
Genotyping of *Cryptosporidium* Species Found in Stools of Infected Children

Thesis

Submitted in Partial Fulfillment of
M.D. Degree of
Basic medical science (Parasitology)

By

Aisha Abbas El-Awady (M.B.,B.Ch.;M.Sc.)
Assistant lecturer of parasitology, Faculty of Medicine,
Cairo University

Under Supervision of:

Prof. Dr. Hoda Helmy ElRahimy

Professor of Parasitology
Faculty of Medicine, Cairo University

Prof. Dr. Hadir Ahmed El-Mahallawy

Professor of Clinical Pathology
National Cancer Institute, Cairo University

Dr. Hager Abd AlHameed Shaheen

Lecturer of Parasitology
National Hepatology and Tropical
Medicine Research Institute

Faculty of Medicine

Cairo University

2007

Abstract

The present work constitutes a prospective study on a total of 97 pediatric patients; 65 cancerous patients and 32 non-cancerous patients suffering from diarrhea. All faecal specimens were examined parasitologically using the MZN and EIA techniques. Samples testing positive for *Cryptosporidium* were examined using the PCR-RFLP technique which amplified the *Cryptosporidium* oocyst wall protein (COWP) gene. Among 97 diarrheic pediatric cases, 47 (48.5%) were positive by nested PCR. *Rsa* I digestion of nested PCR product of COWP gene revealed the presence of 2 genotypes: genotype 1 in 32 (68%) and genotype 2 in 15 (32%) of cases. In this study, comparable correlations were observed between the clinical parameters associated with the 2 genotypes as there were no statistically significant associations between genotypes and age, sex, vomiting, abdominal pain or fever or dehydration. The fact that Genotype 1 was found to be relatively more prevalent than genotype 2 among all groups of patients examined suggests a relatively greater risk of human source of infection than zoonosis.

Key words: COWP, *Cryptosporidium parvum*, genotyping

ACKNOWLEDGEMENT

The completion of this work would not have been possible without the support and assistance of a number of people. In the beginning, I wish to express my sincere gratitude to **Prof. Dr. Olfat Mahmood AlMatarawy**, Professor and Chairman of the Parasitology Department, Faculty of Medicine, Cairo University, for her extremely valuable guidance and constant encouragement.

No words can fulfil my feelings of appreciation to and respect for **Prof. Dr. Hoda Helmy ElRahimy**, Professor of Parasitology, Faculty of Medicine, Cairo University, for her endless support and help, continuous guidance and valuable advice in this work, and for her overwhelming kindness and maternal compassion. I wish to express my extreme gratitude for everything she has done for me.

I would also like to convey my profound gratitude and everlasting appreciation to **Prof. Dr. Hadir Ahmed El-Mahallawy**, Professor of Clinical Pathology, National Cancer Institute, Cairo University, for her unlimited efforts, encouragement, and continuous guidance and support throughout this work.

I offer my deepest appreciation to **Dr. Hager Abd AlHameed Shaheen**, Lecturer of Parasitology, National Hepatology and Tropical Medicine Research Institute, for her highly valuable supervision, helpful guidance and useful advice.

I owe special thanks to **Dr. Laila Ahmed Rashed** Lecturer of Biochemistry, Faculty of Medicine, Cairo University, for providing the Biochemistry Department facilities and for all the assistance she offered for the successful completion of this work.

I also wish to thank all staff members and my colleagues in the Parasitology Department, Faculty of Medicine, for their constant encouragement and cooperation during my work.

And finally my gratitude goes to my family, without whose support I could not have continued my studies. I owe a debt of gratitude to my father for his endless encouragement, support, guidance, and assistance.

