In Vitro Antimicrobial Susceptibility patterns of *Blastocystis*

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Running Head: *Blastocystis* susceptibility testing

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ABSTRACT

Blastocystis is the most 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, nystatin and itraconazole) at 10 different concentrations *in vitro*. 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.

INTRODUCTION

Blastocystis is the most common enteric protist found in humans with rates of infection ranging from 2-100% in developed and developing countries (1, 2). There have been 17 subtypes (ST) identified from humans and animals with ST1- 9 being 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 have found varying rates of efficacy with ranges of 0% to 100% (10, 14-16). Other antimicrobials which have been used to treat *Blastocystis* infection include iodoquinol, ketoconazole, nitazoxanide, paromomycin, tinidazole and trimethoprim-sulfamethoxazole all with varying 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, nitazoxonide, 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 common 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. All patients had a history of gastrointestinal symptoms including diarrhoea and cramps but had no previous treatment for *Blastocystis.* Samples were identified as positive for *Blastocystis* by microscopy of a permanent Iron Haematoxylin stain and confirmed by PCR using a previously published

method (26). For culture purposes 10mg 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 four days in the same media and incubated at 35˚C.

***Blastocystis* subtyping-** DNA was extracted from *Blastocystis* cultures using the Bioline Isolate fecal DNA kit as per manufacturer's instructions, and were 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 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).

Antimicrobial susceptibility testing- The following agents were used for susceptibility testing: metronidazole, paromomycin, ornidazole, albendazole, ivermectin, trimethoprim- sulfamethoxazole (TMP-SMX), furazolidone, nitazoxonide, secnidazole, fluconazole, nystatin 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 1000µg/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 (28) 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.

Statistical anaylsis- Statistical analysis was performed in R version 3.1.0 with graphics constructed using the *ggplot2* package, Poisson regression fitted using the *glm* function and likelihood ratio testing performed using the *lmtest* package. In the Poisson regression model, concentration is nested within condition. Confidence intervals in Figure(s) 1- 4 (and supplementary file Fig. 5- 13) are obtained using bootstrapping.

Characterisation 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 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 grown on agar plates were identified to species level using routine bacteriological procedures including biochemical testing and identification using the Bruker microflex MALDI-TOF.

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 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 nystatin 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. Fig. 1-4 show the cell counts vs concentration of drug for day 1 for metronidazole, paromomycin, trimethroprim- sulphamethoxazole and ivermectin. Due to the large amount of data received from this study, all other results are presented in a supplementary file.

Subtype dependency- slight differences were noted between subtypes and response to drug concentration as stated below.

Statistical analysis- In Fig. 1- 4 (and the supplementary files Fig. 5-13) 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 one 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 generalised linear model, with the three-way interaction between these variables identified as statistically significant (p-value<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 butyricium, Provotella 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 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 16s 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 125µg/ml as shown in Fig. 1. Metronidazole is the most frequently prescribed antibiotic for *Blastocystis* treatment with high rates of clearance being reported from some clinical studies (15, 30, 31). Metronidazole resistance in *Blastocystis* has been reported since 1996 (32) 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 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 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 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 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) (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 64µg/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 could be an option at the highest concentration for *Blastocystis*. Nitazoxanide was previously shown to have high clearance rates against *Blastocystis* in children with 97-100% 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 500µg/ml. Furazolidone had little effect at 250µg/ml and no effect after the

226 third highest concentration at 125µg/ml. It was previously stated that furazolidone has some
227 activity against *Blastocystis* at 100µg/ml but our results do not agree with this (22). The use
228 of a triple therapy using drugs that possess little anti-parasitic activity on *Blastocystis* is a
229 practice not to be encouraged and can have serious consequences. An overload of antibiotics
230 can have a detrimental effect on the patient causing sickness. Another consequence of the
231 unnecessary use of drugs is the development of drug resistance within the microbial gut flora
232 that may have other consequences for the patient.

