PHENOTYPIC AND PATHOGENIC CHARACTERIZATION OF *BLASTOCYSTIS* ISOLATES FROM EGYPTIAN PATIENTS WITH COLORECTAL CARCINOMA

Thesis

Submitted for partial fulfillment of the M.D. degree
in ***Basic Medical Science (Medical Parasitology)***

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الخواص المظهرية والمسببة للمرض لطفيل المتبرعمة الكيسية (بلاستوسيستس) المعزول من مرضى مصريين مصابين بسرطان القولون والمستقيم

رسالـــــة

توطئة للحصول على درجة الدكتوراه الطبية في العلوم الطبية
(الطفيليات الطبية)

مقدمـــه من

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List of Symbols and Abbreviations

Symbols and abbreviations	Full names
°C	Degree Celsius
µg	Microgram
µg/ml	Microgram / milliliter
µl	Microliter
µm	Micrometer
2^{nd}	Second
3^{rd}	Third
ACF	aberrant crypt foci
ANOVA	Analysis of variance
approx.	Approximate
APS	Ammonium per-sulfate
B.	*Blastocystis*
B. hominis	*Blastocystis hominis*
B. galli	*Blastocystis galli*
B. ratti	*Blastocystis ratti*
Blasto-Ag	*Blastocystis* antigen
BSA	Bovine serum albumin
CaCl2	Calcium chloride
Caco-2	Caucasian colon human epithelial adenocarcinoma cells
CHO cells	Chinese Hamster Ovary cells
cm	Centimeter
CRC	colorectal cancer
CTSB	cathepsin-B
dH_2o	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	Dithiothretol
ELISA	Enzyme-linked immune sorbent assay

ER	Endoplasmic reticulum
F-actin	Filamentous actin.
Fig.	figure
G	Group
GIT	Gastrointestinal tract
gm	Gram
GM-CSF	granulocytes macrophages–colony stimulating factor
GT	Generation time
H & E	Hematoxylin and eosin.
HC84	Human Colorectal Adenocarcinoma Cell Line
HCL	Hydrochloric acid
hr	hour
hrs	hours
H_2SO_4	Sulphuric acid
HT-29	Human intestinal epithelium cell lines
HTC116	Human colorectal carcinoma cell line
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IEC-6	Rodent intestinal cell line.
IECs	Intestinal epithelium cells.
IFN-γ	interferon gamma
IgA	immunoglobulin A
IL-12	Interleukin 12
IL8	interleukin 8
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	inducible nitric oxide synthesize
IP	intestinal permeability
KCl	Potassium chloride
kDa	kilo Dalton
KH_2PO_4	Potassium phosphate, monobasic
lab	laboratory
lbs/in^2	Pounds / inch square

LE	Locke's Egg medium
log	logarithmic
M	Molar
MALDI TOF MS	matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry
MEM	minimum essential medium
mg	Milligram
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentrate
min	Minute
ml	Milliliter
MLC	Minimal lethal concentrate
MLO	mitochondrion-like organelles
mm	Millimeters
mM	Millimolar
mm^3	Cubic millimeter
MTZ	Metronidazole
MTZ^r	Metronidazole-resistant
MTZ^s	Metronidazole-sensitive
MW	Molecular weight
N	number
Na_2HPO_4	Sodium phosphate, dibasic
NaCl	Sodium Chloride
$NaHCO_3$	Sodium bicarbonate
NandII	*Blastocystis* subtype 1 isolate
NC	Negative control
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institute of Health.
NJM	Nelson—Jones' medium
nm	Nanometer.
NO	nitric oxide

