Molecular epidemiology of *Blastocystis* sp.

By

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Certificate of Original Authorship

This study was carried out in the Microbiology Department, St. Vincent's Hospital, Sydney under the supervision of Professor John Ellis and Dr Damien Stark. I certify that this thesis has not been submitted previously as part of any course or degree other than in fulfilment of the requirements of a PhD degree at the University of Technology, Sydney. I certify that this thesis has been written by me and the vast majority of work described was completed by me. All other contributors have been acknowledged throughout this thesis as necessary.

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Peer reviewed Journal Publications

Chapter 1:

Roberts, T., Stark, D., Harkness, J., Ellis, J. 2014. Update on the diagnostic tools and molecular epidemiology of *Blastocystis* sp. Journal of Medical Microbiology and Diagnosis, **3**:131

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Chapter 2:

Roberts, **T.,** Barratt, J., Harkness, J., Ellis, J., and Stark, D. 2011. Comparison of Microscopy, Culture, and Conventional Polymerase Chain Reaction for Detection of *Blastocystis* sp. in Clinical Stool Samples. Am. J. Trop. Med. Hyg., 84(2), 308–312

Chapter 3:

Roberts, **T.,** Stark, D., Harkness, J., and Ellis, J., 2013a. Subtype distribution of *Blastocystis* isolates identified in a Sydney population and pathogenic potential of *Blastocystis*. Eur J Clin Microbiol Infect Dis. 32, 335–343

Chapter 4:

Roberts, **T.,** Stark, D., Harkness, J., and Ellis, J., 2013b. Subtype distribution of *Blastocystis* isolates from a variety of animals from New South Wales, Australia. Veterinary Parasitology, 196, 85-89

Chapter 6:

Roberts, **T.,** Ellis, J., Harkness, J., Marriott, D., and Stark, D. 2014. Treatment failure in patients with chronic *Blastocystis* infection. Journal of Medical Microbiololgy, 63, 252–257

Conference Presentations

Roberts, T., Stark, D., Harkness, J., Ellis, J. *Blastocystis* molecular epidemiology and susceptibility patterns from Sydney, Australia. 6th Asean congress on Tropical Medicine and Parasitology 5-7th Mar 2014. Kuala Lumpar, Malaysia. Oral presentation

Roberts, T., Stark, D., Harkness J., Ellis, J. Molecular epidemiology and subtype susceptibility patterns of *Blastocystis* from Sydney, Australia. 8th European Congress on Tropical Medicine and International Health 10- 13 Sep 2013, Copenhagen, Denmark. Oral presentation

Roberts, T., Stark, D., Harkness, J., Ellis, J. *Blastocystis* subtype distribution from a variety of animals including Australian native animals. World Association for the Advancement of Veterinary Parasitology 25-29 Aug 2013, Perth, Australia. Oral presentation

Roberts, T., Stark, D., Harkness, J., Ellis, J. Subtype distribution of *Blastocystis* from a variety of animals including Australian native fauna. International Congress of Protistology XIV 28 July- 2 Aug 2013, Vancouver, Canada. Oral presentation

Roberts, T., Stark, D., Harkness, J., Ellis, J. Pathogenic potential of *Blastocystis sp.*RNSH Scientific Research Meeting, November 2012, Sydney, Australia. Oral presentation

Roberts, T., Stark, D., Harkness, J., Ellis, J. Molecular epidemiology and clinical aspects of *Blastocystis sp.*- an Australian perspective. American Society of Parasitologists 86th Annual Meeting 1-4 June 2011, Anchorage, USA. Oral Presentation

Roberts, T., Stark, D., Harkness, J., Ellis, J. Phylogenetic Analysis and Pathogenic Potential of *Blastocystis sp.* From Sydney, Australia. American Society for Microbiology 111th General Meeting 21-25 May 2011, New Orleans, USA. Oral presentation

Roberts, T., Stark, D., Harkness, J., Ellis, J. Molecular epidemiology of *Blastocystis sp.* from Sydney, Australia. 12th International Congress of Parastiology 15-21 Aug 2010, Melbourne, Australia. Poster presentation

Roberts, T., Stark, D., Harkness, J., Ellis, J. Genotyping of *Blastocystis* sp. from symptomatic patients in Sydney, Australia. ASP and ARC/NHMRC Research Network for Parasitology Annual Conference 12-15 July 2009, Sydney, Australia. Oral presentation

Abbreviations

AIDS Acquire immunodeficiency syndrome

ART Antiretroviral therapy

bp base pairs

CDC Centre for Disease Control

CPS Cat protection society

DNA Deoxyribonucleic acid

ELSIA Enzyme linked immunosorbant assay

HIV Human immunodeficiency virus

HM Haematological malignancies

IBD Inflammatory bowel disease

IBS Irritable bowel syndrome

IFN Interferon

IgA Immunoglobulin alpha

IgG Immunoglobulin gamma

IL Interleukin

kb Kilobases

kDa Kilo Dalton

MALDI-TOF Matrix-assisted laser desorption/ionisation time-of-flight

MBD Modified Boeck and Drbohlav's

Mb Megabase

mg milligram

MIC Minimum inhibitory concentration

MLC Minimum lethal concentration

ml millilitre/s

MLO Mitochondria like organelle

MLST Multilocus sequence typing

NCBI National center for Biotechnology Information

NJ Neighbour joining

NSW New South Wales

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PCV Peace corps volunteers

rDNA ribosomal Deoxyribonucleic acid

RFLP Restriction fragment length polymorphism

RNA Ribonucleic acid

rRNA ribosomal Ribonucleic acid

qPCR Quantitative polymerase chain reaction

SAF Sodium acetate acetic acid formalin

SNPs Single nucleotide polymorphisms

SSU Small subunit

ST Subtype

SUPAMAC Sydney University Prince Alfred Macromolecular Analysis Centre

TMP-SMX trimethroprim-sulfamethoxazole

TNF Tumor necrosis factor

TYGM-9 Tryptose, yeast extract, glucose, methionine 9

µl microlitre

µg microgram

w/v weight per volume

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Abstract

Blastocystis sp. is the most common enteric protist of the human gastrointestinal tract. There has been continual controversy over the role *Blastocystis* plays in causing gastrointestinal disease in humans. It has been suggested to be a pathogen or an opportunistic commensal and it has also been suggested that pathogenicity could be related to subtype (ST) determined by molecular methods. Until recently there was little known about this protist in terms of epidemiology, pathogenicity and treatment. Clinical diagnosis has traditionally been based on microscopy of wet preparations or permanent stains but there has recently been a push towards more sensitive techniques such as culture and polymerase chain reaction (PCR). The correct diagnosis of *Blastocystis* is necessary for epidemiological and clinical studies which will aid in determining the actual role of this parasite in the gut and in producing disease. Due to the lack of knowledge on the pathogenicity of this parasite, research into treatment options is limited. Metronidazole is a commonly used anti-parasitic drug that has frequently been used for *Blastocystis* treatment. There is evidence that this drug may not actually have much efficacy at all on *Blastocystis* and therefore be the incorrect treatment option.

This project was designed to address some of the shortcomings in the literature surrounding this parasite. The overall aim of the project was to describe the molecular epidemiology of *Blastocystis sp.* from Australia and comment on the pathogenicity of *Blastocystis* in humans. To be able to determine the molecular epidemiology, it was necessary to use the correct diagnostic method and therefore the first aim of this study was to determine the best diagnostic technique used for the detection of *Blastocystis* (aim 1 of this study). Five different techniques were tested for their sensitivity for detecting *Blastocystis* and it was found that microscopy of a permanent stain was the least sensitive at detecting *Blastocystis* and that PCR was the most sensitive technique. Once the most sensitive diagnostic technique was established it was then possible to determine the prevalence of *Blastocystis* within the Sydney population from clinical samples (aim 2 of this study). It was found that there was a 19% incidence of *Blastocystis* in this population and seven subtypes (STs) were identified by sequencing- ST1, ST2, ST3, ST4, ST6, ST7 and ST8. ST3 was found to be the most common ST in this population.

This study then investigated the prevalence of *Blastocystis* in animals and determined the STs present (aim 3 of this study). There were 38 different species of animal from seven different locations investigated for the presence of *Blastocystis* using PCR. There were 80 (18%) positive isolates from 18 species, and nine different STs were identified- ST1, ST2, ST3, ST4, ST5, ST7, ST11, ST12 and ST13. This is the first report of *Blastocystis* from the eastern grey kangaroo, red kangaroo, wallaroo, snow leopard and ostrich. This study has expanded current knowledge on the host range of *Blastocystis*.

Blastocystis is associated with symptoms in humans similar to irritable bowel syndrome (IBS) such as bloating, diarrhoea and abdominal pain and therefore this study aimed to look at the relationship between *Blastocystis* and IBS (aim 4 of this study). This study showed that though there was not a significantly higher percentage of *Blastocystis* seen in the IBS group compared to the control group, there was a difference in the STs present with ST4 only present in the IBS group. This study also highlighted the need for full microbiological work-up before a diagnosis of IBS can be given as *Blastocystis,* along with other microbes, may actually be a contributor to the disease process.

The final part of this study was to look at treatment options for *Blastocystis.* Due to the lack of knowledge on the pathogenicity of *Blastocystis* there have only been a few studies on treatment options and much more information is needed (aim 5 of this study). This study followed 18 patients with chronic *Blastocystis* infection who were treated with a variety of antimicrobials. It was seen that the most common drug treatment of choice, metronidazole, was not effective for the clearance of *Blastocystis.* This study also highlighted the chronic nature of *Blastocystis* infection in the absence of any other infectious agents. This study also carried out *in vitro* testing for four common human *Blastocystis* STs (ST1, ST3, ST4 and ST8) against 12 commonly used antimicrobials- metronidazole, paromomycin, ornidazole, albendazole, ivermectin, trimethoprim- sulfamethoxazole (TMP-SMX), furazolidone, nitazoxonide, secnidazole, fluconazole, nyastatin and itraconazole. Cultures were maintained in media that was determined the best for *Blastocystis* growth from aim 1 of this study. From this *in vitro* study the lack of efficacy of commonly used antimicrobials for the treatment of *Blastocystis* was shown in particular metronidazole, paromomycin and a triple therapy combination of furazolidone,

nitazoxanide and secnidazole. This study did show the efficacy of two drugs- TMP-SMX and ivermectin and suggested the use of these treatments instead of metronidazole.

Each of these studies aims has furthered the knowledge on *Blastocystis* epidemiology, pathogenicity and treatment options. This is the largest molecular epidemiological study to be completed in Australia and also the largest animal study to be undertaken thus far. Overall, this PhD project has contributed significantly by enhancing and extending current knowledge on *Blastocystis* and will hopefully encourage future research on this fascinating protist.

Chapter 1 Introduction

Preface

This chapter comprises two reviews which look at different aspects of *Blastocystis* research. The first section describes the range of molecular tools used for the diagnosis of *Blastocystis* and how these tools have been used to describe the molecular epidemiology of *Blastocystis* throughout the world. The second section focuses on the pathogenicity of *Blastocystis* including the immune response, animal studies and case reports. This section also discusses the lack of efficacy of current treatment options for *Blastocystis.* The objective of these sections is to give a background to *Blastocystis* research and show the range of molecular diagnostic techniques used. This chapter also discusses the controversial idea of *Blastocystis* pathogenicity. These sections show the need for further research on the different aspects of *Blastocystis* in particular the clinical significance of this organism and doing so justify the research which was carried out as part of this PhD project.

1.1 Update on the diagnostic tools and molecular epidemiology of *Blastocystis sp.*

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Certificate

I certify that the following publication is largely my own work although the contributions of other authors are duly recognised.

- All authors contributed in the following way:
    - By providing suggestions on topics to be reviewed
    - By proof reading draft manuscripts
    - By correcting spelling and grammatical errors in drafts
    - By providing suggestions to improve writing style and language

Otherwise the core composition of this work is credited to me.

I hereby certify that the above statements are true and correct:

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1.1.1 Abstract

Blastocystis is the most common enteric protist found in humans. Due to the recent advancements in molecular technologies, up to 17 subtypes have been identified in both humans and animals. Molecular epidemiological studies have shown the large range of subtype (ST) distribution within geographical locations with ST1-9 being identified in humans. ST3 has been identified as the predominant subtype in most epidemiological studies with a considerable absence of ST4 noted in Africa and the Middle East compared to Europe and Australia where this subtype is fairly common. This review summarises the molecular tools used for diagnosis and speciation of *Blastocystis* and comments on advancements in the area over the last 15 years and what future trends may be. This review also describes the geographical distribution of *Blastocystis* and comments on possible intra-subtype evolutionary relationships.

1.1.2 Introduction

For a protist that was discovered over 100 years ago there is still so much unknown about *Blastocystis* in regards to its pathogenicity, transmission, lifecycle and susceptibility to antimicrobials. Symptoms associated with *Blastocystis* infection include diarrhoea, abdominal pains and bloating (Roberts et al., 2014, Jones et al., 2009). Until recently it was not possible to differentiate between the subtypes of *Blastocystis* and many different molecular techniques have been developed to distinguish between them. In turn this has resulted in different terminologies being used for the identification and designation of *Blastocystis* subtypes. So far 17 subtypes have been identified within the *Blastocystis* clades existing in mammals, birds and reptiles. Due to recent studies it has been shown that no group exclusive to humans exists and that human isolates predominantly lie within subtype 1-4 (Noel et al., 2005, Scicluna et al., 2006, Parkar et al., 2007). Until recently, all human isolates of *Blastocystis* were commonly referred to as *Blastocystis hominis* and all other animal isolates were called *Blastocystis sp.* but these recent molecular results now show that all samples identified through only microscopy should be called *Blastocystis sp.* due to there not being a single subtype specific to humans. From many early studies completed on the subtyping of *Blastocystis*, lack of consistency in the designation of subtypes resulted in confusion and made it difficult for comparison and collaboration within studies. A study on the consensus for the

terminology of *Blastocystis* compared all the previously published data which showed many discrepancies with subtype allocation (Stensvold et al., 2007b). This paper proposed that all isolates fall within nine subtypes and concluded that a standardisation within the nomenclature would make future epidemiological and clinical studies much easier. Since this paper was published, a further eight subtypes have been identified from animals (Stensvold et al., 2009a, Parkar et al., 2010, Alfellani et al., 2013a). Recently the whole genome sequence for subtype (ST) 7 was completed (Denoeud et al., 2011). This new information has shed some light on many of the aspects of *Blastocystis* including the metabolic pathways, virulence factors and possible drug resistance mechanisms.

1.1.3 Typing methods

Clark and colleagues were among the first to employ molecular techniques to distinguish the various subtypes of *Blastocystis* present in human stool samples. They used restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) amplified small subunit (SSU) rDNA for the rapid comparison of multiple isolates. Through this technique they identified seven different *Blastocystis* ribodemes, and suggested that *Blastocystis* was a zoonosis (Clark, 1997). In the last 15 years there has been extensive work done in developing molecular techniques to distinguish the different subtypes of *Blastocystis* and 17 subtypes have been identified from humans and animals. Using PCR and RFLP analysis, which relies on the digestion of PCR products with restriction endonucleases to yield variable gel banding products, up to five different subtypes were identified from different geographical locations (Hoevers et al., 2000, Bohm-Gloning et al., 1997, Kaneda et al., 2001, Rivera and Tan, 2005). The use of RFLP can be problematic in that mixed infections may be missed and if there is a mutation at any of the bases that make up the restriction recognition sequence isolates could be incorrectly identified (Stensvold et al., 2007c).

The application of subtype- specific primer pairs was developed to distinguish between seven different subtypes but as more subtypes have been identified this method for typing is not reliable as it does not cover all the subtypes and therefore infections may be missed or incorrectly assigned to a different subtype (Abe et al., 2003a, Yan et al., 2006, Yoshikawa et al., 2004b). PCR with subsequent

dideoxy sequencing of amplified PCR products, targeting typically the 18S SSU rRNA, is now the most common way for identifying the different subtypes isolated from samples as this method allows for all subtypes to be identified, given that correct primer pairs have been chosen that do not show subtype preference (Scicluna et al., 2006).

Pyrosequencing technology combined with a nested PCR was developed to differentiate between seven subtypes (Stensvold et al., 2007c). The pyrosequencing technology system is based on the pyrophosphate released when a nucleotide is introduced to the DNA-strand and is suited for detecting single or multiple nucleotide polymorphisms within short-read DNA sequences (Ronaghi, 2001, Ahmadian et al., 2006) This technique showed a high specificity and sensitivity and is a fairly fast typing method. This technique does have problems though with possible nucleotide miscalling in short reads and is also not possible to discriminate between subtypes found in mixed infections.

There have only been three studies reported which have developed a quantitative PCR (qPCR) for *Blastocystis*. The first study (Jones et al., 2008) was able to detect ST1, ST3 and ST4 but it is unknown whether other subtypes are able to be detected using this assay and therefore the specificity and sensitivity is unknown. The second study designed a genus- specific PCR which was able to detect all known subtypes so far identified in humans (Poirier et al., 2011). This test only had 95% specificity though, and the amplicon size of 339bp is much longer than usually desired for qPCR sensitivity. The most recent study developed a TaqMan assay which is highly sensitive and specific for subtypes 1-9 of *Blastocystis* (Stensvold et al., 2012a). This appears to be the most sensitive and specific qPCR so far developed but still does not differentiate between the subtypes. At the moment it appears that qPCR could be the most useful tool for the screening of *Blastocystis* but subtyping of isolates by conventional PCR is still the only way to designate subtypes. The development of qPCR techniques which have the ability to discriminate between the different subtypes within a sample will be beneficial for screening and epidemiological studies for *Blastocystis.* qPCR would also prove to be more time and cost effective with extraction and qPCR theoretically being able to be completed within only a few hours.

More recently multilocus sequence typing (MLST) of the mitochondria-like organelle (MLO) of *Blastocystis* has been used to show the differences in diversity within subtypes (Stensvold et al., 2012b). There were five loci of ST3 MLST sequences studied and six loci for the ST4 MLST. The authors showed that most human ST3 infections occur through human- human transmission as most human infections were restricted to a single clade (MLO clade 1) within the ST3 group. Animal infections and people exposed to animals (in particular animal handlers) were found to lie within three other clades which shows the result of zoonotic transmission. This study showed the intra-subtype genetic variability within ST3 and ST4. The study also suggested that ST4 has a more recent history of colonising humans than ST3 due to the lack of variation within the sequences and also because ST4 is common in European populations but is rarely found elsewhere.

The use of the "barcode region" for sequencing appears to be the most effective for the designation of *Blastocystis* subtypes and is the best technique used if phylogenetic information is to be obtained (Scicluna et al., 2006). DNA barcoding refers to a method proposed to produce a unique identifier for all living species and has been used previously to study a variety of animals (Hebert et al., 2004a, Hebert et al., 2004b). The *Blastocystis* barcode region amplifies the 5' 600bp region of the *Blastocystis* SSU-rDNA using the primer pair of RD5, a primer with broad eukaryotic specificity, and BhRDr which is more specific to *Blastocystis* and was designed for subsequent sequencing. This PCR does have the possibility for false positives though if only used for *Blastocystis* screening. The PCR has the ability to detect other eukaryotes particularly fungi which makes this technique not desirable when sequencing of positives is not available.

A substantial number of sequences in Genbank use the "barcode region" and have been submitted to the *Blastocystis* Subtype (18S) and Sequence Typing (MLST) Database (www.pubmlst.org/blastocystis) which makes comparison and designation of subtypes simpler and keeps integrity within the subtype allocations. The advantage of the *Blastocystis* Subtype (18S) and Sequence Typing (MLST) Database is that it uses the consensus subtype nomenclature (discussed below), unlike GenBank, and also assigns the allele to the SSU-rDNA barcode sequence (Stensvold, 2013). This database currently has 255 sequences and 64 MLST profiles and is continually updated.

The use of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has recently revolutionised the clinical microbiology laboratory with accurate, rapid results available for the identification of microorganisms. A recent study showed the accuracy of MALDI-TOF MS for the speciation of five different *Blastocystis* STs (Martiny et al., 2013). Axenic cultures speciated by SSU-rDNA analysis were used to create profiles for the different *Blastocystis* subtypes. It was found that speciation was better after extraction using the ethanol/formic acid procedure. Axenic cultures were shown to be the preferred sample used with xenic broth cultures not having as high ID scores. This study did have a small study number though and showed the limitations of the xenic cultures. This study does however highlight an exciting area of research for the future which could substantially cut costs for *Blastocystis* detection and speciation.

Although there is now a standardised nomenclature, there is still not a standardised method for subtyping with people doing sequencing on different regions of the SSU rDNA or using Sequence-Tagged-Site PCR for subtype designation. Through the application of these different techniques it has been shown that there is substantial antigenic and genetic heterogeneity among *Blastocystis* isolates within and among geographical regions. Special care must be taken when choosing the primers used in PCR where certain primers can have an effect on the final result. Some primers exhibit subtype preferential amplification, which could result in other subtypes being missed in the analysis. Also some primers have been developed to work with cultured samples, whereas others work better with direct extraction of DNA from the stool so this needs to be taken in to consideration when using molecular techniques for diagnosis (Stensvold et al., 2009c). Table 1.1.1 shows the different primers that have been used for the different PCR techniques. There is still much more work needed to develop a simple and effective method for the detection and typing of *Blastocystis sp.* A qPCR that is able to detect and allocate all of the subtypes would be the most useful for diagnosis as well as epidemiological studies whereas the “barcode region” may be the most effective technique for phylogenetic studies and showing evolutionary relationships.

1.1.4 Epidemiology

The prevalence of *Blastocystis* infection was stated as being higher in developing countries than in developed countries (Tan, 2008). Due to varying modes of diagnostic techniques used in epidemiological studies of *Blastocystis,* it is difficult to determine the actual prevalence of *Blastocystis* due to some studies relying solely on microscopy for diagnosis. It was shown that molecular techniques have the highest sensitivity rates for diagnosis of this organism (Stensvold et al., 2007a, Roberts et al., 2011) and the use of only microscopy in diagnosis and epidemiological studies limits interpretation of data on prevalence of *Blastocystis* infections.

A study performed to show the prevalence of intestinal parasites among immigrants in food handling and housemaid jobs in Qatar showed that of the 1337 people surveyed from the Philippines, Indonesia, Indian sub- continent and Africa over two years, there were 588 individuals positive for at least one of the parasites studied. Of all the parasites studied, *Blastocystis* was shown to have the second highest incidence rate at 13.2% for all the nationalities put together with only a slightly higher incidence in African samples than the other nationalities. There was a much higher prevalence in women than men (Abu-Madi et al., 2008).

A study on the Orang Asli aborigine community in Pahang, Malaysia showed that *Blastocystis* had the highest prevalence rate (52.3%) followed by *G. lamblia* (29.2%). Overall there was a 72.3% positive parasitic infection rate for this community. These high rates of parasitic infection can be attributed to the low standard of sanitation and hygiene in this area with there being no electricity, running water or toilets inside their dwellings and water from the river being used for all purposes including cooking, bathing, swimming and washing vegetables (Noor Azian et al., 2007).

In a Zambian school, 53.8% of children were reported to be infected with *Blastocystis* with some being co-infected with *Endolimax nana* (which is another parasite not considered to be pathogenic to humans) with almost half the children suffering from diarrhoea. This study supports the argument that *Blastocystis* infections are associated with inadequate sanitation and low hygiene standards and can contribute to diarrhoea in children in developing countries (Graczyk et al., 2005).

For travellers, it was shown that there is an increased rate of infection while travelling to tropical and under-developed countries, with 30% infection rates found

in tourists in Nepal. *Blastocystis* is frequently identified in stools from foreign workers in South-east Asia and Taiwan, with the incidence of infection being attributed to poor personal and environmental hygiene, lack of water supply and poor sewage and rubbish removal (Sohail and Fischer, 2005). For 36 Peace Corps volunteers (PCVs) in Guatemala for a length of two years or greater there was a 38.8% infection rate with an intestinal parasite. *Blastocystis* was the most common parasite in these samples with a progressive increase in infection from 11.1% of PCVs at the start to 37.9%, 53.1% and then 77.8% at the end of the 2 year study. Most of the *Blastocystis* infections were found to be asymptomatic and last several weeks. The prevalence of intestinal parasites found in these PCVs in Guatemala could be attributed to them being repeatedly exposed to potentially risky diets. Diarrheal illness is the most common medical disorder encountered by travellers from developed countries to developing countries and *Blastocystis* infection should be considered as a potential cause for disease (Herwaldt et al., 2001).

In an orphanage in Thailand over a one year period the rate of infection varied between 6.6%- 27.4% with samples being taken every two months (Pipatsatitpong et al., 2012). These varying results could have been due to the shedding effect seen in many cases of parasitic infection as well as the diagnostic technique (microscopy of a permanent smear and culture) being used not being sensitive enough. These results also highlight the easy transmission of *Blastocystis* in places with poor hygiene.

Through the use of molecular techniques for epidemiological studies, ST3 was found to be the predominant subtype isolated in humans with a range of 40-92% of isolates belonging to this group (Clark, 1997, Kaneda et al., 2001, Souppart et al., 2009). There have been six studies where ST1 was the predominant subtype(Malheiros et al., 2011, Ramirez et al., 2013, Alfellani et al., 2013a, Motazedian et al., 2008, Leelayoova et al., 2008) . There has been four studies where ST4 was the predominant subtype with 37% and 76% in Denmark, 84% in Nepal and 94% in Spain, with no ST3 identified in the Spanish population (Stensvold et al., 2009b, Stensvold et al., 2011, Lee et al., 2011, Dominguez-Marquez et al., 2009).

In Singapore the prevalence of *Blastocystis* is at 3.3% of the studied population with ST1 and ST3 being the only subtypes isolated. There was a 78%

prevalence of ST3 and a 22% incidence of ST1. This prevalence of ST3 in humans in an urbanised area such as Singapore is consistent with the argument that ST3 is of human origin (Wong et al., 2008).

An investigation of isolates of *Blastocystis* in Japan, Pakistan, Bangladesh, Germany and Thailand showed that ST3 was the most common varying from 41.7%-92.3% positives with the 2nd most common being ST1 (7.7-25%) and ST4 (10-22.9%). In Bangladesh an almost equal number of symptomatic and asymptomatic isolates were tested but there was no major percentage difference between ST1 and ST3.

The incidence of *Blastocystis* in a province in China showed a prevalence of 32.6% in studied people with ST3 being the predominant subtype in 70.5% of the positive population, followed by ST1 with 20.5%. The significance of hygiene practices was shown to have an effect on the type of infection with ST3 corresponding to the drinking of unboiled water and ST1 relating to the consumption of raw water plants (Li et al., 2007b).

In Lebanon there was a 19% prevalence of *Blastocystis* from a population of symptomatic and asymptomatic people (El Safadi et al., 2013). Four subtypes were identified with almost equal numbers of ST3 (33.3%), ST2 (33.3%) and ST1 (30.6%) and only one patient with ST4 (2.8%). There was an association seen between ST1 and symptoms. Table 1.1.2 summarises the different subtypes isolated in different countries.

From all the epidemiological studies a pattern can be seen where there is a much higher incidence of ST4 in Europe and Australia compared to Africa and the Middle East where very low numbers, if any, are seen. Why this is occurring is unknown, but it could be due to several reasons. The use of diagnostic tool may have an effect on the STs identified, as previously discussed, with some primer pairs showing ST preferential. Another reason may be due to the recent evolution of ST4. There is a much higher homogeny among ST4 samples compared to ST3 samples suggesting that this may be a relatively new ST (Stensvold et al., 2012b).

1.1.5 Conclusions

The future of *Blastocystis* diagnostics and epidemiology is an exciting area. With the possibility of new genomes being available for the different subtypes, this may help

improve targets for qPCR that will be beneficial for both diagnosis and differentiation between subtypes. As diagnosis and speciation becomes easier, more epidemiological studies will be possible which may potentially show the large range of *Blastocystis* distribution around the world. As more samples are studied, the relationship between geographical location and subtype might become apparent as well as intra-subtype evolutionary relationships.