233 Paromomycin is currently one of the recommended treatment options by the Centre
234 for Disease Control (CDC) and the Australian Therapeutic Guidelines for several enteric
235 parasites including *Blastocystis*. There have been several case studies that have shown the
236 effectiveness of paromomycin (19, 29, 37, 38). An *in vitro* study contradicts these by
237 showing paromomycin to be completely ineffective (22). Our study agrees with Mirza et al
238 (2011) in that paromomycin did not have a lethal effect even at the highest concentrations as
239 shown in Fig. 2. Paromomycin was the only drug where the lower concentrations did not
240 outgrow the control but there was also high numbers of cells seen even at the highest
241 concentration. Paromomycin is a poorly absorbed aminoglycoside and from this study and
242 the previous *in vitro* study it cannot be recommended as a suitable treatment.

243 A recent review on antimicrobial treatments for *Blastocystis* suggested that TMP-
244 SMX is a good alternative to metronidazole with less side effects and being more cost
245 effective (34). It states that it is not known if TMP-SMX has a direct effect on the
246 *Blastocystis* or on the gut bacteria which is essential for *Blastocystis* survival. In this study we
247 examined the bacteria present before and after treatment from these cultures and found that
248 there was no difference in the bacteria present at the different concentrations which suggests
249 that the death of *Blastocystis* was not due to the removal of the bacteria, but this is not
250 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 are able to be cultured by routine microbiology testing. TMP-SMX was seen to be highly effective up to a concentration of 500µg/ml/100µg/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 500µg/ml/100µg/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 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 albendazole had a lethal concentration up to 250µg/ml and ivermetcin up to 125µg/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 anti-fungal drugs used in this study (fluconazole, nystatin and itraconazole) had little to no effect after the highest concentration of 500µg/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 a ST was shown however in one previous study (22). 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

276 was a suggestion that some STs are more pathogenic than others and that some STs may be
277 more resistant to drugs than others. One study showed that ST3 had the highest increase in
278 cell numbers after treatment with metronidazole suggesting this ST is more pathogenic and
279 resistant to treatment but that was not seen in this current study (25). Another study compared
280 ST4 and ST7 and showed that ST7 was resistant to metronidazole and sensitive to emetine,
281 while ST4 was sensitive to metronidazole and resistant to emetine (22). Another study
282 showed the inability of both metronidazole and TMP-SMX to clear ST1, ST3, ST4 and ST6
283 (40). In this study we noted that there is a slight variation in the efficacy of different
284 antibiotics against STs as shown where TMP-SMX is more effective against ST3 and
285 albendazole is more effective against ST1 and ST4 over the other STs. We also noted that
286 there were minor differences even within each ST. From these results we cannot conclusively
287 say that any one ST is more resistant than the others but there is a statistically significant
288 interaction between ST, cell count and concentration of drug that may play a role in
289 *Blastocystis* treatment failure but further studies are needed. Intra-subtype differences shown
290 by the alleles present may also play a role in the different reactions to drugs. Unfortunately
291 for this study we were unable to identify the different alleles in the isolates but this is
292 something to consider for further testing. The website http://pubmlst.org/blastocystis/ is able
293 to designate isolates in to STs and find alleles present for each ST.

294 The draft genome from the NandII ST1 (unpublished) isolate and the full genome for
295 ST7 (41) have been described. The information from these genomes may be useful for
296 developing new drug therapies by identifying genes that may be involved in drug absorption
297 pathways. There appears to be quite a lot of genetic differences between the ST1 and ST7
298 genomes with a higher GC% content in ST1 but also ST1 has a substantially smaller genome
299 than ST7 (16.4 Mb and 18.8Mb respectively). The difference in genomes may mean that a
300 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 (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.

CONCLUSION

This study shows that metronidazole should not be used 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 is no significant 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.

