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Advantage of using colonic washouts for Blastocystis detection in colorectal cancer patients

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Abstract

Background: There have been previous studies associating microorganisms to cancer and with our recent findings of Blastocystis antigen having a higher *in vitro* proliferation of cancer cells strengthens the suspicion. Collecting faecal samples alone to associate this parasite with cancer may not be accurate due to the phenomenon of irregular shedding and the possible treatment administered to the cancer patients. Hence, this become the basis to search for an alternate method of sample collection. Colonic washout is an almost complete washed up material from colon and rectum which includes various microorganisms such as Blastocystis and other lodged material within the villi. The detection of parasite in colonic washouts will give a better reflection on the association between Blastocystis and CRC.

Methods: Blastocystis detection was made by *in vitro* culture method using Jones' medium, formal ether concentration technique and conventional polymerase chain reaction (PCR) on faecal samples and colonic washouts of 204 CRC patients from colonoscopy procedure. Faecal samples and colonic washouts from 221 normal individuals served as control.

Results: We observed an increased detection of Blastocystis using colonic washouts (n = 53, 12.47%) than faecal samples (n = 26, 6.12%). Eleven faecal samples showed positive results for Blastocystis which were also found in colonic washouts using the PCR technique. This study for the first time showed a significant Blastocystis infection among CRC patients (n = 43, 21.08%) compared to the asymptomatic normal individuals (n = 22, 9.95%). Blastocystis subtype 3 infection was found to be significantly more prevalent (n = 26, 12.75%) compared to other subtypes namely subtype 1: n = 9 (4.41%), subtype 2: n = 1 (0.49%), subtype 5: n = 1 (0.49%) and mixed subtype: n = 6 (2.94%) among the CRC patients.

Conclusion: The study showed that colonic washouts provide a better alternative for Blastocystis detection in CRC patients compared to faecal samples as this prevents treatment regime and the phenomenon of irregular shedding from influencing the detection results obtained from faecal samples.

Keywords: Colorectal cancer, Blastocystis, Subtypes

Background

Blastocystis is one of the most commonly detected microorganisms in the human gut [1]. It is known to cause many non-specific symptoms such as stomach bloating and diarrhea [2]. Among colorectal cancer (CRC) patients, the prevalence of Blastocystis is yet to be determined probably due to the lack of evidence to show its pathogenic role.

Considering the fact that human intestine is often exposed to various microorganisms, the putative role of infectious agents in causing gastrointestinal disorders including CRC is undeniable. In addition, nearly 18% of all cancers worldwide were associated with infectious agents [3]. Human colon can easily allow the growth of over 500 different species of bacteria due to its environment which is rich in nutrients [4].

Blastocystis infection was also reported to be frequent in cancer and HIV/AIDS patients with gastrointestinal symptoms [5]. Although ten different subtypes of Blastocystis (Subtype 1 – Subtype 10) have been identified thus far

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[6-8], epidemiological studies related to Blastocystis have often been hampered by the poor sensitivity of standard methods available to detect Blastocystis genotypes in faecal samples. While a previous *in vitro* study have demonstrated that the solubilized antigen of Blastocystis could proliferate colon cancer cells [3], the present study attempts to assess the prevalence of Blastocystis in CRC patients. However, detection of Blastocystis becomes harder in this cohort as these patients are likely to undergo chemotherapy and other drug treatments that probably could have killed the organism. The irregular shedding reported previously [9] raises the possibility that Blastocystis detection in faecal samples could be missed. Therefore in the present study we attempted to compare the prevalence of Blastocystis using colonic washouts and faecal samples to identify the Blastocystis genotype present. It is highly probable that a better Blastocystis occurrence in patients with CRC can be obtained when colonic washouts are used as sample material. Patients resort to colonoscopy only as a last resort to finding the cause when the initial screening and treatment do not relieve symptoms. The possibility of re-covering parasites from colonic washout is higher as it contains an almost complete washed up material from colon and rectum which includes various microorganisms such as Blastocystis and other lodged material within villi.

Methods

Patients

This is a hospitalized-based cross-sectional study of 425 patients who underwent diagnostic colonoscopy in University of Malaya Medical Centre (UMMC). The samples were collected over a 2-year period, between 2010 and 2012. Colonoscopy is usually recommended for screening and prevention of colorectal cancer (CRC). Over 751 potential participants were briefed about this study but only 425 patients were interested. They were provided with a consent form in person. They belonged to two cohorts- a group of 221 patients who came for normal screening and 204 patients presenting colorectal malignancies. This study was approved by the Medical Ethics Committee of the UMMC in accordance with the declaration of Helsinki.