O_2^-	Superoxide
Org	Organism
p53	pro-apoptotic protein 53
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PC	Personnel computer
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
PM	Pavlova's medium
PML	polymorphonuclear leucocytes
PMNCs	Polymorphonuclear cells
PVA	Polyvinyl alcohol
P-value	Probability value
RFLP	restriction fragment length polymorphism
RN94-9	*Blastocystis* sp. non-invasive strain in rats
ROS	reactive oxygen species
rpm	Round per minute.
RPMI	Roswell Park Memorial Institute medium
s (in 18s rDNA)	Svedberg unit
SD	standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sec	seconds
SEM	Scanning electron microscope
SG	Subgroup
sp.	Species (singular)
spp.	Species (plural)
SPSS	Statistical package for Social Science
SSU-rDNA	small subunit ribosomal deoxyribonucleic acid
SSU rRNA	small subunit ribosomal ribonucleic acid
STs	subtypes

STS	sequence-tagged-site
T-84	Human intestinal epithelium cell lines
TBRI	Theodor Bilharz Research Institute
TEF	Trans-epithelial flux
TEM	transmission electron microscopy
TEMED	Tetra methyl ethylene diamine
TER	Trans-epithelial resistance
TGF-β	transforming growth factor β
Th1	T-helper 1.
Th2	T-helper 2.
TJ	Tight junction.
TJs	tight junctions
TNFα	Tumor necrosis factor α
U	Unit
UK	United Kingdom
USA	United State of America
UV	Ultraviolet
UVR	Ultraviolet rays
V	Volts
v/v	Volume / Volume
VO	viable organisms
w/v	Weight / Volume
WR1	a *Blastocystis ratti* isolate with zoonotic potential
ZO1	Zonula Occludens-1

ABSTRACT

Previous studies had related *Blastocystis* sp. to cancer colon, as *Blastocystis* sp. antigen facilitates the proliferation of cancer cells *in vitro* and the organisms caused enhancement of drug-induced carcinogenesis *in vivo*.

The present work aimed to investigate the phenotypic and pathogenic characters and pathogenic effects of human-derived *Blastocystis* isolates from patients with colorectal carcinoma and from carriers without colorectal carcinoma (CRC) both symptomatic and asymptomatic.

Nineteen *Blastocystis* sp. isolates were recruited from CRC patients, and apparent Non-CRC symptomatic and asymptomatic carriers. For each isolate the following was done: growth kinetics study and MTZ-sensitivity assay in LE medium, examination of surface ultrastructure of organisms under SEM, analysis of protein profile and zymography by SDS-page. Experimental blastocystosis have been induced in mice to examine histopathological sections of large intestine for pathogenic effects of different isolates

Statistical significant differences existed between CRC and Non-CRC isolates as regards the surface ultrastructure, showing a coarse, intensely folded rough surface of CRC isolates, in contrast to, slightly rough and smooth surface of symptomatic and asymptomatic isolates, respectively, from Non-CRC carriers. A significant presence of two protein bands of 230 and 32 kDa could differentiate between CRC and Non-CRC isolates by SDS-page protein analysis. Significant differences in invasive proliferative pathogenic effects were recorded in histopathological sections of large intestine of mice infected by isolates from CRC patients and isolates from Non-CRC carriers. Zymography showed statistical non-significant increased number of protease bands in CRC isolates than Non-CRC isolates. MTZ-sensitivity was nearly similar for CRC and Non-CRC asymptomatic isolates, both are significantly higher than in symptomatic isolates. *In vitro* growth kinetics of CRC and Non-CRC symptomatic isolates were nearly similar with higher peaks than the slower growing Non-CRC-asymptomatic isolates. In conclusion, phenotypic and pathogenic differentiating characters exist between CRC and Non-CRC *Blastocystis* isolates.

Keywords: *Blastocystis* sp. – Colorectal carcinoma – Symptomatic – Asymptomatic – Phenotypic characters – Pathogenic effects.

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Protocol of

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INTRODUCTION

Blastocystis is an enteric protistan parasite with zoonotic potential (**Tan, 2008**). It is one of the most common parasites colonizing the human gut, with prevalence ranging between 10% of the population in developed countries and 50% in developing countries (**Wong *et al*., 2008**). The name *Blastocystis hominis* was previously used in the designation of the species infecting humans. Recent phylogenetic analysis has shown that *Blastocystis hominis* as a unique entity does not exist, and more than one species of *Blastocystis* infects humans and suggest the existence of zoonotic strains of the parasite (**Noël *et al*., 2005**). Thus, it is preferable to use the term "*Blastocystis* species" instead (**Stensvold *et al*., 2007**).

