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A NEW MODIFIED TECHNIQUE FOR CONCENTRATING INTESTINAL PARASITES

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Abstract

The present study evaluated the diagnostic performance of a modification of the formol ethyl acetate concentration technique, with the addition of 25% acetic acid as compared with formol ethyl acetate concentration technique (FEA) and fecal parasite concentrator kit Fresh fecal material, free of ova and parasites, was pooled in a ratio of 1:4 with 10% buffered formalin to prepare a standardized specimen. Sufficient volumes of formalin-fixed suspension of *Giardia lamblia* cysts, *Entamoeba histolytica* cysts, *Cryptosporidium* oocysts; *Ascaris lumbricoides* ova, *Necator americanus*, *Taenia spp*. and *Hymenolepis nana* were used to seed individually 3-ml portions of the fecal specimen. The 3-ml samples were split in three parts, one processed by FEA, a second part with FPC and the third part by the modified FAEA; six smears from each sediment were examined by light microscopy. FAEA technique gave the clearest sediments and the highest numbers in most of the parasites. FAEA resulted in a higher percentage of *H. nana, Taenia spp., N. americanus*, and *G. lamblia* per one ml of stool compared with FEA method. When compared with FPC, the same results were achieved in addition to *E. histolytica*.

Keywords: Modified formol ethyl acetate concentration technique.

Introduction

Intestinal parasites are a major public health problem in developing countries (Bundy, 1997). Over 80% of all deaths were due to infectious and parasitosis or more than 3 million annually (WHO, 1999). Protozoa and helminthiasis affected 3.5 billion people worldwide (Khan and Alkhalife, 2005; AlKhalife, 2006). Diagnosis depended on microscopic detection of the parasitic stages in stool by a simple smear in normal saline or iodine stain solution using light microscopy and/or by a concentration technique (WHO, 1991). Most laboratories used the concentration techniques to allow for an accurate diagnosis infection (Parija and Srinivasa, 1999; Mandong and Madaki , 2005). The concentration procedures included sedimentation in which the eggs and cysts deposited down and, the flotation in which the eggs and cysts float on surface (Ukaga *et al*., 2002). Ritchie (1948) gave the original concentration sedimentation procedure for parasitic infections. Some modifications were used in order to improve the efficacy and the safety of the technique (Allen and Ridley, 1970; Knight *et al*., 1976) as the use of ethyl acetate instead of ether (Traunt *et al*., 1981; Al-Braiken *et al.*, 2003). The commercially available fecal concentrated devices standardized the concentration procedure and improved the parasite recovery and identification (Perry *et al*., 1990). Of them, Fecal concentrator kit, fecal parasite concentrator, Para-pak macrocon and Trend fekal contrate when compared with formalin-ethyl acetate gave good result and sediment clarity (Perry *et al*., 1990).

This study evaluated a new modification of formol ethyl acetate concentration technique by addition of acetic acid to the steps of the technique as previous studies documented its efficiency as fat solvent (Bellis *et al*., 1967), disinfectant (Sadjjadi *et al*., 2006) and its ability to reduce the adhesion of the fecal forces to the parasite stages.

Material and Methods

Formalin-fixed suspensions of *Giardia lamblia* cysts, *Entamoeba histolytica* cysts, *Cryptosporidium* spp. oocysts, ova of *Ascaris lumbricoides*, *Necator americanus*, *Taenia* spp, and *Hymenolepis nana* were obtained commercially (Scientific Device Laboratories, Inc., Gl-

enview, III). These parasites were selected due to their variations in size and represented different zoonotic parasites. The parasite concentrations in stock suspensions were determined by counting and averaging their numbers in six direct smears of 10 μl (0.01 ml/smear) each.

Seeding of fecal specimen: A fresh parasite-free fecal material with variations in mucus, cellular content, and consistency was mixed with 10% buffered formalin (1:4) to prepare a standardized specimen. A volume of 0.15-0.4 ml formalin-fixed suspension of each parasite was used to seed individually 3-ml portions of standardized fecal specimens for detection of at least one parasite per 22x22 mm cover slip by microscopic examination of six smears (0.01 ml each) (Tab. 1). The seeded 3 ml sample was sieved through one layer wet gauze to remove debris, and then divided into three equal parts in 15 ml plastic centrifuge tubes; each one for one concentration technique.