Table 1.1.1 Primer pairs used for PCR

PCR assay	Gene target	Amplification product	Primers	Sequence (5'- 3')	References
Single tube	SSU rDNA	1.8Kbp	RD5	GGAAGCTTATCTGGTTGATCCTGCCAGTA	(Clark, 1997) (Kaneda et al., 2001)
			RD3	GGGATCCTGATCCTTCCGCAGGTCACCTAC	
Single tube	Small-subunit rRNA	1780bp	SR1F	GCTTATCTGGTTGATCCTGCCAGTAGT	(Yoshikawa et al., 2000) (Rivera and Tan, 2005)
			SR1R	TGATCCTTCCGCAGGTTCACCTA	
Single tube	SSU rRNA	1.1kbp	Forward B	GGAGGTAGTGACAATAAATC	(Bohm-Gloning et al., 1997) (Wong et al., 2008, Stensvold et al., 2007a)
			Reverse B	ACTAGGAATTCCTCGTTCATG	
Subtype classification	SSU rRNA				
	ST1	351bp	SB83F	GAAGGACTCTCTGACGATGA	(Yoshikawa et al., 1998)
			SB83R	GTCCAAATGAAAAGGCAGC	
	ST2	650bp	SB155F	ATCAGCCTACACAATCTCCTC	(Yoshikawa et al., 2004b)
			SB155R	ATCGCCACTTCTCCAAT	
	ST3	526bp	SB227F	TAGGATTTGGTGTTTGGAGA	(Yoshikawa et al., 2000)
			SB227R	TTAGAAGTGAAGGAGATGGAAG	
	ST4	338 bp	SB332F	GCATCCAGACTACTATCAACATT	
			SB332R	CCATTTTCAGACAACCACTTA	
	ST5	704 bp	SB340F	TGTTCTTGTGTCTTCTCAGCTC	(Yoshikawa et al., 2003)
			SB340R	TTCTTTCACACTCCCGTCAT	
	ST6	317 bp	SB336F	GTGGGTAGAGGAAGGAAAACA	
			SB336R	AGAACAAGTCGATGAAGTGAGAT	
	ST7	487 bp	SB337F	GTCTTTCCCTGTCTATTCTGCA	
			SB337R	AATTCGGTCTGCTTCTTCTG	

Table 1.1.1 Primer pairs used for PCR (*Continued*)

PCR assay	Gene target	Amplification product	Primers	Sequence (5'- 3')	References
Single tube	SSU rRNA	459bp	BLF	TAACCGTAGTAATTCTAGGGGC	(Grabensteiner and Hess, 2006) (Menounos et al., 2008)
			BLR	AACGTTAATATACGCTATTGG	
Single tube	SSUrRNA	600 bp	Blasto 1F	ATCTGGTTGATCCTGCCAG	(Scicluna et al., 2006) (Souppart et al., 2009)
			Blasto 1R	GAGCTTTTTAACTGCAACAAC	
			Blasto 2F	TCTGGTTGATCCTGCCAGT	
Single tube "Barcode region"	SSUrRNA	600bp	BhRDr	GAGCTTTTTAACTGCAACAACG	(Scicluna et al., 2006) (Clark, 1997)
			RD5	ATCTGGTTGATCCTGCCAGT	
Single tube	SSU rRNA	310 bp	b11400ForC	GGAATCCTCTTAGAGGGACACTATACA	(Stensvold et al., 2006)
			b11710RevC	TTACTAAAATCCAAAGTGTTCATCGGAC	
Single tube	SSU rRNA	550-585 bp	F1	GGAGGTAGTGACAATAAATC	(Stensvold et al., 2007a)
			BHCRseq3	TAAGACTACGAGGGTATCTA	
Single tube	SSU rRNA	1.7kbp	413-S	GCAGGCGCGTAAATTACCCA	(Rivera, 2008)
			827-S	CATGGAAGCAAGGTTAAAAG	
			1256-2	CCGTTCTTAGTTGGTGGAGT	
			1602-2	ACACACCGCCCGTCGCACCT	
Single Tube	SSUrDNA	445-464bp	Blast 505-532	GGAGGTAGTGACAATAAATC	(Santin et al., 2011)
			Blast 998-1017	TGCTTTCGCACTTGTTCATC	
Nested PCR Pyrosequ-encing	SSU rRNA	170bp	RSBHpyro 2F	GCGAAAGCATTTACCAAGGATGT	(Stensvold et al., 2007c)
			RSBHpyro 2R	CCGGAACCCAAAGACTTTGAT	
Real-time fluorescent LC PCR		152bp	prMSJ2-F	CACCTGTGATTCTCGGG	(Jones et al., 2008)
			prMSJ2-R	GAAATGGAAGATGGAATTGATGAC	
			prb-blasto3-1	CACCTCGATCTCGATCTGCTCCCA	
			prb-blasto3-2 LC RED	TTCCGATTCTCTTCACTCATTTGCTCAATCTCAC	
Real-time quantitative PCR	SSU rRNA	320-342 bp	BL18SPPF1	AGTAGTCATACGCTCGTCTCAAA	(Poirier et al., 2011)
			BL18SR2PP	TCTTCGTTACCCGTTACTGC	

Table 1.1. 2 Subtype classification of human *Blastocystis* sp. isolates from different countries

Country/region (no. Of individuals studied)	No. Of positive isolates	Subtypes identified (%)										References
		1	2	3	4	5	6	7	8	9	unknown/ mixed	
Australia (513)	91	28	5	40	12	0	6	1	2	0	0	(Roberts et al., 2013b)
Australia	12	41	0	33	16	0	8	0	0	0	8	(Nagel et al., 2012)
Bangladesh	26	7.7	0	92.3	0	0	0	0	0	0	0	(Yoshikawa et al., 2004b)
Brazil (382)	66	41	32	17	0	0	0	0	0	0	10	(Malheiros et al., 2011)
China, Yunnan (239)	78	20.5	1.3	70.5	1.3	0	0	0	0	0	5	(Li et al., 2007b)
Colombia	125	56	32	12	0	0	0	0	0	0	0	(Ramirez et al., 2013)
Denmark	29	3.4	20.7	51.7	24.1	0	0	0	0	0	0	(Stensvold et al., 2006)
Denmark (126)	24	8.3	12.5	25	37.5	0	0	0	0	12.5	0	(Stensvold et al., 2009b)
Denmark (444)	25	4	16	4	76	0	0	0	0	0	0	(Stensvold et al., 2011)
Egypt	44	18.2	0	54.5	0	0	18	9.1	0	0	0	(Hussein et al., 2008)
Egypt	100	15	0	49	0	0	33	13	0	0	10	(Fouad et al., 2011)
France	40	25.6	9.3	53.5	9.3	0	0	2.3	0	0	7.5	(Souppart et al., 2009)
Germany (67)	12	25	0	41.7	0	17	0	17	0	0	0	(Yoshikawa et al., 2004b)
Germany	171	23.4	0.6	69	7	0	0	0	0	0	3	(Bohm-Gloning et al., 1997)
Greece	45	20	13.3	60	2.2	0	2.2	2.2	0	0	0	(Menounos et al., 2008)
Italy (34)	34	8.8	20.6	47.1	17.7	0	0	2.9	2.9	0	4	(Meloni et al., 2011)
Iran	40	50	10	40	0	0	0	0	0	0	12	(Motazedian et al., 2008)
Iran	141	34	23	37	0	0	0	5	0	0	23	(Moosavi et al., 2012)
Japan	64	15.6	20.3	48.4	10.9	0	0	0	0	0	4.7	(Kaneda et al., 2001)
Japan (2,037)	50	8	0	52	4	0	22	10	0	4	0	(Yoshikawa et al., 2004b)
Lebanon (220)	42	30.6	33.3	33.3	2.8	0	0	0	0	0	0	(El Safadi et al., 2013)
Liberia	25	28	28	32	12	0	0	0	0	0	20	(Alfellani et al., 2013a)
Libya	38	50	7.9	39.5	0	0	0	2.6	0	0	0	(Alfellani et al., 2013a)

Table 1.1. 2 Subtype classification of human *Blastocystis* sp. isolates from different countries (*Continued*)

Country/region (no. Of individuals studied)	**No. Of positive isolates**	**Subtypes identified (%)**										**References**
		1	**2**	3	4	5	6	7	8	9	Unknown/ Mixed	
Malaysia	40	12.5	5	50	27.5	0	0	0	0	0	2	(Tan et al., 2009)
Nepal (82)	20	20	20	60	0	0	0	0	0	0	0	(Yoshikawa et al., 2009)
Nepal (241)	63	63.5	12.7	0	84.1	0	0	0	0	0	70	(Lee et al., 2011)
Netherlands (442)	103	22	22	42	0	0	1	1	0	0	0	(Bart et al., 2013)
Nigeria	22	45.5	0	40.9	13.6	0	0	0	0	0	0.4	(Alfellani et al., 2013a)
Pakistan	10	20	0	70	0	0	10	0	0	0	0	(Yoshikawa et al., 2004b)
Philippines	5	20	0	80	0	0	0	0	0	0	0	(Rivera, 2008)
Singapore (276)	9	22.2	0	77.8	0	0	0	0	0	0	0	(Wong et al., 2008)
Spain	51	2	3.9	0	94.1	0	0	0	0	0	0	(Dominguez-Marquez et al., 2009)
Sweden	63	15.9	14.3	47.6	20.6	0	0	1.6	0	0	0	(Forsell et al., 2012)
Thailand (30)	5	40	0	40	0	0	20	0	0	0	20	(Yoshikawa et al., 2004b)
Thailand (675)	127	77.9	22.1	0	0	0	0	0	0	0	0	(Leelayoova et al., 2008)
Turkey (286)	92	20	24	59.7	0	0	0	0	0	0	4	(Dogruman-Al et al., 2008)
Turkey	87	9.2	13.8	75.9	1.1	0	0	0	0	0	0	(Ozyurt et al., 2008)
Turkey	25	36	24	10	0	0	0	0	0	0	0	(Eroglu and Koltas, 2010)
UK	55	5.4	16.4	40	31	0	0	1.8	5.4	0	0	(Scicluna et al., 2006)
UK	271	12.5	9.6	42.1	31.4	0.7	0.4	1.5	1.8	0	0	(Alfellani et al., 2013a)
USA	21	11.1	0	66.7	0	0	0	0	0	0	22.2	(Jones et al., 2009)

1.2 Update on the pathogenic potential and treatment options for *Blastocystis sp.*

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Certificate

I certify that the following publication is largely my own work although the contributions of other authors are duly recognised.

- All authors contributed in the following way:
    - By providing suggestions on topics to be reviewed
    - By proof reading draft manuscripts
    - By correcting spelling and grammatical errors in drafts
    - By providing suggestions to improve writing style and language

Otherwise the core composition of this work is credited to me.

I hereby certify that the above statements are true and correct:

Tamalee Roberts (PhD candidate): _____

Date: _____

1.2.1 Abstract

Although *Blastocystis* is one of the most common enteric parasites, there is still much controversy surrounding the pathogenicity and potential treatment options for this parasite. In this review we look at some of the evidence supporting *Blastocystis* as a pathogen as shown by numerous case studies and several *in vivo* studies. We describe the chronic nature of some infections and show the role of *Blastocystis* in immunocompromised patients and the relationship between irritable bowel syndrome and *Blastocystis* infection. There have been several studies that have suggested that pathogenicity may be subtype related with subtype 1 and 4 being implicated in disease. Metronidazole is the most widely accepted treatment for *Blastocystis* but several cases of treatment failure and resistance have been described. Other treatment options which have been suggested include paromomycin and trimethroprim-sulfamethoxazole.

1.2.2 Introduction

Blastocystis is one of the most common intestinal protists of humans. *Blastocystis* was first described 100 years ago but still surprisingly little is still known about the pathogenicity, genetic diversity, host range and treatment. First classified as a yeast, *Blastocystis* was then subsequently classified as a protist and has now been placed within the Stramenopiles (Zierdt, 1991, Zierdt et al., 1988, Arisue et al., 2002, Tan, 2008). *Blastocystis* has a world-wide distribution with higher numbers being found in developing countries probably due to poor sanitation (Graczyk et al., 2005). *Blastocystis* has been found in a wide range of animals including non-human primates, other mammals, birds and amphibians. Up to 17 subtypes have been described with subtype (ST) 1-9 being found in humans (Parkar et al., 2010). ST3 is the predominant ST found in most human epidemiological studies (Roberts et al., 2013b, Alfellani et al., 2013b). Due to the lack of knowledge about this parasite, there is still controversy about whether to treat infections as they may just be opportunistic colonisation. There have been conflicting results about the efficacy of treatments and this is an area where much more research is needed. *Blastocystis* is transmitted by the faecal oral- route by human- human or animal- human transmission. There have been several studies that have shown possible transmission by contaminated water and it has been stated that the poor provision of basic amenities plays an important role in transmission (Ithoi et al., 2011, Banaticla and

Rivera, 2011, Anuar et al., 2013). Figure 1.2.1 describes a current view of the lifecycle of *Blastocystis*.

1.2.3 Pathogenicity

There is still much debate about the pathogenicity of *Blastocystis* in humans. Though many authors have given credit to it as a pathogen (Andiran et al., 2006, Leelayoova et al., 2004, Carrascosa et al., 1996, Levy et al., 1996), there are still many that doubt the role of *Blastocystis* in human disease (Leder et al., 2005, Tungtrongchitr et al., 2004). The most common symptoms associated with *Blastocystis* infection include diarrhoea, abdominal pain and vomiting. There are many reports of patients that show there was no other cause of sickness in patients with *Blastocystis.*

There have been several case reports suggesting that *Blastocystis* is related to urticaria (Tan, 2008). The amoeboid forms of *Blastocystis* ST3 were found in a case of acute urticaria and the authors suggested that cutaneous symptoms may be caused by disruptions to the immune homeostasis as the host produces an inflammatory response against the amoeboid forms (Katsarou-Katsari et al., 2008). Another case showed the presence of *Blastocystis* ST2 in a severe case of gastrointestinal symptoms and chronic urticaria in the absence of any other infectious agent. Symptoms persisted after initial antibiotic therapy but were finally eradicated after combined metronidazole and paromomycin treatment (Vogelberg et al., 2010). Case reports are summarised in Table 1.2.1.

It was suggested that gastrointestinal symptoms related to *Blastocystis* might be ST related but results remain inconclusive (Bohm-Gloning et al., 1997, Kaneda et al., 2001, Ozyurt et al., 2008). It was suggested that ST1 may be related to pathogenicity with a higher subtype-symptom relationship being noted (Yan et al., 2006).

There have been conflicting reports on the pathogenicity of ST2 with some studies showing high symptom- infection rates (Ozyurt et al., 2008, Vogelberg et al., 2010) whereas others have seen no link (Yoshikawa et al., 2004b, Dogruman-Al et al., 2008). A study in Colombia showed that 100% of patients with diarrhoea had ST2 where asymptomatic people all had ST1 (Ramirez et al., 2013). There have been two previous studies that have suggested ST4 to be a pathogenic strain due to the high incidence of this ST in patients with severe diarrhoea (Dominguez-Marquez et

al., 2009, Stensvold et al., 2011). It was also suggested that ST8 could be a pathogenic strain. ST8 is a rare subtype found in humans and in two studies has been related to severe symptoms (Stensvold et al., 2008, Roberts et al., 2013b). Even though ST3 is the most common ST found in humans, there is a low association between this ST and symptoms shown by patients (Roberts et al., 2013b). An animal study in rats showed that ST1 was statistically related to pathogenicity and that there may be pathogenic and non- pathogenic strains within ST3 and ST4 (Hussein et al., 2008). These studies highlight the need for more research in to the relationship between ST and symptoms.

The whole genome for ST7 has been described (Denoeud et al., 2011). This genome has shed light on some important processes identifying genes coding for proteins that are responsible for host protease inhibition. Proteins like these may modulate the host protease activity thereby disturbing the intestinal homeostasis (Parija and Jeremiah, 2013). Further information will be gathered as more genomes are described that can help show the role of genes in the potential pathogenicity of *Blastocystis*.

The study of host immunity to *Blastocystis* is under represented in the literature and offers many opportunities for future study. For example, the study of physiological or genetic factors of the host that may affect the outcome of *Blastocystis* infection as a possible pathogenic organism.

1.2.4 Immune response and animal studies

Several lines of evidence shed light on the mechanisms of possible pathogenesis. *Blastocystis* express cysteine proteases which localise to the parasite central vacuole and have been shown to be sensitive to the inhibitors iodoacetamide and E-64 in azoceasein assays (Puthia et al., 2008). Cysteine proteases play important functional roles in invasion of host cells, immune evasion, pathogenesis, virulence and cell cycle regulation. It was shown that proteases from *Blastocystis* isolates can degrade human secretory immunoglobulin A (Puthia et al., 2005) and that *Blastocystis* WR1 ST4 induces contact-independent apoptosis, F-actin rearrangement and barrier function disruption in IEC-6 cells (Puthia et al., 2006). There was shown to be extensive variation in morphology and protease activity between the two different STs, 4 and 7, of *Blastocystis* (Mirza and Tan, 2008) with

the avian (ST7) isolates having almost twice as much cysteine protease activity compared to the smaller rodent (ST4) isolates. These two STs were also shown to cleave secretory IgA with cysteine and aspartic protease activities respectively. These results suggest the possibility of *Blastocystis* proteases as virulence factors and that they contribute to parasite survival *in vivo* by degrading neutralising mucosal antibodies. Another study was able to identify two cysteine proteases (a cathepsin B and a legumain) secreted by ST7 which could be helpful in the development of virulent and diagnostic markers as well as targets for chemotherapy (Wawrzyniak et al., 2012). One study suggested that 32kDa proteases of ST3 could be virulence factors responsible for protein degradation (Abdel-Hameed and Hassanin, 2011) while another study suggested that the 29kDa *Blastocystis* antigen could be used as a marker for pathogenicity and differentiate between symptomatic and asymptomatic infections (Abou Gamra et al., 2011). Higher levels of IgA in symptomatic individuals with *Blastocystis* compared to healthy asymptomatic carriers has also been described (Mahmoud and Saleh, 2003). A recent study on the effect of *Blastocystis* on the expression of interferon gamma and proinflammatory cytokines in the cecal mucosa of rats showed a significantly upregulated amount of gene transcription of type 1 and proinflammatory cytokines IFN-γ, IL-12 and TNF-α. This suggests that *Blastocystis* infection in rats stimulates specific local host responses including T cells, monocytes/macrophages and/or natural killer cells when exposed to antigens (Iguchi et al., 2009). Several mice studies have highlighted the effect of *Blastocystis* on infected mice with weight loss and diarrhoea occurring when mice were inoculated with high doses of *Blastocystis* (Moe et al., 1997, Yao et al., 2005, Zhang et al., 2006). Another study showed that *Blastocystis* can invade the lamina propria, submucosa and muscle layers (Elwakil and Hewedi, 2010) while another study found elevated levels of hyaluronidase in rat urine infected with *Blastocystis* which suggests invasion of the colonic epithelium with *Blastocystis* (Chandramathi et al., 2010). One study highlighted the use of rats as a good animal model for *Blastocystis* infection. They showed how rats infected with ST1 showed histopathological changes at all the different doses given and suggested that ST1 infection has pathogenic potential with individual variation (Li et al., 2013). These studies show how animals can be used as a good model for pathogenicity but it is

important to take in to consideration that mice are not naturally infected with *Blastocystis* unlike rats which are commonly found to harbour *Blastocystis*.

1.2.5 *Blastocystis* infection in immunocompromised patients

Infections of the gastrointestinal tract play a fundamental role in the morbidity and mortality of acquired immunodeficiency syndrome (AIDS) and human immunodeficiency virus (HIV) patients. A much higher rate of gastrointestinal tract infections has been described since the first cases of HIV and AIDS were reported including diarrhoea associated with parasitosis (Stark et al., 2007a, Cimerman et al., 1999). Diarrhoea is one of the clinical manifestations in HIV infection and usually tends to be chronic. Parasite induced diarrhoea is prominent in AIDS patients and there is varying infection rates due to geographical location with a high incidence in developing countries (eg. up to 95% of infected people in Africa and only up to 50% in developed countries). Suppressed immunological responses at the mucosal level that hinder the intestinal non- specific defence mechanisms in the gastrointestinal tract play a major role in AIDS pathogenesis.

There have been several studies into the prevalence of intestinal parasites in HIV and AIDS infected people with varying results and in particular the incidence of *Blastocystis* in these study populations. A study in Brazil found 40% of patients in an HIV positive population to be infected with at least one enteropathogen and some with two or more present (Cimerman et al., 1999). In this study though, only one patient was infected with *Blastocystis* which suggests that this protist may not be an opportunistic parasite in HIV infected people. Another study in northern India found only two patients (7.7%) of the study population to be infected with *Blastocystis* with 19 of the 26 people studied having parasitic infections (Prasad et al., 2000). Though this is not a high incidence of the parasite in the population, it was shown that in these two patients there were 10 or more organisms seen per field of view and the presence of no other pathogens suggested that *Blastocystis* was the cause of diarrhoea in these patients. This is in comparison to studies done in Africa which showed *Blastocystis* infection to be at a higher rate in HIV positive patients compared to a control group. A study in Senegal found *Blastocystis* in only HIV infected patients with all but one suffering from diarrhoea and with no other pathogens found in the samples. This study suggested that *Blastocystis* should be

considered an opportunistic parasite (Gassama et al., 2001). Another African study in an Ethiopian teaching hospital found there to be an incidence of 14.1% of *Blastocystis* infection in HIV/AIDS patients. There were no statistically significant differences in the prevalence of parasites amongst cases and controls except that of *Blastocystis* which was significantly higher in HIV/AIDS patients. They concluded that *Blastocystis* was a possible pathogenic agent in immunocompromised patients (Hailemariam et al., 2004). A more recent study of intestinal parasites in HIV/AIDS patients in Ethiopia showed that *Blastocystis* was the third most common parasite identified in 10.6% of the study population of 248 patients (Alemu et al., 2011). There were no *Blastocystis* infections seen in the HIV negative group. Diarrhoea was a clinical finding in 80.9% of the parasite positive patients. Another study in Ethiopia showed that the presence of intestinal parasite infections were significantly higher among HIV positive people not on Antiretroviral Treatment (ART) compared to those on ART (Adamu et al., 2013). *Blastocystis* was the second most common parasite identified in the non ART group at 12.8% positive and there was a significant association between *Blastocystis* infection and symptoms of diarrhoea. A study in Iran showed that the occurrence of parasites in HIV positive patients was not as high as seen in African countries with an infection rate of only 18.4%. Of the parasites seen in this study though, *Blastocystis* was the second most prevalent at 4.4% with most of these cases being seen in diarrhoea positive patients (Zali et al., 2004). In Indonesia a total of 318 HIV positive patients were investigated for parasites and *Blastocystis* was identified as the most common parasite occurring in 73.6% of the patients (Kurniawan et al., 2009). *Blastocystis* was found to be present in all $CD4^+$ groups with either high or low counts. A study in China identified *Blastocystis* as the most common enteric parasite in both the HIV positive and HIV negative groups but it was observed that there was actually a higher percentage in the HIV negative group (Tian et al., 2013). This study also observed that co-infection with *Blastocystis* and HIV created lower CD4 levels and higher IL-2 levels compared to the other co-infections with parasites. A recent study on the STs found in HIV/AIDS patients identified 19.8% of patients positive for *Blastocystis* with ST3 being the most common subtype with 55% of isolates followed by ST4 with 25%, ST1 with 15% and ST2 with 5% (Tan et al., 2009). The majority of isolates

belonging to ST3 is consistent with results from most molecular epidemiological studies conducted around the world.

Most of these studies show that *Blastocystis* is not higher in the HIV/AIDS population than what was previously found in normal populations with *Blastocystis* incidence ranging from 6-70% in developing counties. There are also some problems in relation to accuracy of these results with most studies relying on techniques such as the less sensitive microscopy and culture. Although these studies give varying results in respect to *Blastocystis* infection in HIV/AIDS patients, this parasite should still be considered as a cause of diarrhoea in these cases and shows the significance of parasite infection in immunosuppressed patients.

A study on cancer patients and *Blastocystis* infection showed that *Blastocystis* was acquired after the commencement of chemotherapy treatment. This study raises the possibility of opportunistic infections of *Blastocystis* in immunocompromised people (Chandramathi et al., 2012). Another study showed that 7.7% of cancer patients were infected with *Blastocystis* with a slightly higher rate of detection found in the pre-treatment group (9.7%) as opposed to the post-treatment group (6.7%) (Tan et al., 2009). Another study in France compared the occurrence of *Blastocystis* in immunocompromised patients with haematological malignancies (HM) and a non immunocompromised control group. The study showed that there was not a high level of difference between the two groups with prevalence values of 16% for the HM group and 13% for the control group but there was a difference in STs found within the groups. ST4 was the most common ST found in both the HM and control group (66.7% and 58.3% respectively) followed by ST3 (20%), ST6 (6.7%) and ST7 (6.7%) in the HM group. In the control group the second highest was ST7 (16.7%) followed by equal number of ST1, ST2 and ST3 (8.3%) (Poirier et al., 2011). These studies show how *Blastocystis* can easily be an opportunistic infection.

1.2.6 Irritable bowel syndrome and the role of *Blastocystis*

There have been several hypotheses and increasing studies in the last few years relating the incidence of *Blastocystis* infections with the prevalence of irritable bowel syndrome (IBS) in patients. Irritable bowel syndrome is described as a chronic functional gastrointestinal condition. Due to *Blastocystis* causing symptoms similar to those attributed to IBS such as diarrhoea, abdominal pains, cramps and nausea it is

easily seen why an association with this parasite and IBS patients could be made. It is also possible that the change in the environment in the intestine caused by IBS may allow for the conditions favoured by *Blastocystis* for growth. It has been proposed that a possible mechanism for the IBS- like symptoms might be the low-grade inflammation through persistent antigenic exposure in a chronic *Blastocystis* infection (Stark et al., 2007b). It has also been suggested that polymorphisms in genes encoding inflammatory cytokines might have a role in the pathophysiology of IBS. A recent study has suggested that there is a role in the etiology of IBS from the association between IL-8 and IL-10 gene polymorphisms in IBS- *Blastocystis* carriers (Olivo-Diaz et al., 2012). One study showed a possible link between *Blastocystis* and IBS (with 95 IBS patients and 55 control cases) where there was an infection rate of 46% in IBS patients and only 7% in the control group (Yakoob et al., 2003). There have been several other studies which have shown the high number of *Blastocystis* positive individuals in the IBS group compared to the control group with rates of 71%, 76% and 49% with less than 20% in the control groups (Yakoob et al., 2010b, Dogruman-Al et al., 2010, Yakoob et al., 2010a).

A recent study performed in Mexico on IBS patients showed an association between *Blastocystis* and pathogenicity with 31% of IBS patients found to harbour *Blastocystis* (Jimenez-Gonzalez et al., 2011). This study showed a high number of ST1 and ST3 infections within this population which are also common in most non-IBS populations. This study therefore does not show an association between subtype and IBS. A different study on the STs associated with IBS showed a much higher incidence of ST1 in the IBS group compared to the control group but with an equal number of ST3 from both groups (Yakoob et al., 2010b). Another study from Egypt highlights the prevalence of ST1, ST3 and ST4 in IBS patients with ST1 only being detected in the IBS group and not the control group and also showed that ST1 was statistically more relevant to pathogenicity than the other STs (Ahmadian et al., 2006). In Colombia, 100% of IBS patients with *Blastocystis* were identified as harbouring ST3 (Ramirez et al., 2013). The differences from these studies highlights that more research needs to be done on IBS and *Blastocystis* STs associated with disease but does suggest that there may be a role of *Blastocystis* in IBS.

1.2.7 Treatment

Due to the controversy surrounding the potential pathogenicity of *Blastocystis* and the self-limiting nature of symptoms, the treatment of this disease is equivocal. Metronidazole is the most frequently prescribed antibiotic for infections (Gupta and Parsi, 2006, Lucia et al., 2007, Moghaddam et al., 2005). Various drug treatments using metronidazole have been prescribed ranging from 250- 750 mg three times a day for 10 days (Nassir et al., 2004, Valsecchi et al., 2004) or used in combination with other drugs including paromomycin (Pasqui et al., 2004) or trimethroprim-sulfamethoxazole (TMP-SMX) (Andiran et al., 2006). There have been reports of resistance to metronidazole (Yakoob et al., 2004a, Haresh et al., 1999) and the cyst form has been shown to have resistance up to 5mg/ml (Zaman and Zaki, 1996). Nitazoxanide, a broad-spectrum 5-nitrothiazole antiparasitic agent has also been reported to be effective in treatment (Cimerman et al., 2003, Diaz et al., 2003). Other studies have shown the efficacy of emetine, furazolidone, TMP-SMX, iodochlorhydroxyquin and pentamidine (Moghaddam et al., 2005, Rossignol et al., 2005). One study also showed the potential benefits of *Saccharomyces boulardii* treatment on *Blastocystis* infected children in Turkey (Dinleyici et al., 2010). A case study in Australia of 18 patients showed that clearance of *Blastocystis* and symptoms did not occur after treatment with either metronidazole, iodoquinol or triple combination therapy consisting of nitazoxanide, furazolidone and secnidazole showing the lack of efficacy of several commonly used antimicrobials for the treatment of *Blastocystis* (Roberts et al., 2014). It has also been proposed that the different STs of *Blastocystis* have varying susceptibility to antimicrobial drugs (Stensvold et al., 2010). There have been four *in vitro* studies looking at susceptibility patterns of *Blastocystis*. Although these studies had a small number of study isolates, it was apparent that different STs show different susceptibility patterns and that metronidazole is not the most effective treatment for *Blastocystis* infection (Mirza et al., 2011a, Mirza et al., 2011b, Dunn et al., 2012, Dhurga et al., 2012).

Due to the uncertainty of whether this parasite is a pathogen or not does make it difficult for physicians to decide whether to treat the infection. There are several online resources including The Blastocystis Research Foundation (www.bhomcenter.org) which is helpful for both physicians and patients with

information about symptoms and treatments and also presents some of the implications of infection in relation to transmission within families and households.

Treatment should be considered if there are chronic symptoms of diarrhoea and abdominal pain in the absence of other pathogens identified from the stool sample. There may be a correlation between ST and sensitivity to drugs which is yet to be addressed in studies.

1.2.8 Conclusions

It is clear from all these studies that there is still much work to be done in the areas of pathogenicity, treatment and control. As more information from the genome is gathered there will be more opportunity to identify possible genes that transcribe for pathogenicity and treatment resistance. A simple antimicrobial susceptibility test for *Blastocystis* could help to assure the correct drug is administered and not allow for resistance to develop. Diagnosis should be made by the use of PCR and treatment should be considered when no other infectious agent can be identified.

Table 1.2.1 Selected Case Reports

Patient	Clinical history	Diagnosis	Treatment	Outcome	Study
One 11 y.o male and one 12 y.o male	Both presented with right lower quadrant tenderness, anorexia, abdominal pain, nausea, vomiting	Initially diagnosed with appendicitis. Stool examination showed *Blastocystis* and patients were then diagnosed with *Blastocystis* infection	Metronidazole and co-trimoxazole	Complete recovery	(Andiran et al., 2006)
24 y.o female	Nine week history of urticaria, hives, chronic diarrhoea, IBS	Initially diagnosed with cellulitis. Treated with non-steroidal cream with no recovery of symptoms. Presented with hives and diagnosed as urticaria. Extensive investigation showed 4 + *Blastocystis* in her stool.	Metronidazole	All urticarial and IBS symptoms cleared	(Gupta and Parsi, 2006)
45 y.o female	Four month history of erythematous and pruriginous lesions on trunk and limbs, mild gastoenteric complaints	Diagnosed with urticaria. Extensive investigation showed the stool postive for *Blastoycstis*.	Paromomycin and metronidazole	All urticarial and gastrointestinal symptoms cleared	(Pasqui et al., 2004)

Table 1.2.1 Selected Case Reports (*Continued*)

Patient	Clinical history	Diagnosis	Treatment	Outcome	Study
32 y.o female	Four year history of allegic rhinitis and chronic urticaria, swelling in pressure sites	Diagnosed with delayed pressure urticaria. Treated with systemic corticosteroids with only partial clearance of symptoms. Stool examination positive for *Blastocystis*	Metronidazole	All urticarial symptoms cleared	(Cassano et al., 2005)
60 y.o female	Four year history of anaphylactoid reactions, severe asthma and generalised urticaria	Diagnosed with chronic urticaria. Extensive investigation identified *Blastocystis* in the stool	Paromomycin	All urticaria symptoms cleared	(Biedermann et al., 2002)
74 y.o male	Diarrhoea, abdominal pain, nausea, fatigue and fever	Hospitalised. Stool was positive for *Blastocystis*	Metronidazole	Clearence of symptoms after 10 days	(Levy et al., 1996)
29 y.o female	Six month history of morning stiffness, pain and swelling of joints, elbows, ankles, knees, diarrhoea, abdominal pain and vomiting	Treated for presumed infectious arthiritis of the knee. No improvement. Microscopy of the synovial fluid and stools both showed the presence of *Blastocystis.*	Metronidazole	After two weeks knee inflammation subsided and all abdominal pain and diarrhoea were cleared	(Lee et al., 1990)
24 y.o male	Six week history of diffuse abdominal pain and diarrhoea	Stool examination was positive for *Blastocystis* and *Endolimax nana.*	Metronidazole	Complete resolution of symptoms 10 days later	(Shah et al., 2012)

Table 1.2.1 Selected Case Reports (*Continued*)

Patient	Clinical history	Diagnosis	Treatment	Outcome	Study
19 y.o male	Three week history of hives, abdominal pain for 2.5 months	Diagnosed with acute urticaria. Routine testing showed the presence of *Blastocystis* in the stool ST3	Metronidazole	10 days after treatment both urticaria and abdominal discomfort were cleared	(Katsarou-Katsari et al., 2008)
20 y.o male	Urticaria and flatulence	Treated with antihistamines with no success. Further investigation showed *Blastocystis* ST2 in the stool. Initially treated with metronidazole but treatement failure appears to have occured. Then treated with co-trimoxazole with no success and finally treated with combination metronidazole and paramomycin	Metronidazole then co-trimoxazole followed by paromomycin	All symptoms cleared 10 days later	(Vogelberg et al., 2010)
40 y.o female	Hospitalised due to severe diarrhoea and fever	*Blastocystis* ST8 infection diagnosed from stool cultures. Treated with metronidazole. Symptoms persisted and the patient also noted bloating, flatulence and abdominal pain. Further treated with co-trimoxazole	Metronidazole then co-trimoxazole	All symptoms cleared	(Stensvold et al., 2008)

Table 1.2.2 Summary of treatments and efficacy for *Blastocystis*

Treatment (Dose)	Efficacy	Reference
Iodoquinole (650mg t.i.d)	0%	(Markell and Udkow, 1986)
Emetine (100μg/ml)	50%	(Mirza et al., 2011a)
Metronidazole (2000mg s.i.d)	0%	(Armentia et al., 1993)
Metronidazole (1500mg s.i.d)	100%	(Cassano et al., 2005)
Metronidazole (750mg t.i.d)	100%	(Gupta and Parsi, 2006)
Metronidazole (750mg t.i.d)	100%	(Katsarou-Katsari et al., 2008)
Metronidazole (500mg t.i.d)	100%	(Lucia et al., 2007)
Metronidazole (250- 750mg t.i.d)	33%	(Moghaddam et al., 2005)
Metronidazole (750mg t.i.d)	100%	(Nassir et al., 2004)
Metronidazole (1500mg s.i.d)	80%	(Nigro et al., 2003)
Metronidazole (800mg t.i.d)	0%	(Stensvold et al., 2008)
Metronidazole (30mg/kg twice daily)	67%	(Dinleyici et al., 2010)
Nitazoxanide (500mg t.i.d)	100%	(Cimerman et al., 2003)
Nitazoxanide (100-200mg b.i.d for children <12yr, 500mg b.i.d for >11yr)	86%	(Rossignol et al., 2005)
Nitazoxanide (500mg t.i.d)	100%	(Romero Cabello et al., 1997)
Ornidazole (500mg t.i.d)	50%	(Hahn, 1985)
Paromomycin (25mg/kg t.i.d)	100%	(Armentia et al., 1993)
Paromomycin (500mg t.i.d)	100%	(Kick et al., 2002)
Paromomycin (25mg/kg t.i.d)	100%	(Valsecchi et al., 2004)
Paromomycin (1000mg b.i.d) & MZ (750mg t.i.d)	100%	(Pasqui et al., 2004)
Saccharomyces boulardii (250mg b.i.d)	78%	(Dinleyici et al., 2010)
Trimethroprim-SMX	22%	(Moghaddam et al., 2005)

Table 1.2.2 Summary of treatments and efficacy for *Blastocystis* (*Continued*)

Treatment (Dose)	**Efficacy**	**Reference**
Trimethroprim-SMX (6mg/kg TMP, 30mg/kg SMX s.i.d)	95%	(Ok et al., 1999)
Trimethropim- SMX (320mg TMP, 1600mg SMX s.i.d)	93%	(Ok et al., 1999)
Trimethroprim- SMX (80mg TMP, 400mg SMX t.i.d)	100%	(Stensvold et al., 2008)

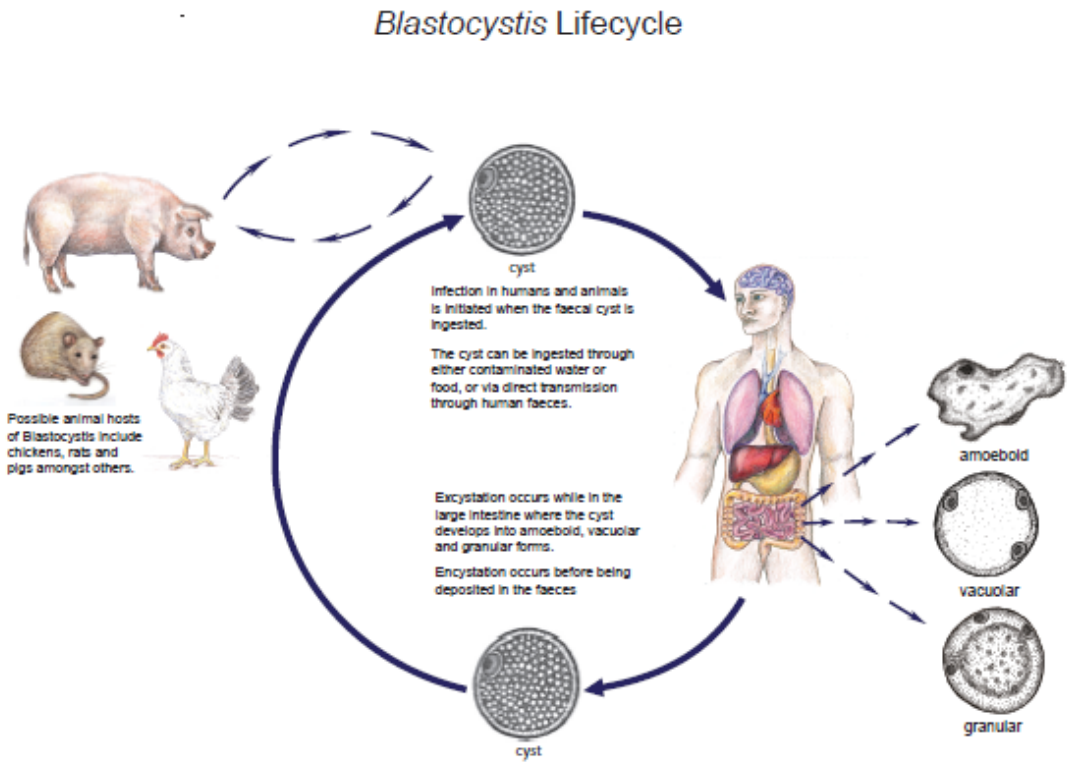

Figure 1.2.1 Lifecycle of *Blastocystis* sp.

