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COMPARISON BETWEEN MODIFIED ZIEHL NEELSEN AND SAFRANIN METHYLENE BLUE STAINING TECHNIQUES FOR DIAGNOSIS OF CRYPTOSPORIDIUM INFECTION AMONG DIARRHEIC PATIENTS IN RABAK CITY, WHITE NILE STATE, SUDAN

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ABSTRACT

One hundred and twenty stool samples were collected from diarrheic patients at Rabak teaching hospital, white Nile state and examined to compare the results of *Cryptosporidium* infection obtained by Ziehl Neelsen and safranin methylene blue staining techniques, to determine the prevalence of the disease among those patients and to determine the other intestinal parasitic diseases in the study area. Samples were examined using direct saline, formal ether concentration and the two staining technique. Direct saline and formal ether concentration techniques give negative result for cryptosporidiosis, the prevalence were found to be 43(35.8%) and 38(31.7%) using modified Ziehl Neelsen and safranin methylene blue stain respectively. The study showed that the modified Ziehl Neelsen staining technique is most sensitive and accurate than safranin methylene blue staining technique. High prevalence of parasite was detected in male 25(58.1%) and 22(57.9%) than female, in age group one 32(74.4%) and 31(81.6%), in those with history of fever and weight loss 27(63%) and 25(65.8%) and in patient consuming pipeline water 39(90.7%) and 36(94.7%). Other intestinal parasites detected using direct and formal ether concentration techniques were, *G. lamblia, E . histolytica, E . coli* and *H. nana*. The study recommended to include the detection of Cryptosporidium oocysts in routine ova and parasite diagnosis using modified Ziehl Neelsen as first choice and safranin methylene blue stain as alternated technique and raise awareness of cryptosporidiosis in public health including personal hygiene and sanitation to limit its transmission.

KEYWORDS: *Cryptosporidium*, Ziehl Neelsen stain, safranin methylene blue stain, Risk factors, Diarrheic patients, Prevalence.

INTRODUCTION

Cryptosporidium is a genus of protozoan parasites with species that infect fish, amphibians, reptiles, birds and mammals.[1] It has gained much attention in the last 20 years as a clinically important human pathogen. Unlike other intestinal pathogens, it can survive most environments for long periods of time due to its "hardy cyst" and inhabits all climates and locales.[2] The first case of human cryptosporidiosis was reported in 1970 involved a 3-year girl from rural Tennessee who suffered from severe gastroenteritis for two weeks.[3] It has been recognized as important human enteric parasite since 1976 many cases of nonbacterial, non-viral infectious human diarrhea are commonly attributed.[4] It is associated with infection in person with immune deficiency syndrome, AIDs or other condition which reduce normal immune response include treatment with immune suppressive drugs.[5,6] In most surveys it is among the four major pathogens causing acute and chronic diarrheal diseases in developing countries specially in children associated with malnutrition and high morbidity and mortality rates.[7,8,9] This parasite is recognized as highly infectious enteric pathogen and is transmitted mainly by the fecal-oral route.[10,11] It is more prevalent in developing countries (5% to 10%) than in developed countries (1% to 3%), since it responsible for 8–19% of cases of diarrheal disease with a significant effect on mortality. In part of Asia and Africa as many as 31.5% of all children less than 2 years of age are infected, the parasite posting a significant health risk.[7,11,12] Reports from West Mumbai and Bengal showed the parasite to be prevalent in 4.45% and 5.5% of children with diarrhea respectively.[13] Although initially associated with a compromised immunity, it is now clear that persons with normal immunologic functions are affected as well. Many studies in different parts of the world show that children are more affected

than adults.[14] Estimates from United States public health records suggest that 2% of all stools tested by health care providers are positive for *Cryptosporidium* and 15 million annual visits for diarrhea, infection might be expected in 300 000 persons.[15]

Diagnosis of *Cryptosporidium Parvum* is carried out by using either acid fast (modified Ziehl-Neelsen method) or immunofluorescence staining on un-concentrated fecal smears. Several enzyme linked immunosorbent assays are also available for detection of specific cryptosporidial oocysts antigens. New methods involving PCR may help to detect the species in water supplies or asymptomatic carriers. All these methods are sensitive but also time consuming.[10] Choice of diagnostic techniques depends on available equipment and reagents, experience, and considerations of time and cost.[16]

The Ziehl-Neelsen staining method was first used to detect *Cryptosporidium* oocysts in feces in 1981. It introduced for staining cryptosporidial oocysts by veterinary workers who had found that it was associated with scouring of calves.[17,18] It recommended to use with the concentration floatation technique for the accurate diagnosis and rapid identification.[19,20] Advantages of the technique are that; low cost, good for screening large number of samples and permanent stain making it possible to send doubtful or scanty positive slides to a reference laboratory for confirmation. On the other hand the limitations are that; low sensitivity, low specificity, time-consuming procedure, requires intensive training and experience to interpret the results and distinguishing *Cryptosporidium* oocysts from other elements such as yeast.[13,18]

