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INVESTIGATIONS ON INTESTINAL AMOEBA USING VARIOUS LABORATORY DIAGNOSTIC METHODS AMONG STUDENTS IN KOSTI CITY, WHITE NILE STATE SUDAN

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ABSTRACT

Two hundred stool samples were collected from four basic school children in Kosti city, Kosti Province, White Nile State and examined to assess the use of poly vinyl alcohol (P.V.A) in smears prepared from Balamuth's medium stool culture, to compare the results of different direct methods with culture method and to determine the prevalence of amoebic infection among those children. The samples were examined using normal saline method, formal ether concentration method, culture method without P.V.A and culture method with P.V.A. The total number of intestinal amoeba detected by normal saline method was 42 (21%), by formal- ether concentration method was 70 (35%), by culture method without P.V.A was 79 (39.5%) and by culture method with P.V.A was 86 (43%). The study showed that the culture method is more sensitive than the conventional methods (P>0.000). Stool culture when fixed with P.V.A was more reliable and effective in detecting trophozoites and cysts of intestinal amoeba than the same technique without P.V.A and the two are more efficient than wet preparation and formal ether concentration techniques.

KEYWORDS: Poly vinyl alcohol, Balamuth's medium, Smear, Stool culture, Intestinal amoeba, Normal saline method, Formal- ether concentration method, Kosti city.

INTRODUCTION

The amoeba was first discovered in 1873 by Lösch in Russia. Nearly 40 years passed before it was generally accepted that an intestinal amoeba can cause disease. Schaudinn named this group *Entamoeba histolytica* in 1903.[1] The genera and species are differentiated on the basis of size and nuclear structure, the species have naked protoplasm during their trophic stage and characteristically lobose pseudopodia used as locomotors organelles.[2,3] It causes about 50 million infections with a death rate of over 100,000 worldwide annually.[4,5] Infection occurs through ingestion of infective cysts or invasion of motile trophozoites.[6] The disease is widely reported in developing countries like India and tropical Africa, the incidence is increasing in non-endemic areas such as the USA and European countries due to the ease of world travel and immigration of people from endemic areas.[7] The infection causes a variety of clinical presentations, from asymptomatic colonization to invasive amoebic dysentery and extra intestinal amoebiasis. The problem is compounded by the lack of reliable and practical diagnostic tools.[8] Historically light microscopy has been the method of choice for the diagnosis of amoebiasis in fresh or fixed stool samples. In the absence of haematophagous trophozoite, the sensitivity of microscopy is limited by its inability to distinguish between samples infection with *E. histolytica* and those infected with *E. dispar* which is 10 times more common and morphologically identical to *E. histolytica* and does not require treatment.[9,10,11] The wet saline smear reveals many helminthic eggs and larvae and it may reveal motile trophozoite and non-motile cysts, after addition of iodine, it may reveal some additional morphological details.[12] However, the method has its disadvantages like; it is not suitable for examination under oil emersion, does not reveal adequate morphology of protozoa and if the preparation is too thick, protozoa as well as eggs of helminth will probably be missed.[13] Formal ether technique has greater sensitivity in detecting most parasites, but it requires the use of ether which may present storage, handling and disposal problems.[2] Concerning the culture method, three types are available for cultivation of *Entamoeba* species: 1\ Xenic cultivation, where the parasite is grown in the presence of an undefined flora; 2\ Monoxenic cultivation where the parasite is grown in the presence of a single species of organism; and 3\ Axenic cultivation where the

parasite is grown in the absence of any other metabolizing cells. The term polyxenic is sometimes erroneously used as a synonym for xenic. *E. histolytica* has never been grown axenically without first being established in culture with other organisms usually with a complex of undefined bacterial flora.[2] Cleveland and Sanders first accomplished monoxenic cultivation of *E. histolytica* in a diphasic medium with a single species of bacterium (*Crithidia fasciculate*). While Philips and Rees were the first to eliminate the bacteria from amoeba cultures replacing them with *Trypanosome cruzi* for monoxenic cultivation. The media commonly used for xenic cultivation of amoebae are LE medium; Robinson's medium and TYSGM-9 medium.[14] Properly prepared slides of specimens fixed in P.V.A solution are essential to ensure good trichrome stains and to facilitate microscopic examination. The specimen should be fixed in ratio of at least three parts fixative to one part stool sediment. Poly vinyl alcohol (P.V.A) is a water soluble plastic which, when combines with Schoudinn's fixative, provides a good preservative for protozoan trophozoites and cysts.[2] P.V.A fixative serves not only as a preservative but also as an adhesive agent during the staining process. Both concentration and permanent staining procedures can be performed on P.V.A fixed specimens, maintains the morphology as well as staining capability of the parasites, allowing excellent long- term preservation of protozoan trophozoites and easily mixed with specimen.[12,15] Based on studies utilizing culture of faecal samples for detection of *E. histolytica* trophozoites or cysts, culture techniques provide up to four-fold increase in sensitivity compared to microscopy. It was found to be useful in clinical amoebiasis in detecting *E. histolytica* in specimens which were negative by both direct and concentration technique.[16,17,18,19]

