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Clinical Evaluation For the Wet Mount Preparations Versus Ziehl–Neelsen Staining Modifications For Diagnosis And Severity Scoring Of Cryptosporidium Parvum In Children Under Five Years

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Abstract :

Aim : Current study aims to investigate the Clinical Evaluation for the wet mount preparations versus Ziehl–Neelsen staining modifications for Diagnosis of cryptosporidium parvum in children under 5 years in Baqubah-Diyala province

Methods : fecal samples were collected from (100)diarrheic children and stained by Lugol's iodine solution; concentration using Sheather's Sugar solution ;Ziehl–Neelsen staining (ZN) ,cold &hot techniques and Kinyoun's staining method for detection of *C. parvum*

Results:

both of sheather's sugar solution concentration technique and ZN hot staining were identical in diagnosis of negative *C. parvum* among diarrhea cases in children . Significant difference as well as correlation regarding the detection and scoring of *C. parvum* oocysts among children was reported between sheather's sugar concentration and ZN hot staining procedure (P value=0.000).Very good agreement (kappa =0.832, p value= 0.000),was reported between ZN hot staining procedure and sheather's sugar concentration technique for diagnosis of *C. parvum* oocysts in children .

both of lugol's iodine technique and ZN hot staining were identical in diagnosis of negative *C. parvum* among diarrhea cases in children . Significant difference as well as correlation regarding the detection and scoring of *C. parvum* oocysts among children was reported between lugol's iodine and ZN hot staining procedure (P value=0.000).Very good agreement (kappa =0.659, p value= 0.000),was reported between ZN hot staining procedure and lugol's iodine for diagnosis of *C. parvum* oocysts in children .

Conclusion : Wet mount preparations (lugol's iodine and sheather's sugar concentration technique considered as a very good alternative to ZN hot staining procedure for diagnosis of *C. parvum* oocysts among children in primary health care centres and hospitals.

Keywords : cryptosporidium parvum, Diagnosis, diarrhea, Scoring

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Introduction

Cryptosporidium is intracellular protozoan parasite that live in the gastrointestinal tract of mankind and many other vertebrates animals including mammals, reptiles, birds and fish[1]. They are the widespread source of diarrheal disease among both immunocompetent and immunocompromised individuals throughout the world leading to considerable morbidity and mortality, especially in developing countries[2] and in individuals less than five years[3] .

Cryptosporidiosis is a diarrheal disease caused by microscopic parasites of the genus Cryptosporidium [4, 5]. The parasite is protected by an outer shell that allows it to survive outside the body for long periods of time and makes it very resistant to chlorine disinfectants. Both the disease and the parasite are commonly known as (Crypto)[1] .Most cases of human cryptosporidiosis are due to infections with the human specific C. hominis or the zoonotic *C. parvum* [6].Other Cryptosporidium species have also been detected in humans, although less frequently [7] . Current evidence indicates that ruminants are a reservoir of zoonotic Cryptosporidium from where humans get infected by contaminated food and water or through direct contact with livestock, for example animal handlers [1]

Twenty six Cryptosporidium species and nearly 50 genotypes have been recognized and described and still new genotypes are being discovered [8].At least ten Cryptosporidium species and four genotypes can infect humans. C. hominis and *C. parvum* are internationally the most commonly species infecting humans.[8, 9] . Humans can acquire cryptosporidium infections through several transmission routes such as person to person transmission, zoonotic transmission, food borne transmission and waterborne transmission [10].A single oocyst is sufficient to cause infection and disease [11].

When excreted, Oocysts are directly infectious and are able to survive for up to 6 months in a moist and cool environment. In water, oocysts remain viable for 140 days [12]. In immunocompetent persons, cryptosporidium infection usually asymptomatic. in children under the age of five and in immunosuppressed people, the infection leads to severe diarrhea. Nausea, vomiting, discomfort and low-grade fever are other clinical symptoms which may occur during an infection with Cryptosporidium [13] . Symptoms in immunocompromised patients can be very severe and even death has been described[14] .In developing countries 45% of the children are experiencing an infection before the age of two[3]

Current study aims to investigate the Clinical Evaluation for the wet mount preparations versus Ziehl–Neelsen staining modifications for Diagnosis of *C. parvum* in children under 5 years in Baqubah-Diyala province

Material and Methods

Study area and study population

This study was conducted on newborn to less than 5 years old Iraqi children, living in the Baqubah city -Diyala province 33°45'34.71"N; 44°36'23.97"E ,Northeast.