Three concentration techniques used: Fecal Parasite Concentrator (FPC) Kit (FPC; Evergreen Scientific, Los Angeles, CA) according to the manufacturer's instructions, formalin-ethyl acetate concentration sedimentation technique (FEA) after Traunt *et al.* (1981) and modified formalin-acetic acid ethyl acetate concentration sedimentation technique (FAEA), a modification of modified Ritchie concentration technique (Traunt *et al.*, 1981). The modified technique FAEA was achieved by adding acetic acid to the steps of FEA technique, given the abbreviation FAEA. A series of acetic acid (10%, 15%, 20%, 25% & 50%) were tested to determine the effective one regarding the number of parasites and sediment clarity. The best results were obtained with 25% acetic acid–formalin suspension (25 ml acetic acid, 10 ml 10% formalin & 65 ml saline), and was used experimentally.

The three concentration techniques were started by distributing one ml of the seeded samples of each parasite into a separate graduated centrifuge tube. In case of FPC & FEA, 10% formalin was added to the 1 ml seeded feces to bring the total volume of the tubes to the 7 ml. whereas 25% acetic acid–formalin suspension was added to the FAEA concentration technique tube to bring the total volume to the 7 ml. FPC procedure was done according to the manufacturer's instructions But, the followed steps of FEA & FAEA concentration techniques were performed (Traunt *et al.*, 1981). After centrifugation and decanting, an estimate of the sediment volume was approximated to 0.5 ml for all tubes. Sediment was thoroughly mixed with wooden

applicator sticks. The sediment of each parasite concentration was examined microscopically by preparing six separate slides (0.01ml/ sample). The entire cover slip was examined by 10x & 40x objectives. *Cryptosporidium* oocysts were similarly processed and pipetted onto slides, spread into thin films, stained by a modified Kinyon acid-fast stain (Zierdt, 1984), and examined by 40x & 100x objectives; all measurements were recorded. Comparison between parasite numbers/ 0.01 ml of concentrated sediment of each method and background clarity was the efficacy basis. Parasite numbers /0.01 ml of non concentrated seeded fecal sample and concentrated ones were compared.

Results

Lower concentrations (10% & 15%) recovered few parasites, but high ones provided high sediment volume which interfered with the clarity of the sediment. The best number of parasites and sediment clarity was obtained with 25% acetic acid. There was a notable variation in the effectiveness of the three methods. FAEA concentration method gave the clearest sediment and highest volume. *H. nana* eggs were the best parasite concentrated by all methods (96.1%), followed by *E. histolytica* (88.8%), then (83.3%) for *N. americanus* & *Taenia* spp. *Cryptosporidium* spp. (75%), and *G. lamblia* (71.8%). The lowest one was *A. lumbricoides* (66.6%), FAEA technique gave the high detection numbers in most of parasites (Tab. 2).

Combined averages of all parasites detected per 0.01 ml of FEA and FAEA sediments were compared with a similar average by direct examination of non concentrated seeded material (Tab. 3) and *H. nana*, *Taenia* spp, *N. americanus* and *G. lamblia* gave the highest diagnostic percentage and were effectively concentrated with FAEA. There was only a slight difference between the two methods in *E. histolytica* and *A. lumbricoides*, but, the FEA method was more effective for *Cryptosporidium* oocysts than the FAEA. Comparison between the FPC kit and FAEA (Tab. 4), gave the priority for FAEA in addition to *E. histolytica*, and the two methods equally concentrated *A. lumbricoide* ova. Besides, the FPC was better in concentrating *Cryptosporidium* oocysts than FAEA. So, FAEA was the best method for concentrating *H. nana*, *Taenia* spp., *N. americanus* and *G. lamblia*.

