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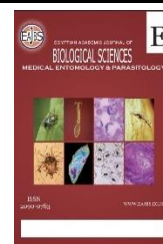
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Assessment of Protease Enzymatic Activity Among Symptomatic Blastocystosis Patients

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ABSTRACT

Blastocystis is the most common eukaryotic parasite of the human gastrointestinal tract. Its pathogenicity remains a matter of debate, however, many recent studies suggest that this organism is a pathogen. Some authors reported that cysteine protease plays important role in the pathogenicity of *Blastocystis* spp. The study investigated the protease activity of *Blastocystis* isolates obtained from stool samples of symptomatic and asymptomatic individuals using gelatin SDS-PAGE and azocasein assay. The present study was carried out on 62 subjects positive for *Blastocystis* whether presenting with gastrointestinal symptoms or not. The symptomatic group (cases, GI) included 42 cases while the asymptomatic (control, GII) group included 20 subjects.

Using gelatin SDS-PAGE analysis, the protease profiles of *Blastocystis* isolates showed 14 protease bands of both high and low molecular weights with significant differences between symptomatic and asymptomatic groups at 35, 60 and 140 kDa MW bands. Statistical analysis of the protease profile of *Blastocystis* isolates showed a significant difference (P value < 0.05) between the two groups. Using Azocasein assay, *Blastocystis* isolates from symptomatic cases show quantitatively higher protease activity than asymptomatic cases but without significant difference between both groups.

INTRODUCTION

Blastocystis spp. is the most common eukaryotic parasite detected in human faecal samples. The prevalence of blastocystosis in the human intestinal tract is recorded to be 0.5% to 60% (Zaneti *et al.*, 2020). The pathogenicity of *Blastocystis* spp. remains controversial since *Blastocystis* spp. were described in symptomatic and asymptomatic cases. *Blastocystis* infection is associated with gastrointestinal symptoms, abdominal pain, diarrhoea, nausea, and flatulence. In addition, *Blastocystis* spp. is also suggested to be involved in irritable bowel syndrome. Differences in the disease-causing potential of *Blastocystis* may be related to high morphological and genetic diversity and thus a significant challenge in researching its pathogenicity (Yakoob *et al.*, 2010) *Blastocystis* spp. produce cysteine protease that breaks up IgA antibodies. Thus, protease allows survival and *Blastocystis* colonization in the human intestinal tract and stimulates inflammatory cytokines that cause tissue damage and gastrointestinal symptoms (Puthia *et al.*, 2005 and 2008).

Blastocystis amoebic form was suggested to have a sticky surface that allows the adherence to the intestinal epithelium and thus the release of proteases to break down the extracellular matrix (Tan and Suresh, 2006). The aim of the work is to investigate the protease activity of *Blastocystis* isolates obtained from stool samples of symptomatic and asymptomatic individuals using gelatin SDS-PAGE and azocasein assay.

MATERIALS AND METHODS

This case-control study was performed in the period from September 2017 to February 2018 on 62 patients who attended the Parasitology Diagnostic and Research Unit, Kasr Al-Aini, Faculty of Medicine, Cairo University. Cases of both sexes included in the study were selected after history taking and stool examination. Subjects with *Blastocystis* infection (n=62) were further classified according to the clinical history into GI (cases group), 42 patients complaining of gastrointestinal (GIT) symptoms, e.g., diarrhea, abdominal pain, constipation and abdominal distention; GII (control group), 20 subjects without GIT symptoms.

In-vitro Culture and Purification:

The stool samples were subjected to in-vitro cultivation to confirm the diagnosis of *Blastocystis* and to amplify *Blastocystis* organisms for subsequent use in the protein profile study. Cultures were done by inoculation in Jones' medium supplemented with 10% of horse serum (Jones, 1946). Cultures were incubated at 37°C for 48 – 72 hours, then examined microscopically for *Blastocystis* organisms. *Blastocystis* was isolated from the culture medium by centrifugation, then samples were purified using density gradient centrifugation on Ficoll-Hypaque to reduce contamination of bacteria (Parkar *et al.*, 2007).

