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

Thesis

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رسالة

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﴿ علم الطفيليات الطبية﴾

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List of Abbreviations

Abbreviation	Full name
AF	Acid fast
AFLP	Amplified fragment length polymorphism
AIDS	Acquired immunodeficiency syndrome
AP	Auramine-phenol
C.	*Cryptosporidium*
CD	Cluster of differentiation
CDPKs	Calcium-dependent protein kinases
COWP	*Cryptosporidium* oocyst wall protein
DFA	Direct fluorescent-antibody
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucloetide 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

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 *alpha*
%	Percentage
^{o}C	Celsius degree
µl	Micro litre
µm	Micro meter

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Marmar Ahmed Hanafy

Contents

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 immunodefeciency 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 occurrance, 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 rapidily 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.