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Growth Kinetics and Metronidazole Sensitivity of *Blastocystis* Sp. Isolated from Colorectal Carcinoma (CRC) and Non-CRC Patients

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ABSTRACT

Blastocystis sp. is the most common protist detected in human fecal samples in numerous studies globally. Extreme debate regarding *Blastocystis* sp. pathogenicity exists. Some studies have speculated a possible correlation between *Blastocystis* and CRC. No previous study has investigated the presence of non-genotypic differences in *Blastocystis* sp. isolated from CRC and non-CRC patients. The present work aimed to investigate the growth kinetics (G.K) and metronidazole (MTZ) sensitivity in *Blastocystis* isolates from CRC and apparent non-CRC whether symptomatic and asymptomatic *Blastocystis* carriers. Seven isolates from CRC patients and 6 from symptomatic and 6 from asymptomatic non-CRC carriers were cultured in Locke's Egg (LE) medium supplemented with bovine serum and antibiotic mixture and incubated at 37◦ C. Mean viable organisms counts of each isolate were counted every 24 hours in medium to follow their GK and after exposure to different MTZ concentrations to perform drug sensitivity assay. *In vitro* growth kinetics of CRC and Non-CRC symptomatic isolates were nearly similar with higher peaks attained at 72 hours incubation than by the slower growing non-CRC-asymptomatic isolates. MTZ-sensitivity of CRC isolates was nearly similar to that of non-CRC asymptomatic isolates, both were significantly more sensitive than the symptomatic isolates especially at high drug concentration of 200 µg/ml of the medium.

Keywords: *Blastocystis* sp., Colorectal carcinoma, Symptomatic, Asymptomatic, growth kinetics, metronidazole sensitivity.

INTRODUCTION

Blastocystis sp. is the most common protists detected in human fecal samples in numerous studies globally [1, 2]. It is a pleomorphic organism existing in multiple forms: the vacuolar, granular, amoeboid and cystic [3]. The extreme debate regarding *Blastocystis* sp. pathogenicity had led many researchers to attempt finding out differentiating characters between asymptomatic and symptomatic human-derived *Blastocystis* isolates [4]. Different growth profiles characterizing isolates from different clinical groups were previously reported [4, 5] demonstrating phenotypic differences between isolates recruited from different clinical groups.

Metronidazole (MTZ) is the first-line prescribed drug with various rates of efficacy ranging from 0% to 100% depending on the dose administered [6]. Although MTZ demonstrates effectiveness in some individuals [7], it has also been shown to exhibit side effects and resistance in others [8]. MTZ induces apoptosis in *Blastocystis* sp. as a defensive mechanism to ensure that some of the cells survive to propagate the genome [9].

Accumulating epidemiological data suggests that *Blastocystis* is associated with various gastrointestinal conditions including colorectal cancer (CRC) [10, 11]. Incidence of *Blastocystis* infection was (36%) in healthy individuals and (34%) in patients with colorectal adenoma. However, the incidence was significantly increased (53%) in patients with colorectal carcinoma [10].

In the available literatures, no work was carried out to investigate *Blastocystis* sp. isolates from CRC patients. The existence of extreme genotypic diversity among *Blastocystis* isolates from different clinical groups of carriers, necessitates the extrapolation of possible different biological and phenotypic characters between isolates.

The aim of the present work was to study the growth kinetics (G.K) and MTZ sensitivity in *Blastocystis* isolates from CRC and non-CRC patients.

MATERIALS AND METHODS

Blastocystis sp. isolates*:*

The present work was done in the period from September 2015 to October 2017. **The study was approved by the Ethics Board of Ain Shams University.**

A total of 19 *Blastocystis* sp. isolates from CRC, and apparent non-CRC patients attending the outpatient clinics of El Demerdash Hospitals of Faculty of Medicine, Ain Shams University were studied. *Blastocystis* sp. isolates were grouped in 3 groups; 7 isolates from early diagnosed CRC patients before receiving any chemotherapeutic drugs (GI or CRC group), and 12 isolates from apparent non-CRC carriers (GII or Non-CRC

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group). GII included 6 isolates from symptomatic patients with gastrointestinal tract symptoms suggestive of blastocystosis (GIIa) and 6 isolates from asymptomatic carriers (GIIb).