List of Abbreviations

A:	adenine
ABC:	avidin biotin complex
Abd. Pain:	abdominal pain
Approx:	approximately
bp:	base pair
°C:	degree celcius
C.:	<i>Cryptosporidium</i>
Caco-2:	human colonic carcinoma cell line
cAMP:	cyclic adenosine monophosphate
CD:	cluster of differentiation
CHEF:	contour-clamped homogeneous electric field
COWP:	<i>Cryptosporidium</i> oocyst wall protein
Cox:	cyclooxygenase
Cpgp:	<i>Cryptosporidium parvum</i> glycoprotein
CSA:	<i>Cryptosporidium</i> specific antigen
DAB:	diaminobenzidine
DFA	direct fluorescent-antibody
DHFR:	dihydro folate reductase
DNA:	deoxyribonucleic acid
dNTP:	deoxynucleotide triphosphate
E:	extended
EB:	ethidium bromide
EDTA:	ethylenediamine tetraacetic acid
EIA:	enzyme immunoassay
ELISA:	enzyme linked immunosorbant assay
EM:	electron microscope
f:	female
FEA:	formalin-ethyl acetate
fg	femtogram
G:	glycine
g:	gravity
gm:	gram
GST:	glutathione S-transferase
h:	hour
HAART:	highly active anti-retroviral therapy
HCl:	hydrochloric acid

HIV:	human immunodeficiency virus
<i>hsp</i> :	heat shock protein gene
H ₂ O ₂ :	Hydrogen peroxide
IF:	immunofluorescence
Ig:	immunoglobulin
IL:	interleukin
IMS:	immunomagnetic bead separation
Inc:	incorporation
ITS:	internal transcribed spacer
Kb:	kilo base
KCl:	potassium chloride
KDa:	kilo dalton
L:	litre
Ltd:	limited
m:	male
M:	mole
mm:	millimetre
mM:	millimole
mAbs:	monoclonal antibodies
Mbp:	million base pair
mg:	milligram
MgCl ₂ :	magnesium chloride
Min:	minute
ml:	millilitre
MZN:	modified Ziehl-Neelsen
N:	nested
NaCl:	sodium chloride
Neg:	negative
NF:	nuclear factor
No:	number
<i>NotI</i> :	<i>Nocardia otitidiscaviarum</i>
nr:	nuclear ribosomal
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PFGE:	pulsed field gradient electrophoresis
pH:	hydrogen potential
pmoles:	pico moles
Pos:	positive
P value:	probability value
r:	ribosomal

RAPD:	random amplified polymorphic deoxyribonucleic acid
<i>RasI</i> :	<i>Rhodobacter sphaeroides</i> I
RFLP:	restriction fragment length polymorphism
RPH:	reverse passive haemagglutination
rpm:	revolutions per minute
rRNA:	ribosomal ribonucleic acid
SCID:	severe combined immunodeficient
<i>SfiI</i> :	<i>Streptomyces fimbriatus</i> I
Spp:	species
SSF:	sheather sucrose flotation
SSrRNA:	small subunit ribosomal ribonucleic acid
S rDNA:	small subunit ribosomal deoxyribonucleic acid
S rRNA:	small subunit ribosomal ribonucleic acid
SSU:	small subunit
Std:	standard
T:	thymine
TAE:	tris-acetate ethylenediamine tetraacetic acid
<i>Taq</i> :	<i>Thermus aquaticus</i>
TE:	tris ethylenediamine tetraacetic acid
TNF:	tumour necrosis factor
TRAP-C:	thrombospondin-related adhesive protein of <i>Cryptosporidium</i>
tRNA:	transfer ribonucleic acid
UK:	United Kingdom
USA:	United States of America
UV:	ultra violet
v/v:	volume/ volume
w/v:	weight/volume
μ:	micron
μL:	microlitre
μm:	micrometer

CONTENTS

	Page
ACKNOWLEDGEMENT	<i>i</i>
LIST OF ABBREVIATIONS	<i>iii</i>
CONTENTS	<i>vi</i>
LIST OF FIGURES	<i>ix</i>
LIST OF TABLES	<i>xi</i>
INTRODUCTION	1
AIM OF THE WORK	4
REVIEW OF LITERATURE:	
• HISTORICAL BACKGROUND AND CURRENT STATUS	5
• TAXONOMY	7
• EPIDEMIOLOGY	10
• UNIQUENESS OF <i>Cryptosporidium parvum</i>	15
• PATHOGENESIS AND CLINICAL PICTURE	19
• SCOPE OF METHODS OF DETECTION OF <i>CRYPTOSPORIDIUM</i>	23
1- Conventional and immunological methods	23
2- Molecular methods	26
• GENETICS OF CRYPTOSPORIDIUM	27
1- The Nucleus	27

