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Development of Human *Blastocystis* ST3 and ST1 Multiplex Detection Primers from Unique SSU rRNA Gene

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Abstract: *Blastocystis*, a single-celled eukaryote is a relatively common parasite that is found in the environment. Upon making contact with a human or animal host, it thrives in the lower gastrointestinal tract and spreads through the host's stool. From the 17 recorded Subtypes (STs), ST1 and ST3 are most commonly found in human stool samples. In addition, these two subtype are widespread in Southeast Asian countries. Medical practices at large still utilize old-school methods for the detection of these subtypes despite their limitations. Therefore, this study aims to develop multiplex PCR primers for a more efficient detection of *Blastocystis* ST1 and ST3. The primers were designed for each STs using PerlPrimer and validated in silico using Oligo analyzer. Reference sequences were acquired from the NCBI database. PCR and gel electrophoresis were carried out, whereby the specificity of the primers were tested using non-ST1 and ST3 subtypes as well as a few common non-*Blastocystis* pathogens. A sensitivity test was also carried out through 10-fold dilutions of the ST1 and ST3 DNA samples. The detection primers were successfully tested, yielding target band sizes of 138 and 233 bp for ST1 and ST3, respectively. Specificity tests have confirmed that the target subtypes were detected without any false positives from non-enteric, non-*Blastocystis* and non-ST1 and non-ST3 variants. Primer dimers and ghosting were also ruled out from the findings. The sensitivity test has confirmed a clear detection with as low as 7 ng/μL of extracted ST1 DNA and 70 ng/μL for ST3. The multiplex PCR primers developed in this study has the potential to reduce the turnaround time, cost and labor in the detection of these human parasites. Furthermore, its use can be extended to detection of possible sources of infection, e.g., water sources, soil and even animals such as poultry and livestock. The use of these primers can greatly aid in the containment and eradication of any ongoing outbreaks as well as the prevention of reinfection due to difficulties in identifying the root cause of an epidemic.

Key words: Blastocysytis, detection, multiplex, PCR, subtype, enteric, stool

INTRODUCTION

The genus *Blastocystis* is a group of single-celled eukaryotic parasites known to infect the human intestinal tract (Stenzel and Boreham, 1996). These protozoans are transmitted through waterborne routes, typically as a result of contamination, poor hygiene and a lack of proper water-treatment (Lee *et al*., 2012). The life cycle of these parasites require that they be transmitted while in their cysts form through an infected host's fecal matter (Zierdt, 1973). Although, zoonotic infections are possible, it is also relatively common for transmission to occur from human to human, generally in rural areas. Various studies have been carried out to confirm the prevalence of these parasites in human hosts from a number of Malaysian aboriginal tribes (Anuar *et al*., 2013; Sinniah *et al*., 2012, Al-Delaimy *et al*., 2014). Areas with an overcrowded population and a low socio-economic status are also more likely to be prone to contamination and repeated reinfection. The lack of proper detection, treatment and prevention measures has resulted in difficulties in completely removing these parasites from the population.

Blastocystis sp. exists in four major forms; vacuolar, granular, cyst and ameboid. Typically, under laboratory culture conditions, these parasites have been observed to exist in vacuolar form, although, the latter have also been reported to be prevalent in patients. Most commonly,

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Blastocystis in the ameboid form has been identified from patients exhibiting acute gastrointestinal syndromes. However, due to their irregular morphology, they are easily mistaken for macrophages and neutrophils and are missed during microscopic assays (Boreham and Stenzel, 1993).

Infections by *Blastocystis* sp. are usually overlooked during diagnosis due to their nondescript signs and symptoms. Clinical consequences following infection includes abdominal pains, diarrhea, nausea, vomiting, flatulence, dizziness and even weight-loss. However, carriers of the parasite may not exhibit any signs or symptoms (Sheehan *et al*., 1986).

For years, medical facilities have relied on the old-school detection methods such as trichrome staining, typically following formalin-ether sedimentation. Due to the pleiomorphic nature of the parasite's morphology, it is often confused with fat globules or other unrelated organisms such as yeast, *Cyclospora* sp. (Thompson and Smith, 2011, Ahmed and Karanis, 2018).