Stool Samples collection and processing

The stool samples were collected from 100 children less than 5 years of age suffering from gastrointestinal illness. Sample collection took place from November 2016 to June 2017. The inclusion criterion was diarrhoea, defined as passage of three or more loose or liquid stools per day, or more frequently than is normal for the individual[15]. The

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samples were obtained from Albatul teaching hospital-Baqubah . An ethical consideration and consent by the parents or guardians of the children was signed before getting the samples . The samples were collected in a special tightly capped leak proof containers. Each sample was labeled with the child's name, Sex and age[16]. Each sample was divided into two portions, one used for immediate examination ,other one preserved and stored in 10% formalin. One volume of the fecal sample was mixed thoroughly using wooden applicator stick, with 3 volumes of 10% formalin. The sample was mixed again, and the specimen containers were sealed well[6]. All samples were reinforced with parafilm, the container was inserted in a plastic bag, and samples were stored at 4ºC in the clinical pathology laboratory at college of veterinary medicine ,Diyala university

Methods:

Direct Microscopy

Lugol's iodine solution

All stool samples were examined directly by emulsifying a small portion (approximately 0.1 ml) in a drop of Lugol's iodine on a separate slide. These wet preparations were covered with a glass cover slip and examined visually under magnifications of x 100 and x400 with reduced light for a minimum of 3 min per preparation, covering approximately 100 fields. Oocysts were visible as round or oval refractile particles with diameters of 2 to 6 ,um. They failed to take up iodine; this is helpful in distinguishing them from yeast cells, which they resemble on wet preparations [17, 18]. Concentration by flotation in Sheather's sugar solution according to [19-21] .

Staining Techniques :

Ziehl–Neelsen staining (ZN) ::Cold method of ZN Staining Of Fecal Smears (Modified Kinyoun's Acid-Fast Stain) This technique was applied according to [6, 22]. Thin smears of fecal sediment were made on a clean glass slide and air-dried. Then, the smears were fixed by rapid movement over a flame for 2-4 seconds . The smears were flooded with basic fuchsin-phenol stain, and allowed to stand at room temperature for 10 minutes. The smears were then washed in tap water for 1-2 min. Then, decolonization of the slides with 5% sulfuric acid for 30 second and counterstained with 3% methylene blue for 1 min. ,sand air-dried. A total of 200 fields was examined using 40× and 100× to confirm the diagnosis according to oocysts morphology[23].

Hot method of ZN staining of fecal smears : In the hot method, Thin smears of fecal sediment were made on a clean grease free glass slide and air-dried. Then, the smears were fixed transiently over a flame. The smears were flooded with basic fuchsin-phenol stain. The slide was heated until the steam appeared without boiling at room temperature for 10 minutes. The smears were then washed in running water for 1-2 min. Then, the slides were decolorized in 5% sulfuric acid for 30 second and counterstained with 3% methylene blue for 1 minute and air-dried. A total of 200 fields was examined using 40× and 100× to confirm the diagnosis according to oocysts morphology.[6, 22, 23].

Kinyoun's staining method :Solutions for the Kinyoun's staining method according to[24] .

Kinyoun's staining Procedure:

Kinyoun's staining was done as per the procedure [25]. The thin smear of fecal sediment was made and air-dried. The smear was fixed with absolute methanol for 1 min. Then, the slide was flooded with Kinyoun's carbol fuchsine stain for 5 min. After staining, the slide was rinsed with 50% ethanol for 3-5 second and later with distilled water. Stained smear decolorized with 1% sul-

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phuric acid for 2 min and was counterstained with 1% methylene blue for 1 min. Then examined microscopically under 100x.

