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Accuracy of Ziehl Neelsen and Safranin Methylene Blue Staining Modalities for Detection Of *C.parvum* Infection In Children under Five years

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Abstract :

Aim : To investigate the agreements between Ziehl Neelsen and Safranin Methylene Blue Staining modalities staining techniques for detection of *C.parvum* infection in children under 5 years in Baqubah-Diyala province -Iraq

Methods :one hundred diarrheic children under 5 years were included .stool samples were examined after stained with hot Ziehl –Neelsen ;Safranin Methylene Blue Staining modalities

Results :

Both of ZN cold and ZN hot , Kinyoun's , SMB staining were identical in diagnosis of negative *C.parvum* among diarrhea cases in children , (26%).A total of (12%) have low score in both techniques, (19%) were moderate and (33%) have heavy score in both techniques. Very good agreement (kappa =0.862),was reported between ZN hot and ZN cold for diagnosis of *C.parvum* oocysts. A total of (11%) of cases have low score in both techniques, (12%) were moderate and (22%) heavy score in ZN hot and Kinyoun's staining techniques. Very good agreement (kappa =0.614), between ZN hot and Kinyoun's for diagnosis of *C.parvum* oocysts in children. A total of (11%) have low score in both techniques ,(12%) were moderate and (18%) with heavy score in ZN hot technique and SMB techniques. Moderate agreement (kappa =0.567),was reported between ZN hot and SMB for diagnosis of *C.parvum* oocysts in children .A total of (10%) of cases have low score in both techniques, (7%) were moderate and (20%) with heavy score in ZN hot and modified SMB staining . Moderate agreement (kappa =0.514),was reported between ZN hot and modified SMB for diagnosis of *C.parvum* oocysts in children .

Conclusion : All Alternative stains for Hot ZN(ZN cold ; Kinyoun's, SMB and modified SMB staining have identical specificity (100%) for diagnosis of negative *C.parvum* among diarrhea cases in children . According to very good agreement between ZN hot and ZN cold ; ZN hot and Kinyoun's these stains can be used as first line alternative to hot ZN stain .while SMB and modified SMB used as a second alternatives due to moderate agreement with ZN hot staining for diagnosis of *C.parvum* oocysts in children which represent good alternatives in rural areas and low income countries

Key words :cryptosporidium parvum , Ziehl Neelsen and Safranin Methylene Blue, diarrhea

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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, 3] and in individuals less than five years[4, 5] . Cryptosporidiosis is a diarrheal disease caused by microscopic parasites of the genus Cryptosporidium [2] . 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[6, 7]. Most cases of human cryptosporidiosis are due to infections with the human specific *C. hominis* or the zoonotic *C. parvum* [8].Other Cryptosporidium species have also been detected in humans, although less frequently [3, 9] . 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 [10, 11].

Twenty six Cryptosporidium species and nearly 50 genotypes have been recognized and described and still new genotypes are being discovered [12].At least ten Cryptosporidium species and four genotypes can infect humans. *C. hominis and C. parvum* are internationally the most commonly species infecting humans.[12, 13] . Humans can acquire cryptosporidium infections through several transmission routes such as person to person transmission, zoonotic transmission, food borne transmission and waterborne transmission [14].A single oocyst is sufficient to cause infection and disease [15, 16]. 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 [17]. 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 [18] . Symptoms in immunocompromised patients can be very severe and even death has been described [11] .In developing countries 45% of the children are experiencing an infection before the age of two[4]

Current study aims to investigate the clinical compatibility between Ziehl Neelsen and Safranin Methylene Blue Staining modalities For Detection Of *C.parvum* Infection In Children under 5 years in Baqubah-Diyala province

Material and Methods

Study area and study population

This study was conducted on 100 newborn to less than 5 years old Iraqi children, living

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in the Baqubah city -Diyala province 33°45'34.71"N; 44°36'23.97"E ,Northeast [19, 20] .