The genetic diversity of the *Blastocystis* had led to their categorization into multiple Subtypes (STs) according to the phylogenetic relationship of their Small Subunit rRNA gene (SSU rRNA) (Vassalos *et al*., 2010). To date, 17 STs have been classified originating from various avian and mammalian hosts. However, isolates from human origins are limited to subtypes 1-9 (Stensvold *et al*., 2007). Furthermore, only subtypes 1 and 3 have been determined to be commonly occurring in human hosts in Malaysia.

The use of PCR technology has been lauded as the gold standard for many detection protocols, due to its sensitivity, accuracy, efficiency and cost-effectiveness. Most importantly, it requires a relatively minimal skill level as there is no need for an extensive knowledge on the various morphologies of the parasite to ensure accurate diagnosis. In the recent years, development of molecular detection methods such as PCR for these parasites have gain traction where several subtype-specific primers targeting individual STs have been reported. In addition, multiplex PCR technology further improves on the efficiency and benefits of singleton PCR (McAuliffe *et al*., 2013). However, the development of multiplex PCR primers for the detection of *Blastocystis* subtypes, especially, those tailored for strains more commonly found in Malaysia are still currently lacking. Therefore, in this study, we aimed to design multiplex PCR primers for the simultaneous detection of *Blastocystis* ST1 and ST3.

MATERIALS AND METHODS

DNA extraction: Sample DNA was extracted from previously tested positive ST1 and ST3 strains. Extractions were performed using E.Z.N.A.®Blood DNA Mini Kit (OMEGA bio-tek, USA) following manufacturer's instructions. Non-Blastocystis samples *E.coli*, *B.subtilis*, *S. tyhpi*, *S. flexneri* and *V. parahaemolyticus* were also extracted for use as negative samples. The DNA samples were then quantified using a NanoDrop™ 2000cc spectrophotometer (Thermo Scientific, USA) and the purity was assessed at 260/280 nm absorbance.

Primer design: Reference DNA sequences for *Blastocystis* ST1 (Acession number AF166086) and *Blastocystis* ST3 (Accession number AF166088) were acquired from the NCBI database. Primer design was performed using PerlPrimer. The melting temperature range was set to 58-60°C and allowing for 2°C difference. The primer length was set to be between 18 and 24 bp. Following generation of potential primers, pairs with suggested full dimer dG closer to zero were selected for screening. The primer binding sites were curated using PerlPrimer's built-in BLAST function and was reconfirmed using NCBI's Primer BLAST. Risk of primer dimers were assessed using the Oligo Analyzer Software. Following design and curation, primers ST1g_F:5'-AGA AGA AAC GGT TCT GAT CAC-3', ST1g_R:5'- ATT GAC AAG ACG GCT CCT-3', ST3e_F:5'-CTT TGC CTT TCG GAT AAT CGT-3', ST3e_R:5'-ACC AAG TGC TCT ATC GCA-3' were then synthesized by Integrated DNA Technologies (IDT).

PCR and gel electrophoresis: Primers were first reconstituted before use, following manufacturer's instructions. The multiplex mixture was prepared as shown in Table 1. A gradient PCR run was first performed to determine optimal annealing temperature using TaKaRa Thermal Cycler (TaKaRa, Japan). After which a final confirmation run was performed using the protocol shown in Table 2.

Table 1: PCR multiplex recipe

Reagents	Volume (mL)
Deionised water	14.0
Buffer	2.5
$MgCl_2$	1.0
dNTP	0.5
ST1g_F	1.0
ST1g_R	1.0
ST3e_F	1.0
ST3e_R	1.0
Taq polymerase	1.0
ST1 DNA sample	1.0
ST3 DNA sample	1.0

Table 2: PCR amplification protocol

Profile	Temperature (°C)	Time	Cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	35
Annealing	59	30 sec	
Extension	72	1 min	
Final extension	72	10 min	1

Upon completion of PCR run, 4 μL of the amplicons were loaded into 1.5% (w/v) agarose gel in 1×TBE and 6 μL Ethidium Bromide (EtBr). The samples were then electrophoresed at 145 V for 60 min. The gel was then viewed using Geliance 600 Gel Documentation System (PerkinElmer, USA).