Table 1: Parasites number of stock fecal suspensions and volume needed for seeding.

Parasite	[a]No. of parasites in 1ml stock	[b]Volume of stock for seeding	[c]No. of parasites in seeded feces (3ml)	[d] No. of parasites in unconcentrated seeded feces (1ml)
G. lamblia cysts	13000	0.15 ml	1950	350
E. histolytica cysts	6000	0.3 ml	1800	300
Cryptosporidium oocysts	10000	0.2 ml	2000	300
A.lumbricoides eggs	3500	0.3 ml	1050	100
N. americanus eggs	600	0.4 ml	240	50
Taenia spp. Eggs	1800	0.4 ml	720	100
H. nana eggs	1300	0.4 ml	520	100

[a]Mean numbers of parasites in one ml of stock fecal suspensions. (six readings of 0.01 ml of stock fecal suspensions). [b]Volume of formalin-fixed stock suspensions of each parasite to seed individually 3-ml portions of standardized fecal specimens. [c]Total number of parasites /3ml of seeded fecal suspension. [d]Mean numbers of parasites in 1 ml of seeded feces (six readings of 0.01ml of seeded samples) before concentration.

Table 2: Outcome results of different methods.

Parasite	[a]No. of parasites in seeded feces (3ml)	[b] No. of parasites in 1ml concentrated sediment of each method			[c]Total
		FEA M (%)	FAEA M (%)	FPC M (%)	
G. lamblia cysts	1950	400 (20.5)	600 (30.8)	400 (20.5)	1400 (71.8)
E. histolytica cysts	1800	700 (38.8)	600 (33.3)	300 (16.7)	1600 (88.8)
Cryptosporidium oocysts	2000	600 (30)	300 (15)	600 (30)	1500 (75)
A. lumbricoides eggs	1050	300 (28.6)	200 (19)	200(19)	700 (66.6)
N. americanus eggs	240	50 (20.8)	100 (41.7)	50 (20.8)	200 (83.3)
Taenia spp. eggs	720	100 (13.8)	300 (41.7)	200 (27.8)	600 (83.3)
H. nana eggs	520	100 (19.2)	300 (57.7)	100 (19.2)	500 (96.1)

[a]Total number of parasites /3ml of seeded fecal suspension. [b]Data obtained from six reading of concentrated sediments (0.01 ml /smear) from each technique. M=mean numbers of parasites in 1 ml. [c]Total=the sum of the mean numbers and the percentages recorded for each concentration technique

Table 3: Comparison between FEA and FAEA.

Parasite	No. of parasites in seeded feces (3ml)	No. of parasites in 1 ml of FEA sediment (%)	No. of parasites in 1 ml of FAEA sediment (%)
G. lamblia cysts	1950	400 (20.5)	600 (30.8)
E. histolytica cysts	1800	700 (38.8)	600 (33.3)
Cryptosporidium spp oocysts	2000	600 (30)	300 (15)
A. lumbricoides ova	1050	300 (28.6)	200(19)
N. americanus ova	240	50 (20.5)	100 (41.7)
Taenia spp. ova	720	100 (13.8)	300 (41.7)
H. nana ova	520	100 (19.2)	300 (57.7)

Data obtained from six reading of concentrated sediments (0.01 ml /smear) of each method.

Table 4: Comparison between FPC and FAEA.

Parasite	No. of parasites into seeded feces (3ml)	No. of parasites in 1 ml of FPC sediment (%)	No. of parasites in 1 ml of FAEA sediment (%)
G. lamblia cysts	1950	400 (20.5)	600 (30.8)
E. histolytica cysts	1800	300 (16.7)	600 (33.3)
Cryptosporidium spp oocysts	2000	600 (30)	300 (15)
A. lumbricoides ova	1050	200 (19)	200(19)
N. americanus ova	240	50 (20.5)	100 (41.5)
Taenia spp. ova	720	200 (27.8)	300 (41.7)
H. nana ova	520	100 (19.2)	300 (57.7)