Blastocystis is a polymorphic protozoan. In addition to the three commonly recognized morphological forms: vacuolar, granular and amoeboid, (**Vdovenko, 2000**), later studies described several additional forms; cystic, avacuolar and multi-vacuolar (**Tan, 2008** and **Stensvold *et al*., 2009**) with the vacuolated form being the most common and the small cyst form with a thick surface coat, surrounding the cells, the transmissible form of the parasite.

Clinical symptoms associating *Blastocystis* infection in humans (**Windsor *et al*., 2002** and **Boorom *et al.,* 2008**) vary as some patients are asymptomatic while others display severe gastrointestinal symptoms with abdominal cramps diarrhea, nausea, vomiting, and bloating. Many reports also found a significant association between infection with *Blastocystis* and irritable bowel syndrome (IBS) **(Dapoigny, 2009** and **Poirier *et al*., 2012).** Although metronidazole is the treatment of choice for *Blastocystis* infections, resistance to metronidazole was reported **(Yakoob *et al*., 2004** and **Stein, 2007).** The virulence factors, pathogenicity and other risk factors involved in disease manifestation are still obscure (**Scanlan, 2012**). Several studies linked the pathogenicity to interaction between parasite products (e.g. cysteine protease) and enterocytes influencing host inflammatory and immunological responses. Secretion of proteases and other hydrolytic enzymes by *Blastocystis* have been identified and attributed to be responsible for the pathogenesis of gastrointestinal symptoms (**Puthia *et al*., 2008** and

Abdel-Hameed and Hassanin, 2011) and cleavage of human secretory immunoglobulin A thereby helping in immune evasion and promoting parasite survival in vivo (**Puthia *et al*., 2005**).

Studies on the genetic and molecular characterization of *Blastocystis* sp. isolates derived from human showed that humans are natural hosts of nine subtypes (ST1 through ST9), of which ST1 to ST4 are by far the most common (**Stensvold *et al*., 2007** and **Stensvold, 2013**). Genotyping using partial small subunit ribosomal RNA (ssrRNA) analysis of isolates from Egyptian symptomatic patients identified a total of five STs (ST1, ST2, ST3, ST4, and ST6) of which ST3 was the most common ST (61.90%) followed by ST1 (19.05%) and ST2 (19.05%)(**Souppart *et al.*, 2009**).

Colorectal cancer (CRC) is a common cancer worldwide. It is the third most common cancer worldwide after lung and breast cancers (**CDC, 2011**). CRC affects men and women of all racial and ethnic groups, and is most often found in people aged 50 years or older in developed countries. CRC was diagnosed in 14% of colonoscopies performed in Egypt. The mean age of patients was 51 years with 25% of cancers occurring in patients aged less than 40 years (**Gado *et al.*, 2014**).

Several studies have shown a correlation between the inflammation that is caused by infectious agents such as parasites and the development of cancer in human **(Fitzpatrick, 2001).** Evidence of *Blastocystis* parasite facilitating cancer cell growth was proved through recording the cytopathic effect, cellular immunomodulation, and apoptotic responses of *B*. *hominis*, especially in malignancy. Significant ultra-structural lesions on the ileocecal mucosa in mice infected with *B. hominis* were reported **(Zhang *et al*., 2006).** Also**,** an intense inflammatory reaction and precancerous polyps were later described in caecum and proximal colon tissues in rats infected with *Blastocystis* species **(Hussein *et al*., 2008)**. **Chandramathi *et al*. (2009)** have suggested that *B. hominis* may possess the ability to induce the growth of colorectal cancer cells by inhibiting the apoptotic effect of colon cancer cells, and in **2010, Chandramathi *et al.*,** reported that solubilized *Blastocystis* antigen facilitated the proliferation of colon cancer cells HCT116. Also, **Chan *et al.***

