

**Evaluation of Different Copro-Preservation
Conditions on DNA Extraction and PCR
Detection of *Cryptosporidium* species**

Thesis

Submitted For partial fulfillment of Master Degree of
Science in **Medical Parasitology**

By

Marmar Ahmed Hanafy

M.B, B.Ch

Demonstrator of Parasitology

Faculty of Medicine, Ain Shams University

Under supervision of

Prof. Dr/ Iman Moawad Abdelsalam

Professor of Medical Parasitology

Faculty of Medicine, Ain Shams University

Dr/ Rania Mohammad Sarhan

Lecturer of Medical Parasitology

Faculty of Medicine, Ain Shams University

Dr/ Hanan Helmy

Assistant Professor

Research and Training Center on Vectors of Disease

Ain Shams University

Medical Parasitology Department

Faculty of Medicine

Ain Shams University

2015

تقييم الطرق المختلفة لحفظ البراز وتأثيرها على استخلاص الحمض النووي والتشخيص الجزئي للكريبتوسبورidium بارفام

رسالة

توطئة للحصول على درجة الماجستير في العلوم الطبية
﴿ علم الطفيليات (الطبية) ﴾

مقدمة من

مرمر أحمد حنفي

معيدة بقسم علم الطفيليات الطبية، كلية الطب، جامعة عين شمس

(المشرفون)

الأستاذة الدكتورة/ إيمان معوض عبد السلام

أستاذ بقسم علم الطفيليات الطبية

كلية الطب- جامعة عين شمس

د. / رانيا محمد سرحان

مدرس بقسم علم الطفيليات الطبية

كلية الطب- جامعة عين شمس

أستاذ مساعد/ حنان حلمي

مركز أبحاث ناقلات الأمراض، جامعة عين شمس

قسم علم الطفيليات الطبية

كلية الطب

جامعة عين شمس

٢٠١٥

List of Abbreviations

Abbreviation	Full name
AF	Acid fast
AFLP	Amplified fragment length polymorphism
AIDS	Acquired immunodeficiency syndrome
AP	Auramine-phenol
C.	<i>Cryptosporidium</i>
CD	Cluster of differentiation
CDPKs	Calcium-dependent protein kinases
COWP	<i>Cryptosporidium</i> oocyst wall protein
DFA	Direct fluorescent-antibody
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
dsDNA	Double stranded DNA
EIAs	Enzyme immunoassays
ELISA	Enzyme linked immunosorbent assay
FISH	Fluorescence in situ hybridization
GC	Guanine and cytosine
GP60	60kDa glycoprotein
H & E	Hematoxylin and eosin
HAART	Highly active anti-retroviral therapy
HIV	Human immunodeficiency virus
HSP 70	70 KDa heat shock protein
ICT	Immunochromatographic test
IFN-γ	Interferon gamma
IL	Interleukin
KDa	Kilo Dalton
Kdichromate	Potassium dichromate
LAMP	Loop-mediated isothermal amplification
mAbs	Monoclonal antibodies
mg	Milli gram

List of Abbreviations

.....

Abbreviation	Full name
MHC	Major histocompatibility complex
MLST	Multilocus sequence typing
mm³	Cubic Millimeter
MZN	Modified Ziehl-Neelsen
NK	Natural killer cells
PCR	Polymerase chain reaction
PCR-RFLP	PCR-Restriction fragment length polymorphism
PVA	Polyvinyl alcohol
RAPD	Random amplification of polymorphic DNA
RT	Room temperature
SAF	Sodium acetate formaldehyde
SAM	S-adenosyl-methionine synthetase
SNP	Single nucleotide polymorphism
Spp.	Species
SsDNA	Single stranded DNA
SSU rRNA	Small subunit ribosomal RNA
Th	T helper sells
TNF-α	Tumor necrosis factor <i>alpha</i>
%	Percentage
°C	Celsius degree
µl	Micro litre
µm	Micro meter

List of Tables

Number		Page
Table(1):	Primers used for the amplification of <i>Cryptosporidium</i> oocyst wall protein COWP gene.	82
Table(2):	Components of the 1ry and 2ry PCR reaction assays.	83
Table(3):	The thermocycler program showing the used cycling conditions.	83
Table(4):	Description of sensitivity of studied preservatives after 10, 20 and 30 days individually.	104
Table(5):	Description of overall sensitivity of studied preservatives along whole duration.	105
Table(6):	Comparison between different preservatives as regard overall performance.	105
Table(7):	Comparison between different preservatives after 10, 20 and 30 days of preservation.	106
Table(8):	Comparison between -20 °C and other preservatives as regard performance after 10, 20 and 30 days.	109
Table(9):	Comparison between K dichromate at RT and K dichromate at 4°C as regard performance after 10, 20 and 30 days.	110
Table (10):	Comparison between different preservatives along different intervals of extraction.	111

