Blastocystis* sp. isolated from healthy individuals and symptomatic patients (Tan et al., 2008; Chan et al., 2012) which was attributed to subtype differences. Results from Chapter 4 showed a distinct phenotypic variation within *Blastocystis* sp. ST3 isolated from three different groups' namely asymptomatic individuals, symptomatic and irritable bowel syndrome (IBS) patients. Subtype 3 (ST3) has been previously reported to be one of the predominant subtypes which influences the pathogenicity of the parasites. The predominance of ST3 was also clearly seen in studies carried out in Singapore (Wong et al., 2008) and the United States (Jones et al., 2009).

Studies have also shown that *Blastocystis* sp. could be a causative agent for IBS (Boorom et al., 2008) since both share the same clinical symptoms, including constipation, abdominal pain, diarrhea, cramps, nausea and fatigue (Graczyk et al., 2005). Intestinal parasites especially *Blastocystis* sp. could further cause inflammation in the intestinal tract of IBS patients (Gwee, 2005). This parasite could also cause enteritis (Gallagher & Venglarcik, 1985) and terminal ileitis (Tsang et al., 1989). Invasion and mucosal inflammation of the intestine due to *Blastocystis* sp. infection was seen to cause degradation of extracellular matrix proteins by damaging the colonic

epithelial cells in gnotobiotic guinea pigs (Zierdt, 1991). Hyaluronidase, was shown to be released during the invasion stage which caused tissue histolysis and mucosal invasion (Hotez et al., 1994). Increasing intestinal permeability during the invasion of parasites has also been suggested to damage the gut wall.

Hence, the present study aimed to elucidate the pathogenic effects of *Blastocystis* sp. ST3 in experimentally infected *Wistar* rats by comparing the degree of mucosal sloughing, inflammation, necrosis of tissue seen in the ileum, caecum, colon and rectum from three different groups i.e. asymptomatic individual, symptomatic and IBS patients.

5.2 Materials and methods

5.2.1 Cysts concentration

Blastocystis sp. ST3 cysts for the rat's inoculation were isolated from asymptomatic individuals, symptomatic and IBS patients. Ficoll-Paque technique was used to isolate cysts according (Zaman & Khan, 1994). The fecal sample was dissolved in phosphate buffered solution (PBS) pH 7.4 and filtered using gauze. The samples were then centrifuged at 3000rpm for 10 min. The supernatant was discarded and 5ml of the sediment were then layered on 6ml Ficoll-Paque solution. The sample was the centrifuged at 3600rpm for 20 min. Four layers were formed and the cysts were harvested using a fine pipette from the second top layer and washed twice with PBS. *Blastocystis* sp. ST3 cysts were counted using a hemocytometer chamber (Improved Neubauer, Hausser Scientific Inc., Horsham, PA, USA) with 0.4 % trypan blue dye exclusion (Sigma-Aldrich, Saint Louis, Missouri, USA).

5.2.2 Inoculation of *Blastocystis* sp. ST3 cysts in *Wistar* rats

Four week old *Wistar* rats with a mean weight of 70 g were used in this study. A total of 64 rats were divided into four groups, a) control, b) asymptomatic, c) symptomatic and d) IBS group; n=16 each group respectively. Cyst suspensions were prepared in two concentrations i.e. $1x10^4$cysts/ml and $1x10^6$ cysts/ml respectively and were orally inoculated into each rat in the respective groups (Figure 5.1). Control rats were fed with 1ml of PBS. This study has been approved by the Institutional Animal Care and Use Committee (IACUC), University Malaya. The reference numbers are PAR/29/06/2012/LIL(R) and PAR/23/05/2013/HC(R).

5.2.3 Detection of *Blastocystis* sp. in rat's stool samples

Fresh stool samples were collected daily from all the rats post-inoculation for 7 days. The stool samples were examined microscopically and cultured in 3ml Jones' medium supplemented with 10% horse serum (Suresh & Smith, 2004). The rats were considered positive for *Blastocystis* sp. infection if vacuolar or granular form were observed under light microscopy and using the *in vitro* culture method.

5.2.4 Histological assessment

The rats were dissected on the 1st and 4th week after inoculation based on their respective groups (Figure 5.1). Tissue sections from ileum, caecum, colon and rectum were washed with PBS and fixed in 10% formal saline. The paraffin embedded tissues were sectioned and stained with hematoxylin–eosin (H&E). The slides were examined by a pathologist who was not told of the source of the cysts. The histological score system (0, +1, +2 and +3) was based on the severity of mucosal sloughing (Figure 5.2 A-D), inflammation of tissue (Figure 5.3 A-D), and necrosis of tissue (Figure 5.4 A-D). The score 0, for none; +1, at least 20% of the affected area in the H&E slide showing mild histopathological changes (mucosal sloughing, inflammation and necrosis of tissue); followed by moderate (+2; 21%-50%); and extensive histopathological changes (+3; < 51%). The scores for each tissue were recorded based on the histological score system (Table 5.1).

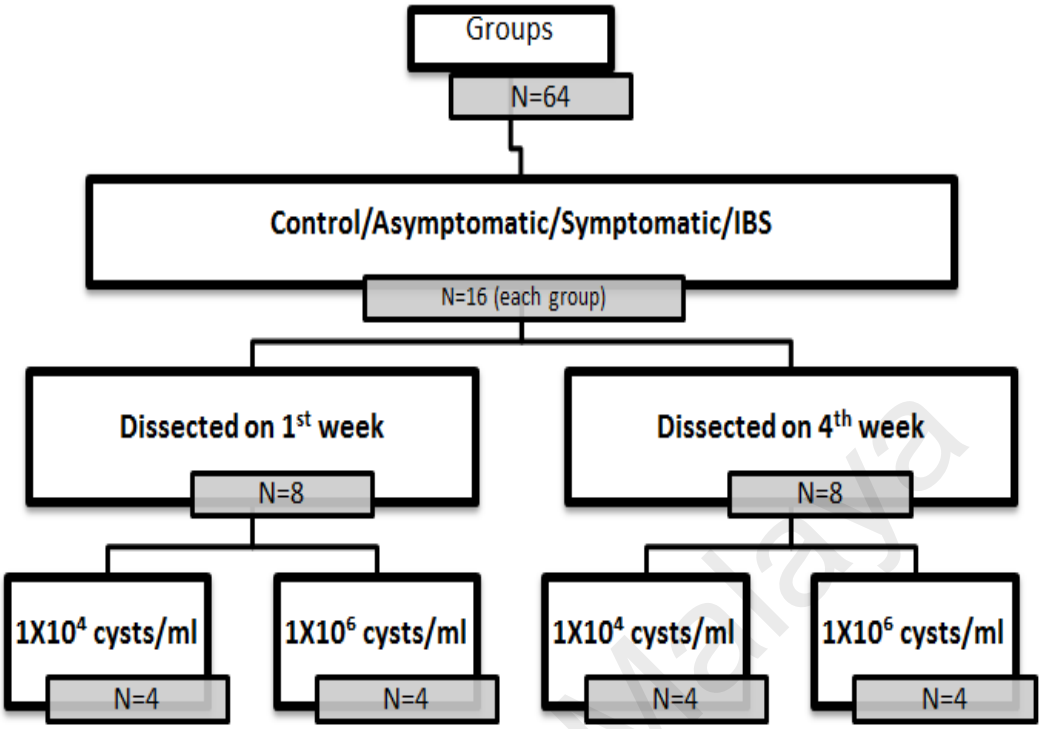

Figure 5.1: Schematic representation of grouping for inoculation of *Blastocystis* sp. ST3 cysts in *Wistar* rats

Table 5.1 Histological score system

Scores	Mucosal sloughing	Inflammation	Necrosis	Percentage of tissue affected
0	None	None	None	0
+1	Mild sloughing	Mild inflammation	Focal Necrosis	<20%
+2	Moderate sloughing	Moderate inflammation	Moderate Necrosis	21%-50%
+3	Extensive sloughing	Extensive inflammation	Extensive Necrosis	>51%

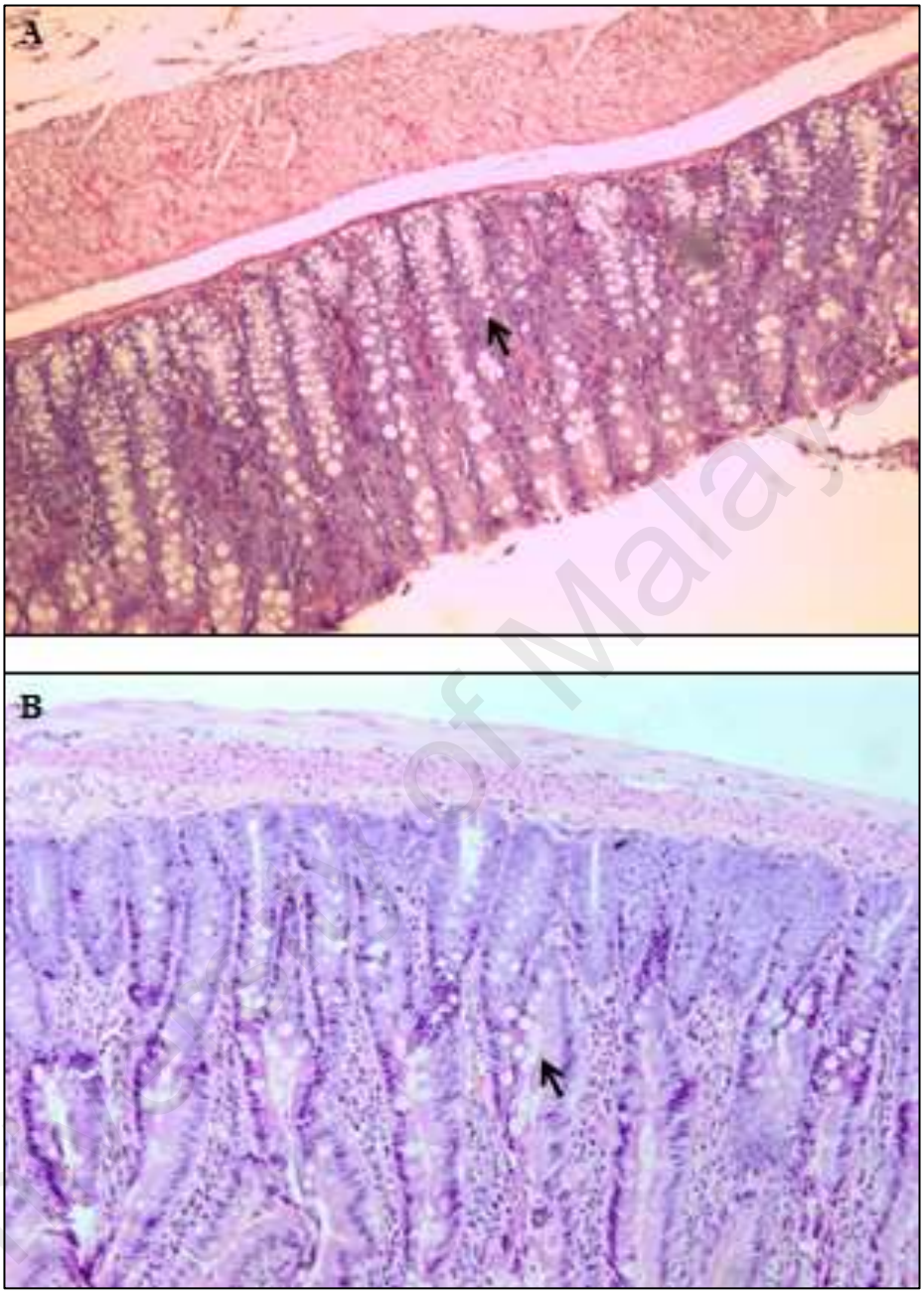

Figure 5.2: Histological score system based on mucosal sloughing H&E tissues. (A) 0: none, H&E (x 10 magnification), (B) +1: mild mucosal sloughing, H&E (x 10 magnification) (see arrows).

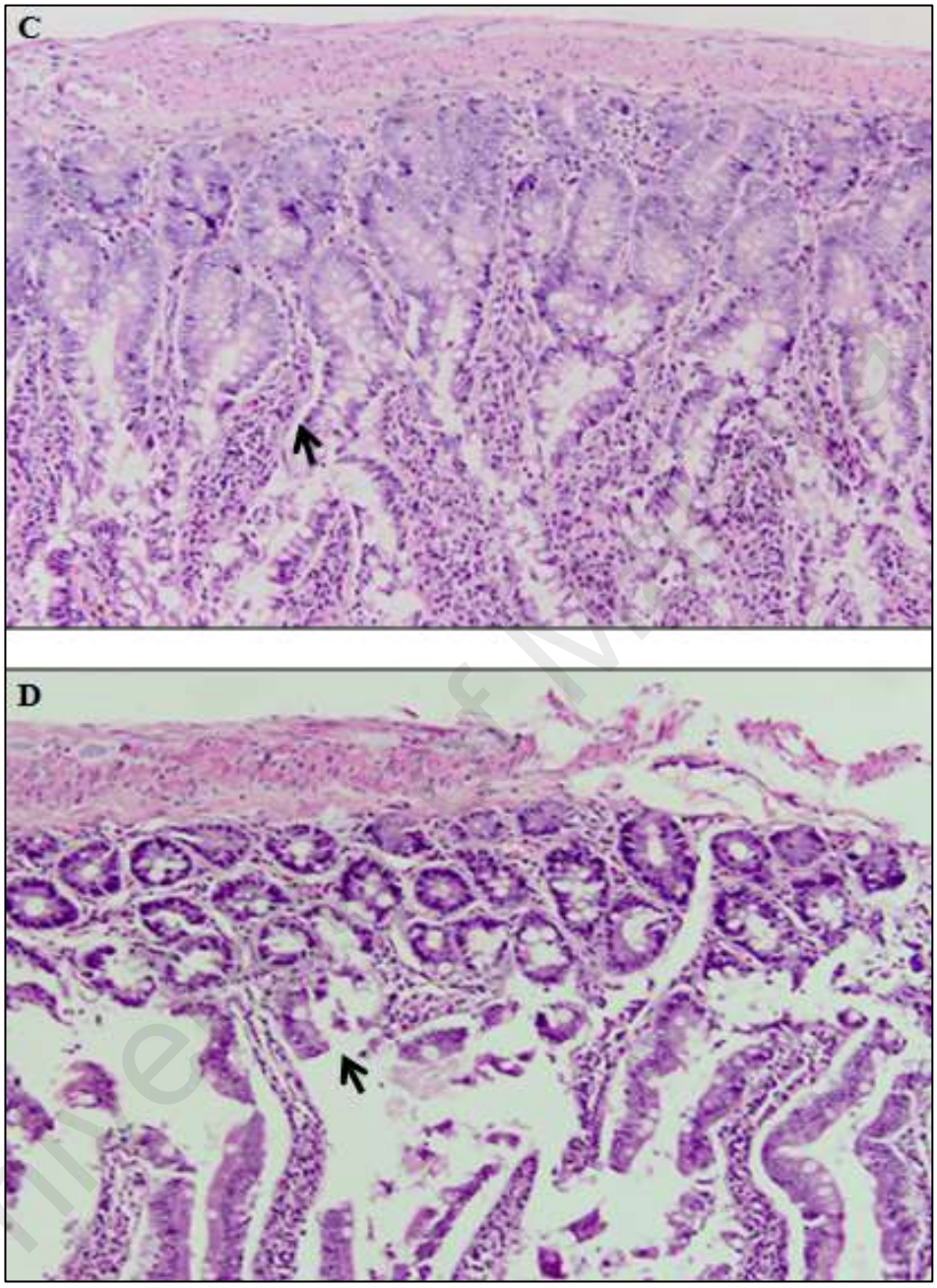

Figure 5.2, continued Histological score system based on mucosal sloughing H&E tissues. (C) +2: moderate mucosal sloughing, H&E (x10 magnification), (D) +3: extensive mucosal sloughing, H&E (x 10 magnification) (see arrows).

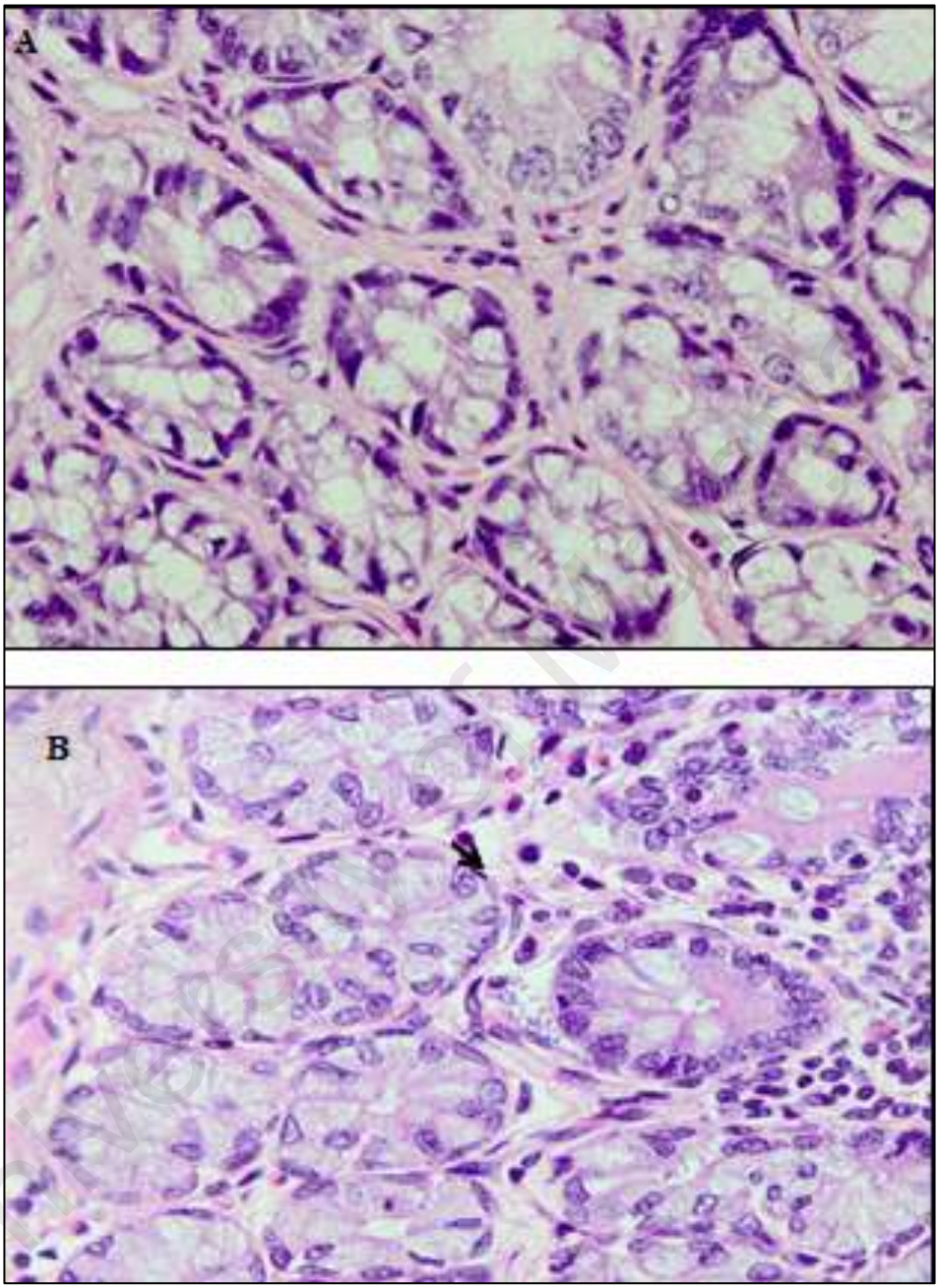

Figure 5.3: Histological score system based on inflammation H&E tissues. (A) 0: none, H&E (x 40 magnification), (B) +1: mild inflammation, H&E (x 40 magnification) (see arrows).

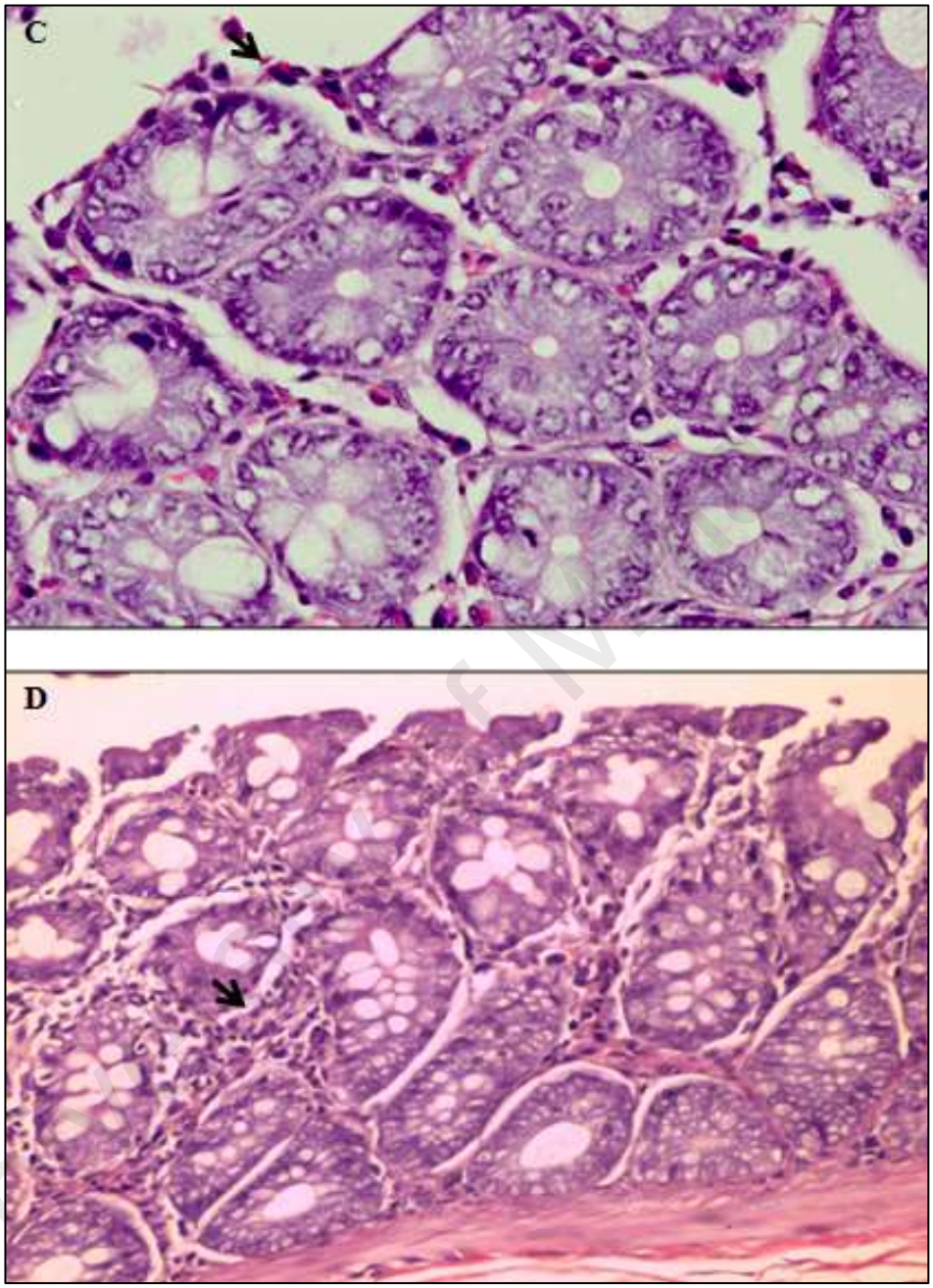

Figure 5.3, continued Histological score system based on inflammation H&E tissues. (C) +2: moderate inflammation, H&E (x 40 magnification), (D) +3: extensive inflammation H&E (x 40 magnification) (see arrows).

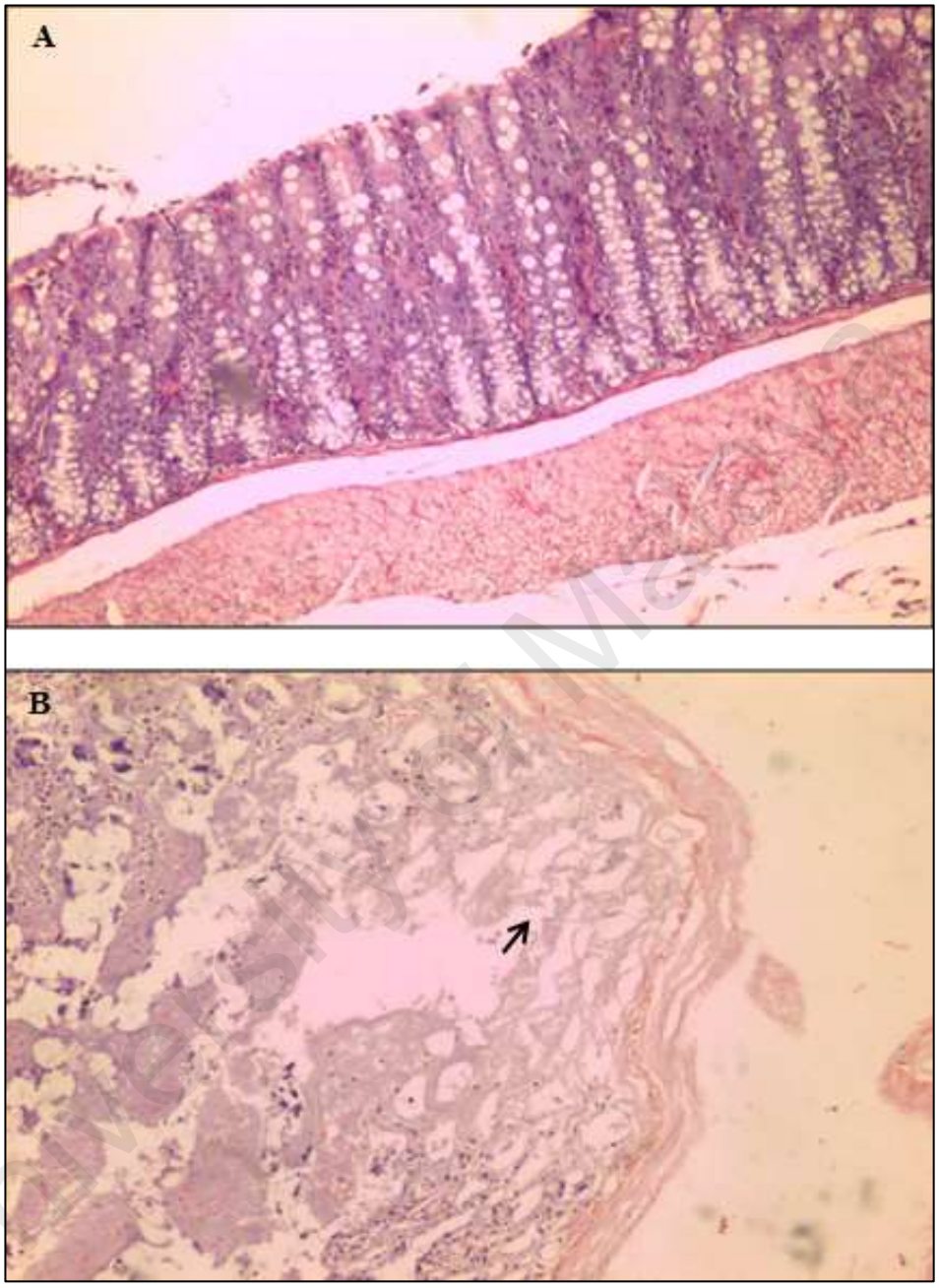

Figure 5.4: Histological score system based on necrosis H&E tissues. **(**A) 0: none, H&E (X 10 magnification), (B) +1: focal necrosis, H&E (x10 magnification) (see arrows).

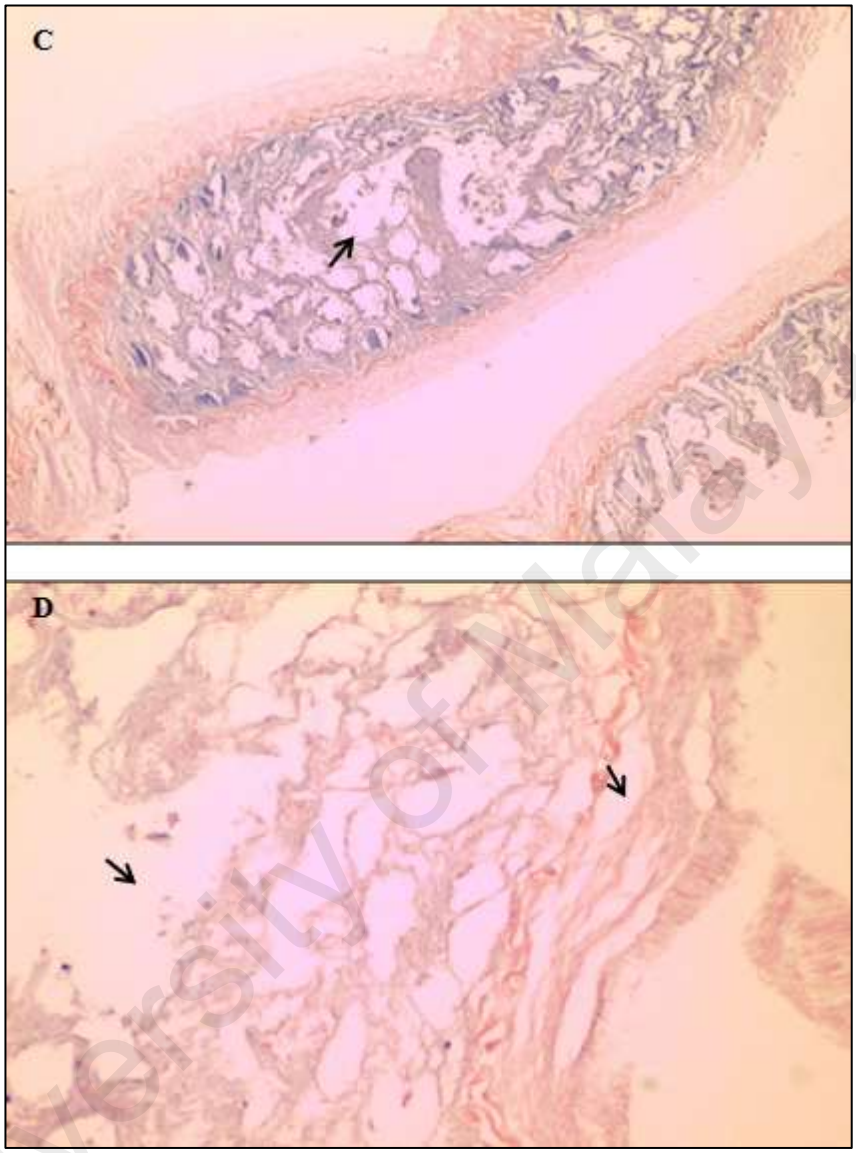

Figure 5.4, continued Histological score system based on necrosis H&E tissues. (C) +2: moderate necrosis, H&E (x10 magnification), (D) +3: extensive necrosis sloughing, H&E (x10 magnification) (see arrows).

5.3 Results

5.3.1 Histopathological sections of intestinal tract

The stools collected from the rats infected with *Blastocystis* sp. ST3 cysts of IBS patients were found to be more soft and watery compared to rats infected with *Blastocystis* sp. cysts isolated from asymptomatic and symptomatic patients. Stool samples from the control rats showed negative results for *Blastocystis* sp. for the entire investigation. The histopathological sections of intestinal tracts from control, asymptomatic, symptomatic and IBS groups were assessed. An intense convolution and reddishness was seen in the intestines of rats inoculated with cysts of *Blastocystis* sp. ST3 from IBS patients (Figure 5.5D) as compared to intestines from control rats, asymptomatic individuals (Figure 5.1B) and symptomatic patients (Figure 5.1C).

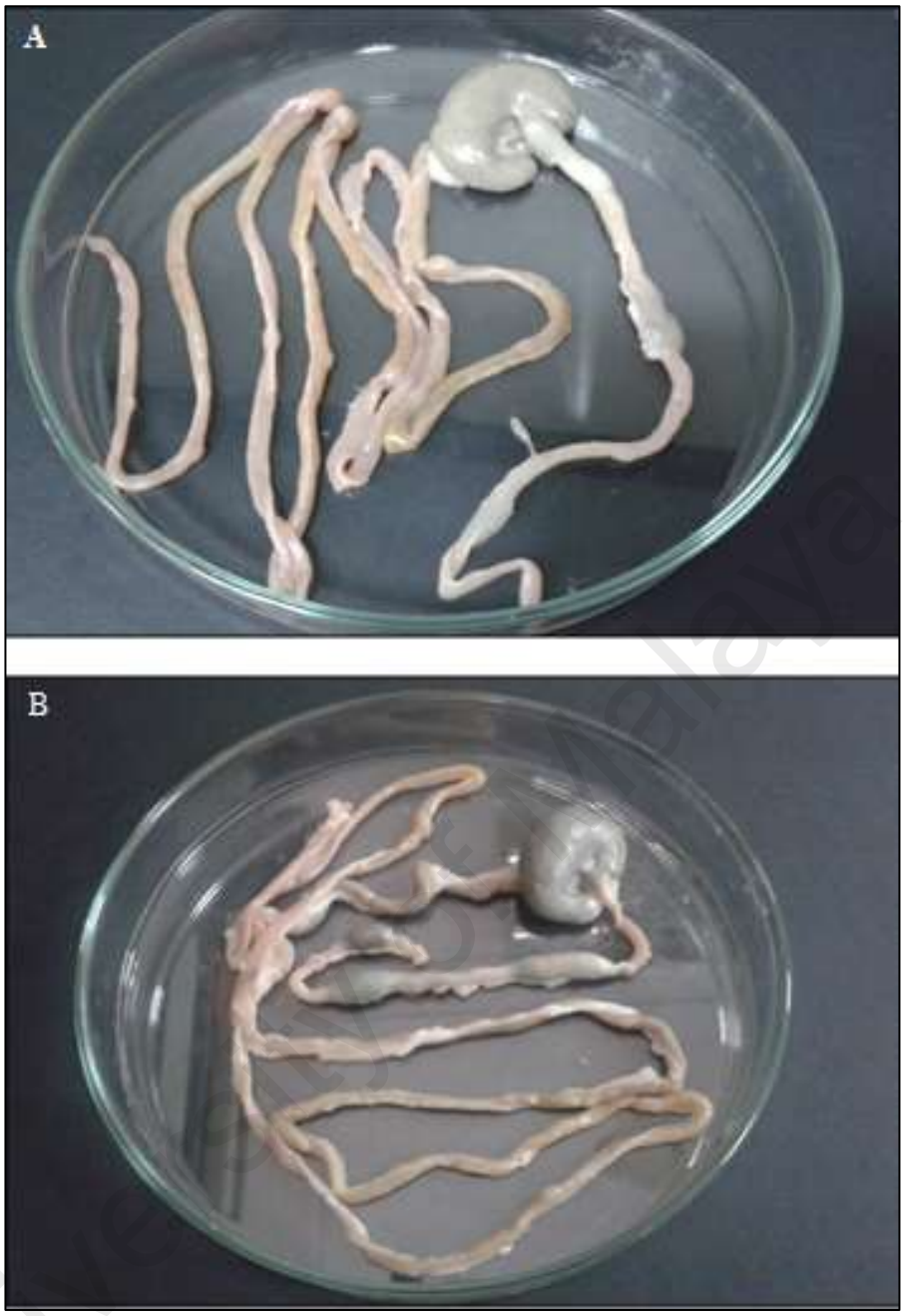

Figure 5.5: **Histopathological sections of intestinal tract**. Intestine of a control rat (A) and absence of convolution and reddishness in the intestines of rats inoculated with cysts of *Blastocystis* sp. ST3 from asymptomatic individuals (B).

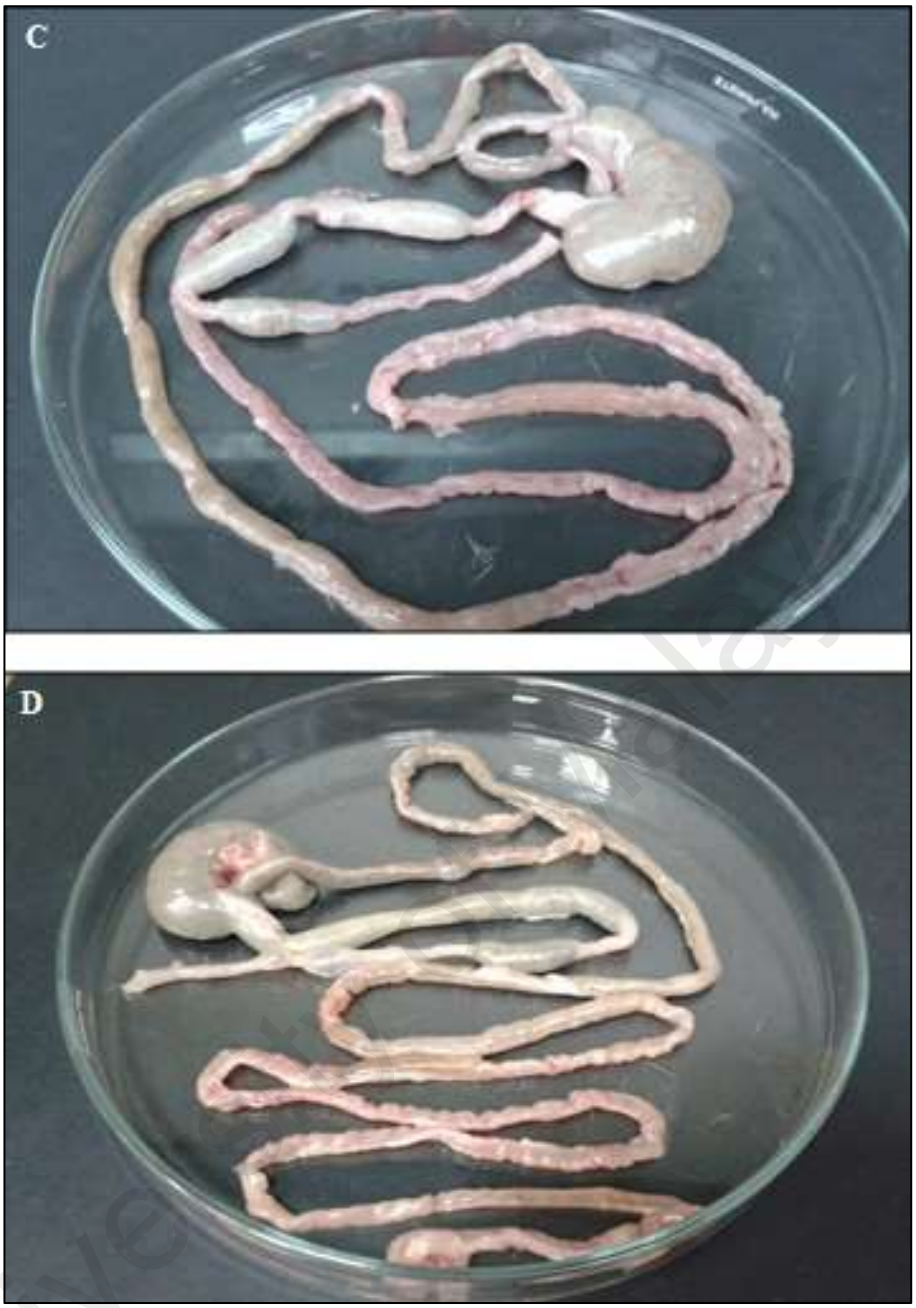

Figure 5.5, continued Histopathological sections of intestinal tract. Absence of convolution and reddishness in the intestines of rats inoculated with cysts of *Blastocystis* sp. ST3 from symptomatic patient (C). Note intense convolution and reddishness seen in the intestines of rats inoculated with cysts of *Blastocystis* sp. from IBS patients (D).

