Diyala Journal for Veterinary sciences
Open Access Journal Published by College of Veterinary Medicine
University of Diyala, Iraq
P-ISSN: 2410-8863
Vol. 1, NO. 2, June 2021
Proceedings of 2nd National & 1st International Scientific Conference
Of Veterinary Medicine & Science , (NISCVMS-2021)

Clinical Agreements Between Ziehl Neelsen And Methylene Blue Staining Modifications For Detection Of *C.parvum* Infection In Claves

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Received: 1 -4-2021 Accepted: 25-5-2021 Published: 1-7-2021

Abstract :

Aim : To investigate the agreements between Ziehl Neelsen and Safranin Methylene Blue Staining modalities staining techniques for detection of *C.parvum* infection in calves during first year of life in Diyala province

Methods :Fifty diarrheic calves under 1 year were included .stool samples were examined after stained with Ziehl –Neelsen ;Safranin Methylene Blue Staining modalities

Results:

Both of ZN hot stains and ZN cold , Kinyoun's technique ; Safranin Methelyne Blue (SMB) and Modified Safranin Methelyne Blue (Modified SMB) were identical in diagnosis of (34%), negative *C.parvum* among diarrhea cases. A total of (20%) have low score ; (22%) moderate scores and (8%) heavy score in both techniques. Good agreement (kappa =0.780),was reported between ZN hot staining procedure and ZN cold technique for diagnosis of *C.parvum* oocysts in calves .A total of (24%) , have low score ,(8%) moderate and (4%) with heavy score in both techniques (Hot ZN & Kinyoun's technique). Moderate agreement (kappa =0. 586),was reported between ZN hot staining and Kinyoun's technique for diagnosis of *C.parvum* oocysts in calves. A total of (24%) have low score,(14%) moderate and (8%) with heavy score in both techniques(ZN&SMB). Good agreement (kappa =0. 699),was reported between ZN hot staining procedure and SMB technique for diagnosis of *C.parvum* oocysts in calves.

A total of (20%) have low score,(18%) moderate and (8%) with heavy score in both techniques. Good agreement (kappa = 0.726),was reported between ZN hot staining procedure and Modified SMB technique for diagnosis of *C.parvum* oocysts .

Conclusions : 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 calves . According to good agreement between ZN hot and(ZN cold ; SMB , Modified SMB) ,these stains can be used as first line alternative to hot ZN stain in diagnostic laboratory .While Kinyoun's staining technique used as a second alternatives due to moderate agreement with ZN hot staining for diagnosis of *C.parvum* oocysts in calves which represent good alternatives in rural areas and low income countries

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

How to cite this article :

AL-Ezzy; AIA, Khadim; AT, Humadi; AA. Clinical Agreements Between Ziehl Neelsen And Methylene Blue Staining Modifications For Detection Of C.Parvum Infection In Claves. Diyala Journal For Veterinary Sciences. 2021;1(2):145-58.

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Introduction

Cryptosporidium is intracellular protozoan parasite that live in the gastrointestinal tract of human and many vertebrates animals [1-3]. 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 [4]and in individuals less than five years[1, 2] .Cryptosporidiosis is a diarrheal disease caused by microscopic parasites of the genus Cryptosporidium [1, 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[6]. Both the disease and the parasite are commonly known as "Crypto[7, 8]. Most cases of human cryptosporidiosis are due to infections with the human specific *C. hominis* or the zoonotic *C. parvum* [6, 9].Other Cryptosporidium species have also been detected in humans, although less frequently [10] . 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 [6, 11]. Humans can acquire cryptosporidium infections through several transmission routes such as person to person transmission, zoonotic transmission, food borne transmission and waterborne transmission [2, 12].A single oocyst is sufficient to cause infection and disease [13]. 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 [14]. 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 [15] . Symptoms in immunocompromised patients can be very severe and even death has been described[16] .In developing countries 45% of the children are experiencing an infection before the age of two[2, 17].Current study aims to investigate the clinical compatibility between Ziehl Neelsen and Safranin Methylene Blue Staining modalities For Detection Of *C.parvum* Infection In calves during first year of life in Baqubah-Diyala province.

Material and Methods

Study area and study population

This study was conducted on 50 newborn to less than 1 years old Iraqi calves, hosted in the Baqubah city -Diyala province 33°45'34.71"N; 44°36'23.97"E ,Northeast [3, 18]

Stool Samples collection and processing

Fifty Diarrheic and watery fecal samples were collected using disposable spatula. Samples were labeled with details of age, sex, place and date of collection. Each animal was sampled once only and the collected samples were transported to the laboratory for further processing[19].