2- Chemical and functional organization of the DNA	27
3- Karyotype	28
4- Characterized genes	29
5- Repetitive sequences	31
6- Ribosomal genes	32
7- Extra-nuclear genomes	33
8- The <i>Cryptosporidium</i> Oocyst Wall Protein genotypes	35
• <i>CRYPTOSPORIDIUM</i> SPECIATION AND SUBSPECIATION	37
1- Phenotypic basis for <i>Cryptosporidium</i> speciation	37
2- Genotypic characterization	40
3- Valid <i>Cryptosporidium</i> Species	41
4- <i>Cryptosporidium</i> species infecting humans	43
• MOLECULAR GENOTYPING	46
1- Genetic markers of <i>Cryptosporidium</i>	46
2- Common Molecular approaches for genotyping	47
• POLYPHASIC TYPING OF <i>CRYPTOSPORIDIUM</i> SPECIES	50
• IMPORTANCE OF GENOTYPING	53
1- Epidemiological significance of strain variation	53
2- Medical significance of <i>C. parvum</i> strain variation	55
3- Revised taxonomy	55
MATERIALS AND METHODS	57

• PARASITOLOGICAL EXAMINATION	58
• IMMUNOLOGICAL STUDIES	61
• MOLECULAR DIAGNOSIS AND GENOTYPING OF <i>CRYPTOSPORIDIUM</i>	66
RESULTS	74
DISCUSSION	95
CONCLUSION AND RECOMMENDATIONS	117
SUMMARY	120
REFERENCES	123
ARABIC SUMMARY	

List of Figures

Figure	Page
Fig. (1): Life cycle of <i>Cryptosporidium parvum</i>	16
Fig. (2): Structural organization of COWP of <i>C. parvum</i>	35
Fig. (3): Restriction maps of the PCR products amplified with the oligonucleotides cry-15 and cry-9.....	36
Fig. (4): The conventional ABC technique.....	63
Fig. (5): Thermal cycler.....	71
Fig. (6): Hybrid horizontal gel tank.....	72
Fig. (7): Percentages of cryptosporidiosis cases among the studied groups.....	74
Fig. (8): Occurrence of vomiting among non-cryptosporidial and cryptosporidial diarrheic pediatric cases.....	77
Fig. (9): Occurrence of abdominal pain among non-cryptosporidial and cryptosporidial diarrheic pediatric cases.....	78
Fig. (10): Occurrence of dehydration among non-cryptosporidial and cryptosporidial diarrheic pediatric cases.....	80
Fig. (11): Occurrence of fever among non-cryptosporidial and cryptosporidial diarrheic pediatric cases.....	82
Fig. (12): An agarose gel electrophoresis showing nested PCR products of the <i>Cryptosporidium</i> COWP gene.....	83
Fig. (13): Photomicrographs of a stool specimen stained with MZN stain showing red-staining oocysts at a	

microscopic magnification of x1000.....	83
Fig. (14): Photomicrographs of a stool specimen after EIA staining.....	84
Fig. (15): Percentages of cryptosporidiosis among diarrheic pediatric cases as detected by PCR, MZN and EIA tests	85
Fig. (16): An agarose gel electrophoresis showing RFLP products after digestion with <i>RsaI</i> endonuclease.....	86
Fig. (17): Distribution of COWP genotypes among pediatric cases with cryptosporidiosis.....	87
Fig. (18): Occurrence of vomiting among cryptosporidial pediatric cases harbouring COWP genotypes 1 and 2...	90
Fig. (19): Occurrence of abdominal pain among cryptosporidial pediatric cases harbouring COWP genotypes 1 and 2...	91
Fig. (20): Occurrence of dehydration among cryptosporidial pediatric cases harbouring COWP genotypes 1 and 2.....	92
Fig. (21): Occurrence of fever among cryptosporidial pediatric cases harbouring COWP genotypes 1 and 2.....	94