5.3.2 Histopathological changes for mucosal sloughing, inflammation and necrosis of tissue against the number of rats.

In present study, three tables were created to present the results for histopathological changes observed. The first table is on the status of the *Wistar* rats for mucosal sloughing, inflammation and necrosis of tissue (Table 5.2). The number of rats which had the highest score of +3 for mucosal sloughing and necrosis of tissue were recorded. Meanwhile, the highest score for the inflammation of tissue is only +2 in this study (Table 5.2). The second and third table mainly focus on histopathological changes of tissues (ileum, caecum, colon and rectum) on rats inoculated with $1x10^6$ cysts/ml demonstrating extensive mucosal sloughing (+3) and moderate inflammation (+2) at 4^{th} week (Table 5.3 and 5.4)

5.3.2.1 Histopathological changes against the number of rats for mucosal sloughing.

Table 5.2 shows the histopathological changes for mucosal sloughing, inflammation and necrosis of tissue, and the respective tabulations for mucosal sloughing against the number of rats and the severity of such changes measured by the histological score system (Figure 5.2 A-D)

Mucosal sloughing was seen as early as one week after infection. In all three groups infected with $1x10^4$cysts/ml, one rat from each group showed extensive mucosal sloughing of a score of +3. However, on the 4^{th} week, three out of four rats (75%) from asymptomatic and symptomatic groups showed extensive mucosal sloughing (+3) but all four rats from the IBS group (100%) with a score of +3. Meanwhile only one rats

inoculated with $1x10^6$cysts/ml only one (25%) on 1st week, in asymptomatic group and three rats (75%) out of four rats from symptomatic and IBS group were seen to have extensive mucosal sloughing, (+3). For the rats infected with $1x10^6$cysts/ml in the 4th week, two rats (50%) out of four rats from the asymptomatic group showed to have a score of +3 but all four rats from the symptomatic and IBS group (100%) scored +3.

5.3.2.2 Histopathological changes against the number of rats for inflammation.

Table 5.2 shows the histopathological changes for mucosal sloughing, inflammation and necrosis of tissue and the respective tabulations for inflammation against the number of rats and the severity of such changes measured by the histological score system (Figure 5.3 A-D)

Inflammation was also seen as early as one week after infection. In all three groups infected with $1x10^4$cysts/ml, two rats (50%) from asymptomatic group showed moderate inflammation of a score of +2 but all four rats from the symptomatic and IBS group (100%) scored +2. However, on the 4^{th} week, three out of four rats from asymptomatic and symptomatic (75%) showed moderate inflammation (+2) but all four rats from the IBS group (100%) scored +2. In rats inoculated with $1x10^6$cysts/ml, two rats (50%) on 1^{st} week, in asymptomatic group and three out of four (75%) from symptomatic scored +2; but all four rats from the IBS group (100%) scored +2. For the rats infected with $1x10^6$cysts/ml at the 4^{th} week, three rats out of four rats (75%) from the asymptomatic group showed to have a score of +2 but all four rats from the symptomatic and IBS group (100%) scored +2.

5.3.2.3 Histopathological changes against the number of rats for necrosis of tissue.

Table 5.2 shows the histopathological changes for mucosal sloughing, inflammation and necrosis of tissue and the respective tabulations for necrosis of tissue against the number of rats and the severity of such changes measured by the histological score system (Figure 5.4 A-D)

Only one out of four rats (25%) from the IBS group inoculated with $1x10^6$cysts/ml and dissected on the 4^{th} week showed to have extensive necrosis, (+3) of ileum tissue but no rats infected with cysts from IBS patients showed necrosis compared with rats inoculated with cysts derived from asymptomatic and symptomatic patients (Table 5.2).

Table 5.2: Pathological status of the rats for mucosal sloughing, inflammation and necrosis of tissue

Groups	Week	Concentration (cysts/ml)	n	Number of rats, n (%)		
				Mucosal sloughing (3+)	Inflammation (2+)	Necrosis (3+)
Asymptomatic	1st	$1x10^4$	4	1 (25)	2 (50)	0 (0)
	4th	$1x10^4$	4	3 (75)	3 (75)	0 (0)
	1st	$1x10^6$	4	1 (25)	2 (50)	0 (0)
	4th	$1x10^6$	4	4 (100)	3 (75)	0 (0)
Symptomatic	1st	$1x10^4$	4	1 (25)	4 (100)	0 (0)
	4th	$1x10^4$	4	3 (75)	3 (75)	0 (0)
	1st	$1x10^6$	4	3 (75)	3 (75)	0 (0)
	4th	$1x10^6$	4	2 (50)	4 (100)	0 (0)
IBS	1st	$1x10^4$	4	1 (25)	4 (100)	0 (0)
	4th	$1x10^4$	4	4 (100)	4 (100)	0 (0)
	1st	$1x10^6$	4	3 (75)	4 (100)	0 (0)
	4th	$1x10^6$	4	4 (100)	4 (100)	1 (25)

5.3.3 Histopathological changes for mucosal sloughing and inflammation in small (ileum) and large intestine (caecum, colon, and rectum).

5.3.3.1 Histopathological changes for mucosal sloughing in small (ileum) and large intestine (caecum, colon, and rectum)

The severity of mucosal sloughing (+3) in intestine was then compared based on the small (ileum) and large intestine (caecum, colon, and rectum). In all three groups infected with $1x10^6$cysts/ml on 4^{th} week, ileum of all four rats (100%) from asymptomatic group scored +3. Figure 5.6 A shows the extensive mucosal sloughing ileum of rat's inoculated with cysts from asymptomatic individual. *Blastocystis* like-organisms were seen in figure 5.6 A and figure 5.6 B shows a higher magnification of numerous *Blastocystis* like-organisms seen in the ileum. The *Blastocystis* like-organisms were small and vacuolated. Meanwhile, only ileum of two rats from the symptomatic and IBS group (50%) scored +3.

Extensive mucosal sloughing was seen in caecum, colon, and rectum in rats infected with cysts from IBS patients (Figure 5.6 C-E) compared with rats inoculated with cysts obtained from asymptomatic individual and symptomatic patient. Caecum and colon of one rat (25%) and two rats out of four rats (50%) from symptomatic and IBS group respectively showed extensive mucosal sloughing, +3. Of all three groups, only rectum of one rat (25%) from IBS group scored +3 (Table 5.3).

Table 5.3: Pathological status of rats inoculated with $1x10^6$ *Blastocystis* cysts/ml in small (ileum) and large intestine (caecum, colon, and rectum) demonstrating extensive mucosal sloughing (+3) at 4^{th} week

Tissues	n	Number of Rats, n (%)		
		Asymptomatic	Symptomatic	IBS
Ileum	4	4 (100)	2 (50)	2 (50)
Caecum	4	0 (0)	1 (25)	2 (50)
Colon	4	0 (0)	1 (25)	2 (50)
Rectum	4	0 (0)	0 (0)	1 (25)

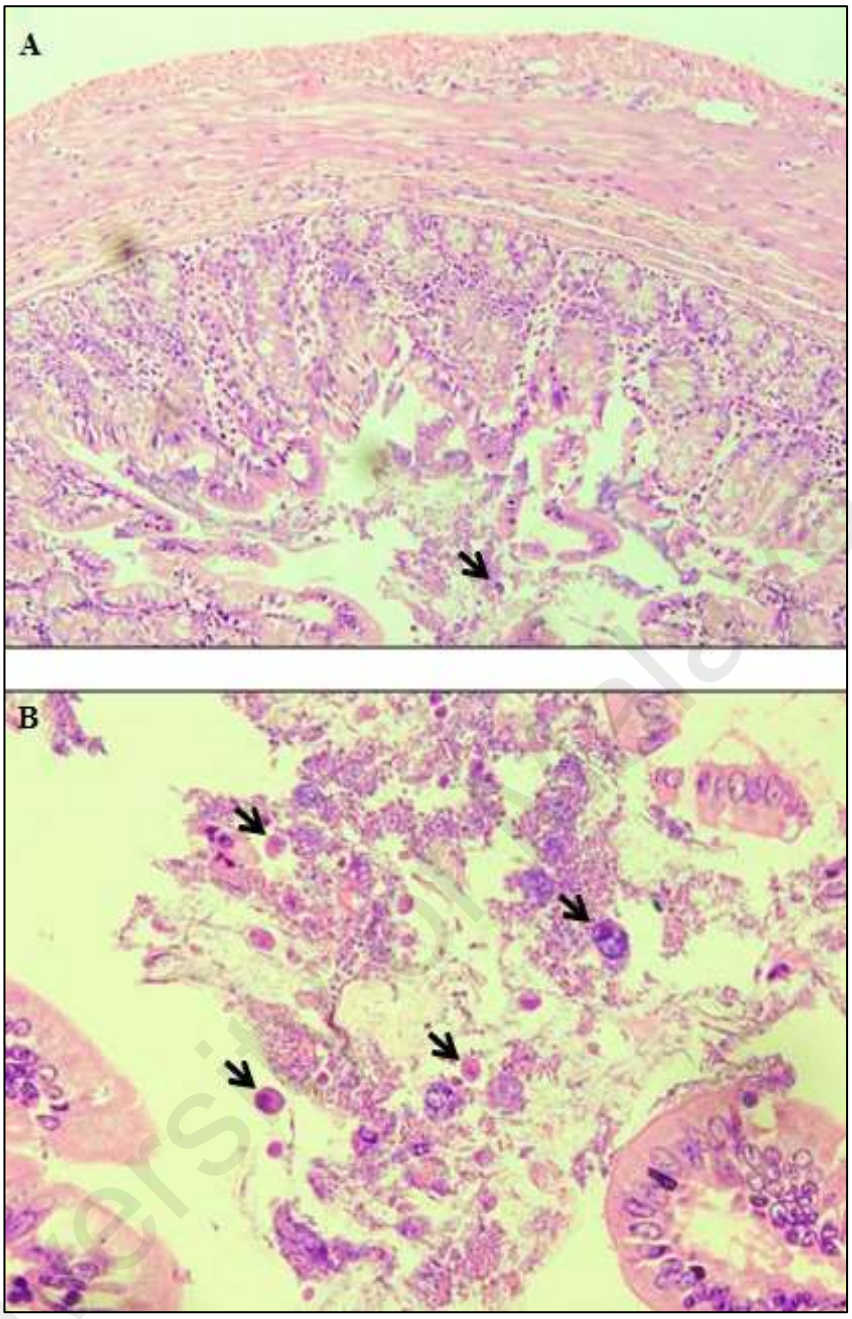

Figure 5.6: Histological H&E staining for mucosal sloughing of tissues

(A) Sloughing of the mucosal epithelium shed into the lumen. *Blastocystis* sp. like-organism in ileum tissue of rat inoculated with *Blastocystis* sp. ST3 cysts from asymptomatic individual was seen (arrow) (x10 magnification). (B) Higher magnification, showing more details of the *Blastocystis* sp. like-organism, and numerous *Blastocystis* sp. like-organisms were also seen (x40 magnification)

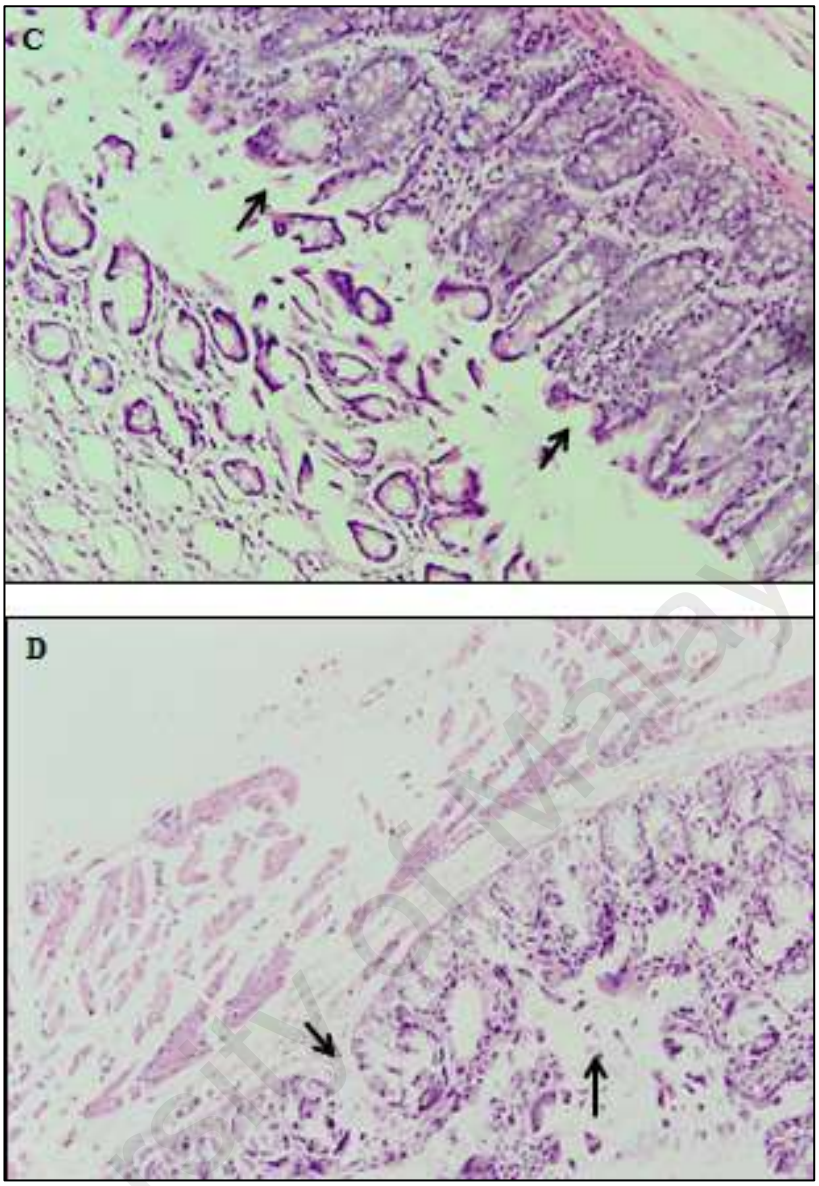

Figure 5.6, continued Representative histological H&E staining for mucosal sloughing of tissues

(C) Extensive mucosal sloughing of colon (C) (x10 magnification) and (D) sloughing of the mucosal epithelium of the villus of rectum (x10 magnification) was seen in rats inoculated with cysts from IBS patients.

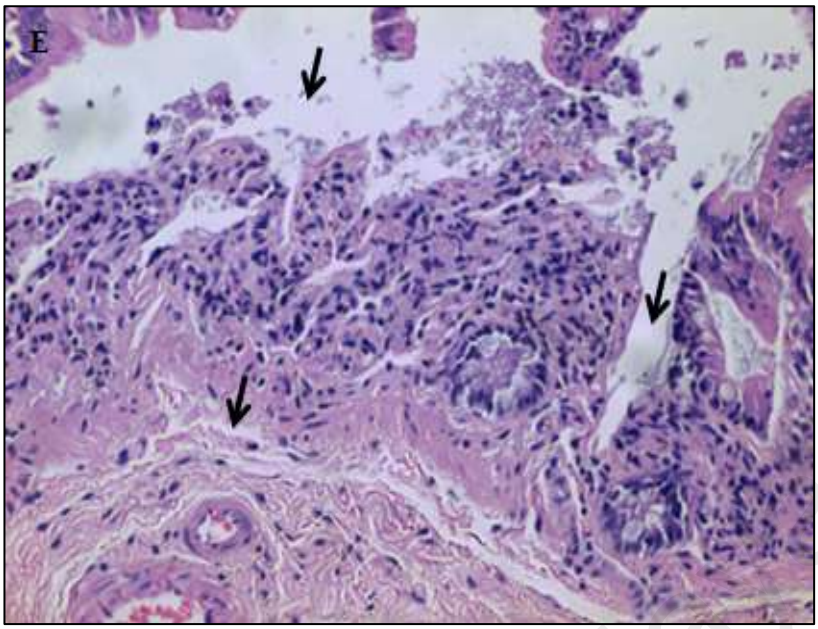

Figure 5.6, continued Representative histological H&E staining for mucosal sloughing of tissues

(E) Extensive mucosal sloughing of caecum (x10 magnification) (see arrows).

5.3.3.2 Histopathological changes for inflammation in small (ileum) and large intestine (caecum, colon, and rectum)

For the asymptomatic group, ileums from three rats (75%) were seen to have moderate inflammation, (+2) whilst no inflammation was seen for caecum, colon and rectum. Ileum from four rats (100%) and one rat (25%) out of four rats for the colon tissue have moderate inflammation, (+2) but no inflammation was seen in the caecum and rectum for the symptomatic group. However, inflammation was seen in the ileum of three rats (75%), with one rat (25%) showing inflammation in caecum, colon and rectum (moderate inflammation, +2) for the rats inoculated with cysts from IBS patients (Figure 5.7 A and B) (Table 5.4). A dense lymphocytic infiltrate in the rectum was seen in rats inoculated with cysts from IBS patients (Figure 5.7 B).

Table 5.4: Pathological status of rats inoculated with $1x10^6$ *Blastocystis* cysts/ml in small (ileum) and large intestine (caecum, colon, and rectum) demonstrating moderate inflammation (+2) at 4^{th} week.

Tissues	N	Number of Rats, n (%)		
		Asymptomatic	Symptomatic	IBS
Ileum	4	3 (75)	4 (100)	3 (75)
Caecum	4	0 (0)	0 (0)	1 (25)
Colon	4	0 (0)	1 (25)	1 (25)
Rectum	4	0 (0)	0 (0)	1 (25)

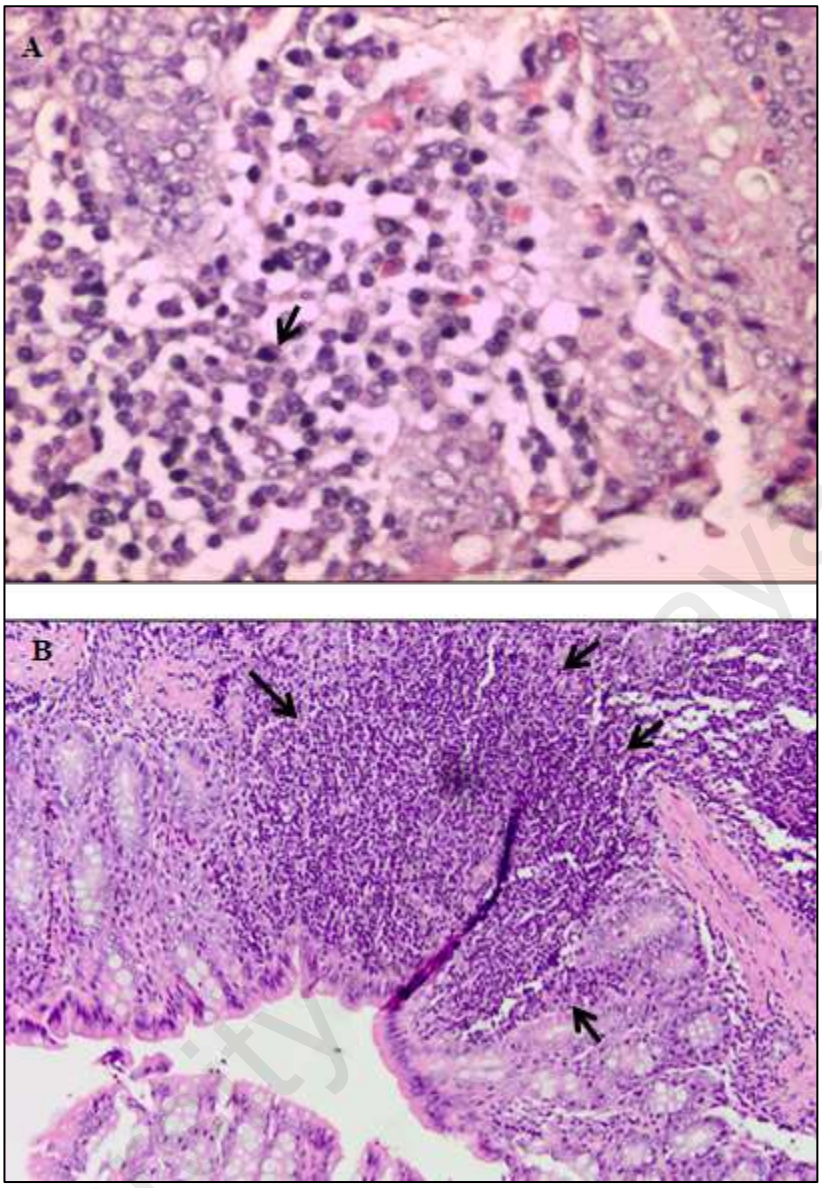

Figure 5.7: Representative histological H&E staining for inflammation of tissue

(A) Oral submucosa with inflammatory infiltrate (blue/purple) (x40 magnification) and (B) dense lymphocytic infiltrate was seen in the rectum of seen in colon of rats infected with cysts from IBS patients (x10 magnification).

5.3.3.3 Histopathological changes for necrosis in small (ileum) and large intestine (caecum, colon, and rectum)

As mentioned previously in 5.3.2.3, only one rat (25%) inoculated with $1x10^6$ *Blastocystis* cysts/ml derived from IBS patients showed extensive necrosis, (+3) of ileum tissue on the 4th week and was not seen in other tissues (caecum, colon, rectum) (Figure 5.8 A). Extensive mucosal sloughing and inflammation of ileum tissue was also seen in the same rat (Figure 5.8 B and C).

Moreover, none of the rats from asymptomatic and symptomatic showed necrosis of tissues (ileum, caecum, colon and rectum) at 4th week post-inoculation (Table 5.5).

Table 5.5: Pathological status of rats inoculated with $1x10^6$ *Blastocystis* cysts/ml in small (ileum) and large intestine (caecum, colon, and rectum) demonstrating necrosis of tissue (+3) at 4^{th} week.

Tissues	n	Number of Rats, n (%)		
		Asymptomatic	Symptomatic	IBS
Ileum	4	0 (0)	0 (0)	1 (25)
Caecum	4	0 (0)	0 (0)	0 (0)
Colon	4	0 (0)	0 (0)	0 (0)
Rectum	4	0 (0)	0 (0)	0 (0)

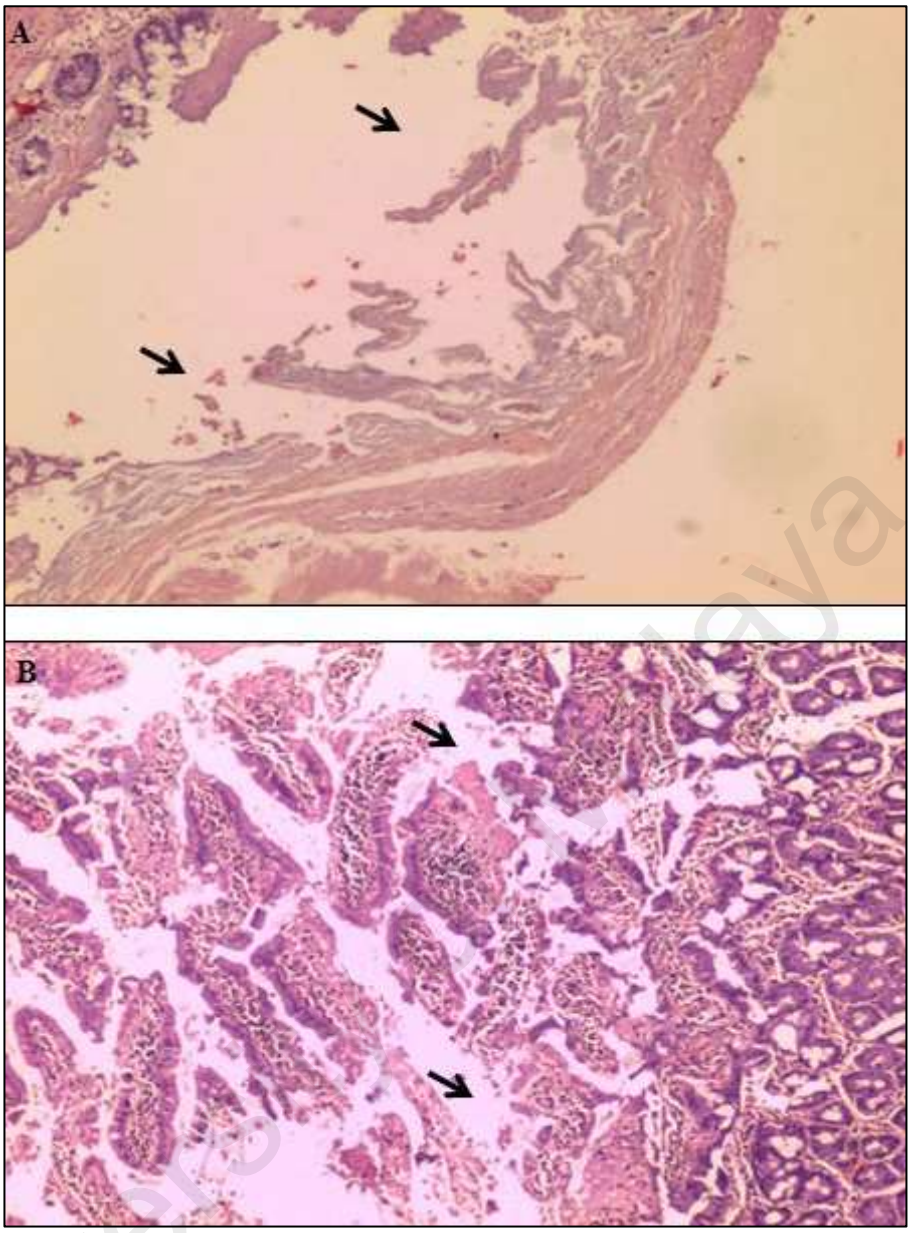

Figure 5.8: Representative histological H&E staining of necrosis of tissue

(A) Extensive necrosis of mucosa and mucosa and muscularis mucosa was seen in the rat inoculated with *Blastocystis* cysts from IBS patients (x10 magnification). (B) Extensive mucosal sloughing of ileum with inflammation was seen in the same rat (x10 magnification).

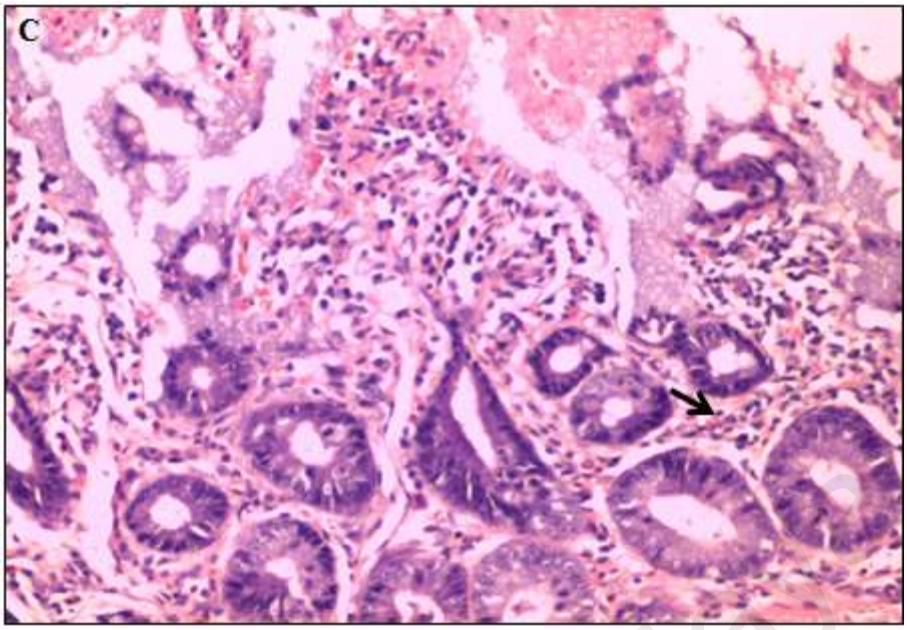

Figure 5.8, continued Representative histological H&E staining of necrosis of tissue

(C) Higher magnification, showing more details of the infiltration of the lamina propia with inflammatory cells in the same rat inoculated with *Blastocystis* cysts from IBS patient which demonstrate the extensive necrosis and mucosal sloughing of tissue (X10 magnification)

5.4 Discussion

Recent studies carried out showed that inflammation in the IBS gut does play a role in contributing to the gastrointestinal symptoms. A study done by Hilmi, et al. (2013) provided evidence on the biopsies of colonic inflammation based on the microscopic colitis which was a histopathological diagnosis for IBS-diarrhea patients. Changes of bacterial flora in the IBS gut, alteration in gut motility, and visceral hypersensitivity have been suggested to be possible mechanisms for IBS (Barbara et al., 2004; Talley et al., 2006; Barbara et al., 2011). Intestinal inflammation does play an important role in clinical manifestation in IBS patients (Gwee, 2005). Intestinal parasites, especially *Blastocystis* sp. are also known to cause mucosal inflammation which can contribute to the gastrointestinal symptoms (Ustün & Turgay, 2006). Studies have shown that *Blastocystis* sp. infection coupled with intestinal disorder could further cause effect in contributing to the GI symptoms (Chandramathi et al., 2012). Thus far, there are limited animal models to study the infectivity of *Blastocystis* sp. cysts. The parasite's mode of transmission was suggested to be by the fecal oral route (Moe et al., 1997). Yoshikawa et al. (2004) reported that 10 cysts were sufficient to cause an infection in an experimental rat. Hence in this study *Wistar* rats were successfully infected with *Blastocystis* sp. cysts isolated from asymptomatic individuals, symptomatic and IBS patients.

This is the first study carried out to elucidate histopathological aspects of the rats inoculated with *Blastocystis* sp. ST3 cysts from three different groups i.e. asymptomatic, symptomatic and IBS. In the present study, the appearance of the histopathological features seen in the intestines of rats inoculated with *Blastocystis* cysts from IBS patients were different compared to rats inoculated with cysts from

asymptomatic individual and symptomatic patient. Rats inoculated with cysts isolated from IBS patient showed the intestines to be highly convoluted and reddish compared to that seen in the control, asymptomatic and symptomatic groups of rats. This present study also showed that the size of the inoculum ($1x10^4$cysts/ml and $1x10^6$cysts/ml) and the duration of infection (week 1 to 4) do influence the histopathological changes such as mucosal sloughing, inflammation and necrosis of tissue (Table 5.2). The numbers of rats demonstrating extensive mucosal sloughing (+3) for all three groups dissected on 4^{th} week was seen to be higher in rats inoculated with $1x10^4$cysts/ml compared to seen on the 1^{st} week. However, the number of rats inoculated with $1x10^6$cysts/ml for symptomatic group was lower at 4^{th} week compared to 1^{st} week but for asymptomatic and IBS group, the number of rats with extensive mucosal sloughing (+3) was higher in 4^{th} week compared to 1^{st} week. Rats inoculated with $1x10^4$cysts/ml with the highest score +2, moderate inflammation showed an increase from 1^{st} week to 4^{th} week in asymptomatic group but decreased eventually from 1^{st} week to 4^{th} week for the symptomatic group. However, the number of rats demonstrating a moderate inflammation was seen to increase from 1^{st} to 4^{th} week for the asymptomatic and symptomatic rats inoculated with $1x10^6$cysts/ml. In the IBS group, all the rats demonstrated moderate inflammation, (+2).

The results revealed that a longer exposure of *Blastocystis* sp. infection in the intestinal gut could possibly cause a greater mucosal sloughing over time. The detrimental effect of cysts towards the intestinal gut could be seen more in the 4^{th} week after infection. The rats inoculated with cysts from the IBS patients showed a comparatively greater effect of mucosal sloughing and inflammation compared to other groups, asymptomatic and symptomatic.

Examination of the tissues stained with H&E revealed that both mucosal sloughing and inflammation were seen to be the highest in the ileum compared to caecum, colon and rectum, with the greatest severity seen in rats from the asymptomatic group. At the 4th week, all four rats (100%) inoculated with $1x10^6$cysts/ml from asymptomatic and IBS group showed extensive mucosal sloughing, +3. This suggests that excystation of cysts and the higher rate of multiplication of vacuolar forms in the intestine might have caused this consequence seen in the ileum even the rats from asymptomatic group. The findings correlate with a previous study which showed that asymptomatic isolates had the highest growth rate in cultures when compared to symptomatic (Tan et al., 2008). The finding concurred with the findings shown in chapter 5 of the present study where asymptomatic isolates had the highest growth compared to the symptomatic and IBS isolates (Chapter 5). Hence the extensive sloughing seen in rats infected with cysts from asymptomatic isolates must be due to the effects exerted by the highly proliferative forms from this group of cysts. The extent of mucosal sloughing and inflammation was seen to be more pronounced in caecum, colon and rectum in rats infected with cysts from IBS patients compared to cysts from the symptomatic and asymptomatic patients. Furthermore, extensive necrosis was seen in one rat inoculated with $1x10^6$cysts/ml, dissected on the 4th week, from the IBS group, and was also seen to have extensive mucosal sloughing. This was not seen in the other asymptomatic and symptomatic groups. This probably explains why IBS patients harboring *Blastocystis* sp. show greater symptoms than the symptomatic and asymptomatic patients.

The *Blastocystis* sp. ST3 derived from IBS patients could be more pathogenic resulting in different histopathological changes compared to asymptomatic and symptomatic groups despite being from the same subtype, ST3. This study concurs with

a previous finding in chapter 4, that there is a variation within the same subtype, ST3 depending on the host individual and the gut environment. Apart from that, our study showed that the cysts of *Blastocystis* sp. ST3 can infect the *Wistar* rats via oral inoculation suggesting that they can be used as experimental animal models to study the pathogenic potential in comparing the host individual variation.

Mucosal sloughing in intestine will cause destruction to the surface area resulting in poor absorption, which in turn will cause digestive problems. Higher numbers of inflammation markers and mast cells were seen in patients suffering from IBS (O'sullivan et al., 2000; Guilarte et al., 2007; Piche et al., 2008; Cremon et al., 2009). Another study done in Sri Lanka reported that IBS patients have been shown to have low grade inflammation and higher eosinophils with other chronic inflammatory cells (De Silva et al., 2012). Protease from *Blastocystis* sp. has also been associated with the pathogenicity of the parasite (Sio et al., 2006; Abdel-Hameed & Hassanin, 2011) in which immunoglobulin A (IgA) have been shown to be degraded. Proteases have been attributed to contribute to the virulence factor which facilitates the colonization of the gut, invoking an immune response and causing the disruption of barrier function (Puthia et al., 2005; Puthia et al., 2008; Mirza & Tan, 2009). A study done previously by Abdel-Hameed & Hassanin (2011) showed that *Blastocystis* sp. ST3 derived from symptomatic patients possesses protease activity at 32kDa which has been identified to contribute to the pathogenicity of the parasite. In the present study the protease from *Blastocystis* sp. ST3 from the IBS patient has been shown to play an important role in the pathogenicity of the parasite which results in the histopathological changes seen for the mucosal sloughing, inflammation and necrosis.

5.5 Conclusion

This is the first study to provide evidence that the occurrence of mucosal sloughing, inflammation and necrosis was higher in rats infected with *Blastocystis* cysts isolated from IBS group compared to symptomatic and asymptomatic groups. This study supports the earlier findings that intestinal inflammation does play an important role in the clinical manifestation of IBS patient. Studies previously carried out showed that long term inflammation could lead to cancer especially colorectal cancer (Husain, 2007). Further studies need to investigate the interaction between the host's intestine and parasite and what causes the mucosal sloughing, inflammation and necrosis to occur. This study also justifies further that parasites in adapting to the gut environment could exert different pathologic effects.

Chapter 6: Molecular studies to assess the effects of *Blastocystis* sp. ST3 antigens derived from non-IBS and IBS patients on the growth of colon cancer cells.

6.1 Introduction

6.1.1 Inflammation and *Blastocystis* sp.

There are studies that have associated *Blastocystis* sp. and inflammation in gnotobiotic guinea pigs which showed invasion and mucosal inflammation of the intestine (Zierdt, 1991). Previous reports have suggested a link to persistent bowel dysfunction, which was associated with extensive penetration of *Blastocystis* sp. causing greater mucosal inflammation (Carrascosa et al., 1996; Ghosh et al., 1998). An endoscopic report showed the presence of inflammation in the colon due to *Blastocystis* sp. infection (Gallagher & Venglarcik, 1985). Inflammatory disorders due to gut infections can also disrupt the gut sensorimotor function and further increase sensory perception (Collins, 1996). Long et al. (2001) had postulated that inflammatory cytokines have the ability to influence human epithelial cells in the presence of *Blastocystis* sp. which could further downregulate the immune system.

Blastocystis sp. was also shown to trigger the cytopathic and cellular immune responses to occur in which interleukin-6 (IL-6) and interleukin-8 (IL-8) was upregulated. There was downregulation of tumor necrosis factor-alpha (TNF- α) were seen after the peripheral blood mononuclear cells (PBMCs) were exposed to solubilized antigens of *Blastocystis* sp. culture filtrate (Blasto-Ag) (Chandramathi et al., 2010).