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 [7, 20] .The 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[21, 22] . The samples were collected in a special tightly capped leak proof containers. Each sample was labeled with the child's name, gender and age[22]. 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[23]. 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:

Staining Techniques

1) Ziehl–Neelsen staining (ZN)

A. Cold method of ZN Staining Of Fecal Smears (Modified Kinyoun's Acid-Fast Stain) This technique was applied according to [24].A total of 200 fields was examined using 40× and 100× to confirm the diagnosis according to oocysts morphology.

B. Hot method of ZN staining of fecal smears Solutions for the hot ZN staining method according to [25, 26] .

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 counter-stained 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.

2) **Kinyoun's staining method :**Solutions for the Kinyoun's staining method according to[27]

3) **Safranin methylene blue staining (SMB):** The SMB staining technique was followed as per method of [28, 29] .

4) Modified Safranin Technique (Hot Method) according to [30]

A. Scoring system and reporting of oocyst results :

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Scoring system for positive sample was used, based on the number of oocyst under x40/ x100 objective lens [31]

Low (+): only one oocysts per high power field x40/ x100.

Moderate (++):2-10 oocysts per high power field x40/ x100.

Heavy (+++):11 or more oocysts per high power field x40/ x100

Statistical Analysis

Demography and cross tabulation were calculated by Statistical analysis using SPSS for windows TM version 17.0[32]. Chi square was used to verify possible differences between scores of infection and staining techniques[33].Values were considered to be statistically significant when the p-value obtained was less than 0.05[11].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 value [34]. a) Poor agreement = Kappa value Less than 0.20 (b)Fair agreement = Kappa value 0.20 to 0.40; c) Moderate agreement = Kappa value 0.40 to 0.60 (d) Good agreement = Kappa value 0.60 to 0.80; e) Very good agreement = Kappa value 0.80 to 1.00

Results :

Agreement between ZN cold Score and ZN hot staining technique for detection of *C.parvum* infection in Children

As shown in Table(1), both of ZN cold 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, (19%) were moderate and (33%) 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 **ZN cold** 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 **ZN cold** and have heavy oocysts score using ZN hot staining procedure. A total of (3%) of cases were reported as having moderate score of *C.parvum* oocysts using **ZN cold** and have heavy oocysts score using ZN hot staining procedure. A total of (3%) of cases were reported as having heavy score of *C.parvum* oocysts using **ZN cold** 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 **ZN cold** and ZN hot staining procedure (P value=0.000).Very good agreement (kappa =**0.862**, p value= 0.000),was reported between ZN hot staining procedure and **ZN cold** for diagnosis of *C.parvum* oocysts in children .

Agreement between Kinyoun's Score and ZN hot staining technique for detection of *C.parvum* infection in children

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As shown in Table(2), both of ZN hot technique and Kinyoun's staining were identical in diagnosis of negative *C.parvum* among diarrhea cases in children , (26%).A total of 11/100 ,(11%) of cases were recorded as positive with low score in both techniques, 12/100, (12%) were moderate and (22%) were recorded as positive with heavy score in both techniques. A total of (11%) of cases were reported as having low score of *C.parvum* oocysts using Kinyoun's and have moderate oocysts score using ZN hot staining procedure. A total of (3%) of cases were reported as having low score of *C.parvum* oocysts using Kinyoun's and have heavy oocysts score using ZN hot staining procedure. A total of (1%) of cases were reported as having moderate score of *C.parvum* oocysts using Kinyoun's and have low oocysts score using ZN hot staining procedure. A total of (12%) of cases were reported as having moderate score of *C.parvum* oocysts using Kinyoun's and have heavy oocysts score using ZN hot staining procedure. A total of (2%) of cases were reported as having heavy score of *C.parvum* oocysts using Kinyoun's 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 Kinyoun's and ZN hot staining procedure (P value=0.000).Very good agreement (kappa =0.614, p value= 0.000),was reported between ZN hot staining procedure and Kinyoun's for diagnosis of *C.parvum* oocysts in children

Table(1): Agreement between ZN cold Score and ZN hot staining technique for detection of *C.parvum* infection in Children

