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IJPBS |Volume 4| Issue 1 |JAN-MAR|2014|14-18

Research Article
Biological Sciences

THE EFFECT OF FREEZING-THAWING ON DNA EXTRACTION FROM *CRYPTOSPORIDIUM* OOCYSTS IN FECAL SAMPLES

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ABSTRACT

The DNA of Cryptosporidium Oocysts was extracted from fecal samples by using Commercial Wizard Genomic DNA purification general kit, tow freezing- thawing protocols have been compared in this study: deep freezer for three cycles and liquid nitrogen for fifteen cycles, the results indicate that the second protocol was more influential in obtaining a sufficient amount of DNA from Oocysts of Cryptosporidium spp in fecal samples.

KEY WORDS

DNA, Cryptosporidium Oocysts, tow freezing- thawing protocols

INTRODUCTION

Feces are abundant; its collection can be totally noninvasive ; and a single gram of feces contains huge quantities of host's DNA from millions of sloughed intestinal mucosal cell and other microorganism such as bacteria, fungus, yeast, protozoa, and even helminths or / and its larval stage or ova (1,2). Fecal specimens remain the most difficult and least studied with respect to the development of extraction methods that would allow DNA diagnosis. Feces samples are processed for the detection of various enteric microbial pathogens such as *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Vibrio cholerae*, *Giardia* spp., *Cryptosporidium* spp and *Entamoeba histolytica*.(3,4)

A standard, maximized method for DNA extraction from *Cryptosporidium* oocysts is essential both for detecting small numbers of oocysts and for evaluating the sensitivity of detection by PCR (5). DNA extraction in some countries is restricted to academic studies, mostly using commercial kits for the extraction of genomic DNA, or in collaboration with external reference centers, rendering the methods expensive and limited (6). No recommended method for extracting *Cryptosporidium* DNA from oocyst exists, and sensitivity of the most described methods has not been addressed fully (7), especially if extracted from fecal sample because it remains the most difficult and least studied with respect to the development of extraction methods that would allow DNA diagnosis (8). DNA loss can be a consequence of subsequent DNA using commercial purification kits, but normally there should be an adequate number of oocysts present in the sample to extract sufficient *Cryptosporidium* DNA for PCR techniques (7, 9, 10).

The selection of a suitable DNA extraction technique is the most important step in determining the final sensitivity of *Cryptosporidium* DNA detection (5, 7). This paper addresses a method for DNA extraction includes the effect of freezing- thawing from fecal sample containing oocyst of *Cryptosporidium spp*.

MATERIALS AND METHODS

Sampling and preservation:

Fecal specimens were collected from 57 patients from AL-Noor primary health care center, aged 1 month to 55 years, diagnosed according to clinical symptoms consistent with cryptosporidiosis. From each specimen an aliquot was processed using the standard formalin-ethyl acetate concentration

procedure and examined microscopically for the presence of *Cryptosporidium* oocysts on modified acid-fast Ziehl-Neelsen stained slides (7). Then, the positive fecal samples for *Cryptosporidium* were diluted in 2.5% potassium dichromate solution and kept at 4 ^{0}C until subsequent procedures (11).

DNA extraction:

Washing:

1. Fecal samples were vortexed
2. Approximately 200 µl of fecal sample was transferred to extraction tube.
3. Deionised distilled water (800 µl) was added to the fecal sample.
4. The sample was centrifuged with 13.000 rpm for 10 minutes.
5. Supernatant was aspirated and discarded.
6. Deionised distilled water (1000 µl) was added, then vortex the tube to break up the pellet.
7. The sample was centrifuged with 13.000 rpm for 10 minutes.
8. Supernatant was aspirated and discarded.
9. Steps (6-8) were repeated for 5 times.

Freezing thawing

Tow protocols of freezing -thawing was used as follows:

Protocol I (12)

1. The cell lysis solution was added to freshly washed sample.
2. Samples were frozen at (-70 ^{o}C) by deep freezer for 15 minutes and thawed at (56 ^{o}C) for 5 minutes.
3. Step (2) was repeated for 3 cycles.
4. Samples were centrifuged at 13.000 rpm for 10 minutes, and supernate were removed.
5. The nuclei lysis solution was added to the pellet.
6. Samples were processed in the subsequent steps of DNA extraction.

Protocol II “modified protocol”

1. The nuclei lysis solution was added to freshly washed samples.
2. Samples were frozen at (-170 ^{o}C) by liquid nitrogen and thawed at (98 ^{o}C) for 3 minutes.
3. Step (2) was repeated for 3 cycles.
4. Samples were centrifuged at 13.000 rpm for 10 minutes, and supernatant was removed.
5. Samples were processed in the subsequent steps of DNA extraction.

DNA Extraction final steps:

Concentrated fecal pellets were used for the DNA extraction after several cycles of freezing-thawing as previously mentioned. DNA was extracted from freeze-thawed fecal pellets using a DNA purification kit (Wizard Genomic DNA purification kit; Promega) (12) according to the following steps:

1. Protein precipitation solution (100 µl) was added to the sample and vortex vigorously at high speed for 20 seconds.
2. The sample was let on ice for 5 minutes.
3. The sample was centrifuged at 13.000 rpm for 3 minutes.
4. Supernatant containing the DNA was transferred to a clean 1.5 at 13.000 rpm for 3 minutes. microcentrfuge tube containing 300 µl of room temperature isopropanol. And it was mixed gently until the thread –like strands of DNA from visible mass.
5. The microcentrfuge tube was centrifuged at 13.000 rpm for 2 minutes.
6. The supernatant was carefully decanted and the tube was drained on cleaning absorbent paper.
7. Approximately (300 µl) of 70% ethanol was added and the tube was gently inverted several times to wash the DNA pellet.
8. The tube was centrifuged at 13.000 RPM for 2 minutes. Ethanol then was aspirated carefully.
9. The tube was drained on clean absorbent paper, and the pellets were allowed to air-dry for 10-15 minutes.
10. DNA Rehydration solution (50 µl) was added.
11. DNA was rehydrated by incubating at 56 ^{0}C for 1 hour, the solution was mixed by gently tapping the tube, and alternatively DNA was rehydrated by incubating the solution overnight at room temperature or at 4 ^{0}C.
12. The DNA was stored at 2-8 ^{0}C.