The proteases from *Blastocystis* sp. were shown to have the ability to degrade immunoglobulin A (Ig A) and disrupt the intestinal barrier function (Puthia et al., 2005, Puthia et al., 2008; Mirza & Tan, 2009; Abdel-Hameed & Hassanin, 2011). *Blastocystis* sp.'s ability to colonize the IBS patient's gut competently was shown to better than a normal individual's gut (Clark et al., 2013).

6.1.2 Inflammation in *Blastocystis* sp. infected IBS patients

Ultrastructural studies of *Blastocystis* sp. ST3 isolated from IBS patients (Chapter 4) showed that the organism possesses a thicker surface coat with coarse and uneven surface which could influence the cytopathic effect of this parasite towards the intestinal lining of the gut. These results correspond to the high FITC-Con A binding showed by the parasite isolated from IBS patients as well the aggregation and clumping that was seen in stained smears which clearly showed that this parasite has a sticky surface. This explains the adherence of *Blastocystis* sp. towards the intestinal wall due to the sticky surface which can further exacerbate the gut inflammation.

It was also evident from the *in vivo* experiments conducted in chapter 5 that *Blastocystis* sp. ST3 cysts derived from IBS patients inoculated in *Wistar* rats able to cause a greater mucosal sloughing, inflammation and necrosis of tissue compared to cysts from asymptomatic individuals and symptomatic patients. Therefore, such a scenario is expected to be seen in IBS patients as there are reports that intestinal inflammation due to *Blastocystis* sp. infection could play a role in contributing to the gastrointestinal symptoms. The question that arises here is whether prolonged mucosal inflammation caused by *Blastocystis* sp. in an IBS patient could lead to colon cancer. To answer this, the study further investigated the pathogenicity of *Blastocystis* sp. ST3

antigens (Blasto-Ag ST3) towards colon cancer cells, HCT116, in comparison with normal cells, CCD-18Co, and their involvement in Wnt signaling pathway.

6.1.3 Inflammation and Wnt signaling pathway

During a gut infection, innate immunity is activated immediately to protect the host cells from intrusion and infection. The first response of the immune system is the inflammation. Generally, mediators of inflammation can cause modification of genes which include the mutation of tumor suppressor genes, changes in gene expression and trigger posttranslational alterations leading to cancer (Hussain & Harris, 2007). Different signaling transduction pathways regulate the expression of various genes that are responsible for eliminating any invading microorganisms and regulate inflammation. Any failure in the precise control of inflammatory response can lead to uncontrolled inflammation, generating a pathologically conducive microenvironment that may favour the initiation and progression of cancer (Hussain & Harris, 2007). Out of many other signaling pathways activated, the Wnt signaling pathway is very much associated with colon cancer. The Wnt signaling pathway has become known as one of the essential regulators of inflammation and plays a role in the etiology of colon cancer.

6.1.4 Wnt signaling pathway

The Wnt signaling pathway was first known for its role in carcinogenesis, but later recognized for its role in embryonic development (Moon et al., 1997; Wodarz & Nusse, 1998). It is evolutionary conversed and plays a major role in the progression of many other organ systems (Mikesch et al., 2007). During normal development process, the Wnt ligands which are also known as secreted glycoprotein play an important role in cell proliferation, patterning, and fate determination (Lee et al., 2004). Abnormal activation of Wnt signaling has been shown in many other human developmental disorders (Moon et al., 2004) and in cancerous growth of the colon, skin, plasma, brain and prostate (Polakis, 2000; Moon et al., 2004; Reya & Clevers, 2005; Clevers et al., 2006); importantly activation of Wnt signaling pathway is observed in more than 90% of colon cancers (Giles et al., 2003). It has many elements influencing the pleiotropic and excess effects, including ligands, receptors, co-receptors, and inhibitors (Velasco et al., 2010) (Table 6.1). There are 19 Wnt ligands which bind to a membrane receptor complex formed by a frizzled protein (FZD) and two lipoprotein receptors related proteins (LRPs), LRP5 and LRP6 (Miller, 2002).

The signaling pathway can be divided into two pathways which are the canonical pathway and non-canonical pathway. The non-canonical pathway can be further divided into two which are the non-canonical planar cell polarity pathway and the non-canonical calcium pathway.

Table 6.1 Elements of Wnt signaling pathway

Types	Examples
Wnt ligands	WNT1, WNT2, WNT2B, WNT3, WNT3A, WNT4, WNT5, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A, WNT10A,WNT11, WNT16
Wnt receptors/co-receptors	LRP5, LRP6, frizzled (FZD) 1-8
Wnt binding proteins	SFRP1, SFRP4,WIF1
Cytoplasmic proteins	DVL, GSK3β, β-catenin
Nuclear transcription factors and co-factors	BCL9, BTRC, TCF7L1, TLEs, EP300
*Target genes	FOSL1/FRA1, FZD7, c-Myc, N-Myc, cyclin D1

*Transcription of target genes is activated by the β catenin-TCF complex in the nucleus

6.1.4.1 Canonical pathway

The canonical Wnt pathway is determined when the Wnt pathway triggers the accumulation of β-catenin in the cytosol and influences the transcription of TCF/LEF target genes (Clevers, 2006). Absence of binding of Wnt ligands to frizzled receptors will result in degradation of β-catenin (Figure 6.2 A). The canonical pathway is activated with the activation of Disheveled (Dvl), resulting in the phosphorylation of Dvl. The Dvl then disrupts a β-catenin destruction complex which consists of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase GSK3-β which degrades β-catenin through phosphorylation (Davis et al., 2008). The binding between Wnts, frizzled and LRP5/6 allows β-catenin to stabilize and accumulate in the cytosol and nucleus (Lee et al., 2008). The β-catenin/T-cell factor (TCF) complex will then initiate the regulation of gene transcription which is responsible for cell proliferation and differentiation (Giles et al., 2003; Taketo, 2004). This influences the expression of Wnt target genes like c-Myc, N-Myc, and cyclin D1, which has been found to be a triggering factor for cancer progression in human (Figure 6.2 B). Accumulation of β catenin occurs and enters the nucleus to displace the Groucho gene from TCF/LEF. β catenin will then bind with TCF to form β-catenin/T-cell factor (TCF) complex to activate the transcription of target genes (Figure 6.1)

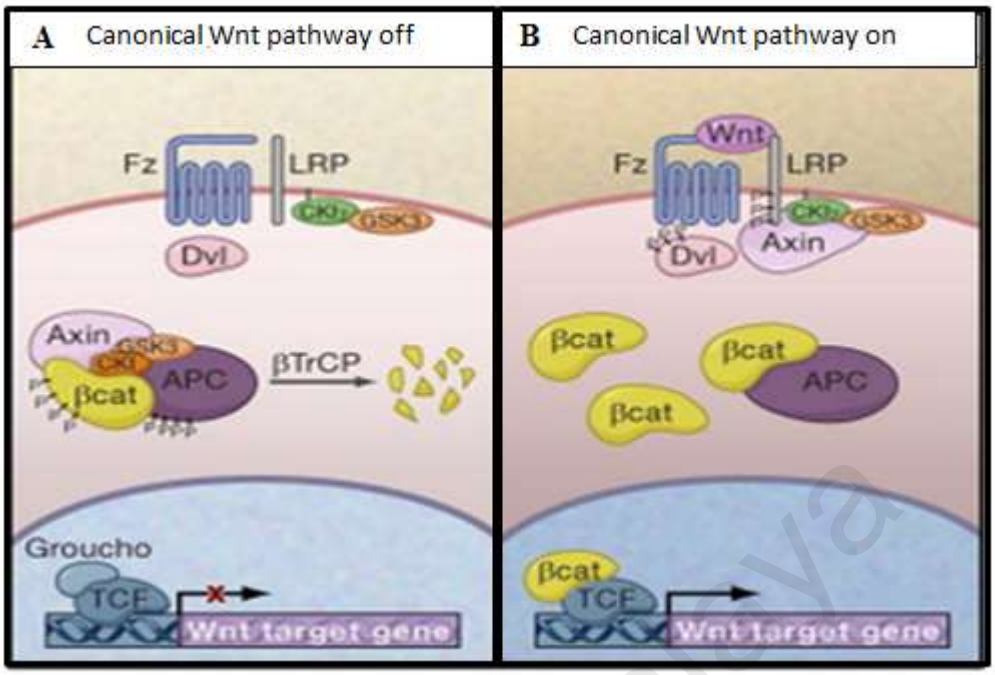

Figure 6.1: Transcriptional activation of target genes in canonical Wnt pathway

(A) Shows the canonical Wnt pathway in its off state, meanwhile (B) shows the accumulation of β catenin which enters the nucleus and bind with TCF to form β-catenin/T-cell factor (TCF) complex to activate the transcription of target genes.

6.1.4.2 Non-canonical pathway

In contrast, the non-canonical pathway does not involve β-catenin (Veeman, et al., 2003) in which it also activates Disheveled (Dvl) (Park et al., 2005) (Figure 6.2 C). Dvl then regulates the proteins which are responsible for the movement of cells such as cell migration and cell polarity (Huelsken & Behrens, 2002; Veeman et al., 2003; Jessen & Solnica-Krezel, 2005; Montcouquiol et al., 2006). Hence, the non-canonical planar cell polarity pathway plays an important role in regulating the cytoskeleton causing the shape differences in cells which is very much associated with cell migration. During gastrulation, the non-canonical planar cell polarity pathway also helps in the cell movements (Heisenberg et al., 2000). Meanwhile, the non-canonical calcium pathway regulates the calcium release from the endoplasmic reticulum inside the cells which plays a role in cell movements during gastrulation which was first discovered in zebrafish and *Xenopus* (Kühl et al., 2001).

In this study, we attempt to assess the effects of *Blastocystis* sp. ST3 antigen (Blasto-Ag ST3) derived from non-IBS and IBS patients respectively on the growth and gene expression of cancer cells, HCT116, *in vitro* in comparison with normal cells, CCD-18Co. A cell proliferation assay was carried out to study the effects of the Blasto-Ag ST3 on the cell growth *in vitro* of colon cancer cells compared to normal cells. A cell migration assay was carried out to measure the number of cells traversing a porous membrane, in response to the Blasto-Ag ST3 from non IBS an IBS isolates. Meanwhile, for the gene expression studies, we aim to study the expression of 75 genes involved in the Wnt signaling pathway in both the cell lines (normal cells and cancer cells) exposed to Blasto-Ag ST3 from non-IBS and IBS patients.

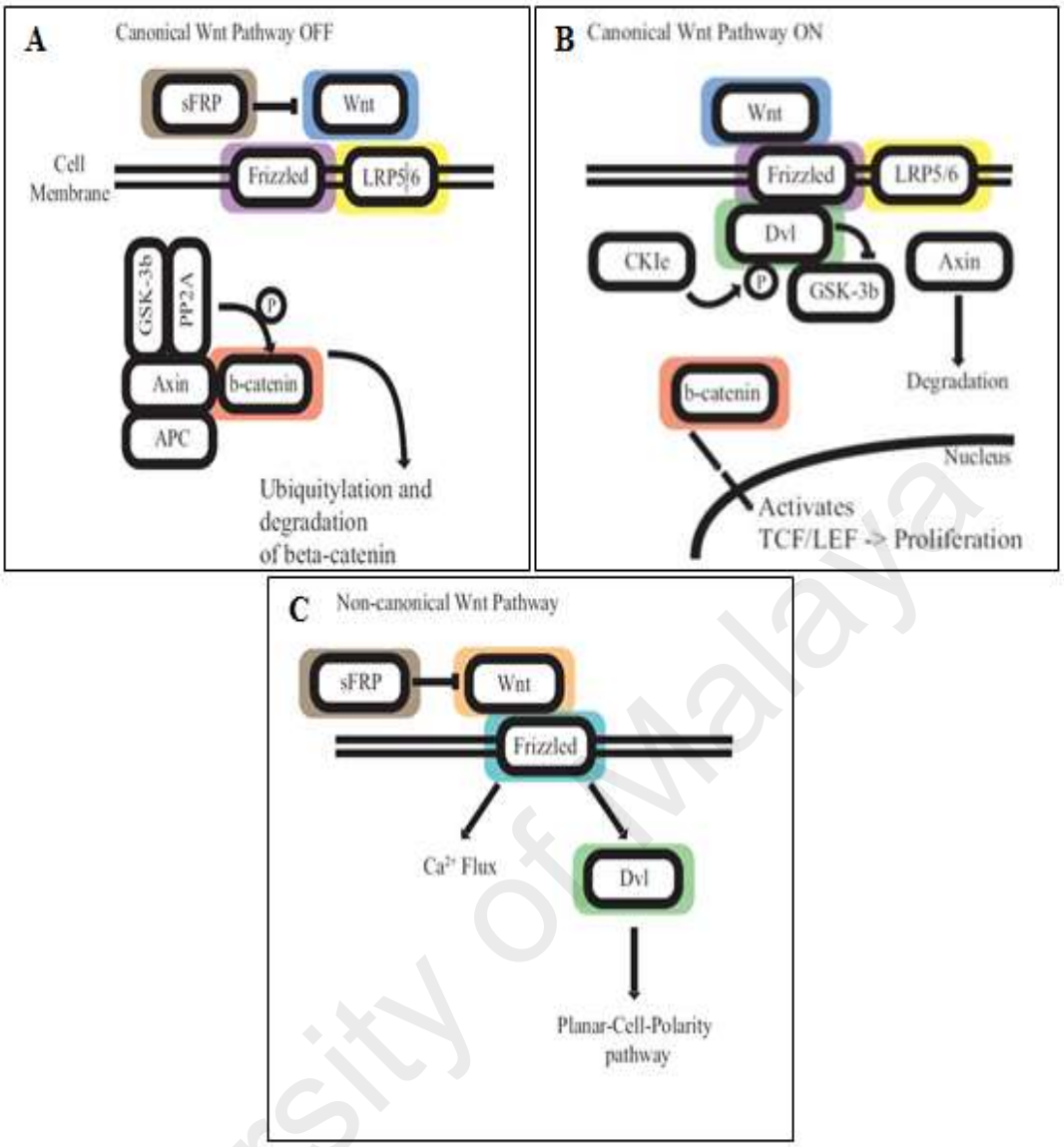

Figure 6.2: Schematic representation of the canonical and non-canonical Wnt pathways. SFRPs are inhibitors of both the canonical and non-canonical branches of the Wnt pathway. (A) Canonical Wnt pathway in its off state. (B) Canonical Wnt pathway in its on state. (C) Non-canonical Wnt pathway.

Colour represents genes that were used in this study. Grey: SFRP1, SFRP2, SFRP3, SFRP4, SFRP5; Blue: WNT1, WNT3A; Purple: FZD1; Yellow: LRP5, LRP6; Red: CTNNB1; Orange: WNT5A, WNT11; Teal: FZD2, FZD3, FZD6; Green: DVL2.

[Adapted from Lee et al., 2008]

6.2 Materials and methods

6.2.1 Source of *Blastocystis* sp. ST3 isolates

A total of eight *Blastocystis sp.* ST3 isolates were obtained from four non-IBS patients (asymptomatic and symptomatic), NIBS1-4 and four IBS patients, IBS1-4 at a gastrointestinal clinic, University Malaya Medical Centre (UMMC). Irritable bowel syndrome was defined based on the Rome III criteria (Longstreth et al., 2006). The stools were screened for other intestinal parasites such as hookworm, *Trichuris trichiura, Dientamoeba fragilis, Ascaris lumbricoides, Entamoeba histolytica and Giardia lamblia.* Patients infected with any of these parasites were excluded from this study. The isolation of parasites was done by culturing in 3ml Jones' medium supplemented with 10% horse serum (Gibco Laboratories, Life Technologies).

6.2.2 Preparation of solubilized antigen from *Blastocystis* sp. ST3 (Blasto-Ag ST3)

Ficoll-Paque density gradient centrifugation method was used to isolate all the eight cultures as previously described (Chandramathi et al., 2010; Chan et al., 2012; Kumarasamy et al., 2013). The lysates were sonicated at a frequency of 60Hz and 0.5 amplitude for 10 cycles. The lysate was screened under a microscope to observe that the lysis had taken place. The sonicated samples were kept overnight at 4^{o}C and were centrifuged at 60,000xg for 15 min. The supernatants were then filter sterilized, and the protein concentrations were determined using the Bradford assay (Bio-Rad, USA).

6.2.3 Cultivation and collection of normal cell (CCD-18Co).

Colon normal cells, CCD-18Co, were purchased from the American Type Culture Collection (ATCC) and maintained in 25cm³ culture flasks containing 5ml growth medium of CCD supplemented with 40% fetal bovine serum (FBS), 1% 2 mM L-glutamine, 1% 100 U/ml penicillin–streptomycin, 1% non-essential amino acid and 1% sodium pyruvate incubated in an incubator set to 100% humidity, atmosphere containing 5% carbon dioxide, and a temperature of 37°C. The cells in the culture flask were liberated from the substratum during the harvesting process using 0.25% trypsin–EDTA. Phosphate buffered saline (PBS) was used to wash the detached cells in the growth medium before introducing it to antigens isolated from *Blastocystis* sp. ST3

6.2.4 Cultivation and collection of Human colorectal carcinoma cells (HCT116)

Human colorectal carcinoma cell line, HCT116, was purchased from the American Type Culture Collection (ATCC) and maintained in 25cm³ culture flasks containing 5ml growth medium of RPMI 1640 supplemented with 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin–streptomycin, and 2.5 μg/ml fungizone and incubated in an incubator set with 100% humidity, atmosphere containing 5% carbon dioxide and a temperature of 37°C. The cells in the culture flask were liberated from the substratum during the harvesting process using 0.25% trypsin–EDTA. PBS was used to wash the detached cells in the growth medium before introducing it to antigens isolated from *Blastocystis* sp. ST3.

6.2.5 Introduction of non-IBS and IBS Blasto-Ag ST3 into normal cell lines, CCD-18Co.

Harvested normal cells, CCD-18Co (50, 000 cell/ml) in 4ml of CCD and growth medium with 20% FBS were seeded into 25cm^3 flasks. After the overnight incubation in a carbon dioxide (CO_2) incubator containing 5% CO_2 at 37°C, Blasto-Ag ST3 isolated from non-IBS and IBS individuals, at final concentration of 5µg/ml was added to each flask containing normal cells and were further incubated for 48 hours. Then, the cells were washed, harvested and counted to see the proliferation/viability of the cells.

6.2.6 Introduction of non-IBS and IBS Blasto-Ag ST3 into colorectal cancer cell lines, HCT116.

Harvested cancer cells (50, 000 cells/ml) in 4ml of RPMI and growth medium with 5% FBS were seeded into 25cm^3 flasks. After the overnight incubation in a CO_2 incubator containing 5% CO_2 at 37°C, Blasto-Ag ST3 from *Blastocystis* sp. isolated from non-IBS and IBS individuals at final concentration of 5µg/ml was added to each flask containing HCT116 cells and which were further incubated for 48 h. Then, the cells were washed, harvested and counted to see the proliferation/viability of the cells.

6.2.7 Cell migration

The cells were starved in a serum-free medium supplemented with 0.5% FBS. After 24 hours, the cells were harvested, and centrifuged at 10, 000 rpm for 10 min. The supernatant were removed and washed with 1X wash buffer and counted for 30, 000 cells in 50 µl for one well (30, 000 cells in 50 µl for 50 wells). 50µl of cells were added per well to the top chamber. 150µl of medium per well were added to the clear bottom chamber with 10% of 1µg/ml of non-IBS and IBS Blasto-Ag ST3. The chamber was incubated at 37˚C for 48 hours. The top chamber was then carefully aspirated and each well was washed with 100µl of 1X Wash Buffer. The bottom chamber was aspirated and washed with 200µl 1X Wash Buffer. 100µl of Cell Dissociation Solution/Calcein-Am were added to bottom chamber. The cell migration assay was then assembled and incubated again at 37˚C in a CO_2 incubator for one hour. The top chamber was then removed and the plate was read at 485nm excitation, 520nm emission. The percent of migration was determined.

6.2.8 Wnt signaling pathway

QuantiGene 2.0 Plex Magnetic Separation Assay kit was used (Affymetrix) for this study. The assay was done based on the manufacturer's instructions. 20 µL of working bead mix were added into each well of the 96-well hybridization plate. 80 µL of lysate were then added to each well of the Hybridization Plate containing Working Bead Mix. The hybridization plate was sealed using a pressure seal. The plate was incubated for 18-22 hours at 54°C ± 1°C at 600 rpm. The hybridization plate was centrifuged at 240 x g for one min at room temperature. The seal was removed and the

lysate bead mix was pipetted up and down for five times and transferred to a magnetic separation plate.

6.2.9 Statistical analysis

The statistical analysis was conducted with IBM Statistical package for Social Sciences for Windows SPSS (version 21). The level of significance between the two different groups'- induced cell proliferations, cell migration and gene expression studies were determined using Student's t-test. Results are presented as the mean value. A p-value<0.05 was considered as the minimum threshold of significance. All statistical analyses were performed using the SPSS software version.

6.3 Results

6.3.1 Cell proliferation rate

In the present study, the optimum concentration of antigen used was 5µg/ml. The cell proliferation rate was seen to be significantly higher in the normal cell line, 32.38% upon exposure of Blasto-Ag ST3 from IBS isolates compared to non-IBS Blasto-Ag ST3, 23.35% (Figure 6.3). Similar results were seen for the colorectal carcinoma cell line (HCT116) when introduced with Blasto-Ag ST3 from IBS isolates and non-IBS Blasto-Ag ST3. The proliferation rate of colon cancer cells when introduced with IBS Blasto-Ag ST3 was 38.10%, significantly higher than the proliferation rate of cancer cells introduced with non-IBS Blasto-Ag ST3, 15% (Figure 6.4).

6.3.2 Cell migration

The percentages of cell migration of normal and colorectal cancer cells were observed. The cell migration was seen to be significantly higher in normal cells upon exposure of IBS Blasto-Ag ST3, compared to non-IBS Blasto-Ag ST3 (Figure 6.5). The cell migration was also seen to be significantly higher in cancer cells introduced with IBS Blasto-Ag ST3 compared to cells introduced with non-IBS Blasto-Ag ST3 (Figure 6.6).

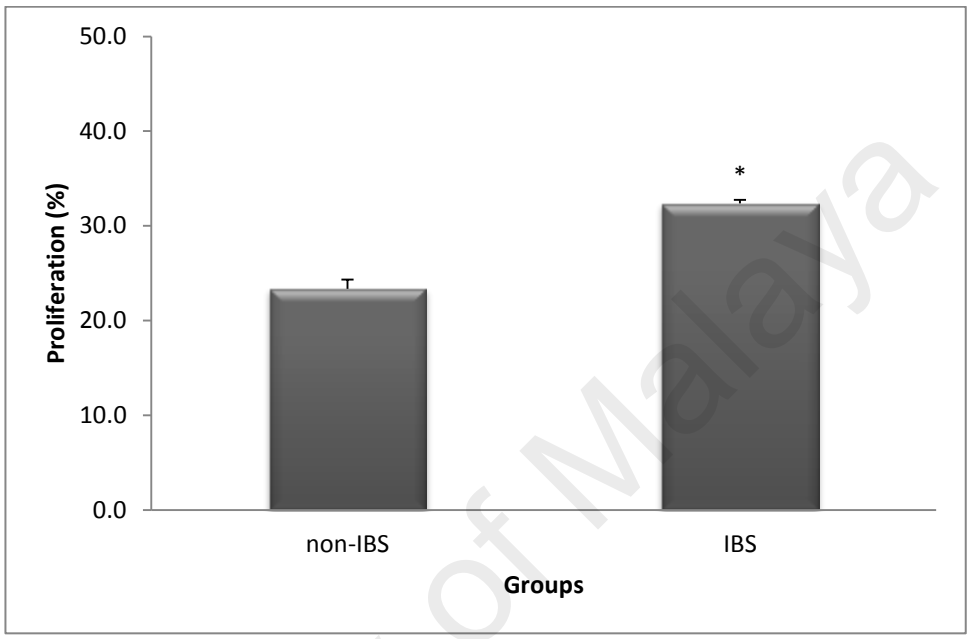

Figure 6.3: Proliferation of normal cells introduced with both the non-IBS and IBS Blasto-Ag ST3. Values are given in mean± SE. *P<0.05 is the comparison done against non-IBS group.

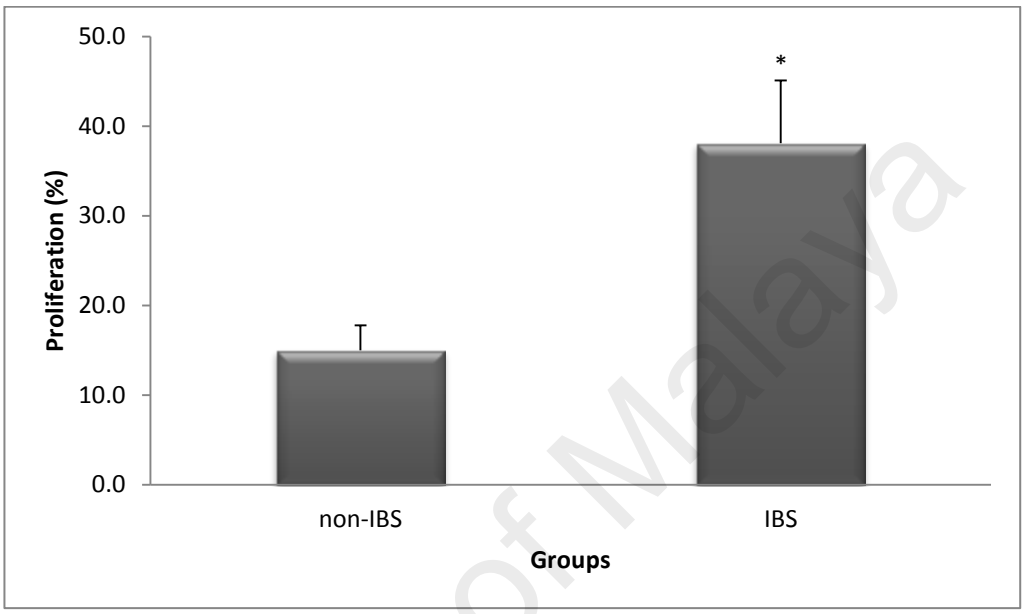

Figure 6.4: Proliferation of cancer cells introduced with both the non-IBS and IBS Blasto-Ag ST3. Values are given in mean± SE. *P<0.05 is the comparison done against non-IBS group.

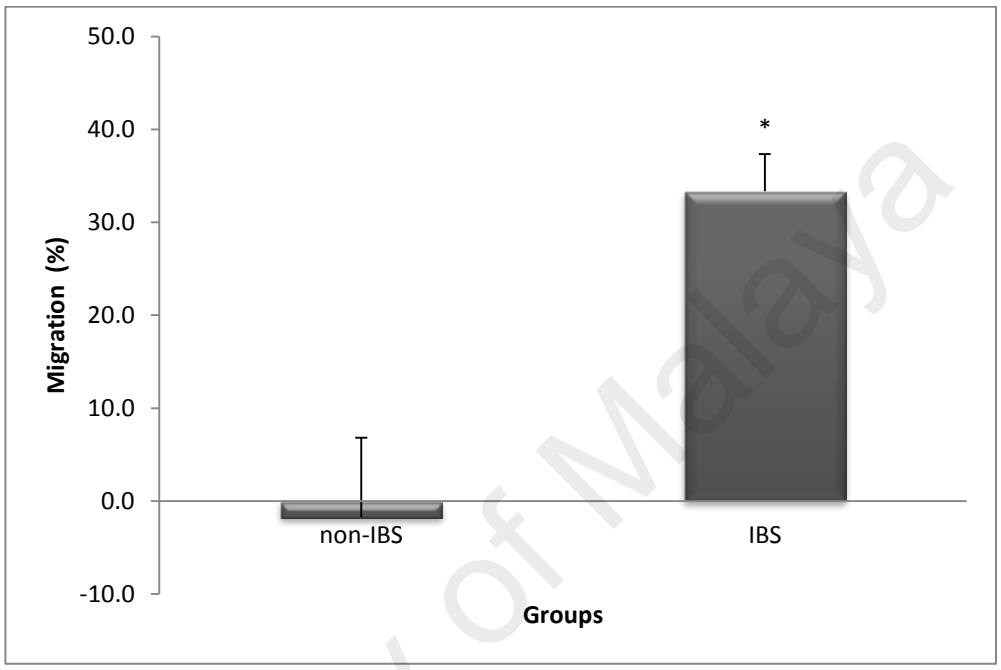

Figure 6.5: Migration of normal cells upon exposure of both the non-IBS and IBS Blasto-Ag ST3. Values are given in mean± SE. *P<0.05 is the comparison done against non-IBS group.

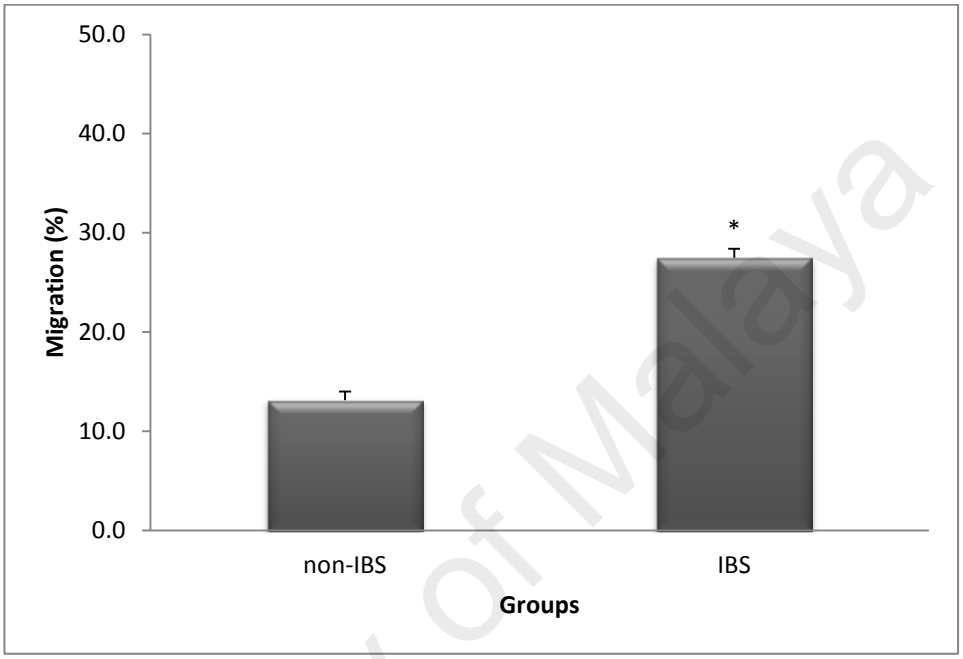

Figure 6.6: Migration of cancer cells upon exposure of both the non-IBS and IBS Blasto-Ag ST3. Values are given in mean± SE. *P<0.05 is the comparison done against non-IBS group.

6.3.3 Wnt QuantiGene

6.3.3.1 Gene expression for the normal cells (CCD-18Co)

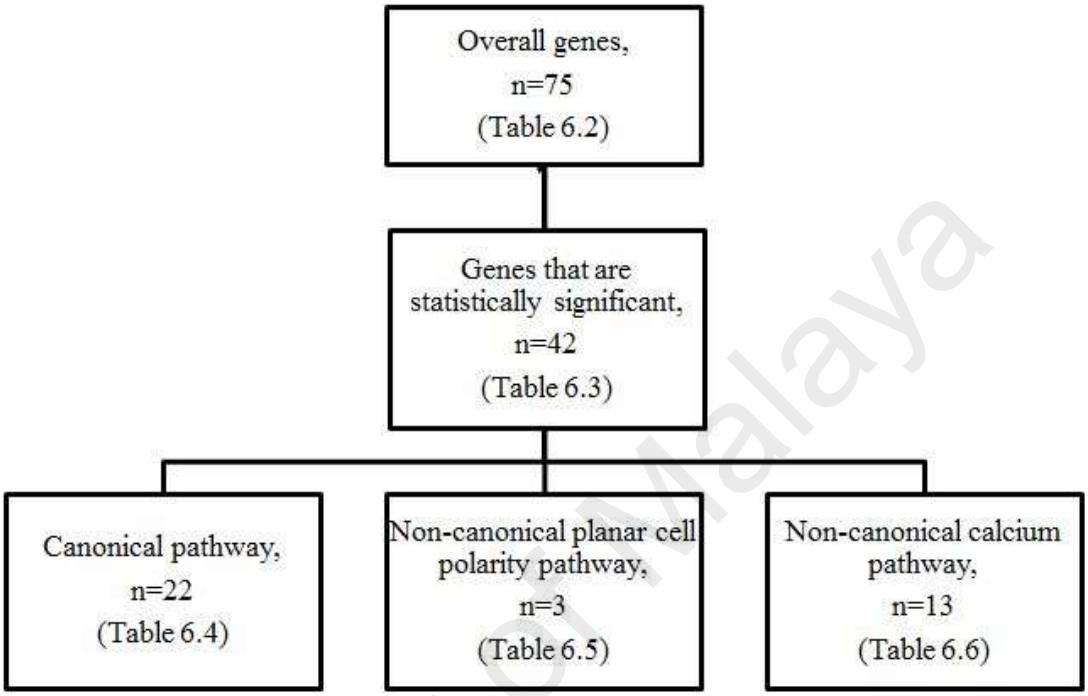

Figure 6.7: Classification of genes for normal cells.

The expression data of the Wnt signaling genes in normal cells were analyzed using Student's t-test and was classified based on their respective classification (Figure 6.7). A total of 75 genes were analyzed when Blasto-Ag ST3 derived from non-IBS and IBS patients were introduced to the normal cells (Table 6.2). Out of 75 genes, 42 genes were statistically significant between two study groups (non-IBS and IBS) at p-value 0.05 (Table 6.3). They include Wnt ligands, receptors, co-receptors, frizzleds (Fzd), Wnt binding proteins, cytoplasmic proteins, nuclear transcription factors and cofactors as well as other target genes (Table 6.1). The 42 genes which were statistically significant between two groups (non-IBS and IBS) are AXIN1, BCL9, CCND2, CER1, CREBBP, CSKN2A1, CXXC4, DVL2, FGF4, FRAT1, FZD3, FZD4, FZD7, FZD8, JUN, MMP7, NKD1, NKD2, PITX2, PORCN, PYGO1, SFRP4, SOX17, TCF7, TCF7L1, TLE1, TLE2, WIF1, WNT1, WNT10A, WNT11, WNT16, WNT2, WNT2B, WNT3, WNT3A, WNT4, WNT6, WNT7A, WNT7B, WNT8A and WNT9A (Table 6.3). These genes were further categorized based on canonical pathway, non-canonical planar cell polarity pathway and non-canonical calcium pathway. A total of 22 genes were categorized under canonical pathway which include DVL2, FRAT1, FZD4, FZD8, NKD, PORCN, SFRP4, SOX17, TCF7, TCF7L1, WIF1, WNT1, WNT10A, WNT2, WNT2B, WNT3, WNT3A, WNT4, WNT6, WNT7A, WNT7B, WNT8A (Table 6.4). Meanwhile, only three genes were categorized under the non-canonical planar cell polarity pathway, DVL2, NKD1 and WNT9A (Table 6.5). 13 genes categorized under the non-canonical calcium pathway are WNT1, WNT10A, WNT11, WNT2, WNT2B, WNT3, WNT3A, WNT4, WNT6, WNT7A, WNT7B, WNT8A and WNT9A (Table 6.6).

Table 6.2: Genes showing differential expression in normal cells

No	Gene	non-IBS	IBS	p-value
1	AES	0.437	0.802	0.075
2	APC	1.046	1.547	0.315
3	AXIN1	0.503	0.964	0.035
4	BCL9	0.690	1.491	0.010
5	BTRC	0.568	0.864	0.227
6	CCND1	0.758	1.174	0.262
7	CCND2	0.388	0.938	0.014
8	CER1	0.344	1.071	0.006
9	CHD8	0.518	0.909	0.086
10	CREBBP	0.430	0.881	0.028
11	CSKN1A1	0.470	0.801	0.146
12	CSKN2A1	0.443	1.126	0.001
13	CTBP1	0.531	0.891	0.127
14	CTBP2	0.505	0.740	0.321
15	CTNNB1	0.521	0.724	0.390
16	CTNNBIP1	0.584	0.989	0.107
17	CXXC4	0.514	1.491	0.003
18	DKK1	0.747	1.068	0.393
19	DVL1	0.601	0.925	0.207
20	DVL2	0.495	1.140	0.030
21	EP300	0.543	0.966	0.105
22	FBXW2	0.524	0.906	0.116
23	FGF4	0.486	1.866	0.001
24	FOSL1	0.629	1.159	0.083
25	FRAT1	0.425	1.020	0.005
26	FZD1	0.681	1.346	0.072
27	FZD2	0.441	0.703	0.155
28	FZD3	0.564	1.551	0.000
29	FZD4	0.398	0.915	0.020
30	FZD5	0.503	1.016	0.062

Table 6.2, continued Genes showing differential expression in normal cells

No	Gene	non-IBS	IBS	p-value
31	FZD6	0.547	0.745	0.430
32	FZD7	0.456	0.963	0.012
33	FZD8	0.641	1.323	0.055
34	GSK3B	0.481	0.722	0.291
35	JUN	0.445	0.952	0.020
36	KREMEN1	0.644	1.086	0.157
37	LEF1	0.650	1.141	0.096
38	LRP5	0.426	0.743	0.119
39	LRP6	0.529	0.845	0.235
40	MMP7	0.228	0.957	0.001
41	MYC	0.448	0.838	0.071
42	NKD1	0.382	1.042	0.005
43	NKD2	0.474	1.008	0.030
44	NLK	0.438	0.669	0.271
45	PITX2	0.696	1.549	0.003
46	PORCN	0.521	1.149	0.030
47	PPP2CA	0.600	0.965	0.149
48	PPP2R1A	0.478	0.809	0.120
49	PYGO1	0.612	1.140	0.038
50	SENP2	0.506	0.807	0.178
51	SFRP1	0.608	0.837	0.372
52	SFRP4	0.744	1.899	0.003
53	SMAD2	0.463	0.786	0.148
54	SOX17	0.536	1.231	0.004
55	TCF7	0.743	1.358	0.030
56	TCF7L1	0.485	0.972	0.032
57	TLE1	0.423	0.915	0.022
58	TLE2	0.382	1.198	0.008
59	WIF1	0.487	1.584	0.002
60	WNT1	1.216	2.403	0.056

Table 6.2, continued Genes showing differential expression in normal cells

No	Gene	non-IBS	IBS	p-value
61	WNT10A	0.601	1.807	0.008
62	WNT11	0.739	2.512	0.010
63	WNT16	0.629	1.545	0.005
64	WNT2	0.173	0.587	0.040
65	WNT2B	0.370	0.768	0.054
66	WNT3	0.435	1.245	0.003
67	WNT3A	0.380	1.125	0.001
68	WNT4	0.537	1.407	0.013
69	WNT5A	0.529	0.880	0.111
70	WNT5B	0.535	0.896	0.095
71	WNT6	0.420	1.263	0.000
72	WNT7A	0.662	1.789	0.004
73	WNT7B	0.227	0.994	0.011
74	WNT8A	0.315	1.159	0.008
75	WNT9A	0.441	1.070	0.005

Data are shown in mean values of four biological replicates. P-values are obtained from the comparison between non-IBS and IBS using Student's t-test.