ZN cold 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%)	19(19%)	3 (3%)	22(22%)
Heavy	0(0%)	0(0%)	3(3%)	33(33%)	36(36%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100(100%)
$\chi 2$	226.917				
P value	0.000				
R	0.95625				
P value	0.000				
Kappa	0.862				
P value	0.000				

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Table(2): Agreement between Kinyoun's Score and ZN hot staining technique for detection of *C.parvum* infection in children

Kinyoun's 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%)	11(11%)	11(11 %)	3(3%)	25(25%)
Moderate	0(0%)	1(1%)	12(12%)	12(12%)	25(25%)
Heavy	0(0%)	0(0%)	2(2%)	22(22%)	24(24%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100(100%)
$\chi 2$	154.7783				
P value	0.000				
R	0.887				
P value	0.000				
Kappa	0.614				
P value	0.000				

Agreement between Safranin Methelyne Blue Score and ZN hot staining technique for detection of *C.parvum* infection in children

As shown in Table(3), both of ZN hot technique and SMB staining were identical in diagnosis of negative *C.parvum* among diarrhea cases in children ,(26%).A total of (11%) of cases were recorded as positive with low score in both techniques (12%) were moderate and (18%) were recorded as positive with heavy score in both techniques. A total of (12%) of cases were reported as having low score of *C.parvum* oocysts using SMB and have moderate oocysts score using ZN hot staining procedure. A total of (6%) of cases were reported as having low score of *C.parvum* oocysts using SMB and have heavy oocysts score using ZN hot staining procedure. A total of (1%) of cases were reported as having moderate score of *C.parvum* oocysts using SMB and have low oocysts score using ZN hot staining procedure. A total of (13%) of cases were reported as having moderate score of *C.parvum* oocysts using SMB 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 SMB 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 SMB and ZN hot staining procedure (P value=0.000).Moderate agreement (kappa =0.567, p value= 0.000),was reported between ZN hot staining procedure and SMB for diagnosis of *C.parvum* oocysts in children .

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Agreement between Modified Safranin Methelyne Blue Score and ZN hot staining technique for detection of *C.parvum* infection in children.

As shown in Table(4), both of ZN hot technique and modified SMB staining were identical in diagnosis of negative *C.parvum* among diarrhea cases in children (26%).A total of (10%) of cases were recorded as positive with low score in both techniques, (7%) were moderate and (20%) were recorded as positive with heavy score in both techniques. A total of (16%) of cases were reported as having low score of *C.parvum* oocysts using modified SMB and have moderate oocysts score using ZN hot staining procedure. A total of (6%) of cases were reported as having low score of *C.parvum* oocysts using modified SMB and have heavy oocysts score using ZN hot staining procedure. A total of (1%) of cases were reported as having moderate score of *C.parvum* oocysts using modified Safranin Methelyne Blue and have low oocysts score using ZN hot staining procedure. A total of (11%) of cases were reported as having moderate score of *C.parvum* oocysts using modified SMB 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 modified SMB and have low oocysts score using ZN hot staining procedure. A total of (2%) of cases were reported as having heavy score of *C.parvum* oocysts using modified SMB 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 modified SMB and ZN hot staining procedure (P value=0.000).Moderate agreement (kappa =0.514, p value= 0.000),was reported between ZN hot staining procedure and modified SMB for diagnosis of *C.parvum* oocysts in children

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Table(3): Agreement between Safranin Methelyne Blue Score and ZN hot staining technique for detection of *C.parvum* infection in children

Safranin Methelyne Blue 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%)	11(11%)	12(12%)	6(6%)	29(29%)
Moderate	0(0%)	1(1%)	12(12%)	13(13%)	26(26%)
Heavy	0(0%)	0(0%)	1(1%)	18(18%)	19(19%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100(100%)
$\chi 2$	144.3279				
P value	0.000				
R	0.8571				
P value	0.000				
Kappa	0.567				
P value	0.000				

Table(4): Agreement between Modified Safranin Methelyne Blue Score and ZN hot staining technique for detection of *C.parvum* infection in children