Statistical Analysis :

Demography and cross tabulation were calculated by Statistical analysis using SPSS for windows TM version 17.0[26]. Chi square was used to verify possible association between infection and exposure with different factors[27-29]. Values were considered to be statistically significant when the p-value obtained was less than 0.05[30, 31].The concordance of the Zn hot and other staining techniques was studied using the Cohen's kappa index of agreement. The level of confidence limits was 0.095 and Here is one possible interpretation of Kappa value15[32].Poor agreement = Kappa value Less than 0.20 (b)Fair agreement = Kappa value 0.20 to 0.40 ;Moderate agreement = Kappa value 0.40 to 0.60 (d) Good agreement = Kappa value 0.60 to 0.80;Very good agreement = Kappa value 0.80 to 1.00[6, 23, 33]

Results :

As shown in table (1),the typical *C. parvum* oocysts was detected in (74%) children suffered from diarrhea by Lugol's Iodine wet preparation. Low score of *C. parvum* oocysts were reported in ,(16%), while moderate and heavy score reported in (29%) as shown in figure (1). As shown in table (2),the typical *C. parvum* Oocysts was detected by concentration using Sheather's Sugar solution in ,(74%),children suffered from diarrhea .low score of *C. parvum* oocysts were reported in (16%), moderate score, were reported in (28%).Heavy score of *C. parvum* oocysts was reported in (30%)of positive cases as shown in figure (2). As shown in table (3),the typical *C. parvum* oocysts was detected in,(74%),children suffered from diarrhea by using ZN cold staining technique. low score of *C. parvum* oocysts were reported in (16%), moderate score, were reported in(28%).Heavy score of *C. parvum* oocysts was reported in (30%)of positive cases as shown in figure (3). As shown in table (4),the typical *C. parvum* oocysts was detected in,(74%),children suffered from diarrhea by ZN hot staining technique .low score of *C. parvum* oocysts were reported in (12%), moderate score, were reported in (25%).Heavy score of *C. parvum* oocysts was reported in (37%)of positive cases as shown in figure (4). As shown in table (5),the typical *C. parvum* oocysts was detected in,(74%),children suffered from diarrhea using Kinyoun's staining technique .Low score of *C. parvum* oocysts were reported in (25%), moderate score, were reported in (25%).Heavy score of *C. parvum* oocysts was reported in (24%)of positive cases as shown in figure (5).

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Table(1): Detection of *C. parvum* infection in children by lugol's iodine wet preparation

Sample source	Lugol's iodine wet mount Preparation	score				Total
		Negative	Low	Moderate	Heavy	
Chil-dren	Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
	Positive	0(0%)	16(16%)	29(29%)	29(29%)	74(74%)
	Total	26(26%)	16(16%)	29(29%)	29(29%)	100(100 %)

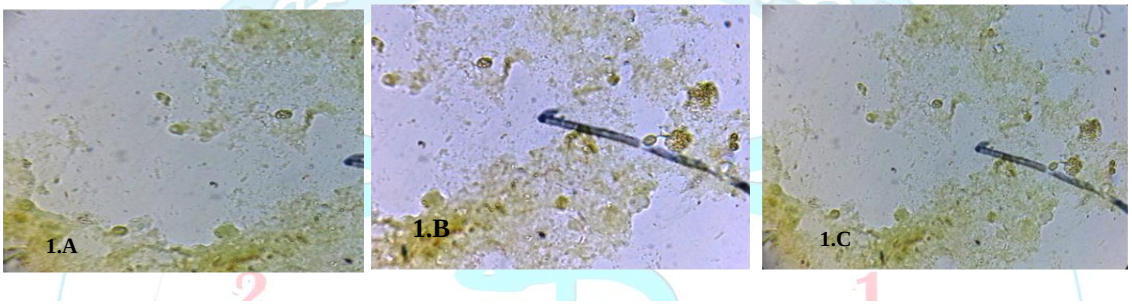

Figure (1): Detection of *C. parvum* infection in children by lugol's iodine wet preparation .A) low score ,B)moderate; c)Heavy score