Table 6.3: Genes that are statistically significant between non-IBS and IBS in normal cells

No	Gene	non-IBS	IBS	p-value
1	AXIN1	0.503	0.964	0.035
2	BCL9	0.690	1.491	0.010
3	CCND2	0.388	0.938	0.014
4	CER1	0.344	1.071	0.006
5	CREBBP	0.430	0.881	0.028
6	CSKN2A1	0.443	1.126	0.001
7	CXXC4	0.514	1.491	0.003
8	DVL2	0.495	1.140	0.030
9	FGF4	0.486	1.866	0.001
10	FRAT1	0.425	1.020	0.005
11	FZD3	0.564	1.551	0.000
12	FZD4	0.398	0.915	0.020
13	FZD7	0.456	0.963	0.012
14	FZD8	0.641	1.323	0.050
15	JUN	0.445	0.952	0.020
16	MMP7	0.228	0.957	0.001
17	NKD1	0.382	1.042	0.005
18	NKD2	0.474	1.008	0.013
19	PITX2	0.696	1.549	0.003
20	PORCN	0.521	1.149	0.030
21	PYGO1	0.612	1.140	0.038
22	SFRP4	0.744	1.899	0.003
23	SOX17	0.536	1.231	0.004
24	TCF7	0.743	1.358	0.030
25	TCF7L1	0.485	0.972	0.032
26	TLE1	0.423	0.915	0.022
27	TLE2	0.382	1.198	0.008
28	WIF1	0.487	1.584	0.002
29	WNT1	1.216	2.403	0.050
30	WNT10A	0.601	1.807	0.008

Table 6.3, continued Genes that are statistically significant between non-IBS and IBS in normal cells

No	Gene	non-IBS	IBS	p-value
31	WNT11	0.739	2.512	0.010
32	WNT16	0.629	1.545	0.005
33	WNT2	0.173	0.587	0.040
34	WNT2B	0.370	0.768	0.054
35	WNT3	0.435	1.245	0.003
36	WNT3A	0.380	1.125	0.001
37	WNT4	0.537	1.407	0.013
38	WNT6	0.420	1.263	0.000
39	WNT7A	0.662	1.789	0.004
40	WNT7B	0.227	0.994	0.011
41	WNT8A	0.315	1.159	0.008
42	WNT9A	0.441	1.070	0.005

Data are shown in mean values of four biological replicates. P-values are obtained from the comparison between non-IBS and IBS using Student's t-test.

Table 6.4: Genes that are categorized based on the canonical pathway in normal cells.

No	Gene	non-IBS	IBS	p-value
1	DVL2	0.495	1.140	0.030
2	FRAT1	0.425	1.020	0.005
3	FZD4	0.398	0.915	0.020
4	FZD8	0.641	1.323	0.055
5	NKD1	0.382	1.042	0.005
6	PORCN	0.521	1.149	0.030
7	SFRP4	0.744	1.899	0.003
8	SOX17	0.536	1.231	0.004
9	TCF7	0.743	1.358	0.030
10	TCF7L1	0.485	0.972	0.032
11	WIF1	0.487	1.584	0.002
12	WNT1	1.216	2.403	0.056
13	WNT10A	0.601	1.807	0.008
14	WNT2	0.173	0.587	0.040
15	WNT2B	0.370	0.768	0.054
16	WNT3	0.435	1.245	0.003
17	WNT3A	0.380	1.125	0.001
18	WNT4	0.537	1.407	0.013
19	WNT6	0.420	1.263	0.000
20	WNT7A	0.662	1.789	0.004
21	WNT7B	0.227	0.994	0.011
22	WNT8A	0.315	1.159	0.008

Data are shown in mean values of four biological replicates. P-values are obtained from the comparison between non-IBS and IBS using Student's t-test.

Table 6.5: Genes that are categorized based on the non-canonical planar cell polarity pathway in normal cells.

No	Gene	non-IBS	IBS	p-value
1	DVL2	0.495	1.140	0.030
2	NKD1	0.382	1.042	0.005
3	WNT9A	0.441	1.070	0.005

Data are shown in mean values of four biological replicates. P-values are obtained from the comparison between non-IBS and IBS using Student's t-test

Table 6.6: Genes that are categorized based on the non-canonical calcium pathway in normal cells.

No	Gene	non-IBS	IBS	p-value
1	WNT1	1.216	2.403	0.056
2	WNT10A	0.601	1.807	0.008
3	WNT11	0.739	2.512	0.010
4	WNT2	0.173	0.587	0.040
5	WNT2B	0.370	0.768	0.054
6	WNT3	0.435	1.245	0.003
7	WNT3A	0.380	1.125	0.001
8	WNT4	0.537	1.407	0.013
9	WNT6	0.420	1.263	0.000
10	WNT7A	0.662	1.789	0.004
11	WNT7B	0.227	0.994	0.011
12	WNT8A	0.315	1.159	0.008
13	WNT9A	0.441	1.070	0.005

Data are shown in mean values of four biological replicates. P-values are obtained from the comparison between non-IBS and IBS using Student's t-test.

6.3.3.2 Gene expression for the colon cancer cells (HCT116)

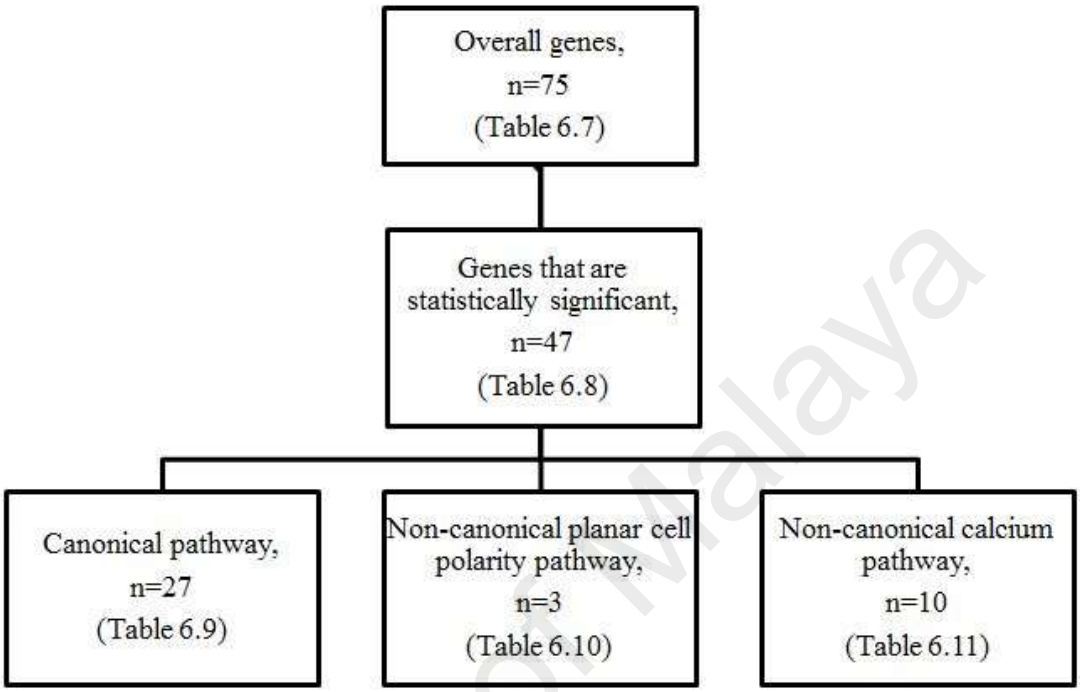

Figure 6.8: Classification of genes for colon cancer cells.

The expression data of the Wnt signaling genes in colon cancer cells were analyzed using Student's t-test and was classified based on their respective classification (Figure 6.8). A total of 75 genes were analyzed when Blasto-Ag ST3 derived from non-IBS and IBS patients were introduced to the colon cancer cells (Table 6.7). Out of 75 genes, 47 genes were statistically significant between two study groups. They include AES, APC, AXIN1, BCL9, BTRC, CCND2, CSKN2A1, CTNNB1, DKK1, DVL1, EP300, FBXW2, FGF4, FOSL1, FRAT1, FZD1, FZD4, FZD5, FZD6, FZD7, GSK3B, JUN, KREMEN1, LEF1, LRP5, MYC, NKD1, PITX2, PYGO1, SFRP1, SFRP4, SMAD2, SOX17, TCF7, TLE2, WIF1, WNT10A, WNT11, WNT16, WNT2, WNT2B, WNT3, WNT3A, WNT5A, WNT6, WNTA and WNT9A (Table 6.8). A total of 27 genes were categorized under the canonical pathway, APC, CTNNB1, DKK1, DVL1, EP300, FRAT1, FZD1, FZD4, FZD5, FZD6, FZD7, GSK3B, LEF1, LRP5, NKD1, SFRP1, SFRP4, SOX17, TCF7, WIF1, WNT10A, WNT2, WNT2B, WNT3, WNT3A, WNT6 and WNT7A (Table 6.9). Meanwhile, only three genes were categorized under the non-canonical planar cell. They include DVL2, NKD1 and WNT9A (Table 6.10). Ten genes were categorized under the non-canonical planar calcium pathway, WNT10A, WNT11, WNT2, WNT2B, WNT3, WNT3A, WNT5A, WNT6, WNT7A and WNT9A (Table 6.11).

Table 6.7: Genes showing differential expression in colon cancer cells

No	Gene	non-IBS	IBS	p-value
1	AES	2.191	1.531	0.028
2	APC	2.211	1.151	0.004
3	AXIN1	0.834	1.447	0.021
4	BCL9	2.383	1.589	0.005
5	BTRC	2.055	1.268	0.011
6	CCND1	1.998	1.579	0.085
7	CCND2	0.176	0.702	0.046
8	CER1	0.625	1.360	0.233
9	CHD8	1.016	1.361	0.057
10	CREBBP	2.000	1.592	0.087
11	CSNK1A1	1.901	1.422	0.074
12	CSNK2A1	0.252	0.741	0.000
13	CTBP1	1.202	1.429	0.277
14	CTBP2	4.179	1.467	0.000
15	CTNNB1	2.919	1.226	0.001
16	CTNNBIP1	2.919	1.226	0.219
17	CXXC4	0.300	0.578	0.089
18	DKK1	2.382	1.481	0.013
19	DVL1	2.437	1.259	0.001
20	DVL2	1.522	1.420	0.594
21	EP300	2.714	1.407	0.001
22	FBXW2	2.010	1.332	0.028
23	FGF4	0.248	1.004	0.001
24	FOSL1	0.504	1.505	0.039
25	FRAT1	0.437	0.720	0.018
26	FZD1	2.340	1.495	0.012
27	FZD2	0.409	0.682	0.087
28	FZD3	1.042	0.776	0.135
29	FZD4	0.275	0.595	0.009
30	FZD5	0.347	0.902	0.001

Table 6.7, continued Genes showing differential expression in colon cancer cells.

No	Gene	non-IBS	IBS	p-value
31	FZD6	2.255	1.072	0.004
32	FZD7	0.535	0.867	0.018
33	FZD8	0.176	0.275	0.423
34	GSK3B	1.666	0.856	0.005
35	JUN	0.424	1.217	0.028
36	KREMEN1	1.526	0.839	0.015
37	LEF1	0.150	0.565	0.011
38	LRP5	1.697	0.940	0.000
39	LRP6	4.318	3.878	0.881
40	MMP7	0.271	0.646	0.138
41	MYC	2.428	1.595	0.018
42	NKD1	0.956	1.395	0.044
43	NKD2	0.847	0.967	0.524
44	NLK	1.729	1.173	0.056
45	PITX2	0.297	0.642	0.019
46	PORCN	0.287	0.404	0.200
47	PPP2CA	1.135	1.564	0.129
48	PPP2R1A	2.080	1.557	0.116
49	PYGO1	0.219	0.593	0.000
50	SENP2	1.682	1.348	0.053
51	SFRP1	0.381	0.939	0.007
52	SFRP4	0.176	0.444	0.010
53	SMAD2	2.017	1.397	0.000
54	SOX17	0.277	0.827	0.024
55	TCF7	1.725	1.032	0.010
56	TCF7L1	0.476	0.587	0.163
57	TLE1	1.349	1.351	0.986
58	TLE2	2.271	1.393	0.003
59	WIF1	0.206	0.703	0.002
60	WNT1	0.308	1.127	0.205

Table 6.7, continued Genes showing differential expression in colon cancer cells.

No	Gene	non-IBS	IBS	p-value
61	WNT10A	0.200	0.564	0.014
62	WNT11	0.172	0.476	0.025
63	WNT16	1.359	1.029	0.022
64	WNT2	0.159	0.522	0.001
65	WNT2B	0.474	0.748	0.045
66	WNT3	0.361	1.236	0.009
67	WNT3A	0.222	0.484	0.029
68	WNT4	0.408	0.677	0.215
69	WNT5A	0.279	0.776	0.002
70	WNT5B	0.841	0.349	0.075
71	WNT6	0.239	0.763	0.001
72	WNT7A	0.573	1.198	0.002
73	WNT7B	1.407	1.300	0.706
74	WNT8A	0.228	0.543	0.097
75	WNT9A	0.284	0.610	0.005

Data are shown in mean values of four biological replicates. P-values are obtained from the comparison between non-IBS and IBS using Student's t-test.

Table 6.8: Genes that are statistically significant between non-IBS and IBS in colon cancer cells

No	Gene	non-IBS	IBS	p-value
1	AES	2.191	1.531	0.028
2	APC	2.211	1.151	0.004
3	AXIN1	0.834	1.447	0.021
4	BCL9	2.383	1.589	0.005
5	BTRC	2.055	1.268	0.011
6	CCND2	0.176	0.702	0.046
7	CSKN2A1	0.252	0.741	0.000
8	CTNNB1	2.919	1.226	0.001
9	DKK1	2.382	1.481	0.013
10	DVL1	2.437	1.259	0.001
11	EP300	2.714	1.407	0.001
12	FBXW2	2.010	1.332	0.028
13	FGF4	0.248	1.004	0.001
14	FOSL1	0.504	1.505	0.039
15	FRAT1	0.437	0.720	0.018
16	FZD1	2.340	1.495	0.012
17	FZD4	0.275	0.595	0.009
18	FZD5	0.347	0.902	0.001
19	FZD6	2.255	1.072	0.004
20	FZD7	0.535	0.867	0.018
21	GSK3B	1.666	0.856	0.005
22	JUN	0.424	1.217	0.028
23	KREMEN1	1.526	0.839	0.015
24	LEF1	0.150	0.565	0.011
25	LRP5	1.697	0.940	0.000
26	MYC	2.428	1.595	0.018
27	NKD1	0.956	1.395	0.044
28	PITX2	0.297	0.642	0.019
29	PYGO1	0.219	0.593	0.000
30	SFRP1	0.381	0.939	0.007

Table 6.8, continued Genes that are statistically significant between non-IBS and IBS in colon cancer cells

No	Gene	non-IBS	IBS	p-value
31	SFRP4	.176	.444	.010
32	SMAD2	2.017	1.397	.000
33	SOX17	.277	.827	.024
34	TCF7	1.725	1.032	.010
35	TLE2	2.271	1.393	.003
36	WIF1	.206	.703	.002
37	WNT10A	.200	.564	.014
38	WNT11	.172	.476	.025
39	WNT16	1.359	1.029	.022
40	WNT2	.159	.522	.001
41	WNT2B	.474	.748	.045
42	WNT3	.361	1.236	.009
43	WNT3A	.222	.484	.029
44	WNT5A	.279	.776	.002
45	WNT6	.239	.763	.001
46	WNT7A	.573	1.198	.002
47	WNT9A	.284	.610	.005

Data are shown in mean values of four biological replicates. P-values are obtained from the comparison between non-IBS and IBS using Student's t-test.

Table 6.9: Genes that are categorized based on the canonical pathway in colon cancer cells.

No	Gene	non-IBS	IBS	p-value
1	APC	2.211	1.151	.004
2	CTNNB1	2.919	1.226	.001
3	DKK1	2.382	1.481	.013
4	DVL1	2.437	1.259	.001
5	EP300	2.714	1.407	.001
6	FRAT1	0.437	0.720	.018
7	FZD1	2.340	1.495	.012
8	FZD4	0.275	0.595	.009
9	FZD5	0.347	0.902	.001
10	FZD6	2.255	1.072	.004
11	FZD7	0.535	0.867	.018
12	GSK3B	1.666	0.856	.005
13	LEF1	0.150	0.565	.011
14	LRP5	1.697	0.940	.000
15	NKD1	0.956	1.395	.044
16	SFRP1	0.381	0.939	.007
17	SFRP4	0.176	0.444	.010
18	SOX17	0.277	0.827	.024
19	TCF7	1.725	1.032	.010
20	WIF1	0.206	0.703	.002
21	WNT10A	0.200	0.564	.014
22	WNT2	0.159	0.522	.001
23	WNT2B	0.474	0.748	.045
24	WNT3	0.361	1.236	.009
25	WNT3A	0.222	0.484	.029
26	WNT6	0.239	0.763	.001
27	WNT7A	0.573	1.198	.002

Data are shown in mean values of four biological replicates. P-values are obtained from the comparison between non-IBS and IBS using Student's t-test.

Table 6.10: Genes that are categorized based on the non-canonical planar cell polarity pathway in colon cancer cells.

No	Gene	non-IBS	IBS	p-value
1	DVL1	2.437	1.259	.001
2	NKD1	0.956	1.395	.044
3	WNT9A	0.284	0.610	.005

Data are shown in mean values of four biological replicates. P-values are obtained from the comparison between non-IBS and IBS using Student's t-test.

Table 6.11: Genes that are categorized based on the non-canonical calcium pathway in colon cancer cells.

No	Gene	non-IBS	IBS	p-value
1	WNT10A	.200	.564	.014
2	WNT11	.172	.476	.025
3	WNT2	.159	.522	.001
4	WNT2B	.474	.748	.045
5	WNT3	.361	1.236	.009
6	WNT3A	.222	.484	.029
7	WNT5A	.279	.776	.002
8	WNT6	.239	.763	.001
9	WNT7A	.573	1.198	.002
10	WNT9A	.284	.610	.005

Data are shown in mean values of four biological replicates. P-values are obtained from the comparison between non-IBS and IBS using Student's t-test

6.4 Discussion

6.4.1 Cell proliferation

Previously, an increase in cell proliferation rate was observed in colon cancer cells (HCT116) inoculated with Blasto-Ag from a single isolate at a certain concentration, indicating that the parasite has the ability to facilitate the growth of cancer cells *in vitro* (Chandramathi et al., 2010). Another study done by Chan et al. (2012) revealed that Blasto-Ag from symptomatic isolates introduced to colon cancer cells showed higher proliferation rate compared to Blasto-Ag derived from asymptomatic isolates. The study suggests that symptomatic isolates were seen to be more pathogenic and have the ability to weaken the immune system and aid in the growth of colorectal cancer cells compared to asymptomatic isolates. However neither study compared the subtype influence on the level of cancer cell proliferation. Following this, Kumarasamy et al., (2013) showed that Blasto-Ag ST3 triggers a higher proliferation rate of colon cancer cells compared to Blasto-Ag from five other subtypes.

The present study is the first to evaluate the proliferation rate of both normal and cancer cells induced with Blasto-Ag ST3 from non-IBS and IBS isolates by keeping the subtype constant that is ST3. Chapter 4 indicated that different phenotypic characteristics showed by *Blastocystis* sp. ST3 from IBS patients compared to the isolate derived from non-IBS patients (symptomatic patients and asymptomatic individuals) could be due to the gut condition of IBS patient. Therefore, it is important to investigate if differences can be seen on the growth of normal and colon cancer cells introduced with Blasto-Ag ST3 derived from non-IBS and IBS isolates.

Results from figure 6.4 and 6.5 showed that Blasto-Ag ST3 from non-IBS and IBS groups could increase the proliferation rate for both normal and colon cancer cells. Moreover, the proliferation rate for both normal and cancer cells were significantly higher when induced with Blasto-Ag ST3 derived from IBS patients compared to Blasto-Ag ST3 from non-IBS patients.

6.4.2 Cell migration

Initially, Wnt signaling pathway was found to play an important role during embryogenesis which were firmly regulated (Yamaguchi, 2001). This pathway is important for the development of important tissues which include bone, muscle and heart through proliferation of cells, cell migration, body axis patterning and cell fate specification. However, defective Wnt signaling can results in many human diseases such as breast cancer, colon cancer, skeletal deficiencies and human birth defect conditions (Logan & Nusse, 2004). Figure 6.5 and 6.6 showed that percentage of cell migration was significantly higher in normal and colon cancer cells upon exposure of IBS Blasto-Ag ST3, compared to non-IBS Blasto-Ag ST3. Wnt signaling does play a role in regulating cell migration. The migration of Chinese hamster ovary cells and myeloma, a type of cancer had been seen to be triggered by higher expression of WNT3a (Endo et al., 2005). Very similar results were obtained in this study in which WNT3A gene was upregulated in normal cell upon exposure of IBS Blasto-Ag ST3. WNT3A gene was in contrast downregulated in normal cells upon exposure of non-IBS Blasto-Ag ST3 and also in cancer cells upon exposure of non-IBS and IBS Blasto-Ag ST3.

6.4.3 Wnt signaling pathway

6.4.3.1 Canonical pathway

Previous studies have shown that the primary mutation of genes which lead to cancer targets single transduction pathways such as vascular endothelial growth factor (VEGF signaling pathway) (McMahon, 2000), cyclic adenosine monophosphate (cAMP) pathway (Huang et al., 2005), p53 pathway (Vazquez et al., 2008), TGF-β pathway (Derynck et al., 2001) and Wnt signaling pathway (Giles et al., 2003). Wnt signaling pathway is one of the signal transduction pathways which plays an important role in the pathophysiology of inflammation (Bienz & Clevers, 2000; Giles et al., 2003). Besides that, this pathway has also been associated with cell proliferation and migration (Toyama et al., 2010). This pathway is made up of proteins that pass signals from outside of a cell through cell surface receptors to the inside of the cell. Numerous studies have associated Wnt pathway with the pathogenesis of cancer, namely colorectal cancer (Bienz & Clevers, 2000; Giles et al., 2003). To date, this is the first study to investigate the role of Blasto-Ag ST3 in Wnt signaling pathway of colon cancer cells exposed to the solubilized antigen extracted from the parasite and the observation was compared with normal cells.

In the present study, the expression data of the 75 genes in normal cells and colon cancer cells were analyzed. 42 genes and 47 genes were found to be significantly different between two groups (upon exposure of non-IBS and IBS Blasto-Ag ST3) for normal and cancer cells respectively (Table 6.3 and Table 6.8). The genes were further categorized based on the canonical, non-canonical planar cell polarity and non-canonical calcium pathway. The data shows that for normal cells, 22, 3 and 13 genes

fall under canonical, non-canonical planar cell polarity and non-canonical calcium pathway respectively. Meanwhile for cancer cells 27, 3 and 10 genes fall under canonical, non-canonical planar cell polarity and non-canonical calcium pathway respectively.

In the present study, APC gene was significantly upregulated in cancer cells exposed to non-IBS Blasto-Ag ST3 (fold difference= 2.2, Table 6.8) compared to IBS Blasto-Ag ST3 (fold difference= 1.2, Table 6.8). APC gene is known as a tumor suppressor gene however its actual function at molecular level is still poorly studied (Bienz & Clevers, 2000). Mutation and loss of APC has been indicated in cell migration, cell proliferation and tumor progression (Bienz & Clevers, 2000). In this study, the upregulation of APC gene seen in cancer cells especially when exposed to non-IBS Blasto-Ag ST3 may be a reason for decreased level of migration and proliferation seen in the cancer cells exposed to the same antigen as shown in figures 6.3 and 6.4. In other words, the Blasto-Ag ST3 from IBS isolates has only marginal effect on the APC gene and this could be a reason for higher exacerbation of cell growth seen in cancer cells.

Previous studies have reported on several canonical Wnts that are highly expressed in Wnt signaling pathway which cause human cancer in breast, colon cancer and lung cancer. The canonical ligands that are upregulated in breast cancer cells are WNT3A, WNT4, WNT6, WNT7B, WNT8B, WNT9 and WNT10B (Wong et al., 1994; Shimizu et al., 1997; Katoh & Katoh, 2005; Person et al., 2005; Benhaj et al., 2006). WNT2, WNT3, WNT4, WNT5, WNT6, WNT7A, WNT10A and WNT11 genes were also seen to be upregulated in cancer cells of colon, stomach gastric, prostrate, pancreas

and ovary cancer (Katoh, 2001a; Holcombe et al., 2002; Kirikoshi & Katoh, 2002; Thompson et al., 2010; Nambotin et al., 2012; Nishioka et al., 2013)

To date, this is the first study to investigate the regulation of Wnt genes in both normal and cancerous colon cells lines upon exposure to solubilized antigens of an intestinal protozoan parasite. The present data has also provided information on some of the genes that are significantly expressed in the canonical pathway. Wnt ligands such as WNT3A, WNT4, WNT6 and WNT10A genes were found to be upregulated when introduced with IBS Blasto-Ag ST3 in normal cells but downregulated in others (Table 6.4 and 6.9). Past studies have reported on the high expression of these genes in colon (Kiroshi et al., 2001) and breast cancer cell lines (Benhaj et al., 2006). Observation from the current study indicates that *Blastocystis* sp. in IBS patients has the tendency to alter the normal colon cells to become oncogenic by stimulating the Wnt ligands that will increase gene transcription and subsequently activate its proliferative properties. This correlates with our previous data shown in the first part of this chapter in which cell proliferation was significantly higher in both normal and cancer cells introduced with IBS Blasto-Ag. This implies that these genes can play a role in causing a higher proliferation.

Although these genes were downregulated in cancer cells, other canonical pathway related genes especially WNT3 and WNT7A genes were seen to be upregulated in both normal and cancer cells when exposed to IBS Blasto-Ag ST3 (Table 6.4 and 6.9). These two genes are usually used as key markers for canonical pathway in cancer (Davis et al., 2008; David et al., 2010) compared to other Wnt ligands (e.g.: WNT3A, WNT4, WNT6 and WNT10) that may play dual role in canonical and non-canonical pathways (Table 6.4 and 6.9).

In addition to this, WNT3 gene was implicated as a proto-oncogene that may be a key factor in some cancers of breast, rectal, lung and gastric cancer by initiating the Wnt- β catenin-TCF signaling pathway (activates gene transcription and cell propagation) (Katoh, 2001b). The WNT7A gene was also upregulated in ovarian cancer, colorectal cancer cells, pancreatic cancer and in gastric cancer cells via activation of β catenin (canonical) pathway. (Kirikoshi & Katoh, 2002; Yoshioka et al., 2012).

In the present study, the fold difference of WNT7A caused by IBS Blasto-Ag ST3 is much higher in normal cells (fold differences= 1.79) compared to cancer cells (fold differences= 1.20), leading to the speculation that this gene has greater influence in promoting β catenin-targeted cell proliferation in normal compared to cancer cells (Table 6.4 and 6.9). Whereas the WNT3 gene was upregulated equally in both the normal (fold differences= 1.24) and cancer cells (fold differences= 1.24) when introduced with IBS Blasto-Ag ST3. Overall, WNT3 and WNT7A genes could be used as biomarkers to investigate the role of *Blastocystis* sp. in causing inflammation in IBS patients with and without colorectal cancer.

As mentioned earlier, WNT3 has been correlated with the TCF signaling pathway. The binding Wnt ligand will inhibit the phosphorylation of β catenin. This will allow the regulation of target gene at nuclear level. This corresponds to the current study, in which TCF7 gene was upregulated in normal cells when introduced with IBS Blasto-Ag ST, and also in cancer cells when introduced with both the non-IBS and IBS Blasto-Ag ST3. This further concurs with the notion that the Blasto-Ag ST3 from IBS isolates could influence the β catenin (canonical) pathway in IBS patients by regulating WNT3 and TCF signaling.

The β catenin-TCF complex will then regulate the nuclear target genes which are directly involved in proliferation and migration of cells (Clevers, 2006). In present study, the target gene FOSL1 (also known as FRA1) was found to be upregulated only in cancer cells exposed to IBS Blasto-Ag ST3. This shows that *Blastocystis* sp. from IBS patients appear to be more pathogenic and has a tendency to further exacerbate cancer especially in IBS patient with colorectal cancer. This coincides with our previous data in this chapter that the proliferation and migration of cells *in vitro* were significantly increased in both normal and cancer cells introduced with IBS Blasto-Ag ST3 (Figure 6.3-6.6).

Apart from the involvement of Wnt ligands in the canonical pathway, target genes as well as receptors and co-receptors (FZD1, FZD3, FZD6 and FZD8) (Table 6.1) do play a vital role in the inhibition of β catenin phosphorylation in cells (β catenin phosphorylation will inhibit gene transcription in the nucleus) (Figure 6.1). Previous studies had reported on the upregulation of FZD1, FZD3, FZD6 and FZD8 in gastric cancer cells, hepatocellular carcinoma cells (HCC) and in breast cancer cells (Kirikoshi et al., 2001; Holcombe et al., 2002; Milovanovic et al., 2004; Bengochea et al., 2008). In the present study, FZD3 and FZD8 were significantly upregulated in normal cells introduced with IBS Blasto-Ag ST3. Meanwhile, FZD1 and FZD8 were upregulated upon exposure of both the non-IBS and IBS Blasto-Ag. Frizzled genes basically act as receptors for Wnt ligands in order to initiate the signaling cascade (Bhanot et al., 1996). This will lead to the inhibition of β catenin phosphorylation which then regulates the target gene in the nucleus which results in abnormal growth of cells (cell proliferation and cell migration).

6.4.3.2 Non-canonical pathway

Apart from that, non-canonical Wnts (WNT5A and WNT11) activate the non-canonical planar cell polarity pathway which is responsible for the cell movement (Heisenberg et al., 2000). Studies have shown that the non-canonical Wnts can trigger antagonism of the canonical pathway (Torres et al., 1996; Kühl et al., 2001; Ishitani et al., 2003). WNT11 gene, known as endogenous ligand helps in activating the pathway in *Xenopus laevis* (Tao et al., 2005) and was seen to be upregulated in prostate cancer patients (Thompson et al., 2010). The WNT11 gene was also reported to be highly expressed in hepatocellular carcinoma cells (HCC) (Kim et al., 2008). Meanwhile, the functional role of WNT5A still remains unclear as it was found to be downregulated in pancreatic cancer but upregulated in breast, lung and prostate cancer (Iozzo et al., 1995; Lejeune et al., 1995; Crnogorac-Jurcevic et al., 2001).

In this research, WNT11 which is the key gene of the calcium pathway (sub-category of non-canonical pathway) was significantly upregulated in normal cells introduced with IBS Blasto-Ag ST3 compared to non-IBS Blasto-Ag ST3 (Table 6.5). This shows that Blasto-Ag ST3 derived from IBS patients does play a role in calcium pathway which is involved in cell signaling in normal cells. Calcium also known as a second messenger plays an important role in the Wnt signaling pathway. Prolonged increase in calcium in cells can result in proliferation while short term in increases of calcium can cause cell death (Mattson & Chan, 2003; Orrenius et al., 2003; Lipskaia et al., 2009). This could be a probable method employed by the IBS Blasto-Ag ST3 in order to enhance the proliferative and migration effect seen in normal cells as depicted by figure 6.3 and figure 6.5. In contrast to WNT11, WNT5A did not show any significant difference in normal cells. In cancer cells, WNT5A and WNT11 were

downregulated upon exposure to both non-IBS and IBS Blasto-Ag ST3 suggesting less effect played by the Blasto-Ag on the key genes of the calcium pathway. In other words, in colorectal cancer cells, *Blastocystis* sp. plays greater role in Wnt-canonical pathway compared to non-canonical pathway for the exacerbation of cell proliferation and migration. This speculation correlates with reports of numerous findings that pathogenesis of colorectal cancer is triggered by mutation and dysregulation in canonical Wnt pathway (Kirikoshi & Katoh, 2002; Yoshioka et al., 2012).

Apart from the calcium pathway, genes involved in the planar cell polarity pathway (another subgroup of non-canonical pathway), involved in cell morphology and migration such as DVL1, DVL2, NKD1 and NKD2 were also assessed in this study. In normal cells, DVL2, NKD1 and NKD2 genes were upregulated upon exposure of IBS Blasto-Ag ST3 compared to non-IBS Blasto-Ag (downregulated). Similar to this observation, previous studies have reported on the upregulation of DVL2, NKD1 and NKD2 genes in human and mouse colorectal cancers tissues (Yan et al., 2001; Caldwell et al., 2010; Huang et al., 2013; Chang et al., 2015). This implies that the IBS Blasto-Ag ST3 has the ability to trigger the cancer features of normal colon cells by changing cell polarity which subsequently elevates the ability to migrate as evident in the graph of (Figure 6.5)

Meanwhile, in cancer cells, the NKD1 gene was upregulated when introduced with IBS Blast-Ag ST3, whereas the DVL1 gene was significantly upregulated upon exposure of both the non-IBS and IBS Blasto-Ag ST3. High expression of DVL1 in prostate (Mizutani et al., 2005) and non-small lung cancers (Wei et al., 2008) have been reported previously. In the current research project, the NKD and DVL genes (DVL1, DVL2, NKD1 and NKD2) have been listed in both canonical and non-canonical planar

cell polarity pathways (Table 6.4, 6.5, 6.9, and 6.10). This is due to the fact that these genes are recognized as a family of important regulators which act as intermediates in canonical Wnt/β-catenin signaling as well as in non-canonical Wnt signaling pathways. Nevertheless, studies commonly recognize their role in non-canonical planar cell polarity.

6.5 Conclusion

IBS Blasto-Ag ST3 triggers a higher proliferation and migration of cells in both normal and cancer cells. The effect was seen higher in both cells upon exposure of IBS Blasto-Ag ST3. This implies that *Blastocystis* sp. ST3 may play a role in advancing inflammation in IBS patients with and without colorectal cancer. This could also result in tumor progression. Higher expression of genes in the canonical pathway can be the key factors in the cell proliferation and migration of cells which can eventually lead to cancer progression.

Observation from the Wnt analysis showed that a large number of genes were involved in the canonical pathway (inhibition of β catenin phosphorylation) in both normal (56%) and cancer cells (55%) regardless of the groups (non-IBS and IBS). These results correspond to numerous studies done demonstrating that canonical Wnts in the canonical pathway play a major role in cancer progression compared to the non-canonical pathway. Therefore, this is the first study to show the role of *Blastocystis* sp. in regulating the β catenin/canonical pathway especially in relation to colorectal cancer.

IBS Blasto-Ag ST3 was shown to have a higher effect in both normal and cancer cells. Greater effects exhibited by IBS Blasto-Ag ST3 suggests that it can play a role in the activation of the Wnt canonical pathway which regulates the transcription of target gene in the nuclear level resulting in abnormal cell proliferation and cell migration (seen in the first part of this chapter). This shows that *Blastocystis* sp. ST3 from IBS patients could be more pathogenic in causing the activation of the canonical pathway. Chapter 4 showed that *Blastocystis* sp. ST3 appeared to be phenotypically different compared to *Blastocystis* sp. from asymptomatic and symptomatic isolates. This could be due to the

differing gut conditions. Therefore, such gut conditions could result in different expression of genes in which the IBS Blasto-Ag ST3 had greater effects than Blasto-Ag from non-IBS patients (asymptomatic and symptomatic isolates). Moreover, *Blastocystis* sp. ST3 cysts derived from IBS patients inoculated in rats were shown to results in have a greater mucosal sloughing, inflammation and necrosis of tissue. This could be due to the activation of certain genes that may play a role in pathogenesis of inflammation.

In this study, several canonical Wnt genes were highly upregulated in normal cells compared to cancer cells upon exposure to IBS Blasto-Ag. Canonical Wnts such as WNT3A, WNT3, WNT4, WNT6, WNT7A and WNT10A were seen to be significantly upregulated in normal cells when introduced with IBS Blasto-Ag. Besides that, both non-canonical planar cell polarity (NKD1, NKD2 and DVL2) and calcium pathway (WNT11) were also seen to be upregulated in normal colon cells. Generally, the non-canonical planar cell polarity pathway plays an important role in regulating the cytoskeleton causing the shape differences in cells which is very much associated with cell migration. During gastrulation, the non-canonical planar cell polarity pathway also helps in the cell movements (Heisenberg et al., 2000). Meanwhile the non-canonical calcium pathway regulates calcium release from the endoplasmic reticulum inside the cells which is important in cell movements during gastrulation (Kühl et al., 2001). Hence, results from this chapter show that IBS Blasto-Ag ST3 can trigger the cell signaling in normal cells which can promote cell proliferation and cell migration (Figure 6.4 and 6.6). Therefore, the canonical and non-canonical (planar cell polarity and calcium pathways) play a significant oncogenic role in causing normal cells to become cancerous upon exposure to *Blastocystis* sp. ST3 from IBS patients.

Meanwhile, in cancer cells, exposure to Blasto-Ag ST3 had an influence on genes of the canonical and the non-canonical planar cell polarity pathways. Canonical Wnts such as WNT3 and WNT7A were the only genes upregulated in both normal and cancer cell upon exposure of IBS Blasto-Ag ST3. Besides that, NKD1 and DVL1 genes were found to be upregulated in cancer cells upon exposure of IBS Blasto-Ag ST3. Blasto-Ag ST3 from IBS isolates could influence the β catenin (canonical) pathway as well as non-canonical planar cell polarity pathway in IBS patients by regulating these genes.