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 [20].A total of 200 fields was examined using 40× and 100× to confirm the diagnosis according to oocysts morphology[1, 8].
B. Hot method of ZN staining of fecal smears

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Solutions for the hot ZN staining method according to [21-23] .

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.

2) **Kinyoun's staining method :**Solutions for the Kinyoun's staining method according to[24]

3) **Safranin methylene blue staining (SMB):** The SMB staining technique was followed as per method of [23, 25, 26] .

4) Modified Safranin Technique (Hot Method) according to [27]

A. Scoring system and reporting of oocyst results :

Scoring system for positive sample was used, based on the number of oocyst under x40/ x100 objective lens [8, 28, 29]

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[30, 31]. Chi square was used to verify possible differences between scores of infection and staining techniques[32].Values were considered to be statistically significant when the p-value obtained was less than 0.05[3, 33].The concordance of the Zn hot and other staining techniques was studied using the Cohen's kappa index of agreement[30]. The level of confidence limits was 0.095 and Here is one possible interpretation of Kappa value [34].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

Results :

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

As shown in Table(1), both of ZN cold Score technique and ZN hot staining were identical in diagnosis of negative *C.parvum* among diarrhea cases, (34%).A total of 10/50 ,(20%) , of cases were recorded as positive with low and (22%) moderate scores in both techniques and (8%) were recorded as positive with heavy score in both techniques. A total of (6%)of cases were reported as having moderate score of *C.parvum* oocysts using ZN cold technique and have heavy oocysts score using ZN hot staining procedure.

A total of 4%) of cases were reported as have low score of *C.parvum* oocysts using ZN cold technique ,while they have moderate score in ZN hot staining procedure . on the other hand , (2%) have low score of *C.parvum* oocysts using ZN cold technique and have heavy oocysts score using ZN hot staining procedure. A total of cases reported with moderate score in ZN cold staining procedure have low score(

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2%) and heavy score (2%) when screening via ZN hot staining technique. A total of 3/50 of cases reported with moderate score in ZN cold staining procedure have low score(2%) and moderate score (4%) when screening via ZN hot staining technique.

Significant difference as well as correlation regarding the *C.parvum oocysts* scoring was reported between ZN cold technique and ZN hot staining procedure (P value=0.000). Good agreement (kappa =0.780 , p value= 0.000),was reported between ZN hot staining procedure and ZN cold technique for diagnosis of *C.parvum* oocysts in calves .

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

ZN cold Score	ZN hot score in calves				
	Negative	Low	Moderate	Heavy	Total
Negative	17(34%)	0(0%)	0(0%)	0(0%)	17(34%)
Low	0(0%)	10(20%)	2(4%)	1(2%)	13(26%)
Moderate	0(0%)	1(2%)	11(22%)	1(2%)	13(26%)
Heavy	0(0%)	1(2%)	2(4%)	4(8%)	7(14%)
Total	17(34%)	12(24%)	15(30%)	6(12%)	50(100%)
$\chi 2$	87.253				
P value	.000				
R	0.873				
P value	.000				
Kappa	0.780				
P value	.000				

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

As shown in Table(2), both of Kinyoun's Score technique and ZN hot staining were identical in diagnosis of negative *C.parvum* among diarrhea cases, (34%).A total of (24%) , of cases were recorded as positive with low and ,(8%) moderate scores in both techniques and (4%) were recorded as positive with heavy score in both techniques. A total of (22%)of cases were reported as having low score of *C.parvum* oocysts using Kinyoun's technique and have moderate oocysts score using ZN hot staining procedure. A total of (6%) of cases were reported as have low score of *C.parvum* oocysts using Kinyoun's technique ,while they have heavy score in ZN hot staining procedure .

on the other hand (2%) have moderate score of *C.parvum* oocysts using Kinyoun's technique and have heavy oocysts score using ZN hot staining procedure.