Chapter 2 Comparison of Microscopy, Culture and Conventional PCR for the Detection of *Blastocystis sp.* in clinical stool samples

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Keywords

Diagnosis; PCR; culture; *Blastocystis*

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Certificate

I certify that the following publication is largely my own work although the contributions of other authors are duly recognised.

- Dr Joel Barratt (a research assistant at the time the study was conducted) assisted with the collection, culture and DNA extraction from clinical stool samples.
- All authors contributed in the following way:
    - By providing suggestions on topics to be reviewed
    - By providing advice on appropriate experimental design
    - By proof reading draft manuscripts
    - By correcting spelling and grammatical errors in drafts
    - By providing suggestions to improve writing style and language

Otherwise the experimental work and the core composition of this work is credited to me.

I hereby certify that the above statements are true and correct:

Tamalee Roberts (PhD candidate): _____

Date: _____

Preface

To determine the molecular epidemiology of *Blastocystis sp.* from Sydney, it was first necessary to find the most appropriate diagnostic method. Until recently microscopy of permanent stains had been the gold standard for the diagnosis of *Blastocystis* from stools in the clinical laboratory. The use of more advanced techniques including culture and PCR are gaining popularity in clinical laboratories as well as in epidemiological studies. The aim of this chapter was to determine the most sensitive diagnostic tool for the detection of *Blastocystis* from human stool samples and to comment on the prevalence of *Blastocystis* in the Sydney population.

2.1 Abstract

A total of 513 stool samples from patients from Sydney, Australia were tested for the presence of *Blastocystis* by five different diagnostic techniques, including microscopy of a permanently stained smear using a modified iron haematoxylin stain, two xenic culture systems (a modified Boeck and Drbohlav's medium [MBD] and TYGM-9) and two previously published conventional PCR methods targeting the small subunit (SSU) ribosomal (r) DNA. Ninety-eight (19%) samples were positive for *Blastocystis* in one or more of the diagnostic techniques. The PCR 2 method was the most sensitive at detecting *Blastocystis* with a sensitivity of 94%, and the least sensitive was microscopy of the permanent stain (48%). Subtype 3 was the most predominant subtype present with 43% of samples assigned to this group. This study highlights the low sensitivity of microscopy when used as the sole diagnostic modality for the detection of *Blastocystis sp.*

2.2 Introduction

Blastocystis is a single- celled enteric protist that has a world-wide distribution (Stenzel and Boreham, 1996). *Blastocystis* is the most common parasite isolated from human stool samples in both developing and developed countries (Tan et al., 2002, Tan, 2004). Rates of infection vary from 3.3% in developed countries (Wong et al., 2008) to 53.8% in developing countries (Graczyk et al., 2005). *Blastocystis* is found in both humans and animals. There has been some controversy over whether this protozoan is considered pathogenic in humans but many recent *in vivo* and *in vitro*

studies strongly suggest that this organism is a pathogen (Stark et al., 2007b, Boorom et al., 2008, Tan, 2008).

The wide range in prevalence of *Blastocystis* seen between countries can be attributed to several factors such as socioeconomic conditions, but also to the different diagnostic methods used for detection. The most common diagnostic technique used world-wide for the identification of *Blastocystis* is the permanent stain. The use of xenic cultures, where *Blastocystis* is grown *in vitro* with non-specific microorganisms, has been shown to be more sensitive in detecting *Blastocystis* but it is not commonly used by the diagnostic laboratory (Zman and Khan, 1994, Leelayoova et al., 2002, Suresh and Smith, 2004). Molecular diagnosis by PCR using the small subunit (SSU) ribosomal (r)RNA gene is gaining popularity for the detection of enteric parasites. Though this technique is more costly, it is known to be more sensitive than both the direct smear and xenic culture (Stensvold et al., 2007a). Since considerable diversity exists in the rDNA of *Blastocystis*, as the result of diversification of this species into a wide range of subtypes, the choice of primers is crucial from a diagnostic perspective. Some primers may amplify specific subtypes preferentially, which could result in some subtypes being missed in the analysis. Also it is preferable that the PCR/primers are compatible with DNA extracted directly from the stool.

There is a considerable degree of genetic heterogeneity shown within *Blastocystis* and currently human, mammalian, avian and reptilian isolates can be assigned to one of 17 subtypes (Noel et al., 2005, Noel et al., 2003, Parkar et al., 2010, Stensvold et al., 2007a), but it is still not clear as to whether any of these subtypes are specific to human disease. The majority of human *Blastocystis* infections were shown to be attributed to subtype 3, having been reported in 40% to 92% of faecal samples (Yoshikawa et al., 2004b, Scicluna et al., 2006). Although, in some countries this is not the case as seen in Spain where subtype 4 was the most common subtype isolated from a human study group and no isolates were assigned to subtype 3 (Dominguez-Marquez et al., 2009).

Most epidemiological studies rely on molecular analysis from cultures containing *Blastocystis*. Such studies are limited by the fact they do not include *Blastocystis* that do not grow in culture. In addition, there have only been a few

studies that compare the sensitivity of the diagnostic techniques used for the identification of *Blastocystis sp*. (Termmathurapoj et al., 2004, Stensvold et al., 2006). Consequently the aim of this study was to compare five different diagnostic techniques (microscopy of a permanent stain using a modified iron haematoxylin stain, two xenic culture systems (a modified Boeck and Drbohlav's medium [MBD] and TYGM-9) and two previously published conventional PCR methods targeting the SSU rDNA) for the detection of *Blastocystis sp.* in stool samples.

2.3 Materials and Methods

2.3.1 Stool specimens

All stool specimens (n= 513) submitted to St. Vincent's Hospital, Sydney (Microbiology Department) from symptomatic and asymptomatic patients from March 2008 to December 2008 were included in the study. Samples were divided into three aliquots. A portion of the sample was mixed with sodium acetate acetic acid formalin (SAF) preservative and fixed overnight in preparation for permanent staining. A 10mg sample was taken from the fresh faecal samples for culture and a portion frozen at -20˚C to be kept for DNA extraction and further PCR analysis.

2.3.2 Microscopy

Samples fixed in SAF were stained using a modified iron haematoxylin stain (Fronine, Australia) according to the manufacturer's instructions and examined by oil-immersion microscopy. Diagnosis of *Blastocystis* was based on the morphology of the parasites observed in the permanent stained smears.

2.3.3 Culture

All fresh faecal samples were inoculated into two different culture systems upon arrival into the laboratory. Approximately 10mg of the stool sample was put into a monophasic xenic media TYGM-9 and a diphasic xenic system using a dorset egg slope overlaid with approximately 5ml of an in house made growth media containing 90% PBS, 9% sterile horse serum and 1% of 20% w/v bacteriological peptone, with 1mg rice starch and 500 microliters of penicillin-streptomycin solution. Tubes were incubated at 35°C and examination of a drop of sediment was examined every 2 days for the duration of 1 week under phase-contrast microscopy for the presence of

parasites. When parasites were seen in the sediment, a portion was fixed in SAF and stained with modified iron- haematoxylin stain for identification of the organism under oil-immersion microscopy.

2.3.4 DNA extraction

DNA was extracted from all frozen stool samples using the QIAamp™ DNA stool minikit (Qiagen, Hilden, Germany) as per the manufacturer's instructions

2.3.5 PCR for *Blastocystis sp.*

All DNA from the stool specimens underwent PCR for *Blastocystis* using two different, previously described protocols (Stensvold et al., 2006, Stensvold et al., 2007a). Briefly, PCR 1 used primers b11400ForC (5'GGA ATC CTC TTA GAG GGA CAC TAT ACA T-3') and b11710RevC (5'-TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3') that amplifies a 310-base pair fragment of the SSU rDNA. The following reaction was used- thirty- five cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min.

PCR 2 used primers F1 (5'GGA GGT AGT GAC AAT AAA TC-3') (Bohm-Gloning et al., 1997) and BHCRseq3 (5'-TAA GAC TAC GAG GGT ATC TA-3') (Stensvold et al., 2007a) that also target the SSU rDNA of *Blastocystis*. PCR involved denaturation at 95°C for 7min, 35 cycles of 94°C for 60s, 56°C for 45s, followed by a final extension step of 72°C for 7min. Amplicons of 550 to 585 base pairs seen on an agarose gel (Ready Agarose Gels- Bio- Rad, Marnes la Coquette, France) were considered positive.

PCR reactions of 25µl were performed using pure- Taq Ready-To-Go (Amersham Pharmacia Biotech) PCR beads (each containing – 1.5 units Taq DNA polymerase, 10mM Tris-HCl pH9, 50 mM KCl, 1.5 mM $MgCl_2$, 200µM of each dNTP and stabilizers, including BSA), 2µl of genomic DNA extract and 0.5µm of each PCR primer.

Inhibition controls were run to exclude inhibition as a contributor to negative samples. Briefly, samples were spiked with an equal volume of genomic DNA from a known *Blastocystis* positive control, confirmed by sequencing, and run in parallel with an unspiked specimen.

2.3.6 Sequence analysis

DNA sequence analysis was performed on all PCR positive samples. PCR products were purified using the QIAquick™ PCR purification Kit (Qiagen) as per the manufacturer's instructions. The PCR products were then sequenced in both directions on an ABI prism 3700 automated sequencer at the SUPAMAC facility (Royal Prince Alfred Hospital, Sydney). The SSU rDNA sequences were then compared to those available in the GenBank database using the BLASTN program run on the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST).

2.4 Results

A total of 513 samples from 462 patients were included in the study over a 10 month period. The specimens came from 235 males and 227 females with an age range of 3 months to 96 years with an average age of 46.2 years. Ninety-eight (19%) samples were positive for *Blastocystis* in one or more of the diagnostic methods, where a true positive was defined as a sample identified as positive from the use of one or more of the diagnostic techniques. When only PCR was positive, sequence analysis of the PCR product was used as a confirmatory test.

Seventy- eight samples were positive by the initial permanent modified iron haematoxylin stain for any parasite and 47 (9%) of these were positive for *Blastocystis* (results summarised in Table 2.1). Thirty- five samples were positive for *Blastocystis* alone and 12 had a co-infection with one or more other parasites. *Endolimax nana* was the most common protozoan parasite found in conjunction with *Blastocystis* (six), followed by *Giardia intestinalis* and the *Entamoeba histolytica/dispar/moshkovskii* complex (two each). A list of parasites other than *Blastocystis* found in the samples is summarised in Table 2.2. Thirty- eight (38.7%) of the 98 patients with *Blastocystis* infection reported some form of gastrointestinal symptom and five had a previous history of travel. There were 69 patients where *Blastocystis* was the only infectious agent detected. Figure 2.1 shows *Blastocystis* in culture and stained by Iron Haematoxylin.

Of all the diagnostic techniques used, PCR 2 was the most sensitive method for detecting *Blastocystis,* with 92/98 samples positive by any of the diagnostic techniques. The MBD media had 81 positives from stool samples, while 80 were

positive using the TYGM-9 media. Sixty- five samples were found positive by PCR 1 and microscopy of permanent stains was shown to be the least effective at the detection of *Blastocystis* with only 44 positives. The results are summarised in Table 2.3. There was an almost equal specificity for all three diagnostic techniques with PCR and culture having 100% specificity and microscopy 99.9% specificity. Less than 1% of samples were considered inhibitory where inhibition controls failed to produce an amplified product in 5/513 samples.

Although PCR 2 was the more sensitive method at detecting *Blastocystis*, PCR 1 was able to detect four positives which PCR 2 did not. These samples were not found to be subtype specific. There were four samples which were positive in both permanent stain and culture but were initially negative in both PCR techniques. On repeat these samples were found positive in PCR 2. These false-negatives were all *Blastocystis* subtype 1.

While there were an almost equal number of positives from both culture techniques, more prolific growth occurred in the MBD media than the TYGM-9 media. There were two samples which were positive in the permanent stain and also positive in the PCR but were negative in both culture techniques. These samples were both found to have been stored at 4°C overnight before being cultured. Isolates in the MBD culture system lasted for many more passages than the TYGM-9 media where cultures only lasted 3-4 passages with lower numbers seen after each passage. The MBD media produced high numbers of cells after every passage and most cultures lasted for longer than ten passages. Only a few cultures were maintained beyond this amount of time due to storage constraints. All positive cultures were confirmed by microscopy of wet preparations and permanent stains.

There were eight samples where both permanent stain and culture were negative but PCR was positive. There was no correlation between subtype and negative culture with four samples belonging to subtype 3, and two belonging to both subtype 1 and 4. There was no correlation between a negative diagnosis (by initial stain) and a positive PCR result with almost equal numbers of each subtype found when there was a negative stain or a positive stain.

From the *Blastocystis* positive patients there were 11 people that submitted more then one sample. Four of these patients duplicate samples gave different results

from each other. The first patients sample was PCR 1 negative, PCR 2 positive while the second sample was both PCR 1 and 2 positive. *Blastocystis* was seen in both samples cultures but was only seen in the permanent stain for the second sample. The second patient's first sample was positive in both PCR techniques but negative four days later. Both culture and permanent stain were negative for this sample. The third patient had a negative result initially but 12 days later was found to have a PCR positive result but both culture and permanent stain were negative. The fourth patient initially had *Blastocystis* in the permanent stain and culture and both PCR techniques gave a positive result and after sequencing it was shown to be subtype 1. The second sample from this person a day later was negative in the permanent stain but positive for culture and both PCR techniques but after sequencing the isolate was found to belong to subtype 3.

In the initial permanent stain only vacuolar morphological types were seen. During the stages of culture vacuolar and granular types were the main morphological type seen but in older cultures amoeboid cells were also present.

Sequence analysis was completed on all PCR positive samples. 91 readable sequences were obtained from the 95 samples that were positive by either of the PCR techniques. There were six different subtypes identified and subtype (ST) 3 was the most predominant subtype found in clinical samples from this Sydney population with 41 (43%) isolates belonging to this group. ST1 was the second most common subtype with 28 (29%) isolates in this group. This was then followed by ST4 with 12 (12%) isolates, ST2 with 6 (6%) isolates, ST6 with 3 (3%) isolates and just one isolate belonging to ST8 (1%).

Table 2.1 List of other parasites found in-conjunction with *Blastocystis* by use of the permanent stain

Parasites	**No.**
Blastocystis sp	35
Blastocystis + Endolimax nana	4
Blastocystis + Giardia intestinalis	2
Blastocystis + Dientamoeba fragilis	1
Blastocystis + Entamoeba histolytica complex[1]	1
Blastocystis + Enteromonas hominis +Chilomastix mesnili	1
Blastocystis + Iodamoeba butschlii + E. nana + E.histolytica complex[1]	1
Blastocystis + E.nana + Entamoeba coli + Entamoeba hartmanii	1
Blastocystis + D.fragilis + E. coli + I. butschlii + E. nana	1
Total	**47**

[1] *Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii* or *Entamoeba bangladeshi*

Table 2.2 Number and prevalence of parasites other than *Blastocystis* in the clinical specimens studied using the permanent stain

Parasite	**No.**	**%**
G. intestinalis	11	24.4
E. nana	9	20.5
D. fragilis	7	15.5
E. histolytica complex	4	8.8
E. coli	3	6.6
Cryptosporidium	3	6.6
E. hartmanii	2	4.4
I.butshlii	2	4.4
E. hominis	2	4.4
C. mesnili	1	2.2
Strongyloides sp	1	2.2
Total	**45**	**100**

Table 2.3 Comparison of diagnostic techniques for the identification of *Blastocystis*

Diagnostic Method	**Permanent stain**	**MBD culture**	**TYGM-9 cutlure**	**PCR 1**	**PCR 2**
No. Positives *Blastocystis*	47	81	80	65	92

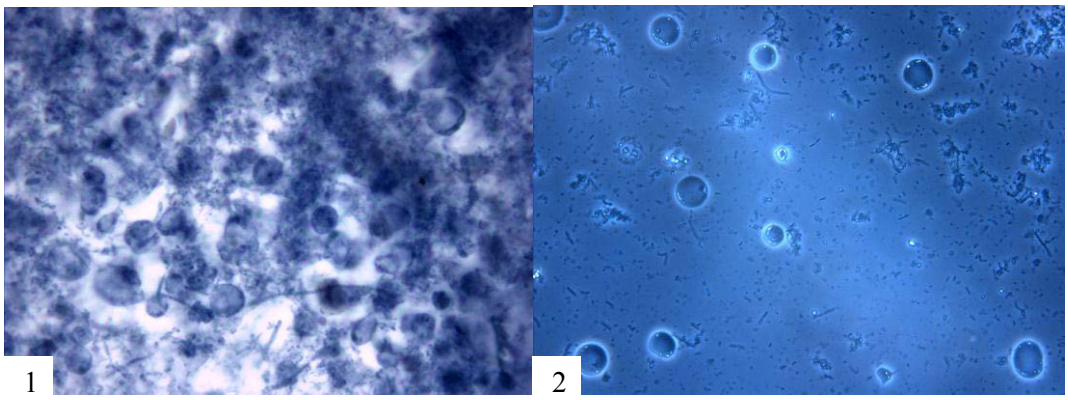

Figure 2.1 1.*Blastocystis* stained with Iron Haematoxylin under oil immersion 2. Blastocystis grown in culture shown under 40x phase contrast

2.5 Discussion

From this study it was shown that PCR 2 was the most effective method for detecting *Blastocystis* in clinical stool samples. While PCR was the most sensitive method, it does have limitations in that it can be expensive, time consuming and labour intensive due to the manual extraction of DNA and specialised equipment needed. In a normal clinical diagnostic laboratory permanent stains are the gold standard for the diagnosis of enteric parasites. While this is effective for the identification of a wide variety of parasites, it was shown in this study that over 50% of the infections with *Blastocystis sp.* are missed. The low sensitivity of this method may result from the parasite being present in low numbers in the samples and so are not detected by the staining procedure. The higher detection rates seen in the cultures may be attributed to the organism having longer to grow and replicate. The results from this study with PCR being the most effective form of diagnosis agree with previous studies which conclude that molecular analysis is the most efficient for the detection of *Blastocystis* (Stensvold et al., 2007a, Parkar et al., 2007). This is in contrast to another study (Termmathurapoj et al., 2004) that suggested *in vitro* culture was superior to direct PCR from stool samples.

This study also demonstrated that in the Sydney population there is a 19% prevalence of *Blastocystis* in people with gastrointestinal symptoms. This is similar to results reported from around the world with countries such as Germany, Thailand and China reporting prevalences of 17.9%, 13.3% and 32.6% respectively (Li et al., 2007b, Yoshikawa et al., 2004b). These studies all used molecular techniques for the diagnosis of *Blastocystis*. *Blastocystis* has been reported in much higher numbers in studies where molecular tools were not used and results were reliant on microscopy alone- 53.8% in Zambia (Graczyk et al., 2005), 52.3% in Malaysia (Noor Azian et al., 2007) and 30% in travelers in Nepal (Sohail and Fischer, 2005). These results are extremely high and could be attributed to the fact that these are studies from developing countries where there is usually a higher prevalence of intestinal parasites due to poor hygienic practices. The misidentification of *Blastocystis* as other organisms such as *Dientamoeba fragilis* or yeasts is a common problem and often the cyst form is difficult to see which may confound studies limited to the use of microscopy alone (Stark et al., 2006). From our study it is shown that for

epidemiological studies PCR should be the method of choice, but if this is not possible due to financial constraints, at least two different diagnostic techniques should be used in conjunction with each other.

The sensitivity of the two different PCR techniques varied greatly in this study. PCR 2 had a sensitivity of 94% while PCR 1 only had a sensitivity of 66%. These results show how important it is to choose the right set of primers and reaction conditions for the detection of *Blastocystis* DNA by PCR.

There have been many studies which use Jones medium successfully as the medium of choice for xenic culture growth of *Blastocystis* (Leelayoova et al., 2002, Suresh and Smith, 2004, Parkar et al., 2007, Stensvold et al., 2007a). In this study two different media, MBD and TYGM-9, were used for the comparison of growth of *Blastocystis* in culture. These culture media were chosen for use instead of Jones medium as it has already been noted that *Blastocystis* grows successfully in Jones medium. These other media were used so as to investigate which other media can be used for the cultivation of *Blastocystis*. There is the possibility that other media could be more suited for the diagnosis of *Blastocystis* than the MBD and TYGM-9 used in this study (giving a higher growth yield) and this should be taken in to consideration when considering further studies.

There were no mixed subtype infections found. There was one patient that submitted two samples within two days and had a different subtype isolated from each sample, ST1 from the initial sample and ST3 from the second sample. This could mean that there was a mixed infection in both of these samples but the PCR primers were only able to detect one subtype from each. There is also the possibility that there are different shedding rates for the different subtypes and this is why two different subtypes were detected from the one person. There were three other people that submitted more then one sample which all had different results. The first patient was initially PCR 1 negative, PCR 2 positive. The second sample was then found to be positive for both PCR 1 and 2. The low sensitivity of PCR 1 can attribute to the fact that it did not detect *Blastocystis* from the first sample. The second patient sample was positive only from PCR 2 from the first sample but was negative for *Blastocystis* in all diagnostic techniques for the second sample which was submitted 4 days later. This could be due to the self-limiting effect of *Blastocystis* and also the

patient may have been treated for another form of disease and this has in effect removed the *Blastocystis*. The final patient was initially negative in all diagnostic techniques for *Blastocystis* but then tested positive by both PCR techniques 12 days later. This was an admitted patient so there is the possibility that the patient either acquired the disease from contaminated food or water brought in to the hospital. It is also possible that the patient had intermittent shedding of *Blastocystis*. This shows the importance of submitting more then one sample over several days to ensure that an accurate diagnosis is made.

There was no correlation seen between permanent stain and a negative culture or with PCR positive results, this suggests that the culture systems used in this study do not discriminate between subtypes of *Blastocystis*. These results do show, though, that it is necessary to do PCR on all culture and microscopy negative samples to get a true perspective of prevalence of *Blastocystis sp.* with isolates possibly being missed if molecular techniques are only applied to positive culture samples. Many epidemiological studies reported only do further molecular analysis when there have been samples found positive by culture (Hoevers et al., 2000, Kaneda et al., 2001, Li et al., 2007b, Rivera, 2008, Wong et al., 2008, Yoshikawa et al., 2004b). An obvious conclusion is that these studies are not providing a true representation of the prevalence of *Blastocystis* infections in human population studies where some culture systems used may not allow certain strains of *Blastocystis* to grow.

From the sequence analysis completed on all PCR positive samples it was found that ST3 is the most common subtype present in the Sydney population. This is the first molecular epidemiological study to look at the subtype distribution of *Blastocystis sp.* in an Australian population. These results with ST3 being the predominant subtype are similar to studies from around the world where ST3 has been the most common subtype isolated in almost all epidemiological studies (Li et al., 2007a, Ozyurt et al., 2008, Yoshikawa et al., 2009, Souppart et al., 2009, Souppart et al.).

2.6 Conclusion

In conclusion, this is the first large scale study to compare five diagnostic techniques used in a clinical laboratory for the diagnosis of *Blastocystis sp.* PCR was the most superior of the diagnostic techniques studied whereas microscopy detected only 48%

of the positive samples. In people from Sydney, Australia we report a prevalence of 19% for *Blastocystis* and show that subtype 3 is the most predominant subtype found in this population with 43% of samples isolated to this group. If the use of PCR is not feasible for a diagnostic laboratory it is recommended that at least two different diagnostic techniques be used for the detection of *Blastocystis*. These results show the need to be cautious when interpreting prevalence reports especially where studies rely solely on microscopy.

Chapter 3 Subtype distribution of *Blastocystis* isolates identified in a Sydney population and pathogenic potential of *Blastocystis*

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Certificate

I certify that the following publication is largely my own work although the contributions of other authors are duly recognised.

- All authors contributed in the following way:
    - By providing suggestions on topics to be reviewed
    - By providing advice on appropriate experimental design
    - By proof reading draft manuscripts
    - By correcting spelling and grammatical errors in drafts
    - By providing suggestions to improve writing style and language

Otherwise the experimental work and the core composition of this work is credited to me.

I hereby certify that the above statements are true and correct:

Tamalee Roberts (PhD candidate): _____

Date: _____

Preface

Once PCR was demonstrated as the most sensitive technique for the detection of *Blastocystis* from aim 1 of this project, it was possible to use this knowledge to determine the molecular epidemiology of *Blastocystis* from the Sydney population. This is the first large scale study to look at the molecular epidemiology of *Blastocystis* from Australia. The information gathered from this study can aid in the global picture of *Blastocystis* subtypes present in humans. Due to the controversy over whether *Blastocystis* should be considered a pathogen there is limited data on subtype- symptom relationships. This study has added to the information on subtype pathogenicity and shows that some subtypes may be considered pathogenic.

3.1 Abstract

Blastocystis is one of the most common enteric parasites present in humans. There is still much uncertainty about the pathogenic potential of this parasite and it was suggested that pathogenicity could be subtype related. This report aimed to study 98 *Blastocystis* isolates found in human stool specimens to identify the subtypes present and do phylogenetic analysis on these isolates. This study also aimed to show the relationship between subtype and symptoms. Five- hundred and thirteen stool samples were subjected to five different diagnostic techniques for the detection of *Blastocystis*. PCR positive samples were then sequenced and the SSU rDNA sequences were aligned and submitted to phylogenetic analysis. Ninety- eight samples were positive by any of the diagnostic methods for *Blastocystis* and 96 were positive by PCR. There were seven different subtypes (1, 2, 3, 4, 6, 7 and 8) identified by PCR and sequencing. This is the first large scale study to look at the occurrence of *Blastocystis* in Australia. This study reports the high prevalence of subtype 3 (44%) in this population and discusses the emerging idea of subtype dependent pathogenicity.

3.2 Introduction

Blastocystis is a single-celled enteric parasite that is commonly found in humans and a variety of animals including primates, other mammals, birds, reptiles and amphibians (Abe et al., 2002, Stenzel and Boreham, 1996, Tan et al., 2002, Yoshikawa et al., 2004a). *Blastocystis* has a world-wide distribution with higher

infection rates seen in developing countries, with more than 50% of people infected (Graczyk et al., 2005). The most common form of transmission of *Blastocystis* is the faecal-oral route, and *Blastocystis* can also be transmitted by the consumption of contaminated food or water (Stenzel and Boreham, 1996, Smith and Nichols, 2006, Cheng et al., 2006, Karanis et al., 2007).

There has been much debate about the pathogenicity of *Blastocystis*. Symptoms related to *Blastocystis* infection include diarrhoea, abdominal cramps, vomiting and flatulence. It was recently suggested that pathogenicity may be related to subtype (ST) allocation but results remain unclear (Bohm-Gloning et al., 1997, Kaneda et al., 2001, Ozyurt et al., 2008, Hussein et al., 2008). Subtype 3 is the most common subtype found in the majority of human epidemiological studies. There has been a low association of ST3 with symptoms in these study populations suggesting that it may have minimal pathogenic potential (Tan, 2008, Jones et al., 2009). There has been a much higher degree of symptom- subtype association seen within patients that have ST1 (Yan et al., 2006) and it was therefore suggested that this subtype has pathogenic potential. Conflicting results exist over the pathogenic potential of ST2 with some studies showing no link to symptoms (Dogruman-Al et al., 2008) whereas others have shown high symptom- infection intensity (Ozyurt et al., 2008, Vogelberg et al., 2010). Intrasubtype differences in lifecycle stage morphology may also be related to pathogenicity with amoeboid forms potentially related to symptoms (Tan and Suresh, 2006, Vassalos et al., 2010).