Table(2): Detection of *C. parvum* infection in Children by Sheather's Sugar solution

Sample source	Sheather's Sugar solution wet mount Preparation	score				Total
		Negative	Low	Moderate	Heavy	
Children	Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
	Positive	0(0%)	16(16%)	28(28%)	30(30%)	74(74%)
	Total	26(26%)	16(16%)	28(28%)	30(30%)	100(100%)

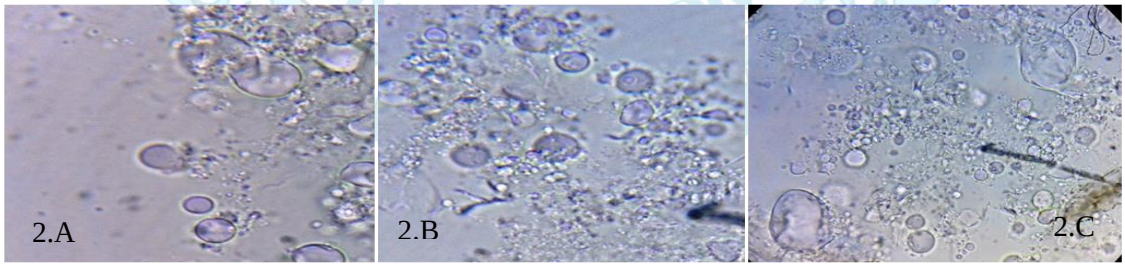

Figure (2):Detection of *C. parvum* infection in children by Sheather's Sugar solution . A) low score ,B)moderate; c)Heavy score

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Table(3): Detection of *C. parvum* infection in Children by ZN cold staining Technique

Sample source	ZN cold staining Technique	score				Total
		Negative	Low	Moderate	Heavy	
Children	Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
	Positive	0(0%)	16(16%)	22(22%)	36(36%)	74(74%)
	Total	26(26%)	16(16%)	22(22%)	36(36%)	100(100%)

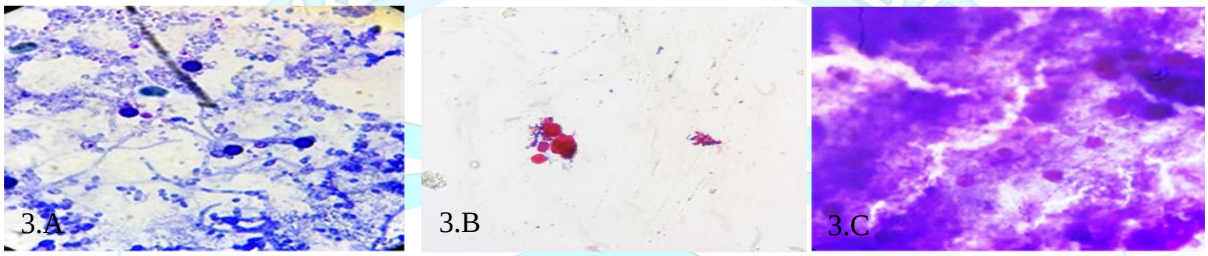

Figure (3):Detection of *C. parvum* infection in children by ZN cold staining technique . A) low score ,B)moderate; c)Heavy score

Table(4): Detection Of *C. parvum* Infection In Children By ZN Hot Staining Technique

Sample source	ZN cold staining Technique	score				Total
		Negative	Low	Moderate	Heavy	
Children	Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
	Positive	0(0%)	12(12%)	25(25%)	37(37%)	74(74%)
	Total	26(26%)	12(12%)	25(25%)	37(37%)	100(100%)