Significant difference as well as correlation regarding the *C.parvum oocysts* scoring was reported between Kinyoun's technique and ZN hot staining procedure (P value=0.000). Moderate agreement (kappa =0. **586** , p value= 0.000),was reported between ZN hot staining procedure and Kin-

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youn's technique for diagnosis of *C.parvum* oocysts in calves

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

Kinyoun's Score	ZN hot score in calves				
	Negative	Low	Moderate	Heavy	Total
Negative	17(34%)	0(0%)	0(0%)	0(0%)	17(34%)
Low	0(0%)	12(24%)	11(22%)	3(6%)	26(52%)
Moderate	0(0%)	0(0%)	4(8%)	1(2%)	5(10%)
Heavy	0(0%)	0(0%)	0(0%)	2(4%)	2(4%)
Total	17(34%)	12(24%)	15(30%)	6(12%)	50(100%)
χ^2	70.474				
P value	.000				
R	.828				
P value	.000				
Kappa	.586				
P value	.000				

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

As shown in Table(3), both of Safranin Methelyne Blue and ZN hot staining were identical in diagnosis of negative *C.parvum* among diarrhea cases, (34%).A total of (24%) , of cases were recorded as positive with low and ,(14%) moderate scores in both techniques and (8%) were recorded as positive with heavy score in both techniques.

A total of (14%)of cases were reported as having low score of *C.parvum* oocysts using Safranin Methelyne Blue technique and have moderate oocysts score using ZN hot staining procedure. A total of (2%) of cases were reported as have low score of *C.parvum* oocysts using Safranin Methelyne Blue technique ,while they have heavy score in ZN hot staining procedure . On the other hand (4%) have heavy score of *C.parvum* oocysts using Safranin Methelyne Blue technique and have low to moderate oocysts score using ZN hot staining procedure ,(2%) for each score.

Significant difference as well as correlation regarding the *C.parvum oocysts* scoring was reported between Safranin Methelyne Blue and ZN hot staining procedure (P value=0.000). Good agreement (kappa =0. 699 , p value= 0.000),was reported between ZN hot staining procedure and Safranin Methelyne Blue technique for diagnosis of *C.parvum* oocysts in calves

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

Safranin Methelyne Blue Score	ZN hot score in calves				
	Negative	Low	Moderate	Heavy	Total
Negative	17(34%)	0(0%)	0(0%)	0(0%)	17(34%)
Low	0(0%)	11(22%)	7(14%)	1(2%)	19(38%)
Moderate	0(0%)	0(0%)	7(14%)	1(2%)	8(16%)
Heavy	0(0%)	1(2%)	1(2%)	4(8%)	6(12%)
Total	17(34%)	12(24%)	15(30%)	6(12%)	50(100%)
$\chi 2$	80.501				
P value	.000				
R	.845				
P value	.000				
Kappa	.699				
P value	.000				

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

As shown in Table(4), both of Modified Safranin Methelyne Blue and ZN hot staining were identical in diagnosis of negative *C.parvum* among diarrhea cases, (34%).A total of (20%) , of cases were recorded as positive with low and,(18%) moderate scores in both techniques and (8%) were recorded as positive with heavy score in both techniques.

A total of (8%)of cases were reported as having low score of *C.parvum* oocysts using Modified Safranin Methelyne Blue technique and have moderate oocysts score using ZN hot staining procedure. A total of (2%) of cases were reported as have low score of *C.parvum* oocysts using Modified Safranin Methelyne Blue technique ,while they have heavy score in ZN hot staining procedure . on the other hand (4%) have moderate score of *C.parvum* oocysts using Modified Safranin Methelyne Blue technique and have low to heavy oocysts score using ZN hot staining procedure ,(2%) for each score. A total of (6%) of cases were reported as have heavy score of *C.parvum* oocysts using Modified Safranin Methelyne Blue technique ,while they have low(2%) to moderate(4%) score in ZN hot staining procedure .

Significant difference as well as correlation regarding the *C.parvum oocysts* scoring was reported between Modified Safranin Methelyne Blue and ZN hot staining procedure (P value=0.000). Good agreement (kappa = 0.726 , p value= 0.000),was reported between ZN hot staining procedure and Modified Safranin Methelyne Blue technique for diagnosis of *C.parvum* oocysts

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

Modified Safranin Methelyne Blue Score	ZN hot score in calves				
	Negative	Low	Moderate	Heavy	Total
Negative	17(34%)	0(0%)	0(0%)	0(0%)	17(34%)
Low	0(0%)	10(20%)	4(8%)	1(2%)	15(30%)
Moderate	0(0%)	1(2%)	9(18%)	1(2%)	11(22%)
Heavy	0(0%)	1(2%)	2(4%)	4(8%)	7(14%)
Total	17(34%)	12(24%)	15(30%)	6(12%)	50(100%)
$\chi 2$	79.118				
P value	.000				
R	0.854				
P value	.000				
Kappa	0.726				
P value	.000				