The most common form of identification for the diagnosis of *Blastocystis* is by microscopy. The use of xenic culture is also a common way of diagnosis and is more often used in epidemiological studies as it was shown to be more sensitive for the diagnosis of *Blastocystis* than microscopy directly from a stool (Zman and Khan, 1994, Leelayoova et al., 2002, Suresh and Smith, 2004, Stensvold et al., 2007a). Molecular diagnosis by PCR using the small subunit (SSU) ribosomal (r)RNA gene target is gaining popularity for the identification of enteric parasites such as *Blastocystis*. Though this technique is considered to be costly and time consuming, it was shown to be the most sensitive method for the detection of *Blastocystis sp.* and can also aid in the identification of the various subtypes (Roberts et al., 2011, Stensvold et al., 2007a). There have been several epidemiological studies that rely on

PCR of extracted DNA from cultured samples (Wong et al., 2008, Yoshikawa et al., 2004b, Li et al., 2007b, Hoevers et al., 2000). These studies do not account for *Blastocystis* that do not grow in culture, and thus they may not necessarily reflect the true picture of the populations present. The choice of PCR/ primer pairs can also have an effect on the subtypes that are identified by PCR. Some PCR/ primer combinations only detect specific subtypes and therefore do not give a true representation of the subtypes in the study group (Stensvold et al., 2009c). PCR inhibitors can also affect the outcome of a PCR with possible false- positive results. There have only been two reported studies on the development of a quantitative PCR assay (Jones et al., 2008). Though highly sensitive, these assays can still miss subtypes if they are only developed for a certain subtype and therefore there is still a need for the introduction of more highly sensitive quantitative PCR methods which are validated for all subtypes.

The aim of this study was to determine the subtypes of *Blastocystis* isolates present in stools from a Sydney population that were submitted for parasitological examination to St. Vincent's Hospital. A further aim was to determine if intestinal symptoms were related to *Blastocystis* subtype.

3.3 Materials and methods

3.3.1 Stool specimens

A total of 513 stool samples were collected from patients at St. Vincent's Hospital between March and December 2008. Samples were collected from symptomatic people as well as routine screening samples from asymptomatic inpatients. Human ethics was approved by the institution. Samples were screened for bacterial pathogens including *Salmonella sp, Shigella sp, Vibrio cholerae, Campylobacter sp, Clostridium difficile* and *Aeromonas sp* by culture and screened for intestinal parasites by microscopy of a permanent modified iron haematoxylin stain. Isolates were then submitted to five different diagnostic techniques for the detection of *Blastocystis sp.* and a fresh portion was frozen for DNA extraction.

3.3.2 Microscopy

Microscopy was performed on fresh SAF fixed faecal samples stained with a permanent modified iron haematoxylin stain according to the manufacturer's

instructions (Fronine, Australia). Approximately 20 fields of view were examined under 100x oil immersion.

3.3.3 Culture

Culture was performed in two xenic culture systems- a diphasic modified Boeck and Drbohlav's medium [MBD] overlaid with approximately 5ml of an in house made growth media containing 90% PBS, 9% sterile horse serum and 1% of 20% w/v bacteriological peptone, with 1mg rice starch and 500 microlitres of penicillin-streptomycin solution, and the monophasic TYGM-9 media with no additives. Approximately 10mg of fresh stool was put in to each culture system and tubes were incubated at 35°C. A drop of sediment was examined every 2 days for the duration of 1 week under phase-contrast microscopy for the presence of parasites.

3.3.4 DNA extraction and PCR for *Blastocystis sp.*

DNA was extracted from all fresh frozen stool samples using the QIAamp™ DNA stool minikit (Qiagen, Hilden, Germany) as per the manufacturer's instructions which was modified for optimal elimination of PCR inhibitors All extracted PCR was performed using two previously published conventional PCR methods targeting the SSU rDNA (Stensvold et al., 2007a, Stensvold et al., 2006, Roberts et al., 2011) . PCR 1 used primer pair b11400ForC (5'GGA ATC CTC TTA GAG GGA CAC TAT ACA T-3') and b11710RevC (5'-TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3') (Stensvold et al., 2006) and PCR 2 used primer pair F1 (5'GGA GGT AGT GAC AAT AAA TC-3') (Bohm-Gloning et al., 1997) and BHCRseq3 (5'-TAA GAC TAC GAG GGT ATC TA-3') (Stensvold et al., 2007a). Inhibition controls were run to exclude inhibition as a contributor to negative samples. Briefly, samples were spiked with an equal volume of genomic DNA from a *Blastocystis* control and run in parallel with an unspiked specimen. Samples were also tested for *Dientamoeba fragilis* by PCR using a previously published method (Stark et al., 2010).

3.3.5 Sequence and phylogenetic analysis

DNA sequence analysis was performed on all PCR positive samples. PCR products were purified using the QIAquick™ PCR purification Kit (Qiagen) as per the manufacturer's instructions. The PCR products were then sequenced in both

directions on an ABI prism 3700 automated sequencer at the SUPAMAC facility (Royal Prince Alfred Hospital, Sydney). The SSU rDNA sequences were then compared to those available in the GenBank database using the BLASTN program run on the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST). Subtypes were determined by an exact match (or closest similarity) with sequence data from known *Blastocystis* subtypes. Each sequence was then aligned with a panel of reference sequences from Genbank using the ClusalW (http://npsapbil.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html) program to determine sequence similarity.

Sequence data from all samples, along with 35 reference *Blastocystis* GenBank sequences (Table 3.1) were aligned using the ClustalW program and gaps and ambiguous sequences were removed from the alignment. A phylogenetic tree was constructed on the phylogeny.fr platform using the BioNJ program for neighbour-joining (NJ) distances with the kimura-two parameters and branch reliability was assessed using bootstrap analyses of 1,000 replicates as shown at the nodes (http://www.phylogeny.fr/). The phylogenetic tree rendering was performed with TreeDyn (v198.3). Clusters in the phylogenetic tree were recorded if they had branch support values of over 70%. The Stramenopile *Proteromonas lacertae* GenBank accession U37108 was used as the outgroup as used for previous phylogenetic studies (Jones et al., 2008, Menounos et al., 2008).

3.4 Results

Of the 513 stool samples collected, 98 were positive for *Blastocystis* by any of the diagnostic methods. Sixty-five samples were from males and 33 were from females with an age range of 1-94 years old with the average age of 46.2 years. It was found that PCR 2 had a much higher sensitivity than PCR 1 detecting 92/98 positives, where PCR 1 only detected 65/98. There were four isolates which PCR 1 detected which PCR 2 did not. Two of these were ST3, one was ST4 and one was ST8. There were two samples which were positive only through microscopy and culture. Both the culture mediums had a sensitivity of 82% and microscopy had the lowest sensitivity at 48%.

All 96 PCR positive samples were sequenced, however only 91 readable sequences were obtained and therefore statistical results are based on the 91 samples. There were seven different subtypes found by BLAST searching. Subtype 3 was the predominant subtype identified with 40 isolates in this group, followed by ST1 with 28 isolates, ST4 with 12 isolates, ST2 with five isolates, ST6 with three isolates, ST7 with one isolate and two isolates from ST8 (Table 3.2).

In Table 3.3 it is shown that *Blastocystis* was the only parasite found in 71 of the 91 samples sequenced. *Blastocystis* was the only parasite found in all of ST6 and ST8 infections. There is an even spread of co-infections between ST1 and ST3 with other parasites. *Cryptosporidium* was only found in a ST2 specimen and this specimen was also the only isolate positive for *Dientamoeba fragilis* by PCR which was not seen in the microscopy. All ST2, ST7 and ST8 patients noted having symptoms. 50% of ST4, 41% of ST3 and 33% of both ST1 and ST6 noted any symptoms present (Table 3.4). There were seven patients that also had a pathogenic bacterial infection- four with *Campylobacter jejuni*, one with *Shigella sonnei*, one with *Shigella flexneri* and one with *Clostridium difficile.* These bacterial infections were found with ST1, ST3, ST4 and ST8 and 5/7 of these patients had symptoms (results summarised in Table 3.5).

There was an even distribution of subtype allocations that were not identified by the Iron Haematoxylin stain apart from all of ST2 where none were identified by the permanent stain. 14/28 ST1 were negative in the Iron Haematoxylin stain, 5/5 ST2, 22/40 ST3, 5/12 ST4 and 1/3 ST6, 1/1 ST7 and 0/2 ST8 (Table 3.6). There were nine samples that were only positive by PCR, two from ST2 and ST4 and five from ST3.

The phylogeny identified seven separate clades from patient isolates and related GenBank sequence subtypes as well as three clades from other GenBank sequence subtypes. Each sequence showed high similarity (96-100%) to homologous sequences from previous reported *Blastocystis* isolates stored in GenBank. From the phylogenetic analyses (Figure 3.1) the vast difference between ST7 and ST8 from the rest of the samples was shown. The ST7 group has a completely separate branch from the rest of the samples and the ST8 clade containing two ST8 Genbank sequences and the two clinical samples has a very long branch. It was seen that the

clades containing ST1 and ST2, and ST3 and ST4 group together. The phylogeny shows that ST3 and ST4 samples are monophyletic and are derived from a common ancestor.

Table 3.1 List of GenBank sequences used for phylogeny comparison

Host	Country of origin	Accession number	Subtype	Reference
Human	Japan	AB023578	1	(Arisue et al., 2002)
Pig	Japan	AB107961	1	(Abe, 2004)
Human	Japan	AB107962	1	(Abe, 2004)
Human	Denmark	AM275354	1	(Stensvold et al., 2007c)
Human	Denmark	AM275356	1	(Stensvold et al., 2007c)
Human	Denmark	AM275357	1	(Stensvold et al., 2007c)
Human	Denmark	AM275359	1	(Stensvold et al., 2007c)
Human	Philippines	EU445492	1	(Rivera, 2008)
Human	Turkey	AM779054	1	(Ozyurt et al., 2008)
Human	Denmark	AM275371	2	(Stensvold et al., 2007c)
Human	Denmark	AM275386	2	(Stensvold et al., 2007c)
Human	Denmark	AM712467	2	(Stensvold et al., 2007a)
Human	Denmark	AM712470	3	(Stensvold et al., 2007a)
Human	Denmark	AM712471	3	(Stensvold et al., 2007a)
Human	Turkey	AM779043	3	(Ozyurt et al., 2008)
Human	Turkey	AM779046	3	(Ozyurt et al., 2008)
Human	France	AY135402	3	(Noel et al., 2003)
Human	Philippines	EU445495	3	(Rivera, 2008)
Human	Philippines	EU445496	3	(Rivera, 2008)

Table 3.1 List of GenBank sequences used for phylogeny comparison (*Continued)*

Host	**Country of origin**	**Accession number**	**Subtype**	**Reference**
Human	Japan	AB070988	3	(Arisue et al., 2003)
Human	Thailand	AY618268	3	(Thathaisong et al., 2003)
Human	Denmark	AM275393	4	(Stensvold et al., 2007c)
Pig	Japan	AB091248	5	(Arisue et al., 2003)
Turkey	France	AY135411	6	(Noel et al., 2003)
Chicken	Philippines	EU445485	6	(Rivera, 2008)
Chicken	Japan	AB091242	6	(Arisue et al., 2003)
Swan Goose	Japan	AB107973	7	(Abe, 2004)
Chicken	France	AY135410	7	(Noel et al., 2003)
Human	Denmark	AM495096	8	(Stensvold et al., 2008)
Pheasant	Japan	AB107971	8	(Abe, 2004)
Human	Japan	AF408425	9	(Yoshikawa et al., 2004b)
Human	Japan	AF408426	9	(Yoshikawa et al., 2004b)
Australia	Elephant	GU256932	11	(Parkar et al., 2010)
Australia	Giraffe	GU256905	12	(Parkar et al., 2010)
Australia	Quokka	GU256935	13	(Parkar et al., 2010)

Table 3.2 Subtype distribution of *Blastocystis* isolates from this study

Subtype	**1**	**2**	**3**	**4**	**6**	**7**	**8**
Number of samples	28	5	40	12	3	1	2
%	31%	5%	44%	13%	3%	1%	2%

Table 3.3 Co-infection of *Blastocystis* subtypes with other parasites found by microscopy

Parasite	Subtype							Total
	1	**2**	**3**	**4**	**6**	**7**	**8**	
Only *Blastocystis*	23	4	28	11	3	0	2	71
Blastocytsis + *Giardia*	1	0	3	0	0	1	0	5
Blastocystis + *Dientamoeba fragilis*	0	0	1	1	0	0	0	2
Blastocystis + *Endolimax nana*	1	0	3	0	0	0	0	4
Blastocystis + *Cryptosporidium*	0	1	0	0	0	0	0	1
Blastocystis + *Entamboeba coli*	0	0	1	0	0	0	0	1
Blastocystis + *Entamoeba hitolytica/dispar complex*	1	0	0	0	0	0	0	1
Blastocystis + 2 others	0	0	2	0	0	0	0	2
Blastocystis + 3 others	0	0	3	0	0	0	0	3
Blastocystis + 4 others	1	0	0	0	0	0	0	1

Table 3.4 *Blastocystis* subtype and symptoms described

Subtype	Abdominal pain	Vomiting	Nausea	Diarrhoea	Multiple symptoms described	No symptoms noted	% with symptoms
Subtype 1	1	0	1	9	2	19	32
Subtype 2	3	0	0	4	1	0	100
Subtype 3	1	2	0	15	2	24	40
Subtype 4	3	0	1	6	4	6	50
Subtype 6	0	0	0	1	0	2	33
Subtype 7	1	0	0	1	1	0	100
Subtype 8	0	0	0	2	0	0	100

Table 3.5 Bacterial infections found in conjunction with *Blastocystis* subtypes and symptoms

Bacteria	**Subtype**	**Symptom**
Campylobacter jejuni	1	Diarrhoea
Campylobacter jejuni	3	Diarrhoea
Campylobacter jejuni	8	Diarrhoea
Campylobacter jejuni	3	No symptoms
Shigella flexneri	1	No symptoms
Shigella sonnei	3	Diarrhoea
Clostridium difficile	4	Diarrhoea

Table 3.6 Number of *Blastocystis* subtypes identified by permanent stain and culture from this study

Method of identification	**Subtype**						
	1	**2**	**3**	**4**	**6**	**7**	**8**
Positive by microscopy of permanent Iron Haematoxylin stain	14	0	18	7	2	0	2
Positive by culture	26	6	35	10	3	1	2
Total number of isolates for each subtype	28	6	40	12	3	1	2

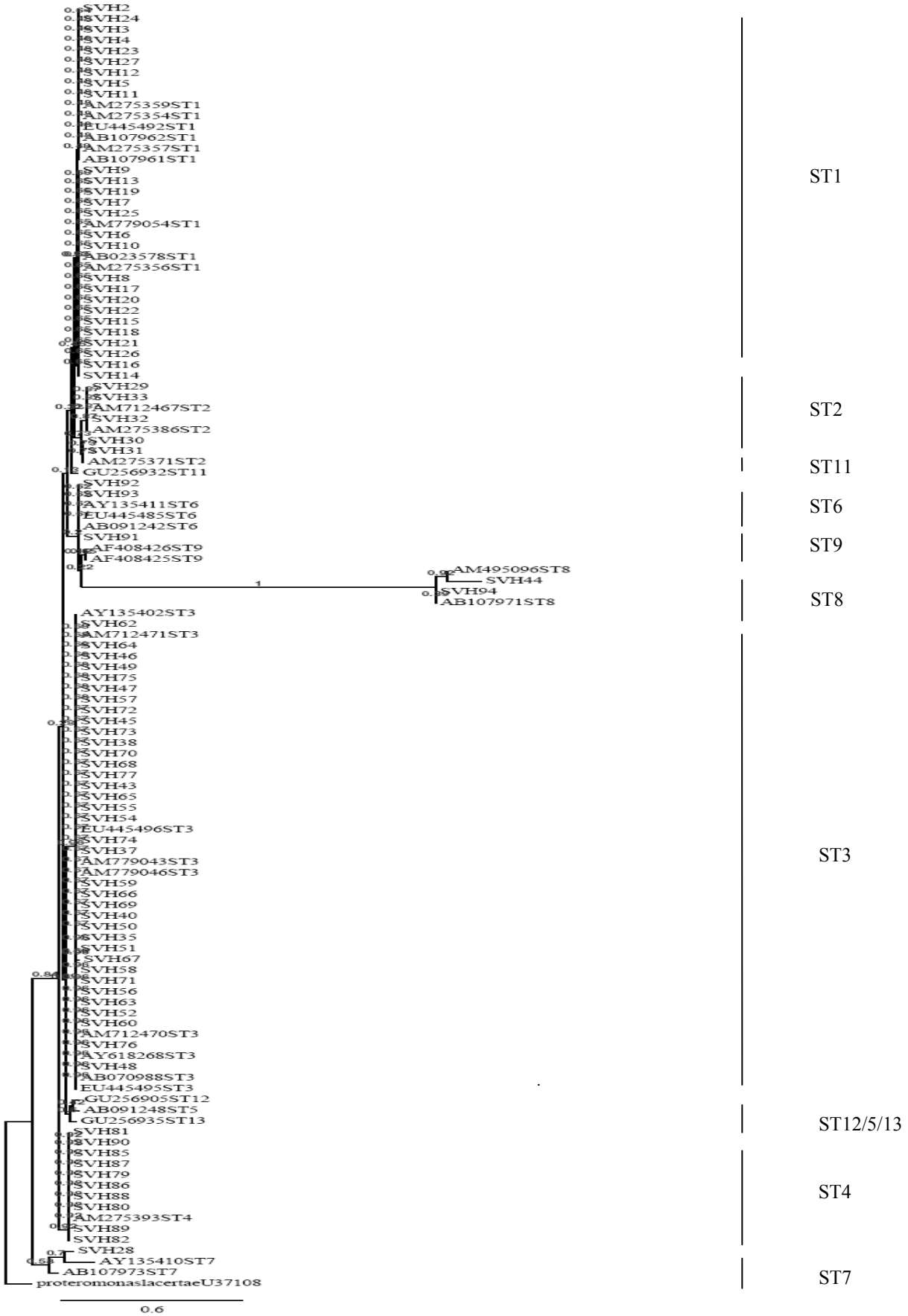

Figure 3.1 Rooted phylogenetic analysis of the *Blastocystis* SSUrDNA sequences from this study and GenBank reference sequences. The tree is inferred using the neighbour-joining method with bootstrap values indicated at the internal nodes (1,000 replicates). The scale bar represents the number of changes along the branch. The position of the subtypes in the tree is indicated on the right of the tree. *Proteromonas lacertae* (accession number U37108) is used as the outgroup.

3.5 Discussion

Blastocystis sp. is commonly found in human stool yet its role as a pathogen is still unclear (Stensvold et al., 2009c). A wide variety of subtypes were previously identified by PCR and DNA sequencing (ST1-ST17) of the 18S ribosomal DNA (Parkar et al., 2010). In this study the identity of the *Blastocystis* subtypes present in the Sydney population was investigated by PCR and sequencing, as well as any association with clinical signs of infection. It was anticipated that this study would provide further knowledge on the pathogenicity of the different *Blastocystis* subtypes, as well as a link to human gastrointestinal disease.

This study revealed the high prevalence of ST3 in the Sydney population studied at St. Vincent's Hospital from people with suspected gastrointestinal problems. Epidemiological studies from other locations around the world also indicate that ST3 is commonly, but not exclusively, the most predominant subtype isolated from a variety of symptomatic and asymptomatic people (Clark, 1997, Kaneda et al., 2001, Yoshikawa et al., 2004b, Li et al., 2007b, Ozyurt et al., 2008, Wong et al., 2008, Souppart et al., 2009).

Most studies have identified ST3 as the predominant subtype in humans, although a few studies have identified other subtypes as the predominant subtype. Three studies have identified ST4 as the predominant subtype. In Valencia, Spain, 94.1% of isolates were ST4 with none belonging to ST3 (Dominguez-Marquez et al., 2009). Another study from France identified ST4 as the predominant ST with 63% of isolates belonging to this group with ST3 being the second most predominant group (Poirier et al., 2011). In Nepal it was found that 84% of isolates belonged to ST4 (Lee et al., 2011). There are two studies that have identified ST1 as the predominant subtype present in humans from China and Brazil with 51.4% and 41% of isolates belonging to ST1 respectively (Yan et al., 2006, Malheiros et al., 2011). These results show that the ST present in humans may vary with the geographical distribution of *Blastocystis.* This may result from a wide range of contributing factors. For example, the subtype distribution could be related to different rural settings and the different animals that are found living nearby to humans in these settings (Lee et al., 2011).

Seven different clades were identified by the phylogeny analyses of the clinical samples that included seven different subtypes. ClustalW sequence alignment shows the sequences to be similar with a relatively high number of nucleotide differences between the subtypes. There were smaller numbers of nucleotide differences observed between ST3 and ST4, and ST1 and ST2 with much larger nucleotide differences between ST6 with ST3 and ST4. These differences are congruent with the clustering identified in the phylogeny analysis.

The neighbour-joining distance tree showed how different the ST7 and ST8 groups were from the rest of the subtypes with both having long branch distances from the rest of the groups. This shows the large genetic variability that exists within the *Blastocystis* subtypes.

All the phylogenetic analysis indicate that ST1 and ST2 share a common ancestor. The tree also shows that ST6 and ST9 are related and that ST3 and ST4 are closely related. Previous studies have also noted the close relationship seen between ST1 and ST2 as well as ST3 and ST4 (Lee et al., 2011, Stensvold et al., 2009a, Stensvold et al., 2007b) An interesting observation though is that the ST8 group is closer related to ST6 and ST9 as seen in the phylogeny tree but it has previously been seen that ST8 groups with the ST3 and ST4 group in other phylogenetic studies (Stensvold et al., 2007b, Ozyurt et al., 2008, Petrasova et al., 2011). From the NJ tree it is possible to see the differences that lie within all the subtypes other than ST4 with quite apparent genetic variability within the subtypes which is not observed within the ST4 clade. This has previously been observed and has been suggested that this could indicate that the ST4 group expanded more recently on an evolutionary scale (Lee et al., 2011)

Subtype 3 is rarely found in animals other than humans and this could suggest that this subtype has human to human transmission and may indicate a high host specificity occurring within this subtype. Sydney is an urban centre where contact with animals and contaminated water is limited. This could explain why there is not a high incidence of other subtypes present in this population. In other studies from around the world, there were higher frequencies of other subtypes and more subtypes have been identified from a single study population. This could be attributed to the socioeconomic position of these people, possibly living in rural areas where water

and food can be contaminated by livestock and wild animals. ST1 was found in a number of different animals including monkeys, apes, chickens and humans (Abe, 2004). This shows that this subtype is not host specific and there are many possible animal reservoirs to pass this subtype on to humans. It was suggested that ST6 is of animal origin having only been found in one other human case previous to this study. Subtype 6 is predominantly found in cattle and pigs (Abe et al., 2003a, Abe et al., 2003b, Abe et al., 2003c, Yoshikawa et al., 2003, Yoshikawa et al., 2004b, Yoshikawa et al., 2004a, Rene et al., 2009).

There does not seem to be a relationship between subtype and co-infection with there being a wide range of different parasites found in conjunction with *Blastocystis* within the subtypes. There are a larger number of co-infections seen within ST1 and ST3 but that is more likely to occur considering the higher number of samples found within those subtypes.

It was previously suggested that ST1 is a pathogenic strain of *Blastocystis* (Yan et al., 2006). In this study only 33% of people with ST1 noted having any form of illness. Two patients had other pathogenic protozoa in their stool samples (*Entamoeba histolytica complex* and *Giardia intestinalis)* but only the patient with *Giardia intestinalis* reported having any intestinal symptoms. One patient with ST1 also had *Campylobacter jejuni* infection which could have been the main cause of intestinal disease. From our results it is not possible to conclude that ST1 is a pathogenic strain.

All patients with ST2 also noted some form of gastrointestinal disease. Although one of these five patients noted having other pathogenic intestinal protozoa present, *Cryptosporidium* and *Dientamoeba fragilis*, the other four patients had no other infectious agent present. There is conflicting evidence about the pathogenicity of ST2 with two studies showing the high infection intensity of ST2 (Ozyurt et al., 2008, Vogelberg et al., 2010) while other studies state there is no link between ST2 and pathogenicity (Yan et al., 2006, Yoshikawa et al., 2004b). Our results suggest that there could be a link between ST2 and pathogenicity.

Subtype 3 is the most common subtype found in this study group and is suggested to be of human origin. Approximately 40% of ST3 patients noted some

sort of gastrointestinal disease with the strongest association of symptom being with diarrhoea.

Of the 50% of ST4 patients that had symptoms, all of them noted having diarrhoea. There was only one patient who had a co-infection with another parasite (*Dientamoeba fragilis*) and one patient had an infection with *Clostridium difficile*. It was suggested that ST4 is a pathogenic strain, but our results do not agree with this suggestion (Dominguez-Marquez et al., 2009).

Blastocystis was the only parasite found in both ST6 and ST8 samples. Only one of three patients with ST6 noted any symptoms. There were two patients found to harbour ST8 and both of these patients had symptoms of diarrhoea. One of these patients also had *Campylobacter jejuni* bacterial infection. This observation suggests ST8 may be pathogenic, but as this is a rare subtype in humans it is difficult to judge if this conclusion is true, though there was a case in Denmark of a lady who also had ST8 infection and symptoms (Stensvold et al., 2008).

There have been several previous studies that highlight the lack of sensitivity for microscopy of permanent stains as a diagnostic technique (Stensvold et al., 2007a, Parkar et al., 2007). A previous study showed that microscopy only detects approximately 50% of *Blastocystis* infections (Roberts et al., 2011). In this study no ST2 were identified by Iron Haematoxylin staining. The lack of identification of this subtype could be attributed to the possible morphological forms and size of this subtype. If this subtype is smaller than other subtypes it may be missed in the microscopy or if it is a different morphological form they could be confused with other protozoa or other faecal matter.

There are many problems associated with molecular diagnosis of enteric parasites. There are several different extraction kits available on the market for stool DNA isolation and previous studies have shown the differences in sensitivity between the kits for the detection of other parasites (Stauffer et al., 2008). There are many inhibitors in stools which can affect the outcome of analysis. Extraction can also be time consuming and costly which can add to work labour. There are several studies that have performed extraction and PCR analysis only from cultured samples (Yoshikawa et al., 2004b, Li et al., 2007b, Wong et al., 2008). There is one main problem associated with this approach which is the possibility of some culture

systems having subtype preference and therefore certain subtypes might be missed during molecular analysis and not give a true subtype representation in a population (Stensvold et al., 2009c). This is why it is suggested that for epidemiological studies, PCR should be performed on DNA extracted directly from fresh stool samples. There is also the choice of primer pairs which can affect the sensitivity of a PCR with the possibility of some primer pairs being more subtype specific. This can also affect epidemiological studies with the possibility that a true representation of the different subtypes may not be detected. In this study it was found that the PCR 1 primers are strongly ST3 affinitive which may decrease PCR sensitivity, whereas the primers used in PCR 2 are quite sensitive but with relatively low specificity.

The present method for subtyping *Blastocystis* relies on the DNA target being informative in terms of the number of nucleotide differences occurring between the groups under study. The present region of the 18S SSU rDNA that is currently used has a relatively low number of nucleotide differences occurring between subtypes. A much faster evolving DNA target may provide in the future much more robust phylogenies of the *Blastocystis* subtypes.

3.6 Conclusion

In conclusion, this is the first large scale molecular study in Australia on the presence of *Blastocystis* in human clinical specimens. It was found that subtype 3 is the predominant subtype found in this population. The phylogeny highlights the high level of genetic variability present within the *Blastocystis* species at the 18S SSU rDNA studied. This study also describes the possible pathogenic potential of *Blastocystis sp.* and shows that it may be subtype related.

Chapter 4 Subtype distribution of *Blastocystis* isolates from a variety of animals from New South Wales, Australia

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Certificate

I certify that the following publication is largely my own work although the contributions of other authors are duly recognised.

- All authors contributed in the following way:
    - By providing suggestions on topics to be reviewed
    - By providing advice on appropriate experimental design
    - By proof reading draft manuscripts
    - By correcting spelling and grammatical errors in drafts
    - By providing suggestions to improve writing style and language

Otherwise the experimental work and the core composition of this work is credited to me.

I hereby certify that the above statements are true and correct:

Tamalee Roberts (PhD candidate): _____

Date: _____

Preface

To truly understand the epidemiology and transmission of *Blastocystis* it is necessary to determine the prevalence and subtypes found in animals. There have been several studies looking at the prevalence of *Blastocystis* in animals with a few also describing the subtypes present. This chapter aims to expand on that knowledge as well as commenting on the occurrence of *Blastocystis* in Australian native animals.

4.1 Abstract

A total of 438 stool samples from 38 different species of animal from seven different locations were studied for the presence of *Blastocystis.* PCR analysis was completed on all samples and DNA sequence data from the rDNA were submitted to subtype allocation. There was a total of 80 (18%) sequences from 18 species, and nine different subtypes were identified- ST1, ST2, ST3, ST4, ST5, ST7, ST11, ST12 and ST13. This is the first report of *Blastocystis* from the eastern grey kangaroo, red kangaroo, wallaroo, snow leopard and ostrich. This study highlights the need for further investigation in to the genetic diversity of *Blastocystis* which could help show the zoonotic potential of *Blastocystis.*

4.2 Introduction

Blastocystis is a single celled enteric parasite that is commonly found in humans and a number of different animal species (Stenzel and Boreham, 1996, Tan et al., 2002, Yoshikawa et al., 2004a). *Blastocystis* has shown to exhibit extensive genetic diversity and through many recent studies on the small subunit (SSU) ribosomal (r)RNA gene, 17 subtypes (ST) have been identified from humans and a variety of animals including non-human primates, other mammals and birds. (Parkar et al., 2010, Abe et al., 2003b, Noel et al., 2003, Stensvold et al., 2007a, Stensvold et al., 2009a). There are some subtypes that seem to be more host specific such as ST3 which is the most common subtype isolated in humans (Ozyurt et al., 2008, Wong et al., 2008, Souppart et al., 2009), whereas other subtypes appear to show low host specificity which raises the possibility of zoonotic transmission. The transmission of *Blastocystis* from animals living in a community based environment is feasible via the faecal- oral route and there is supporting evidence that this does occur. For example, a higher incidence of infection rates was reported in zoo keepers who came

in close contact with animal enclosures (Parkar et al., 2010). Though there has been a reasonable number of animal species studied for the identification of *Blastocystis,* more studies need to be carried out to understand the possible zoonotic transmission of this parasite and to identify whether other subtypes of *Blastocystis* do exist.

The aim of this study was to investigate the presence of *Blastocystis* in a variety of animals from several different locations and to genetically characterise them by SSU rDNA sequencing. This data aims to expand our knowledge on the role of animals in the epidemiology of *Blastocystis.*

4.3 Materials and methods

4.3.1 Animal samples

A total of 438 stool samples were collected from 38 different species of animal from seven different locations over a two year period- a farm in western Sydney, a Sydney area veterinary practice, Cat Protection Society (CPS), a rural Aboriginal community (King et al., 2012) a rural field (Cinque et al., 2008), a piggery (Armson et al., 2009), western New South Wales (N.S.W) bush area and Taronga Zoo, Sydney.

4.3.2 DNA extraction and PCR

Stool samples were frozen immediately after collection and DNA was extracted using the Bioline Isolate fecal DNA kit as per manufacturer's instructions. DNA was then submitted to PCR for the detection of *Blastocystis sp.* using a previously described method (Stensvold et al., 2007a) targeting the SSU rDNA specific to the *Blastocystis* region. PCR reactions of 25µl were performed using PuReTaq Ready-To-Go (Amersham Pharmacia Biotech) PCR beads (each containing – 1.5 units Taq DNA polymerase, 10mM Tris-HCl pH9, 50 mM KCl, 1.5 mM MgCl, 200µM of each dNTP and stabilizers, including BSA), 2µl of genomic DNA extract and 0.5µm of each PCR primer (F1 and BHCRSeq3).