Blastocystis sp. were isolated by *in-vitro* cultivation of stool samples in Locke's Egg (LE) medium supplemented with 10% bovine serum and antibiotics at 37°C [12]. Subsequently, parasites were maintained in lab by sub-culturing every 3 to 4 days when organisms were in the log phase of growth.

Growth characteristics:

Starting with an initial concentration of 1×10^4 cells per ml in LE medium, GK studies were performed in triplicate for each isolate. Viable *Blastocystis* organisms were counted daily in the Improved Neubauer haemocytometer chamber (Haussler Scientific) using Trypan blue viability exclusion test [13] where 0.4% Trypan blue solution was used as an indicator of viability: Only viable organisms that exclude the dye and appear with clear cytoplasm were counted. Counting continued till no viable organisms were detected in culture tubes. Average counts of isolates in each group were calculated at 24 hour intervals and an average growth kinetic curve was drawn for each group. The generation time (GT) was calculated in the first 24-h period during which the most rapid growth occurred, according to the following formula [14].

GT= t/n = t/3.3 log (b/B), where:

B: number of *Blastocystis* organisms at start of time period t.

b: number of *Blastocystis* organisms at the end of time period t.

t: time period.

n: number of generations.

log: logarithm to base 10 (common log).

GT: generation time.

In MTZ sensitivity assay [15]: Aqueous working solution of 1 mg/ml metronidazole was prepared and added to the medium to give final concentrations of 10, 25, 50, 100 and 200 µg/ml medium. A duplicate of each concentration was done.

Starting with an initial *Blastocystis* organism's concentration of $5.0x10^5$ org/ml medium and incubated at 37°C with the different metronidazole concentrations. The percentage increase or decrease in growth in the test tubes and the control tubes (with no drug added) were calculated every 24 hours. The minimum inhibitory concentrate (MIC) and the minimal lethal concentrate (MLC) were identified for each isolate.

Statistical analysis

Results are expressed as mean ± standard deviation (SD) for quantitative values and as number and percent for qualitative values. Statistical analyses were carried out using SPSS version 20. One-way test of ANOVA was used for comparison of growth characteristic and MTZ sensitivity of the isolates. $P < 0.05$ was considered statistically significant and $P < 0.001$ was considered statistically highly significant.

RESULTS

Growth characteristics:

Blastocystis sp. vacuolar form was detected in culture of all 19 isolates while the amoeboid form was detected in 43% of isolates of CRC group and 33% of isolates of non-CRC symptomatic and was not detected at all in cultures of non-CRC asymptomatic carriers with no statistical significant difference between the groups as shown in table (1).

Table (1): Frequency of detecting *Blastocystis* sp. forms in culture tubes of isolates in different groups in the studies

***Blastocystis* forms in culture**	**Groups of the study**			**Total number of isolates (N= 19)**	***P* value**
	GI	**GII**			
	(Colorectal carcinoma	**Non- Colorectal carcinoma group**			
	N=7	**GII a Symptomatic (N=6)**	**GII b - Asymptomatic (N=6)**		
	N. (%)	**N. (%)**	**N. (%)**	**N. (%)**	
Vacuolar	7 (100%)	6 (100%)	6 (100%)	19 (100%)	N A
Granular	4 (57%)	5 (83.3%)	3 (50%)	12 (63%)	0.448
Amoeboid	3 (43%)	2 (33.3%)	0 (0%)	5 (26.3%)	0.194

N.: number; (%): percentage; NA: Not available as it is constant.

