In Vitro Antimicrobial Susceptibility Patterns of Blastocystis

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Blastocystis is the most common human enteric protist with controversial clinical significance. Metronidazole is considered a first-line treatment for Blastocystis infection; however, there has been increasing evidence for 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 4 common Blastocystis subtypes (ST1, ST3, ST4, and ST8) against 12 commonly used antimicrobials (metronidazole, paromomycin, ornidazole, albendazole, ivermectin, trimethoprim-sulfamethoxazole [TMP-SMX], furazolidone, nitazoxanide, secnidazole, fluconazole, nystatin, and itraconazole) at 10 different concentrations in vitro. It was found that each subtype 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.

Blastocystis is the most common enteric protist found in humans, with rates of infection ranging from 2% to 100% in developed and developing countries (1, 2). There have been 17 subtypes (STs) identified from humans and animals, with ST1 to ST9 identified in humans (3–5). ST3 is the predominant subtype found in most human studies (6–8). There have been numerous studies that have highlighted the clinical relevance of Blastocystis, and an association between subtype and symptoms has been made (9–12). 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 (13). Most case studies report first-line treatment with metronidazole and found various rates of efficacy with ranges of 0% to 100% (10, 14–16). Other antimicrobials that were used to treat Blastocystis infection included iodoquinol, ketoconazole, nitazoxanide, paromomycin, tinidazole, and trimethoprim-sulfamethoxazole (TMP-SMX), all with varied results (17–21). 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 (22–25). In this study, the in vitro susceptibility patterns of 12 different commonly used antiparasitics and antimicrobials (metronidazole, paromomycin, ornidazole, albendazole, ivermectin, trimethoprim-sulfamethoxazole, furazolidone, nitazoxanide, secnidazole, fluconazole, nystatin, and itraconazole) were examined against 12 clinical isolates of Blastocystis from four different subtypes (ST1, ST3, ST4, and ST8) run in triplicate. These results show the lack of efficacy of the most commonly used drugs for antiparasitic treatment, including metronidazole. This study shows other possible treatment options, including trimethoprim-sulfamethoxazole and ivermectin.

MATERIALS AND METHODS

Blastocystis cultures. Twelve Blastocystis isolates from stool samples submitted to St. Vincent’s Hospital Microbiology Department were used for the study. Each patient had a history of gastrointestinal symptoms, including diarrhea and cramps, but had no previous treatment for Blastocystis infection. Samples were identified as positive for Blastocystis by microscopy of a permanent iron hematoxylin stain and confirmed by PCR using a previously published method (26). For culture purposes, 10 mg of fresh sample was inoculated into a diphasic xenic dorset egg slope (Oxoid) using a previously published method (27). Xenic cultures were maintained by passaging every 4 days in the same medium and were incubated at 35°C.

Blastocystis subtyping. DNA was extracted from Blastocystis cultures using the Bioline Isolate fecal DNA kit as per the manufacturer’s instructions and was submitted to PCR for the detection of Blastocystis sp. using a previously described method (26). 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 each direction. Reads were assembled into a consensus. The small subunit (SSU)-ribosomal DNA (rDNA) sequences were then compared to those available in the GenBank database using the BLASTN program run on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST).

Antimicrobial susceptibility testing. The following agents were used for susceptibility testing: metronidazole, paromomycin, ornidazole, albendazole, ivermectin, trimethoprim-sulfamethoxazole (TMP-SMX), furazolidone, nitazoxanide, secnidazole, fluconazole, nystatin, and itraconazole. Metronidazole (Pfizer, NSW, Australia) in liquid phosphate-buffered saline (PBS) to cover a concentration range of 1,000 μg/ml to 1 μg/ml was used as a stock solution and diluted with phosphate-buffered saline (PBS) to cover a concentration range of 1,000 μg/ml to 1 μg/ml by doubling dilution. Ornidazole (provided by J. Harkness) was prepared as a stock solution at 5 mg/ml was used as a stock solution and diluted with phosphate-buffered saline (PBS) to cover a concentration range of 1,000 μg/ml to 1 μg/ml by doubling dilution.
Upcroft, Queensland Institute of Medical Research) in powder form was dissolved in 50% ethanol to 5 mg/ml and diluted as above. Paromomycin sulfate, 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 5 mg/ml and diluted in the same manner as above. Albendazole tablets (GlaxoSmithKine, VIC, Australia) were dissolved in glacial acetic acid to 5 mg/ml and diluted as above. Ivermectin tablets (Merck Sharp & Dohme Pty Ltd., NSW, Australia) were dissolved in methanol to 5 mg/ml and diluted as above. TMP-SMX in liquid form was diluted to 40 mg/ml sulfamethoxazole and 8 mg/ml trimethoprim with PBS and then diluted as above. Nystatin (Omega- pharm, VIC, Australia) in liquid form was diluted to 5 mg/ml in PBS and diluted as above. One hundred microliters of the respective antibacterial dilutions was inoculated into 96-well microtiter plates, and 100 μl of Blastocystis culture was added to each dilution. A control containing 100 μl of 10% ethanol was performed for all drugs in powder form to rule out any inhibitory effects of the solvent on Blastocystis. One hundred microliters of PBS buffer was used for the metronidazole control, 100 μl of diluted glacial acetic acid was used for the albendazole control, and 100 μl of diluted methanol was used for the ivermectin control. All drug testing was performed in triplicate. Microtiter plates were then incubated in anaerobic conditions at 35°C.