List of figures

Number	Page
Fig. (1): Classification of <i>Cryptosporidium</i> .	8
Fig. (2): <i>Cryptosporidium</i> species	9
Fig. (3): Life cycle of <i>Cryptosporidium parvum</i> .	13
Fig. (4): Ultra structure of Apicomplexa.	14
Fig. (5): Sporulation of <i>Cryptosporidium</i> oocysts and release of four sporozoites.	16
Fig. (6): Mechanism of diarrhea caused by <i>Cryptosporidium</i> .	20
Fig. (7): <i>Cryptosporidium</i> oocysts in wet mount.	34
Fig. (8): Various staining techniques used for staining of <i>Cryptosporidium</i> oocysts in fecal samples.	35
Fig. (9): <i>Cryptosporidium</i> oocysts stained with Modified AF stain.	36
Fig. (10): Hematoxylin and eosin stain of intestinal epithelium.	37
Fig. (11): <i>Cryptosporidium</i> oocysts stained with safranin stain.	37
Fig. (12): <i>Cryptosporidium</i> oocysts stained with Modified acid fast trichrome.	38
Fig. (13): <i>Cryptosporidium parvum</i> oocysts stained with the fluorescent stain auramine-rhodamine.	40
Fig. (14): Transmission electron micrograph of a murine small intestinal epithelial cell infected by <i>Cryptosporidium</i> .	40
Fig. (15): <i>Cryptosporidium</i> spp. oocysts labeled with immunofluorescent antibodies.	42
Fig. (16): Model for ICT.	44

List of Figures

Number	Page
Fig. (17): A diagram illustrating FISH technique.	46
Fig. (18): Conventional PCR.	49
Fig. (19): Nested PCR.	52
Fig. (20): Leak-proof plastic containers used for collection of stool samples.	62
Fig. (21): Basic fuchsin powder and working Carbol fuchsin solution.	69
Fig. (22): Malachite green used for the staining of the background.	69
Fig. (23): Slides after staining with MZN stain.	69
Fig. (24): Principle of ICT.	70
Fig. (25): ICT showing <i>Cryptosporidium</i> positive and negative results.	73
Fig. (26): Preservative solutions used for stool samples preservation: k dichromate, formaldehyde, and ethyl alcohol.	74
Fig. (27): Qiagen stool DNA extraction Mini Kit.	79
Fig. (28): Thermal cycler used in DNA amplification (Professional thermocycler, Biometra, Applied Biosystem, California, USA).	84
Fig. (29): Gel electrophoresis apparatus used for detection of the PCR products.	87
Fig. (30): MZN stained smear for <i>Cryptosporidium</i> positive stool samples.	89
Fig. (31): MZN stained smear from negative stool samples.	89
Fig. (32): Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from fresh stool samples 1, 2, 3, 4 and 5.	90
Fig. (33): Agarose gel electrophoresis for the products of	91

List of Figures

Number	Page
	the nPCR targeting COWP gene at 553 bp, for DNA extracted from fresh stool samples 6, 7, 8, 9 and 10.
Fig. (34):	Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 1, 2 and 3 after 10 days of preservation. 92
Fig. (35):	Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 4 and 5 after 10 days of preservation. 93
Fig. (36):	Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 6 and 7 after 10 days of preservation. 94
Fig. (37):	Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 8, 9 and 10 after 10 days of preservation. 95
Fig. (38):	Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 1, 2, and 3 after 20 days of preservation. 96
Fig. (39):	Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 4 and 5 after 20 days of preservation. 97
Fig. (40):	Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 6 and 7 after 20 days of preservation. 98

List of Figures

Number	Page
Fig. (41): Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 8, 9 and 10 after 20 days of preservation.	99
Fig. (42) Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 1, 2 and 3 after 30 days of preservation.	100
Fig. (43) Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 4 and 5 after 30 days of preservation.	101
Fig. (44) Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 6 and 7 after 30 days of preservation.	102
Fig. (45) Agarose gel electrophoresis for the products of the nPCR targeting COWP gene at 553 bp, for DNA extracted from stool samples 8, 9 and 10 after 30 days of preservation.	103
Fig. (46) Comparison of different preservatives as regards overall sensitivity.	106
Fig. (47) Comparison of sensitivity of different preservatives after 10 days of preservation.	107
Fig. (48) Comparison of sensitivity of different preservatives after 20 days of preservation.	107
Fig. (49) Comparison of sensitivity of different preservative after 30 days of preservation.	108



Acknowledgement

*I wish to express my deep gratitude to **Prof. Dr. Iman Moawad Abdelsalam**, Professor of Medical Parasitology, Medical Parasitology Department, Faculty of Medicine, Ain Shams University, for her faithful supervision, precious advice and meticulous revision of every part of this work,*

*I am much obliged to **Dr. Rania Mohammad Sarhan**, Assistant Professor of Medical Parasitology, Medical Parasitology Department, Faculty of Medicine, Ain Shams University, for her constant guidance, kind supervision and sincere encouragement.*

*I would like to express my deep appreciation to **Dr. Hanan Helmy**, Assistant Professor at Research and Training Center on Vectors of Disease, Ain Shams University for her sincere help, continuous guidance and facilities she provided to perform the study.*

*I would like to thank **Prof. Dr. Hisham Mohammad Hussein**, Head of Medical Parasitology Department, Faculty of Medicine, Ain Shams University, for her great support.*

*I feel much indebted to **Dr. Ayman Abdel-Moamen El-Badry**, Professor of Medical Parasitology, Medical Parasitology Department, Faculty of Medicine, Cairo University, for his guidance and for the facilities he provided to perform the study.*

Many thanks to the members of Diagnostic and Research Unit of Parasitology, Faculty of Medicine, Ain Shams University and the Pediatrics Department, Eldemerdash Hospital, for allowing me to obtain samples for the study.