4.3.3 Sequencing analysis

DNA sequence analysis was performed on all PCR positive samples. PCR products were purified using the QIAquick™ PCR purification Kit (Qiagen) as per the manufacturer's instructions and sent to the Australian Genome Research Facility (Westmead Millennium Institute, Sydney) for sequencing in both directions. The

SSU rDNA sequences were then compared to those available in the GenBank database using the BLASTN program run on the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST).

4.4 Results

A total of 438 stool samples were collected from 38 different species of animal over a two year period from seven different locations within N.S.W, Australia (Table 4.1). Eighty samples (18%) from a total of 18 species of animal were positive for *Blastocystis sp.* from PCR. All PCR positive samples were sequenced and nine different subtypes were identified by Blast searching- ST1, ST2, ST3, ST4, ST5, ST7, ST11, ST12 and ST13 (Table 4.2). There were four mixed infections seen in the chimpanzees with them found to harbour both ST1 and ST11. All of the primate species noted positives as well as four of the five marsupial macropod species. ST1 was the most common subtype isolated from this group of animals with 26%. ST11 was the second most predominant ST with 22% followed by ST4 (21%), ST2 (16%), ST5 (10%), ST3 (3%), ST7 (2%) and ST12 and ST13 (1% each).

Table 4.1 Location and number of animal species included in this study and the number of *Blastocystis* positive samples for each species

Location	Host	Scientific name	Samples (n)	Positive (n)
Farm	Horse	*Equus ferus caballus*	1	0
	Guinea Pig	*Cavia porcellus*	2	0
	Chicken	*Gallus gallus domesticus*	25	1
	Rabbit	*Oryctolagus cuniculus*	1	0
	Guinea fowl	*Numida meleagris*	2	2
Vet/CPS	Cat	*Felis catus*	43	0
	Dog	*Canis lupus familiaris*	11	0
	Possum	*Trichosurus vulpecula*	1	0
	Monkey	*Macaca sp.*	1	1
	Frog	*Litoria ewingii*	1	0
Rural	Dog	*Canis lupus familiaris*	45	0
Field	Deer	*Cervus elaphus*	50	1
Piggery	Pig	*Sus scrofa domesticus*	83	13
Bushland, NSW	Eastern Wallaroo	*Macropus robustus*	3	3
	Swamp Wallaby	*Wallabia bicolor*	1	0
Taronga zoo	Asian Elephant	*Elephas maximus*	20	11
	Tiger	*Panthera tigris*	10	0
	Lion	*Panthera leo*	10	0
	Ostrich	*Struthio camelus*	10	6
	Chimpanzee	*Pan troglodytes*	10	7
	Orang Utan	*Pongo abelii*	10	9

Table 4.1 Location and number of animal species included in this study and the number of *Blastocystis* positive samples for each species (*Continued*)

Location	Host	Scientific name	Samples (n)	Positive (n)
Taronga zoo	Gorilla	*Gorilla gorilla*	10	10
	Snow Leopard	*Panthera uncia*	6	1
	Meerkat	*Suricata suricatta*	10	0
	Kodiak Bear	*Ursus arctos middendorffi*	5	0
	Francois Langur	*Trachypithecus francoisi*	6	5
	Giraffe	*Giraffa camelopardalis*	6	1
	Zebra	*Equus burchellii*	4	0
	Cassowary	*Casuarius casuarius*	10	2
	Brazillian Tapir	*Tapirus terrestris*	3	0
	Southern Hairy Nosed Wombat	*Lasiorhinus latifrons*	3	0
	Common Wombat	*Vombatus ursinus*	2	0
	Western Grey Kangaroo	*Macropus fuliginosus*	2	1
	Eastern Grey Kangaroo	*Macropus giganteus*	4	3
	Red Kangaroo	*Macropus rufus*	4	3
	Short beaked echidna	*Tachyglossus Aculeatus*	1	0
	Long beaked echidna	Zaglossus bartoni	2	0
	Koala	*Phascolarctos cinereus*	10	0
	Tasmanian Devil	*Sarcophilus harrisii*	10	0

Table 4.2 *Blastocystis* subtypes characterised from animals in this study

Animal Host	*Blastocystis* sp. subtype (ST)													
	ST1	**ST2**	**ST3**	**ST4**	**ST5**	**ST6**	**ST7**	**ST8**	**ST9**	**ST10**	**ST11**	**ST12**	**ST13**	**Mixed**
Chicken	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Guinea fowl	-	-	-	-	-	-	2	-	-	-	-	-	-	-
Ostrich	-	-	-	6	-	-	-	-	-	-	-	-	-	-
Cassowary	-	2	-	-	-	-	-	-	-	-	-	-	-	-
Pig	3	-	2	-	8	-	-	-	-	-	-	-	-	-
Deer	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Elephant	-	-	-	-	-	-	-	-	-	-	11	-	-	-
Giraffe	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Snow Leopard	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Chimpanzee	4	-	-	-	-	-	-	-	-	-	7	-	-	4
Orang Utan	-	9	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.2 *Blastocystis* subtypes characterised from animals in this study (*Continued*)

Animal Host	*Blastocystis* sp. subtype (ST)													
	ST1	**ST2**	**ST3**	**ST4**	**ST5**	**ST6**	**ST7**	**ST8**	**ST9**	**ST10**	**ST11**	**ST12**	**ST13**	**Mixed**
Gorilla	9	1	-	-	-	-	-	-	-	-	-	-	-	-
Francois Langur	5	-	-	-	-	-	-	-	-	-	-	-	-	-
Macaca	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Western Grey Kangaroo	-	-	-	-	-	-	-	-	-	-	-	-	1	-
Eastern Grey Kangaroo	-	-	-	3	-	-	-	-	-	-	-	-	-	-
Red Kangaroo	-	-	-	3	-	-	-	-	-	-	-	-	-	-
Wallaroo	-	-	-	3	-	-	-	-	-	-	-	-	-	-

4.5 Discussion

This study reports the incidence of *Blastocystis* in a number of different animal species and describes the presence of *Blastocystis* in a number of animal species not previously known to harbour *Blastocystis*.

Blastocystis was identified in all of the three ape species and two monkey species studied. 10/10 Gorilla, 7/10 Chimpanzee, 9/10 Orang-utan, 5/6 Francois Languar and 1/1 Macaca sp. were positive for *Blastocystis.* There has previously been a high prevalence of *Blastocystis* reported in primates and our results are consistent with this (Abe et al., 2003b, Abe et al., 2002). Ape isolates in this study belonged to subtypes 1, 2, 3, and 11. These results are also similar to previous studies (Yoshikawa et al., 2009, Parkar et al., 2010, Yoshikawa et al., 2004a). The exception to this is the Chimpanzee isolates where all were identified as ST11 and four had a mixed infection with ST1. This is the first time that Chimpanzee isolates have been assigned to ST11, with previous studies identifying Chimpanzees with subtypes 1, 2, 3 and 5 (Abe et al., 2003b, Abe, 2004, Stensvold et al., 2009a).

Blastocystis sp. has previously been identified in several Australian native animals including the southern hairy nosed wombat, the western grey kangaroo, quokka and brushtailed possum (Parkar et al., 2007, Parkar et al., 2010). This current study also noted the presence of *Blastocystis* in the western grey kangaroo but it was not detected in the southern hairy nosed wombat. The *Blastocystis* detected in the western grey kangaroo in this study belonged to the newly defined ST13 which was different to that identified in the western grey kangaroo from a previous study which was identified as ST12 (Parkar et al., 2010). This is the first study to report the presence of *Blastocystis sp*. in the eastern grey kangaroo, red kangaroo and eastern wallaroo. All of these newly identified isolates belonged to ST4. Clearly no specific subtype can be assigned to kangaroos at this time. The more studies that are carried out on Australian native fauna, the greater our knowledge will be about the subtype host specificity of *Blastocystis.*

This is the second study to identify *Blastocystis* in deer species. One Roe deer was found to harbour ST10 during a study in Denmark (Stensvold et al., 2009a). This current study noted 1/50 samples contained *Blastocystis* with the subtype identified as ST4. There have been two other previous studies which have investigated the

presence of *Blastocystis* in six different deer species and neither study was able to identify *Blastocystis* in any of the samples (Abe et al., 2002, Lim et al., 2008). Both studies relied exclusively on microscopy for the identification of *Blastocystis* which has previously been shown to be inefficient in detecting all *Blastocystis* infections (Roberts et al., 2011).

Blastocystis has only once previously been described in Asian Elephants. That study identified a new subtype, ST11, and described the presence of *Blastocystis* in Asian Elephants from five different locations (Parkar et al., 2010). Another previous study was unable to identify *Blastocystis* from Asian Elephants (Abe et al., 2002). This current study identified 11/20 positive samples for *Blastocystis;* all of them were identified as ST11 which could suggest that elephants only harbour this subtype as no other subtype has been identified in elephants.

This study noted 1/6 Giraffes were positive for *Blastocystis*. *Blastocystis* has only once before been described in Giraffes and was identified as a new subtype, ST12 (Parkar et al., 2010). The Giraffe *Blastocystis* from this study also belonged to this new subtype.

There were 6/10 Ostrich samples containing *Blastocystis* which belonged to ST4. This appears to be the first time that *Blastocystis* has been studied in Ostriches. The Guinea fowl also contained *Blastocystis* and these two specimens were identified as ST7. Guinea fowl was previously shown to harbour ST6 (Abe et al., 2003a) and this shows that there is no subtype specific to the Guinea fowl due to there being several subtypes that infect this species. There was one chicken specimen containing *Blastocystis* from the 15 studied. This *Blastocystis* was identified as ST2. There have been several studies completed on chickens and a variety of subtypes have been identified including ST1, 2, 6 and 7 (Arisue et al., 2003, Noel et al., 2003, Abe et al., 2003a).

There were 13/83 pig samples containing *Blastocystis*. There were three different subtypes identified in this group- ST1, ST3 and ST5. There have been several previous studies completed on pigs with four different subtypes identified- ST1, ST2, ST3 and ST5 (Abe et al., 2003c, Arisue et al., 2003, Noel et al., 2003, Navarro et al., 2008, Yoshikawa et al., 2004a).

ST4 has most commonly been identified in rodents and marsupials with a low number from non-human primates. ST4 is also the third most common subtype found in humans (Scicluna et al., 2006, Stensvold et al., 2006, Souppart et al., 2009, Meloni et al., 2011). In this study ST4 was identified in Kangaroos (marsupials) but was also identified in Ostriches and a Snow Leopard showing that there is a greater host range for this subtype than previously described.

Blastocystis has previously been described in domestic cats and dogs but this study relied solely on microscopy with no further molecular analysis being completed (Duda et al., 1998). There have been several studies which have highlighted the need for further molecular analysis due to the poor sensitivity of microscopy (Stensvold et al., 2007a, Roberts et al., 2011). Of the 43 domestic cat, nine domestic dog and 45 rural dog samples that were analysed in this study, there was no evidence for the presence of *Blastocystis.* Several other studies carried out on domestic cats and dogs have highlighted the absence of *Blastocystis* and our results are consistent with this (Chuong et al., 1996, Konig and Muller, 1997, Abe et al., 2002) with only one recent paper showing the relationship between pet carriage and human infection (Nagel et al., 2012).

There were several specimens that were identified as positive by the PCR method used but after sequence analysis was completed it was found that these products actually were derived from fungal species in particular *Mucor spp.* and *Rhizomucor spp*. The pig samples also gave sequence data from *Cryptosporidium sp*. The ability of the PCR and primers used in this study to cross react with species other than *Blastocystis* shows the need for sequence analysis to be performed on all PCR positive samples to confirm the identity of the PCR sequence. This also highlights the difficulty in producing primer pairs specific to *Blastocystis*. Molecular analysis of *Blastocystis* has grown in the last 15 years and there are continuously new subtypes being identified as more animals and people from different geographical locations are studied. This makes the choice of correct PCR technique and primer pairs essential for the possibility to detect all new subtypes. It is possible that a number of undiscovered subtypes are being missed due to the inability of current PCR techniques to detect *Blastocystis.* It is presently recommended to use multiple primer pairs to account for possible sequence variation within primer sites (Stensvold

et al., 2009c). Another problem with correct phylogenetic analysis is if the primers amplify small products. For future phylogenetic and taxonomic analysis it is suggested that only products of over 1000bp be used to show accurate phylogenetic relationships (Parkar et al., 2010). The length of the sequence data used is in itself not enough to guarantee an accurate phylogeny; the sequence also has to contain evolutionary informative data (Morrison and Ellis, 1997). The barcode region should be used as a potential means to standardise subtype calling for all future epidemiological and phylogenetic studies. The barcode region has many benefits including the availability of a public database that can perform analysis on FASTA files as well as perform allele identification (http://pubmlst.org/blastocystis/) (Scicluna et al., 2006).

There does not seem to be any effect on these animals from infection with *Blastocystis* with all animals appearing to be healthy with no noted symptoms of disease. This is quite different to a number of human infections where there are huge symptom- infection rates (Yan et al., 2006, Ozyurt et al., 2008, Vogelberg et al., 2010). This shows the possible relationship where *Blastocystis* does not have a pathogenic role in animals.

Blastocystis was shown to be transmitted through the faecal- oral route as well as through contaminated water (Leelayoova et al., 2004). It is possible to say that the faecal- oral route may be the main route of transmission in the community based groups of animals such as the apes and elephants. The ape communities all eat and defecate in the same areas in their enclosures as well as playing together and grooming each other. If one animal were to harbour *Blastocystis*, it would be easy for that animal to spread it amongst the rest of the group. The Meerkat group were the only animals that live in a large community in the zoo that did not harbour *Blastocystis.* All the other community living animals were almost all positive for *Blastocystis*. This shows the easy transmission of this parasite through a group and how simple it can be spread through the faecal oral route.

Most animal studies have been carried out on domestic animals and animals from zoos. It would be interesting to compare the results of the zoo animals to their wild relatives to determine if the same subtypes and infection rates are found. It is not possible to describe a true representation of *Blastocystis* infection rates and

subtypes present in animals if only zoo animals or domestic animals have been studied and only from specific areas of the world.

4.6 Conclusion

This study highlights the variety of *Blastocystis* subtypes isolated from animals collected from a variety of locations. This study is the first to identify *Blastocystis* in the Snow Leopard, Ostrich and from several Australian native animals- Eastern Grey Kangaroo, Red Kangaroo and Wallaroo. Further studies are still required to show the host specificity of the *Blastocystis* subtypes and determine if more subtypes are present in the animal population. This study also highlights the need for specific *Blastocystis* PCR methods to be developed to stop the occurrence of false positives during diagnosis and the possibility of missed infections.

Chapter 5 The role of *Blastocystis* sp. in Irritable Bowel Syndrome

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Keywords-

Chronic *Blastocystis* infection; subtypes; diarrhoea

Preface

Due to *Blastocystis* infection causing symptoms similar to irritable bowel syndrome (IBS) including diarrhoea, bloating and abdominal pain, an association has been made. The aim of this chapter was to determine the role *Blastocystis* plays in IBS, if at all and compare these results to a healthy control group. The results from this chapter should also help with the overall picture of *Blastocystis* pathogenicity and show that symptoms may be subtype related.

5.1 Abstract

Blastocystis is the most common enteric parasite found in humans. *Blastocystis* can cause symptoms similar to irritable bowel syndrome (IBS) including diarrhoea, abdominal pain, nausea and bloating and has often been identified in patients suffering from IBS. It is because of this that a link has been made between *Blastocystis* and IBS. Therefore the aim of this study was to determine the prevalence, genotyping and clinical relevance of *Blastocystis* in people with irritable bowel syndrome and see if there was an association between the *Blastocystis* subtypes present. In this study we compared 87 patients identified as suffering from IBS and 70 healthy control people for the presence of *Blastocystis* by microscopy of a permanent stain and polymerase chain reaction (PCR). There were 16 patients positive in the IBS group for *Blastocystis* and seven in the control group. Although there was a higher incidence in the IBS group, this was not statistically significant ($p= 0.21$). There were three subtypes identified in the IBS group- 4 subtype (ST) 1, 5 ST3 and 5 ST4 and four subtypes in the control group- 1 ST1, 1 ST2, 4 ST3 and 1 ST6. From this study we suggest there may be an association between ST4 and IBS as this ST was only found in the IBS group. This study also highlights that a full microbiological and parasitological work-up should be carried out before a diagnosis of IBS is made as *Blastocystis* can mimic IBS symptoms leading to an incorrect diagnosis.

5.2 Introduction

Irritable bowel syndrome (IBS) is described as a chronic functional gastrointestinal condition. The Rome criteria describes it as recurrent abdominal pain or discomfort for at least three days/month in the last three months associated with two or more of

the following: improved with defecation; onset associated with a change in frequency of stool; and onset associated with a change in form of stool (Dai et al., 2008). IBS is acknowledged to be a heterogenous, multisystem and multi-domain disorder, with a new emphasis in research on the brain-gut interactions. Inflammation of the gut is among the most important pathophysiologic mechanisms of IBS symptoms (Crentsil, 2005). Symptoms associated with IBS include abdominal discomfort, bloating, flatulence and disturbed defecation with no known cause. IBS is a common condition affecting approximately 12%-15% of people in the western world (Agreus et al., 1995, Mertz, 2003). IBS is the seventh most frequent diagnosis given to patients seeking primary care (Dogruman-Al et al., 2010). There is a higher prevalence of IBS seen in females with a 2:1 female predominance (Stark et al., 2007b). Irritable bowel syndrome affects many peoples quality of life impacting on their work productivity and a wide range of daily activities (Hungin et al., 2005).

It has been suggested that parasites, in particular *Blastocystis*, may play a role in IBS due to this organism causing symptoms similar to those seen in IBS patients including diarrhoea, abdominal pains and cramping, flatulence and bloating. *Blastocystis* has been found in much higher numbers in several IBS patient studies compared to controls with rates of 46%, 71%, 76% and 49% in IBS patients compared to less than 20% in the control groups (Yakoob et al., 2004b, Yakoob et al., 2010b, Dogruman-Al et al., 2010, Yakoob et al., 2010a). However, two other studies showed that there was no significant difference in the number of *Blastocystis* positive samples in the IBS group compared to the control group (Tungtrongchitr et al., 2004, Surangsrirat et al., 2010). Both of these studies however relied solely on microscopy from culture and no molecular tools were used for the detection of *Blastocystis*.

Due to the recent advances in molecular technology for the diagnosis of *Blastocystis,* it has been possible to identify 17 different subtypes of *Blastocystis* from humans and animals. Subtype (ST) 3 is the predominant subtype found in most human epidemiological studies (Li et al., 2007b, Ozyurt et al., 2008, Souppart et al., 2009). Though ST3 is the predominant subtype found in humans, it was suggested that ST1 is a pathogenic strain (Yan et al., 2006, Hussein et al., 2008). A study on the subtypes associated with IBS showed that there was a much higher prevalence of

ST1 in the IBS group than in the control group but there were equal numbers of ST3 in both groups (Yakoob et al., 2010b). This could suggest that ST1 is related to IBS infection.

Therefore, the aim of this study was to determine the prevalence, genotyping and clinical relevance of *Blastocystis* in people with irritable bowel syndrome.

5.3 Materials and Methods

A total of 87 IBS patients and 70 healthy control people were included in the study. IBS patients were diagnosed by a gastroenterologist from the gastroenterology clinic at St. Vincent's Hospital, Sydney between 2010- 2012 according to the Rome III criteria. IBS was determined by the onset of symptoms of abdominal discomfort, diarrhoea, flatulence and constipation that could not be explained by any other disease and had occurred for a period of over six months. Patients with inflammatory bowel disease or Crohns disease were excluded from the study. The control group consisted of healthy volunteers who had no previous abdominal discomfort or diarrhoea in the past year. All IBS patients and controls completed a simple survey about symptoms and previous travel and signed a consent form allowing for their samples to be used in this study. The study was approved by the institutional ethics review committees (University of Technology, Sydney reference number 2011-228A and St. Vincent's Hospital reference number 09/150). One stool sample was collected from all patients and controls and all samples underwent microscopy of a permanent modified Iron Haematoxylin stain for the detection of any parasites present. All samples also underwent a microbiological investigation for the presence of *Shigella* spp*., Salmonella* spp., *Campylobacter* spp*., Clostridium difficile, Vibrio cholerae* and *Aeromonas* spp. using culture. Viral screening was not performed on stool specimens due to cost limitations but as these patients had chronic symptoms, it is unlikely that they had a viral infection caused by rotavirus, adenovirus or norovirus. DNA was extracted from all fresh frozen samples using the Bioline Isolate fecal DNA kit as per manufacturer's instructions. All DNA was then submitted to a previously described PCR method for the detection of *Blastocystis* (Roberts et al., 2013a, Stensvold et al., 2007a). DNA sequence analysis was performed on all PCR positive samples. PCR products were purified using the QIAquick™ PCR purification Kit (Qiagen) as per the manufacturer's instructions and sent to the

Australian Genome Research Facility (Westmead Millennium Institute, Sydney) for sequencing in both directions. The SSU rDNA sequences were then compared to those available in the GenBank database using the BLASTN program run on the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST). Statistical analysis was carried out using graphpad.com.

5.4 Results

A total of 87 IBS samples and 70 control samples were tested for the presence of *Blastocystis sp.* Sixteen samples (18%) were positive for *Blastocystis* from the IBS group and seven (10%) samples were positive from the control group by PCR. There was no statistically significant difference between the two groups (p=0.21). All positive isolates were submitted to sequencing. From the IBS group there were 4 ST1 (25%), 5 ST3 (31%), 5 ST4 (31%) and two samples where no sequence data was available. For the control group there was 1 ST1 (14%), 1 ST2 (14%), 4 ST3 (57%) and 1 ST6 (14%). There were no bacterial pathogens isolated from any of the samples from either group. There were two other parasites identified in the IBS group by microscopy, one patient had *Endolimax nana* while the other had *Iodamoeba butschlii*. Neither of these patients were identified as having *Blastocystis.* In the control group one patient had *Giardia intestinalis* and two other patients had *Dientamoeba fragilis* identified by microscopy with neither of these patients harbouring *Blastocystis.* The average age of the IBS group was 31 with 34 males and 53 females. The average age in the control group was 33 with 30 males and 40 females. Table 5.1 describes IBS patients positive for *Blastocystis* results. Table 5.2 describes control group people positive for *Blastocystis.*

Table 5.1 IBS patients results positive for *Blastocystis*

Patient	Age	Sex	Symptoms	Travel	Microscopy	PCR	Subtype
1	35	M	Diarrhoea	No	3+ *Blastocystis*	Positive	1
2	31	M	Diarrhoea, bloating	South East Asia	3+ *Blastocystis*	Positive	1
3	21	M	Diarrhoea	No	Negative	Positive	1
4	40	F	Diarrhoea	No	Negative	Positive	1
5	32	F	Diarrhoea, abdominal pain, nausea	No	Negative	Positive	3
6	38	F	Constipation, nausea, bloating, abdominal cramps	No	Negative	Positive	3
7	30	F	Diarrhoea, vomiting	No	Negative	Positive	3
8	64	F	Diarrhoea	No	Negative	Positive	3
9	40	M	Diarrhoea	Hong Kong	1+ *Blastocystis*	Positive	3
10	26	M	Diarrhoea, abdominal cramps	South East Asia	3+ *Blastocystis*	Positive	4
11	30	F	Diarrhoea, abdominal pain	South East Asia	3+ *Blastocystis*	Positive	4
12	53	F	Diarrhoea	No	2+ *Blastocystis*	Positive	4
13	50	M	Diarrhoea, flatulence, bloating	World-wide	Negative	Positive	4
14	37	M	Diarrhoea	No	2+ *Blastocystis*	Positive	4
15	62	M	Diarrhoea	No	Negative	Positive	-
16	29	M	Diarrhoea	No	Negative	Positive	-

Table 5.2 Control group results positive for *Blastocystis*

Control	Age	Sex	Travel	Microscopy	PCR	Subtype
1	28	M	South East Asia	2+ *Blastocystis*	Positive	1
2	51	F	No	Negative	Positive	2
3	28	F	South East Asia	2+ *Blastocystis*	Positive	3
4	56	M	World-Wide	Negative	Positive	3
5	65	M	No	Negative	Positive	3
6	35	M	No	Negative	Positive	3
7	34	F	No	Negative	Positive	6

5.5 Discussion

In this study there was a higher incidence of *Blastocystis* present in the IBS group compared to the control group but this was not considered statistically significant (p=0.21). There were three different STs identified in the IBS group and four STs in the control group. Although there were more STs identified in the control group, two of these STs (ST2 and ST6) were not found in the IBS group. ST4 was only found in the IBS group and not in the control group. It was previously suggested that ST4 may be a pathogenic strain and the fact that it was only found in the IBS group in this study, all of whom had gastrointestinal symptoms, could support that argument (Dominguez-Marquez et al., 2009). A previous study also found a higher incidence of ST4 in the IBS group compared to the control group (Alfellani et al., 2013a). ST1 has also been suggested to have pathogenic potential and even though there was one ST1 isolate found in the control group, there were four found in the IBS group suggesting that ST1 may play a role in causing disease. ST3 is the predominant ST found in most human epidemiological studies (Roberts et al., 2013b). The results with ST3 being the most common ST in both the control group and the IBS group therefore is expected. There was a slightly higher prevalence of ST3 in the control group (57%) in comparison to the IBS group (31%) which shows that this ST is more common in healthy people. There has been varying opinions for the role of ST3 in causing disease (Roberts et al., 2013b). One study however, showed that 100% of IBS patients were found to harbour ST3 (Ramirez et al., 2013). There is much intra-subtype variation seen within ST3 and it is possible that this variation may play a role in causing disease (Stensvold et al., 2012b).

Chronic intermittent diarrhoea was the most common symptom described by the IBS patients and there was no difference between the STs and symptoms. There were five patients that noted symptoms started after travel to Asia several years beforehand. It is highly possible that these patients acquired the *Blastocystis* from their travels and have been infected with *Blastocystis* this whole time and been incorrectly diagnosed with IBS. A full stool work-up for any infectious agents including bacteria and parasites should be taken out before a diagnosis of IBS is given. Infection with *Blastocystis* can be chronic and this needs to be taken in to consideration when diagnosing patients with IBS. Three of the seven control

patients' positive for *Blastocystis* also reported travel to Asia the year before. There was no association between travel to Asia and ST recovered (2 ST1, 3 ST3 and 3 ST4).

There have been several studies that have shown the high incidence of *Blastocystis* in an IBS group compared to the control group, including this present study, and this may be due to several reasons (Jimenez-Gonzalez et al., 2011, Yakoob et al., 2004b). It is possible that the *Blastocystis* may be causing symptoms similar to those encountered in IBS such as diarrhoea, abdominal pain and bloating. These symptoms may be due only to the *Blastocystis* infection and symptoms may clear after treatment has been given for the *Blastocystis* suggesting that it was not actually IBS that the patient suffered from. There is also the possibility that *Blastocystis* can colonise the gut of people with IBS more effectively than a healthy person (Clark et al., 2013). This may not mean that *Blastocystis* is the cause of the IBS symptoms, but may just be a secondary diagnosis. Another suggestion is that single nucleotide polymorphisms (SNPs) for interleukin (IL)-8 and IL-10 may change an individual's susceptibility to IBS by increasing the relative risk in the development of IBS in *Blastocystis* infected people (Olivo-Diaz et al., 2012).

This study does have limitations in that there is only a small sample size for both the IBS and control groups and that there are different numbers for these two groups which does make comparisons difficult. This study was completed over a two year period of which all IBS patients that attended the gastroenterology clinic were invited to take part in the study but only a small number chose to partake. Factors such as a willingness to submit a stool sample and fill out a simple survey were the main reasons that people did not choose to join the study for both the IBS and control groups. A higher number of samples for both groups would be advantageous to show a more correct epidemiological survey of *Blastocystis* in IBS patients compared to a control group. A higher number of IBS patients with *Blastocystis* infection would also allow for comparisons between ST and symptoms and therefore make it possible to make a more thorough statement about the relationship between *Blastocystis* and IBS. There have been 12 previous IBS and *Blastocystis* studies, eight of which have also had a study number of less than 100 patients (Poirier et al., 2012, Ramirez et al., 2013, Fouad et al., 2011). This shows the difficulty in collecting samples in this

patient group and how any extra information gathered on IBS patients in relation to *Blastocystis* is important. Another limitation is that only one stool sample was collected from each person. Unfortunately the collection of more than one stool sample was not possible due to almost all patients and controls unwilling to give more than one sample. It is possible that there is a higher rate of parasites including *Blastocystis* in this group that has been missed but the use of PCR in this study, which is highly sensitive for the detection of *Blastocystis* does make this possibility low (Roberts et al., 2011). To clearly show whether symptoms are related to *Blastocystis* in the IBS group it would be ideal to follow patients after a course of treatment had been administered and see if symptoms were alleviated after clearance of *Blastocystis.* When further studies into the relationship between parasites and IBS are carried out, this should be something that should be considered.

5.6 Conclusion

This study highlights the possible role of *Blastocystis* in IBS with higher rates of infection seen in the IBS group compared to the healthy control group. ST4 was only found in the IBS group and all IBS patients described chronic symptoms of diarrhoea, abdominal pain and nausea. This study suggests that though *Blastocystis* may be found in conjunction with IBS-like symptoms, special care must be taken as the *Blastocystis* may be the actual cause of the disease and should be treated before a diagnosis of IBS is given.

Chapter 6 *Blastocystis* susceptibility testing

Preface

The previous chapters from this study have shown that *Blastocystis* can be pathogenic and cause disease in humans. Although this has been widely reported in the literature, there have only been a few studies that have looked at treatment options for *Blastocystis*. Metronidazole appears to be the most common treatment option for *Blastocystis* infection even though there have been several reports of treatment failure. The aim of this chapter is to report the efficacy of common treatment options in people who have chronic *Blastocystis* infection. This chapter also describes *in vitro* susceptibility testing of 12 common antimicrobials against four human *Blastocystis* subtypes. The information gathered from this chapter should help aid clinicians in effective drug therapy treatments for *Blastocystis* infected patients.

6.1 Treatment failure in patients with chronic *Blastocystis* infection

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Certificate

I certify that the following publication is largely my own work although the contributions of other authors are duly recognised.

- All authors contributed in the following way:
    - By providing suggestions on topics to be reviewed
    - By providing advice on appropriate experimental design
    - By proof reading draft manuscripts
    - By correcting spelling and grammatical errors in drafts
    - By providing suggestions to improve writing style and language

Otherwise the experimental work and the core composition of this work is credited to me.

I hereby certify that the above statements are true and correct:

Tamalee Roberts (PhD candidate): _____

Date: _____

6.1.1 Abstract

This article reports long term infection and treatment failure in 18 symptomatic individuals infected with *Blastocystis* spp. Patients were initially treated with either metronidazole, iodoquinol or triple combination therapy consisting of nitazoxanide, furazolidone and secnidazole. Following treatment, resolution of clinical symptoms did not occur and follow up testing revealed ongoing infection with the same subtype. Patients then underwent secondary treatment with a variety of antimicrobial agents with most remaining symptomatic with *Blastocystis* sp. still present in faeces. Sequencing of the SSU rDNA was completed on all isolates and four subtypes were identified in this group- ST1, ST3, ST4 and ST5. This study highlights the lack of efficacy of several commonly used antimicrobial regiments in the treatment of *Blastocystis* and the chronic nature of some infections. It also demonstrates the need for further research into treatment options for *Blastocystis* infection.