Three distinct and different growth profiles representing average growth kinetics of isolates in the three groups starting with an initial inoculation of 1.0 X10^4 cells/ml were shown in figure (1). CRC isolates in GI and symptomatic isolates in GIIa showed nearly similar average higher growth peaks compared to the asymptomatic isolates (GIIb) (Fig. 2). Peak counts in the three groups were reached after 72 hours incubation (Fig. 1) with lowest values those of the asymptomatic isolates in GIIb reaching 188 to 235 x 10^4 organisms/ml (Fig. 2). Higher peak counts were those of CRC isolates in GI isolates, ranging from (213 – 302) x 10^4 organisms/ml, and of non-CRC symptomatic isolates in GIIa with values ranging from (217 – 301) x 10^4 organisms/ml (Fig. 2).

The generation time recorded in the first 24 hours for all isolates showed that CRC isolates (GI) (8.97±1.16 hours) isolates (GIIa) (8.89±1.21 hours) were as nearly similar as non-CRC symptomatic but both were faster in growth than asymptomatic isolates (GIIb) (9.55±0.31 hrs) and thus, both GI and GIIa achieved parasite counts greater than asymptomatic at peak growth.

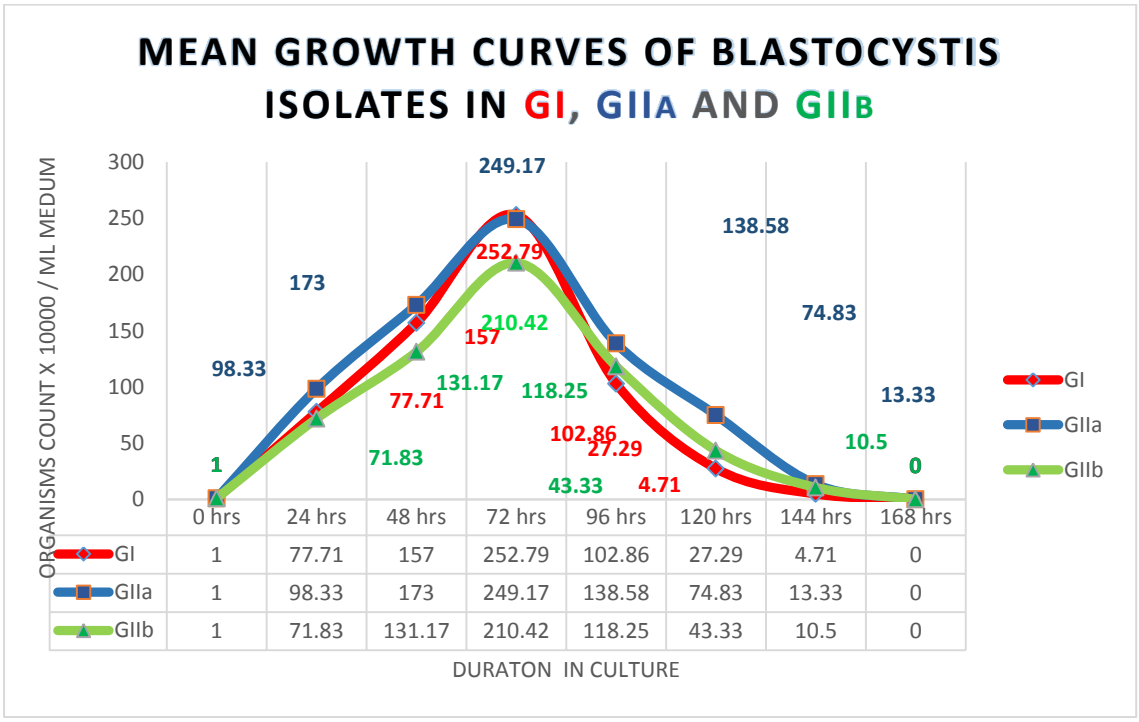

Fig. (1): Line chart for the growth kinetics of *Blastocystis* isolates of GI, GIIa and GIIb organisms in the biphasic LE culture medium at 37°C incubation starting by *Blastocystis* Organisms' concentration of 1.0 x 10^4/ml. **GI**: CRC group, **GIIa**: Non-CRC Symptomatic sub-group and **GIIb**: Non-CRC Asymptomatic sub-group.