Modified Safranin Methelyne Blue 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%)	10(10%)	16(16%)	6(6%)	29(29%)
Moderate	0(0%)	1(1%)	7(7%)	11(11%)	26(26%)
Heavy	0(0%)	1(1%)	2(2%)	20(20%)	19(19%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100(100%)
$\chi 2$	137.110				
P value	0.000				
R	0.82971				
P value	0.000				
Kappa	0.514				
P value	0.000				

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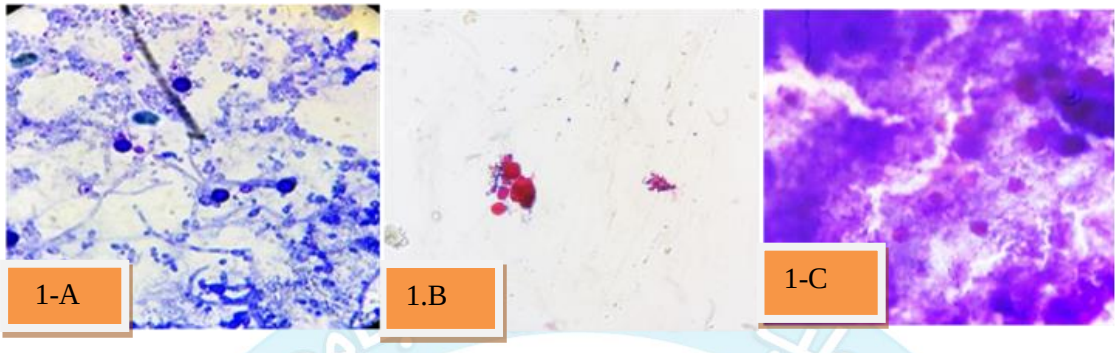

Figure 1. *C.parvum* oocysts detected in diarrheic stool by ZN cold staining technique In children :A .low score ,B. moderate score, C heavy score (100x)

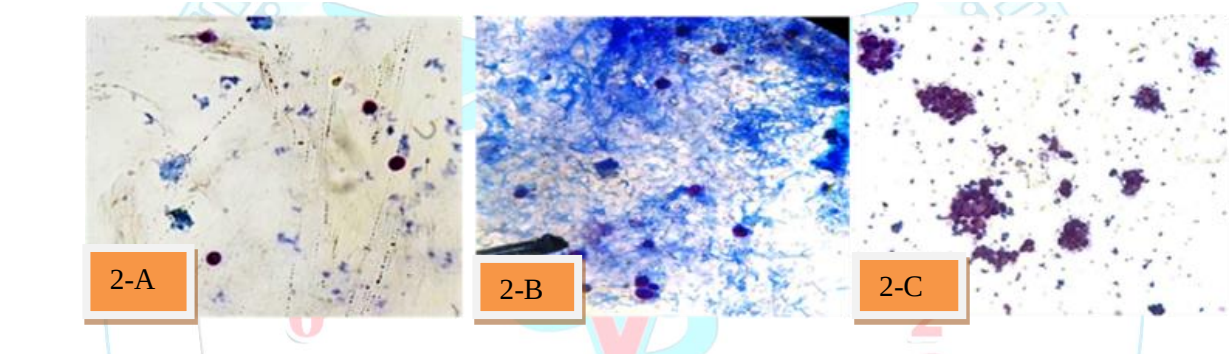

Figure 2. *C.parvum* oocysts detected in diarrheic stool by ZN hot staining technique In children :A .low score ,B. moderate score, C heavy score (100x)