Analysis of the Protease Profile of *Blastocystis* Isolates Using Gelatin-SDS PAGE (Gelatin Zymography):

Stool culture isolates were washed four times with 0.85% NaCl. Then

parasitic lysates were done by the pellet being resuspended in equal volumes of 0.25% (v/v) Triton X-100 and 0.25 M sucrose. Bio-Rad microassay method (Bio-Rad) was used to measure the protein content of the cell lysates. Then gelatin was included in an acrylamide gel. This is an essential step in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method. Gelatin is broken by using electrophoresis by proteases, and then after staining with Coomassie brilliant blue clear bands are seen. Cell lysate of *Blastocystis* was analyzed for the activity of proteases by gelatin-SDS-PAGE, this was done using the SDS-discontinuous buffer system of Laemmli (1970). The concentration of acrylamide in resolving and stacking gel was 12.5% and 5%, respectively. 0.2% gelatin (Sigma) was copolymerized into the resolving gel. Not to destroy the protease enzymatic activity samples were not boiled. A constant current of electrophoresis at 30 mA/gel for 1.5 hours. Then gels were immersed for one hour and shook in 2.5% (v/v) Triton X-100 so as to remove the SDS and for the protease to be more active. Then immersed in an incubation buffer that contains 1 mM DTT for three hours at 37°C until proteinase bands appeared. Staining in 0.12% (w/v) Coomassie brilliant blue R-250 for 30 min. to visualize the bands.

Azocasein Assay for Colorimetric Quantification of Protease Activity:

Parasites were purified as discussed. 15 cycles of freeze and thaw for the cells to be lysed. The tubes with pellets were left at 4°C overnight. Tubes were centrifuged. Bio-Rad Microassay method (Bio-Rad) was used to measure the content of protein. The concentration of each sample was standardized. The cell lysates were supplemented with DTT (2 mM) (Sigma-Aldrich) and incubated at 37°C for 10 minutes to activate proteases. Then 5 mg/ml of Azocasein was prepared at pH 7.4 in PBS. 100 microliters of lysate were mixed with 100 pre-heated (37°C)

azocasein solution. Then incubated at 37°C for one hour. 300 of Ice-cold Trichloroacetic acid was added to stop the reaction. Then the mixture was put on ice for 30 min. To remove undigested azocasein the tubes were centrifuged at 8000 g. The supernatant was added to 500 NaCl (500 mM). Using 440 nm absorbance, the orange solution was measured. Positive (Trypsin) and negative controls (inactivated lysate) were used. This experiment was done in triplicate (Rajamanikam and Govind, 2013).

Statistics:

Statistical package SPSS (Statistical Package for the Social Science) version 23 was used. Mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (percentage) were used for categorical data. Comparisons between quantitative variables were done using the non-parametric Mann-Whitney test. Chi-square (χ^2) test was done to compare categorical data, Exact test was needed when the expected frequency is less than 5 (Chan, 2003). P-values less than 0.05

were considered statistically significant.

RESULTS

Protease Profile Analysis of The Cultured *Blastocystis* Isolates:

Using gelatin-SDS-PAGE analysis, protease bands can be seen as clear bands against a stained dark background. The protease profile of *Blastocystis* isolates showed 14 protease bands of both high (140, 120, 100, 74, 70, 63 and 60 kDa) and low molecular weights (48,45, 39, 35, 33, 32 and 22 kDa) in both cases group GI and control group GII (Fig. 1 and Table 1).

The commonest protease band in both GI and GII was the low molecular weight 32 kDa band which was detected in 92.2% of the parasite isolates of GI and 95% of GII. The least frequent band in GI isolates was the 39 kDa band (19%). While in GII isolates, the least common band was detected at 60 kDa (5%).

Statistical analysis of the protease profile of *Blastocystis* isolates showed a significant difference (P value < 0.05) between the two groups at the low molecular weight 35 kDa band and the two high molecular weights 60 and 140 kDa bands.