A large set canonical Wnts which were expressed in the canonical pathway were also seen to be expressed in the non-canonical pathway, especially calcium pathway, in both normal and colon cancer cells upon exposure to Blasto-Ag, suggesting that these ligands may play a dual role in activating the Wnt signaling pathway by regulating the gene transcription (canonical) and non-canonical calcium pathway. The summary of results and implications obtained from this chapter has been illustrated in figure 6.9 and figure 6.10.

Currently, treatments for colorectal cancer are still lacking and there is a need for a few new therapeutic approaches, as well as improved methods of diagnosis and prognosis. Chemotherapy has been used as an option to decrease colorectal cancer death while studies on developing natural or synthetic chemotherapy drugs are still ongoing (Madka & Rao, 2013). Knowledge on the expression of genes in both normal and colon cancer cells upon exposure of Blasto-Ag ST3 shed some light on new diagnostic and prognostic markers as well as therapeutic relevance.

This study proposed the development of novel Wnts ligands especially WNT3 and WNT7A so that they can be used as novel biomarkers to inhibit the β catenin/TCF complex formation which then further inhibits nuclear gene transcription as well as cell proliferation and cell migration. These genes can also be considered as candidate markers to detect inflammation or cancer progression in IBS patients especially those infected with *Blastocystis* sp. The molecular approach of using biomarkers based on the knowledge of the Wnt pathway can assist in major advancements in diagnosis of colorectal cancer especially in IBS patients as they have a higher risk of developing cancer. Besides that, research on the development of novel anti-tumor drugs pointing its molecular mechanism are increasing (Jackson et al., 1999). Therefore, drugs targeting this pathway can be developed in order to prevent the progression of tumour in IBS patients with and without colorectal cancer.

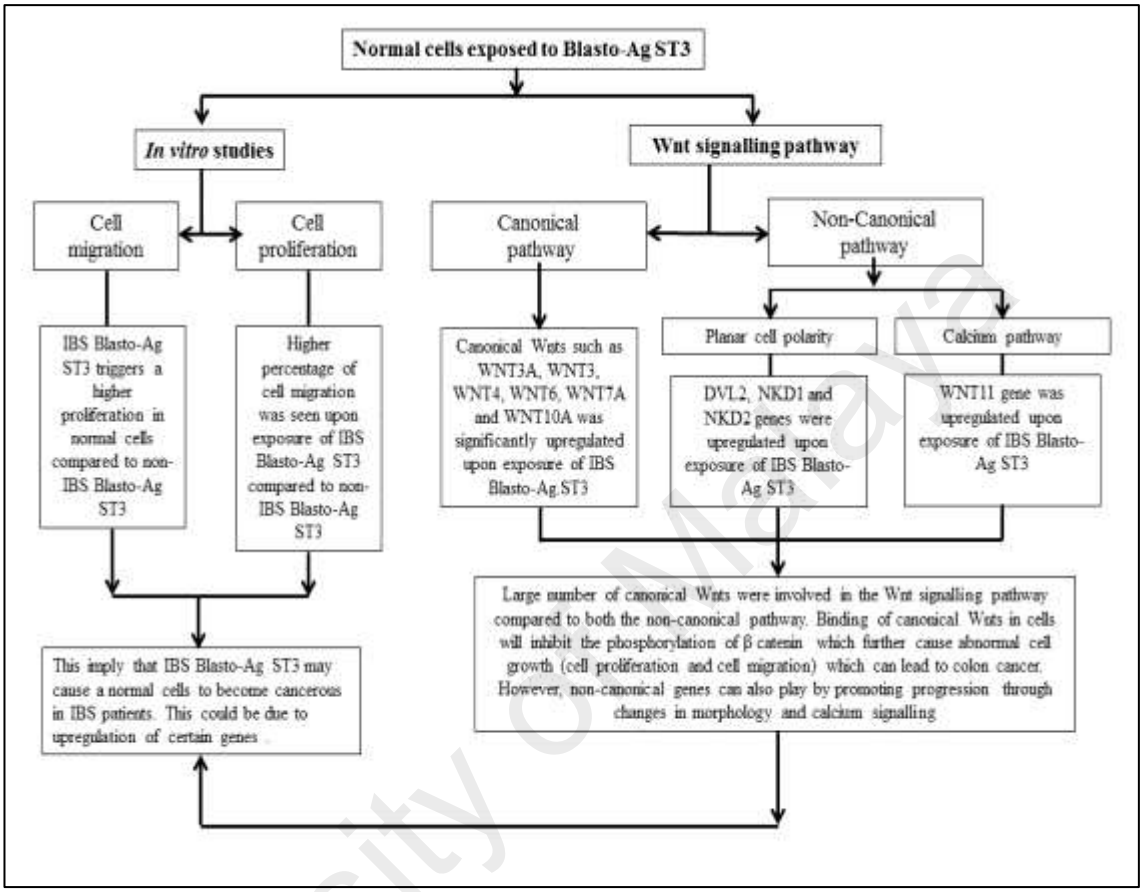

Figure 6.9: Illustration on the overview of results obtained based on normal cells.

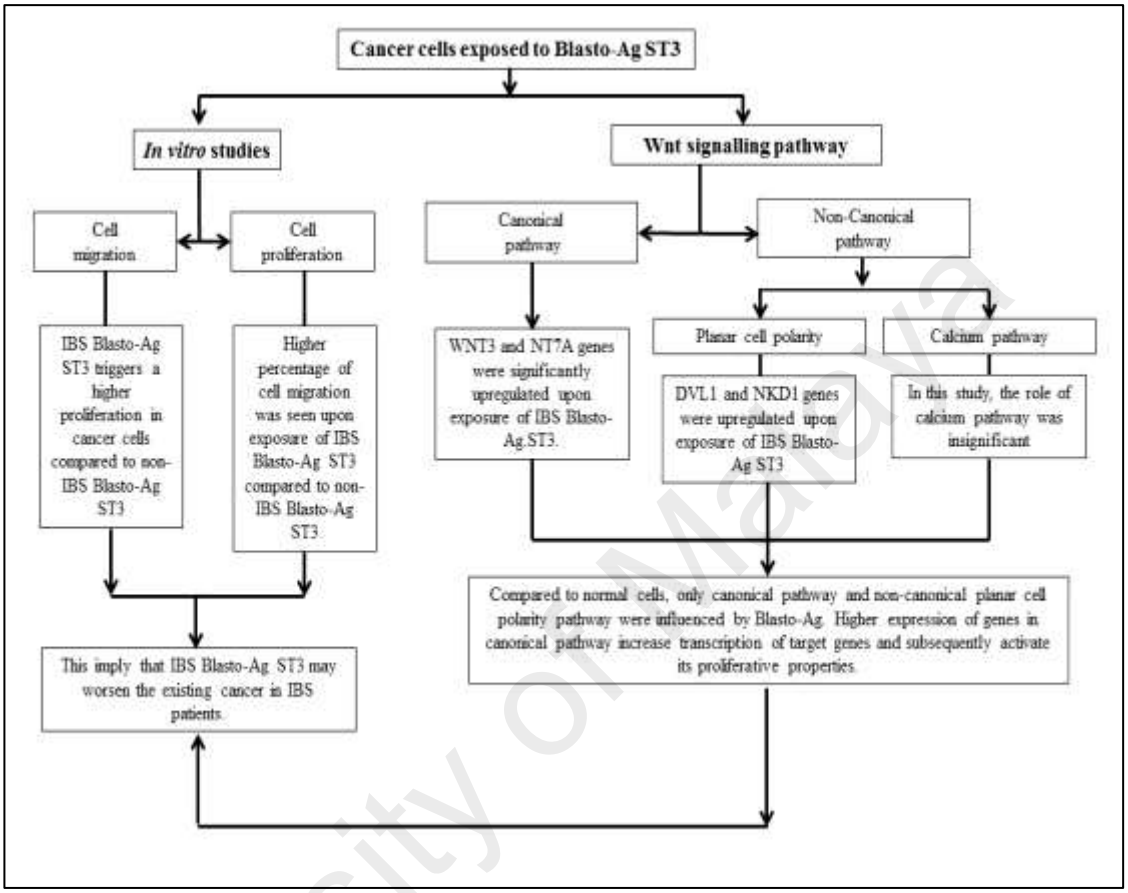

Figure 6.10: Illustration on the overview of results obtained based on cancer cells

Chapter 7: Studies to assess the factors that influence the shedding of *Blastocystis* sp. ST3 cysts in an irritable bowel syndrome patient- An evidence based case study.

7.1 Introduction

In western countries, the most used diagnostic test to screen stool samples for intestinal parasites in parasitology laboratories is by microscopic examination. The phenomenon of irregular shedding reported previously seen in *Blastocystis* sp. can pose a challenge when detecting the parasite during stool test. Despite stools being negative for *Blastocystis* sp. when examined using direct microscopy and *in vitro* culture methods for days, they can yet revert to becoming positive. This will surely influence the detection of the parasite.

The cystic stage of *Blastocystis* sp. is the infective stage and can be transmitted from one host to another. It was shown previously that cyst shedding can be influenced by metronidazole treatment which increased the number of granular forms when stools from treated patients were cultured (Haresh et al., 1999). Balakrishnan and Kumar's (2014) findings concurred with the previous study by showing that metronidazole induced cells to develop granular forms in order to survive. Despite this, metronidazole continues to be considered as the drug of choice for irritable bowel syndrome (IBS) patients (Stensvold et al., 2009) and other patients showing condition such as dermatological disorder and infected with *Blastocystis* sp. (Valsecchi et al., 2004; Katsarou-Katsari et al., 2008). The finding influences the effectiveness in treating patients especially IBS patients infected with *Blastocystis* sp. A lot of money and

resources have been spent seeking alternative treatment approaches but none thus far have shown any effect on the infected patient.

Results from the current previous study (Chapter 3) demonstrate a positive association between *Blastocystis* sp. and IBS. Stool aspirate samples were shown to be an alternative sample source to detect this parasite via the PCR. The most predominant subtype was shown to be ST3. It is important therefore to also assess the factors that could influence the shedding of *Blastocystis* sp. cysts. Stool samples were collected from an IBS patient positive for *Blastocystis* sp. ST3 for duration of 30 days. A questionnaire reflecting the time of the day, consistency of stool, the emotional status and the type of food consumed prior to defecating was developed. The factors assessed were a) the frequency of visiting the toilet in a day, b) the timing of toilet visits, c) the stool forms, d) the type of mood the patient was in whilst frequenting the toilet, and e) food intake (Appendix 5).

7.2 Materials and methods

7.2.1 Patient's background

Apart from the questionnaire, her emotional level was also assessed through direct phone conversations and face to face interviews at her residence. She had a history of abdominal pain which usually extended a few inches particularly in the evening for the past 15 years contributing to discomfort and inconvenience. She has sought medical help on numerous occasions but was not diagnosed for any other pathogenic parasites, bacteria and viruses. In the laboratory, it was shown that *Blastocystis* sp. was the only organism present in her stool.

She was a housewife with two children and finds time to exercise like running for 7 to 9 km in the morning once in a while to keep her fit, and reported to be careful with intake of food to reduce gastrointestinal symptoms. She has visited almost 40 countries as a backpack traveler and has taken the opportunity to visit many rural and outskirt parts of the respective country sides.

7.2.2 Sample source

A total of 79 stool samples were collected over a period of 30 days from a female patient aged 42 suffering from irritable bowel syndrome (IBS) confirmed by Rome III Criteria (Longstreth et al., 2006). The samples were sent to the laboratory within 24 hours of collection.

7.2.3 Culture of sample

Approximately 50mg of sediment was cultured in 3ml Jones' medium supplemented with 10% horse serum and kept at 37°C. The culture was examined for *Blastocystis* sp. after 24, 48 and 72 hours under light microscopy.

7.2.4 Subtyping of *Blastocystis* sp.

The genomic DNA of *Blastocystis* sp. for the stool samples collected were extracted using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) based on the manufacturers' protocol. The sample was subjected to sequence tagged site (STS) primer-polymerase chain reaction (PCR) using the seven sets of primers previously described (Yoshikawa et al., 2004b). Two to five microliters of DNA preparations were used to amplify the genomic sequences in a 20µl reaction containing 1x PCR buffer (Fermentas, USA). PCR conditions consisted of 1 cycle of initial denaturing at 95^0C for 5 min, followed by 40 cycles of denaturing at 95^0C for 1 minute, annealing at 56.3^0C for 1 minute 30 seconds and extending at 72^0C for 1 minute, and an additional cycle of elongation at 72^0C for 10 min (Thermocycler Biorad). The amplified products were then electrophoresed in 1.5% agarose gels (Promega, USA) in Tris–borate-EDTA

buffer. Gels were stained with ethidium bromide and photographed using an ultraviolet gel documentation system (Uvitec, United Kingdom). PCR amplification for each primer pair was done in triplicate.

7.2.5 Cysts counting

Cysts were isolated using the Ficoll-Paque technique according to (Zaman & Khan, 1994). One g of the fecal sample was dissolved in phosphate buffered solution (PBS) pH 7.4 and filtered using gauze. The sample was then centrifuged at 3000rpm for 10 min. The supernatant was discarded and 4ml of the sediment were then layered on 5ml Ficoll-Paque solution (Figure 7.1). The sample was centrifuge at 3600rpm for 20 min. Four layers were formed and the cysts were harvested using a fine pipette from the second top layer and washed twice with PBS. *Blastocystis* sp. cyst count per gram of stool was carried out using a hemocytometer chamber (Improved Neubauer, Hausser Scientific) with 0.4 % trypan blue dye exclusion (Sigma-Aldrich) (Figure 7.2).

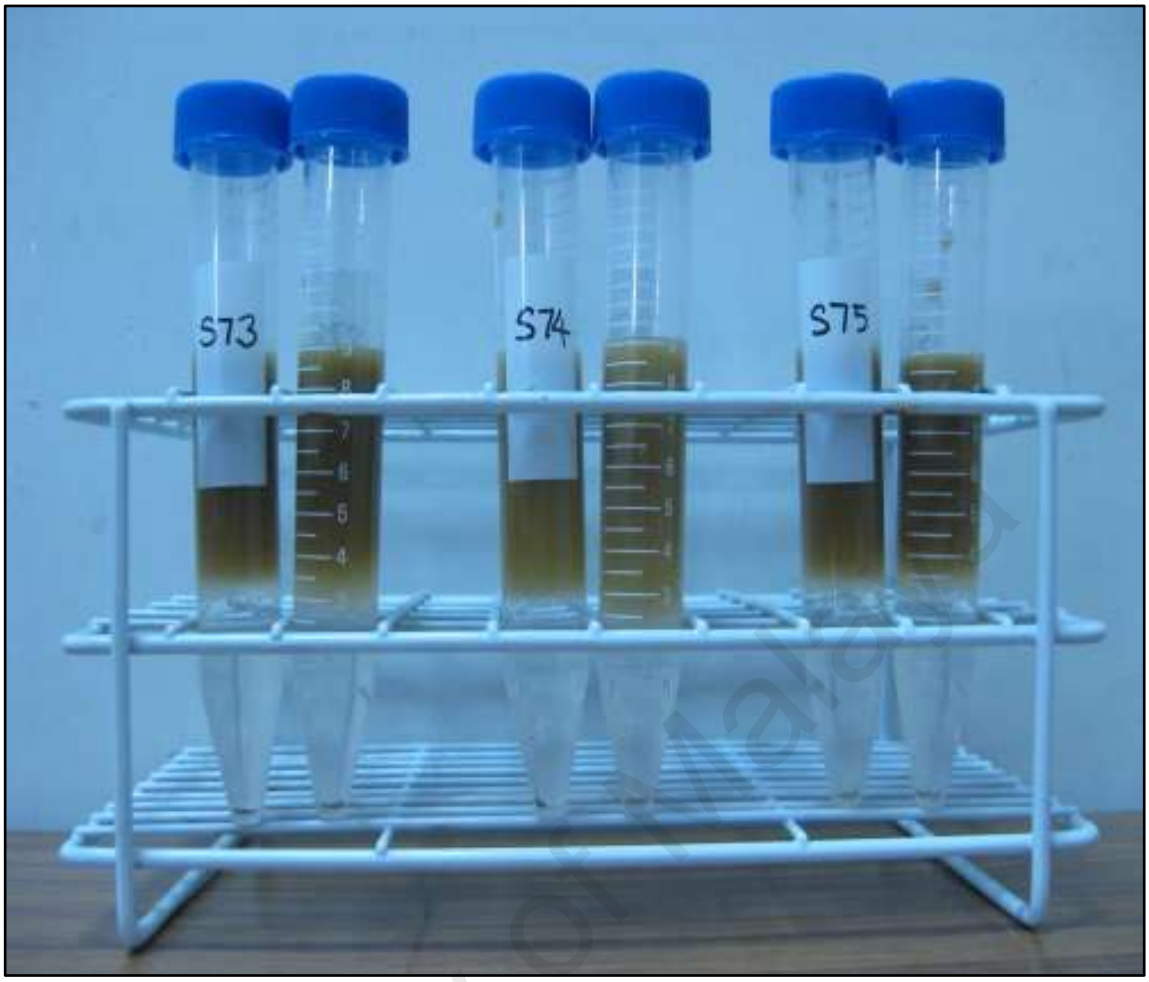

Figure 7.1: Ficoll-Paque of the samples.

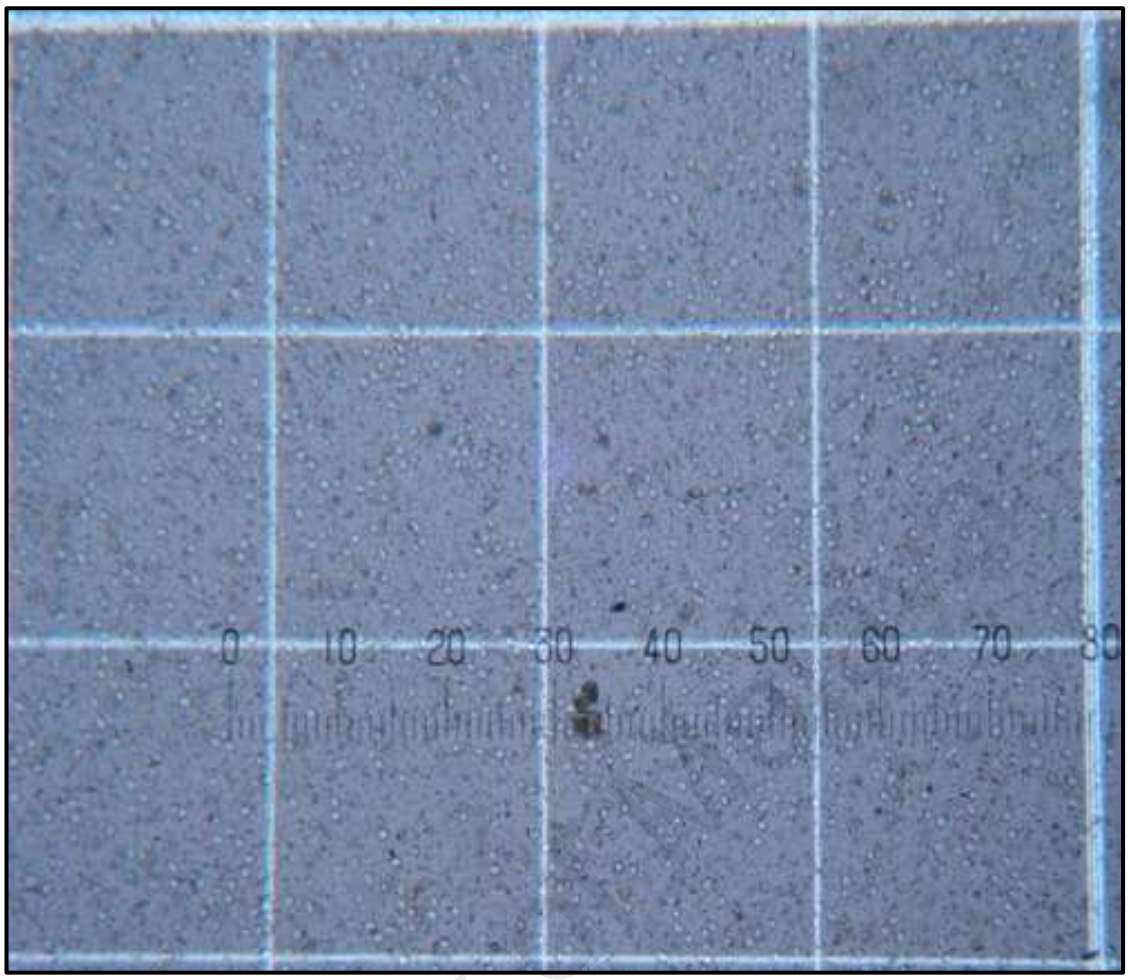

Figure 7.2: *Blastocystis* sp. cyst count per gram of stool using a hemocytometer chamber

7.2.6 Questionnaire

A questionnaire reflecting the time of the day, consistency of stool, the emotional status and type of food consumed prior to frequenting the toilet was developed. The factors assessed were a) the frequency of frequenting the toilet in a day, b) the timing of toilet visits, c) the stool forms, d) the type of mood the patient was in whilst frequenting the toilet, and e) food intake (Appendix 5).

7.2.7 Determining the time of day frequenting the toilet.

The questionnaire categorized into 4 different slots namely between:-

a) 6am-11.59am

b) 12pm-5.59pm

c) 6pm-11.59pm

d) 12pm-5.59am

7.2.8 Determining the stool forms.

The patient was asked to record the form of the stools each time after frequenting the toilet. The stool forms were classified into 4 different categories:-

a) Loose

b) Soft

c) Semisolid

d) Hard form.

7.2.9 Moods and emotions

The patient was requested to fill up the score using the scale provided for her emotional status as below:-

1: very sad/very depressed, very anxious, very frustrated

2: sad/ depressed/ anxious/ frustrated

3: normal/ unemotional

4: very happy

5: happy

6: Others (Any unusual events)

7.2.10 Food intake

The patient was asked to record the type of food consumed before frequenting the toilet each time in a day. Food intake refers to the type of food and not the quantity of food consumed.

7.2.11 Vacuolar form of *Blastocystis* sp.

A direct smear of the specimen was made for each stool sample received in the laboratory and vacuolar forms were counted on an average of 20 fields using 40X light microscopy.

7.3 Results

7.3.1 Stool samples

A total of 79 stool samples were collected over a period of 30 days. 79 questionnaires were respectively filled up whenever patient frequented the toilet. Stool samples were confirmed positive for *Blastocystis* sp. by *in vitro* culture method (Tan & Suresh, 2006). The patient was found negative for all other pathogenic parasites, bacteria and viruses.

7.3.2 Subtyping

Based on subtyping done with the STS primers, the sample amplified with the primer SB227 (~526bp) (Yoshikawa et al., 2004b). The samples were found to be ST3.

7.3.3 Cysts shedding pattern for the period of 30 days

The concentration of cysts shed throughout the 30 days for the 79 stool samples collected was 40.01X10^6cysts/g. The highest number of cysts shed was on day 29, 15.38X10^6cysts/g. The questionnaire when analyzed revealed that the frequency of the patient frequenting the toilet varied from once to five times a day. The frequency of frequenting the toilet once a day was found to occur only once in the 30 days and at this time only 0.09X10^6cysts/g were shed. The number of cysts shed where the patient went to the toilet twice a day was 16.19X10^6cysts/g. The number of cysts shed when frequenting 3, 4 and 5 times were 22.24X10^6cysts/g, 1.26X10^6cysts/g and 0.22 X10^6cysts/g respectively (Table 7.1).

7.3.4 The association between the time of frequenting the toilet with the shedding pattern of cysts.

Another factor that was observed influencing the shedding pattern of cysts was the time of day frequenting the toilet. The concentration of cysts shed from 6am-11.59am was 21.66X10^6cysts/g with total of 51 stool cups, followed by 12pm-5.59pm, 15.41X10^6cysts/g in 9 stool cups collected. 2.94X10^6cysts/g was shed in total of 19 stool cups collected for the timing from 6pm to 11.59pm. The patient did not have the urge to visit the toilet from 12am-5.59am (Table 7.2).

Table 7.1: Concentration of cysts shed based on the frequency frequenting to the toilet

Frequency frequenting to the toilet	Total Days	Concentration of cysts ($X10^6$cysts/g)
Once a day	1	0.09
Twice a day	16	16.19
Three times a day	8	22.24
Four times a day	3	1.26
Five times a day	2	0.22

Table 7.2: The time of toilet visits

Timing	Total stool cups	Concentration of cysts ($X10^6$cysts/g)
6am-11.59am	51	21.66
12.00pm-5.59pm	9	2.94
6.00pm-11.59pm	19	15.41
12am-5.59am	Nil	nil

7.3.5 Irregular shedding of parasite

Irregular shedding of *Blastocystis* sp. cysts were observed in 10 out of 30 days, both by direct microscopy and *in vitro* culture method. On day 2, the cyst range was from 0-0.028X10^6cysts/g, day 3, 0-0.036X10^6cysts/g, day 7, 0-0.012X10^6cysts/g, day 8, 0-0.12X10^6cysts/g, day 11, 0-0.004X10^6cysts/g, day 16, 0-0.012X10^6cysts/g, day 17, 0-1.20X10^6cysts/g, day 22, 0-0.076X10^6cysts/g, day 27, 0-0.68X10^6cysts/g and day 28, 0-0.184X10^6cysts/g (Table 7.3).

7.3.6 The association between the stool form with the shedding pattern of cysts.

An average count of 2.00X10^6cysts/g, was the highest was seen in semi formed stools in 22 stool cups collected. Soft stools in 31 stool cups, showed an average count of 0.42X10^6 cysts/g followed by average number of cysts in 18 stool cups, 0.12X10^6cysts/g in loose formed stools and average number of cysts in 8 stool cups, 0.11X10^6cysts/g in hard formed stool (Table 7.4).

Table 7.3: Range in number of cysts shed during irregular shedding within a day

Day	Cyst range ($X10^6$ cysts/g)
Day 2	0-0.028
Day 3	0-0.036
Day 7	0-0.012
Day 8	0-0.120
Day 11	0-0.004
Day 16	0-0.012
Day 17	0-1.200
Day 22	0-0.076
Day 27	0-0.680
Day 28	0-0.180

Table 7.4: Average number of cysts shed based on the stool form

Stool forms	Total stool cups	Average number of cysts ($X10^6$cysts/g)
Loose	18	0.12
Soft	31	0.42
Semisolid	22	2.00
Hard	8	0.11

7.3.7 The association between moods and emotions with the shedding pattern of cysts.

21 (63.3%) out of 30 days stool sample collection based on questionnaire recorded a range of emotions such as anxiety, stress, sadness, frustration, anger, before surgery, post-surgical anxiety, exhaustion, and discomfort (this was due to an unrelated surgery she had to undergo during this period) whilst the remaining days were stool samples collected when the patient felt normal and happy. The average daily cyst count during the days of emotional fluctuation ranged from 0 cyst to $5.13X10^6$ cysts/g of stool compared to $0.001X10^6$ - $0.144X10^6$ cysts/g seen on normal days when the patient was happy or unemotional.

Five peaks were seen with a gradual increase of peaks as days of collection proceeded. There were recorded on days 5, 10, 13, 19 and 29. The average cyst count were $0.32X10^6$ cysts/g, $0.40X10^6$ cysts/g, $1.02X10^6$ cysts/g, $2.25X10^6$ cysts/g and $5.13X10^6$ cysts/g respectively (Figure 7.3). The five peaks in the cyst counts correlated with the patient's emotional state which ranged from high levels of anxiety and unusual bouts of worry and stress (Table 7.5).

The patient was feeling very anxious during the first peak. The second peak corresponded to extreme sadness and inner discomfort due to issues pertaining to her children. The third peak corresponded to the patient being stressed. The fourth peak to sadness as the patient was forced to bid goodbye to her good friends who were leaving the country for good. She also recorded exhaustion and noted that her stomach was bloated. The highest peak on day 29 corresponded to the patient showing no appetite to eat and felt an anxiety as she had to remove her stiches from her face following day due

to a surgery she had undergone. She was feeling exhausted, could not sleep well and was feeling extreme discomfort due to a swollen face, a result of optional plastic surgery she subjected herself.

7.3.8 Food intake

There was no association between food intake and the shedding pattern of cysts. However, based on the food intake diary, it was observed that the food consumed throughout the 30 days had a high content of carbohydrates, proteins, biogenic amines, fats and fibers.

7.3.9 Vacuolar form

The average number of vacuolar forms in 20 fields ranged from 0-13 for 30 days; however the numbers were not consistent and had no correlation to the shedding pattern of cysts.

Table 7.5: The five peaks in the cyst counts correlated with the patient's emotional state

Day	Moods and Emotions	Average daily cysts count ($X10^6$cysts/g)
5	Anxious	32.4
10	Upset/Stress	40.0
13	Stress	101.6
19	Sad	225
29	Tired / Anxious / Pain	512.6

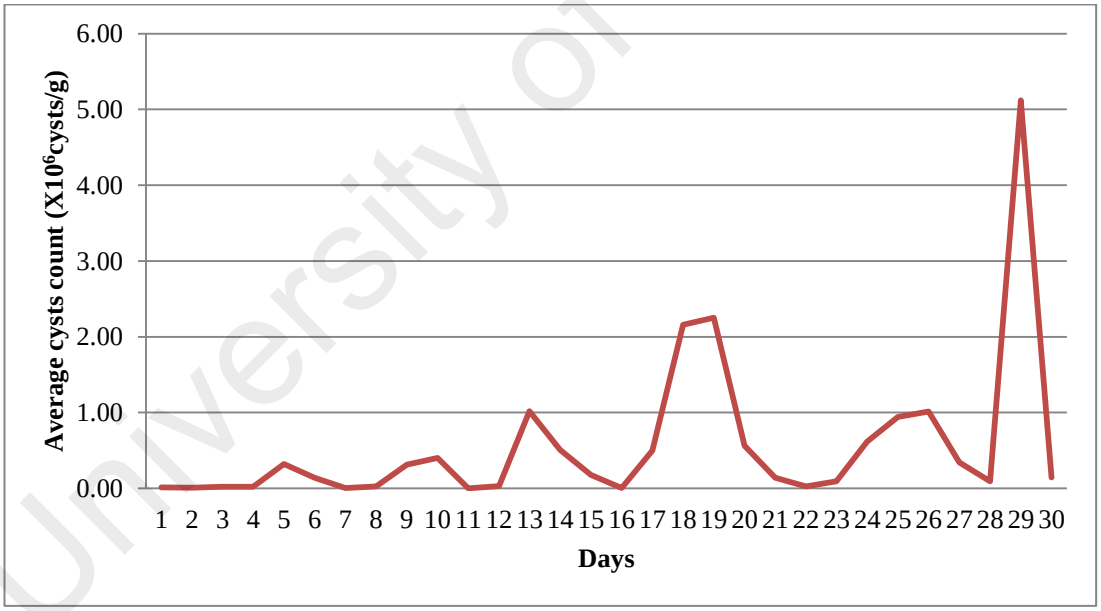

Figure 7.3: Average daily cyst count ($X10^6$cysts/g)

7.4 Discussion

Several studies have suggested that pathogenicity of *Blastocystis* sp. is subtype related notably subtype 3 (ST3) where this association was evident in Malaysia (Tan et al., 2008), Singapore (Wong et al., 2008) and the United States (Jones et al., 2009). Tan et al. (2008) also suggested that ST3 was pathogenic by comparing the phenotypic characteristics between symptomatic and asymptomatic isolates of *Blastocystis* sp. Antigen derived from *Blastocystis* sp. ST3 (Blasto-Ag ST3) was among the five other subtypes studied that triggered higher proliferation rates when introduced into colorectal cancer cells (Kumarasamy et al., 2013). There is also considerable evidence that *Blastocystis* sp. could be pathogenic and opportunistic in immunocompromised populations (Chandramathi et al., 2012; Rivero-Rodríguez et al., 2013; Paboriboune et al., 2014).

This study followed up on one IBS patient infected with *Blastocystis* sp. ST3 for the period of 30 days. The limitation of this study was that conclusions were made from one individual. It is a challenge to get patients to volunteer their stools for an entire month. Moreover the assessments on five different factors that influence cyst shedding have been consistent and it is obvious that a pattern appears to exist in the shedding of the cysts. The cyst form of *Blastocystis* sp. is known to be the infective stage and the route of transmission is by the fecal-oral route (Yoshikawa et al., 2000). The parasite will excyst in the large intestines and evolve into vacuolar forms upon ingestion. The encystation of parasites will occur in the large intestine and the cysts will be excreted in the feces (Moe et al., 1997). *Blastocystis* sp. could cause mucosal inflammation which can lead to exacerbation of the symptoms (Barbara et al., 2012).

Frequenting the toilet as much as 3 times a day saw the highest number of cysts, $22.24x10^6$cysts/g shed. Such high shedding can cause transmission if personal hygiene is not prioritized. The shedding during the morning visits to the toilet excreted $21.66x10^6$cysts/g. A cyclic pattern of the number of cysts shed peaks in the morning, reduced in the afternoon and subsequently increases slightly at night, with the numbers again reaching a peak the following morning.

The shedding becomes irregular within a day and this was observed in 10 out of 30 days where the widest range recorded on day 17, showed between 0 and $1.2x10^6$cysts/g (Table 7.3). Vennila et al. (1999) reported that stool samples should be collected 3 days consecutively due to the irregular shedding of cysts. In this present study, it was found out there was irregular shedding of cysts even within a day, implying that stool samples should be collected whenever an IBS patient frequents the toilet even within a day. The highest concentration of cysts shed was $15.38x10^6$ cysts/g on day 29 which may results in the greatest possibility of transmitting *Blastocystis* sp. ST3 infection to others especially from patients lacking personal hygiene or in close contact with others. Stool samples were collected from the patient's husband and her two children. Her husband showed positive for *Blastocystis* sp. ST3 and negative for other pathogenic parasites. Her husband however did not report of any gastrointestinal symptoms and was categorized as asymptomatic. He was calmer, did not have worries and took things as they came by. It is likely that he too like the wife whilst travelling to many countries must have acquired the infection through contaminated food and water. Chapter 4 showed phenotypic variation within ST3 and it is likely that IBS conditions exacerbated by moods and emotional fluctuations can cause microenvironment changes especially in the gut flora and this could thereby influence to trigger a pathogenic variation in ST3 isolated from both husband and wife. Amoeboid forms have been shown previously

only in ST3 isolates (Tan & Suresh, 2006) and therefore it is a possibility that these forms in IBS patients could invade the mucosal membrane, causing inflammation which then can trigger leaky gut syndrome to activate the immune system. Leaky gut syndrome occurs due to damaged intestinal wall caused not only by parasites but also infection or undigested food which leak through triggering an immune response (Kiefer and Ali-Akbarian, 2003).

Long term emotional instability, mood swings and stress can create imbalance in the body system resulting in the passing of loose and soft forms of stools with accompanying gastrointestinal symptoms. The peaks of cyst count corresponded to the days the patient experienced stress and anxiety. The average cyst count ranged from 0 to $5.13X10^6$ cysts/g when she was feeling stressed and anxious compared to $0.001X10^6$ to $0.144X10^6$ cysts/g shed during the duration when she was happy and emotionally stable. IBS patients are most likely to shed higher number of cysts when their emotional state such as anxiety disorder, depression and fear can further influence the gut motility and exacerbate the IBS symptoms (Mayer et al., 2001). Exacerbation of symptoms has been shown to occur right after food ingestion followed by sudden abdominal pain together with the urge to defecate (Simren et a., 2001). These are usually common in IBS patients. IBS patients tend to worry about the abdominal discomfort and this might influence gastrointestinal symptoms. However the present study showed no correlation between food intake and the shedding of *Blastocystis* sp. ST3 cysts.

7.5 Conclusion

The study conclusively provides evidence for the first time that the factors such as the frequency of frequenting the toilet in a day, the timing of toilet visits, the stool forms, and moods as well as emotions can influence the shedding pattern of cysts in an IBS patient. This study also suggests that stool samples for diagnostic purposes be collected in the morning and to be aware that emotions do influence shedding pattern. It is also recommended that stool samples be collected every time one frequents the toilet even within a day to avoid obtaining false results. Semi-solid stools have more cysts compared to other stool forms. The study proves that *Blastocystis* sp. ST3 identified as pathogenic in many previous studies is largely influenced by host conditions and more studies need to be carried to associate genotypic, phenotypic and pathophysiological aspects for better understanding of patient management in IBS patients infected with *Blastocystis* sp.

Chapter 8: General discussion and conclusion

8.1 General discussion

Blastocystis sp. a common intestinal gut parasite can also be an opportunistic parasite when it is present in immunocompromised patients, especially in cancer and AIDS (Horiki et al., 1999; Kurniawan et al., 2009; Chandramathi et al., 2012). Several research studies carried out have suggested that there is a possible association between *Blastocystis* sp. and irritable bowel syndrome (IBS) (Tan, 2008).

This present study aimed to assess the prevalence, immunology, biology, histopathology, and molecular biology of *Blastocystis* sp. ST3 derived from IBS patients comparing with *Blastocystis* sp. ST3 from asymptomatic individuals and symptomatic patients. This study was divided into five main parts in order to accomplish the objectives above. First, is to assess the prevalence of *Blastocystis* sp. in irritable bowel syndrome (IBS) patients by comparing the prevalence of *Blastocystis* sp. from stool and stool aspirate samples obtained from IBS and non-IBS patients. Non-IBS patients are healthy individuals. This also includes subtyping the parasite in order to assess the most predominant subtype associated with IBS. Besides this, the interleukin level (IL-3, IL-5 and IL-8) in serum samples from four different groups; non-IBS group, IBS group, non-IBS patients infected with *Blastocystis* sp. (non-IBS Blasto) group and IBS patients infected with *Blastocystis* sp. (IBS Blasto) group were measured. Second, the study was aimed at elucidating the phenotypic characteristics of *Blastocystis* sp. ST3 isolated from asymptomatic individuals, symptomatic and irritable bowel syndrome (IBS) patients. Third is to assess the histopathological changes during *in vivo* experimental infection with *Blastocystis* sp. ST3 isolated from three different groups (asymptomatic individual, symptomatic and IBS patients) in the intestinal tract of

Blastocystis sp. ST3 inoculated *Wistar* rats. The fourth is to assess the effects of solubilized antigen of *Blastocystis* sp. ST3 (Blasto-Ag ST3) derived from non-IBS and IBS patients respectively on the growth and gene expression of colon cancer cells, HCT116 *in vitro* (in comparison with normal cells, CCD-18Co). Finally, a case study was done to investigate the factors that influence the shedding of *Blastocystis* sp. ST3 cysts in an IBS patient.