Cell concentration and viability were determined quantitatively by the trypan blue dye exclusion method (28) by counting each dilution using Kova slides viewed under phase-contrast microscopy and then counted every day for 4 days. As numbers of Blastocystis cells in negative controls decline after 92 h, susceptibility testing with each compound was performed for only 4 days. The MIC was determined by the concentration of drug where there were lower numbers of growth compared to the control, if there was a change in the bacteria before and after antibiotic treatment, this would be able to confirm conclusively if there was a change in the bacteria before and after antibiotic treatment.

Characterization of bacteria present in xenic cultures. All samples were tested for enteric bacterial pathogens in the clinical laboratory prior to parasite culture. During parasite culture, the bacterial flora present in each sample was characterized 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 CO₂ at 35°C for 24 to 48 h while the anaerobic plates were incubated for 48 h under anaerobic conditions using an AnaXomat Mark II system (Mart Microbiology) with the following gas composition: 0.16% O₂, 5% H₂, 10% CO₂, and 85% N₂. All bacteria grown on agar plates were identified to species level using routine bacteriological procedures, including biochemical testing and identification using a Bruker microflex matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer.

RESULTS

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

Antimicrobial testing. There was a progressive reduction in the number of Blastocystis cells seen during the 4 days at all concentrations, which were comparable to the control. There was variation seen between each isolate even within the subtypes. The MIC values for the compounds were 250 μg/ml to 64 μg/ml (metronidazole), 125 μg/ml to 32 μg/ml (ornidazole), 64 μg/ml to 16 μg/ml (secnidazole), 1 μg/ml (paromomycin), 64 μg/ml to 16 μg/ml (albendazole), 250 μg/ml to 125 μg/ml (furazolidone), 500 μg/ml to 250 μg/ml (nitazoxanide), 500 μg/ml to 250 μg/ml (fluconazole), 500 μg/ml to 250 μg/ml (itraconazole), and 250 μg/ml (nystatin). Due to time and space constraints and the obvious lack of efficacy after the 2nd concentration, the antifungals were only tested over 3 days for four different concentrations. Ivermectin had an MLC of 64 μg/ml to 32 μg/ml, and TMP-SMX had an MLC of 100/500 μg/ml to 12/64 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. Paromomycin was the only drug observed where the lower concentrations did not outgrow the control. Figures 1 to 4 show the cell counts versus drug concentrations for day 1 for metronidazole, paromomycin, trimethoprim-sulfamethoxazole, and ivermectin. Due to the large amount of data received from this study, all other results are presented in the supplemental material.

Subtype dependency. Slight differences were noted between subtypes and responses to drug concentration as stated below.

Statistical analysis. In Fig. 1 to 4 (see Fig. S5 to S13 in the supplemental material), the mean number of counts is indicated by a symbol, and the lines represent confidence intervals for the mean cell counts. We observe that there are large differences in the reaction to the different concentrations of each agent between the subtypes. For example, TMP-SMX is more effective for ST3 than the other subtypes at lower concentrations, but albendazole is more effective for ST1 and ST4 than ST3. For most agents, the cell counts after 1 day are very low for high concentrations of the agent, and differences between subtypes cease to exist. This interaction between agent, concentration, and subtype on cell count is confirmed using a generalized linear model with the three-way interaction between these variables identified as statistically significant (P < 0.0001).

Bacteria present in cultures. There were no enteric bacterial pathogens identified from clinical laboratory testing. The bacteria isolated from the cultures were as follows: Escherichia coli, Enterococcus faecalis, Clostridium butyricum, Prevotella spp., and Citrobacter freundii. There appeared to be no effect on the bacteria present in the cultures before and after treatment and within the subtypes from the bacteria that were identified from culturing. It is likely that there are numerous amounts of gut bacteria that we were unable to identify through routine microbiological testing, and only 165 rRNA testing would be able to confirm conclusively if there was a change in the bacteria before and after antibiotic treatment.

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 (29). 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 antiparasitic agent as shown in this study.