No viable organisms were found after 168 hrs (7 days) incubation in all isolates in all groups (Fig. 1).

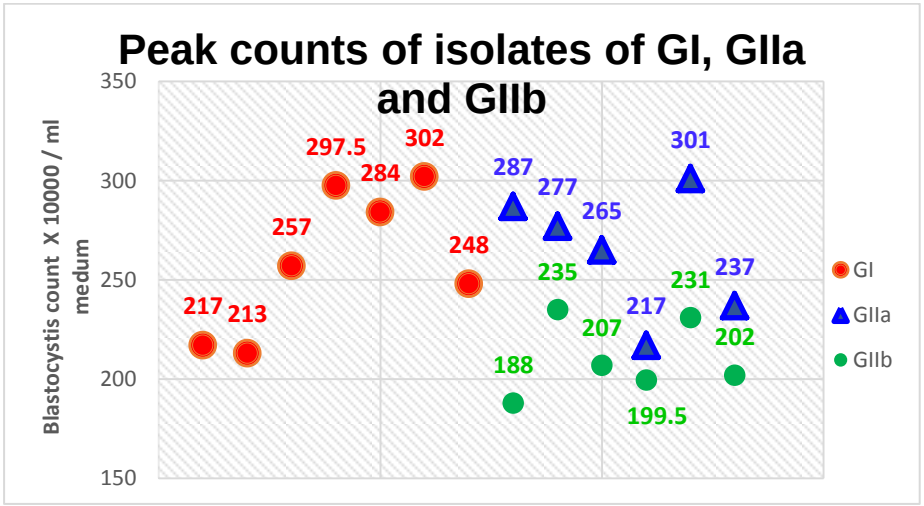

Fig. (2): Scatter chart for the growth peaks of isolates of the three groups **GI**: CRC group, **GIIa**: Non-CRC Symptomatic sub-group and **GIIb**: Non-CRC Asymptomatic sub-group.

Table (2): Mean generation time of *Blastocystis* isolates in GI, GIIa and GIIb

	GI (CRC group)	GIIa (Non-CRC Symptomatic subgroup)	GIIb (Non-CRC Asymptomatic subgroup)	F	*P*-value
Generation time (Hour)					
Mean±SD	8.97±1.16	8.89±1.21	9.55±0.31	0.804	0.465
Range	6.4-9.63	6.43-9.65	9.2-10		

SD: Standard deviation
Comparison using One-way ANOVA test where;
P= probability value
P>0.05 = non-significant difference, P < 0.05 = significant difference and P<0.01 = highly significant difference

Metronidazole susceptibility assay (Table 3 and 4):

The number of organisms in the control tubes with no drug added and with the same conditions as the test tubes started to decline after 48 hrs incubation (data not shown). For that reason the assay was carried out over only 2 days duration. Great reduction (GI, 94.21%, GIIa, 90.31% and GIIb, 94.13 %) in growth of all *Blastocystis* isolates in the three groups occurred during the first 24 hrs exposure to different doses of MTZ varying between 10 µg/ml to 200 µg/ml. This progressed for every isolates till the end of 48 hours of the assay. GIIa (Non-CRC Symptomatic isolates) were the least to be affected than the isolates in GI (CRC group) and in GIIb (Non-CRC Asymptomatic sub-group). MIC value for all the isolates was 200 µg / ml. No minimal lethal dose (MLC) was recorded as no complete death of organisms in culture was achieved.