The study aimed to assess the use of poly vinyl alcohol (P.V.A) in smears prepared from Balamuth's medium stool culture, to compare the results of direct saline method and formal-ether concentration methods with culture method and to determine the prevalence of amoebic infection among those children using the different methods.

MATERIALS AND METHODS

Sample collection and ethics

This study was carried out in Kosti city among basic school children aged between 9 - 15 years. Four basic schools were selected for the study; these schools are Aboshareef (Area1), Omama Bint Alharith (Area2), Zat Alnetagan (Area3) and Abozar ALgufari (Area4). Each of these schools has eight classes with different numbers of pupils. A total of two hundreds samples were examined i.e. 50 samples for each school. About 10 gm of fresh faeces, uncontaminated with urine or water were collected from each pupil and placed into a clean, dry, wide- mouthed plastic stool container with screw cap. The container was cleaned to free it from detergent or soap or any other materials, which may affect the parasites. The samples were labeled clearly with the pupil name, identifying number, name of the school and time of collection. Pupil's informed consent was obtained before inclusion in the study which was reviewed and approved by the Ethical Committee of Faculty of Medical Laboratory Sciences, Elimam Elmahdi University and education administration in the state.

Diagnostic methods of the parasite

Wet preparation was made out of the each stool sample and screened systematically with the low power of the microscope for the presence of amoeba cysts or trophozoites.[2] The rest of the sample was preserved in 10% formal saline for further examination using formal ether concentration technique. Briefly about one gm of faeces was emulsified in 4ml of 10% formal saline contained in a screw-capped tube. Then further 3-4 ml of 10% formal saline was added. 3- 4 ml of diethyl ether were added and the contents were stoppered, shaken for one minute and then the top of the tube was wrapped and centrifuged immediately for one minute at 3000 rpm. After centrifugation the tube was rapidly inverted to discard ether, faecal debris and formal saline and returned to its up-right position to allow the fluid from the sides to drain to the bottom. The sediment was mixed by Pasteur pipette and transferred to a clean slide, covered with cover glass and examined microscopically using 10 X then the 40 X objective was used to identify small cysts.[9]

Part of the sample was cultured immediately while in the school in 5- 7 ml of Balamuth's medium contained in screw- capped container and incubated at 37C° for 48 hrs. The medium was then examined using wet preparation technique without fixing the slide with P.V.A to confirm the growth of amoeba. When a negative result was obtained, subcultures were made at 48 hrs intervals, as sometimes the subcultures may be positive. The PVA fixative was added to the culture sediment in a ratio of 3 - 1 and the mixture were shaken by hand to mix the content. The mixture was transferred to a labelled glass slide, spread, placed at room temperature and allowed to dry overnight and stained using Wheatley's trichrome staining technique as follows; The slides were washed in 70% alcohol plus iodine for 3- 5 minutes, and then transferred to two successive washes into 70% alcohol for 3- 5 minutes each. The slide was allowed to dry and then transferred to trichrome stain for 6- 10 minutes, then to acidify alcohol for 20 seconds or until the stain ran from the smear .The slides were transferred to two successive washes in absolute alcohol for 2- 5 minutes each, then two zylene washes for 5- 10 minutes each. The smears were mounted with cover glass using D.P.X medium.[2]

Statistical analysis

Associations between the different methods were tested using Chi- square test. P values < 0.05 were considered significant for all statistical analysis.