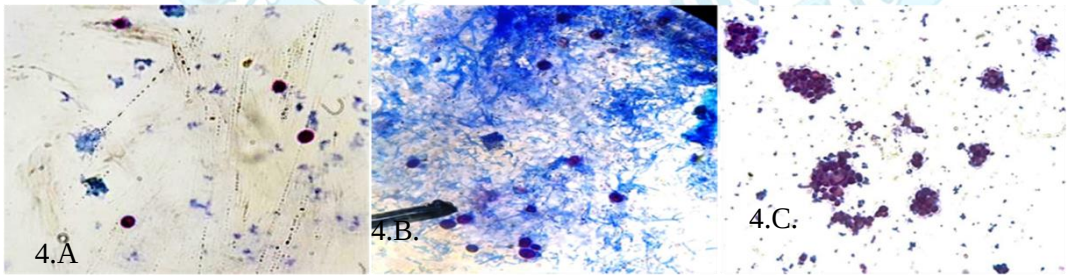

Figure 4 . *C. parvum* oocysts detected in diarrheic stool by ZN hot staining technique In children :A .low score ,B. moderate score, C heavy score (100x)

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Table(5): Detection of *C. parvum* infection in Children by Kinyoun's staining Technique

Sample source	Kinyoun's staining Technique	score				Total
		Negative	Low	Moderate	Heavy	
Children	Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
	Positive	0(0%)	25(25%)	25(25%)	24(24%)	74(74%)
	Total	26(26%)	25(25%)	25(25%)	24(24%)	100(100%)

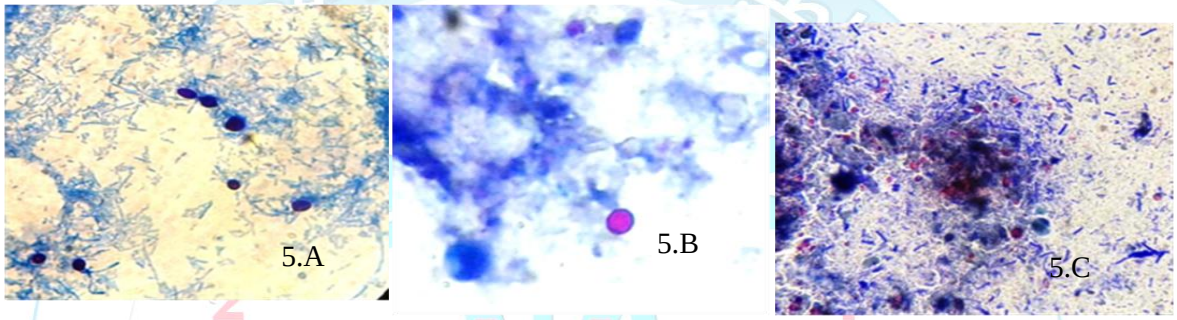

Figure 5. *C. parvum* oocysts detected in diarrheic stool by Kinyoun's staining technique in children :A .low score ,B. moderate score, C heavy score (100x).

As shown in Table(6), both of sheather's sugar solution concentration technique and ZN hot staining were identical in diagnosis of negative *C. parvum* among diarrhea cases in children , (26%).A total of (12%) of cases were recorded as positive with low score in both techniques, (21%) were moderate and,(30%) were recorded as positive with heavy score in both techniques. A total of (3%) of cases were reported as having low score of *C. parvum* oocysts using sheather's sugar concentration and have moderate oocysts score using ZN hot staining procedure. A total of (1%) of cases were reported as having low score of *C. parvum* oocysts using sheather's sugar concentration and have heavy oocysts score using ZN hot staining procedure. A total of (7%) of cases were reported as having moderate score of *C. parvum* oocysts using sheather's sugar concentration and have heavy oocysts score using ZN hot staining procedure. A total of (1%) of cases were reported as having heavy score of *C. parvum* oocysts using sheather's sugar concentration and have moderate oocysts score using ZN hot staining procedure.

Significant difference as well as correlation regarding the detection and scoring of *C. parvum* oocysts among children was reported between sheather's sugar concentration and ZN hot staining procedure (P value=0.000).Very good agreement (kappa =0.832, p value= 0.000),was reported between ZN hot staining procedure and sheather's sugar concentration technique for diagnosis of *C. parvum* oocysts in children .