Data obtained from six reading of concentrated sediments (0.01 ml /smear) from each technique

Discussion

The direct smear method was not recommended solely for the routine examination of the suspected parasitic infections because the parasites usually shed in scanty amount (Oguoma and Ekwunife, 2007) but direct smear was demanded for the observation of motile protozoan trophozoites and the examination of cellular exudates. So, there was a need to concentrate the fecal samples to increase the chance of finding the parasites for an accurate diagnosis. Though the direct stool smear technique was easy, rapid and inexpensive when compared with other concentration techniques but it might be critical in giving false results in consequence of misdiagnosis due to decrease

the shedding number of parasites (Mandong and Madaki, 2005). The most concentration sedimentation procedures were adapted from Ritchie (1948). The modifications of formol ether sedimentation method were developed to improve diagnosis of parasites (Wang, 1998; Siripanth *et al.,* 2002; Parija *et al.,* 2003). Besides, the traditional concentration ones different commercial devices were used to improve the probability of diagnosing parasitic infection (Perry *et al.,* 1990; Weitzel *et al.,* 2007; Polaczyk *et al.,* 2008).

In the present study, formol ethyl acetate as a traditional concentration technique (FEA), fecal parasite concentrator (FPC) a commercial device with modified formalin acetic acid ethyl acetate (FAEA), concentration technique were used. Acetic acid as fat solvent (Bellis *et al.,* 1967) helped in declaring the sediment from fat soluble substances. The simulated samples were used rather than patient's samples to justify and insure the number of parasites. FAEA showed highest performance regarding the clarity of the sediment; due to the acetic acid which reduced adhesive fecal forces, and gave better recovery of the parasitic stages. Loughlin and Stoll (1946) recorded that the efficiency of fecal concentration increased by adding acids like hydrochloric acid that permitted easy sedimentation ova. Pavliukobv and Berezantsev (1991) used vinegar with ether in precipitation of helminthes eggs and obtained good results. Also, Sadjjadi *et al.* (2006) reported the ability of vinegar in inactivation of *Giardia* cyst. Sodium acetate acetic acid formalin (SAF) preserved stool, but was not accepted by Troll *et al.* (1997) to be used in processing stool samples for PCR as it inhibited the enzymes activity, but not with using acetic acid.

In the present study, acetic acid was adjusted to be 25% of total volume of the reagents gave reasonable amount of sediment not exceed that precipitated by others. But, any concentration more than or less than 25% was not valuable. The ability of FAEA in detecting the parasites rate was highest with *H. nana* (57.7%) followed by *Taenia*, and *N. americanus* (41.7%), *E. histolytica* (33.3%), *G. lamblia* (30.8%), *A. lumbricoides* (19%) and least one was *Cryptosporidium* (15%). So, FAEA had the advantage over FEA in high rate detection of parasites except *Cryptosporidium,* meanwhile there was only a mild decrease in the results of *E. histolytica* and *A. lumbricoides* with FAEA. Surprising was the results of comparing the FAEA & the FPC

whereas all tested parasites detection rate was high in the former test over the later except in case of *A. lumbricoides* where the rate of detection was equal in the both techniques and the detection rate for the *Cryptosporidium* spp. was high with FPC technique.

The inability of FAEA to detect *A. lumbricoides* in high rate as expected might be explained on the reports by Crites (1958) who declared that the acetic acid initiates swelling of *A. lumbricoides* outer mucoprotein coat , but did not dissolve it. This might led to its floatation in the working solution and inability to be detected. In the same way, low detection rate of *Cryptosporidium* was astonishing since acetic acid is known to act as good surfactant dislodging the parasitic stages from fecal material, but it seemed that *Cryptosporidium* was easily masked by debris and needed more treatment of the debris by floating it to achieve clear background (Weber *et al.*,1992).

In conclusion, the modified formalin-acetic acid ethyl acetate concentration sedimentation technique (FAEA) proved to increase the success of laboratory diagnosis of intestinal parasites, particularly cestodes. Also, it kept some cysts inactive during the manipulation.

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