Epidemiological studies to associate *Blastocystis* sp. and IBS patients will provide a better understanding on the role of this parasite in causing IBS. Thus far, two studies carried out in Thailand showed no association between *Blastocystis* sp. and IBS patients (Tungtrongchitr et al., 2004; Surangsrirat et al., 2010). Prevalence data of *Blastocystis* sp. in IBS patients can be influenced by the type of detection methods used. Besides this, the phenomenon of irregular shedding of this parasite reported previously can influence epidemiological data (Vennila et al., 1999). In order to accomplish the first objective, stool and stool aspirate samples were collected from non-IBS and IBS patients who were undergoing colonoscopy to assess the prevalence of *Blastocystis* sp. in IBS patients (Chapter 3). Four different methods were employed, direct microscopy, *in vitro* cultivation, formalin ether concentration technique (FECT) and polymerase chain reaction (PCR) to detect the presence of *Blastocystis* sp.

In the present study, the results were statistically significant in which 17% of IBS patients were infected with *Blastocystis* sp. compared to 5.5% in non-IBS patients. Moreover, the study also showed that the PCR method used on stool aspirate samples were more sensitive than direct microscopy, *in vitro* culture method and FECT in detecting *Blastocystis* sp. Parasite in the intestine might be flushed out due to laxatives and rapid colonic transit before colonoscopy resulting in an initial negative result for

direct microscopy, *in vitro* culture method and FECT. Nevertheless, in the present study, DNA of this parasite was detected via PCR method demonstrating that PCR could be a more sensitive method in detecting this parasite in IBS patients who were undergoing colonoscopy compared to other three methods.

Hence, this is the first study in Southeast Asia to demonstrate a positive significant association between *Blastocystis* sp. infection and IBS. The study introduces stool aspirate samples for the first time as another source of sample collection other than stools for the identification of *Blastocystis* sp. This offers an opportunity to capture data on the occurrence of *Blastocystis* sp. in stool aspirate samples. Stool aspirate samples collected during colonoscopy are usually performed after confirming that the patient having IBS. The samples therefore would be a better indicator for correlating the parasite with IBS. On the other hand, stool samples collected from the patients were found to be negative for *Blastocystis* sp. The result obtained indeed support the phenomenon of irregular shedding which was seen in the case of *Blastocystis* sp. Chapter 3 also revealed that the most predominant subtype for *Blastocystis* sp. was ST3. Genotype of the parasite has also been linked to pathogenicity of the parasite (Souppart et al., 2009). Moreover, many studies have been done demonstrating ST3 to be one of the most predominant subtypes found in their respective cohort group (Wong et al., 2008).

Following this, a case study was done to assess factors that can influence the shedding of *Blastocystis* sp. cysts in an IBS patient. The IBS patient was positive for *Blastocystis* sp. ST3. In present study, a total of 72 stool cups were collected from the IBS patients for over a period of 30 days (Chapter 7). Based on the questionnaire, the factors assessed were a) the frequency of frequenting the toilet in a day, b) the timing of

toilet visits, c) the stool forms, and d) the type of mood the patient was in whilst frequenting the toilet.

Shedding of *Blastocystis* sp. cysts became irregular within a day and this was observed in 10 out of 30 days. The widest range recorded was on day 17, in which a total of 3 stool cups were collected and the range was between 0 and $1.2x10^6$cysts/g. Vennila e al. (1999) had previously suggested that stools should be collected 3 days consecutively due to the irregular shedding of cysts. This study for the first time showed that there was irregular shedding of cysts even within a day. Thus, this might be due to the reason why there is a challenge in detecting this parasite in the stool which may then affect the epidemiological data. Besides that, the cysts shed were seen to be in a cyclic pattern. The number of cysts peaked in the morning, reduced in the afternoon and subsequently increased slightly at night, with the numbers again reaching a peak the following morning. Frequenting the toilet as much as 3 times a day and semi solid stool form saw the highest number of cysts shed. Therefore, Chapter 7 suggested that stool samples for diagnostic purpose should be collected in the morning, semi solid form and should be collected whenever an IBS patient frequents the toilet even within a day. This will help to minimize the chances in missing this parasite during stool testing. These findings will have implications on the diagnosis and a more reliable prevalence data to associate IBS and B*lastocystis* sp. can be obtained. The factors such as irregular shedding of cysts, stool form, the timing, and frequency frequenting the toilet seen in this present study (Chapter 7) could be due to the gut condition of the IBS patient. *Blastocystis* sp. ST3 may play a pathogenic role in worsening the condition of the IBS patients. Moreover, the gut of IBS patients has been reported to be more sensitive with altered central processing and abnormal gastrointestinal motility compared to healthy individuals (Kanazawa et al., 2011). Emotional turbulence such as anxiety disorder, and

depression in an IBS patient, can result in an imbalance in the body systems which influence the passing of stool with accompanying gastrointestinal symptoms (Mayer et al., 2001).

This study (Chapter 7) correlates emotional fluctuation to cyst shedding. A count as high as 5 million cyst shed in a day during the time when the patients had high bouts of emotional turbulence were recorded. This can provide an incredible opportunity for the transmission of cysts to others especially if there is a lack of personal hygiene.

The patient's husband was positive for *Blastocystis* sp. ST3 and negative for other pathogenic parasites. Her husband was categorized as asymptomatic because he did not report of any gastrointestinal symptoms. He was calmer, did not have worries and took things as they came by. It is likely that he too like the wife whilst travelling to many countries must have acquired the infection through contaminated food and water. This is the first study to demonstrate that moods as well as emotions can influence the shedding pattern of cysts in an IBS patient. IBS patients should be aware of their emotional state especially when submitting their sample for a stool test. The influence of moods and emotions on shedding pattern of cysts can provide a better understanding of the transmission patterns. Several studies reported that there is a pathophysiological association between the emotions and the gut which is still unclear. Therefore Chapter 7 provides clear evidence that differences of gut flora and emotional state of the patient can influence the pathogenicity of this parasite.

Previous studies done by Tan et al., 2008 demonstrated that *Blastocystis* sp. from asymptomatic individuals and symptomatic patients were phenotypically different. This could be due to the different conditions of the gut. However, the isolates used in

the study done by Tan et al. (2008) were without any molecular characterization. Hence, in Chapter 4, the study aimed to elucidate the phenotypic characteristics of *Blastocystis* sp. isolated from three different groups (asymptomatic, symptomatic and IBS) by keeping the subtype constant which is ST3. Therefore, this study allowed us to investigate if the gut environment of three different conditions, namely asymptomatic, symptomatic and IBS conditions can influence the phenotypic characteristics of *Blastocystis* sp. ST3. IBS patients have a different gut flora compared to the gut from non-IBS patients (asymptomatic individuals and symptomatic patients). *Blastocystis* sp. ST3 was isolated from the three different groups (asymptomatic individuals, symptomatic and IBS patients) through a clinical and field survey. Growth profile, generation time and average size of *Blastocystis* sp. ST3 from three different groups were assessed. The results revealed that *Blastocystis* sp. ST3 isolated from IBS patients have a distinct growth profile which falls between the asymptomatic and symptomatic growth profile. The average generation time for asymptomatic isolates is 5.97±1.52 h indicating it grew faster than the IBS isolates (average generation time: 7.56±1.06 h) and symptomatic isolates (average generation time: 9.87±2.97 h). Meanwhile, the average size of parasites from IBS isolates showed the largest diameter with a mean of 18.43±2.22μm compared to parasites of symptomatic isolates 15.54±3.02μm and asymptomatic isolates 11.76±0.82μm.

These results correspond to a study done by Tan et al., (2008) in which growth profiles and generation time have been used previously to demonstrate phenotypic differences between asymptomatic and symptomatic isolates. This present study provides evidence that the rate, and possibly the biology of reproduction could be influenced by gut conditions. Moreover, the average size of isolates derived from IBS patients are relatively larger compared to parasites from symptomatic and asymptomatic

individuals suggesting that it may have an effect on the growth conditions in IBS gut would have results in variety of size, growth rate and generation time.

Notable clumping was seen in parasites isolated from IBS patients using Modified Fields' stain but this was absent totally in all the other asymptomatic isolates. However this was partially seen in symptomatic isolates. The parasites from three different groups (asymptomatic, symptomatic and IBS) were stained using Fluorescein isothiocyanate-labelled Concanavalin A (FITC-Con A). IBS isolates showed a high FITC-Con A binding with 88-100% of cells, showing affinity fluorescence unit (AFU) of 4+ compared to symptomatic and asymptomatic isolates which showed only 3+ and 1+ respectively. Ultrastructural studies of *Blastocystis* sp. ST3 isolates from three different groups using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were done to further study the surface characteristics of this parasite. The surface of parasites from IBS isolates appear to be folded and coarse as seen by SEM and its surface coat seen more prominently thicker by TEM studies compared to the other two groups (asymptomatic and symptomatic). The parasites from IBS isolates also showed to have an electron dense material which was absent in any of the parasites from both the asymptomatic and symptomatic isolates.

The clumping of the parasite seen in IBS isolates stained with Modified Fields' stain could be due to the sticky surface. IBS isolates showing high FITC-Con A binding further confirmed that this parasite had a prominent surface since it showed AFU of 4+ compared to symptomatic and asymptomatic isolates. Ultrastructural studies provide more evidence on the characteristics of the surface of parasites from IBS patients. The coarse and uneven surface with folding seen in parasites of IBS isolates supports the previous results showing high FITC Con A binding of cell surface carbohydrate. This

implies that the surface texture of the cell wall could facilitate the adherence of bacteria to the parasite surface coat which could influence the parasite's pathogenicity. Apart from that, aggregation and clumping was seen in stained smears provides evidence for a sticky surface. This sticky surface could assist in the adherence of bacteria as well as to the intestinal lining which can exacerbate the inflammation of gut and gastrointestinal symptoms in IBS patients. Moreover, the thicker surface coat seen in IBS isolates suggests that it can play a role by influencing the cytopathic effect of *Blastocystis* sp. towards the intestinal lining of the gut. The *Blastocystis* sp. ST3 in the IBS patient reported in the case study done in Chapter 7 could possess the same characteristics which were seen in Chapter 4. Therefore, the parasite in the IBS patient could play a pathogenic role in contributing to the development of gastrointestinal symptoms.

This study is the first to investigate the muscle twitching differences using ileum of Balb/C mice and rabbit. The ileum of Balb/C mice and rabbit were introduced with five different concentrations of solubilized antigen of *Blastocystis* sp. ST3 (Blasto-Ag ST3) derived from three different groups (asymptomatic, symptomatic and IBS). The concentrations of Blasto-Ag ST3 (0.2, 0.4, 0.6, 0.8 and 1.0mg/ml) were introduced directly to the ileum which was suspended in an organ bath connected to Powerlab Programme. The optimal muscle twitching was based on the sensitivity of both the ileum in response towards the Blasto-Ag ST3. An average twitching rate per minute of both the rabbit and Balb/C mice's ileum upon exposure of the Blasto-Ag ST3 was recorded. Rabbit's ileum showed no effect of muscle twitching upon exposure of 0.2, 0.4, 0.6 and 0.8mg/ml for the Blasto-Ag ST3 derived from three different groups (asymptomatic, symptomatic and IBS) but 1mg/ml Blasto-Ag ST3 showed to a have an effect on the rabbit's ileum. Moreover, rabbit's ileum exposed to IBS Blasto-Ag ST3 showed to have the highest number of muscle twitching compared to the Blasto-Ag ST3 from the other

two groups. However, for Balb/C mice ileum, all five concentration of Blasto-Ag ST3 showed to have an influence on the muscle twitching of the mice's ileum. Ileum introduced with IBS Blasto-Ag ST3 showed to have the highest twitching per minute when 1.0mg/ml of Blasto-Ag ST3 was introduced compared to Blasto-Ag ST3 derived from asymptomatic and symptomatic isolates.

Several studies reported that the destruction of the epithelial barrier could be due to visceral pain associated with IBS which causes increased in gut sensitivity and motility. Para-cellular permeability which was associated with the destruction of tight junctions (TJs) was seen to be increased in colonic biopsies obtained from IBS patients. (Piche et al., 2009). Furthermore, visceral hypersensitivity in IBS patients are more consistent resulting in high-amplitude in stimulating contractions compared to healthy individuals (Kellow et al., 1987; Simrén et al., 2000). Activation of mast cells (degranulation) due to enteric infection can further trigger the hypersensitivity of the gut in IBS patients. Therefore, the present study postulated that *Blastocystis* sp. ST3 derived from the IBS patients might have secreted biologically active products such as serotonin and proteases during the mast cell degranulation in the infection site. Studies have shown the association between proteases and the pathogenicity of *Blastocystis* sp. (Sio et al., 2006; Abdel-Hameed & Hassanin, 2011). In the present study, increases in the number of muscle twitching per minute of rabbit's and mice's ileum introduced with IBS Blasto-Ag ST3 (Chapter 4) was seen which could be due to the pathogenic effect of *Blastocystis* sp. ST3 derived from IBS the effect of mast cell degranulation. Furthermore, degranulation of mast cells due to gut infection can further release a large amount serotonin and proteases (active biology products due to mast cell degranulation) which influence the gut motility and further exacerbate the gastrointestinal symptoms. This implies that mast cell degranulation due to *Blastocystis* sp. infection can play a major role in regulating

intestinal movement by increasing the gut contraction and further causing pathophysiologic disturbances in IBS patients, which was seen the muscle twitching of the ileum. This could be the reason why the IBS patients frequent the toilet up to five times a day (Chapter 7).

Therefore, this is the first study to show the association between the hypersensitivity of ileum relating in IBS patients infected with *Blastocystis* sp. by using an animal model and IBS patient. This is also the first study to suggest that Balb/C mice can be a better animal model than using the ileum of rabbit as it is cheaper and easier to handle. An optimal response was seen in Balb/C mice even with the lowest concentration of Blasto-Ag ST3, 0.2mg/ml but such response was not seen in rabbit's ileum (Chapter 7). This implies that the *Blastocystis* sp. from IBS patients is more pathogenic compared to *Blastocystis* sp. derived from asymptomatic and symptomatic.

Chapter 4 provides evidence that the gut condition can influence the phenotypic characteristics of this parasite. This is the first study done demonstrating the phenotypic variation within the same subtype of ST3. Moreover, *Blastocystis* sp. ST3 isolated from IBS patients were more pathogenic and has the ability to influence the cytopathic effect on the intestinal lining of the host, which can result in inflammation of the gut compared to asymptomatic and symptomatic isolates.

Innate immunity is activated immediately to protect the host cells from intrusion and infection during a gut infection. Therefore, the first response of the immune system is inflammation. Adherence of parasite towards the intestinal lining as well as the presence of proteases and serotonin secreted by the parasite could lead to inflammation of intestinal gut. Chapter 4 had showed that *Blastocystis* sp. ST3 derived from IBS patients

possessed different phenotypic characteristics which may facilitate the adherence of this parasite towards the intestinal gut. Evidence on the sticky surface of the *Blastocystis* sp. ST3 from IBS patients (Chapter 4) can further enhance the adherence of this parasite. Therefore, the invasion of this parasite to sub-mucosa can occur due to increased intestinal permeability which then triggers inflammation. Besides that, amoeboid forms have been shown previously to be present only in ST3 isolates (Tan and Suresh 2006). This amoeboid form can play a possible role in mucosal membrane invasion, causing inflammation which then can trigger leaky gut syndrome to activate the immune system in an IBS patient. Several studies had also suggested that amoeboid forms can be linked with protease activities which possess a higher pathogenic potential by further exacerbating the gastrointestinal symptoms in the host (Tan & Suresh, 2006; Zhang et al., 2012; Rajamanikam & Govind, 2013). The present study was able to demonstrate an association between the activation of the immune system and inflammation in IBS patients infected with *Blastocystis* sp.

The results (Chapter 3) demonstrate that the interleukin levels (IL-3, IL-5 and IL-8) were higher in IBS than non-IBS group. This implies that *Blastocystis* sp. infection can have an effect on inflammatory cytokines released by the cells and may influence the immune system in IBS patients. Chapter 4 provides more evidence on the activation of the immune system due to *Blastocystis* sp. ST3 infection in IBS patients. The present study elucidates the histopathological changes such as mucosal sloughing, inflammation and necrosis of tissue of the rats inoculated with *Blastocystis* sp. ST3 cysts isolated from asymptomatic individual, symptomatic and IBS patients in the intestinal tract of *Blastocystis* sp. ST3 inoculated *Wistar* rats (Chapter 5).The degree of mucosal sloughing, inflammation, necrosis of tissue seen in the ileum, caecum, colon

and rectum from three different groups i.e. asymptomatic individual, symptomatic and IBS patients were then compared.

The results revealed that the histopathological features seen in the intestines of rats inoculated with cysts from IBS patients were different compared to rats inoculated with cysts from asymptomatic individual and symptomatic patient. The intestine of rats inoculated with cysts isolated from IBS patient showed to be highly convoluted and reddish compared to that seen in the control, asymptomatic and symptomatic group of rats. Chapter 4 of this study provides evidence that the surface of the parasites isolated from IBS patients is sticky but whether the surface of the parasite found to very rough and folded as seen in SEM has a correlation is uncertain. It is likely that the high convolution and reddishness of the infected intestines seen in *Wistar* rats inoculated with cysts from IBS patients is due to the adherence by this sticky surface of this parasite as reported in Chapter 4. The present study (Chapter 5) showed that the size of the inoculum ($1x10^4$cysts/ml and $1x10^6$cysts/ml) and the duration of infection (weeks 1 to 4) do have an effect on the histopathological changes such as mucosal sloughing, inflammation and necrosis of tissue.

The results revealed that a longer exposure of *Blastocystis* sp. ST3 infection in the intestinal gut could possibly cause a greater mucosal sloughing over time. Histopathological changes of the intestinal gut were seen more in the 4^{th} week after infection. A comparatively greater effect of mucosal sloughing and inflammation was seen in the rats inoculated with cysts from the IBS patient compared to other groups, asymptomatic and symptomatic. Meanwhile, histopathological changes were more marked in the ileum compared to caecum, colon and rectum, with the greatest severity seen in rats from the asymptomatic group. 100% of the rats inoculated with

$1X10^6$cysts/ml *Blastocystis* sp. ST3 cysts on 4^{th} week, showed extensive mucosal sloughing compared to 50% of rat inoculated with cysts from symptomatic and IBS patients. The result coincided with the findings shown in chapter 4 that the asymptomatic isolates had the highest growth rate compared to the symptomatic and IBS isolates. The findings concur with a previous study which showed that asymptomatic isolates had the highest growth rate in cultures when compared to symptomatic ones (Tan et al., 2008). The higher rate of multiplication of vacuolar forms of the asymptomatic isolates could account for the extensive mucosal sloughing seen in rats infected with asymptomatic isolates. Therefore, rats infected with cysts from asymptomatic isolates showed extensive sloughing and this could be due to the effects exerted by the highly proliferative forms from this group of cysts. However, severe histopathological changes were seen in rats inoculated with *Blastocystis* sp. ST3 cysts isolated from IBS patients.

The degree of mucosal sloughing and inflammation was seen to be more marked in caecum, colon and rectum in rats infected with cysts from IBS patients compared to cysts from the symptomatic and asymptomatic patients. Moreover, extensive necrosis was seen in one rat inoculated with $1x10^6$cysts/ml, dissected on the 4^{th} week, from the IBS group showed extensive mucosal sloughing and moderate inflammation. This was not seen in asymptomatic and symptomatic groups. This perhaps explains why IBS patients infected with *Blastocystis* sp. ST3 show greater symptoms than the symptomatic and asymptomatic patients.

The substantive evidence point to *Blastocystis* sp. ST3 derived from IBS patients to be more pathogenic resulting in greater severity seen when assessing histopathological changes compared to asymptomatic and symptomatic groups despite

being from the same subtype. This study concurs with the previous finding in Chapter 4, that there are phenotypic differences even within the same ST3 depending on whether individuals are symptomatic, asymptomatic or IBS patients. Moreover, in Chapter 4, *Blastocystis* sp. ST3 showed to be phenotypically different in which this parasite possesses a sticky surface which was seen in the Modified Field's stain, FITC-Con A, SEM and in TEM. Therefore, *Blastocystis* sp. ST3 can play a pathogenic role in IBS patients due to its distinct characteristics which can result in the invasion of sub-mucosa and hence, contributing to the development of inflammation. The release of proteases by the parasite reported previously (Puthia et al., 2008) could play a major role in the pathophysiology of inflammation in the IBS patients. Due to this, increased interleukin level was observed in IBS patients infected with *Blastocystis* sp. compared to non-IBS patients (Chapter 3). Moreover, decreased surface area due to mucosal sloughing can cause poor absorption which in turn will cause digestive problems. Therefore, this effect can be seen in the case study done in the IBS patient (Chapter 7) suggesting that *Blastocystis* sp. ST3 have a detrimental effect on her gut which may worsen her condition. On the other hand, the study also established that, *Wistar* rats can be used as an experimental animal model to investigate the pathogenicity of *Blastocystis* sp. ST3. This *in vivo* experimental study has shown the role of *Blastocystis* sp. ST3 derived from IBS patient in causing a greater effect on the intestine compared to *Blastocystis* sp. ST3 derived from asymptomatic and symptomatic patient. Large numbers of inflammatory cells seen in the lamina propia of the rat's ileum infected with *Blastocystis* sp. ST3 from IBS (Chapter 5) demonstrate that *Blastocystis* sp. does evoke an immune response

Chapter 3 showed increased interleukin levels in IBS patients infected with *Blastocystis* sp. compared to non-IBS patients (Chapter 3) implying that *Blastocystis* sp. also triggers the immune response. Such condition can be seen in IBS patients infected

with *Blastocystis* sp. which results in different gene expression due to activation of the immune system and inflammation of the intestine. Long term inflammation of the intestine can lead to cancer (Husain, 2007). Out of many other signaling transduction pathways, Wnt signaling pathway is associated with inflammation and plays a role in the etiology of cancer, especially in colon cancer.

Hence, the present study (Chapter 6) investigated the effects of *Blastocystis* sp. ST3 antigen (Blasto-Ag ST3) derived from non-IBS (asymptomatic and symptomatic) and IBS patients on the growth and gene expression of both the normal and cancer cells. The proliferation rate for both normal and cancer cells were significantly higher when induced with Blasto-Ag ST3 derived from IBS patients compared to Blasto-Ag ST3 from non-IBS patients. Meanwhile, the percentage of cell migration was significantly higher in normal and colon cancer cells upon exposure of IBS Blasto-Ag ST3, compared to non-IBS Blasto-Ag ST3. IBS Blasto-Ag ST3 has the ability to cause a higher cell proliferation and migration *in vitro* implying that it may play a role in causing normal cells to become cancerous cells in IBS patients with and without colorectal cancer. Cell proliferation and migration have been associated with Wnt signaling pathway. Upregulation of genes can cause abnormal cell proliferation and migration which leads to cancer progression.

The second part of Chapter 6 studied the gene expression of 75 genes involved in Wnt signaling pathway in both the cell lines (normal cells and cancer cells) introduced with Blasto-Ag ST3 from non-IBS and IBS. Thus far, this is the first study to investigate the role of Blasto-Ag ST3 in the Wnt signaling pathway of colon cancer cells exposed to the solubilized antigen (Blasto-Ag) extracted from the parasite, compared with normal cells.

Wnt pathway analysis showed that the canonical pathway plays a major role in regulating the transcription of target gene in the nuclear level by inhibiting the phosphorylation of β catenin compared to the non-canonical pathway upon exposure to both non-IBS and IBS Blasto-Ag ST3. These results correspond to the previous study done showing the association between canonical pathway and cancer especially colorectal cancer (Giles et al., 2003). Therefore, this is the first study to demonstrate the role of *Blastocysti*s sp. ST3 in IBS patients in facilitating the cancer progression by regulating the canonical pathway especially in association with colorectal cancer.

IBS Blasto-Ag ST3 showed to have a higher effect on both the normal and cancer cells compared to non-IBS Blasto Ag ST3. Such differences seen could be due to the differing gut condition. Chapter 4 provides evidences on the distinct phenotypic characteristics of the *Blastocystis* sp. ST3 derived from IBS patients. The sticky surface of the parasite could enhance the adherence towards the intestinal gut resulting in intestinal inflammation. Moreover, chapter 3 showed an increase in interleukin levels (IL-3, IL5 and IL-8) in IBS patients infected with *Blastocystis* sp. This shows that *Blastocystis* sp. ST3 from IBS patients could be more pathogenic resulting in destruction of intestinal lining causing an inflammation at the infection site. As seen in Chapter 5, the rats inoculated with *Blastocystis* sp. ST3 derived from IBS patients showed to have a greater mucosal sloughing, inflammation and necrosis of tissue compared to the other two groups (asymptomatic and symptomatic). This means that, inflammation can trigger mutation and gene modification as well as changes in gene expression which can facilitate cancer progression in *Blastocystis* sp. ST3 infected IBS patients. Such an effect therefore triggers the gene regulation via the canonical pathway suggesting that long term inflammation can lead to cancer, especially colorectal cancer which was seen in Chapter 6.

In the normal cells, several canonical Wnts such as WNT3A, WNT3, WNT4, WNT6, WNT7A and WNT10A were seen to be significantly upregulated in normal cells upon exposure of IBS Blasto-Ag ST3 compared to cancer cells. Apart from that, both the non-canonical planar cell polarity (NKD1, NKD2 and DVL2) and calcium pathway (WNT11) were upregulated in normal cells upon exposure of IBS Blasto-Ag ST3 in normal cells. Therefore, both the canonical and non-canonical pathways play an important role in regulating the genes. Generally, the β catenin (canonical) pathway regulates the cell proliferation, migration, morphology and cell adhesion by inhibiting β catenin phosphorylation (Widelitz, 2005). Abnormal activation of this pathway can lead to tumorigenesis. Moreover, cell population can attract more cells through the paracrine action of growth as well as cell adhesion of molecules (Widelitz, 2005). Adhesion of parasites towards the gut due to sticky surface (Chapter 4) can be associated with β catenin (canonical) pathway. On the other hand, regulation of non-canonical planar cell polarity modulates cytoskeleton and cell migration. The initial part of Chapter 6 showed the percentage of cell migration was significantly high upon exposure to IBS Blasto-Ag ST3 compared to non-IBS Blasto-Ag ST3 in both the normal and cancer cells. The non-canonical calcium pathway also contributes to the development of cancer by regulating the calcium releases from the endoplasmic reticulum. Hence, both the pathways play a significant oncogenic role causing a rapid and uncontrolled cell proliferation and migration facilitating the normal cells to become cancerous upon the exposure of *Blastocystis* sp. ST3 from IBS patients.

In cancer cells, only β catenin (canonical) (WNT3 and WNT7A) and non-canonical planar cell polarity pathways (NKD1 and DVL1) were found to be regulated upon exposure of IBS Blasto-Ag ST3. This implies that *Blastocystis* sp. from IBS patients with colorectal cancer may play a role in regulating both canonical and non-

canonical planar cell polarity by inhibiting the phosphorylation of β catenin as well as modulation of cytoskeleton.

WNT3A and WNT7A was the only genes found to be significantly upregulated in both normal and cancer cells introduced with IBS Blasto-Ag ST3. Both WNT3 and WNT7A genes were able to inhibit the phosphorylation of β-catenin in the cell allowing the accumulation of β catenin to occur. β catenin will bind to T-cell factor (TCF) cells forming a complex (β catenin-TCF complex) to activate the transcription of target genes. This subsequently activates the proliferation and migration of cells which was seen in the first part of Chapter 6. Therefore, WNT3 and WNT7a can be used as novel biomarkers to inhibit the β catenin/TCF complex formation. Knowledge on the Wnt signaling pathway provides better understanding on the biomarkers used which can assist in major advancement sin diagnosis of colorectal cancer especially in IBS patients as they have a higher risk of developing cancer.

8.2 Conclusion

1. The study has proven that stool aspirate samples can be a suitable alternate to stool samples for the detection of *Blastocystis* sp. using PCR method. ST3 was found to be the most predominant subtype. The study also confirms elevated levels of interleukin (IL-3, IL-5 and IL-8) in *Blastocystis sp.* infected IBS patients demonstrating that *Blastocystis* sp. does have an effect on the immune system. The study is the first in South East Asian countries to show a positive association between IBS and *Blastocystis* sp.

   (Nanthiney D.R., Suresh, K.G., Tan T.C., Sanjiv, M. & Ho S.H. (2015). *Blastocystis* sp. In Irritable Bowel Syndrome – Detection In Stool Aspirates During Colonoscopy. *PloS one*, 10:9)

2. The present finding for the first time demonstrated obvious phenotypic differences even within the same subtype which is ST3. It is highly possible that different gut environments can influence phenotypic expression of even the same subtype. The study has diagnostic implications and it is important to ensure that a proper and a detailed study be undertaken before forming any definite conclusion as gut environment does play a part in expressing and influencing phenotypic characteristic.

   (Nanthiney, D.R., Suresh, K.G., Tan T.C. & Sanjiv M. (2014). Phenotypic variation in Blatocystis sp. ST3. *Parasites and vector*, 7:404.)

3. The *in vivo* study provides evidence that *Blastocystis* sp. ST3 cysts derived from IBS patients can cause extensive mucosal sloughing, necrosis and moderate inflammation of tissues (ileum, caecum, colon and rectum) compared to *Blastocystis sp*. ST3 cysts derived from two other groups (asymptomatic and

symptomatic) . The present finding has important implications as it forms the basis for an association for long term inflammation leading to colorectal cancer. (Nanthiney et al., 2015: Manuscript in preparation)

4. The *in vitro* studies showed that the IBS Blasto-Ag ST3 has the ability to cause a higher cell proliferation and migration in both normal and cancer cells. These results imply that it may play a role in may play a role in advancing inflammation in IBS patients with and without colorectal cancer which could result in tumor progression. This is the first study to demonstrate the role of *Blastocystis* sp. ST3 derived from IBS patients to have a higher effect on both cells compared to *Blastocystis* sp. ST3 derived from non-IBS (asymptomatic and symptomatic).

   (Nanthiney et al., 2015: Manuscript in preparation)

5. Based on the Wnt pathway analysis, a large number of genes were involved in the canonical pathway (inhibition of β catenin phosphorylation) in both normal (56%) and cancer cells (55%) regardless of the groups (non-IBS and IBS). IBS Blasto-Ag ST3 exhibited a greater effect on both cells compared to non-IBS Blasto-Ag, suggesting that it can play a role in the activation of the Wnt canonical pathway which regulates the transcription of target gene in the nuclear level resulting in abnormal cell proliferation and cell migration. *Blastocystis* sp. ST3 from IBS patients could be more pathogenic in causing the activation of the canonical pathway. Therefore, this is the first study to show the role of *Blastocystis* sp. in regulating the β catenin/canonical pathway especially in relation to colorectal cancer.

   (Nanthiney et al., 2015: Manuscript in preparation)

6. The present study showed *Blastocystis* sp. ST3 derived from IBS patients plays a role in regulating all three pathways (canonical, non-canonical planar cell polarity and calcium pathway) in normal cells. Canonical Wnts such as WNT3A, WNT3, WNT4, WNT6, WNT7A and WNT10A were significantly upregulated upon exposure of IBS Blasto-Ag ST3. Meanwhile, non-canonical planar cell polarity (NKD1, NKD2 and DVL2) and calcium pathway (WNT11) were also seen to be upregulated in normal cells upon exposure of IBS Blasto-Ag ST3. Binding of canonical Wnts in cells will inhibit the phosphorylation of β catenin which further causes abnormal cell growth (cell proliferation and cell migration) which can lead to colon cancer. Moreover, non-canonical genes can play an important role by promoting progression through changes in shape and cell signaling. These imply that IBS Blasto-Ag ST3 may cause normal cells to become cancerous in IBS patients. This could be due to upregulation of certain genes.

   (Nanthiney et al., 2015: Manuscript in preparation)

7. In cancer cells, *Blastocystis* sp. ST3 derived from IBS patients was also seen to play a role in regulating two pathways (canonical and non-canonical planar cell polarity). Canonical Wnts such as WNT3 and NT7A genes were significantly upregulated upon exposure to IBS Blasto-Ag ST3. Meanwhile, for non-canonical planar cell polarity (DVL1 and NKD1) were upregulated in normal cells upon exposure of IBS Blasto-Ag ST3. Higher expression of genes in canonical pathway increases transcription of target genes and subsequently activates its proliferative properties. These imply that IBS Blasto-Ag ST3 may worsen the existing cancer in IBS patients.

   (Nanthiney et al., 2015: Manuscript in preparation)

8. In the present study, WNT3 and WNT7A genes were the only genes found to be significantly upregulated in both the normal and cancer cells upon exposure of IBS Blasto-Ag ST3. Therefore, this study proposes the development of novel biomarkers to inhibit the β catenin/TCF complex formation which then further inhibit the nuclear gene transcription as well as cell proliferation and cell migration. Hence, this study for the first time proposes WNT3 and WNT7A genes to be used as a candidate marker to detect inflammation or cancer progression in IBS patients especially those infected with *Blastocystis* sp. (Nanthiney et al., 2015: Manuscript in preparation).

9. This study for the first time provides evidence that the factors such as the frequency of frequenting the toilet in a day, the timing of toilet visits, the stool forms and moods as well as emotions can influence the shedding pattern of cysts in an IBS patient. This study suggest that stool samples should be collected in the morning, should be a semi solid form, should be collected every time one frequents the toilet even within a day. This is the first study to correlate patient's emotions and mood with the shedding of *Blastocystis* sp. cysts.

   Nanthiney, D.R., Suresh, K.G., Tan T.C. & Sanjiv M. (2015). Factors that influences the shedding of *Blastocystis* sp. cysts in an irritable bowel syndrom (IBS) patients- an evidence-based case study. *Parasitology Research*. 1-7

References

Abdel-Hameed, D. M., & Hassanin, O. M. (2011). Proteaese activity of *Blastocystis hominis* subtype 3 in symptomatic and asymptomatic patients. *Parasitology Research, 109*(2), 321-327.

Abdulsalam, A. M., Ithoi, I., Al-Mekhlafi, H. M., Ahmed, A., Surin, J., & Mak, J. W. (2012). Drinking water is a significant predictor of *Blastocystis* infection among rural Malaysian primary schoolchildren. *Parasitology, 139*(8), 1014-1020.

Abe, N. (2004). Molecular and phylogenetic analysis of *Blastocystis* isolates from various hosts. *Veterinary parasitology, 120*(3), 235-242.

Afzan, M. Y., Sivanandam, S., & Kumar, G. S. (2010). Modified Field's staining—a rapid stain for *Trichomonas vaginalis*. *Diagnosis Microbiology Infectious Disease, 68*(2), 159-162.

Al, F. D., & Hökelek, M. (2006). Is *Blastocystis hominis* an opportunist agent?. *Turkiye parazitolojii dergisi/Turkiye Parazitoloji Dernegi= Acta parasitologica Turcica/Turkish Society for Parasitology, 31*(1), 28-36.

Albrecht, H., Stellbrink, H.-J., Koperskl, K., & Greten, H. (1995). *Blastocystis hominis in human immunodeficiency virus-related diarrhea*. *Scandinavian Journal of Gastroentorology, 30*(9), 909-914.

Alexeieff, A. (1911). Sur la nature des formations dites kystes de *Trichomonas intestinalis*. *CR Soc Biol, 71*, 296-298.

Alfellani, M. A., Stensvold, C. R., Vidal-Lapiedra, A., Onuoha, E. S. U., Fagbenro-Beyioku, A. F., & Clark, C. G. (2013a). Variable geographic distribution of *Blastocystis* subtypes and its potential implications. *Acta tropica, 126*(1), 11-18.

Alfellani, M. A., Taner-Mulla, D., Jacob, A. S., Imeede, C. A., Yoshikawa, H., Stensvold, C. R., & Clark, C. G. (2013b). Genetic Diversity of *Blastocystis* in Livestock and Zoo Animals. *Protist, 164*(4), 497-509.

Amin, O. M. (2002). Seasonal prevalence of intestinal parasites in the United States during 2000. *The American journal of tropical medicine and hygiene, 66*(6), 799-803.

Andiran, N., Acikgoz, Z. C., Turkay, S., & Andiran, F. (2006). *Blastocystis hominis*—an emerging and imitating cause of acute abdomen in children. *Journal of Pediatric Surgery, 41*(8), 1489-1491.

Arisue, N., Hashimoto, T., & Yoshikawa, H. (2003). Sequence heterogeneity of the small subunit ribosomal RNA genes among *Blastocystis* isolates. *Parasitology, 126*(01), 1-9.

Arisue, N., Hashimoto, T., Yoshikawa, H., Nakamura, Y., Nakamura, G., & Nakamura, F. (2002). Phylogenetic position of *Blastocystis hominis* and of stramenopiles inferred from multiple molecular sequence data. *Journal of Eukaryotic Microbiology, 49*(1), 42-53.

Ashford, R., & Atkinson, E. (1992). Epidemiology of *Blastocystis hominis* infection in Papua New Guinea: age-prevalence and associations with other parasites. *Annals of tropical medicine and parasitology, 86*(2), 129-136.

Barbara, G., Cremon, C., De Giorgio, R., Dothel, G., Zecchi, L., Bellacosa, L., Carini, G., Stanghellini, V. & Corinaldesi, R. (2011). Mechanisms underlying visceral hypersensitivity in irritable bowel syndrome. *Current Gastroenterology Reports, 13*(4), 308-315.

Barbara, G., De Giorgio, R., Stanghellini, V., Cremon, C., Salvioli, B., & Corinaldesi, R. (2004). New pathophysiological mechanisms in irritable bowel syndrome. *Alimentary pharmacology & therapeutics, 20*(s2), 1-9.

Barbara, G., Zecchi, L., Barbaro, R., Cremon, C., Bellacosa, L., Marcellini, M., De Giorgio, R., Corinaldesi, R. & Stanghellini, V. (2012). Mucosal permeability and immune activation as potential therapeutic targets of probiotics in irritable bowel syndrome. *Journal of clinical gastroenterology, 46*, S52-S55.