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Table(6): Agreement between Sheather's sugar solution and ZN hot staining technique for detection of *C. parvum* infection in Children

Sheather's sugar solution Score	ZN hot score in children				
	Negative	Low	Moderate	Heavy	Total
Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
Low	0(0%)	12(12%)	3(3%)	1(1%)	16(16%)
Moderate	0(0%)	0(0%)	21(21%)	7(7%)	28(28%)
Heavy	0(0%)	0(0%)	1(1%)	29(29%)	30(30%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100(100%)
$\chi 2$	221.048				
P value	0.000				
R	0.952				
P value	0.000				
Kappa	0.832				
P value	0.000				

As shown in Table(7), both of lugol's iodine technique and ZN hot staining were identical in diagnosis of negative *C. parvum* among diarrhea cases in children , (26%).A total of (12%) of cases were recorded as positive with low score in both techniques (15%) were moderate and (22%) were recorded as positive with heavy score in both techniques.

A total of (3%) of cases were reported as having low score of *C. parvum* oocysts using lugol's iodine and have moderate oocysts score using ZN hot staining procedure. A total of (1%) of cases were reported as having low score of *C. parvum* oocysts using lugol's iodine and have heavy oocysts score using ZN hot staining procedure.

A total of (14%) of cases were reported as having moderate score of *C. parvum* oocysts using lugol's iodine and have heavy oocysts score using ZN hot staining procedure. A total of (7%) of cases were reported as having heavy score of *C. parvum* oocysts using lugol's iodine and have moderate oocysts score using ZN hot staining procedure.

Significant difference as well as correlation regarding the detection and scoring of *C. parvum* oocysts among children was reported between lugol's iodine and ZN hot staining procedure (P value=0.000).Very good agreement (kappa =0.659, p value= 0.000),was reported between ZN hot staining procedure and lugol's iodine for diagnosis of *C. parvum* oocysts in children .

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Table(7): Agreement between lugol's iodine and ZN hot staining technique for detection of *C. parvum* infection in Children

lugol's iodine Score	ZN hot score in children				
	Negative	Low	Moderate	Heavy	Total
Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
Low	0(0%)	12(12%)	3(3%)	1(1%)	16(16%)
Moderate	0(0%)	0(0%)	15(15%)	14(14%)	29(29%)
Heavy	0(0%)	0(0%)	7(7%)	22(22%)	29(29%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100(100%)
$\chi 2$	178.586				
P value	0.000				
R	0.906				
P value	0.000				
Kappa	0.659				
P value	0.000				

Discussion :

Current study is the first of its kind in Iraq in terms of work scores for the severity of infection . Detection of *C. parvum* infection by wet mount preparations has several advantages such as simplicity, availability of materials, minimum time requirement , low cost , no need for complex equipments and well trained microbiologist .All of these factors make them preferred and advisable technique in area where the poverty is outstanding feature[4, 15].The typical *C. parvum* oocysts was detected by lugol's iodine in (74%) of children. Low score of *C. parvum* oocysts were reported in (16%), while moderate and heavy score reported in (29%) . From the microscopic examination it was found that using direct wet mount by saline and iodine, the oocysts appear as a small spherical bodies and their sizes were about the size of some fungi but can be differentiated from fungi by their shapes which were oval. Also, the oocysts of Cryptosporidium contain granules inside them, while in iodine preparation the oocyst were colorless with the appearance of sporozoites inside some of them[34]. In Sulimani *C. parvum* oocysts recovered from 13.6% of diarrheic children by using direct wet mount[34] without detection for the intensity of infection by scoring which come in agreement with [7, 35] .

One of the most outstanding features of Sheather's Sugar solution concentration technique that it leads to pink coloration of oocysts when examined under light microscope and this mean simple distinguish between oocysts and fecal remnants[15, 36]. The use of sugar solution with a (1.266)specific gravity for detection of oocysts can keep pink color of the oocysts for a long time[36].Beside pink coloration of the oocysts using a sugar solution with 1.2 of SG ,centrifugation of fecal sample leads to removing of fecal remnants makes microscopic observation easier. As approved by current study, this method has the ability for detection of the oocysts from different scores which come in counteract with that reported by [36],where they claim that small number of oocysts could not reported clearly using this method. Current study reported

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that in children, low score of *C. parvum* oocysts were reported in (16%), moderate score, were reported in (28%) while heavy score was reported in (30%)of positive cases. Detection of oocysts with low score in infected children have critical importance due to low infective dose of C.parvum. The floatation method do not influenced by duration of and the number of oocysts included in clinical samples [36] .