6.1.2 Introduction

Blastocystis is the most common enteric parasite present in humans (Tan et al., 2002). There have been up to 17 subtypes described in humans and animals with subtype (ST) 3 being the predominant subtype identified in most epidemiological studies (Parkar et al., 2010, Forsell et al., 2012, Meloni et al., 2011, Roberts et al., 2013b). Transmission has been attributed to the faecal-oral route as well as consumption of contaminated food and water (Leelayoova et al., 2004). Although there is still some debate about the pathogenicity of *Blastocystis*, symptoms attributed to *Blastocystis* infection include diarrhoea, abdominal pain, bloating and vomiting (Boorom et al., 2008). Due to the lack of knowledge of *Blastocystis* pathogenicity, treatment may not be offered resulting in ongoing symptoms and possible transmission to family and household members. Metronidazole is the most commonly prescribed drug for the treatment of *Blastocystis* with a large variation in efficacy ranging from 0%- 100% (Moghaddam et al., 2005, Nigro et al., 2003, Stensvold et al., 2008, Nagel et al., 2012). Other antimicrobial agents which have been used to treat *Blastocystis* infection include paromomycin, nitazoxanide, iodoquinol and trimethoprim- sulfamethoxazole with varying results (Cimerman et al., 2003, Andiran et al., 2006, Pasqui et al., 2004, Rossignol et al., 2005)

In this study, 18 symptomatic patients infected with *Blastocystis* were followed to determine the efficacy of antimicrobial treatment. We report emergence of treatment failure with metronidazole involving four different *Blastocystis* subtypes.

6.1.3 Methods and Materials

Stool samples were collected from 18 individuals complaining of intestinal symptoms including diarrhoea, abdominal cramps and bloating. All samples were submitted to microscopy of a permanent modified iron haematoxylin stain according to the manufacturer's instructions (Fronine, Australia). DNA was extracted using the Bioline Isolate fecal DNA kit as per manufacturer's instructions, and underwent PCR for the detection of *Blastocystis sp.* using a previously described method (Stensvold et al., 2007a). DNA sequence analysis was performed on all PCR products generated. PCR products were purified using the QIAquick™ PCR purification Kit (Qiagen) as per the manufacturer's instructions and sent to the Australian Genome Research Facility (Westmead Millennium Institute, Sydney) for sequencing in both directions and reads were assembled in to a consensus. The SSU rDNA sequences were then compared to those available in the GenBank database using the BLASTN program run on the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST). Samples also underwent PCR for the detection of *Dientamoeba fragilis* using a previously published method (Stark et al., 2010). PCR was performed on all samples positive for *Entamoeba histolytica* complex by microscopy to speciate (Fotedar et al., 2007b). All samples were also screened for bacterial pathogens including *Salmonella sp, Shigella sp, Vibrio cholerae, Campylobacter sp, Clostridium difficile* and *Aeromonas sp* by culture. Household members and pets were also screened for the presence of *Blastocystis.*

6.1.4 Results

Eighteen patients were identified as infected with *Blastocystis spp.* by microscopy and confirmed by PCR. All patients were symptomatic. A total of four different subtypes (ST) were identified by sequencing- ST1 (n=1), ST3 (n=14), ST4 (n=2) and ST5 (n=1). No bacterial pathogens were isolated. Due to the chronic nature of infection no viral testing was performed. The cohort consisted of eight females and

10 males with an average age of 37 years (6-62 years). Results summarised in Table 6.1.

Patients 1-3 were family members living at the same residence. Symptoms of diarrhoea were described after the ingestion of water from a water tank from a property in rural New South Wales, Australia which was suggested as being contaminated. *Blastocystis* sp. and *Dientamoeba fragilis* were detected by microscopy in all three patients. The patients were prescribed a dose of metronidazole 400mg three times daily for ten days but remained symptomatic following treatment. Follow- up testing one month post- therapy revealed that all patients were still infected with *Blastocystis* and patient 2 was still positive for *D. fragilis*. Subtyping of the isolates demonstrated the same subtypes were present in both the pre and post treatment samples (ST5 in patient 1 and ST3 in patients 2 and 3). All three patients were then treated with paromomycin (25mg/kg three times daily for ten days). Follow-up clinical consultation and subsequent stool samples revealed clearance of *Blastocystis* (and *Dientamoeba fragilis* in patient 2) with resolution of symptoms.

Patient 4 was positive for *Blastocystis* ST1. This patient also had *E. histolytica* complex by microscopy which was subsequently confirmed as the non-pathogenic *E. dispar* by PCR. The patient complained of diarrhoea, nausea and abdominal pain. Initial treatment with metronidazole 400mg three times daily for seven days resulted in the clearance of *E. dispar*; however *Blastocystis* was still present and symptoms persisted. The patient was then prescribed a single dose of tinidazole but remained symptomatic and failed to clear *Blastocystis*. Finally norfloxacin was administered for four weeks. The patient reported a slight reduction in gastrointestinal symptoms and follow-up samples six months later showed that while the patient was no longer symptomatic, *Blastocystis* was still present in the stool but in very low numbers. The patient's household contacts were tested for *Blastocystis* and both the housemate and pet dog tested negative for *Blastocystis* by microscopy and PCR.

Patient 5 first presented with intestinal symptoms of diarrhoea and abdominal cramps after travelling to Borneo. A laboratory diagnosis of *Blastocystis* infection was made but the physician did not prescribe anti-parasitic treatment. The patients'

gastrointestinal symptoms continued for 12 months. The patient was subsequently diagnosed with *Blastocystis* (ST4) in the absence of any other pathogens. Treatment was commenced immediately with metronidazole 400mg of three times daily for ten days. Three months following treatment the patient was still symptomatic and follow-up samples confirmed that *Blastocystis* (ST4) was still present. The patient was then treated with ciprofloxacin. However, one year later gastrointestinal symptoms remained and the faeces was confirmed positive for *Blastocystis* ST4 by microscopy and PCR.

Patient 6 had a history of abdominal cramps, vomiting, diarrhoea and bloating for three years. This patient saw several gastroenterologists and was diagnosed with irritable bowel syndrome without having any stool samples collected to exclude infective pathogens. After another year a stool sample was finally obtained and *Blastocystis* ST4 was identified. The patient was prescribed metronidazole 400mg three times daily. Symptoms partially subsided after treatment but two years later the patient had ongoing gastrointestinal symptoms and *Blastocystis* ST4 was still present. The patient had several animals which were all tested for *Blastocystis.* One chicken was positive for *Blastocystis* ST2 and two guinea fowl were positive for ST7.

Patients 7-18 were all diagnosed with *Blastocystis* ST3 infection after seeing a gastroenterologist complaining of diarrhoea and abdominal pain. Five patients were initially treated with metronidazole (400mg three times daily) for 10 days, two with iodoquinol (630mg three times daily) and doxycycline (50mg twice daily) for 20 days, and five with a triple therapy of nitazoxanide, furazolidone and secnidazole for 10 days. After treatment all patients continued to describe ongoing gastrointestinal symptoms and after a year were still positive for *Blastocystis*. None of these patients owned pets or had other household members complaining of gastrointestinal symptoms.

Table 6.1 *Blastocystis* subtype results, treatment and household contacts for patients

Patient	Age (years)	Subtype	Symptoms	Initial Treatment	Clearance of symptoms	2nd line treatment	Clearance of symptoms	Time elapsed between initial and latest positive sample	Household Contacts and subtype	Travel
1	6	5	Diarrhoea	Metronidazole	No	Paromomycin	Yes	7mths	2 ST3	Rural N.S.W
2	44	3	Diarrhoea	Metronidazole	No	Paromomycin	Yes	7mths	1 ST5, 1 ST3	Rural N.S.W
3	16	3	Diarrhoea	Metronidazole	No	Paromomycin	Yes	7mths	1 ST5, 1 ST3	Rural N.S.W
4	31	1	Diarrhoea, nausea and abdominal pains	Metronidazole	No	Tinidazole, norfloxacin	No	4 yrs	Housemate and dog both PCR negative	South East Asia
5	30	4	Diarrhoea and abdominal pains	Metronidazole	No	Ciprofloxacin	No	3yrs	No	Borneo
6	27	4	Diarrhoea, nausea, bloating and abdominal pains	Metronidazole	No	-	-	4yrs	Chicken ST2, Guinea fowl ST7	-
7	34	3	Diarrhoea and pains	Metronidazole	No	-	-	1yr	No	-

Table 6.1 *Blastocystis* subtype results, treatment and household contacts for patients (*Continued*)

Patient	Age (years)	Subtype	Symptoms	Initial Treatment	Clearance of symptoms	2nd line treatment	Clearance of symptoms	Time elapsed between initial and latest positive sample	Household Contacts and subtype	Travel
8	44	3	Diarrhoea and abdominal pains	Metronidazole	No	-	-	1yr	No	-
9	54	3	Diarrhoea and abdominal pains	Iodoquinol, doxycycline	No	-	-	1yr	No	-
10	48	3	Diarrhoea and abdominal pains	Iodoquinol, doxycycline	No	-	-	1yr	No	-
11	49	3	Diarrhoea and abdominal pains	Metronidazole	No	-	-	1yr	No	-
12	26	3	Diarrhoea and abdominal pains	Metronidazole	No	-	-	1yr	No	-
13	38	3	Diarrhoea, abdominal pains	Metronidazole	No	-	-	1yr	No	-

Table 6.2 *Blastocystis* subtype results, treatment and household contacts for patients (*Continued*)

Patient	Age (years)	Subtype	Symptoms	Initial Treatment	Clearance of symptoms	2nd line treatment	Clearance of symptoms	Time elapsed between initial and latest positive sample	Household Contacts and subtype	Travel
14	54	3	Diarrhoea and abdominal pains	Triple therapy-nitazoxanide, furazolidone, secnidazole	No	-	-	1yr	No	-
15	29	3	Diarrhoea and abdominal pains	Triple therapy-nitazoxanide, furazolidone, secnidazole	No	-	-	1yr	No	-
16	39	3	Diarrhoea and abdominal pains	Triple therapy-nitazoxanide, furazolidone, secnidazole	No	-	-	1yr	No	-
17	51	3	Diarrhoea and abdominal pains	Triple therapy-nitazoxanide, furazolidone, secnidazole	No	-	-	1yr	No	-
18	62	3	Diarrhoea and abdominal pains	Triple therapy-nitazoxanide, furazolidone, secnidazole	No	-	-	1yr	No	-

6.1.5 Discussion

There is increasing debate over the pathogenicity of *Blastocystis* with some studies stating that it is not pathogenic while others argue the validity of *Blastocystis* being considered a pathogen (Stark et al., 2007b, Tan, 2008, Roberts et al., 2013b). One viewpoint is that some subtypes of *Blastocystis* may be pathogenic. This study's results are consistent with that viewpoint, as we demonstrated by the presence of chronic symptoms in the absence of any other infectious agents (Ozyurt et al., 2008, Hussein et al., 2008, Roberts et al., 2013b). This study reports treatment failure for 18 individuals identified with chronic *Blastocystis* infection. All patients complained of intestinal symptoms including diarrhoea, abdominal cramps and nausea. All patients were infected with *Blastocystis* and four different subtypes were identified from this group- one ST1, fourteen ST3, two ST4, and one ST5.

ST3 is the most common subtype found in most epidemiological studies and there has been a low association between subtype and symptoms. There have been several previous studies that have shown approximately 40- 60% of patients with ST3 have some sort of gastrointestinal symptom (Jantermtor et al., 2013, Roberts et al., 2013b, Jones et al., 2009). ST3 was the most common subtype isolated in this group and all patients had symptoms suggesting that this subtype is pathogenic. Although four patients had other parasites present on initial testing, after the first treatment these parasites were cleared while intestinal symptoms still persisted. No other pathogens were identified which suggests that *Blastocystis* was the probable cause of these symptoms.

The three family members that presented with *Blastocystis* stated that symptoms started after the consumption of water from a water tank whilst on holiday in rural Australia. There were four members of the family that were infected with *Blastocystis* but after the initial treatment, one patient had resolution of symptoms and diagnostic methods confirmed that infection had been cleared. There is the possibility that re-infection rather than treatment failure occurred with the other three members of the family, but as two different subtypes were isolated within this group and that the fourth member did not have any further symptoms, this appears unlikely. It has been previously suggested that treatment failure could be mistaken for re-

infection. This should be considered in all cases where symptoms persist (Stensvold et al., 2010).

Although there have been several publications that have reported the eradication of *Blastocystis* with metronidazole, there have been few studies that have examined treatment failure in relation to subtype. Metronidazole is considered first-line treatment but reported success rates vary between 0%-100% (Stensvold et al., 2010, Nagel et al., 2012). Metronidazole treatment failure has been reported in one patient with ST2 related urticaria and gastrointestinal disease (Vogelberg et al., 2010), a patient with severe intestinal symptoms associated with ST8 (Stensvold et al., 2008), and six ST3 and one ST1 infections in patients whom presented with both urticarial and gastrointestinal symptoms (Jones et al., 2009). A study of 11 symptomatic patients (five with ST1, four ST3, four ST4 and one ST6 with three mixed infections) treated with either metronidazole or trimethoprim/ sulfamethoxazole reported that no infection was cleared by treatment (Nagel et al., 2012). The 11 patients in our study who were initially treated with metronidazole were shown to have treatment failure along with the other seven patients treated with combination therapy. It has previously been suggested that some subtypes might be more resistant or are more likely to fail treatment than others (Stensvold et al., 2010). However our study, where four different subtypes were identified, suggests that any subtype could result in treatment failure. These results also highlight the fact that *Blastocystis* should be considered a pathogen as all patients noted severe symptoms in the absence of any other pathogen.

This study also highlights the need for other treatment options for *Blastocystis* infection. One study has demonstrated the efficacy of *Saccharomyces boulardii* (250mg twice a day, Reflor) (Dinleyici et al., 2010) while others have reported treatment with trimethoprim/sulfamethoxazole with varying results; 22% eradication (Moghaddam et al., 2005), 95% clearance (Ok et al., 1999) and 100% efficacy (Stensvold et al., 2008). Other studies suggest paromomycin is the most effective agent for clearing *Blastocystis* with up to 100% efficacy and in our study paromomycin also appeared to be effective (Armentia et al., 1993, Kick et al., 2002, Valsecchi et al., 2004, Pasqui et al., 2004, van Hellemond et al., 2012). A number of other studies have highlighted the variable efficacy of a number of antimicrobial

agents including nitazoxanide, iodoquinol, tinidazole, emetine, pentamidine, iodochlorohydroxyquine and furazolidone (Rossignol et al., 2005, Romero Cabello et al., 1997, Mirza et al., 2011a, Markell and Udkow, 1986, Moghaddam et al., 2005).

Both the Centre for Disease Control (CDC) and the Australian Therapeutic Guidelines refer to the clinical significance of *Blastocystis* as controversial. The CDC recommends treatment with metronidazole, trimethoprim/sulfamethoxazole or nitazoxanide, while the Therapeutic Guidelines recommend *Blastocystis* is treated with tinidazole, metronidazole or nitazoxanide. There is a minor comment that says that pregnant woman should be treated with paromomycin. Unfortunately paromomycin is not readily available in Australia and is accessed on a case by case basis via the Special Access Scheme. From this and other studies it would appear that the recommended treatments should be revised and that further studies are required to determine the most effective treatment options for *Blastocystis* infection. *Blastocystis* should be considered a potential pathogen when in the presence of symptoms and the absence of any other infectious agents. Metronidazole should no longer be considered the first line treatment prescribed.

Host factors such as age and ethnicity may play a role in the severity and length of *Blastocystis* infection. The average age for patients in this group was 37 with only one child in the group. A previous study showed that the time till clearance of *Blastocystis* infection increase as age increases and this could be a factor in this group with almost all of the patients above the age of 27 (Pipatsatitpong et al., 2012). It has previously been shown that IL-8 and IL-10 single nucleotide polymorphisms (SNPs) play a role in *Blastocystis* infection (Olivo-Diaz et al., 2012). SNPs at IL-10 have shown to vary between populations and this may play a role in this Australian Caucasian study group in terms of disease and clearance (Meenagh et al., 2002).

6.1.6 Conclusion

This study reports the failure of treatment to clear *Blastocystis* infections in 18 patients treated with a number of different antimicrobial agents, in particular the recommended treatment agent, metronidazole. This study identified four different subtypes- ST1, ST3, ST4 and ST5 and demonstrated that there is not one particular subtype that has a higher rate of treatment failure. This study also highlights the pathogenic role of *Blastocystis* and reasserts that it should be considered a pathogen

when found in conjunction with symptoms and no other infectious agents. Treatment failures highlight the need for further antimicrobial testing to be performed to expand therapeutic options for the management of *Blastocystis* when treatment failure does occur.

6.2 *In vitro* Susceptibility patterns of *Blastocystis sp.*

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Keywords-

Blastocystis; antimicrobials; subtypes

6.2.1 Abstract

Blastocystis is a common human enteric protist with controversial clinical significance. Metronidazole is considered first- line treatment for *Blastocystis* infection however there has been increasing evidence on the lack of efficacy of this treatment. Treatment failure has been reported in several clinical cases and recent *in vitro* studies have suggested the occurrence of metronidazole resistant strains. In this study we tested 12 *Blastocystis* isolates from four common *Blastocystis* subtypes (ST1, ST3, ST4 and ST8) against 12 commonly used antimicrobials (metronidazole, paromomycin, ornidazole, albendazole, ivermectin, trimethoprim- sulfamethoxazole, furazolidone, nitazoxonide, secnidazole, fluconazole, nyastatin and itraconazole) at 10 different concentrations *in vitro* in triplicate. It was found that all subtypes showed little sensitivity to the commonly used metronidazole, paromomycin and triple therapy (furazolidone, nitazoxanide and secnidazole). This study highlights the efficacy of other potential drug treatments including trimethoprim- sulfamethoxazole and ivermectin and suggests that current treatment regimens be revised.

6.2.2 Introduction

Blastocystis is the most common enteric protist found in humans with rates of infection ranging from 2-30% in developed countries and up to 50% in developing countries (Tan, 2008). There have been 17 subtypes (ST) identified from humans and animals with ST1- 9 being identified in humans (Alfellani et al., 2013b, Ramirez et al., 2013, Parkar et al., 2010). ST3 is the predominant subtype found in most human studies (Forsell et al., 2012, Meloni et al., 2011, Yoshikawa et al., 2004b) . There have been numerous studies that have highlighted the clinical relevance of *Blastocystis* and an association between subtype and symptoms has been made (Dominguez-Marquez et al., 2009, Stensvold et al., 2008, Roberts et al., 2013b, Jones et al., 2009). Although the pathogenic potential of this parasite has long been documented, there is still debate on whether *Blastocystis* infections should be treated and therefore only a small number of studies have looked at treatment options for *Blastocystis* infection. Most case studies report first line treatment with metronidazole and have found varying rates of efficacy with ranges of 0% to 100% (Moghaddam et al., 2005, Nigro et al., 2003, Stensvold et al., 2008, Gupta and Parsi, 2006). Other antimicrobials which have been used to treat *Blastocystis* infection

include iodoquinol, ketoconazole, nitazoxanide, paromomycin, tinidazole and trimethoprim- sulfamethoxazole all with varying results (Andiran et al., 2006, Cimerman et al., 2003, Cohen, 1985, Pasqui et al., 2004, Rossignol et al., 2005). There have only been four previous studies to look at *in vitro* susceptibility patterns of *Blastocystis* all of which have had a small number of study isolates. From these studies though, it is apparent that different subtypes show different susceptibility patterns and that metronidazole is not the most effective treatment for *Blastocystis* infection (Mirza et al., 2011a, Mirza et al., 2011b, Dunn et al., 2012, Dhurga et al., 2012). In this study the *in vitro* susceptibility patterns of 12 different commonly used antimicrobials (metronidazole, paromomycin, ornidazole, albendazole, ivermectin, trimethoprim- sulfamethoxazole, furazolidone, nitazoxonide, secnidazole, fluconazole, nyastatin and itraconazole) were examined against 12 clinical isolates of *Blastocystis* from four different subtypes (ST1, ST3, ST4 and ST8) in triplicate. These results show the lack of efficacy of the most commonly used drugs for anti-parasitic treatment including metronidazole. This study suggests other possible treatment options including trimethoprim- sulfamethoxazole and ivermectin.

6.2.3 Materials and Methods

6.2.3.1 *Blastocystis* cultures

Twelve *Blastocystis* isolates from stool samples submitted to St. Vincent's Hospital Microbiology Department were used for the study. Samples were identified as positive for *Blastocystis* by microscopy of a permanent Iron Haematoxylin stain and confirmed by PCR using a previously published method (Stensvold et al., 2007a). 10mg of fresh sample was inoculated into a diphasic xenic dorset egg slope using a previously published method (Roberts et al., 2011) and cultures were maintained by passaging every 4 days.

6.2.3.2 *Blastocystis* subtyping

DNA was extracted from *Blastocystis* cultures using the Bioline Isolate fecal DNA kit as per manufacturer's instructions, and underwent PCR for the detection of *Blastocystis* sp. using a previously described method (Stensvold et al., 2007a). DNA sequence analysis was performed on all PCR products generated. PCR products were purified using SureClean Plus (Bioline) as per the manufacturer's instructions and

sent to the Australian Genome Research Facility (Westmead Millennium Institute, Sydney) for sequencing in both directions. Reads were assembled into a consensus. The SSU rDNA sequences were then compared to those available in the GenBank database using the BLASTN program run on the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST).

6.2.3.3 Antimicrobial susceptibility testing

The following agents were used for susceptibility testing: metronidazole, paromomycin, ornidazole, albendazole, ivermectin, trimethoprim- sulfamethoxazole (TMP-SMX), furazolidone, nitazoxonide, secnidazole, fluconazole, nyastatin and itraconazole. Metronidazole (Pfizer, NSW, Australia) in liquid form at 5mg/ml was used as a stock solution and diluted with phosphate-buffered saline (PBS) to cover a concentration range of 500µg/ml to 1µg/ml by doubling dilution. Ornidazole (provided by J. Upcroft, Queensland Institute of Medical Research) in powder form was dissolved in 50% ethanol to 5mg/ml and diluted as above. Paromomycin sulphate, furazolidone, nitazoxanide, secnidazole (Sigma-Aldrich, Sydney, NSW, Australia) fluconazole (Diflucan, Pfizer, NSW, Australia) and itraconazole (Sporanox, Janssen Pharmaceuticals Inc, NSW, Australia) in powder form were suspended in 10% ethanol to make stock solutions of 5mg/ml and diluted in the same manner as above. Albendazole tablets (GlaxoSmithKline, VIC, Australia) were dissolved in glacial acetic acid to 5mg/ml and diluted as above. Ivermectin tablets (Merck Sharp & Dohme Pty Ltd, NSW, Australia) were dissolved in methanol to 5mg/ml and diluted as above. TMP-SMX in liquid form was diluted to 40mg/ml sulfamethoxazole and 8mg/ml trimethoprim with PBS and then diluted as above. Nystatin (Omegapharm, VIC, Australia) in liquid form was diluted to 5mg/ml in PBS and diluted as above. 100µl of the respective antibiotic dilutions were inoculated in to 96 well microtitre plates and 100µl of *Blastocystis* culture was added to each dilution. A control containing 100µl of 10% ethanol was performed for all drugs in powder form to rule out any inhibitory effects of the solvent on *Blastocystis*. 100µl of PBS buffer was used for the metronidazole control, 100µl of diluted glacial acetic acid for the albendazole control and 100µl of diluted methanol for the ivermectin control were used. All drug testing was performed in triplicate. Microtitre plates were then incubated in anaerobic conditions at 35˚C.

Cell concentration and viability was determined quantitatively by the trypan blue dye exclusion method (Strober, 2001) by counting each dilution using Kova slides viewed under phase-contrast microscopy and then counted every day for 4 days. As *Blastocystis* numbers in negative controls decline after 92 hours, susceptibility testing with each compound was only performed for 4 days. The minimal inhibitory concentration (MIC) was determined by the concentration of drug where there were lower numbers of growth compared to the control and the minimal lethal concentration (MLC) was determined to be the concentration at which no *Blastocystis* cells were observed.

6.2.3.4 Bacterial characterisation of flora present in xenic cultures

The bacterial flora present in each culture was characterised before antibiotic testing and at the end of the 4 days. Supernatant from each *Blastocystis* culture was inoculated onto the following bacteriological media: Brilliance UTI agar, MacConkey agar and anaerobic agar (Thermofisher Scientific Australia Pty Ltd., VIC, Australia). Aerobic plates were incubated in CO_2 at 35˚C for 24 to 48h while the anaerobic plates were incubated for 48h under anaerobic conditions using an Anoxomat Mark II system (Mart Microbiology) with the following gas composition: 0.16% O_2, 5% H_2, 10% CO_2, and 85% N_2. All bacteria were identified to species level using routine bacteriological procedures including biochemical testing and identification using the Bruker microflex MALDI-TOF.

6.2.4 Results

6.2.4.1 *Blastocystis* subtyping

There were four subtypes identified by sequencing and BLAST searching- five ST1, four ST3, two ST4 and one ST8.

6.2.4.2 Antimicrobial testing

There was a progressive reduction in the number of *Blastocystis* cells seen during the four days at all concentrations which were comparable to the control. There was a variation seen between each isolate even within the subtypes. The MIC values for the compounds were- metronidazole 250 µg/ml- 64 µg/ml, ornidazole 125 µg/ml – 32 µg/ml, secnidazole 64 µg/ml- 16 µg/ml, paromomycin 1 µg/ml, albendazole 64

µg/ml- 16 µg/ml, furazolidone 250 µg/ml- 125 µg/ml, nitazoxanide 500 µg/ml- 250 µg/ml, fluconazole 500 µg/ml- 250 µg/ml, itraconazole 500 µg/ml- 250 µg/ml and nyastatin 250 µg/ml. Due to time and space constraints and the obvious lack of efficacy after the 2nd concentration, the anti-fungals were only tested over 3 days for four different concentrations. Ivermectin had an MLC of 64 µg/ml- 32 µg/ml, and TMP-SMX had an MLC of 100 µg/ml/500 µg/ml- 12 µg/ml/ 64 µg/ml. TMP-SMX and ivermectin were the only drugs where there was no growth at the two highest concentrations for all the isolates. Secnidazole was the only other drug which had no growth at the highest concentration for most of the isolates. Paramomycin was the only drug observed where the lower concentrations did not outgrow the control. *Blastocystis* cell growth numbers are summarised in Table 6.2.1- 6.2.12

6.2.4.3 Subtype dependency

There was not any significant difference between subtypes to response to drug concentration.

6.2.4.4 Bacteria present in cultures

The bacteria isolated from the cultures were as follows- *Escherichia coli, Enterococcus faecalis, Clostridium butyricium, Prevotella* sp. and *Citrobacter freundii.* There did not appear to be any effect on the bacteria present in the cultures before and after treatment and within the subtypes. It was observed however, that when there was a higher number of bacteria present in the culture, there were lower numbers of *Blastocystis* cells.

Table 6.2. 1 *Blastocystis* sp. cell numbers at different concentrations of metronidazole

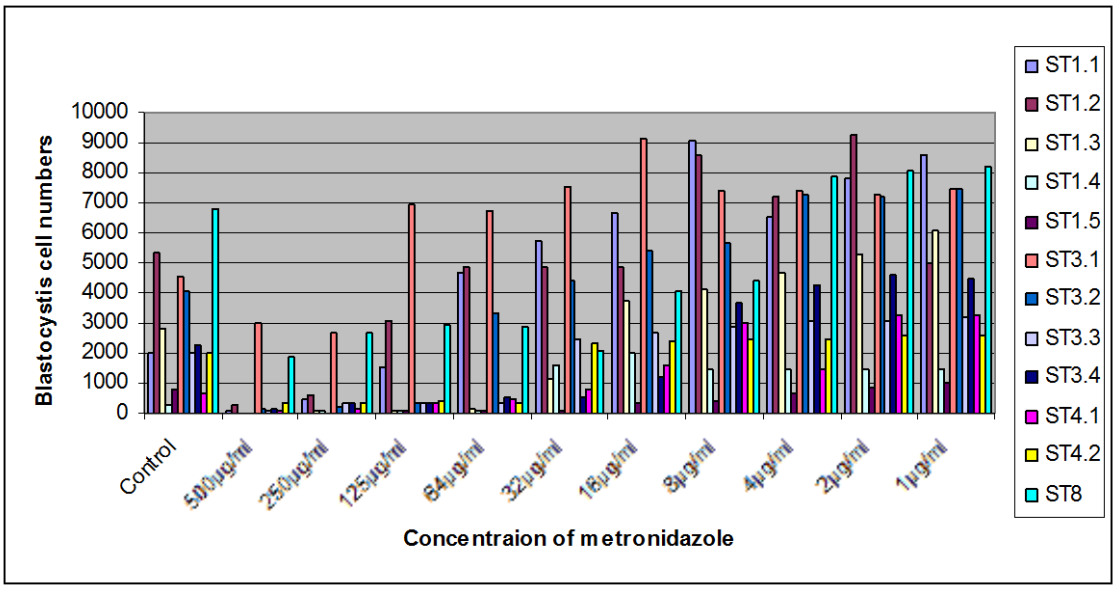


Table 6.2. 2 *Blastocystis* sp. cell numbers at different concentrations of paromomycin

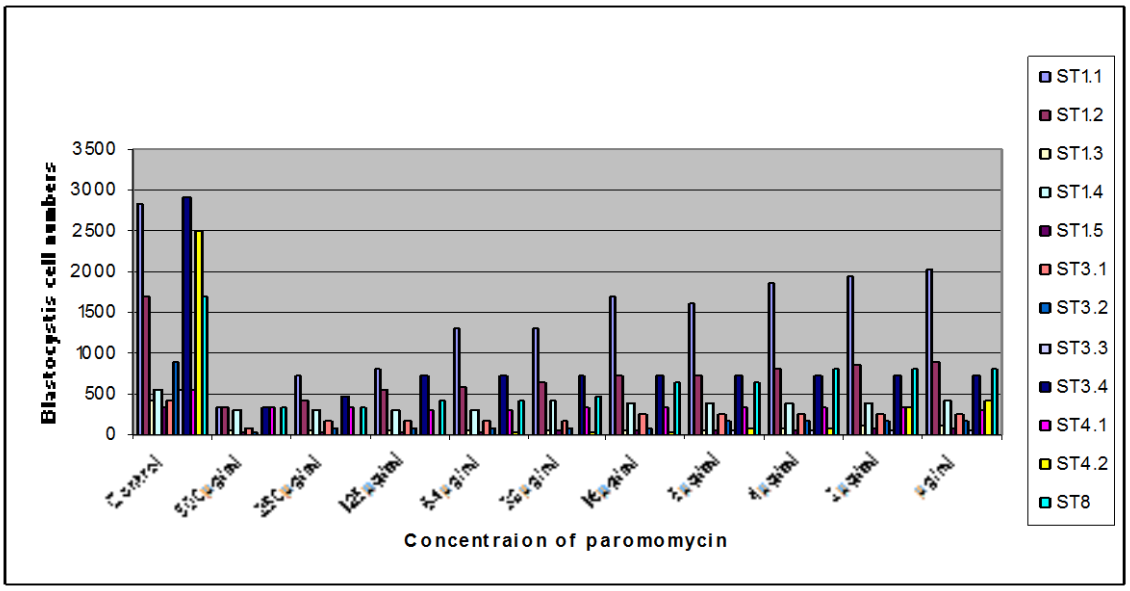


Table 6.2. 3 *Blastocystis* sp. cell numbers at different concentrations of ornidazole