Baron, S., & Yaeger, R. G. (1996). Protozoa: Structure, Classification, Growth, and Development. 4th Edition *Medical Microbiology*

Barrett, J. (1981). *Biochemistry of parasitic helminths*: MacMillan Publishers Ltd.

Basualdo, J. A., Córdoba, M. A., Luca, M. M. d., Ciarmela, M. L., Pezzani, B. C., Grenovero, M. S., & Minvielle, M. C. (2007). Intestinal parasitoses and environmental factors in a rural population of Argentina, 2002-2003. *Revista do Instituto de Medicina Tropical de São Paulo, 49*(4), 251-255.

Belova, L., & Krylov, M. (1997). The distribution of *Blastocystis* according to different systematic groups of hosts. *Parazitologiia, 32*(3), 268-276.

Bengmark, S. (1998). Ecological control of the gastrointestinal tract. The role of probiotic flora. *Gut, 42*(1), 2-7.

Bengochea, A., De Souza, M., Lefrancois, L., Le Roux, E., Galy, O., Chemin, I., Kim., M., wands, J.R., Trepo, C. & Hainaut, P. (2008). Common dysregulation of Wnt/Frizzled receptor elements in human hepatocellular carcinoma. *British Journal of cancer, 99*(1), 143-150.

Benhaj, K., Akcali, K. C., & Ozturk, M. (2006). Redundant expression of canonical Wnt ligands in human breast cancer cell lines. *Oncology reports, 15*(3), 701-707.

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J.-C., Wang, Y., Macke, J. P., Andrew, D., Nathan, J. & Nusse, R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. *Nature, 382*, 225-330.

Bienz, M., & Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. *Cell, 103*(2), 311-320.

Böhm-Gloning, B., Knobloch, J., & Walderich, B. (1997). Five subgroups of *Blastocystis hominis* isolates from symptomatic and asymptomatic patients revealed by restriction site analysis of PCR-amplified 16S-like rDNA. *Tropical Medicine & International Health, 2*(8), 771-778.

Bonfrate, L., Tack, J., Grattagliano, I., Cuomo, R., & Portincasa, P. (2013). Microbiota in health and irritable bowel syndrome: current knowledge, perspectives and therapeutic options. *Scandinavian Journal of Gastroentorology, 48*(9), 995-1009.

Boorom, K. F., Smith, H., Nimri, L., Viscogliosi, E., Spanakos, G., Parkar, U., Li, L.H., Zhou, X.N., Ok, U.Z., Leelayoova, S. & Jones, M.S (2008). Oh my aching gut: irritable bowel syndrome, *Blastocystis*, and asymptomatic infection. *Parasites & Vectors, 1*(1), 40.

Borda, C. E., Rea, M. J. F., Rosa, J. R., & Maidana, C. (1996). Intestinal parasitism in San Cayetano, Corrientes, Argentina. *Bulletin of the Pan American Health Organization, 30*(3), 227-233.

Boreham, P. F., & Stenzel, D. J. (1993). *Blastocystis* in humans and animals: morphology, biology, and epizootiology. *Advances in parasitology, 32*, 2-2.

Brumpt, E. (1912). *Blastocystis hominis* n. sp. et formes voisines. *Bulletin de la Societe de Pathologie Exotique. 5*, 725-730.

Budéjovioe, C. (1991). Occurrence of *Blastocystis* sp. in pigs. *Folia parasitologica, 38*, 297-301.

Bueno, L. (2008). Protease activated receptor 2: a new target for IBS treatment. *Eur Rev Medical and Pharmacological Sciences, 12*(Suppl 1), 95-102.

Caldwell, G., Jones, C., Ashley, A., Wei, W., Hejmadi, R., Morton, D., & Matthews, G. (2010). Wnt signaling in adenomas of familial adenomatous polyposis patients. *British journal of cancer, 103*(6), 910-917.

Camilleri, M., & CHOI, M. G. (1997). Review article: irritable bowel syndrome. *Alimentary pharmacology & therapeutics, 11*(1), 3-15.

Carbajal, J., Del Castillo, L., Lanuza, M. D., Villar, J., & Borras, R. (1997). Karyotypic diversity among *Blastocystis hominis* isolates. *International Journal for Parasitology, 27*(8), 941-945.

Carrascosa, M., Martinez, J., & Perez-Castrillon, J. L. (1996). Hemorrhagic proctosigmoiditis and *Blastocystis hominis* infection. *Annals of internal medicine, 124*(2), 278-279.

Cavalier S. T. (1998). A revised six-kingdom system of life. *Biological Reviews, 73*(3), 203-266.

Chadwick, V. S., Chen, W., Shu, D., Paulus, B., Bethwaite, P., Tie, A., & Wilson, I. (2002). Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology, 122*(7), 1778-1783.

Chan, K. H., Chandramathi, S., Suresh, K., Chua, K. H., & Kuppusamy, U. R. (2012). Effects of symptomatic and asymptomatic isolates of *Blastocystis hominis* on colorectal cancer cell line, HCT116. *Parasitology Research, 110*(6), 2475-2480.

Chandramathi, S., Suresh, K. G., Mahmood, A., & Kuppusamy, U. (2010a). Urinary hyaluronidase activity in rats infected with *Blastocystis hominis*—evidence for invasion? *Parasitology Research, 106*(6), 1459-1463.

Chandramathi, S., Suresh, K., & Kuppusamy, U. R. (2010b). Solubilized antigen of *Blastocystis hominis* facilitates the growth of human colorectal cancer cells, HCT116. *Parasitology Research, 106*(4), 941-945.

Chandramathi, S., Suresh, K., Anita, Z. B., & Kuppusamy, U. R. (2012). Infections of *Blastocystis hominis* and microsporidia in cancer patients: are they opportunistic? *Transactions of the Royal Society of Tropical Medicine and* Hygiene, *106*(4), 267-269.

Chandramathi, S., Suresh, K., Shuba, S., Mahmood, A., & Kuppusamy, U. (2010c). High levels of oxidative stress in rats infected with *Blastocystis hominis*. *Parasitology, 137*(04), 605-611.

Chang, B., Tessneer, K. L., McManus, J., Liu, X., Hahn, S., Pasula, S., Wu, H., Song, H., Chen, Y., Cai, X., Dong, Y., Brophy, M.L., Rahman, R., Ma, J.X., Xia, L. & Cai, X. (2015). Epsin is required for Dishevelled stability and Wnt signaling activation in colon cancer development. *Nature communications, 6* (6380).

Chaudhari, H. S., & Singh, P. P. (2011). Comparative drug susceptibility study of five clonal strains of *Trichomonas vaginalis* in vitro. *Asian Pacific Journal of Tropical Medicine, 4*(1), 50-53.

Chen, X., Singh, M., Ho, L., Tan, S., Ng, G., Moe, K., & Yap, E. (1997). Description of a *Blastocystis* species from *Rattus norvegicus*. *Parasitology Research, 83*(4), 313-318.

Ciferri, R., Redaelli, P., & Visocchi, V. (1938). The histoplasmaceae family. *Mycopathologia, 1*(2), 104-114.

Cirioni, O., Giacometti, A., Drenaggi, D., Ancarani, F., & Scalise, G. (1999). Prevalence and clinical relevance of *Blastocystis hominis* in diverse patient cohorts. *European journal of epidemiology, 15*(4), 387-391.

Clark, C. G., van der Giezen, M., Alfellani, M. A., & Stensvold, C. R. (2013). Recent developments in *Blastocystis* research. [Review]. *Advances in Parasitology, 82*, 1-32.

Clevers, H. (2006). Wnt/β-catenin signaling in development and disease. *Cell, 127*(3), 469-480.

Collins, S. M. (1996). The immunomodulation of enteric neuromuscular function: implications for motility and inflammatory disorders. *Gastroenterology, 111*(6), 1683-1699.

Cremon, C., Gargano, L., Morselli-Labate, A. M., Santini, D., Cogliandro, R. F., De Giorgio, R., Stanghellini, V., Corinaldesi, R. &Barbara, G. (2009). Mucosal immune activation in irritable bowel syndrome: gender-dependence and association with digestive symptoms. *The American journal of gastroenterology, 104*(2), 392-400.

Crnogorac-Jurcevic, T., Efthimiou, E., Capelli, P., Blaveri, E., Baron, A., Terris, B., Jones, M., Tyson, K., Bassi, C., Scarpa, A. & Lemoine, N.R. (2001). Gene expression profiles of pancreatic cancer and stromal desmoplasia. *Oncogene, 20*, 7437-7446.

D'Anchino, M., Orlando, D., & De Feudis, L. (2002). Giardia lamblia infections become clinically evident by eliciting symptoms of irritable bowel syndrome. *Journal of Infection, 45*(3), 169-172.

Daryani, A., Sharif, M., Amouei, A., Ettehad, G., Ziaei, H., Gohardehi, S., & Bastani, R. (2008). *Blastocystis* sp: a Neglected Zoonotic Protozoan. *Proc ASEAN Congr Trop Med Parasitol, 3*, 59-62.

David, M. D., Cantí, C., & Herreros, J. (2010). Wnt-3a and Wnt-3 differently stimulate proliferation and neurogenesis of spinal neural precursors and promote neurite outgrowth by canonical signaling. *Journal of neuroscience research, 88*(14), 3011-3023.

Davis, E. K., Zou, Y., & Ghosh, A. (2008). Wnts acting through canonical and noncanonical signaling pathways exert opposite effects on hippocampal synapse formation. *Neural development, 3*(1), 32.

De Silva, A. P., Nandasiri, S. D., Hewavisenthi, J., Manamperi, A., Ariyasinghe, M. P., Dassanayake, A. S., Jewell, D.P. & de Silva, H.J. (2012). Subclinical mucosal inflammation in diarrhea-predominant irritable bowel syndrome (IBS) in a tropical setting. *Scandinavian Journal of Gastroentorology, 47*(6), 619-624.

Denoeud, F., Roussel, M., Noel, B., Wawrzyniak, I., Da Silva, C., Diogon, M., Viscogliosi, E., Brochier-Armanet, C., Couloux, A., Poulain, J., Segurens, B., Anthouard, V., Texier, C., Blot, N., Poirier, P., Ng, G.C., Tan, K.S., Artiguenave, F., Jaillon, O., Aury, J.M., Delbac, F., Wincker, P., Vivarès, C.P. & El Alaoui, H. (2011). Genome sequence of the stramenopile *Blastocystis*, a human anaerobic parasite. *Genome Biology*, *12*(3), R29.

Derynck, R., Akhurst, R. J., & Balmain, A. (2001). TGF-β signaling in tumor suppression and cancer progression. *Nature genetics, 29*(2), 117-129.

Dogruman-Al, F., Dagci, H., Yoshikawa, H., Kurt, Ö., & Demirel, M. (2008). A possible link between subtype 2 and asymptomatic infections of *Blastocystis hominis*. *Parasitology Research, 103*(3), 685-689.

Dogruman-Al, F., Simsek, Z., Boorom, K., Ekici, E., Sahin, M., Tuncer, C., Kustimur, S. & Altinbas, A. (2010). Comparison of methods for detection of *Blastocystis* infection in routinely submitted stool samples, and also in IBS/IBD Patients in Ankara, Turkey. *PLoS One, 5*(11), e15484.

Dogruman-Al, F., Yoshikawa, H., Kustimur, S., & Balaban, N. (2009). PCR-based subtyping of *Blastocystis* isolates from symptomatic and asymptomatic individuals in a major hospital in Ankara, Turkey. *Parasitology Research, 106*(1), 263-268.

Domínguez-Márquez, M. V., Guna, R., Muñoz, C., Gómez-Muñoz, M. T., & Borrás, R. (2009). High prevalence of subtype 4 among isolates of *Blastocystis hominis* from symptomatic patients of a health district of Valencia (Spain). *Parasitology Research, 105*(4), 949-955.

Drossman, D. A., & Dumitrascu, D. L. (2006). Rome III: New standard for functional gastrointestinal disorders. *Journal of Gastrointestinal and Liver Diseases, 15*(3), 237.

Duda, A., Stenzel, D., & Boreham, P. (1998). Detection of *Blastocystis* sp. in domestic dogs and cats. *Vet Parasitol, 76*(1), 9-17.

Dunn, L., Boreham, P., & Stenzel, D. (1989). Ultrastructural variation of *Blastocystis hominis* stocks in culture. *International Journal for Parasitology, 19*(1), 43-56.

El Safadi, D., Gaayeb, L., Meloni, D., Cian, A., Poirier, P., Wawrzyniak, I., Delbac, F., Dabboussi, F., Delhaes, L., Seck, M., Hamze, M., Riveau, G., Viscogliosi, E. & Seck, M. (2014). Children of Senegal River Basin show the highest prevalence of *Blastocystis* sp. ever observed worldwide. *Biomedcentral infectious diseases, 14*(1), 164.

El-Shazly, A., Abdel-Magied, A., El-Beshbishi, S., El-Nahas, H., Fouad, M., & Monib, M. (2005). *Blastocystis hominis* among symptomatic and asymptomatic individuals in Talkha Center, Dakahlia Governorate, Egypt. *J Egypt Soc Parasitol, 35*(2), 653-666.

Endo, Y., Wolf, V., Muraiso, K., Kamijo, K., Soon, L., Üren, A., Barshishat-Küpper, M. & Rubin, J. S. (2005). Wnt-3a-dependent cell motility involves RhoA activation and is specifically regulated by dishevelled-2. *Journal of Biological Chemistry*, *280*(1), 777-786.

Enzenauer, R. J., Underwood, J. G. H., & Ribbing, J. (1990). Tropical pulmonary eosinophilia. *Southern medical journal*, *83*(1), 69-72.

Eroglu, F., Genc, A., Elgun, G., & Koltas, I. S. (2009). Identification of *Blastocystis hominis* isolates from asymptomatic and symptomatic patients by PCR. *Parasitology Research*, *105*(6), 1589-1592.

Gallagher, P. G., & Venglarcik, J. S. (1985). *Blastocystis hominis* enteritis. *The Pediatric Infectious Disease Journal*, *4*(5), 556.

Gecse, K., Roka, R., Ferrier, L., Leveque, M., Eutamene, H., Cartier, C., Ait-Belgnaoui A, Rosztóczy, A., Izbéki, F., Fioramonti, J., Wittmann, T. & Bueno, L. (2008). Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. *Gut*, *57*(5), 591-599.

Gericke, A. S., Burchard, G. D., Knobloch, J., & Walderich, B. (1997). Isoenzyme patterns of *Blastocystis hominis* patient isolates derived from symptomatic and healthy carriers. *Tropical Medicine & International Health*, *2*(3), 245-253.

Ghosh, S., May, M. J., & Kopp, E. B. (1998). NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annual review of immunology*, *16*(1), 225-260.

Giacometti, A., Cirioni, O., Fiorentini, A., Fortuna, M., & Scalise, G. (1999). Irritable bowel syndrome in patients with *Blastocystis hominis* infection. *European Journal of Clinical Microbiology and Infectious Diseases*, *18*(6), 436-439.

Giannella, R., Broitman, S., & Zamcheck, N. (1972). Gastric acid barrier to ingested microorganisms in man: studies in vivo and in vitro. *Gut*, *13*(4), 251-256.

Giles, R. H., van Es, J. H., & Clevers, H. (2003). Caught up in a Wnt storm: Wnt signaling in cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, *1653*(1), 1-24.

Govind, S., Khairul, A. A., & Smith, H. V. (2002). Multiple reproductive processes in *Blastocystis*. *Trends Parasitology*, *18*(12).

Graczyk, T. K., Shiff, C. K., Tamang, L., Munsaka, F., Beitin, A. M., & Moss, W. J. (2005). The association of *Blastocystis hominis* and *Endolimax nana* with diarrheal stools in Zambian school-age children. *Parasitology Research, 98*(1), 38-43.

Guignard, S., Arienti, H., Freyre, L., Lujan, H., & Rubinstein, H. (2000). Prevalence of enteroparasites in a residence for children in the Cordoba Province, Argentina. *European journal of epidemiology, 16*(3), 287-293.

Guilarte, M., Santos, J., De Torres, I., Alonso, C., Vicario, M., Ramos, L., Cristina, M., Francesc, C., Esteban, S. & Malagelada, J. R. (2007). Diarrhoea-predominant IBS patients show mast cell activation and hyperplasia in the jejunum. *Gut, 56*(2), 203-209.

Guyton AC, J. E. (2006). Guyton and hall textbook of medical physiology: with Student Consult Online Access, 11e: Saunders.

Gwee, K. (2005). Irritable bowel syndrome in developing countries–a disorder of civilization or colonization? *Neurogastroenterology & Motility, 17*(3), 317-324.

Hameed, D. M. A., Hassanin, O. M., & Zuel-Fakkar, N. M. (2011). Association of *Blastocystis hominis* genetic subtypes with urticaria. *Parasitology Research, 108*(3), 553-560.

Heisenberg, C.-P., Tada, M., Rauch, G.-J., Saúde, L., Concha, M. L., Geisler, R., Stemple, D.L., Smith, J.C. & Wilson, S. W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature, 405*(6782), 76-81.

Hilmi, I., Hartono, J. L., Pailoor, J., Mahadeva, S., & Goh, K.-L. (2013). Low prevalence of 'classical'microscopic colitis but evidence of microscopic inflammation in Asian Irritable Bowel Syndrome patients with diarrhoea. *BMC gastroenterology, 13*(1), 80.

Holcombe, R., Marsh, J., Waterman, M., Lin, F., Milovanovic, T., & Truong, T. (2002). Expression of Wnt ligands and Frizzled receptors in colonic mucosa and in colon carcinoma. *Molecular Pathology, 55*(4), 220.

Horiki, N., Kaneda, Y., Maruyama, M., Fujita, Y., & Tachibana, H. (1999). Intestinal blockage by carcinoma and *Blastocystis hominis* infection. *The American journal of tropical medicine and hygiene, 60*(3), 400-402.

Horiki, N., Maruyama, M., Fujita, Y., Yonekura, T., Minato, S., & Kaneda, Y. (1997). Epidemiologic survey of *Blastocystis hominis* infection in Japan. *The American journal of tropical medicine and hygiene, 56*(4), 370-374.

Hotez, P., Cappello, M., Hawdon, J., Beckers, C., & Sakanari, J. (1994). Hyaluronidases of the gastrointestinal invasive nematodes *Ancylostoma caninum* and *Anisakis simplex*: possible functions in the pathogenesis of human zoonoses. *Journal of Infectious Diseases, 170*(4), 918-926.

Huang, M.-Y., Yen, L.-C., Liu, H.-C., Liu, P.-P., Chung, F.-Y., Wang, T.-N., Lin, S.-R. (2013). Significant overexpression of DVL1 in Taiwanese colorectal cancer patients with liver metastasis. *International journal of molecular sciences, 14*(10), 20492-20507.

Huang, W.-C., Xie, Z., Konaka, H., Sodek, J., Zhau, H. E., & Chung, L. W. (2005). Human osteocalcin and bone sialoprotein mediating osteomimicry of prostate cancer cells: role of cAMP-dependent protein kinase A signaling pathway. *Cancer research, 65*(6), 2303-2313.

Huelsken, J., & Behrens, J. (2002). The Wnt signaling pathway. *Journal of cell science, 115*(21), 3977-3978.

Hulisz, D. (2004). The burden of illness of irritable bowel syndrome: current challenges and hope for the future. *Journal of Managed Care Pharmacy, 10*(4), 299-309.

Hussain, R., Jaferi, W., Zuberi, S., Baqai, R., Abrar, N., Ahmed, A., & Zaman, V. (1997). Significantly increased IgG2 subclass antibody levels to *Blastocystis hominis* in patients with irritable bowel syndrome. *The American journal of tropical medicine and hygiene, 56*(3), 301-306.

Hussein, E. M., Hussein, A. M., Eida, M. M., & Atwa, M. M. (2008). Pathophysiological variability of different genotypes of human *Blastocystis hominis* Egyptian isolates in experimentally infected rats. *Parasitology Research, 102*(5), 853-860.

Iozzo, R. V., Eichstetter, I., & Danielson, K. G. (1995). Aberrant expression of the growth factor Wnt-5A in human malignancy. *Cancer research, 55*(16), 3495-3499.

Ishitani, T., Kishida, S., Hyodo-Miura, J., Ueno, N., Yasuda, J., Waterman, M., Shibuya, H., Moon, R.T., Ninomiya-Tsuji, J. & Matsumoto, K. (2003). The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-

5a/Ca2+ pathway to antagonize Wnt/β-catenin signaling. *Molecular and cellular biology, 23*(1), 131-139.

Jackson, P. E., Cooper, D. P., O'Connor, P. J., & Povey, A. C. (1999). The relationship between 1, 2-dimethylhydrazine dose and the induction of colon tumours: tumour development in female SWR mice does not require a K-ras mutational event. *Carcinogenesis, 20*(3), 509-513.

Jeddy, T., & Farrington, G. (1991). *Blastocystis hominis* complicating ulcerative colitis. *Journal of the Royal Society of Medicine, 84*(10), 623.

Jelinek, T., Peyerl, G., Löscher, T., Von Sonnenburg, F., & Nothdurft, H. (1997). The role of *Blastocystis hominis* as a possible intestinal pathogen in travellers. *Journal of Infection, 35*(1), 63-66.

Jessen, J. R., & Solnica-Krezel, L. (2005). Morphogenetic cell movements shaping the zebrafish gastrula. *Advances in Developmental Biology, 14*, 131-165.

Jiang, J.-B., & He, J.-G. (1993). Taxonomic status of *Blastocystis hominis. Parasitology Today, 9*(1), 2-3.

Jimenez, O. M., Carbonell, A. E., Garcia, O. M., Rodriguez, L. W., Triana, F. P., & Fabian, L. G. (2012). *Blastocystis hominis* in symptomatic celiac patients. *Acta Gastroenterol Latinoam, 42*(3), 175-181.

Jimenez-Gonzalez, D. E., Martinez-Flores, W. A., Reyes-Gordillo, J., Ramirez-Miranda, M. E., Arroyo-Escalante, S., Romero-Valdovinos, M., Stark, D., Souza-Saldivar, V., Martinez-Hernandez, F., Flisser, A., Olivo-Diaz, A. & Maravilla, P. (2012). *Blastocystis* infection is associated with irritable bowel syndrome in a Mexican patient population. *Parasitology Research, 110*(3), 1269-1275.

Johnson, A. M., Thanou, A., Boreham, P. F., & Baverstock, P. R. (1989). *Blastocystis hominis*: phylogenetic affinities determined by rRNA sequence comparison. *Experimental parasitology, 68*(3), 283-288.

Jones, M. S., Whipps, C. M., Ganac, R. D., Hudson, N. R., & Boroom, K. (2009). Association of *Blastocystis* subtype 3 and 1 with patients from an Oregon community presenting with chronic gastrointestinal illness. *Parasitology Research, 104*(2), 341-345.

Joossens, M., Huys, G., Cnockaert, M., De Preter, V., Verbeke, K., Rutgeerts, P., Vandamme, P. & Vermeire, S. (2011). Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut, 60*(5), 631-637.

Kanazawa, M., Hongo, M., & Fukudo, S. (2011). Visceral hypersensitivity in irritable bowel syndrome. *Journal of gastroenterology and hepatology*, 26(s3), 119-121.

Kaneda, Y., Horiki, N., Cheng, X.-j., Fujita, Y., Maruyama, M., & Tachibana, H. (2001). Ribodemes of *Blastocystis hominis* isolated in Japan. *The American journal of tropical medicine and hygiene*, *65*(4), 393-396.

Karanis, P., Kourenti, C., & Smith, H. (2007). Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. *Journal of Water and Health, 5*(1), 1-38.

Katoh, M. (2001a). Frequent up-regulation of WNT2 in primary gastric cancer and colorectal cancer. *International journal of oncology*, *19*(5), 1003-1007.

Katoh, M. (2001b). Molecular cloning and characterization of human WNT3. *International journal of oncology, 19*(5), 977-982.

Katoh, M., & Katoh, M. (2005). Comparative genomics on Wnt8a and Wnt8b genes. *International journal of oncology, 26*(4), 1129-1133.

Kellow, J. E., & Phillips, S. F. (1987). Altered small bowel motility in irritable bowel syndrome is correlated with symptoms. *Gastroenterology*, 92(6), 1885-1893.

Khan, Z., & Alkhalife, I. (2005). Prevalence of *Blastocystis hominis* among" healthy" food handlers in Dammam, Saudi Arabia. *Journal of Egyptian Society Parasitology, 35*(2), 395-401.

Kim, M., Lee, H. C., Tsedensodnom, O., Hartley, R., Lim, Y.-S., Yu, E., Merle, P. & Wands, J. R. (2008). Functional interaction between Wnt3 and Frizzled-7 leads to activation of the Wnt/β-catenin signaling pathway in hepatocellular carcinoma cells. *Journal of hepatology, 48*(5), 780-791.

Kirikoshi, H., & Katoh, M. (2002). Expression of WNT7A in human normal tissues and cancer, and regulation of WNT7A and WNT7B in human cancer. *International journal of oncology, 21*(4), 895-900.

Kirikoshi, H., Sekihara, H., & Katoh, M. (2001). Expression profiles of 10 members of Frizzled gene family in human gastric cancer. *International journal of oncology, 19*(4), 767-771.

Knowles, R., & Gupta, B. D. (1924). On the nature of *Blastocystis hominis*. *Indian Journal of Medical Research, 12*, 31-38.

Kollmar, O., Menger, M. D., & Schilling, M. K. (2006). Macrophage inflammatory protein-2 contributes to liver resection-induced acceleration of hepatic metastatic tumor growth. *World Journal of Gastroenterology: WJG, 12*(6), 858-867.

Kühl, M., Geis, K., Sheldahl, L. C., Pukrop, T., Moon, R. T., & Wedlich, D. (2001). Antagonistic regulation of convergent extension movements in xenopus by Wnt/β-catenin and Wnt/Ca 2+ signaling. *Mechanisms of Development, 106*(1), 61-76.

Kukoschke, K., & Müller, H. (1991). SDS-PAGE and immunological analysis of different axenic *Blastocystis hominis* strains. *Journal of Medical Microbiology, 35*(1), 35-39.

Kumarasamy, V., Kuppusamy, U. R., Samudi, C., & Kumar, S. (2013). *Blastocystis* sp. subtype 3 triggers higher proliferation of human colorectal cancer cells, HCT116. *Parasitology Research, 112*(10), 3551-3555.

Kumarasamy, V., Roslani, A. C., Rani, K. U., & Kumar Govind, S. (2014). Advantage of using colonic washouts for *Blastocystis* detection in colorectal cancer patients. *Parasites & Vectors, 7*, 162.

Kurniawan, A., Karyadi, T., Dwintasari, S., Sari, I., Yunihastuti, E., Djauzi, S., & Smith, H. (2009). Intestinal parasitic infections in HIV/AIDS patients presenting with diarrhoea in Jakarta, Indonesia. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 103*(9), 892-898.

Lakhanpal, S., Cohen, S. B., & Fleischmann, R. M. (1991). Reactive arthritis from *Blastocystis hominis*. *Arthritis & Rheumatism, 34*(2), 251-253.

Lebbad, M., Norrgren, H., Nauclér, A., Dias, F., Andersson, S., & Linder, E. (2001). Intestinal parasites in HIV-2 associated AIDS cases with chronic diarrhoea in Guinea-Bissau. *Acta tropica, 80*(1), 45-49.

Leder, K., Hellard, M. E., Sinclair, M. I., Fairley, C. K., & Wolfe, R. (2005). No correlation between clinical symptoms and *Blastocystis hominis* in

immunocompetent individuals. *Journal of gastroenterology and hepatology, 20*(9), 1390-1394.

Lee, A. Y., He, B., You, L., Dadfarmay, S., Xu, Z., Mazieres, J., Mikami I, McCormick F. & Jablons, D. M. (2004). Expression of the secreted frizzled-related protein gene family is downregulated in human mesothelioma. *Oncogene, 23*(39), 6672-6676.

Lee, B. J., & Bak, Y.-T. (2011). Irritable bowel syndrome, gut microbiota and probiotics. *Journal of neurogastroenterology and motility, 17*(3), 252.

Lee, E. H., Chari, R., Lam, A., Ng, R. T., Yee, J., English, J., & Lam, W. L. (2008). Disruption of the non-canonical WNT pathway in lung squamous cell carcinoma. *Clinical medicine. Oncology, 2*, 169.

Lee, J., Hunter, S., & Bovee, E. (1985). Illustrated Guide to the Protozoa Society of Protozoologists and Allen Press. *Lawrence, Kansas*.

Lee, M., & Stenzel, D. (1999). A survey of *Blastocystis* in domestic chickens. *Parasitology Research, 85*(2), 109-117.

Leelayoova, S., Rangsin, R., Taamasri, P., Naaglor, T., Thathaisong, U., & Mungthin, M. (2004). Evidence of waterborne transmission of *Blastocystis hominis. The American journal of tropical medicine and hygiene, 70*(6), 658-662.

Leelayoova, S., Siripattanapipong, S., Thathaisong, U., Naaglor, T., Taamasri, P., Piyaraj, P., & Mungthin, M. (2008). Drinking water: a possible source of *Blastocystis* spp. subtype 1 infection in schoolchildren of a rural community in central Thailand. *The American journal of tropical medicine and hygiene, 79*(3), 401-406.

Lejeune, S., Huguet, E. L., Hamby, A., Poulsom, R., & Harris, A. L. (1995). Wnt5a cloning, expression, and up-regulation in human primary breast cancers. *Clinical Cancer Research, 1*(2), 215-222.

Levy, Y., George, J., & Shoenfeld, Y. (1996). Severe *Blastocystis* hominis in an elderly man. *Journal of Infection, 33*(1), 57-59.

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S. & Knight, R. (2008). Evolution of mammals and their gut microbes. *Science, 320*(5883), 1647-1651.

Li, L. H., Zhang, X. P., Lv, S., Zhang, L., Yoshikawa, H., Wu, Z., Steinmann, P., Utzinger, J., Tong, X.M., Chen, S.H. and Zhou, X.N. & Chen, S. H. (2007). Cross-sectional surveys and subtype classification of human *Blastocystis* isolates from four epidemiological settings in China. *Parasitology Research, 102*(1), 83-90.

Lipskaia, L., Hulot, J.-S., & Lompré, A.-M. (2009). Role of sarco/endoplasmic reticulum calcium content and calcium ATPase activity in the control of cell growth and proliferation. *Pflügers Archiv-European Journal of Physiology, 457*(3), 673-685.

Logan, C. Y., & Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annual Review Cell Developmental Biology, 20*, 781-810.

Long, H., Handschack, A., König, W., & Ambrosch, A. (2001). *Blastocystis hominis* modulates immune responses and cytokine release in colonic epithelial cells. *Parasitology Research, 87*(12), 1029-1030.

Longstreth, G. F., Thompson, W. G., Chey, W. D., Houghton, L. A., Mearin, F., & Spiller, R. C. (2006). Functional bowel disorders. *Gastroenterology, 130*(5), 1480-1491.

Longstreth, G. F., Wilson, A., Knight, K., Wong, J., Chiou, C.-F., Barghout, V., Frech, F. & Ofman, J. J. (2003). Irritable bowel syndrome, health care use, and costs: a US managed care perspective. *The American journal of gastroenterology, 98*(3), 600-607.

Ma, T. Y. (1997). Intestinal epithelial barrier dysfunction in Crohn's disease. *Experimental Biology and Medicine, 214*(4), 318-327.

Madka, V., & Rao, C. V. (2013). Anti-inflammatory phytochemicals for chemoprevention of colon cancer. *Current cancer drug targets, 13*(5), 542-557.

Malheiros, A. F., Stensvold, C. R., Clark, C. G., Braga, G. B., & Shaw, J. J. (2011). Molecular characterization of *Blastocystis* obtained from members of the indigenous Tapirapé ethnic group from the Brazilian Amazon region, Brazil. *The American journal of tropical medicine and hygiene, 85*(6), 1050-1053.

Malinen, E., Rinttilä, T., Kajander, K., Mättö, J., Kassinen, A., Krogius, L., Saarela, M., Korpela, R. & Palva, A. (2005). Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *The American Journal of Gastroenterology, 100*(2), 373-382.

Mansour, N., Mikhail, E., El Masry, N., Sabry, A., & Mohareb, E. (1995). Biochemical characterisation of human isolates of *Blastocystis hominis*. *Journal of Medical Microbiology, 42*(4), 304-307.

Markell, E. K., & Udkow, M. P. (1986). *Blastocystis hominis*: pathogen or fellow traveler? *American Journal of Tropical Medicine and Hygiene, 35*(5), 1023-1026.

Mattson, M. P., & Chan, S. L. (2003). Calcium orchestrates apoptosis. *Nature cell biology, 5*(12), 1041-1043.

Maxion-Bergemann, S., Thielecke, F., Abel, F., & Bergemann, R. (2006). Costs of irritable bowel syndrome in the UK and US. *Pharmacoeconomics, 24*(1), 21-37.

Mayer, E. A., Naliboff, B. D., Chang, L., & Coutinho, S. V. (2001). V. Stress and irritable bowel syndrome. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 280*(4), G519-G524.

McClure, H., Strobert, E., & Healy, G. (1980). *Blastocystis hominis* in a pig-tailed macaque: a potential enteric pathogen for nonhuman primates. *Laboratory animal science, 30*(5), 890-894.

McEwen, B. S., & Gianaros, P. J. (2010). Central role of the brain in stress and adaptation: links to socioeconomic status, health, and disease. *Annals of the New York Academy of Sciences, 1186*(1), 190-222.

McMahon, G. (2000). VEGF receptor signaling in tumor angiogenesis. *The Oncologist, 5*(Supplement 1), 3-10.

Meloni, D., Sanciu, G., Poirier, P., El Alaoui, H., Chabe, M., Delhaes, L., Fiori, P.L., Di Cave, D. & Viscogliosi, E. (2011). Molecular subtyping of *Blastocystis* sp. isolates from symptomatic patients in Italy. *Parasitology Research, 109*(3), 613-619.

Mikesch, J., Steffen, B., Berdel, W., Serve, H., & Müller-Tidow, C. (2007). The emerging role of Wnt signaling in the pathogenesis of acute myeloid leukemia. *Leukemia, 21*(8), 1638-1647.

Miller, J. R. (2002). The wnts. *Genome Biology, 3*(1), 1-15.

Milovanovic, T., Planutis, K., Nguyen, A., Marsh, J. L., Lin, F., Hope, C., & Holcombe, R. F. (2004). Expression of Wnt genes and frizzled 1 and 2 receptors in normal

breast epithelium and infiltrating breast carcinoma. *International journal of oncology*, *25*(5), 1337-1342.

Mirza, H., & Tan, K. S. (2009). *Blastocystis* exhibits inter-and intra-subtype variation in cysteine protease activity. *Parasitology Research*, *104*(2), 355-361.

Mizutani, K., Miyamoto, S., Nagahata, T., Konishi, N., Emi, M., & Onda, M. (2005). Upregulation and overexpression of DVL1, the human counterpart of the Drosophila dishevelled gene, in prostate cancer. *Tumori journal*, *91*(6), 546.

Moe, K., Singh, M., Howe, J., Ho, L., Tan, S., Chen, X., Ng, G.C. & Yap, E. (1997). Experimental *Blastocystis hominis* infection in laboratory mice. *Parasitology Research*, *83*(4), 319-325.

Mohandas, K., Sehgal, R., Sud, A., & Malla, N. (2002). Prevalence of intestinal parasitic pathogens in HIV-seropositive individuals in Northern India. *Japanese journal of infectious diseases*, *55*(3), 83-84.

Montcouquiol, M., Crenshaw III, E. B., & Kelley, M. W. (2006). Noncanonical wnt signaling and neural polarity 1. *Annual Review of Neuroscience*, *29*, 363-386.

Moon, R. T., Brown, J. D., & Torres, M. (1997). WNTs modulate cell fate and behavior during vertebrate development. *Trends in Genetics*, *13*(4), 157-162.

Moon, R. T., Kohn, A. D., De Ferrari, G. V., & Kaykas, A. (2004). WNT and β-catenin signaling: diseases and therapies. *Nature Reviews Genetics*, *5*(9), 691-701.

Müller, H. E. (1994). Four serologically different groups within the species *Blastocystis hominis*. *Zentralblatt für Bakteriologie*, *280*(3), 403-408.

Nambotin, S., Tomimaru, Y., Merle, P., Wands, J., & Kim, M. (2012). Functional consequences of WNT3/Frizzled7-mediated signaling in non-transformed hepatic cells. *Oncogenesis*, *1*(10), e31.

Nasirudeen, A., Tan, K., Singh, M., & Yap, E. (2001). Programmed cell death in a human intestinal parasite, *Blastocystis hominis*. *Parasitology*, *123*(03), 235-246

Nassir, E., Awad, J., Abel, A., Khoury, J., Shay, M., & Lejbkowicz, F. (2004). *Blastocystis hominis* as a cause of hypoalbuminemia and anasarca. *European Journal of Clinical Microbiology and Infectious Diseases*, *23*(5), 399-402.

Nimri, L., & Meqdam, M. (2004). Enteropathogens associated with cases of gastroenteritis in a rural population in Jordan. *Clinical Microbiology and Infection, 10*(7), 634-639.

Nishioka, M., Ueno, K., Hazama, S., Okada, T., Sakai, K., Suehiro, Y., Okayama, N., Hirata, H., Oka, M., Imai, K. & Dahiya, R. (2013). Possible involvement of Wnt11 in colorectal cancer progression. *Molecular Carcinogenesis, 52*(3), 207-217.

Noel, C., Dufernez, F., Gerbod, D., Edgcomb, V. P., Delgado-Viscogliosi, P., Ho, L.-C., Singh, M., Wintjens, R., Sogin, M.L., Capron, M. & Pierce, R. (2005). Molecular phylogenies of *Blastocystis* isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis. *Journal of Clinical Microbiology, 43*(1), 348-355.

Noel, C., Peyronnet, C., Gerbod, D., Edgcomb, V. P., Delgado-Viscogliosi, P., Sogin, M. L., Capron, M., Viscogliosi, E. & Zenner, L. (2003). Phylogenetic analysis of *Blastocystis* isolates from different hosts based on the comparison of small-subunit rRNA gene sequences. *Molecular and biochemical parasitology, 126*(1), 119-123.