Specimen collection and screening

Colonic washouts were obtained at the time of (or immediately prior to) the diagnosis of CRC. Whereas, faecal samples were obtained via the standard clinical procedures whereby patients will be given a duration of 1-2 weeks to deliver their faecal samples. Colonic washouts were collected in clean disposable bowls. Each sample was centrifuged at $1,400 \times g$ and the pellets obtained were cultured in Jones' medium [10] supplemented with 10% horse serum [11] and incubated at 37°C for 24 h and then screened for Blastocystis. DNA was extracted from the

remaining fresh colonic washout for genotyping purposes. A pea size of the faecal samples collected from the same individuals were directly cultured in Jones' medium and other procedures carried out were similar to that of colonic washout. Predominant Blastocystis subtype was identified using polymerase chain reaction (PCR) technique.

Formal ether concentration technique

Fresh colonic washout and faecal samples were routinely processed by the formal ether concentration technique (FECT) to obtain stool concentrate. Ficoll-Paque density gradient centrifugation method was carried out to isolate Blastocystis cyst from the concentrate [11]. Briefly, the stool concentrate re-suspended in PBS was layered on 5 ml of Ficoll-Paque and centrifuged at $1,600 \times g$ for 20 minutes. Blastocystis cyst layer which was formed after centrifugation was removed into another Falcon tube and re-suspended in 1 ml PBS and observed under microscope for the detection of Blastocystis cyst.

DNA extraction and genotyping

PCR technique was used to detect Blastocystis in addition to standard stool culture technique in both colonic washouts and faecal samples. DNA extraction was conducted using QIAamp[®] DNA Stool Mini Kit (QIAGEN). Briefly, 200 ml of the sediments of colonic washout samples or faecal samples were used to extract DNA. PCR was carried out with specific sequence-tagged site (STS) primers (Table 1). Seven different STS primers were used for genotyping as described previously [6,7,12-18]. The STS primers used were SB83 (351 bp), SB340 (704 bp), SB227 (526 bp), SB337 (487 bp), SB336 (317 bp), SB332 (338 bp) and SB 155 (650 bp) for subtype 1, 2, 3, 4, 5, 6 and 7 respectively based on a recent classification terminology [19]. One microliter of DNA preparation was used to amplify the genomic sequences in a 20 μl PCR cocktail containing 0.2 mM of the four dNTPs, 25 pmol of each primer, $1 \times$ PCR buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(\text{NH}_4)_2\text{SO}_4$, and 0.01% Tween 20), 2.5 mM MgCl, and 1 U Taq DNA polymerase (recombinant) (Fermentas). PCR was carried out with one cycle denaturing at 95°C for 5 min, 42 cycles including annealing at 56.3°C for 90 s, extending at for 60 s, and additional cycle with a 10 min chain elongation at 72°C (Thermal cycler, BIO-RAD, USA). The PCR products and a size marker of a 100-bp ladder were electrophoresed in 1.5% agarose gels (Promega, USA) which were stained with ethidium bromide and photographed using an ultraviolet gel documentation system (Uvitec, UK). PCR amplification for each primer pair was repeated thrice for each positive sample.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences for Windows SPSS (Version 17.0). The Chi

Table 1 Primer sequences used for Blastocystis genotyping

Subtypes	STS primer sets	Product size (bp)	Sequences of forward (F) and reverse (R) primers (5'-3')	Source of primer	GenBank accession no.	Reference	Clade in the SSU rRNA phylogeny ^a
1	SB83	351	F GAAGGACTCTCTGACGATGA R GTCCAAATGAAAGGCAGC	Nand II	AF166086	Yoshikawa et al. [6]	I
2	SB155	650	F ATCAGCCTACAATCTCCTC R ATCGCCACTTCTCCAAT	B	AF166087		VII
3	SB227	526	F TAGGATTGGTGTGGAGA R TTAGAAGTGAAGGAGATGGAAG	HV93-13	AF166088	Yoshikawa et al. [7]	III
4	SB332	338	F GCATCCAGACTACTATCAACATT R CCATTTTCAGACAACCACTTA	HJ96AS-1	AF166091		VI
5	SB340	704	F TGTTCTTGCTCTCTCAGCTC R TTCTTTCACACTCCCCTCAT	HJ96-1	AY048752	Yoshikawa et al. [18]	II
6	SB336	317	F GTGGGTAGAGGAAGGAAAACA R AGAACAAGTCGATGAAGTGAGAT	SY94-3	AY048751		V
7	SB337	487	F GTCTTCCCTGTCTATTCTGCA R AATTCGGTCTGCTTCTCTG	RN94-9	AY048750		IV

squared test was used to determine significance of differences in prevalence of Blastocystis between the healthy individuals and CRC patients. Fisher's Exact test was used to determine the pre-dominant Blastocystis subtype in the colonic washouts of normal and CRC patients. In all the analyses, a probability level of $p < 0.05$ was used to indicate statistical significance.