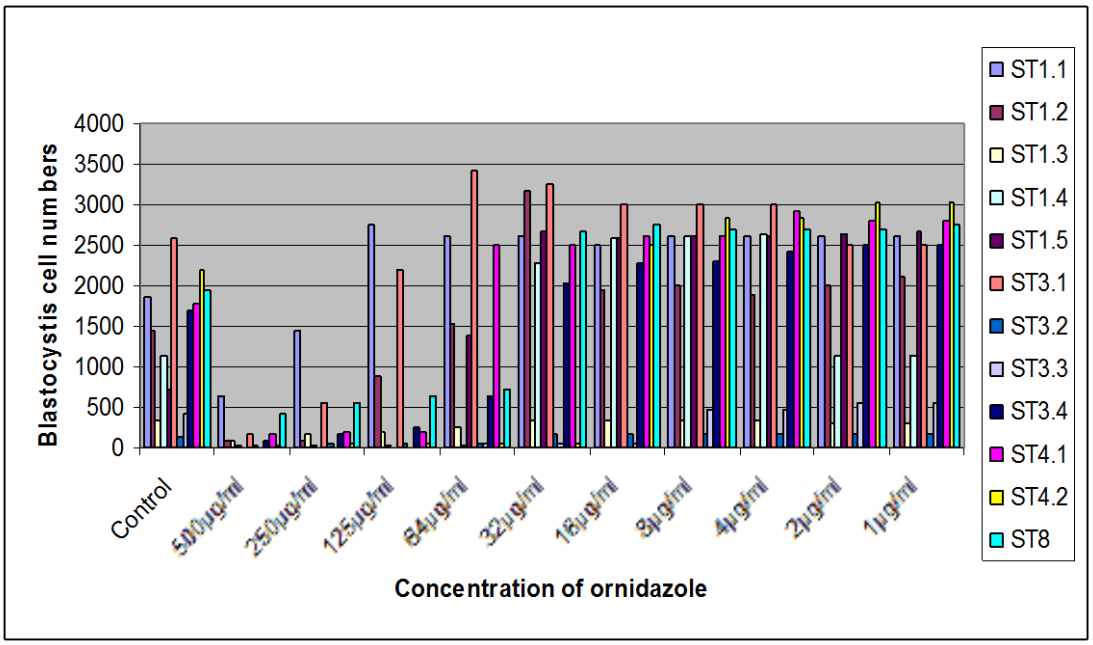


Table 6.2. 4 *Blastocystis* sp. cell numbers at different concentrations of albendazole

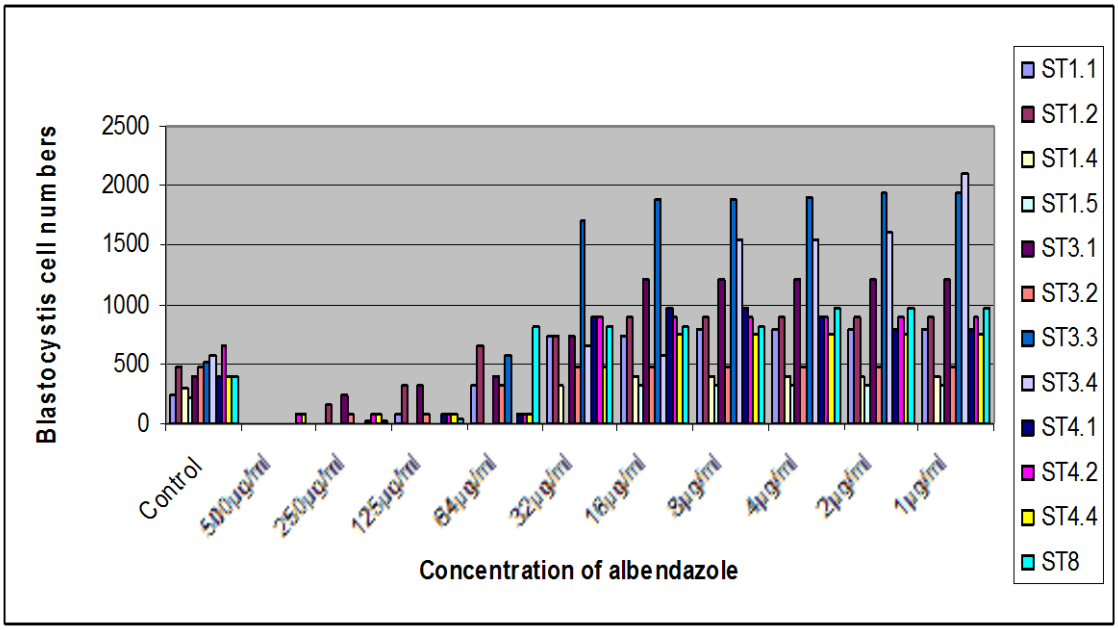


Table 6.2. 5 *Blastocystis* sp. cell numbers at different concentrations of ivermectin

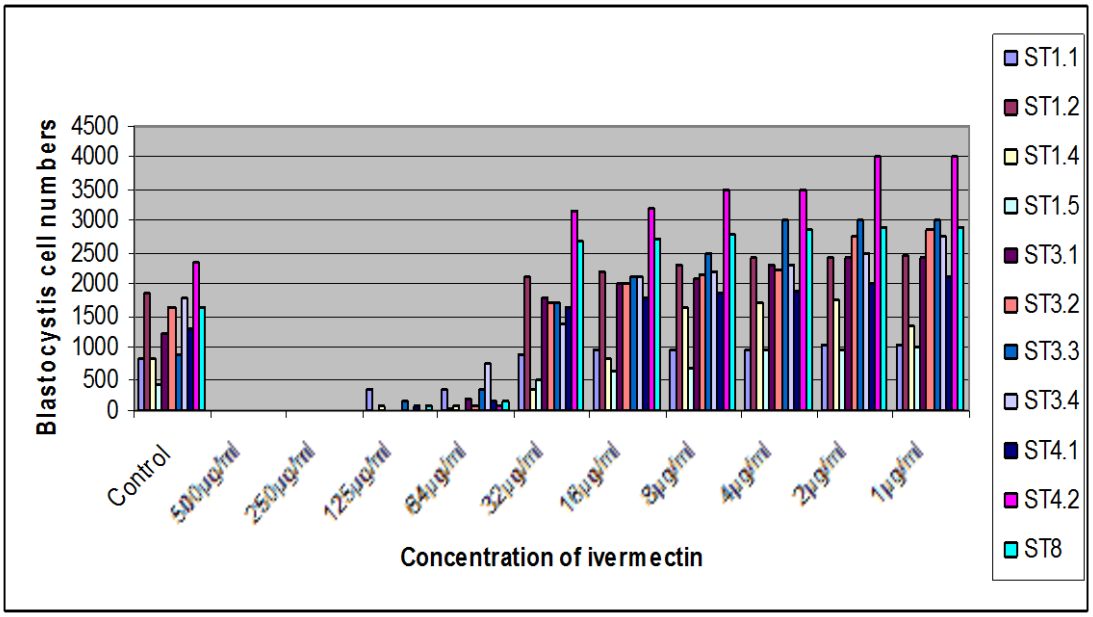


Table 6.2. 6 *Blastocystis* sp. cell numbers at different concentrations of secnidazole

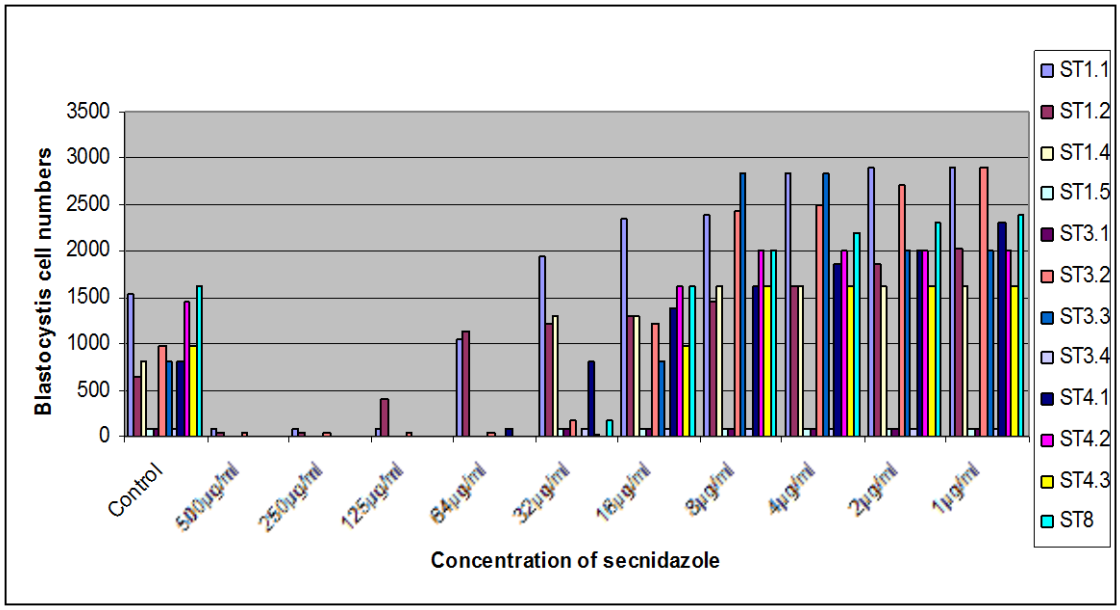


Table 6.2. 7 *Blastocystis* sp. cell numbers at different concentrations of nitazoxanide

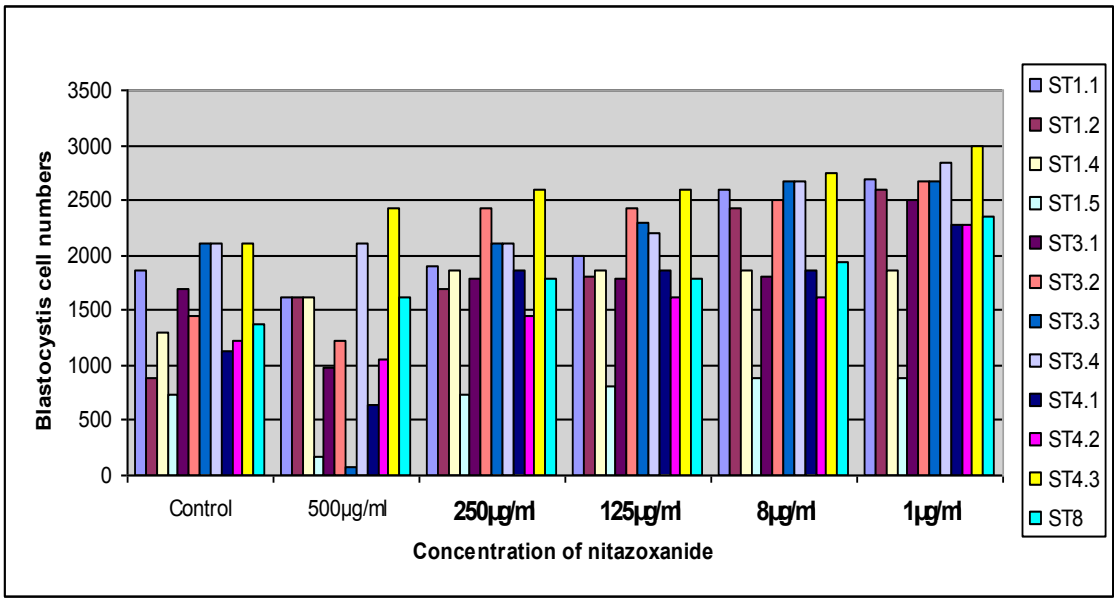


Table 6.2. 8 *Blastocystis* sp. at different concentrations of furazolidone

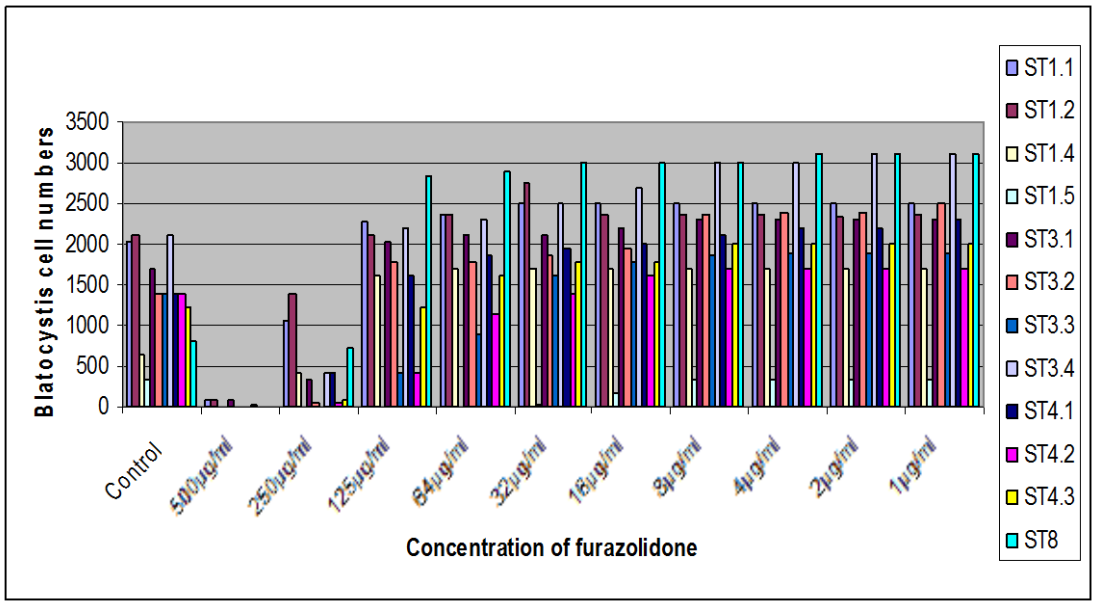


Table 6.2. 9 *Blastocystis* sp. cell numbers at different concentrations of trimethoprim- sulfamethoxazole

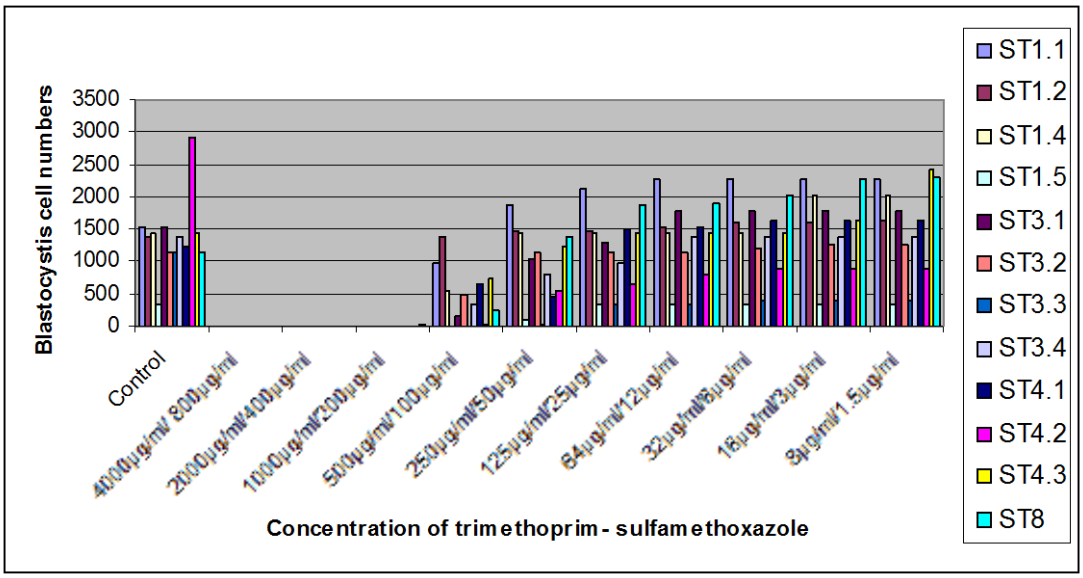


Table 6.2. 10 *Blastocystis* sp. cell numbers at different concentrations of fluconazole

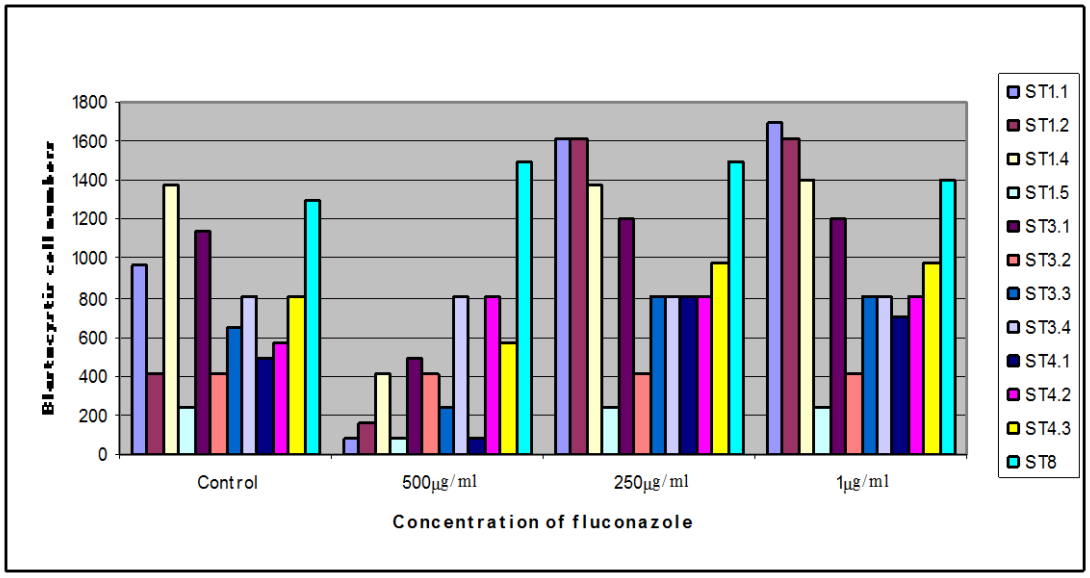


Table 6.2. 11 *Blastocystis* sp. cell numbers at different concentrations of nyastatin

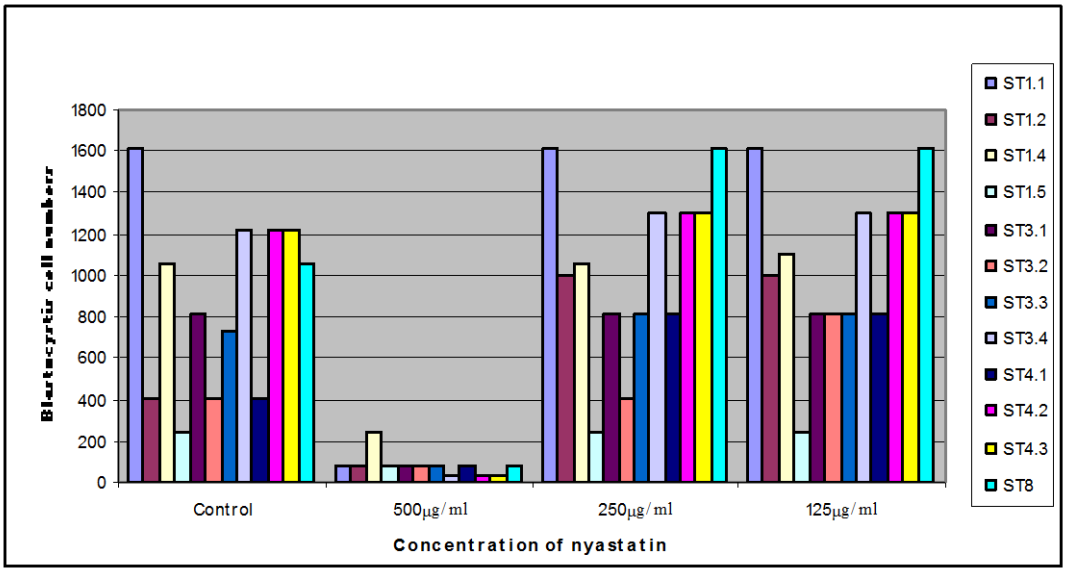
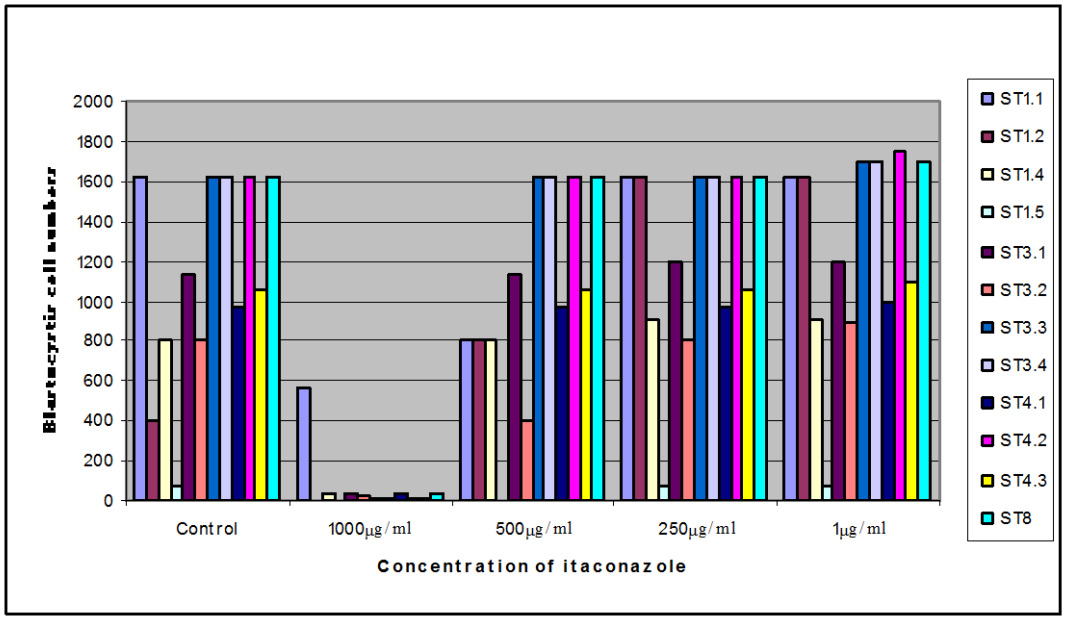


Table 6.2. 12 *Blastocystis* sp. cell numbers at different concentrations of itaconazole



6.2.5 Discussion

Blastocystis is the most common enteric protist found in humans. Though there is still some discussion about the pathogenicity of *Blastocystis*, treatment failure has been widely reported in the literature (Roberts et al., 2014). This study suggests that though metronidazole is the most common drug therapy used for *Blastocystis* treatment, this should be reconsidered as other options such as TMP-SMX or ivermectin are much more effective as an anti-parasitic agent.

Metronidazole was found to have an inhibitory effect only up to the third highest concentration tested of 125µg/ml. Metronidazole is the most frequently prescribed antibiotic for *Blastocystis* treatment with high rates of clearance being reported from some clinical studies (Cassano et al., 2005, Katsarou-Katsari et al., 2008, Nigro et al., 2003). Metronidazole resistance in *Blastocystis* has been reported since 1996 (Zaman and Zaki, 1996) and it was suggested that this could be ST dependent. Our study does not show that one ST is more resistant than others to metronidazole. In this study it was observed that there were much higher cell numbers seen in treated cultures with a concentration of 64µg/ml to the lowest concentration of 1µg/ml compared to the control. One study suggested that there is a mechanism involved in *Blastocystis* that produces higher numbers of viable cells by regulating the apoptotic process in response to treatment with metronidazole which is what was probably witnessed in our study (Dhurga et al., 2012). This indicates that if metronidazole is to be used, it should be used at the highest concentration possible. This is not ideal though with many possible side effects being related to metronidazole treatment such as nausea and vomiting. Also there was never a total clearance of *Blastocystis* noted at even the highest concentration suggesting that metronidazole does not have a complete effect on *Blastocystis.* It is clear that metronidazole should not be the drug of choice for first line treatment of *Blastocystis*.

Ornidazole was shown to be highly effective against other enteric protists including *Dientamoeba fragilis* (Nagata et al., 2012). *Blastocystis* is commonly found in conjunction with *D. fragilis* in stool samples from patients and a drug therapy that cleared both parasites would be beneficial to patients. In this study

ornidazole only had an inhibitory effect up to the third highest concentration at 125µg/ml. This indicates that ornidazole is not ideal for the treatment of *Blastocystis*.

The prescription of a triple drug therapy is becoming common practice by some physicians (using secnidazole, furazolidone and nitazoxanide) (Sekar and Shanthi, 2013). The premise behind a triple therapy is that the combination of three drugs will have the highest possible efficacy against the pathogen. In this study it was found that two of the three drugs used for triple therapy (furazolidone and nitazoxanide) had little to no effect at all on *Blastocystis*. The only drug that did have an effect was secnidazole with an efficacy noted down to a concentration of 64µg/ml but then, like metronidazole, there was a huge increase in cell numbers compared to the control. Secnidazole is a nitroimidazole like metronidazole and ornidazole and therefore the same apoptotic effect may be expected to be seen. Secnidazole was shown to be effective for the treatment of *D. fragilis* infections (Girginkardesler et al., 2003) but this does not appear to be an option for *Blastocystis*. Nitazoxanide was previously shown to have high clearance rates against *Blastocystis* in children with 97-100% efficacy reported (Diaz et al., 2003). This drug has no serious side-effects suggesting it to be a good alternative option for treatment, however in this study it was shown that nitazoxanide had no effect on *Blastocystis* even at the highest concentration of 500µg/ml. Furazolidone had little effect at 250µg/ml and no effect after the third highest concentration at 125µg/ml. It was previously stated that furazolidone has some activity against *Blastocystis* at 100µg/ml but our results do not agree with this (Mirza et al., 2011a). The use of a triple therapy using drugs that possess little anti-parasitic activity on *Blastocystis in vitro* is a practice not to be encouraged and can have serious consequences. An overload of antibiotics can have a detrimental effect on the patient causing sickness. Another consequence of the unnecessary use of drugs is the development of drug resistance within the microbial gut flora that may have other consequences for the patient.

Paromomycin is currently one of the recommended treatment options by the Centre for Disease Control (CDC) and the Australian Therapeutic Guidelines for several enteric parasites including *Blastocystis*. There have been several case studies that have shown the effectiveness of paromomycin (Pasqui et al., 2004, Valsecchi et al., 2004, van Hellemond et al., 2012, Roberts et al., 2014). An *in vitro* study

contradicts these by showing paromomycin to be completely ineffective (Mirza et al., 2011a). Our study agrees with Mirza et al (2011) in that paromomycin did not have a lethal effect even at the highest concentrations. Paromomycin was the only drug where the lower concentrations did not outgrow the control but there was also high numbers of cells seen even at the highest concentration. Paromomycin is a poorly absorbed aminoglycoside and from this study and the previous *in vitro* study it cannot be recommended as a suitable treatment.

A recent review on antimicrobial treatments for *Blastocystis* suggested that TMP-SMX is a good alternative to metronidazole with less side effects and being more cost effective (Sekar and Shanthi, 2013). It states that it is not known if TMP-SMX has a direct effect on the *Blastocystis* or on the gut bacteria which is essential for *Blastocystis* survival. In this study we examined the bacteria present before and after treatment from these cultures and found that there was no difference in the bacteria present at the different concentrations which suggests that the death of *Blastocystis* was not due to the removal of the bacteria. TMP-SMX was seen to be highly effective up to a concentration of 64µg/ml and appears to be the most effective drug against all the STs. TMP-SMX was also the only drug studied that had no growth up to a concentration of 64µg/ml. TMP-SMX was shown to have high clearance rates in previous clinical studies (Ok et al., 1999, Stensvold et al., 2008) and was also shown to have a high efficacy in a previous *in vitro* study (Mirza et al., 2011a). The weight of evidence indicates that TMP-SMX should be the first line treatment for *Blastocystis* infection due to it having a higher efficacy than metronidazole. It also has fewer side effects on patients.

Ivermectin and albendazole are both commonly used anti-helminth treatments. Neither of these drugs has previously been tested *in vitro* against *Blastocystis.* In this study it was found that both of these drugs had a lethal concentration down to 250µg/ml suggesting that taken in high doses these drug are an option for treatment.

Blastocystis has recently been placed within the Stramenopiles which also includes fungi and algae. The three anti-fungal drugs used in this study (fluconazole, nyastatin and itraconazole) did not appear to have an effect on any of the STs. This shows that though *Blastocystis* may be closely related to some fungi, it may not have

the same metabolic pathways and therefore may not respond the same way to treatments.

In this study there was much variation seen for the different drugs even within each of the subtypes. Due to this being the largest *in vitro* study completed so far it is difficult to comment on whether this has been seen in other studies with usually only one or two isolates from each ST being studied. Variation in cell viability within a ST was shown however in one previous study (Mirza et al., 2011a). This variation illustrates how difficult it may be to comment on ST resistance and suggests that perhaps certain STs may not be resistant, but individual isolates within STs may be resistant and therefore each isolate should be treated differently. There was a suggestion that some STs are more pathogenic than others and that some STs may be more resistant to drugs than others. One study showed that ST3 had the highest increase in cell numbers after treatment with metronidazole suggesting this ST is more pathogenic and resistant to treatment (Dhurga et al., 2012). One study compared ST4 and ST7 and showed that ST7 was resistant to metronidazole and sensitive to emetine, while ST4 was sensitive to metronidazole and resistant to emetine (Mirza et al., 2011a). Another study showed the inability of both metronidazole and TMP-SMX to clear ST1, ST3, ST4 and ST6 (Nagel et al., 2012). In this study we did not note any major variation in susceptibility between the STs, but noted that there were minor differences even within each ST. From these results we cannot say that any one ST is more resistant than the others and that no particular ST has a role in *Blastocystis* treatment failure.

The draft genome from the NandII ST1 (unpublished) isolate and the full genome for ST7 (Denoeud et al., 2011) have been described. The information from these genomes may be useful for developing new drug therapies by identifying genes that may be involved in drug absorption pathways. There appears to be quite a lot of genetic differences between the ST1 and ST7 genomes with a higher GC% content in ST1 but also ST1 has a substantially smaller genome than ST7 (16.4 Mb and 18.8Mb respectively). The difference in genomes may mean that a drug that may work in one ST may not have any effect on another ST. The more information gathered from the genomes of the different STs will be highly beneficial for the identification of possible drug therapies. Unfortunately as only these two genomes are currently

available, and that ST7 is rarely seen in humans, only the information gathered from ST1 will be helpful at this time. Axenic cultures are preferred for genome sequencing but it is extremely difficult to axenise *Blastocystis* cultures. One study has shown the role mitochondrion like organelles play in the reduction of ferrodoxins in ST7 in the conversion of metronidazole into its active state. This knowledge about this particular metabolic pathway may help in the development of new drug therapies (Nasirudeen et al., 2004, Lantsman et al., 2008).

The development of a simple antimicrobial susceptibility testing system for *Blastocystis* would be highly beneficial for treatment. Until axenic culture of *Blastocystis* becomes easier though, this may not be possible.

6.2.6 Conclusion

This study shows that metronidazole should be reconsidered for use as first line treatment for *Blastocystis* infections due to its lack of efficacy *in vitro* and its ability to promote cell growth at lower drug concentrations. This study also highlights the lack of efficacy against *Blastocystis* of most commonly used antiprotozoal treatments and shows that there was minimal difference between STs to treatment. From the results presented here and from previous studies, we recommend the use of TMP-SMX as first line treatment as it appears to be the most effective at promoting *Blastocystis* clearance.

Chapter 7 General Discussion

Blastocystis is the most common enteric protist found in humans and has a world-wide distribution (Tan, 2008). *Blastocystis* has been identified in a large number of animals including non-human primates, reptiles, mammals and birds (Parkar et al., 2010, Abe et al., 2003b). Due to recent advancements in molecular technologies including PCR and sequencing, there have been 17 subtypes identified, nine of which have been identified in humans (Clark et al., 2013). Although this is a very common parasite, there are still "holes" in the literature about the molecular epidemiology, pathogenicity and treatment of *Blastocystis.* The aim of this study was to identify the prevalence and molecular epidemiology of *Blastocystis* in Australia and to comment on the pathogenicity and treatment options for this parasite.