Noor, S. O., Ridgway, K., Scovell, L., Kemsley, E. K., Lund, E. K., Jamieson, C., Johnson, I.T. & Narbad, A. (2010). Ulcerative colitis and irritable bowel patients exhibit distinct abnormalities of the gut microbiota. *Biomedcentral gastroenterology, 10*(1), 134.

O'Mahony, S. M., Hyland, N. P., Dinan, T. G., & Cryan, J. F. (2011). Maternal separation as a model of brain–gut axis dysfunction. *Psychopharmacology, 214*(1), 71-88.

Öhman, L., & Simrén, M. (2010). Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions. *Nature Reviews Gastroenterology and Hepatology, 7*(3), 163-173.

Ok, Ü., Cirit, M., Üner, A., Ok, E., Akcicek, F., Basci, A., & Özcel, M. (1997). Cryptosporidiosis and *blastocystosis* in renal transplant recipients. *Nephron, 75*(2), 171-174.

Olivo-Diaz, A., Romero-Valdovinos, M., Gudiño-Ramirez, A., Reyes-Gordillo, J., Jimenez-Gonzalez, D. E., Ramirez-Miranda, M. E., Martinez-Flores, W.A., Martinez-Hernandez, F., Flisser, A. & Maravilla, P. (2012). Findings related to IL-8 and IL-10 gene polymorphisms in a Mexican patient population with irritable bowel syndrome infected with *Blastocystis*. *Parasitology Research, 111*(1), 487-491.

Oppenheim, J., & Ruscetti, F. (2001). Medical immunology. *Lange medical. 10th ed. New York: McGraw-Hill*.

Orrenius, S., Zhivotovsky, B., & Nicotera, P. (2003). Regulation of cell death: the calcium–apoptosis link. *Nature reviews Molecular cell biology, 4*(7), 552-565.

O'sullivan, M., Clayton, N., Breslin, N., Harman, I., Bountra, C., McLaren, A., & O'morain, C. (2000). Increased mast cells in the irritable bowel syndrome. *Neurogastroenterology and Motility, 12*(5), 449-458.

Özyurt, M., Kurt, Ö., Mølbak, K., Nielsen, H. V., Haznedaroglu, T., & Stensvold, C. R. (2008). Molecular epidemiology of *Blastocystis* infections in Turkey. *Parasitology International, 57*(3), 300-306.

Paboriboune, P., Phoumindr, N., Borel, E., Sourinphoumy, K., Phaxayaseng, S., Luangkhot, E., Sengphilom, B., Vansilalom, Y., Odermatt, P., Delaporte, E. & Etard, J.F., Delaporte, E. (2014). Intestinal parasitic infections in HIV-infected patients, Lao People's Democratic Republic. *PLoS One, 9*(3).

Padilla-Vaca, F., & Anaya-Velázquez, F. (2010). Insights into Entamoeba histolytica virulence modulation. *Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders), 10*(4), 242-250.

Paré, P., Gray, J., Lam, S., Balshaw, R., Khorasheh, S., Barbeau, M., Kelly, S. and McBurney, C. R. (2006). Health-related quality of life, work productivity, and health care resource utilization of subjects with irritable bowel syndrome: baseline results from LOGIC (Longitudinal Outcomes Study of Gastrointestinal Symptoms in Canada), a naturalistic study. *Clinical Therapeutics, 28*(10), 1726-1735.

Parija, S. C., & Jeremiah, S. (2013). *Blastocystis*: Taxonomy, biology and virulence. *Tropical Parasitology, 3*(1), 17-25. doi: 10.4103/2229-5070.113894

Park, T. J., Gray, R. S., Sato, A., Habas, R., & Wallingford, J. B. (2005). Subcellular localization and signaling properties of dishevelled in developing vertebrate embryos. *Current Biology, 15*(11), 1039-1044.

Pasqui, A., Savini, E., Saletti, M., Guzzo, C., Puccetti, L., & Auteri, A. (2004). Chronic urticaria and *Blastocystis hominis* infection: a case report. *European review for medical and pharmacological sciences, 8*, 117-120.

Patterson, D. (1989). Stramenopiles: chromophytes from a protistan perspective. *The Chromophyte Algae: Problems and Perspectives.*, 357-379.

Person, A. D., Garriock, R. J., Krieg, P. A., Runyan, R. B., & Klewer, S. E. (2005). Frzb modulates Wnt-9a-mediated β-catenin signaling during avian atrioventricular cardiac cushion development. *Developmental biology, 278*(1), 35-48.

Perwez Hussain, S., & Harris, C. C. (2007). Inflammation and cancer: an ancient link with novel potentials. *International Journal of Cancer, 121*(11), 2373-2380.

Piche, T., Barbara, G., Aubert, P., Des Varannes, S. B., Dainese, R., Nano, J.-L & Galmiche, J. P. (2009). Impaired intestinal barrier integrity in the colon of patients with irritable bowel syndrome: involvement of soluble mediators. *Gut, 58*(2), 196-201.

Poirier, P., Wawrzyniak, I., Albert, A., El Alaoui, H., Delbac, F., & Livrelli, V. (2011). Development and evaluation of a real-time PCR assay for detection and quantification of *Blastocystis* parasites in human stool samples: prospective study of patients with hematological malignancies. *Jornal of Clinical Microbiology, 49*(3), 975-983.

Poirier, P., Wawrzyniak, I., Vivares, C. P., Delbac, F., & El Alaoui, H. (2012). New insights into *Blastocystis* spp.: a potential link with irritable bowel syndrome. *PLoS Pathogen, 8*(3), e1002545.

Polakis, P. (2000). Wnt signaling and cancer. *Genes & development, 14*(15), 1837-1851.

Prasad, K., Nag, V., Dhole, T., & Ayyagari, A. (2000). Identification of enteric pathogens in HIV-positive patients with diarrhoea in northern India. *Journal of Health, Population and Nutrition*, 23-26.

Puthia, M. K., Lu, J., & Tan, K. S. (2008). *Blastocystis* ratti contains cysteine proteases that mediate interleukin-8 response from human intestinal epithelial cells in an NF-κB-dependent manner. *Eukaryotic Cell, 7*(3), 435-443.

Puthia, M. K., Vaithilingam, A., Lu, J., & Tan, K. S. (2005). Degradation of human secretory immunoglobulin A by *Blastocystis*. *Parasitology Research, 97*(5), 386-389.

Qadri, S., al-Okaili, G. A., & al-Dayel, F. (1989). Clinical significance of *Blastocystis hominis*. J*ournal of Clinical Microbiology, 27*(11), 2407-2409.

Rajamanikam, A., & Govind, S. K. (2013). Amoebic forms of *Blastocystis* spp. - evidence for a pathogenic role. *Parasites & Vectors, 6*(1), 295.

Rajendra, S., & Alahuddin, S. (2004). Prevalence of irritable bowel syndrome in a multi-ethnic Asian population. *Alimentary pharmacology & therapeutics, 19*(6), 704-706.

Rajilić–Stojanović, M., Biagi, E., Heilig, H. G., Kajander, K., Kekkonen, R. A., Tims, S., & de Vos, W. M. (2011). Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology, 141*(5), 1792-1801.

Rajilić-Stojanović, M., Smidt, H., & De Vos, W. M. (2007). Diversity of the human gastrointestinal tract microbiota revisited. *Environmental microbiology, 9*(9), 2125-2136.

Ramirez-Miranda, M. E., Hernandez-Castellanos, R., Lopez-Escamilla, E., Moncada, D., Rodriguez-Magallan, A., Pagaza-Melero, C. & Maravilla, P. (2010). Parasites in Mexican patients with irritable bowel syndrome: a case-control study. *Parasites & Vectors, 3*(96).

Requena, I., Hernández, Y., Ramsay, M., Salazar, C., & Devera, R. (2003). Prevalence of *Blastocystis hominis* among food handlers from Caroni municipality, Bolivar State, Venezuela. *Cadernos de Saúde Pública, 19*(6), 1721-1727.

Rey, E., & Talley, N. (2009). Irritable bowel syndrome: novel views on the epidemiology and potential risk factors. *Digestive and liver disease, 41*(11), 772-780.

Reya, T., & Clevers, H. (2005). Wnt signaling in stem cells and cancer. *Nature, 434*(7035), 843-850.

Rivero-Rodríguez, Z., Hernández, A., Bracho, Á., Salazar, S., & Villalobos, R. (2013). Prevalence of intestinal microsporidia and other intestinal parasites in hiv positive patients from Maracaibo, Venezuela. *Biomedica, 33*(4), 538-545.

Russo, A. R., Stone, S. L., Taplin, M. E., Snapper, H. J., & Doern, G. V. (1988). Presumptive evidence for *Blastocystis hominis* as a cause of colitis. *Archives of internal medicine, 148*(5), 1064-1064.

Sainsbury, A., & Ford, A. C. (2011). Treatment of irritable bowel syndrome: beyond fiber and antispasmodic agents. *Therapeutic advances in gastroenterology, 4*(2), 115-127.

Scanlan, P. D., & Marchesi, J. R. (2008). Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture-dependent and-independent analysis of faeces. *International Society for Microbial Ecology, 2*(12), 1183-1193.

Schmulson, M. J., Ortiz-Garrido, O. M., Hinojosa, C., & Arcila, D. (2006). A single session of reassurance can acutely improve the self-perception of impairment in patients with IBS. *Journal of psychosomatic research, 61*(4), 461-467.

Scicluna, S. M., Tawari, B., & Clark, C. G. (2006). DNA barcoding of *Blastocystis*. *Protist, 157*(1), 77-85.

Sekar, U., & Shanthi, M. (2013). *Blastocystis*: Consensus of treatment and controversies. *Tropical Parasitology, 3*(1), 35.

Shimizu, H., Julius, M. A., Giarre, M., Zheng, Z., Brown, A., & Kitajewski, J. (1997). Transformation by Wnt family proteins correlates with regulation of beta-catenin. *Cell growth & differentiation: the molecular biology journal of the American Association for Cancer Research, 8*(12), 1349-1358.

Shlim, D. R., Hoge, C. W., Rajah, R., Rabold, J. G., & Echeverria, P. (1995). Is *Blastocystis hominis* a cause of diarrhea in travelers? A prospective controlled study in Nepal. *Clinical infectious diseases, 21*(1), 97-101.

Sienzel, D., Boreham, P., & McDougall, R. (1991). Ultrastructure of *Blastocystis hominis* in human stool samples. *International Journal for Parasitology, 21*(7), 807-812.

Silberman, J. D., Sogin, M. L., Leipe, D. D., & Clark, C. G. (1996). Human parasite finds taxonomic home. *Nature, 380*(6573), 398-398.

Simrén, M., Barbara, G., Flint, H. J., Spiegel, B. M., Spiller, R. C., Vanner, S., Verdu, E.F., Whorwell, P.J. & Zoetendal, E. G. (2012). Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut*, gutjnl-2012-302167.

Simrén, M., Castedal, M., Svedlund, J., Abrahamsson, H., & Bjõrnsson, E. (2000). Abnormal propagation pattern of duodenal pressure waves in the irritable bowel syndrome (IBS). *Digestive diseases and sciences*, 45(11), 2151-2161.

Singh, M., Ho, L., Yap, A., Ng, G., Tan, S., Moe, K., & Yap, E. (1996). Axenic culture of reptilian *Blastocystis* isolates in monophasic medium and speciation by karyotypic typing. *Parasitology Research, 82*(2), 165-169.

Singh, M., Suresh, K., Ho, L., Ng, G., & Yap, E. (1995). Elucidation of the life cycle of]the intestinal protozoan *Blastocystis hominis. Parasitology Research, 81*(5), 446-450.

Sinha, P., Ghoshal, U., Choudhuri, G., Naik, S., Ayyagari, A., & Naik, S. (1997). Does Entamoeba histolytica cause irritable bowel syndrome? *Indian journal of gastroenterology: official journal of the Indian Society of Gastroenterology, 16*(4), 130-133.

Sio, S. W., Puthia, M. K., Lee, A. S., Lu, J., & Tan, K. S. (2006). Protease activity of *Blastocystis hominis*. *Parasitology Research, 99*(2), 126-130.

Souppart, L., Sanciu, G., Cian, A., Wawrzyniak, I., Delbac, F., Capron, M., Dei-Cas, E., Boorom, K., Delhaes, L. & Viscogliosi, E. (2009). Molecular epidemiology of human *Blastocystis* isolates in France. *Parasitology Research, 105*(2), 413-421.

Spiller, R., & Garsed, K. (2009). Postinfectious irritable bowel syndrome. *Gastroenterology, 136*(6), 1979-1988.

Steck, N., Mueller, K., Schemann, M., & Haller, D. (2013). Republished: Bacterial proteases in IBD and IBS. *Postgraduate medical journal, 89*(1047), 25-33.

Stensvold, C. R. (2013). *Blastocystis*: Genetic diversity and molecular methods for diagnosis and epidemiology. *Tropical Parasitology, 3*(1), 26-34. doi: 10.4103/2229-5070.113896

Stensvold, C. R., Arendrup, M. C., Jespersgaard, C., Mølbak, K., & Nielsen, H. V. (2007). Detecting *Blastocystis* using parasitologic and DNA-based methods: a comparative study. *Diagnosis Microbiology and Infectious Disease, 59*(3), 303-307.

Stensvold, C. R., Suresh, G. K., Tan, K. S., Thompson, R. A., Traub, R. J., Viscogliosi, E., Yoshikawa, H. & Clark, C. G. (2007). Terminology for *Blastocystis* subtypes–a consensus. *Trends Parasitology, 23*(3), 93-96.

Stensvold, C., Lewis, H., Hammerum, A. M., Porsbo, L. J., Nielsen, S., Olsen, K., . . . Mølbak, K. (2009). *Blastocystis*: unravelling potential risk factors and clinical significance of a common but neglected parasite. *Epidemiology and infection, 137*(11), 1655-1663.

Stensvold, C., Traub, R., von Samson-Himmelstjerna, G., Jespersgaard, C., Nielsen, H., & Thompson, R. (2007). *Blastocystis*: Subtyping isolates using pyrosequencing™ technology. *Experimental parasitology, 116*(2), 111-119.

Stenzel, D. J., & Boreham, P. F. L. (1996). *Blastocystis hominis* revisited. *Clinical Microbiology Reviews, 9*(4), 563-584.

Stenzel, D., Boreham, P., & McDougall, R. (1991). Ultrastructure of *Blastocystis hominis* in human stool samples. *International Journal for Parasitology, 21*(7), 807-812.

Stenzel, D., Cassidy, M., & Boreham, P. (1994). Morphology of *Blastocystis* sp. from domestic birds. *Parasitology Research, 80*(2), 131-137.

Stenzel, D., Lee, M., & Boreham, P. (1997). Morphological differences in *Blastocystis* cysts–an indication of different species? *Parasitology Research, 83*(5), 452-457.

Sundin, J., Rangel, I., Hultgren Hörnquist, E., & Brummer, R. (2012). Increased number of double positive CD3+ CD8+ CD4+ lamina propria T lymphocyte in gut mucosa of post infectious IBS patients compared to healthy controls.*The European Society for Clinical Nutrition and Metabolism, September 8-11, Barcelona, Spain*.

Surangsrirat, S., Thamrongwittawatpong, L., Piyaniran, W., Naaglor, T., Khoprasert, C., Taamasri, P., Mungthin, M. & Leelayoova, S. (2010). Assessment of the association between *Blastocystis* infection and irritable bowel syndrome. *Journal of the Medical Association of Thailand, 93*(6), 119-124.

Suresh, K., & Smith, H. (2004). Comparison of methods for detecting *Blastocystis hominis. European Journal of Clinical Microbiology and Infectious Diseases, 23*(6), 509-511.

Suresh, K., Chong, S., Howe, J., Ho, L., Ng, G., Yap, E., & Singh, M. (1995). Tubulovesicular elements in *Blastocystis hominis* from the caecum of experimentally-infected rats. *International Journal for Parasitology, 25*(1), 123-126.

Suresh, K., Howe, J., Ng, G. C., Ho, L. C., Ramachandran, N. P., Loh, A. K. & Singh, M. (1994). A multiple fission-like mode of asexual reproduction in *Blastocystis hominis*. *Parasitology Research, 80*(6), 523-527.

Suresh, K., Khairul Anuar, A., Saminathan, T., Ng, K., & Init, I. (1997a). In vitro culture technique: a better diagnostic tool for *Blastocystis hominis*. *Journal of International Medical Research, 1*, 5-7.

Suresh, K., Mak, J., Chuong, L., Ragunathan, T., & Init, I. (1997b). Sac-like pouches in *Blastocystis* from the house lizard Cosymbotus platyurus. *Parasitology Research, 83*(6), 523-525.

Svenungsson, B., Lagergren, Å., Ekwall, E., Evengård, B., Hedlund, K. O., Kärnell, A., Löfdahl, S., Svensson, L. & Weintraub, A. (2000). Enteropathogens in adult patients with diarrhea and healthy control subjects: a 1-year prospective study in a Swedish clinic for infectious diseases. *Clinical infectious diseases, 30*(5), 770-778.

Swellengrebel, N. (1917). Observations on *Blastocystis hominis*. *Parasitology, 9*(04), 451-459.

Taamasri, P., Mungthin, M., Rangsin, R., Tongupprakarn, B., Areekul, W., & Leelayoova, S. (2000). Transmission of intestinal *Blastocystosis* related to the quality of drinking water. *Southeast Asian journal of tropical medicine and public health, 31*(1), 112-117.

Taketo, M. M. (2004). Shutting down Wnt signal–activated cancer. *Nature genetics, 36*(4), 320-322.

Talley, N. J., Locke, G. R., Lahr, B., Zinsmeister, A. R., Tougas, G., Ligozio, G., Rojavin, M.A. & Tack, J. (2006). Functional dyspepsia, delayed gastric emptying, and impaired quality of life. *Gut, 55*(7), 933-939.

Tan, H., & Zierdt, C. (1973). Ultrastructure of *Blastocystis hominis*. *Zeitschrift für Parasitenkunde, 42*(4), 315-324.

Tan, K. S. (2004). *Blastocystis* in humans and animals: new insights using modern methodologies. *Vetiranary Parasitoly, 126*(1), 121-144.

Tan, K. S. (2008). New insights on classification, identification, and clinical relevance of *Blastocystis* spp. *Clinical Microbiology Reviews, 21*(4), 639-665.

Tan, K. S., & Stenzel, D. J. (2003). Multiple reproductive processes in *Blastocystis*: proceed with caution. *Trends in parasitology, 19*(7), 290-291.

Tan, K. S., Singh, M., & Yap, E. H. (2002). Recent advances in Blastocystis hominis research: hot spots in terra incognita. *International Journal for Parasitology, 32*(7), 789-804.

Tan, K., Howe, J., Yap, E., & Singh, M. (2001). Do *Blastocystis hominis* colony forms undergo programmed cell death? *Parasitology Research, 87*(5), 362-367.

Tan, T., & Suresh, K. (2006). Predominance of amoeboid forms of *Blastocystis hominis* in isolates from symptomatic patients. *Parasitology Research, 98*(3), 189-193.

Tan, T., & Suresh, K. (2007). Evidence of plasmotomy in *Blastocystis hominis*. *Parasitology Research, 101*(6), 1521-1525.

Tan, T., Suresh, K., & Smith, H. (2008). Phenotypic and genotypic characterisation of *Blastocystis hominis* isolates implicates subtype 3 as a subtype with pathogenic potential. *Parasitology Research, 104*(1), 85-93.

Tan, Y. M., Goh, K. L., Muhidayah, R., Ooi, C. L., & Salem, O. (2003). Prevalence of irritable bowel syndrome in young adult Malaysians: a survey among medical students. *Journal of gastroenterology and hepatology, 18*(12), 1412-1416.

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D. & Heasman, J. (2005). Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. *Cell, 120*(6), 857-871.

Taşova, Y., Sahin, B., Koltaş, S., & Paydaş, S. (2000). Clinical significance and frequency of *Blastocystis hominis* in Turkish patients with hematological malignancy. *Acta medica Okayama, 54*(3), 133-136.

Teow, W., Ng, G., Chan, P., Chan, Y., Yap, E., Zaman, V., & Singh, M. (1992). A survey of *Blastocystis* in reptiles. *Parasitology Research, 78*(5), 453-455.

Thathaisong, U., Worapong, J., Mungthin, M., Tan-Ariya, P., Viputtigul, K., Sudatis, A., Noonai, A & Leelayoova, S. (2003). *Blastocystis* isolates from a pig and a horse are closely related to *Blastocystis hominis*. *Journal of clinical microbiology, 41*(3), 967-975.

Thompson, R., Reynoldson, J., & Mendis, A. (1993). Giardia and giardiasis. *Advances in Parasitology, 32*, 71-160.

Thompson, V. C., Hurtado-Coll, A., Turbin, D., Fazli, L., Lehman, M. L., Gleave, M. E., & Nelson, C. C. (2010). Relaxin drives Wnt signaling through upregulation of PCDHY in prostate cancer. *The Prostate, 70*(10), 1134-1145.

Thompson, W., Heaton, K., Smyth, G., & Smyth, C. (2000). Irritable bowel syndrome in general practice: prevalence, characteristics, and referral. *Gut, 46*(1), 78-82.

Torres, M. A., Yang-Snyder, J. A., Purcell, S. M., DeMarais, A. A., McGrew, L. L., & Moon, R. T. (1996). Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus development. *The Journal of cell biology, 133*(5), 1123-1137.

Toyama, T., Lee, H. C., Koga, H., Wands, J. R., & Kim, M. (2010). Noncanonical Wnt11 inhibits hepatocellular carcinoma cell proliferation and migration. *Molecular Cancer Research, 8*(2), 254-265.

Tsang, T.-K., Levin, B. S., & Morse, S. R. (1989). Terminal ileitis associated with *Blastocystis hominis* infection. *The American journal of gastroenterology, 84*(7), 798-799.

Tungtrongchitr, A., Manatsathit, S., Kositchaiwat, C., Ongrotchanakun, J., Munkong, N., Chinabutr, P., Leelakusolvong, S. & Chaicumpa, W. (2004). *Blastocystis hominis* infection in irritable bowel syndrome patients. *Southeast Asian journal of tropical medicine and public health, 35*, 705-710.

Udkow, M. P., & Markell, E. K. (1993). *Blastocystis hominis*: prevalence in asymptomatic versus symptomatic hosts. *Journal of Infectious Diseases, 168*(1), 242-244.

Ustün, S., & Turgay, N. (2006). *Blastocystis hominis* and bowel diseases. *Turkiye parazitolojii dergisi/Turkiye Parazitoloji Dernegi= Acta parasitologica Turcica/Turkish Society for Parasitology, 30*(1), 72-76.

Van Gool, T., Weijts, R., Lommerse, E., & Mank, T. (2003). Triple faeces test: an effective tool for detection of intestinal parasites in routine clinical practice. *European Journal of Clinical Microbiology and Infectious Diseases, 22*(5), 284-290.

Vazquez, A., Bond, E. E., Levine, A. J., & Bond, G. L. (2008). The genetics of the p53 pathway, apoptosis and cancer therapy. *Nature reviews Drug discovery, 7*(12), 979-987.

Veeman, M. T., Axelrod, J. D., & Moon, R. T. (2003). A second canon: functions and mechanisms of β-catenin-independent Wnt signaling. *Developmental cell, 5*(3), 367-377.

Velasco, J., Zarrabeitia, M., Prieto, J., Perez-Castrillon, J., Perez-Aguilar, M., Perez-Nuñez, M. & Ortiz, F. (2010). Wnt pathway genes in osteoporosis and osteoarthritis: differential expression and genetic association study. *Osteoporosis international, 21*(1), 109-118.

Vennila, G., Kumar, G. S., Anuar, A. K., Rajah, S., Saminathan, R., Sivanandan, S., & Ramakrishnan, K. (1999). Irregular shedding of *Blastocystis hominis*. *Parasitology Research, 85*(2), 162-164.

Walderich, B., Bernauer, S., Renner, M., Knobloch, J., & Burchard, G. D. (1998). Cytopathic effects of *Blastocystis hominis* on Chinese hamster ovary (CHO) and adeno carcinoma HT29 cell cultures. *Tropical Medicine & International Health, 3*(5), 385-390.

Wei, Q., Zhao, Y., Yang, Z.-Q., Dong, Q.-Z., Dong, X.-J., Han, Y. & Wang, E.-H. (2008). Dishevelled family proteins are expressed in non-small cell lung cancer and function differentially on tumor progression. *Lung cancer, 62*(2), 181-192.

Whitehead, W. E., Palsson, O., & Jones, K. R. (2002). Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? *Gastroenterology, 122*(4), 1140-1156.

WHO. (2005). *World Health Statistics 2005*: Geneva: World Health Organization.

Widelitz, R. (2005). Wnt signaling through canonical and non-canonical pathways: recent progress. *Growth Factors*, 23(2), 111-116.

Wilairatana, P., Radomyos, P., Radomyos, B., Phraevanich, R., Plooksawasdi, W., Chanthavanich, P., Viravan, C. & Looareesuwan, S. (1996). Intestinal sarcocystosis in Thai laborers. *The Southeast Asian journal of tropical medicine and public health, 27*(1).

Wilson, S., Roberts, L., Roalfe, A., Bridge, P., & Singh, S. (2004). Prevalence of irritable bowel syndrome: a community survey. *British Journal of General Practice, 54*(504), 495-502.

Windsor, J. (2007). *Blastocystis hominis* and *Dientamoeba fragilis*: neglected human protozoa. *The Biomedical Scientist, 64*, 524-527.

Windsor, J. J., & Johnson, E. H. (1999). *Dientamoeba fragilis*: the unflagellated human flagellate. *British journal of biomedical science, 56*(4), 293.

Windsor, J., Macfarlane, L., Hughes-Thapa, G., Jones, S., & Whiteside, T. (2002). Incidence of *Blastocystis hominis* in faecal samples submitted for routine microbiological analysis. *British journal of biomedical science, 59*(3), 154.

Wodarz, A., & Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annual review of cell and developmental biology, 14*(1), 59-88.

Wong, K. H., Ng, G., Lin, R. T., Yoshikawa, H., Taylor, M. B., & Tan, K. S. (2008). Predominance of subtype 3 among *Blastocystis* isolates from a major hospital in Singapore. *Parasitology Research, 102*(4), 663-670.

Yakoob, J., Jafri, W., Beg, M. A., Abbas, Z., Naz, S., Islam, M., & Khan, R. (2010b). *Blastocystis hominis* and *Dientamoeba fragilis* in patients fulfilling irritable bowel syndrome criteria. *Parasitology Research, 107*(3), 679-684.

Yakoob, J., Jafri, W., Beg, M. A., Abbas, Z., Naz, S., Islam, M., & Khan, R. (2010a). Irritable bowel syndrome: is it associated with genotypes of *Blastocystis* hominis. *Parasitology Research, 106*(5), 1033-1038.

Yakoob, J., Jafri, W., Jafri, N., Khan, R., Islam, M., Beg, M. A., & Zaman, V. (2004). Irritable bowel syndrome: in search of an etiology: role of *Blastocystis hominis*. *The American journal of tropical medicine and hygiene, 70*(4), 383-385.

Yamaguchi, T. P. (2001). Heads or tails: Wnts and anterior–posterior patterning. *Current Biology, 11*(17), R713-R724.

Yan, D., Wallingford, J. B., Sun, T.-Q., Nelson, A. M., Sakanaka, C., Reinhard, C. & Williams, L. T. (2001). Cell autonomous regulation of multiple Dishevelled-dependent pathways by mammalian Nkd. *Proceedings of the National Academy of Sciences, 98*(7), 3802-3807.

Yan, Y., Su, S., Lai, R., Liao, H., Ye, J., Li, X. & Chen, G. (2006). Genetic variability of *Blastocystis hominis* isolates in China. *Parasitology Research, 99*(5), 597-601.

Yoshikawa, H., Abe, N., & Wu, Z. (2004b). PCR-based identification of zoonotic isolates of *Blastocystis* from mammals and birds. *Microbiology, 150*(5), 1147-1151.

Yoshikawa, H., Abe, N., Iwasawa, M., Kitano, S., Nagano, I., Wu, Z., & Takahashi, Y. (2000). Genomic analysis of *Blastocystis hominis* strains isolated from two long-term health care facilities. *Journal of Clinical Microbiology, 38*(4), 1324-1330.

Yoshikawa, H., Morimoto, K., Nagashima, M., & Miyamoto, N. (2004c). A survey of *Blastocystis* infection in anuran and urodele amphibians. *Vetiranary Parasitology, 122*(2), 91-102.

Yoshikawa, H., Wu, Z., Howe, J., Hashimoto, T., Geok-Choo, N., & Tan, K. S. (2007). Ultrastructural and phylogenetic studies on *Blastocystis* isolates from cockroaches. *Journal of Eukaryotic Microbiology, 54*(1), 33-37.

Yoshikawa, H., Wu, Z., Kimata, I., Iseki, M., Ali, I. K. M., Hossain, M. B. & Takahashi, Y. (2004a). Polymerase chain reaction-based genotype classification among human *Blastocystis hominis* populations isolated from different countries. *Parasitology Research, 92*(1), 22-29.

Yoshikawa, H., Wu, Z., Nagano, I., & Takahashi, Y. (2003). Molecular comparative studies among *Blastocystis isolates* obtained from humans and animals. *Journal of parasitology, 89*(3), 585-594.

Yoshikawa, H., Yoshida, K., Nakajima, A., Yamanari, K., Iwatani, S., & Kimata, I. (2004d). Fecal-oral transmission of the cyst form of *Blastocystis hominis* in rats. *Parasitology Research, 94*(6), 391-396.

Yoshioka, S., King, M. L., Ran, S., Okuda, H., MacLean, J. A., McAsey, M. E., & Hayashi, K. (2012). WNT7A regulates tumor growth and progression in ovarian cancer through the WNT/β-catenin pathway. *Molecular Cancer Research, 10*(3), 469-482.

Zali, M. R., Mehr, A. J., Rezaian, M., Meamar, A. R., Vaziri, S., & Mohraz, M. (2004). Prevalence of intestinal parasitic pathogens among HIV-positive individuals in Iran. *Jpn J Infect Dis, 57*(6), 268-270.

Zaman, V., & Khan, K. (1994). A concentration technique for obtaining viable cysts of *Blastocystis hominis* from faeces. *Journal-Pakistan Medical Association, 44*, 220-220.

Zaman, V., Howe, J., & Ng, M. (1995). Ultrastructure of *Blastocystis hominis* cysts. *Parasitology Research, 81*(6), 465-469.

Zaman, V., Ng, G., Suresh, K., Yap, E., & Singh, M. (1993). Isolation of *Blastocystis* from the cockroach (Dictyoptera: Blattidae). *Parasitology Research*, *79*(1), 73-74.

Zhang, X., Qiao, J., Wu, X., Da, R., Zhao, L., & Wei, Z. (2012). In vitro culture of *blastocystis hominis* in three liquid media and its usefulness in the diagnosis of *blastocystosis*. *Int J Infect Dis*, *16*(1), e23-28.

Zhang, X., Zhang, S., Qiao, J., Wu, X., Zhao, L., Liu, Y., & Fan, X. (2012). Ultrastructural insights into morphology and reproductive mode of *Blastocystis hominis*. *Parasitology Research*, *110*(3), 1165-1172.

Zierdt, C. (1973). Studies of *Blastocystis hominis*. *Journal of Eukaryotic Microbiology*, *20*(1), 114-121.

Zierdt, C. (1988). *Blastocystis hominis*, a long-misunderstood intestinal parasite. *Parasitology Today*, *4*(1), 15-17.

Zierdt, C. H. (1991). *Blastocystis hominis*--past and future. *Clinical Microbiology Reviews*, 4(1), 61.

Zierdt, C. H., & Tan, H. K. (1976). Ultrastructure and light microscope appearance of *Blastocystis hominis* in a patient with enteric disease. *Zeitschrift für Parasitenkunde*, 50(3), 277-283.

Zierdt, C. H., Zierdt, W. S., & Nagy, B. (1995). Enzyme-linked immunosorbent assay for detection of serum antibody to *Blastocystis hominis* in symptomatic infections. *J Parasitol*, 127-129.

Zierdt, C., Rude, W., & Bull, B. (1967). Protozoan characteristics of *Blastocystis hominis*. *American Journal of Clinical Pathology*, 48(5), 495.

List of publications and papers presented

List of publications

1. Lee, I.L., Tan, T.C., Tan, P.C., **Nanthiney, D.R.**, Biraj M.K, Nabin, K.T., Surendra, K.M. & Suresh, K.G. (2011). **Predominance of *Blastocystis* sp. Subtype 4 in Rural Communities, Nepal.** *Parasitology Research*. 110(4):1553-1562

2. **Nanthiney, D.R.**, Suresh, K.G., Tan T.C. & Sanjiv M. (2014). **Phenotypic variation in *Blastocystis* sp. ST3.** *Parasites and vector*, 7:404.

3. **Nanthiney, D.R.**, Suresh, K.G., Tan T.C. & Sanjiv M. (2015). **Factors that influences the shedding of *Blastocystis* sp. cysts in an irritable bowel syndrome (IBS) patients- an evidence-based case study.** *Parasitology Research*. 1-7

4. **Nanthiney D.R.,** Suresh, K.G., Tan T.C., Sanjiv, M. & Ho S.H. (2015). ***Blastocystis* sp. In Irritable Bowel Syndrome – Detection In Colonic Aspirates During Colonoscopy**. *PloS one*, 10:9.

5. Vinoth K., Suresh, K.G., Umah R., Chandramathi S. & **Nanthiney D.R.** (2015). **Cytotoxic efficacy of 5-Fluorouracil (5-FU) against human colon cancer cells, HCT116 in the presence of solubilized antigen of *Blastocystis* sp.** *Plos One*. Submitted.

6. **Nanthiney D.R.,** Suresh, K.G., Tan T.C. & Jaya, P. (2015) **Pathophysiology of *Wistar* rats infected with *Blastocystis* sp. ST 3 cysts derived from asymptomatic individual, symptomatic and irritable bowel syndrome (IBS) patients.** Manuscript in preparation.

7. **Nanthiney D.R.,** Suresh, K.G., Tan T.C. & Chandramathi S. (2015) **The effects of *Blastocystis* antigen ST3 (Blasto-Ag ST3) derived from non-IBS and IBS patients respectively on the growth and gene expression of cancer cells, HCT116 in vitro (in comparison with normal cells).** Manuscript in preparation.

8. Hemalatha, C., Suresh, K.G., Tan T.C., Jaya, P., Chandrawathani., P., Premaalatha., B. & **Nanthiney D.R. (**2015) **Biochemical, hematological and histopathological changes in rats infected with *Blastocystis* sp**. Manuscript in preparation.

List of papers presented.

1. **Nanthiney D.R**., Suresh, K.G., Tan T.C., Sanjiv, M. & Ho S.H. (2012).*Blastocystis hominis* in Irritable Bowel Syndrome - Detection in Stool Aspirates during Colonoscopy. *48th Annual Scientific Seminar of Malaysian Society of Parasitology and Tropical Medicine (MSPTM),* 27 & 28th March 2012, Kuala Lumpur, Malaysia. (Oral Competition-2nd prize winner)

2. **Nanthiney D.R**., Suresh, K.G., Tan T.C., Sanjiv, M. (2012). Surface differences in *Blastocystis* isolated from asymptomatic individuals, symptomatic and irritable bowel syndrome patients (IBS) patients. *48th Annual Scientific Seminar of Malaysian Society of Parasitology and Tropical Medicine (MSPTM),* 27 & 28th March 2012, Kuala Lumpur, Malaysia. (Poster presentation).

3. **Nanthiney D.R.,** Suresh, K.G., Tan T.C., Sanjiv, M. & Ho S.H. (2012). *Blastocystis* sp. In Irritable Bowel Syndrome – Detection In Stool Aspirates During Colonoscopy. *Asian Pacific Digestive Week (APDW),* 5-8th December 2012, Bangkok, Thailand.

4. **Nanthiney D.R.,** Suresh, K.G., Tan T.C., Sanjiv, M. (2013). The correlation between IBS and *Blastocystis*: Brain Gut Axis. *49th Annual Scientific Seminar of Malaysian Society of Parasitology and Tropical Medicine (MSPTM),* 19 & 20th March 2013, Kuala Lumpur, Malaysia. (Poster presentation).

5. Gaythri T., Suresh K.G., Subha, B., Kalyani, R. & **Nanthiney, D.R.**(2013). **Stress responsing gene in *Blastocystis* sp.** *49th Annual Scientific Seminar of Malaysian Society of Parasitology and Tropical Medicine (MSPTM),* 19 & 20th March 2013, Kuala Lumpur, Malaysia. (Poster presentation).

6. **Nanthiney, D.R.**, Suresh, K.G., Tan T.C. & Sanjiv M. (2014). Phenotypic variation in *Blastocystis* sp. ST3. *50th Annual Scientific Seminar of Malaysian Society of Parasitology and tropical Medicine (ACTMP*), 5-7th March 2014, Kuala Lumpur, Malaysia. (Oral presentation).

7. **Nanthiney, D.R.**, Suresh, K.G., Tan T.C. & Sanjiv M. (2014). Ultrastructural studies of *Blastocystis* isolated from asymptomatic, symptomatic and irritable bowel syndrome (IBS) patients. *Australian Society for Parasitology 50th Anniversary Conference,* 30th June- 3rd July 2014, Canberra, Australia. (Oral presentation).

8. **Nanthiney, D.R.**, Suresh, K.G., Tan T.C. & Sanjiv M. (2014). *Blastocystis* ST3 and irritable bowel syndrome (IBS). *Australian Society for Parasitology 50th Anniversary Conference,* 30th June- 3rd July 2014, Canberra, Australia. (Oral presentation).

9. **Nanthiney, D.R.**, Suresh, K.G., Tan T.C. & Sanjiv M. (2014). Factors influencing the shedding pattern of *Blastocystis* cysts in an irritable bowel syndrome patient- An evidence based case study. *Joint International Tropical Medicine Meeting (JITMM 2014),* 2-4th December 2014, Bangkok, Thailand. (Oral and Poster Presentation).

10. **Nanthiney D.R.,** Suresh, K.G., Tan T.C., Sanjiv, M. (2015). Shedding of *Blastocystis* cysts in an irritable bowel syndrome patient. *51th Annual Scientific Seminar of Malaysian Society of Parasitology and Tropical Medicine (MSPTM),* 3 & 4th March 2015, Kuala Lumpur, Malaysia. (Oral competition).