Specificity and sensitivity tests: To ensure primer specificity, common enteric non-*Blastocystis* isolates listed previously were tested individually. Once confirmed, a sensitivity run was performed by 10-fold serial dilution of the DNA samples. Positive primer controls using the reference primers by Bohm-Gloning *et al*. and Stenvold *et al*. (2009).

RESULTS AND DISCUSSION

Primer design: The finalized primersST1g_F:5'-AGA AGA AAC GGT TCT GAT CAC-3', ST1g_R:5'- ATT GAC AAG ACG GCT CCT-3', ST3e_F:5'-CTT TGC CTT TCG GAT AAT CGT-3' and ST3e_R:5'-ACC AAG TGC TCT ATC GCA-3' were successfully designed, producing amplicons of 233 and 138 bp in size, respectively. BLAST results indicate a 100% specificity to *Blastocystis* genome in all designed primers.

Specificity: PCR runs of the designed primers yielded negative results when tested on enteric non-*Blastocystis* samples as shown in Fig. 1.

Sensitivity: Upon completing PCR runs on 10-fold dilutions of DNA templates, sensitivity of ST1g and ST3e primers were confirmed to be at 70 and 7 ng/μL, respectively as shown in Fig. 2.

Based on the results of the study, the designed primers ST1g and ST3e successfully amplified DNA templates of *Blastocystis* subtypes 1 and 3. PCR runs using non-Blastocystis isolates *E. coli*, *B. subtilis*, *S. thyphi*, *S. flexneri*, *V. parahaemolyticus* and *E. histolytica* confirms their specificity in the presence of enteric species. Amplification using 10-fold DNA also indicates the primers to be sensitive to up to 70 ng/μL (ST1g) and 7 ng/μL (ST3e) DNA templates. Multiplexing of the primers resulted in clearly visible, distinct target bands when tested with internal controls. Similarly, neither false negatives nor false positives were observed during al multiplexed runs.

It should be noted that the amplifications were successful when using isolated DNA extracted and purified from human stool. It is currently not feasible to run DNA amplification of direct stool samples due to the abundance of inhibitory and confounding factors (Stensvold *et al*., 2009). As such, the turnaround time for the detection is currently hindered by the stool sample preparation and DNA extraction processes prior to DNA amplification. Furthermore, the use of PCR requires specific machinery which adds to the cost of the detection method. However, identification of DNA bands require much less expertise compared to

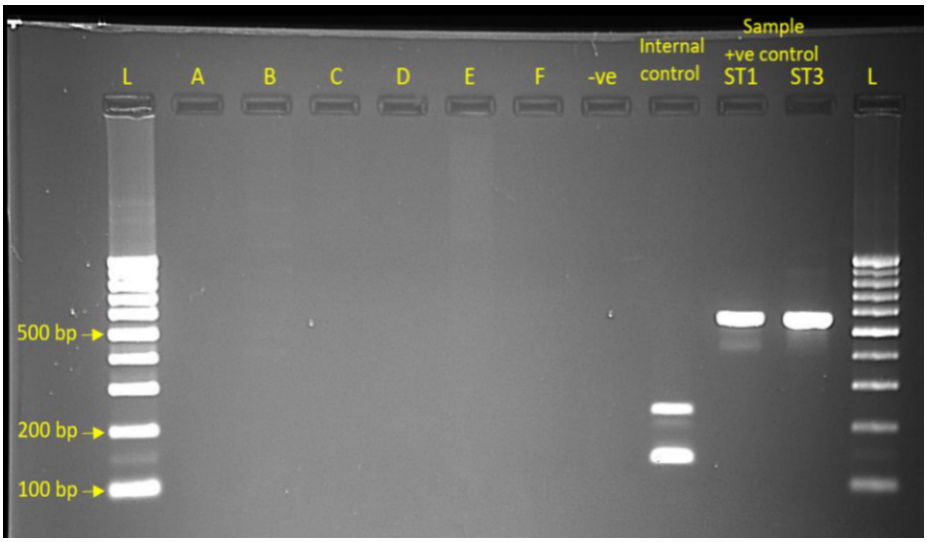

Fig. 1: Specificity test of designed ST1g and ST3 eprimers on enteric non-*Blastocystis* DNA samples, A; *Escherichia coli*, B; *Bacillus subtilis*, C; *Salmonella thyphi*, D; *Shigellaflexneri*, E; *Vibrio parahaemolyticus* and F; *Entamoebahistolytica*