Metronidazole was found to have an inhibitory effect only up to the third highest concentration tested of 125 μg/ml as
shown in Fig. 1. Metronidazole is the most frequently prescribed antibiotic for Blastocystis treatment, with high rates of clearance reported by some clinical studies (15, 30, 31). Metronidazole resistance in Blastocystis has been reported since 1996 (32), and it was suggested that this may 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 64 μg/ml to the lowest concentration of 1 μg/ml than those of the control except for ST8. 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 (25). This indicates that if metronidazole is used, it should be used at the highest concentration possible. This is not ideal, though, with many possible side effects related to metronidazole treatment, such as nausea and vomiting. Also, there was never a total clearance of Blastocystis cells 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 the treatment of Blastocystis.

Ornidazole was shown to be highly effective against other enteric protists, including Dientamoeba fragilis (33). Blastocystis is commonly found in conjunction with D. fragilis in stool samples from patients, and a drug therapy that cleared the two parasites would be beneficial to patients. In this study, ornidazole only had an inhibitory effect up to the third highest concentration at 125 μg/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) (34). 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 up to a concentration of 64 μg/ml, but then, like metronidazole, there was an 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 (35), and this may be an option at the highest concentration for Blastocystis. Nitazoxanide was previously shown to have high clearance rates against Blastocystis in children, with 97% to 100% efficacy reported (36). 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 little effect on Blastocystis even at the highest concentration of 500 μg/ml. Furazolidone had little effect at 250 μg/ml and no effect after the third highest concentration at 125 μg/ml. Furazolidone had little effect at 250 μg/ml and no effect after the third highest concentration at 125 μg/ml. It was previously stated that furazolidone has some activity against Blastocystis at 100 μg/ml, but our results do not agree with this (22). The use of a triple therapy using drugs that possess little antiparasitic activity on Blastocystis 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.
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 (19, 29, 37, 38). An in vitro study contradicts these by showing paromomycin to be completely ineffective (22). Our study agrees with Mirza et al. (22) in that paromomycin did not have a lethal effect even at the highest concentrations as shown in Fig. 2. Paromomycin was the only drug where the lower concentrations did not outgrow the control, but there were 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 fewer side effects and is more cost-effective (34). The review states that it is not known if TMP-SMX has a direct effect on Blastocystis or on the gut bacteria which are 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, but this is not conclusive, as we were not able to identify all the bacteria that might be present in these samples without thorough 16S rRNA testing, which we were not able to complete. These results are just based on the bacteria that were cultured by routine microbiology testing. TMP-SMX was seen to be highly effective up to a concentration of 500/100 μg/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 500/100 μg/ml as shown in Fig. 3. TMP-SMX was shown to have high clearance rates in previous clinical studies (10, 39) and was also shown to have a high efficacy in a previous in vitro study (22). The weight of evidence indicates that TMP-SMX should be the first-line treatment for Blastocystis infection due to its having a higher efficacy than metronidazole. It also has fewer side effects on patients.

Ivermectin and albendazole are commonly used antihelminth treatments. Neither of these drugs has previously been tested in vitro against Blastocystis. In this study, it was found that albendazole had a lethal concentration up to 250 μg/ml and ivermectin had a lethal concentration up to 125 μg/ml (Fig. 4), suggesting that taken in high doses these drugs are an option for treatment.

In this study, we tried to test a wide variety of drugs to see if any had an effect on killing Blastocystis. The three antifungal drugs used in this study (fluconazole, nystatin, and itraconazole) had little to no effect after the highest concentration of 500 μg/ml, showing that these are not good options for Blastocystis treatment.

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 an ST was shown, however, in one previous study (22). This variation illustrates how difficult it may be to comment on...
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, but that was not seen in this current study (25). Another 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 (22). Another study showed the inability of metronidazole and TMP-SMX to clear ST1, ST3, ST4, and ST6 (40). In this study, we noted that there is a slight variation in the efficacy of different antibiotics against STs as shown where TMP-SMX is more effective against ST3 and albendazole is more effective against ST1 and ST4 over the other STs. We also noted that there were minor differences even within each ST. From these results, we cannot conclusively say that any one ST is more resistant than the others, but there is a statistically significant interaction between ST, cell count, and concentration of drug that may play a role in Blastocystis treatment failure, but further studies are needed. Intrasubtype differences shown by the alleles present may also play a role in the different reactions to drugs. Unfortunately, for this study, we were unable to identify the different alleles in the isolates, but this is something to consider for further testing. The website http://pubmlst.org/blastocystis/ is able to classify isolates into STs and find alleles present for each ST.

The draft genome from the NandII ST1 (unpublished) isolate and the full genome for ST7 (41) 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 appear 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 18.8 Mb, respectively). The difference in genomes may mean that a drug that may work in one ST may not have any effect on another ST. 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 as 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 axenize Blastocystis cultures. One study showed the role mitochondrion-like organelles play in the reduction of ferredoxins 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 (42, 43).

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

In conclusion, this study shows that metronidazole should not be used as the 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 is no significant difference between STs in response to treatment. From the results presented here and from previous studies, we recommend the use of TMP-
SMX as a first-line treatment, as it appears to be the most effective at promoting *Blastocystis* clearance.

REFERENCES