A method using safranin and methylene blue has been described by Baxby, *et al*. which stains oocysts red and yeasts and other fecal debris blue.[17,21] The detection of *Cryptosporidium* was nearly similar to modified Ziehl-Neelsen stain while both staining were accurate than the Giemsa stain.[22] Advantages of the method are rapid and simple and it can differentiate yeasts from oocysts. The limitations is necessity for acid-methanol treatment before and vigorous heating during the safranin stage.[13]

The study aimed to compare the results of *Cryptosporidium* infection obtained by Ziehl Neelsen and safranin methylene blue staining techniques, to determine the prevalence of the disease among those patients also to determine the other intestinal parasitic diseases in the study area.

MATERIALS AND METHODS

Sample collection and ethics

This study was carried out in Rabak city among patients from both sexes and different ages with diarrhea who admitted to Rabak teaching hospital and the clinics scattered around it. About 10 gm of fresh feces, uncontaminated with urine or water were collected from 120 participant according to the WHO standard procedure in leak proof, clean plastic containers labelled with individual index number then examined in the laboratory of parasitology using direct preparation. One aliquot of each stool sample was preserved in 10% formal saline for further examination using formal ether concentration, ZN stain and methylene blue safranin staining techniques. Patient informed consent was obtained before inclusion in the study which was reviewed and approved by the Ethical Committee of Elimam Elmahdi University and health 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 the parasites3.[2] In formal ether concentration technique; about one gm of feces 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 X 10 then the X40 objective was used to identify small cysts.[23]

Cold Ziehl- Neelsen stain (acid-fast stain) was held as follow; a drop of fecal suspension was placed on a glass slide and spread to form a thin smear. Slides were air dried and fixed in absolute alcohol for 10 min and then flooded with carbol fuchsin for 10 min. Following washing, the slides were decolorized by flooding with 3% hydrochloric acid in isopropyl alcohol for between 15 seconds and 1 minute, depending on the film thickness. Slides were then washed, counter stained with 1% methylene blue for 4 min, washed, air dried and examined under 40X and100X objectives.[5]

In Safranin- methylene blue staining technique; thin smear of preserved feces was prepared and air- dried then fixed in 3% hydrochloric acid in absolute methanol for 3-5 minutes and washed with clean tap water. The smear was stained with hot 1%w/v aqueous safranin solution for 1 minutes, washed with water, counter stained with 1%w/v Methylene blue for 30 seconds, washed with water and placed in rack to dried then examined microscopically using 40X objective to scan and 100X objective to identified the oocyst.[5]

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

One hundred and twenty stool samples were collected form patient in Rabak teaching hospital and screened for *Cryptosporidium parvum* using direct saline, formal ether concentration technique, modified Ziehl Neelsen and safranin-methylene blue staining techniques. Direct saline and formal ether concentration techniques give negative result for cryptosporidiosis, the numbers of infected cases were found to be 43(35.8%) and 38(31.7%) using modified Ziehl Neelsen and safranin methylene blue stain respectively; table: 1. Infection according to sex is shown in table: 2, while prevalence in relation to age is shown in table: 3. As fever and weight loss are concerned, the number of *Cryptosporidium parvum* detected is shown in table: 4 and table 5 shows the number of infected cases correlated to consuming water from pipeline source. With regard to the other intestinal parasitic infection detected among patient in the study area by direct saline method and formal ether concentration technique, the number and species of parasites were shown in table: 6.

Table 1: The number and percentage of infected and non-infected cases with *Cryptosporidium parvum* using the two different techniques.

Technique / Case	Ziehl Neelsen stain	Safranin-methylene blue stain
Non infected cases	43 (35.8%)	38 (31.7%)
Infected cases	77 (64.2%)	82 (68.3%)
Total	120	120

Table 2: The number and percentage of infected cases with *Cryptosporidium parvum* in relation to sex using the two different techniques.

Technique / Sex	Ziehl Neelsen stain	Safranin-methylene blue stain
Male	25 (58.1%)	22 (57.9%)
Female	18 (41.9%)	16 (42.1%)
Total	43	38

Table 3: The number and percentage of infected cases with Cryptosporidium parvum correlated with age groups using the two different techniques.

Technique / Age	Ziehl Neelsen stain	Safranin-methylene blue stain
Age group one	32 (74.4%)	31 (81.6%)
Age group two	8 (18.6%)	5 (13.2%)
Age group three	3 (7%)	2 (5.2%)

Table 4: The number and percentage of infected cases with *Cryptosporidium parvum* according to history of fever and weight loss using the two different techniques.