List of Tables

Table	Page
Tab. (1): Main structural features of the nuclear genomes of apicomplexan parasites.....	29
Tab. (2): Completely sequenced <i>Cryptosporidium parvum</i> genes.....	30
Tab. (3): Biological differences among putative <i>Cryptosporidium</i> spp.....	38
Tab. (4): Some of the most important species of <i>Cryptosporidium</i> and their hosts.....	42
Tab. (5): Age distribution among cryptosporidial and non-cryptosporidial diarrheic children with and without cancer.....	75
Tab. (6): Sex distribution among cryptosporidial and non-cryptosporidial diarrheic children with and without cancer.....	75
Tab. (7): Occurrence of vomiting among non-cryptosporidial and cryptosporidial diarrheic pediatric cases.....	77
Tab. (8): Occurrence of abdominal pain among non-cryptosporidial and cryptosporidial diarrheic pediatric cases.....	78
Tab. (9): Occurrence of dehydration among non-cryptosporidial and cryptosporidial diarrheic pediatric cases.....	79
Tab. (10): Occurrence of fever among non-cryptosporidial and cryptosporidial diarrheic pediatric cases.....	81
Tab. (11): Results of MZN staining of stool samples of	

	diarrheic pediatric cases, in relation to PCR findings	82
Tab. (12):	Results of the EIA on stool samples of diarrheic pediatric cases, in relation to PCR findings	84
Tab. (13):	Distribution of COWP genotypes among cancerous and non-cancerous pediatric cases with cryptosporidiosis.....	86
Tab. (14):	Age distribution among genotypes detected in both cancerous and non-cancerous patients.....	87
Tab. (15):	Sex distribution among COWP genotypes detected in cryptosporidial pediatric patients.....	88
Tab. (16):	Occurrence of vomiting among cryptosporidial pediatric cases harbouring COWP genotypes 1 and 2.....	89
Tab. (17):	Occurrence of abdominal pain among cryptosporidial pediatric cases harbouring COWP genotypes 1 and 2.....	91
Tab. (18):	Occurrence of dehydration among cryptosporidial pediatric cases harbouring COWP genotypes 1 and 2.....	92
Tab. (19):	Occurrence of fever among cryptosporidial pediatric cases harbouring COWP genotypes 1 and 2.....	93

Introduction

Species of *Cryptosporidium* are protozoan parasites (Apicomplexa) which infect a wide range of vertebrate hosts. The clinical signs of cryptosporidiosis in humans are mainly diarrhoea, dehydration, malabsorption, weight loss and/or wasting. Infection is frequently self-limiting, but chronic infections may establish, particularly (but not exclusively) in young and/or immunodeficient or immunosuppressed individuals (Fayer *et al.*, 2000).

Cryptosporidia are intracellular, extracytoplasmic protozoan parasites with a monoxenous life cycle. They invade the microvillus border of the gastrointestinal and respiratory epithelium of a wide range of vertebrate species, and may cause considerable economic losses in livestock (Sreter *et al.*, 2000). At present, no effective therapy is available (Tzipori, 1998). Environmentally resistant oocysts are transmitted by the fecal-oral route, but zoonotic infection and person-to-person transmission are also known (O'Donoghue, 1995). Cryptosporidia have been reported to cause several waterborne and food-borne outbreaks worldwide (Smith and Rose, 1998), the most severe occurred in Milwaukee, Wisconsin, USA in 1993, where more than 400,000 people were infected (Mac Kenzie *et al.*, 1994). *Cryptosporidium* oocysts are resistant to disinfectants commonly used in drinking water treatment, consequently, they have become a major concern to public health and to the drinking water industry (Xiao *et al.*, 2000) and are recognized as a major cause of waterborne diarrheal disease worldwide (Fayer *et al.*, 1997).