Table (3): Mean counts of viable *Blastocystis* organisms / ml in culture of isolates of GI (CRC group), GIIa (Non-CRC Symptomatic sub-group) and GIIb (Non-CRC Asymptomatic sub-group) after 24 and 48 hrs exposure to 200 µg/ml concentration of metronidazole (starting counts 5.0 X 10^5 organisms /ml)

200µg/ml					
Mean±SD	**6.36±2.23**	**10.08±2.58***	**7.83±2.64**	**2.672**	**0.023***
Range (after 24 hrs exposure)	**3-9**	**8-15**	**4-12**		
200µg/ml					
Mean±SD	**4.64±1.75**	**6.75±2.09***	**5.17±2.32**	**2.196**	**0.038**
Range (after 48 hrs exposure)	**2-7**	**4-10**	**3-9**		

SD: standard deviation.
Comparison by the One-way ANOVA test, where:
P value: Probability value where:
P>0.05 = non-significant difference, *= *P*<0.05 = significant difference , *P*<0.01 = highly significant difference

Table (4): Percentage decrease in mean counts of isolates of GI (CRC group), GIIa (Non-CRC Symptomatic sub-group) and GIIb (Non-CRC Asymptomatic sub-group) in culture after 24 hrs exposure to different concentrations of metronidazole

Drug Concentrations	Percentage decrease of counts after 24 hrs			**F**	***P value***
	GI	**GIIa**	**GIIb**		
10 µg/ml	82.97 %	83.65 %	86.81 %	1.921	0.179
25 µg/ml	85.04 %	85.17 %	89.25 %	0.997	0.391
50 µg/ml	88.10 %	87.74 %	91.19 %	0.490	0.622
100 µg/ml	91.16 %	89.11 %	92.75 %	1.925	0.178
200 µg/ml	94.21 %	*90.31 %	94.13 %	2.196	0.038

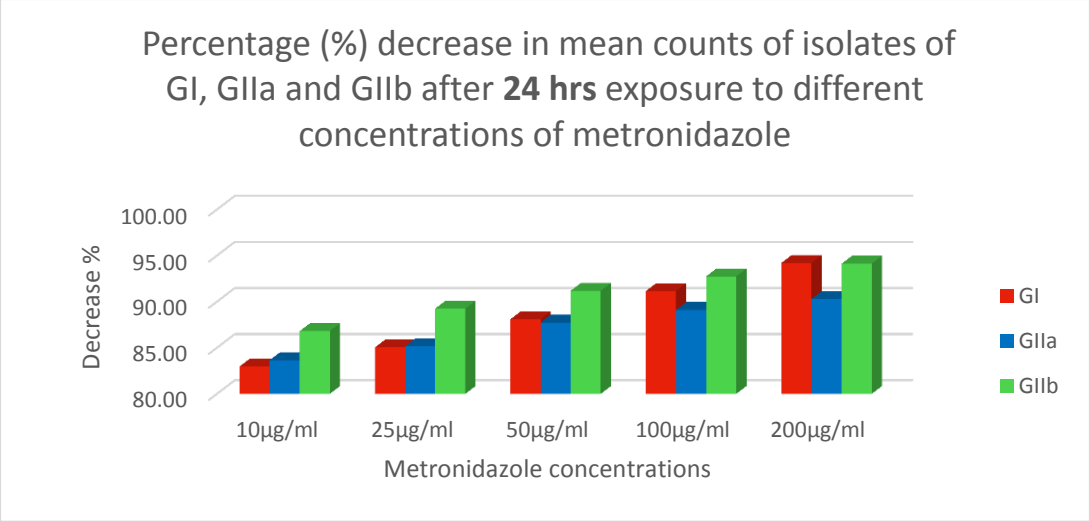

Fig (3): Bar chart for the percentage decrease in mean counts of isolates of **GI** (CRC group), **GIIa** (Non-CRC Symptomatic sub-group) and **GIIb** (Non-CRC Asymptomatic sub-group) in culture after 24 hrs *in vivo* exposure to different concentrations of metronidazole.