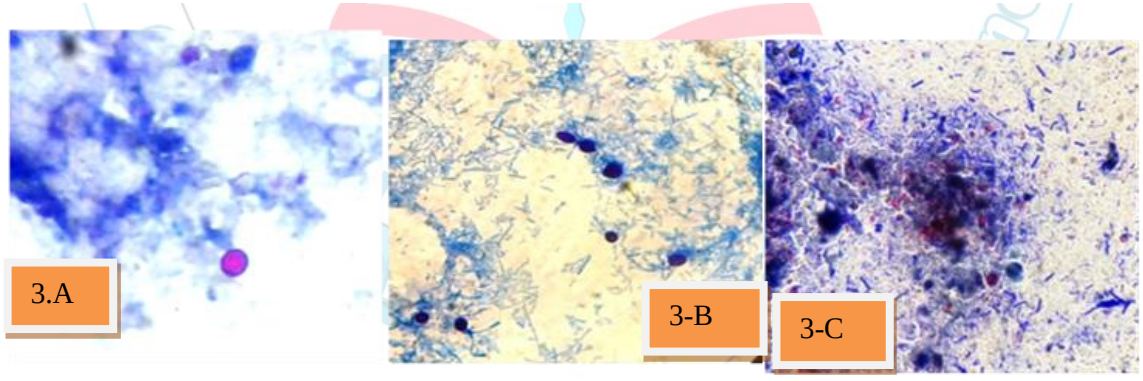

Figure3. *C.parvum* oocysts detected in diarrheic stool by Kinyoun's staining technique In children :A .low score ,B. moderate score, C heavy score (100x)

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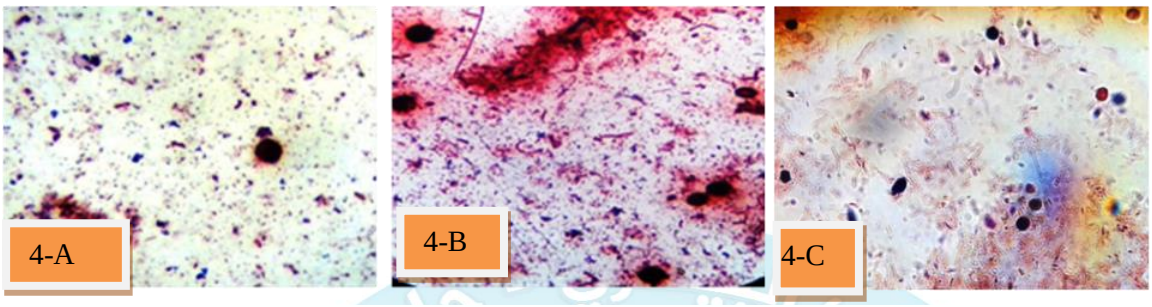

Figure 4. *C.parvum* oocysts detected in diarrheic stool by SMB staining technique In children :A .low score ,B. moderate score, C heavy score (100x)

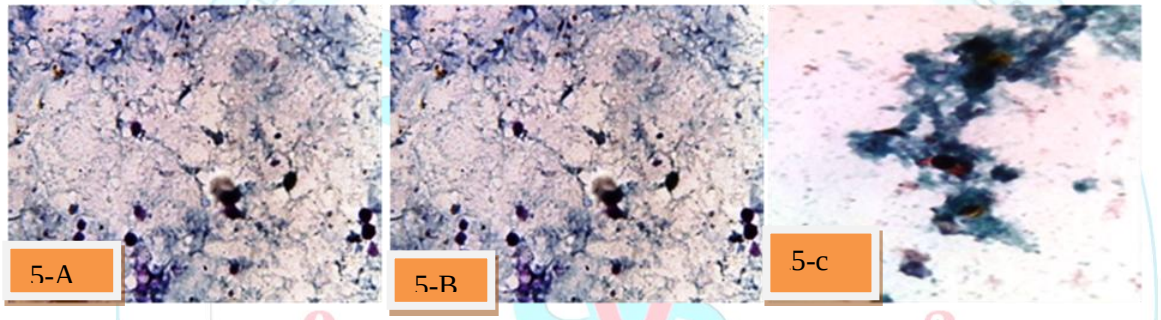

Figure 5. *C.parvum* oocysts detected in diarrheic stool by modified SMB staining technique In children :A .low score ,B. moderate score, C heavy score (100x)

Discussion :