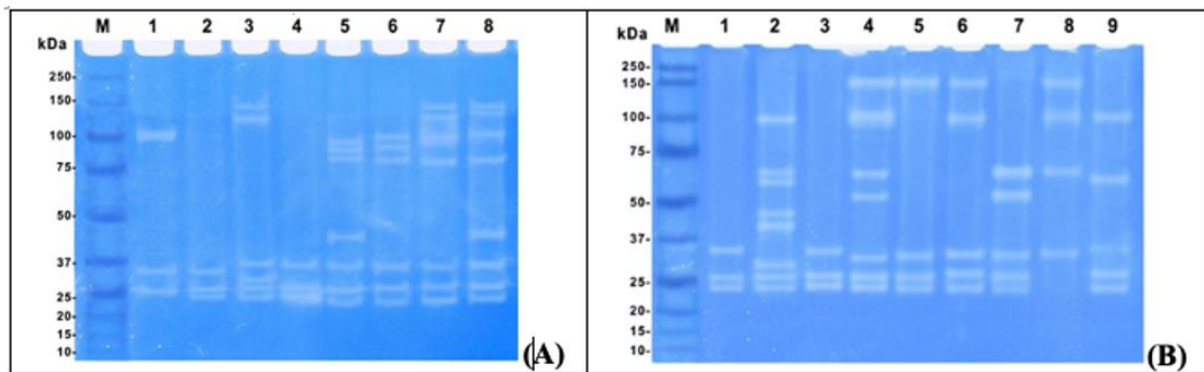


Fig. 1: Gelatin-SDS-PAGE showing the protease profiles of different *Blastocystis* isolates. Protease bands A as clear bands against the blue background. **A:** Lanes 1-2, isolates from the asymptomatic group **GI**. Lanes 3-8, isolates from the symptomatic group **GII**. **B:** Lanes 1, isolates from the asymptomatic group **GII**. Lanes 2-9, isolates from the symptomatic group **GI**.

Table 1: Molecular weight and frequency of protease bands of *Blastocystis* isolate in GI and GII, separated by gelatin SDS-PAGE.

MW of bands in kDa	Group				P value
	GI		GII		
	no	%	no	%	
22	28	66.7%	10	50.0%	0.208
32	39	92.9%	19	95.0%	1
33	14	33.3%	4	20.0%	0.280
35	32	76.2%	8	40.0%	0.005
39	8	19.0%	3	15.0%	1
45	11	26.2%	2	10.0%	0.192
48	16	38.1%	7	35.0%	0.814
60	12	28.6%	1	5.0%	0.045
63	10	23.8%	3	15.0%	0.520
70	21	50.0%	12	60.0%	0.461
74	22	52.4%	10	50.0%	0.861
100	34	81.0%	13	65.0%	0.211
120	26	61.9%	9	45.0%	0.209
140	28	66.7%	8	40.0%	0.047

The table shows a statistically significant difference (P value < 0.05) between the two groups at the 35 kDa band and 60 and 140 kDa bands.

Protease Activity Measurement Using Azocasein Assay:

Protease activity of *Blastocystis* isolates lysate was measured in azocasein units. One azocasein unit is defined as the amount of enzyme producing an increase of 0.01 OD units per hour. Protease activity of GI isolates ranged between 1-20 azocasein units with an arithmetic mean of 6.2, while in GII, the protease activity ranged between 0.1-10 azocasein units with an arithmetic

mean of 4.3 (Fig. 2). Trypsin was used as positive control and the heat-inactivated lysate was used as a negative control.

As regards the relation between protease activity of *Blastocystis* isolates and clinical manifestations in GI (symptomatic), it was found that no statistically significant relationship could be detected between the protease activity and symptoms (P value >0.05) (Table 2).