Table (5): Percentage (%) decrease in mean counts of isolates of GI (CRC group), GIIa (Non-CRC Symptomatic sub-group) and GIIb (Non-CRC Asymptomatic sub-group) in culture after 48 hrs exposure to different concentrations of metronidazole

Drug Concentrations	Percentage decrease of counts after 48 hrs			**F**	**value**
	GI	**GIIa**	**GIIb**		
	%	**%**	**%**		
10 µg/ml	**91.75**	**91.52**	**93.07**	1.921	0.087
25 µg/ml	91.75	91.52	93.07	0.997	0.179
50 µg/ml	94.94	93.78	95.62	0.490	0.391
100 µg/ml	96.50	94.41*	96.36	1.925	0.178
200 µg/ml	97.84	96.35*	97.77	2.196	0.038

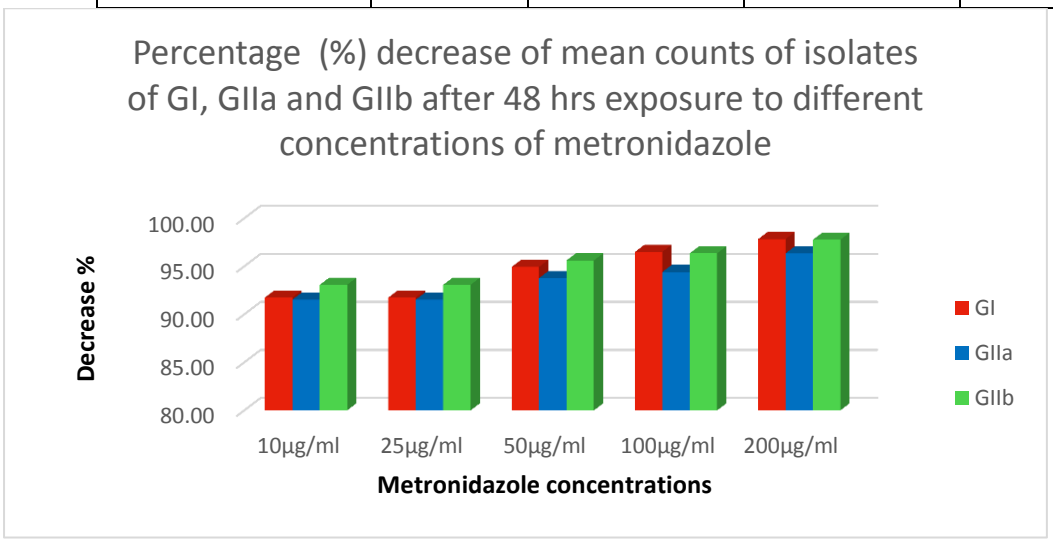

Fig. (4): Bar chart for percentage decreases in the mean organism counts of isolates of **GI** (CRC group), **GIIa** (Non-CRC Symptomatic sub-group) and **GIIb** (Non-CRC Asymptomatic sub-group) after 48 hrs exposure to different concentration of metronidazole.

DISCUSSION

In the present study, the presence of amoeboid forms in culture of 43% of CRC isolates (GI) and 33.3% of non-CRC symptomatic isolates (GIIa) and its complete absence in asymptomatic isolates (GIIb) might be indicative of virulence in some of the isolates in GI and GIIa. This is consistent with previous works [4, 16, 17] where significant presence of the amoeboid form in culture of symptomatic cases only and not in cultures of asymptomatic groups was reported. However, **Souppart *et al.*** [18] detected the amoeboid form in the culture of both symptomatic and asymptomatic *Blastocystis* infected groups with no significant difference between both groups.

Three different growth profiles of the studied groups were detected. Similarly, different growth profiles characterizing groups of isolates from different clinical groups were previously compared [4, 5] demonstrating differences between isolates recruited from different clinical groups.

In the present study, higher peak growth values of symptomatic isolates (GIIa) over the asymptomatic isolates of (GIIb) are in contrast to the findings of some previous studies [4, 5]. In comparing the symptomatic and asymptomatic isolates in the aforementioned works, they reported a higher growth of the asymptomatic isolates over the symptomatic ones reaching peak values after 5 days incubation. This might be due to combined factors composed by the different culture medium used in the three previous studies which was Jones' medium together with the different biology of organisms of the isolates. The role of the associating microbiota in gut of Egyptians on the growth of asymptomatic isolates in the present study could play a role in inhibiting their growth *in vitro*.