RESULTS

200 stool samples were collected from school children and screened for intestinal amoeba, the numbers of infected cases were 42 (21%) by normal saline, 70 (35%) by formal ether concentration technique, 79 (39.5%) by culture technique without P.V.A and 86 (43%) by culture technique with P.V.A, table: (1A and B). Infection according to sex is shown in table: 2 while prevalence of infection in relation to previous history of intestinal diseases is shown in table: 3A and B. As type of latrine is concerned; the number of intestinal amoeba detected is shown in table 4 and table 5 shows the number of infected cases correlated to type of water supply. With regard to the number of infected and non-infected pupils in the study areas, it was found that the study area 1 (Aboshareef school) has the highest infection rate among the study areas; table 6.

Table 1A: The number and percentage of infected and non-infected cases with intestinal amoeba using four different techniques.

Technique / Infection	Normal saline	Concentration technique	Culture with out P.V.A	Culture fixed with P.V.A
Non infected cases	158 (79%)	130 (65%)	121 (60.5%)	114 (57%)
Infected cases	42 (21%)	70 (35%)	79 (39.5%)	86 (43%)
Total	200	200	200	200

Table 1B: The number and percentage of infected cases with intestinal amoeba according to the species using four different techniques.

Technique / Infection	Normal saline	Concentration technique	Culture with out P.V.A	Culture fixed with P.V.A
E. histolytica	33 (78.6%)	57 (81.4%)	65 (82.3%)	70 (81.4%)
E. coli	9 (21.4%)	13 (18.6%)	14 (17.7%)	16 (18.6%)
Total	42	70	79	86

Table 2: total numbers of intestinal amoeba detected using the four different techniques correlated with sex.

Technique / Sex	Normal saline	Concentration technique	Culture with out P.V.A	Culture fixed with P.V.A
Male	18 (42.9%)	21 (30%)	32 (40.5%)	34 (39.5%)
Female	24 (57.1%)	49 (70%)	47 (59.5%)	52 (60.5%)

Table 3A: Number of intestinal amoebae detected using the four different techniques in relation to previous history of intestinal diseases.

Technique / History	Normal saline	Concentration technique	Culture with out P.V.A	Culture fixed with P.V.A
WPH	14 (33.3%)	15 (21.4%)	31 (39.2%)	41 (47.7%)
WTPH	28 (66.7%)	55 (78.6%)	48 (60.8%)	45 (52.3%)

Table 3B: Number of intestinal amoeba detected using the four different techniques correlated with history of infection with diarrheal diseases or intestinal bilharziasis.

Technique / History	Normal saline	Concentration technique	Culture with out P.V.A	Culture fixed with P.V.A
WPH	23 (54.8%)	37 (52.9%)	48 (60.8%)	52 (60.5%)
WTPH	19 (45.2%)	33 (47.1%)	31 (39.2%)	34 (39.5%)

WPH = with previous history of infection.
WTPH = with no previous history of infection.

Table 4: Number of intestinal amoeba detected using four different techniques correlated with type of latrine used.

Technique / Type of latrine	Normal saline	Concentration technique	Culture without P.V.A	Culture fixed with P.V.A
Syphon	15 (35.7%)	12 (17.1%)	30 (38%)	39 (45.3%)
Pit larine	27 (64.3%)	58 (82.9%)	49 (62%)	47 (54.7%)

Table 5: The numbers of parasites detected among pupils with houses receiving drinking water through pipeline supply compared to those having their water needs through transported water.

Technique / Water Source	Normal saline	Concentration technique	Culture without P.V.A	Culture fixed with P.V.A
Transported water	7 (16.7%)	10 (14.3%)	18 (22.8%)	37 (43%)
Pipeline system	35 (83.3%)	60 (85.7%)	61 (77.2%)	49 (57%)

Table 6: Number of infected and non-infected pupils in each school using the four different techniques.

Method / Areas	Normal saline		Concentration technique		Culture without P.V.A		Culture with P.V.A	
	Infect	Non-inf	Infec	Non-inf	Infec	Non-inf	Infec	Non-inf
Area 1	12 (24%)	38 (76%)	35(70%)	15 (30%)	37 (74%)	13 (26%)	38 (76%)	12 (24%)
Area 2	11 (22%)	39 (78%)	13 (26%)	37 (74%)	17 (34%)	33 (66%)	20 (40%)	30 (60%)
Area 3	9 (18%)	41 (82%)	10 (20%)	40 (80%)	11 (22%)	39 (78%)	13 (26%)	37 (74%)
Area 4	10 (20%)	40 (80%)	12 (24%)	38 (76%)	14 (28%)	36 (72%)	15 (30%)	35 (70%)