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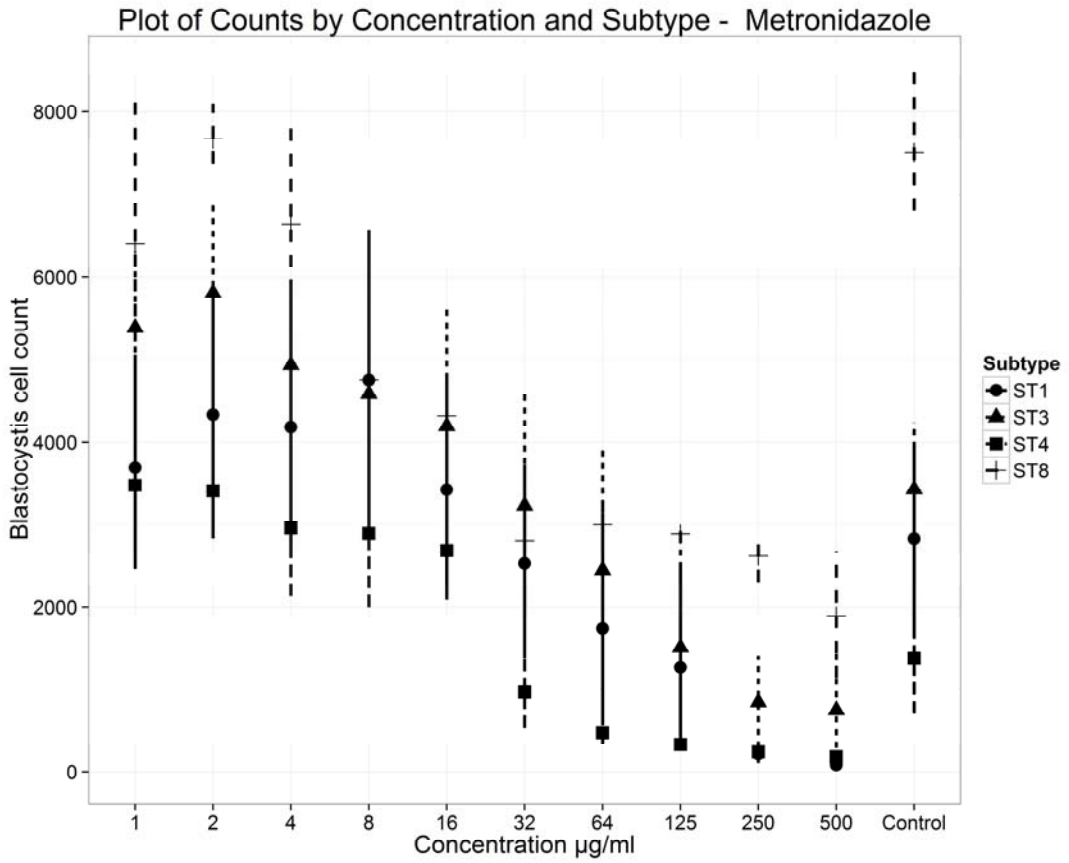

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448 Fig. 1 *Blastocystis* cell counts at different concentrations of Metronidazole on Day 1. Mean
449 cell counts are indicated by a symbol and the lines represent confidence intervals for the
450 mean cell counts for each of the different subtypes.