Results

Blastocystis detection in the faecal samples and colonic washouts

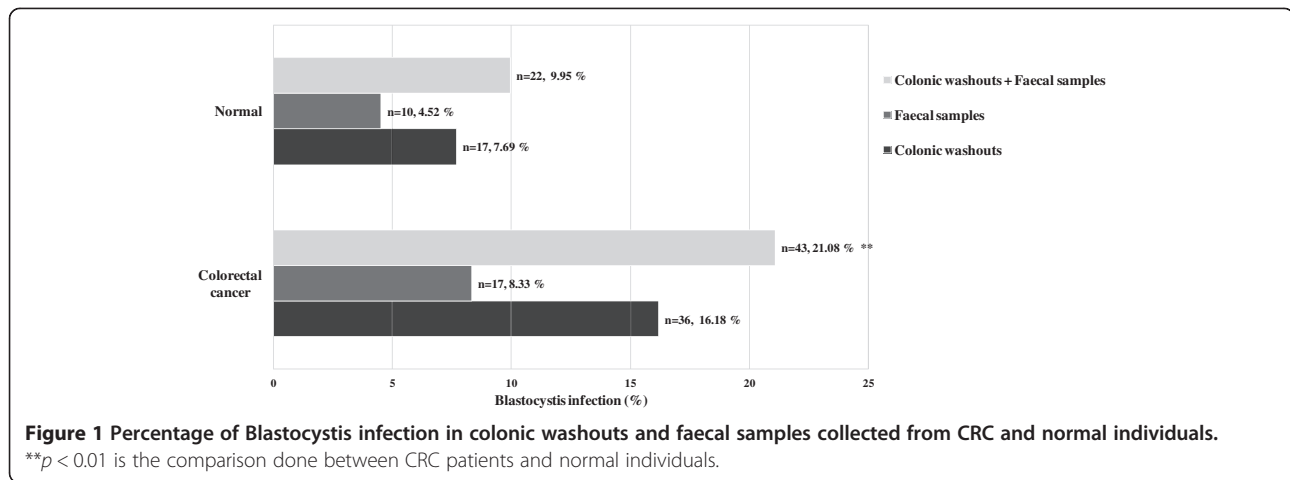
The overall prevalence of Blastocystis infection obtained from the three methods used was 15.29% (65/425). There were 65/425, 4/425, and 4/425 samples (including faecal samples and colonic washouts) detected positive for Blastocystis via PCR, *in vitro* cultivation and formal ether concentration technique respectively. Overlapping positive results were often observed in faecal samples and colonic washouts as well as among the different techniques used. Colonic washouts and faecal samples showed 12.24% ($n = 52$) and 5.65% ($n = 24$) of Blastocystis infection respectively via the conventional PCR technique. Forty-one colonic washouts were positive for Blastocystis, despite the faecal samples from the same patients being negative for Blastocystis. Whereas, 11 faecal samples identified positive for Blastocystis also showed positive for colonic washouts obtained from the same patients using PCR technique. A very small percentage (0.96%, $n = 4$) of faecal samples were found positive via *in vitro* cultivation of faecal samples but none from colonic washouts. Although other parasites such as *Ascaris lumbricoides* and hook worm were detected via formal ether concentration technique but the frequency was negligible and statistically non-significant.

Blastocystis infection and subtype analysis in CRC patients

A total of 43 (21.08%) samples were positive for Blastocystis infection in CRC patients and was significantly higher compared to normal individuals ($n = 22$, 9.95%, $p < 0.01$) (Figure 1). We conducted conventional PCR [20] to classify Blastocystis into certain subtype. In the current study, four different Blastocystis genotypes were identified among all the subjects namely subtype 1 (ST1), subtype 2 (ST2), subtype 3 (ST3), subtype 5 (ST5) and mixed subtypes. Subtype 6 (ST6) and subtype 7 (ST7) were not detected in all cases. ST3 was present at higher levels compared to other subtypes detected in both groups as shown in Table 2. Overall, ST3 was the most prevalent subtype ($n = 30$, 14.71%), whereas ST1, ST2, and ST5 were seen in 5.39% ($n = 11$), 3.43% ($n = 7$) and 0.49% ($n = 1$) of the CRC patients, respectively. Besides that, mixed subtype infections were detected in six samples which were 0.98% ($n = 2$, ST1 and ST2) and 1.96% ($n = 4$, ST2 and ST3) (Table 2). ST3 infection was also found to be statistically significant in CRC patients as compared with the control group (Table 2).