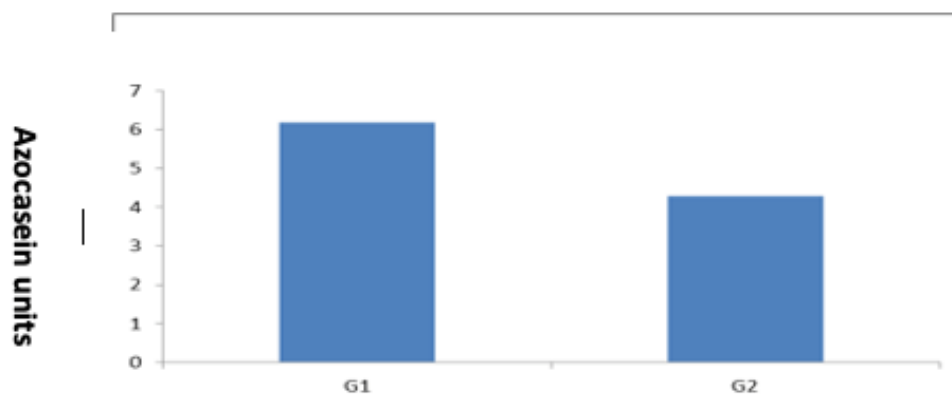


Fig. 2: Mean of protease activity in azocasein units in GI (cases) and GII (control) groups

Table 2: The relation between protease activity of *Blastocystis* isolates and clinical manifestations in GI (cases group)

Symptoms	Protease activity (Azocasein units)		P value
	Mean	Range	
Diarrhea	6.40	1- 20	0.8
Constipation	4.75	3-10	0.9
Abdominal pain	5.41	1- 10	0.3
Abdominal distension	7.67	3 -10	0.4

DISCUSSION

Blastocystis is a single-celled parasite that exists in the gastrointestinal tract of humans. *Blastocystis* has a worldwide distribution. The parasite has been identified as the most common one detected in human faeces (Tan, 2004, Zaneti *et al.*, 2020, and Mossallam and kedr, 2021). The pathogenic role of *Blastocystis spp* is controversial because it has been found in symptomatic and asymptomatic individuals (Kaneda *et al.*, 2000 and Scanlan, 2012)

In Egypt, Rayan *et al.* (2007) recorded a prevalence of 33.3% of *Blastocystis*. Abdel Hameed and Hassanin (2011) detected *Blastocystis* infection in 40% of symptomatic cases. Moreover, Elghareeb *et al.* (2015) in a study on 1200 diarrheic patients in Egypt illustrated *Blastocystis* infection in 22.8% of stool samples.

The gastrointestinal symptoms reported in the present study in cases of GI were abdominal pain in 64.3%, diarrhoea in 47.6%, constipation in 9.5%, abdominal distention in 7.1% and weight loss in 4.8%. Similarly, Kaya *et al.* (2017) reported that abdominal pain was the most frequent symptom in *Blastocystis* infection (76.9%) followed by diarrhoea (50%) and distention (32.6%).

On the other hand, Mahmoud and Saleh (2003), Suresh and Smith (2004) and Hegazy *et al* (2008) found that the main presentation in *Blastocystis* infection is diarrhoea.

Concerning the parasitological methods used to detect *Blastocystis spp.* in the

present work In vitro cultivation using Jones’ medium has been used because it is easy to prepare and yields a bigger number of *Blastocystis* organisms than other culture media as previously demonstrated by many authors (Leelayoova *et al*, 2002, Suresh K Smith, 2004, Souppart *et al.*, 2010, and Yakoob *et al.*, 2010).

Concerning gelatin SDS-PAGE analysis of the protease profile of *Blastocystis* isolates in the present study, 14 protease bands of both high (140, 120, 100, 74, 60, 63 and 60 kDa) and low molecular weights (45, 39, 35, 33, 32 and 22 kDa) have been detected.

Sio *et al.* (2006) in a study on proteases of *Blastocystis* using gelatin SDS-PAGE analysis, revealed nine protease bands, five bands between 44 and 75 kDa and four bands between 20 and 33 kDa.

Bernal-Redondo *et al.* (2008) identified three protease bands between 30 and 50 kDa and two bands between 70 and 100 kD in their study on the protease profiles of *Blastocystis*.

Abdel Hameed and Hassanin (2011) reported in a similar study in Egypt a protease profile similar to that in the present study in the low molecular weight area (32 and 39 kDa) and high molecular weight area (120 and 140 kDa). However, they reported a high MW band of 215 kDa that was not demonstrated in the present work.