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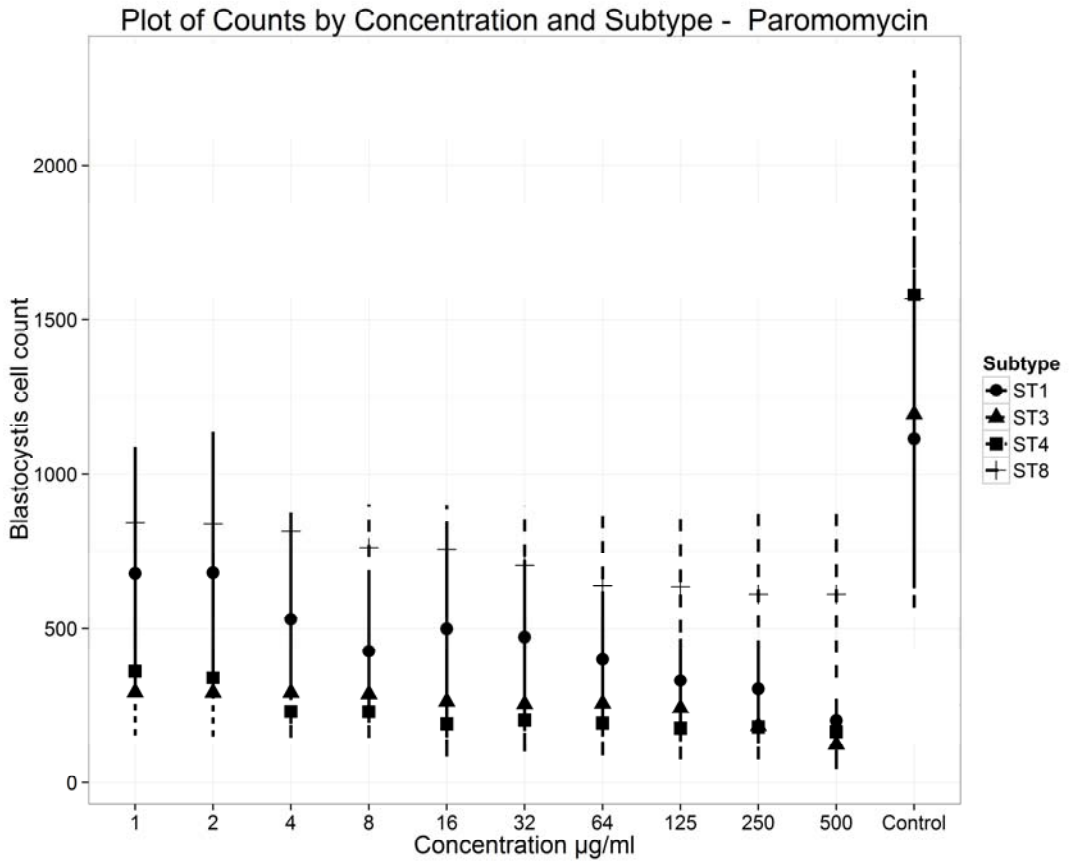

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454 Fig. 2 *Blastocystis* cell counts at different concentrations of Paromomycin on Day 1. Mean
455 cell counts are indicated by a symbol and the lines represent confidence intervals for the
456 mean cell counts for each of the different subtypes.

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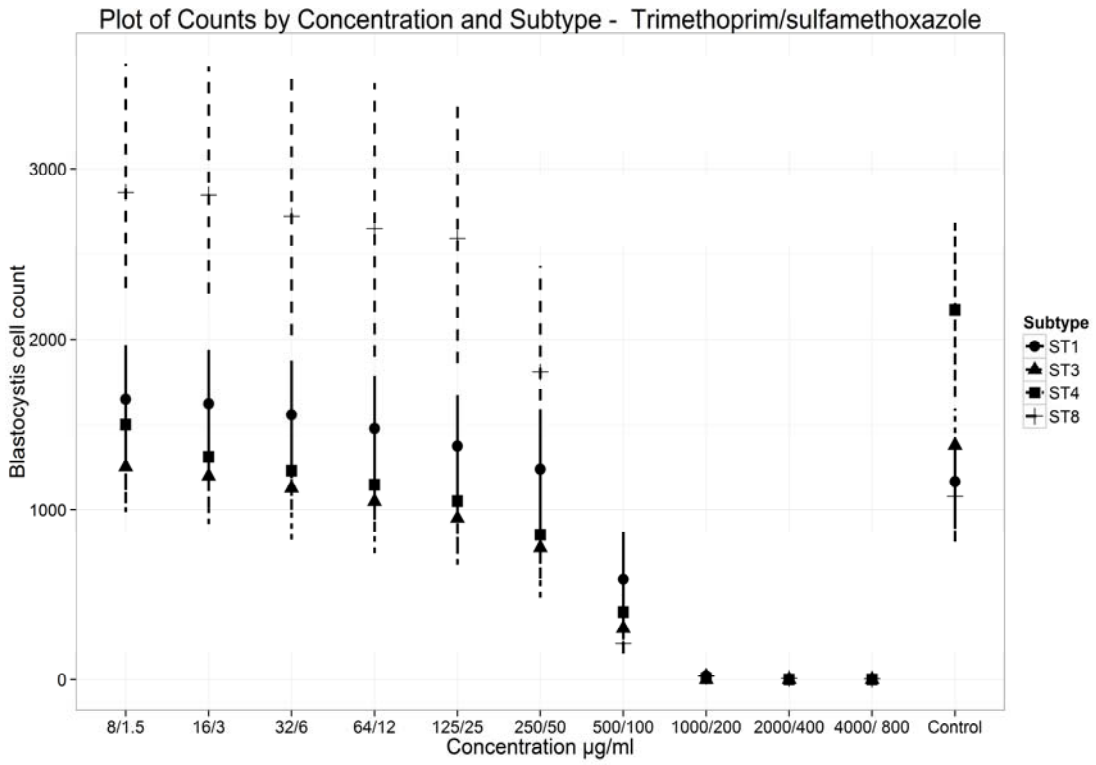