DISCUSSIONS

Wet preparation technique showed less numbers of intestinal amoebae than culture technique. This shows that the technique is less effective for detection of trophozoites or cysts as stated.[17] The technique does not reveal much of the morphology of protozoa besides that the parasites are easily missed if the preparation is too thick as Hiroshi stated in his study of the trichrome stained smears as a screening method for intestinal parasites.[13] The result showed that formal- ether concentration technique is more sensitive than the normal saline technique (P>0.000), since it revealed a larger number of parasites. This is in agreement with the findings of others.[2,14,20,21]

Stool culture as diagnostic aid in the detection of *E. histolytica* in faeces specimen was investigated by Parija and Rao[16] and Abd-Alla, *et- al.*[19] They found that direct saline smear technique detected less numbers of *E. histolytica* trophozoites or cysts in faeces than the culture technique. Culture even without P.V.A fixative, is superior to the wet preparation techniques (P>0.000) and it revealed trophozoites and cysts. The same conclusion was reached by others.[17,19] The culture technique when used with P.V.A fixative is more reliable than all other techniques and this is due to the use of trichrome stain. This stain is able to differentiate between the nucleus, ingested RBCs and inclusion bodies.[20,21,22]

As P.V.A fixative is concerned, this fixative produced a good preparation and provided a good preservation for protozoan cysts and trophozoites. Culture technique when used with P.V.A for trophic stages of *E. histolytica* and *E. coli* was found to be of very high quality.[2,20] The technique was a good preservation method for protozoan trophozoites and was excellent for preparing stained smears for microscopic examination.[12,15] Smears stained with this technique were of high quality and gave well-defined details of amoebic trophozoites and cysts. Culture technique with P.V.A showed increased numbers of positive cases than culture without P.V.A, with significant difference between them (P>0.000). Elamin,[20] emphasized the positive effect of trichrome stain in his study to compare diagnostic staining methods of intestinal protozoan parasites.

As far as sex is concerned, infection with *E. histolytica* and *E. coli* is higher among females than males of the same age group. This may be due to the fact that females may carry the infection under their finger nails beside that females are more confined to indoor life where the areas surrounding houses are more contaminated with cysts of the parasite. This result is in contrast with the finding of Elamin.[20]

The result shows that the greatest numbers of parasites were found in individuals who had no history of previous infection with amoeba confirmed by laboratory diagnosis. This may be due to the development of some sort of resistance or immunity to new infection as stated by F.D.A,[23] but the numbers of parasites were more among individuals with a past history of infection with diarrheal diseases or bilharziasis than in those without such history. This may be due to the relation between these diseases as suggested by Verweij, *et- al.*[24] Previous medical treatment reduced the number of parasites harboured by the patient. This may be due to the residual effect of the drug taken or due to the development of partial immunity or both. The question is whether the present infection in those individuals having previous treatment is a new infection or an old infection not eliminated by previous treatment. This result indicated the importance of a history of medical treatment in reducing the infection with intestinal parasites. The same observation was recorded by others.[14,20,25]

The greater number of parasites detected in those who have a pit latrine in their houses and schools. This may be due to the misuse of this type of latrine which aid in the transmission of the disease because it is a suitable site for houseflies. Those who have no latrine may also aid in transmission of the amoebae through defecation in

the open and contamination of food and water since contamination is the main source of transmission.[23,25,26] The result showed that there are a greater number of parasites in those pupils who have their water supply from the water corporation sources. This may be due to the contamination of the White Nile water from the drainage system due to the slow flow of the river.[14]

With regard to the number of infected and non-infected pupils in the study areas, it was found that the study area 1 (Aboshareef school) has the highest infection rate among the study areas. This may be due to the habit of defecation in the open and around the school due the lack of any sort of latrine and due to the fact that the area around the school is filled with the garbage which is a suitable place for breading of flies besides the contamination resulting from direct contact with it. The lowest was recorded from area 3 (Zat Alnetagan school) which lies in Hai Annasr which is to some extent a modern area and the houses and schools have toilets. In general, there are many factors leading to this result, but the most significant are the poor health condition in the schools, lack of health education and medical care.

As direct smear provides a quick and easy technique it can still recommended to be used as a first line in routine investigation and confirmation of results when needed may be achieved by *invitro* culture of the parasite using P.V.A fixation.

CONCLUSION

Culture when fixed with P.V.A was more reliable and effective in detecting trophozoites and cysts of intestinal amoeba than the same technique without P.V.A and the two are more efficient than wet preparation and formal ether concentration techniques.

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