In the work of **Ragavan *et al.*** [5] a third group of isolates derived from patients with IBS were included in the study and the group had intermediate peak values between the symptomatic and asymptomatic groups. In the present study, the third group of isolates consisting of CRC isolates gave nearly similar curve to that of the sub-group of symptomatic isolates. It is noteworthy to mention that average peak counts and growth curves of groups GI and GIIa were nearly approximating each other and both are higher than GIIb.

The generation time recorded in the present study indicated that CRC isolates (GI) are as nearly similar as non-CRC symptomatic isolates (GIIa) but both are faster in growth than asymptomatic isolates (GIIb) and thus, both GI and GIIa achieved parasite counts greater than asymptomatic at peak growth. Other studies [19-21] reported that the generation time of xenic *Blastocystis* isolates was variable, ranging from 6 to 23 h, depending on the isolate itself and the type of medium used.

In the present work, *in vitro* MTZ sensitivity assay was performed and showed that great reduction occurred in the three groups within 24 hrs, and progressed till the end of 48 hrs of the assay. GIIa isolates were the least to be affected than the isolates in GI (CRC group) and in GIIb (asymptomatic sub-group). Thus, isolates of GIIa, the symptomatic virulent isolates, showed less MTZ sensitivity (MTZ^s). This is in contrast to what should be expected to happen [8, 22]. It was reported that virulent *Blastocystis* isolates are more MTZ sensitive (MTZ^s) isolates than avirulent isolates [8, 22].

In the present study, isolates in the CRC group, GI, are nearly as sensitive as the non-CRC asymptomatic isolates of GIIb and both are more MTZ^s than the non-CRC symptomatic isolates of GIIa. This may be explained by presence of isolates biological variability providing a possible explanation for the diverse clinical outcomes of *Blastocystis* infections.

In the present study, on comparing the growth kinetic studies of the isolates in GI, GIIa and GIIb and the results of MTZ susceptibility assay, the GIIa isolates, non-CRC symptomatic isolates, are both less MTZ^s, otherwise more MTZ^r, meanwhile have rapid growth. This observation is similar to what was previously reported by **Wu *et al.*** [22] who examined the proliferative potential of MTZ^r and MTZ^s strains from growth curves assayed. They noticed that the growth rates of MTZ^r isolates were not always lower than MTZ^s isolates, suggesting that drug resistance in *Blastocystis* might not necessarily be associated in slower growth. The isolates of GIIb, the Non-CRC asymptomatic isolates, are avirulent, producing no-symptoms in their hosts, MTZ^s and slow growing isolates. Those in GIIa, the Non-CRC symptomatic isolates are rapid growing less MTZ^s isolates, and the CRC isolates are rapid growing meanwhile MTZ^s isolates.

In the current study, MTZ caused only inhibition of *in vitro* growth of all the isolates, an observation in accordance with the description of **Zierdt *et al.*** [23] who reported that MTZ is one of the inhibitory antimicrobials and not a lethal drug. This, also, agrees with the reports of **Nasirudeen *et al.*** [24], who explained that MTZ induce apoptosis in *Blastocystis* sp. as a defensive mechanism used by unicellular organisms for the preservation of cell populations to ensure that some of the cells survive to propagate the genome [9]. The survival of resistant isolates could be a result of an efficient and

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effective apoptotic response to MTZ with the formation of granular forms and later release of new progeny of vacuolar forms contained in their vacuoles [(24)]. Also, resistant strains may be due to insufficient dose of MTZ [(24)]. Thus, **Anselmi *et al*.** [(25)] reported that there may be further research needs to be done to investigate the possibility of a lethal effects if higher concentrations of the drug is used and dose-dependent effects can be studied.

In conclusion, studying the growth kinetics and MTZ sensitivity in the present study demonstrated the existence of differences in the biological characters of *Blastocystis* sp. isolates from different clinical groups a possible indication to different pathogenic role.

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