In developing countries, The laboratory diagnosis of Cryptosporidium infection mostly depends on the microscopic examination of stained fecal smears. Using the cold ZN staining procedure ,the oocysts appear as round objects, 4-5 ,µm in diameter, the internal structure stained with red color , and appear an amorphous red mass filling the oocyst and may be appear as multiple, crescentic, sporulated forms[5, 37]. In current study, using cold ZN technique, low score of *C. parvum* oocysts among children were reported in (16%), moderate score (28%) and (30%) were heavy score .

Among children , using hot ZN staining technique, low score of *C. parvum* oocysts were reported in (12%), moderate (25%),heavy score was reported in (37%) of positive cases. Similar observations also made by Bhat et al. In ZNs staining, the oocysts appeared as thickly stained pink bodies against a pale green background, with a clear hallow around the oocysts with four whitish bar like naked sporozoites. The like results were obtained with the hot and cold ZN staining. In India, this technique has been widely used for diagnosis of cryptosporidiosis in animals[38, 39]. Mucus portion of the fecal sample yielded more number of oocysts [40] .

As both cold and hot ZN staining techniques have similar diagnostic efficacy regarding with a status of children, even they differ in their scoring for each case ,this come in line with that reported by [37],that there was no staining method for cryptosporidia is completely effective. This may attributed to the fact that the appearance of cryptosporidial oocysts in faeces is generally sufficiently typical for there to be little difficulty in identifying the organism in most cases. As well as , the inexperienced person may be confused by a variety of objects resembling oocysts in general appearance, whichever staining method is applied[6, 23].

In Kinyoun's staining method, the oocysts appeared as red adjacent to the dark blue background and four sporozoites were visible. A common difficulty with ZN and Kinyoun's staining method is that they cannot distinguish Cryptosporidium oocysts from molds and yeast [15, 22]. The results of current study agree with that reported by [22]that both ZN (hot and cold) as well as Kinyoun's showed similar sensitivity. The main disadvantage was the time required for sample preparation and staining . Decolorization stage was established to be important for both the techniques, which provided good difference between oocysts and background. nevertheless, some oocysts did not get stained due to more than exposure to decolorizer. In the hot method, shrinkage and alteration of oocysts were observed .

Although Significant correlation regarding the detection and scoring of *C. parvum* oocysts among children was reported between SSF and ZN hot staining procedure (P value=0.000). A discrepancy appear in scoring of positive cases when examined by SSF and ZN hot staining procedure which may attributed to the processing of samples with sugar solution of high specific gravity and high osmolarity causing distortion or even destruction of some oocysts was interfere with accurate identification of oocysts ,hence the difference of scores was reported here , with very good agreement between ZN hot staining procedure and SSF technique for diagnosis of *C. parvum* oocysts in children . [41-43] . The results of current study come in contrary with [44] ,reported that SSF has main disadvantage of lower percentages of recovery,

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greater background fecal debris levels, or greater variability in results. SSF characterized by minimum time requirement and low cost and can be utilized everywhere without the need for sophisticated equipment .

Significant correlation and very good agreement regarding the detection and scoring of *C. parvum* oocysts among children was reported between lugol's iodine and ZN hot staining procedure which come in line with [43] Iodine staining and size measurement help greatly in identifying Cryptosporidium spp. oocysts in wet mount preparations .

As the examined cases contain sufficient numbers of oocysts excreted in fecal materials and the examination was done by experts ,beside minimum time requirement and low cost and ability of utilization everywhere without the need for sophisticated equipment making them suitable for large investigation of cryptosporidium infection in community. The need for expert is critical for differentiation between cryptosporidium oocysts and other particles .

Conclusion : Wet mount preparations (lugol's iodine and sheather's sugar concentration technique considered as a very good alternative to ZN hot staining procedure for diagnosis of *C. parvum* oocysts among children in primary health care centres and hospitals

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