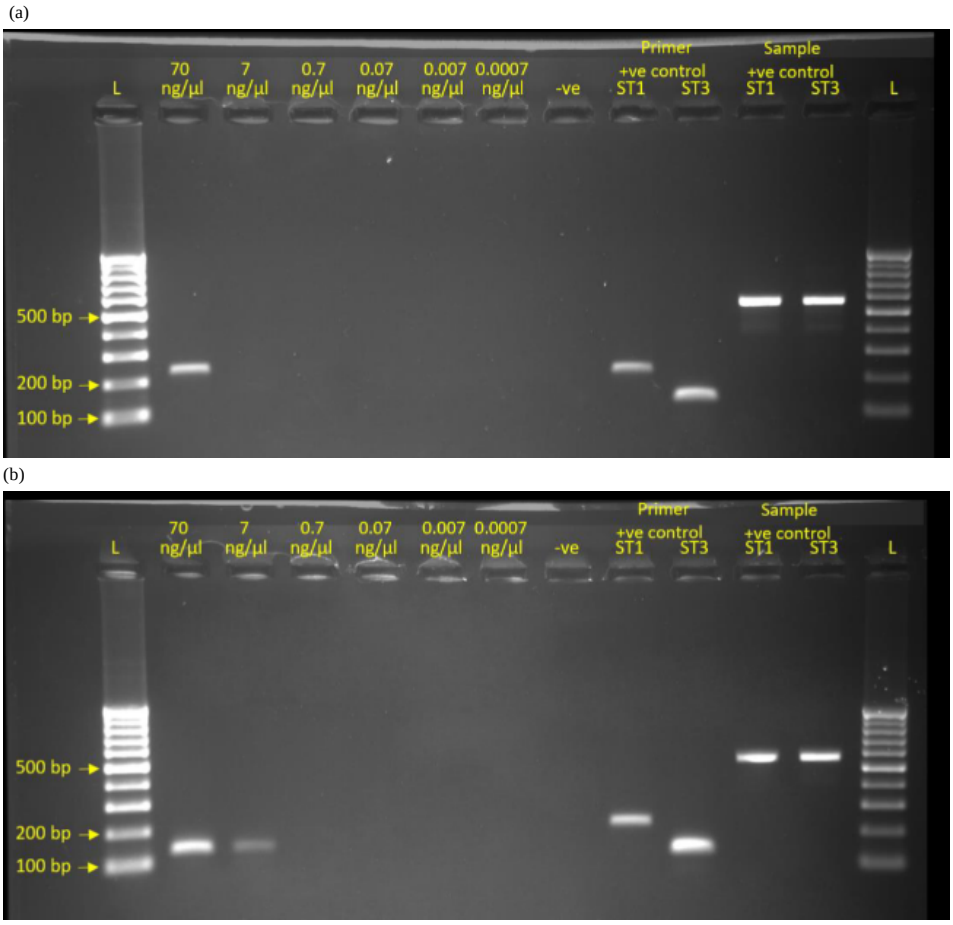

Fig. 2: Specificity test of the designed: a) ST1g and b) ST3e primers on 10-fold dilutions of enteric non-*Blastocystis* DNA samples

screening of *Blastocystis* samples in trichrome stains. Where in the latter, extensive knowledge of the various forms of the parasite in its life cycle is a necessity in order to avoid improper staining and false positives or negatives. While the former simply requires training on the step-by-step protocol for DNA purification, PCR preparation and setup and gel electrophoresis.

CONCLUSION

Multiplex primers for the detection of *Blastocystis* subtypes 1 and 3 were successfully designed and tested to be species specific and sensitive to up to 70 and 7 ng/µL, respectively.

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