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463 Fig. 3. *Blastocystis* cell counts at different concentrations of Trimethoprim-
464 Sulfamethoxazole on Day 1. Mean cell counts are indicated by a symbol and the lines
465 represent confidence intervals for the mean cell counts for each of the different subtypes.

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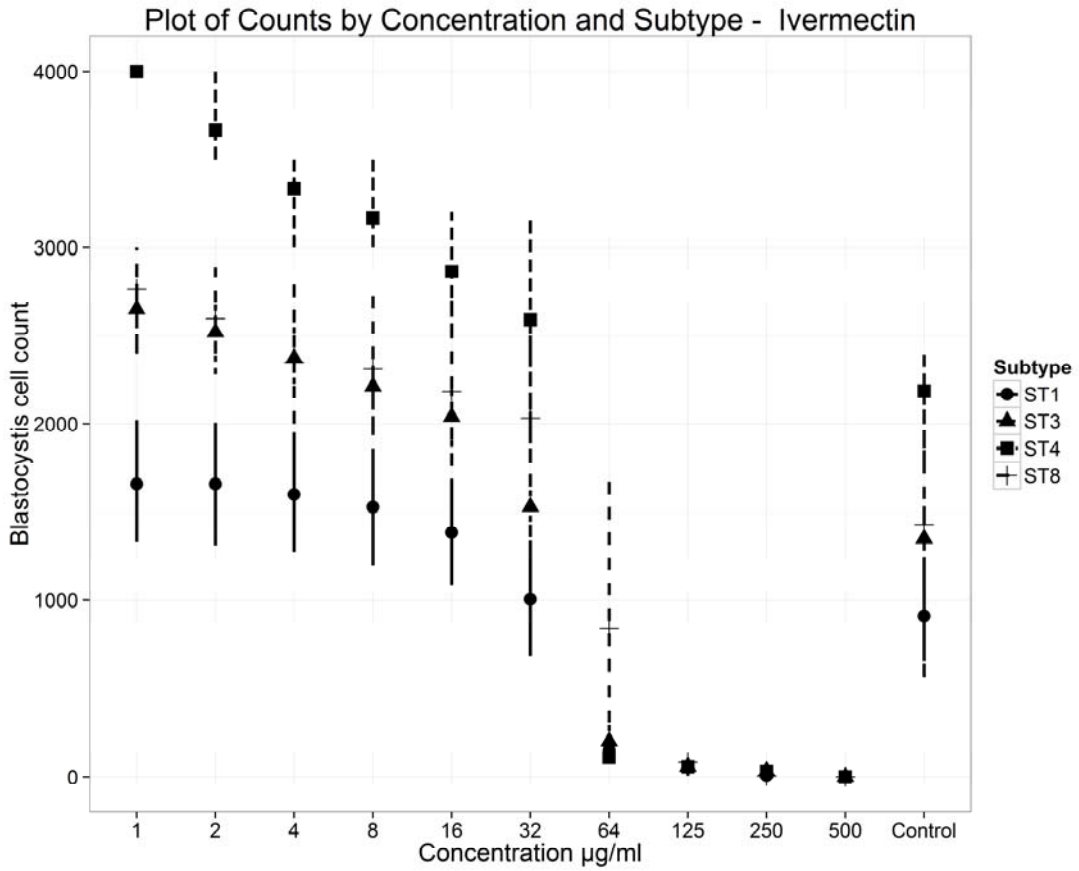

Fig. 4. *Blastocystis* cell counts at different concentrations of ivermectin on Day 1. Mean cell counts are indicated by a symbol and the lines represent confidence intervals for the mean cell counts for each of the different subtypes.