In current study, significant correlation regarding the detection and scoring of *C.parvum* oocysts among children and calves was reported between ZN cold and ZN hot staining procedure ,with obvious differences in scores which come in contrary with previous report by[35] ,who found significant difference in rate of detection between them without utilizing of any scoring system. Current result reveal very good agreement (kappa =0.862, p value= 0.000) between ZN hot staining procedure and ZN cold for diagnosis of *C.parvum* oocysts in children while good agreement (kappa =0.780 , p value= 0.000),was reported between techniques for diagnosis of *C.parvum* oocysts in calves .This come in line with [1, 35],reported that ZN hot and cold techniques was reliable ,easy and relatively simple with low cost and appropriate for detection of oocysts with low numbers. The discrepancy between ZN hot and cold techniques in scores may be attributed to the length of sample treatment and processing ,presence of fecal debris that hidden some of the oocysts beside the effect of heating

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which facilitate the penetration of the stain to the oocysts wall[36].

Cold ZN is simple and easy to learn and practice, it is economical and less cumbersome, is suitable under field conditions, and can be practiced even in remote areas and at periphery where laboratory facilities are limited. There are more practical advantages of cold ZN like, no need of heating in the staining procedure and eliminated the need for separate decolorizing step (requires only two reagents in the staining procedure) .[37]

Agreement between Kinyoun's Score and ZN hot staining technique for detection of *C.parvum* infection in children

In current study significant difference in scores was reported between stains and significant correlation regarding the detection of *C.parvum* oocysts among children and calves was reported between Kinyoun's and ZN hot staining procedure (P value=0.000). Although the recorded scores were variable ,very good agreement (kappa =0.614), in children and moderate agreement (kappa =0. 586) in calves was reported between ZN hot staining and Kinyoun's technique for diagnosis of *C.parvum* oocysts. Differences in score attributed to the several factors include but not limited to ability of oocysts to take-up of stains in variable degree depends on the age of oocysts as well as the developmental stage of oocysts .The use of differential stains like ZN hot technique and Kinyoun's technique which represent modification for the original ZN staining technique facilitate the visual characterization of oocysts from other fecal materials with similar size and shape mainly the yeasts or even bacterial aggregates which take the counter stain(green or blue) versus the oocysts which stained by bright red color[38] . Very good agreement is expected due to staining with both techniques at the same time and short time required for transportation of samples from hospital to laboratory which reduce any harmful effects of formalin 10% preservation on the oocysts. In contrast the moderate agreement between the two techniques may attributed to the effect of long transportation time from rural villages included in the study beside the possible effects of preservation ; aging of oocysts and reduce up-taking of stains which leads to obvious difference in scores and degree of agreement between them.

Agreement between Safranin Methylene Blue (SMB), Modified -SMB Score and ZN hot staining technique for detection of *C.parvum* infection in children

In SMB stain the oocysts stained brilliant reddish orange on a blue background as a results of methylene blue counterstain or a green background in case of modified -SMB as a result of malachite green counterstain. Significant difference and correlation regarding the detection and scoring of *C.parvum* oocysts among children and calves was reported between SMB , modified SMB and ZN hot staining procedure with moderate agreement in children and good agreement in calves was reported between ZN hot staining procedure SMB ;modified SMB technique for diagnosis of *C.parvum* oocysts. The difference in scores could be attributed to the effect of transportation and processing on oocysts up taking of counter stain ,beside the differ-

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ences in the mean of scores of oocysts in the fecal sample taken for preparation of stained samples .In general the agreement between these stains indicate that the diagnosis of oocysts was possible by using alternatives for ZN technique such as SMB , Modified - SMB which is simple ,relatively cheap ,have good discrimination between oocysts and other fecal particles like fungal spores ,bacterial spores and reliable for screening of large scale samples[23, 24, 36, 39]

Conclusion : All Alternative stains for Hot ZN(ZN cold ; Kinyoun's, SMB and modified SMB staining have identical specificity (100%) for diagnosis of negative *C.parvum* among diarrhea cases in children , (26%). According to very good agreement between ZN hot and ZN cold ; ZN hot and Kinyoun's these stains can be used as first line alternative to hot ZN stain .while SMB and modified SMB used as a second alternatives due to moderate agreement with ZN hot staining for diagnosis of *C.parvum* oocysts in children which represent good alternatives in rural areas and low income countries

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