✎ Marmar Ahmed Hanafy



.....

Contents

Subjects	Page
• Introduction	1
• Aim and plan of the Work	4
• Review of literature.....	6
• Materials and Methods	62
• Results	89
• Discussion.....	112
• Summary and conclusion.....	125
• Recommendations.....	131
• References	132
• Arabic summery	

Introduction

Cryptosporidium is an obligate intracellular protozoan causing enteric infection in a wide range of mammals, including humans (*Quilez et al., 2011*). Cryptosporidiosis caused by different *Cryptosporidium* species (spp.) has been increasingly reported world-wide both in immunocompetent and immunocompromised individuals causing a spectrum of diseases ranging from asymptomatic carrier state to severe diarrhea. Infection results in mild self-limited disease in immunocompetent and often lethal diarrhea or extraintestinal disease in immunocompromised individuals, most notably in patients with acquired immunodeficiency syndrome (AIDS) particularly those with low CD4 counts (*Fayer et al., 2000; Chen et al., 2002; Kaushik et al., 2008 and Uppal et al., 2014*).

Despite its worldwide occurrence, cryptosporidiosis is considered a neglected disease by the World Health Organization's Neglected Diseases Initiative 2010, largely due to the lack of studies in developing countries (*Savioli et al., 2006 and Chalmers and Davies, 2010*).

Epidemiologic studies on human cryptosporidiosis are made difficult by the different transmission pathways and by the limitation in identifying species using conventional methods, such as staining and immunological tests. Conventional microscopy is time consuming and needs skilled technician due to the very small oocyst (size: 4-6

µm), which is sometimes difficult to differentiate from the fungal spores which are of the same size and stains red too (*Kurniawan et al., 2009*). In addition, it has relatively low diagnostic sensitivity (*Weber et al., 1991; Mansfield and Gajadhar, 2004 and Chacon-Cruz, 2014*).

Immunological based detection methods have been developed for use in both clinical and environmental monitoring. However, antigenic variability within clinical isolates can result in some infections remaining undetected. These methods also can not be used for species differentiation at varying degrees of sensitivity and specificity. In order to solve the problem, better techniques with higher sensitivity and specificity are necessary such as PCR (*Kaushik et al., 2008*).

Molecular techniques for laboratory diagnosis of cryptosporidiosis were developed and showed excellent specificity and sensitivity, compared with antigen detection and microscopy. This facilitates early detection of *Cryptosporidium* infection which further adds to the knowledge for clinical management and control of the disease (*Tumwine et al., 2003; El-badry et al., 2010 and Salyer et al., 2012*).

DNA isolation from fecal specimen is not as simple as those from blood or other samples; this is due to the presence of inhibitors such as bilirubin, bile acids and mineral ions, in stool that can interfere with the PCR reaction. Apart from that, type of preservative solution and

duration of sample preservation determine the success of the test as the DNA can be rapidly degraded if not appropriately preserved (*Kurniawan et al., 2009 and Kuk et al., 2012*).

So, an effective DNA extraction method is needed to isolate DNA either from fresh stool as quickly as possible, or the stool in question should be appropriately preserved; so preservation time and conditions are important factors in the isolation of DNA from stool samples and its use in molecular approaches to diagnose infection with intestinal parasites and other microorganisms (*Kuk et al., 2012*).

Although various stool preservatives are available and many of them are suitable for sample preservation for further microscopy and immunological tests, preservatives that are compatible with molecular detection are different (*Pietrzak-Johnston et al., 2000 and CDC, 2013*).

The present study aimed to evaluate the best preservation condition for the isolation of *Cryptosporidium* species DNA from stool.

Aim of the work

The Aim of the present study is to:

Evaluate different conditions and timing of fecal preservation for the best outcome of molecular diagnosis of *Cryptosporidium* species.

Plan of the work

Preparatory steps for the study:

- A) Collection of fresh stool samples from patients attending the Diagnostic and Research Unit of Parasitic diseases, Faculty of Medicine, Ain Shams University, the Pediatric department of El-demerdash hospital, Ain Shams University, Abo-elreesh hospital, Cairo University, and the fever hospital in El-abbasya.
- B) Examination of the fresh stool samples by direct wet smear; once by saline and the other by iodine.
- C) Formalin-ethyl acetate sedimentation concentration of the specimens.
- D) Acid fast staining of stool samples with Modified Ziehl-Neelsen technique.
- E) Application of Immunochromatographic test (ICT) to to validate positivity of staining and microscopy.