Technique / Cases	Ziehl Neelsen stain	Safranin-methylene blue stain
With a history	27 (63%)	25 (65.8%)
Without history	16 (37%)	13 (34.2%)

Table 5: The number and percentage of infected cases with *Cryptosporidium parvum* in relation to the source of water supply using the two different techniques.

Technique / Water source	Ziehl Neelsen stain	Safranin-methylene blue stain
Pipeline	39 (90.7%)	36 (94.7%)
Other sources	4 (9.3%)	2 (5.3%)

Table 6: The number and percentage of infected cases with other intestinal parasites detected using direct and formal ether concentration techniques.

Technique / Parasites Detected	Direct saline	Formal ether concentration technique
G. lamblia	20 (42.6%)	45 (45%)
E . histolytica	15 (31.9%)	20 (20%)
E . coli	10 (21.3%)	30 (30%)
H. nana	2 (4.2%)	5 (5%)
Total	47	100

DISCUSSIONS

Cryptosporidium is among the four major pathogens causing diarrheal diseases in developing countries specially in children. This parasite is recognized as highly infectious enteric pathogen and is transmitted mainly by the fecal-oral route.[10,11] The first case of human cryptosporidiosis was reported in 1970.[3] Modified Ziehl- Neelsen, Safranin methylene blue and Trichrome methylene blue were used to stain oocysts which were collected by concentration techniques.[19,20,22] This dissertation focused on the current state of *Cryptosporidium* infections in patients coming to hospitals in Rabak city for ova and parasite examinations aiming to determine the prevalence of *Cryptosporidium parvum* among diarrheic patients and to compare the result obtained by Ziehl Neelsen stain and safranin-methylene blue stain among those subjects. To this 120 of stool samples were collected and screened for the parasites using direct saline, formal ether concentration technique, modified Ziehl Neelsen and safranin-methylene blue staining techniques. Direct saline and formal ether concentration techniques give negative result for cryptosporidiosis, the number of infected cases detected were found to be 43(35,8%) and 38(31.7%) using modified Ziehl Neelsen and safranin methylene blue stain respectively. From these results, it can be assuming that direct saline technique is not suitable for detection of *Cryptosporidium* oocysts. These finding is agreed with that carried out by Abdelrady and Sayed,[19] who reported the direct examination gave very low number of positive samples. In contrast, the staining techniques are advisable since it detect the higher number of parasite in which safranin methylene blue detected oocysts in percentages lower than modified Ziehl Neelsen technique. The results are in agreement with Tamomh, et- al [(24)] who concluded that the modified Ziehl-Neelsen staining is a reliable method for detection of *Cryptosporidium* in smears from fecal samples and in contrast with that reported by others.[22,25,26] who proposed that safranin- methylene blue method is rapid, simple with little source of error and more sensitive than currently recommended Ziehl-Neelsen methods. Also safranin-methylene blue gives better differentiation between oocysts and yeast, it stains oocysts red and yeasts and other fecal debris blue.[17,21,26]

According to sex, the higher number of parasite was detected in male 25(58.1%) and 22(57.9%). This result was disagreed with Tamomh, *et- al*.[24]

The high recovery of parasite was detected in age group one, 32(74.4%)and 31(81.6%). The result was disagreed with Tamomh, *et- al.* [24]

The greater number of parasite was reported in patients having history of fever and weight loss, 27(63%) and 25(65.8%). The clinical manifestations of *C. parvum* include watery diarrhea, abdominal cramps, fever, vomiting and weight loss. It associated with high morbidity and mortality rates in the developing world.[7,8,9] The result was in contrast with report in study conducted in Kenya.[14]

High prevalence is found in people whom consumed pipeline water, 39(90.7%) and 36(94.7%). Oocyst of *C. parvum* is not eliminated by chlorination, common household disinfectants and may persist in post-treatment water supplies. Also the survival of oocysts was greater in membrane filtered river water than in unfiltered water.[1,5,11,13,27]

Distribution of other intestinal parasites detected by direct saline method was 47(39.2%) and that detected by formal ether concentration technique was 100(83.3%). The results was in agreement with that reported by study conducted in Khartoum and Kosti.[28]

It recommended to include the detection of *Cryptosporidium* oocysts in routine ova and parasite diagnosis using modified Ziehl Neelsen as first choice and safranin methylene blue stain as alternated technique and improve public health including personal hygiene and sanitation to limit its transmission.

CONCLUSION

Modified Ziehl Neelsen staining technique was found to be more sensitive and accurate in detecting *C. parvum* oocysts in stool sample. In the study area, *Cryptosporidium* is one of protozoan parasite that causes enteric infection among patient admitted to the hospital, the frequency was higher using both modified Ziehl Neelsen and safranin methylene blue stain.

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