(2012) showed that *B. hominis* isolated from an asymptomatic individual could facilitate the proliferation and growth of existing cancer cells. **Kumarasamy *et al*. (2013)** found that *Blastocystis sp.* subtype 3 triggers higher proliferation of human colorectal cancer cells, and in **2014**, the authors showed a significant *Blastocystis* infection among colorectal carcinoma patients (21.08%) compared to the asymptomatic normal individuals (9.95%)**(Kumarasamy *et al*., 2014).**

Investigation of the pathogenic potential of *Blastocystis* in humans have focused on genotypic analysis **(Kaneda *et al*., 2001** and **Yan *et al*. 2006)** without providing phenotypic information on the isolates studied. Therefore, no comparisons of phenotypic similarities or differences could be made between the isolates **(Tan *et al*., 2008)**.

The existence of extreme genetic diversity among *Blastocystis* isolates necessitates the extrapolation of observations of morphology, drug resistance and pathogenesis from one isolate to another.

AIM OF THE WORK

The aim of the present work is to study the phenotypic and pathogenic characteristics of *Blastocystis* isolates from patients having colorectal carcinoma in comparison with those isolated from infected individuals without colorectal carcinoma.

PLAN OF THE WORK

I. STUDY DESIGN

- A cross sectional study will be done in which *Blastocystis* Isolates collected from 2 groups will be compared for their phenotypic and pathogenic characteristics:
  - **Group 1:** Patients with colorectal carcinoma attending Ain Shams University Hospitals
  - **Group 2:** infected individuals without colorectal carcinoma.
- **Inclusion and exclusion criteria:** The samples will be collected from patients with colorectal carcinoma immediately after diagnosis and before receiving anti-cancer therapy. Samples collected from patients with other intestinal parasites will be excluded from the study.
- The study will be done according to the regulations of the Ain Shams University Ethical Committee that complies with the 1964 Helsinki declaration. The nature of the study will be explained to individuals enrolled in this study and oral consents will be obtained from them before sample collection.
- The sample size included in each group will be determined using appropriate statistical sample size equation methods after consultation of a medical statistician considering the prevalence of colorectal carcinoma and *Blastocystis* in Egypt.

II. SAMPLE COLLECTION AND CULTURING OF THE PARASITE:

- Stool samples will be collected from each patient in a clean container and immediately subjected to parasitological examination by direct and formalin-Ethyl acetate concentration techniques, modified Ziehl–Neelsen- and trichrome-stained smears to diagnose infection with *Blastocystis* or other parasites.
- Positive stool samples for *Blastocystis* will be cultured on suitable medium and axenized by repeated sub-culturing every 2 – 3 days in fresh medium containing antibiotics.
- Study the growth kinetics of the parasite in culture.
- Determination of in-*vitro* metronidazole sensitivity.

III. HARVESTING OF *BLASTOCYSTIS* AND STUDY OF PHENOTYPIC AND PATHOGENIC CHARACTERISTICS OF THE ISOLATES:

Parasite isolates will be maintained by culturing and sub-culturing in suitable medium, the harvested parasites will be subjected to:

i- Studying the surface ultra-structure characters of representatives isolates from both groups by electron microscopy.

ii- Histopathological study by experimental infection of Swiss *albino* mice with the collected isolates according to the method of **Yoshikawa *et al.* (2004)** and **Zhang *et al.* (2006).**

iii- Soluble parasite antigen will be prepared from each isolate according to **Chen *et al.* (1999),** and will be subjected to Sodium-dodecyl-sulphate polyacrylamide gel-electrophoresis (SDS-PAGE) according to **Laemmli (1970)** to study:

- The protein profile.
- The proteinase activity.

IV. COMPARING AND ANALYSIS OF THE RESULTS

The criteria under study for both groups of isolates will be compared using the appropriate statistical technique.