In the present study, 513 stool samples from symptomatic and asymptomatic people were analysed for the presence of *Blastocystis*. Positive samples were then subjected to PCR of the 18S rDNA and DNA sequencing and phylogenetic analysis was completed. There was a 19% prevalence of *Blastocystis* identified in the Sydney population and subtype (ST) 3 was the predominant ST identified. This is the largest study to date to identify the presence of *Blastocystis* in the human population of Australia.

This study (Roberts et al., 2011) compared five different techniques for the diagnosis of *Blastocystis*- microscopy of a permanent modified iron haematoxylin stain, culture in two different xenic culture media (TYGM-9 and modified Boeck and Drbohlav's medium [MBD] egg slopes overlaid with an in house media) and PCR using two different techniques. *Blastocystis* was identified in 98 samples by at least one of the diagnostic techniques. It was found that microscopy of a permanent stain, which is the gold standard for the diagnosis of *Blastocystis* in most clinical laboratories in Australia, was the least effective at detecting *Blastocystis* with only 47 positive samples detected. The second least effective method was the PCR1 technique which only detected 65 samples. The xenic cultures had almost equal number of positives with 80 and 81 positive for the TYGM-9 and MBD slope respectively. Though there was an almost equal number of positives for the two culture mediums, there was a much higher growth yield found in the MBD slope

compared to the TYGM-9 media. PCR2 was found to be the most sensitive for detecting *Blastocystis* with 92 samples positive using this technique.

Blastocystis was the most common parasite identified in the clinical samples with 19% prevalence. *Giardia intestinalis* was the second most common parasite found in the clinical samples with a prevalence of 2%. This was followed by *Endolimax nana* with 1.7% and *Dientamoeba fragilis* with 1.3%. These results reiterate the high prevalence of *Blastocystis* in the population compared to other parasites. This study agrees with other studies world-wide where *Blastocystis* is usually the most common enteric parasite identified (Aguiar et al., 2007, Aksoy et al., 2007, Baldo et al., 2004, Rayan et al., 2007).

Blastocystis can be misidentified using microscopy as other parasites such as *Dientamoeba fragilis* and *Cyclospora* sp. and as yeast (Tan, 2008, Stark et al., 2006). It is important that if PCR cannot be used for the identification of *Blastocystis* in epidemiological studies, at least two techniques should be used in conjunction to reduce the significant number of missed infections when microscopy is employed. Microscopy is the most common diagnostic technique used in clinical laboratories and these results suggest that there should be a movement towards PCR for the diagnosis of intestinal parasites. Previous studies have also shown the lack of sensitivity of microscopy for the diagnosis of common enteric parasites (Stensvold et al., 2007a, Stark et al., 2010, Fotedar et al., 2007a).

It has previously been shown that PCR is the most effective technique for detecting enteric parasites like *Blastocystis* and our study agrees with this (Parkar et al., 2007, Stensvold et al., 2009b). It is important though to use correct primer pairs that do not detect some STs preferentially where some positive samples may be missed by PCR giving incorrect results. Our results show the inconsistency between two different PCR techniques and this must be taken into consideration when developing new PCR techniques. Several different PCR techniques have been developed for the diagnosis of *Blastocystis* with many different primer pairs being used (Clark, 1997, Kaneda et al., 2001, Yoshikawa et al., 2004a, Jones et al., 2009, Poirier et al., 2011).

All PCR positive samples were sequenced in both directions and sequences were compared to those available in the GenBank database using the BLASTN

program. There were seven STs identified in this group by sequencing. ST3 was the predominant ST found with a prevalence of 44%. This was followed by ST1 with 31%, ST4 with 13%, ST2 with 5%, ST6 with 3%, ST8 with 2% and ST7 with 1%.

ST3 is the predominant ST found in most human epidemiological studies and our results agree with this (Yoshikawa et al., 2004b, Li et al., 2007b, Hussein et al., 2008, Nagel et al., 2012, Rivera, 2008, Souppart et al., 2009). There have been a few studies where ST1 was the most common ST identified in a population. In China 51% of isolates were ST1 and in Brazil 41% of isolates were ST1 (Malheiros et al., 2011, Yan et al., 2006). There have been three studies that have identified ST4 as the predominant ST with one study in Spain noting no ST3 (Dominguez-Marquez et al., 2009). A study in France identified 63% of samples as containing ST4 and in Nepal 84% belonged to ST4 (Lee et al., 2011, Poirier et al., 2011). From these studies an interesting observation was made that there is a high prevalence of ST4 in Europe and Australia but a low incidence in Africa and South America. There is little genetic variability seen within ST4 isolates and it is possible that this ST has only recently colonised humans and has not had as much time to have a wider geographical distribution (Stensvold et al., 2012b). It is also possible that a natural animal reservoir of ST4 may not be found in the geographical locations where there are few human ST4 infections. This study has expanded the current knowledge on the world-wide distribution of *Blastocystis.*

There is still much controversy over the pathogenicity of *Blastocystis.* Some studies have shown a relationship between ST and symptoms whereas others have failed to show any relationship at all (Yoshikawa et al., 2004b, Dogruman-Al et al., 2009, Bohm-Gloning et al., 1997). From our study (Roberts et al., 2013b) which looked at all clinical samples submitted to the diagnostic laboratory it was found that all patients with ST2 and ST8 had symptoms. There have been several previous studies that have shown an association with ST2 and symptoms suggesting that this could be a pathogenic ST (Ozyurt et al., 2008, Vogelberg et al., 2010, Ramirez et al., 2013). From our control group though this does not become apparent as ST2 was found in a healthy patient that noted no symptoms. ST8 has rarely been found in human studies and a link with symptoms was reported (Stensvold et al., 2008). There has been some suggestion that ST4 could be pathogenic (Thathaisong et al., 2003). In

our clinical samples only half of ST4 patients noted symptoms. However, in the irritable bowel syndrome (IBS) study conducted it was interesting that ST4 was present in the IBS group but not in the control group. From our case study on chronic *Blastocystis* infections there were also two patients with ST4 that had diarrhoea and abdominal pains. This could mean that a relationship between ST4 and symptoms could be made but more data is necessary. ST3 is the most common ST found in humans. From our study 40% of patients noted some sort of gastrointestinal disease. There was also an almost equal number of ST3 in the control group compared to the IBS group. Considering that this is the most common ST found in most epidemiological studies, it is not likely that this is a pathogenic strain. There has been a suggestion though that ST variability within ST3 and ST4 may play a role in pathogenicity and this could explain why some people with ST3 have symptoms while others do not (Hussein et al., 2008). ST6 was found in the healthy control group as well as in non-symptomatic patients suggesting that this ST is not pathogenic. The whole genome for ST7 has recently been described (Denoeud et al., 2011). Information from this may shed some light on possible genes that encode for pathogenicity but as ST7 is a rare ST found in humans and is not usually related to symptoms, it is possible that this may not be likely. Much more information is needed to be gathered on possible genes that encode for proteins involved in pathogenicity.

In this study there was no significant difference between IBS patients and the control group for the presence of *Blastocystis*. *Blastocystis* has previously been found in much higher numbers in several IBS studies compared to controls with rates of 46%, 71%, 76% and 49% in IBS patients compared to less than 20% in the control groups (Yakoob et al., 2004b, Yakoob et al., 2010b, Dogruman-Al et al., 2010, Yakoob et al., 2010a). There have been however, two other studies where there was no significant difference in the number of *Blastocystis* positive samples in the IBS group compared to the control group (Tungtrongchitr et al., 2004, Surangsrirat et al., 2010). Both of these studies however relied solely on microscopy from culture which we have previously shown is not the most sensitive method for detecting *Blastocystis*. From our study though it was apparent that there were less STs identified in the IBS group compared to the control group and ST4 was only found in

the IBS group. A study on the subtypes associated with IBS showed that there was a much higher incidence of ST1 in the IBS group than in the control group (Yakoob et al., 2010b). There was only a slightly higher number of ST1 infections in the IBS group compared to the control group in our study (25% and 14% respectively) making it difficult to comment on the role of this ST in IBS. It is possible that *Blastocystis* may not play a role in IBS but may actually be the cause of gastrointestinal disease with *Blastocystis* causing chronic symptoms similar to IBS such as diarrhoea, abdominal pains and nausea. It is important that a full microbiological work up including tests for parasites is carried out before a diagnosis of IBS is given. There is also the possibility that *Blastocystis* affects people with IBS more easily than healthy individuals due to it being able to colonise the gut of people with IBS more effectively. Host genetics may also be a contributor to susceptibility of an individual to *Blastocystis*. For example, single nucleotide polymorphisms (SNPs) for interleukin (IL)-8 and IL-10 may change an individual's susceptibility to IBS by increasing the relative risk in the development of IBS in *Blastocystis* infected people (Olivo-Diaz et al., 2012).

Blastocystis has been described since the 1990's in animals such as primates, rodents, birds, reptiles, amphibians and insects (Chen et al., 1997, Teow et al., 1991, Teow et al., 1992, Boreham and Stenzel, 1993, Yoshikawa et al., 1996). Since then a number of studies have looked at the prevalence of *Blastocystis* in animals using different techniques and up to 17 STs have been recognised (Alfellani et al., 2013b, Yoshikawa et al., 2004a, Parkar et al., 2010). This study aimed to expand on the knowledge on the ST distribution of *Blastocystis* in the animal population (Roberts et al., 2013a). In this study we collected 438 stool samples from 38 different species of animal from seven different locations and analysed them for the presence of *Blastocystis* using PCR. There were 80 (18%) positive samples from 18 different species, and nine different subtypes were identified- ST1, ST2, ST3, ST4, ST5, ST7, ST11, ST12 and ST13. This is the first report of *Blastocystis* in the eastern grey kangaroo, red kangaroo, wallaroo, snow leopard and ostrich. This study has expanded the present knowledge on the host range of *Blastocystis*. All of the ape species were positive for *Blastocystis* and were found to harbour STs that are also commonly found in humans- ST1, ST2 and ST3. There have been several previous

studies on apes which have also shown the high incidence of *Blastocystis* (Abe et al., 2002, Abe et al., 2003b). This was the second study to identify *Blastocystis* in the Asian elephant and the same ST was found in this study as was previously- ST11 (Parkar et al., 2010). This could suggest that elephants only harbour ST11. This study also highlighted the large distribution of STs found in avian species as well as pigs with ST2, ST4 and ST7 found in the avian species and ST1, ST3 and ST5 found in the pigs. This shows the large host range for these STs. There has previously been a large number of STs isolated from pigs and our results add further information to the ST distribution within this animal host (Arisue et al., 2003, Noel et al., 2003, Navarro et al., 2008). The large distribution of STs in this animal group that are also commonly found in humans suggests the possibility of zoonotic transmission. To confirm zoonotic transmission though it would be necessary to compare both human and animals sequences and confirm that they have a 100% identity match. Due to the genetic variability seen within the STs, as previously discussed, it is possible that even though the same ST might infect humans and animals, they might be quite dissimilar sequences within the group. The use of the term zoonotic transmission is loosely used in many studies and unless a full work up is done, we are still unable to comment on whether certain *Blastocystis* STs are actually zoonotic.

There were several issues that arose with the speciation of the animal samples. Though there was a high identity to certain ST sequences from GenBank, when phylogeny was completed it was obvious that these isolates were not part of these STs. In particular, the kangaroo species identified as ST4 was not placed nicely within the ST4 clade. It has since been identified that these isolates may actually belong to a new ST. It is difficult however to designate isolates to a new ST if the same PCR technique is not used for all samples. The same technique for speciation should be used to assure that there are no inconsistencies within the speciation. The use of the barcode region has shown to be the best for the designation of STs and also means that integrity can be maintained between the STs (Stensvold, 2013). To designate an isolate as a new ST though, it is suggested that the complete or almost complete SSU-rDNA sequence be used (Clark et al., 2013). The barcode region is not necessarily the best region to use if simply detection of *Blastocystis* is desired. Barcoding uses a primer pair that includes a broad eukaryote primer in combination

with a more specific *Blastocystis* primer but it is possible that other eukaryotes may be positive using this technique. This is why sequencing needs to be done on all positives to confirm they are in fact *Blastocystis.*

Due to the controversy over whether *Blastocystis* should be considered a pathogen or not, research into the efficacy of certain treatment options is limited. There have been several case studies that have reported the clearance of symptoms after treatment suggesting that treatment may be necessary in certain cases (Andiran et al., 2006, Katsarou-Katsari et al., 2008, Gupta and Parsi, 2006, Shah et al., 2012, Biedermann et al., 2002). Metronidazole is the most commonly prescribed drug for *Blastocystis* treatment and a large variation in efficacy has been reported with ranges from 0%- 100% (Moghaddam et al., 2005, Nigro et al., 2003, Stensvold et al., 2008). There have been several reports of metronidazole treatment failure- one patient with ST2 related urticaria and gastrointestinal disease (Vogelberg et al., 2010), a patient with severe intestinal symptoms associated with ST8 (Stensvold et al., 2008), and six ST3 and one ST1 infection in patients whom presented with both urticarial and gastrointestinal symptoms (Jones et al., 2009). Other antimicrobial agents which have been used to treat *Blastocystis* infection include paromomycin, nitazoxanide, iodoquinol and trimethoprim- sulfamethoxazole with varying results (Rossignol et al., 2005, Romero Cabello et al., 1997, Mirza et al., 2011a, Markell and Udkow, 1986, Moghaddam et al., 2005). This study (Roberts et al., 2014) reports treatment failure for 18 individuals identified with chronic *Blastocystis* infection. There were 11 patients initially treated with metronidazole and seven patients treated with combination therapy, all of which showed treatment failure. There were four different STs identified in this group- 1 ST1, 14 ST3, 2 ST4 and 1 ST5. It has previously been suggested that some STs might be more resistant or are more likely to fail treatment than others. In our study there were four different STs identified suggesting that any ST could result in treatment failure. In this study we report treatment failure with metronidazole involving four different *Blastocystis* STs.

It is important that the correct drug is given to stop the emergence of resistant lines. There have only been four previous studies to look at *in vitro* susceptibility patterns of *Blastocystis.* From these studies it has become apparent that metronidazole is not the most effective treatment for *Blastocystis* infection (Mirza et

al., 2011a, Mirza et al., 2011b, Dunn et al., 2012, Dhurga et al., 2012). In this study (chapter 6.2) the *in vitro* susceptibility patterns of 12 different commonly used antimicrobials (metronidazole, paromomycin, ornidazole, albendazole, ivermectin, trimethoprim- sulfamethoxazole [TMP-SMX], furazolidone, nitazoxonide, secnidazole, fluconazole, nyastatin and itraconazole) were examined against 12 clinical isolates of *Blastocystis* from four different STs (ST1, ST3, ST4 and ST8). These results show the lack of efficacy of the most common used drugs for anti-parasitic treatment including metronidazole. Metronidazole was found to only have an inhibitory effect up to the third highest concentration tested- 125µg/ml. In this study it was observed that there were much higher cell numbers seen in treated cultures with a concentration of 64µg/ml to the lowest concentration of 1µg/ml compared to the control for several of the drugs tested including metronidazole, ornidazole, secnidazole and nitazoxanide. One study suggested that *Blastocystis* produce higher numbers of viable cells by regulating the apoptotic process in response to treatment with metronidazole which is probably what was witnessed in our study (Dhurga et al., 2012). There was never a total clearance of *Blastocystis* noted at even the highest concentration for metronidazole suggesting that this drug does not have a complete effect on *Blastocystis in vitro.* The prescription of a triple drug therapy is becoming common practice by some physicians (using secnidazole, furazolidone and nitazoxanide) (Sekar and Shanthi, 2013). In this study it was found that both furazolidone and nitazoxanide had little to no effect at all on *Blastocystis*. The only drug that did have an effect was secnidazole with an efficacy noted up to a concentration of 64µg/ml. Nitazoxanide was previously shown to have high clearance rates with 97-100% reported in children (Diaz et al., 2003) but our results suggest that nitazoxanide is ineffective at inhibiting growth of *Blastocystis*. Furazolidone had little effect at 250µg/ml and no effect after the third highest concentration at 125µg/ml. It was previously stated that furazolidone has some activity against *Blastocystis* at 100µg/ml but our results do not agree with this (Mirza et al., 2011a). From our study on chronic *Blastocystis* infection and treatments there were three patients prescribed paromomycin after initial metronidazole treatment failure. All of the patients noted clearance of symptoms and parasitological work-up showed that *Blastocystis* infection had been cleared. Paromomycin has previously

shown to have efficacy rates up to 100% for the treatment of *Blastocystis* (Vogelberg et al., 2010, Valsecchi et al., 2004, Armentia et al., 1993). An *in vitro* study contradicts these results however by showing paromomycin to be completely ineffective (Mirza et al., 2011a). Our study concurs with this study in that paromomycin did not have a lethal dose even at the highest concentrations *in vitro*. Paromomycin was the only drug however where the lower concentrations did not outgrow the control but there was also high numbers of cells seen even at the highest concentration.

A recent review on antimicrobial treatments for *Blastocystis* suggested that TMP-SMX is a good alternative to metronidazole with less side effects and being more cost effective (Sekar and Shanthi, 2013). Our study shows the high efficacy of TMP- SMX on inhibiting *in vitro* growth and agrees with this previous study that this is a good treatment option. There was a suggestion that some STs are more pathogenic than others and that some STs may be more resistant to drugs than others. One study showed that ST3 had the highest increase in cell numbers after treatment with metronidazole suggesting this ST is more pathogenic and resistant to treatment (Dhurga et al., 2012). One study compared ST4 and ST7 and showed that ST7 was resistant to metronidazole and sensitive to emetine, while ST4 was sensitive to metronidazole and resistant to emetine (Mirza et al., 2011a). Another study showed the inability of both metronidazole and TMP-SMX to clear ST1, ST3, ST4 and ST6 (Nagel et al., 2012). In this study no major variations in susceptibility between the STs was seen but noted that there were minor differences even within each ST group. From all these studies it is seen that there are discrepancies in the literature about the best treatment option for *Blastocystis*. Different techniques used also allow for an inconsistency for interpretation. A simple susceptibility test that could be used in diagnostic laboratories would be highly beneficial to assure correct treatments are given and to allow for the best patient care where patients don't have to go through a trial and error treatment regime.

In conclusion, from all these studies it is obvious that there is still much research needed to be done on *Blastocystis* and shows that more attention needs to be paid to this organism in a clinical setting. The results presented in this thesis show that PCR should be the preferred technique for the diagnosis of *Blastocystis* in

clinical laboratories. This research has shown that there is a high incidence of *Blastocystis* in the Sydney population in comparison to all other enteric parasites. Subtype 3 is the predominant ST found in this population which is comparable to most other world- wide epidemiological studies that have been conducted. This study expanded the knowledge on animal hosts for *Blastocystis* and described the presence of *Blastocystis* in five new animal species. This study highlights the difficulty with ST designation and shows where problems may lie when it comes to the allocation of new STs. This study highlights the problems associated with calling *Blastocystis* a pathogen. While some people have severe and chronic symptoms when infected with *Blastocystis*, other people are completely asymptomatic. This makes the treatment of *Blastocystis* difficult and suggests that treatment should be based on a case-by-case basis. When treatment is prescribed this study highlighted how metronidazole, which is most often the first line treatment suggested, should not be used. This study documented the high treatment failure for metronidazole seen in both *in vivo* and *in vitro* studies. Finally, this research evaluated the efficacy of several other commonly used antimicrobials and suggests that trimethroprim-sulfamethoxazole may be a better first line treatment option for the treatment of *Blastocystis*. These conclusions lead me to believe that this PhD thesis has contributed significantly to clinical microbiology and will lead to changes in clinical practice. The knowledge described here will also provide a solid foundation for future research into *Blastocystis* as a pathogen. This research has shown how *Blastocystis* should be considered a pathogen when symptoms are present and clinicians should come to recognise this. Treatment should be given to chronic *Blastocystis* sufferers and this research has provided the basis for further studies into treatment options. From this research it is apparent that therapeutic guidelines for *Blastocystis* treatment should be reviewed.

Chapter 8 References

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Appendix

Appendix 1. IBS patient information sheet.

All IBS patients that were included in the study were given an information sheet to read and were able to contact Tamalee Roberts if they had any questions.

St. Vincent's Hospital

Department of Microbiology

PATIENT INFORMATION SHEET- IBS STUDY Date: October 2011

Prevalence, Genotyping and Clinical Relevance of *Blastocystis* in Irritable Bowel Syndrome suffering patients

You are invited to take part in a patient research study. It is still unclear as to what causes irritable bowel syndrome (IBS). The enteric parasite *Blastocystis* has been found in higher numbers in IBS suffering people and it has been hypothesised that a certain subtype of *Blastocystis* could be a cause of IBS. *Blastocystis* causes symptoms similar to IBS including diarrhoea, vomiting, abdominal cramps and flatulence. The study is being conducted by the Microbiology department, SydPath, St. Vincent's Hospital in conjunction with the Department of Gastroenterology

AIM: The aim of this study is to determine what role, if any, *Blastocystis sp.* plays in causing irritable bowel syndrome and the rates of infection in the IBS population.

YOUR PARTICIPATION: Your participation is voluntary. If you do agree to be a part of this study, you will be asked to fill in a questionnaire regarding your symptoms and diagnosis. You will be asked to submit a stool sample which will then be sent to the Microbiology department for parasite examination.

OUTCOMES: All data from the study will be held by Tamalee Roberts who is the chief investigator of this study and a post-graduate student in the Microbiology department in SydPath, St. Vincent's Hospital. This data may be used in scientific presentation and for publication in medical journals. All your personal details will be made anonymous.

CONSENT: As part of the procedure for research studies, you will be asked to sign a consent form (see attached forms) that says that you are happy to be involved in this study. You will receive a copy of the consent form to take home with you. All participation is voluntary and you can withdraw consent at any time and it will have no effect on your future treatment. If you have any questions, please do not hesitate to ask your gastroenterologist or Tamalee Roberts.

The principal Researcher at your site is Tamalee Roberts. Please do not hesitate to contact Ms Roberts on (02) 8382 9209 if you have any questions.

If there are concerns or complaints regarding the way this study is being conducted, please contact the Research Office on 8382 2075 and quote HREC file reference number 09/150.

Appendix 2. IBS patient survey.

All IBS patients answered a simple survey so that relevant information could be gathered on the patients' health to be included in the study.

St. Vincent's Hospital

Department of Microbiology

PATIENT SURVEY

A study on the prevalence, genotyping and clinical relevance of *Blastocystis* in Irritable Bowel Syndrome suffering patients

The main purpose of this document is to determine your clinical history and symptoms related to Irritable Bowel Syndrome (IBS). Please try to answer all the questions and tick the correct answer for each question as applicable to you.

Date:

Study ID:

Gender: Male ☐ Female ☐

Age:

How long have you suffered from IBS:

Symptoms (please tick):

Diarrhoea ☐

Vomiting ☐

Nausea ☐

Anorexia ☐

Abdominal Cramps ☐

Flatulence ☐

Other (please specify):

History of travel before IBS symptoms started: Yes ☐ No ☐

If yes, where have you travelled to:

Have you previously been treated for IBS: Yes ☐ No ☐

If yes, what medication have you taken:

Thank you for completing this form. If you have any questions, please contact Tamalee Roberts on 8382 9209. Please return this survey with your stool sample.
Patient survey [1] [16/02/2011]

Appendix 3. IBS patient consent from.

All IBS patients were given a consent form to sign with information regarding the details of the study they were partaking in. All aspects of the study were discussed with the patient by Tamalee Roberts prior to them signing the form.

St. Vincent's Hospital

PARTICIPANT INFORMATION SHEET AND CONSENT FORM

CLINICAL RESEARCH

A study on the prevalence, genotyping and clinical relevance of Blastocystis in patients with Irritable Bowel Syndrome

Invitation

You are invited to participate in a research study into the role in which the enteric parasite *Blastocystis* plays in Irritable Bowel Syndrome.

The study is being conducted by the Department of Microbiology, SydPath, in conjunction with the Department of Gastroenterology, St. Vincent's Hospital. Tamalee Roberts from the Department of Microbiology will be the chief investigator along with Dr. Walsh and Dr. Williams from the Gastroenterology Department.

Before you decide whether or not you wish to participate in this study, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish.

1. **'What is the purpose of this study?'**
The purpose is to investigate whether the enteric parasite, *Blastocystis*, is involved in causing Irritable Bowel Syndrome.

2. **'Why have I been invited to participate in this study?'**
You are eligible to participate in this study because you have been identified as having IBS.

3. **'What if I don't want to take part in this study, or if I want to withdraw later?'**
Participation in this study is voluntary. It is completely up to you whether or not you participate. If you decide not to participate, it will not affect the treatment

you receive now or in the future. Whatever your decision, it will not affect your relationship with the staff caring for you.

If you wish to withdraw from the study once it has started, you can do so at any time without having to give a reason.

However, it may not be possible to return your samples to you or withdraw your data from the study results if these have already had your identifying details removed.

4. 'What does this study involve?'
If you agree to participate in this study, you will be asked to sign the Participant Consent Form.

This study will be conducted over 2 years

Your involvement in the study involves completion of a symptom questionnaire which will take approximately five minutes to complete. You will also be asked to provide a stool sample. A collection container will be given to you to take home and bring back to your next appointment at the clinic.

In addition, the researchers would like to have access to your medical record to obtain information relating to your irritable bowel syndrome including treatment and history of your disease.

No further follow up for this study is required after the above information/sample has been collected.

5. 'How is this study being paid for?'
The study is being funded by St. Vincent's pathology, SydPath

6. 'Are there risks to me in taking part in this study?'
All medical procedures involve some risk of injury. In addition, there may be risks associated with this study that are presently unknown or unforeseeable

7. 'Will I benefit from the study?'
This study aims to further medical knowledge and may improve future treatment of Irritable Bowel Syndrome, however it may not directly benefit you.

8. 'Will taking part in this study cost me anything, and will I be paid?
Participation in this study will not cost you anything and you will not be paid

9. 'What will happen to my stool sample after it has been used?'
Any remaining stool sample/s you provide during the study will be destroyed at the completion of the study.
10. 'How will my confidentiality be protected?'

Of the people treating you, only Dr. Williams and Dr Walsh will know whether or not you are participating in this study. Any identifiable information that is collected about you in connection with this study will remain confidential and will be disclosed only with your permission, or except as required by law. Only the researchers named above will have access to your details and results that will be held securely at the Microbiology department.

11. 'What happens with the results?'

If you give us your permission by signing the consent document, we plan to evaluate the results and possibly publish in a peer journal.

12. 'What should I do if I want to discuss this study further before I decide?'

When you have read this information, the researcher can discuss it with you and any queries you may have. If you would like to know more at any stage, please do not hesitate to contact Tamalee Roberts in the Microbiology department on (02) 83829209

13. 'Who should I contact if I have concerns about the conduct of this study?'

This study has been approved by St Vincent's Hospital HREC. Any person with concerns or complaints about the conduct of this study should contact the Research Office who is nominated to receive complaints from research participants. You should contact them on 02 8382 2075 and quote the HREC File Reference Number 09/150.

Thank you for taking the time to consider this study.

If you wish to take part in it, please sign the attached consent form.

This information sheet is for you to keep.

St. Vincent's Hospital

SYDPATH ST VINCENT'S PATHOLOGY

CONSENT FORM

A study on the prevalence, genotyping and clinical relevance of *Blastocystis* in patients with Irritable Bowel Syndrome

1. I,.....
agree to participate as a participant in the study described in the Participant Information Sheet set out above ***(or: attached to this form).***

2. I acknowledge that I have read the Participant Information Sheet, which explains why I have been selected, the aims of the study and the nature and the possible risks of the investigation, and the information sheet has been explained to me to my satisfaction.

3. Before signing this consent form, I have been given the opportunity of asking any questions relating to any possible physical and mental harm I might suffer as a result of my participation and I have received satisfactory answers.

4. I understand that I can withdraw from the study at any time without prejudice to my relationship with SydPath, St. Vincent's Hospital or Dr. Williams and Dr Walsh.

5. I agree that research data gathered from the results of the study may be published, provided that I cannot be identified.

6. I understand that if I have any questions relating to my participation in this research, I may contact Tamalee Roberts on telephone (02) 8382 9209 who will be happy to answer them.

7. I acknowledge receipt of a copy of this Consent Form and the Participant Information Sheet.

Complaints may be directed to the Research Office, 02 8382 2075

Signature of participant	Please PRINT name	Date

Signature of witness	Please PRINT name	Date

Signature of investigator	Please PRINT name	Date

A study on the prevalence, genotyping and clinical relevance of Blastocystis in patients with Irritable Bowel Syndrome

Appendix 4. IBS patient revocation of consent form. All IBS patients were given a revocation of consent form in the case that they wished to be removed from the study.

St. Vincent's Hospital

REVOCATION OF CONSENT

A study on the prevalence, genotyping and clinical relevance of *Blastocystis* in patients with Irritable Bowel Syndrome

I hereby wish to **WITHDRAW** my consent to participate in the study described above and understand that such withdrawal **WILL NOT** jeopardise any treatment or my relationship with SydPath, St. Vincent's Hospital or my medical attendants.

Signature Date

Please PRINT Name

The section for Revocation of Consent should be forwarded to Tamalee Roberts, Microbiology Department, SydPath, Level 6, St. Vincent's Hospital

A study on the prevalence, genotyping and clinical relevance of *Blastocystis* in patients with Irritable Bowel Syndrome

Patient

Information Sheet [2] [16/02/2011]

Appendix 5. Control group survey.

All people that agreed to submit a sample as part of the control group completed a survey so that knowledge of their current health could be used for the study. If people had any current gastrointestinal symptoms, their sample was not included in the study.

UNIVERSITY OF TECHNOLOGY SYDNEY

University of Technology, Sydney and St. Vincent's Hospital, Sydney

PATIENT SURVEY

A study on the prevalence, genotyping and clinical relevance of Enteric Pathogens in healthy individuals.

The main purpose of this survey is to determine your clinical history and symptoms related to gastrointestinal illness. Please try to answer all the questions and tick the correct answer for each question as applicable to you.

Today's Date: Study ID:

Gender: Male ☐ Female ☐

Age: _____

Country of Birth: _____Country of residence (last 6 months) _____

1. Have you ever been diagnosed with IBS: Yes ☐ No ☐

(If yes, please end here and DO NOT submit a stool specimen; if no continue to the next question.)

2. Have you suffered from or is suffering from any of the following symptoms in the last 4 weeks:

Symptoms (please tick all that apply):

Diarrhoea ☐ (If yes, or if you currently have diarrhoea please end here.)

Vomiting ☐

Nausea ☐

Anorexia ☐

Abdominal Cramps ☐

Flatulence ☐

Crohn's Disease ☐

Other (please specify): ☐

3a. History of travel overseas before symptoms started: Yes ☐ No ☐

3b. If yes, when and where have you travelled to:

_____

4a. Are you currently being treated for any gastrointestinal illness: Yes ☐ No ☐

4b. If yes, what medication have you taken:
_____

Please tell us if you have experienced or was exposed to any of the following situations -up to the last 2 months; (please answer all questions):

5. Work in a Child care or day care centre: Yes ☐ No ☐

6. Wear diapers or care for someone who does? Yes ☐ No ☐

7. Consumed a suspicious food (i.e. any food that you think may have made you ill; eaten at home, take-away, BBQ, party etc)? Yes ☐

8. Drink water from untreated source (such as a river/spring/lake/ pond)? Yes ☐ No ☐

9. Swim in a swimming pool or wading pool a water park? Yes ☐ No ☐

10. Visited a zoo or petted farm, wild, or domestic animals Yes ☐ No ☐

Thank you for completing this form. If you have any questions, please contact Tamalee Roberts on 8382 9209. Please return this survey with your stool sample.