RESULTS AND DISCUSSION

Examination of the samples revealed that 8 (14.03%) patients were positive for *Cryptosporidium* infection.

Theses eight samples of feces were persevered in 2.5% potassium dichromate solution and kept at 4 ^{0}C. Most methods described to extract DNA from oocyst of *Cryptosporidium* spp have not been addressed fully (7). Although there are many different studies on *Cryptosporidium* spp, some were comparative studies on the extraction of DNA from oocyst (6). Two different protocols were used to extract DNA from fecal samples containing oocyst of *Cryptosporidium* spp, to evaluate which protocol can give the maximum yield of DNA for PCR using.

Oocyst viability is retained following storage in 2.5% potassium dichromate or at 4°C, but storage in formalin for extended periods should be avoided if molecular analyses are to be performed since it has a strong inhibitory effect on PCR (13, 14).Potassium dichromate was used for preservation in this study because it has been used successfully to preserve oocysts and faecal samples for subsequent molecular analysis (14).The samples were washed for five times which was recommended to remove the trace of preservative (9). The eight samples were divided in to two groups of sample each contained four samples, the first group of sample were freeze-thawed by deep freezer for three cycles starting with cell lysis solution (Protocol 1) and the second group were freeze-thawed by liquid nitrogen for fifteen cycles starting with nuclei lysis solution (Protocol 2), after DNA extraction the first group of sample showed smear in gel during electrophoresis (**Figure 1**) while the second group showed sharp bands (**Figure 2**). The smearing of DNA may occurred due to low amount of extracted DNA resulting from few cycle of freezing thawing , the deep freeze may not break all the oocyst of the parasites and liberate the cell components. Sharp bands appeared due to several cycle of freezing (fifteen cycles), low temperature provided by liquid nitrogen and the fast heat shock cause rupturing of large number of triple walled oocyst found in feces as well as the role of the detergents found in nuclei lysis solution, these solvents and detergents support the dissolution of lipids in the periplasmic membrane and the outer membrane as well as for solubilization membrane proteins (16).This result agreed with (17) who described a method that maximizes DNA extraction reliably from small numbers of partially purified *Cryptosporidium* spp oocyst, and also agreed with (18) who used a procedure for DNA extraction from *Cryptosporidium* spp oocyst from water sample includes eight cycles of freezing and thawing after incubation of samples at 56ºC. in this study we used general kit for extraction the DNA from fecal sample which is not very expensive when it is compared with specific kit using for fecal sample which is expensive and may not available in all countries. Although freeze-thaw using liquid nitrogen was sufficient to extracted DNA from samples, application of liquid nitrogen is problematic, especially when there are a large number of samples to be examined, other disadvantages of this method are difficulties in its handlings and safety (19), it also may be considered as time consuming method, and the last disadvantage may be not differ from most DNA extraction method used in *Cryptosporidium* spp which may take one to several days for ten to twenty samples (20). As well as this method may overcome some inhibitory factors especially proteins which can be found in the feces (8), by using protein precipitation buffer and boiling at 98^{o}C for 3 minutes several times after freezing, finally we recommended use fifteen cycles freezing thawing by liquid nitrogen before DNA extraction of *Cryptosporidium oocyst in fecal samples.*

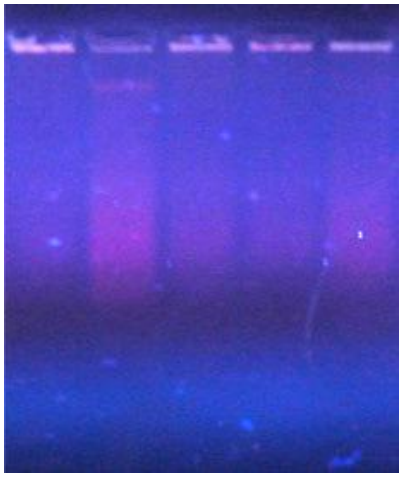

Figure 1: Electrophoresis of the extracted DNA using (protocol 1) showed DNA smearing.
Running conditions: Agarose gel (1.5), 5v/cm for 2 hrs, stained with ethidium bromide.

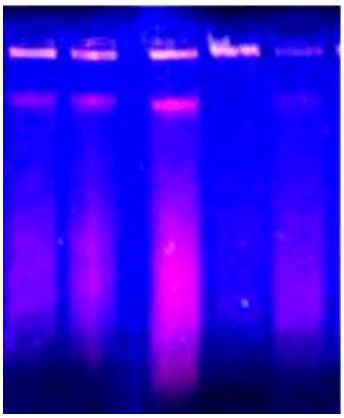

Figure 2: Electrophoresis of the extracted DNA using (Protocol 2) Showed sharp band of extracted DNA. Running conditions: Agarose gel (1.5), 5v/cm for 2 hrs, stained with ethidium bromide

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