Discussion

Faecal sample collection of more than three times from the same person have been shown to significantly raise the possibility of detecting parasites [9]. However this would be too tedious and troublesome to execute. The faecal sample collection was purposely carried out as how it would be for routine screening purpose after the CRC diagnosis regardless of pre or post-treatment regime which could not be avoided. Therefore, the chances of recovering parasites which could have been influenced by the treatment regime cannot be overruled. The irregular



shedding of Blastocystis in faecal sample further compound the challenge for detecting these parasites despite the use of the standard faecal culture technique [9]. In addition, detection using microscope is a greater challenge when parasites present in very small numbers in the faecal samples. As such, the usage of colonic washout probably far more effective as it can be collected almost immediately during diagnosis and the detection without the influence of any previous treatment regime. The diagnostic method to determine the presence of Blastocystis vary widely in sensitivity. Possibility of Blastocystis being eliminated by treatment usually sought by CRC patients for their initial symptoms could have resulted in zero detection in the present study using both formal-ether concentration technique and the gold standard *in vitro* cultivation method. Therefore, PCR technique was employed for the screening of Blastocystis in both faecal samples and colonic washouts. Blastocystis was detected in colonic washout samples from patients who were tested negative for Blastocystis by using faecal sample. This is the first study that detects Blastocystis in colonic washout samples from CRC patients via the conventional

PCR method. These samples were collected at the initial stage of cancer detection which excluded the possibility of the patients undergoing medical treatments such as chemotherapy which could have killed the parasites. Although pathogenicity of Blastocystis is controversial, Blastocystis screening in CRC patients is crucial considering the recent *in vitro* studies providing evidences of the parasite's exacerbating potential in proliferating cancer cells [21-23]. This method probably can be one of the more effective ways to screen for Blastocystis for high risk individuals and those who are suspected of infection but found negative by other less sensitive methods. The collection of colonic washouts is easier as it is a waste product produced from colonoscopy procedure. Furthermore, the chances for cross-contamination can be prevented as it can be collected directly and almost immediately from patients who are undergoing colonoscopy. This method may preferably be used for individuals that go through colonoscopy procedure for other diagnosis purposes such as CRC and those who are suspected of infection but found to be negative by other available methods.

Table 2 Blastocystis genotypes found in CRC patients and normal individuals

Blastocystis subtype	Colorectal cancer patients (n = 204)% (No. positive)	Healthy individuals (n = 221)% (No. positive)
Subtype 1	4.41 (9)*	2.71 (6)
Subtype 2	0.49 (1)**	0.90 (2)*
Subtype 3	12.75 (26)#	3.17 (7)
Subtype 1 + 2	0.98 (2)**	0 (0)
Subtype 2 + 3	1.96 (4)*	1.81 (4)
Subtype 5	0.49 (1)**	1.36 (3)

*p < 0.05; **p < 0.01 are levels of significance between subtype 3 Blastocystis and other subtypes.

#p < 0.05 is the significant difference in Blastocystis (subtype) infection between CRC patients and healthy individuals.

Predominance of Blastocystis ST3 was similar to a previous study conducted among patients in a hospital in Singapore [24]. Blastocystis ST3 was reported to be the only subtype of human origin, while the rest being zoonotic [25]. As an earlier report suggested a possible correlation between ST3 and pathogenic potential [26], it is crucial for subtype identification. This will enable us to understand better, the possible pathogenic role of these subtypes play to the worsening of cancer. Furthermore, the occurrence of other cases such as acute urticaria and gastrointestinal symptoms were reported in patients with ST3 infection [27]. We observed a very low prevalence of ST1 and ST2 in both CRC and healthy individuals. These subtypes were mainly associated with zoonotic transmission. Similarly, ST4, ST6 and ST7 were not found in these patient groups as they are mostly zoonotic microorganisms [28].

Conclusion

In conclusion, this study demonstrated that colonic washouts can be a better alternative to fecal samples to examine for *Blastocystis* infection especially in CRC cases. Our study shows that *Blastocystis* infection is common in CRC patients and it indicates subtype 3 as predominant among these individuals. However, the pathogenic role of this parasite in CRC patients is still unclear. Therefore, further study has to be conducted to determine the correlation between the genotype and symptomatology.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

VK, SKG, ACR and URK participated in the design of the study. VK did the data and sample collections from the patients. VK, SKG, ACR and URK did the data analysis. VK wrote the manuscript. All authors read and approved the final manuscript.

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