The differences in the protease profiles of the *Blastocystis* isolates reported in this work from the previous studies at some high and low molecular weight bands could be due to the difference in the *Blastocystis* isolates as suggested by Abdel-

Hameed and Hassanin (2011). The commonest protease band in the current study was the low molecular weight 32kDa band which was detected in 92.2% of the parasite lysates of the symptomatic group GI and 95% of asymptomatic group GII. The least frequent band in cases of GI isolates was the 39 kDa band (19%). While in asymptomatic group GII, the least frequent band (5%) was detected at 60 kDa. These findings agreed with the work of Abdel Hameed and Hassanin (2011). The authors identified the commonest protease band for *Blastocystis* at 32 kDa, while the least was at 39 kDa. On the other hand, Bernal-Redondo *et al* (2008) detected the high MW 70 kDa protease band in all *Blastocystis* isolates in their study on symptomatic and asymptomatic Mexican children.

Statistical analysis of the protease profiles of *Blastocystis* showed a significant difference between symptomatic and asymptomatic groups at 35, 60 and 140 kDa MW bands. Abdel Hameed and Hassanin (2011) illustrated a significant difference between symptomatic and asymptomatic subjects at 32 kDa and showed that proteases of *Blastocystis* especially at 32 kDa could be identified as a virulence factor responsible for protein degradation and have pathogenic potential in immune evasion.

On the contrary, Bernal-Redondo and his colleagues (2008) reported that protease profiles of *Blastocystis* isolates had not revealed significant differences between symptomatic and asymptomatic subjects.

Using azocasein spectrophotometric assay, in the present study, *Blastocystis* lysates exhibited significant protease activity with azocasein substrate. Protease activity of *Blastocystis* isolates was measured in azocasein units. Protease activity of *Blastocystis* isolates of the cases group ranged between (1- 20 azocasein units) and with an arithmetic mean of 6.1 while the range for isolates of the control group was between (0.1-1

azocasein units) with arithmetic mean (4.3) However, there was no statistical difference between both groups in the average of protease activity.

Few studies were carried out to measure the protease activity of *Blastocystis* isolates using azocasein assay as Sio *et al* (2006), Mirza and Tan (2009) and Rajamanikam and Govind (2013). Sio and his colleagues (2006) studied the protease activity of *Blastocystis* from a patient with gastrointestinal symptoms using azocasein assay and gelatin SDS-PAGE analysis. They proved that *Blastocystis* can produce a cysteine protease with the highest activity at neutral pH that coincides with pH of the colon. The authors of their study also suggested that the proteases secreted by *Blastocystis* might play an important role in the adherence of the parasite and in hydrolyzing proteins of the connective tissue of the intestine.

Puthia *et al.* (2005) reported that *Blastocystis* cysteine protease could cleave human secretory IgA that allows the parasite survival and colonization.

Puthia *et al* (2008) reported that *Blastocystis* proteases induce pro-inflammatory cytokine IL-8 production and thus they are considered potential virulence factors

Mirza and Tan (2009) discussed the protease activity of *Blastocystis* genetic subtypes and demonstrated a quantitative difference between cysteine protease activity of isolates belonging to two different subtypes.

Conclusion:

Using gelatin SDS-PAGE analysis of the protease profiles of *Blastocystis* isolates, 14 protease bands were identified at both high and low molecular weights with significant differences between symptomatic and asymptomatic groups at 35, 60 and 140 kDa MW bands. Using azocasein assay, *Blastocystis* isolates from symptomatic cases show quantitatively higher protease activity than from the asymptomatic subjects with no statistically significant difference between the groups.

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Compliance with Ethical Standards:

Approval of the study design was obtained from the research committee unit, faculty of Medicine, and Cairo university before the commencement of the study. The patients were informed verbally about the purpose of the study and written consent was taken from them before the collection of samples.

Authors Contribution: Hanaa O Fadl did the practical work, wrote the article, revised, and corrected the mistakes. Safeya O. El Bassiouni and Hoda A. El Bolaky revised the writing. Dina Sabry did the practical work, Hanaa O. Fadl, Dina M.H. El Akkad did the practical work, revised, wrote, and submitted the article.

All authors declare that they revised the work and accepted the submission of the article

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Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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