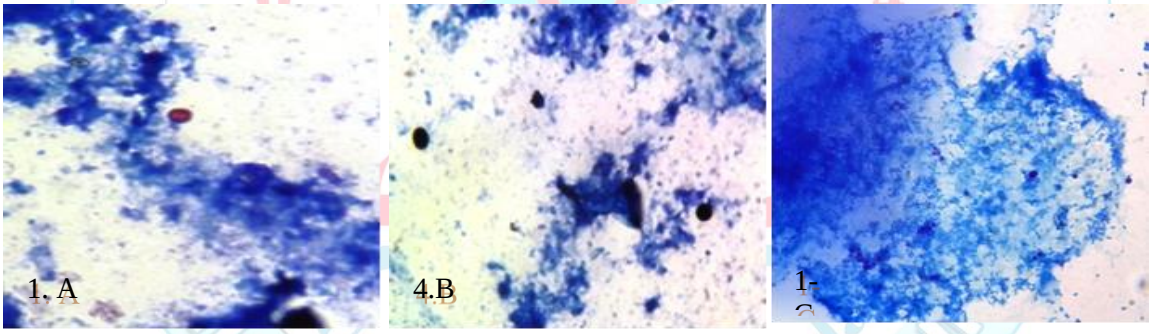

Figure 1. *C.parvum* oocysts detected in diarrheic stool by ZN cold staining technique In calves : A -low score ,B- moderate score, C- heavy score (100x)

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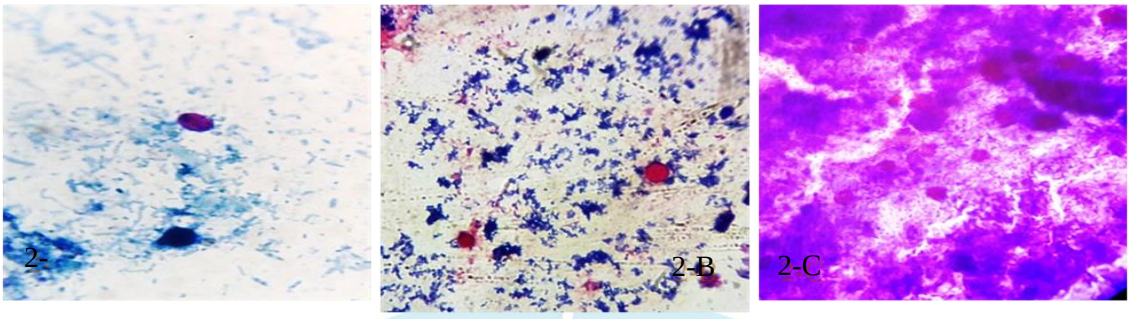

Figure 2. *C.parvum* oocysts detected in diarrheic stool by ZN hot staining technique . In calves :

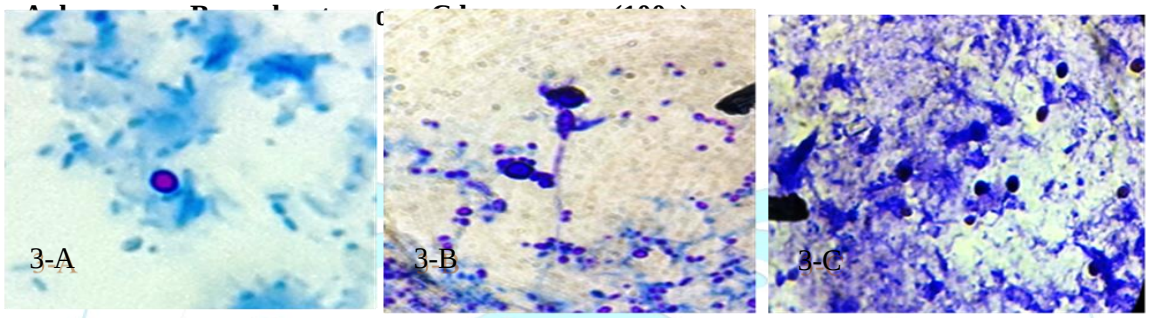

Figure 3. *C.parvum* oocysts detected in diarrheic stool by Kinyoun's staining technique .In calves : A .low score ,B. moderate score, C. heavy score (100x)

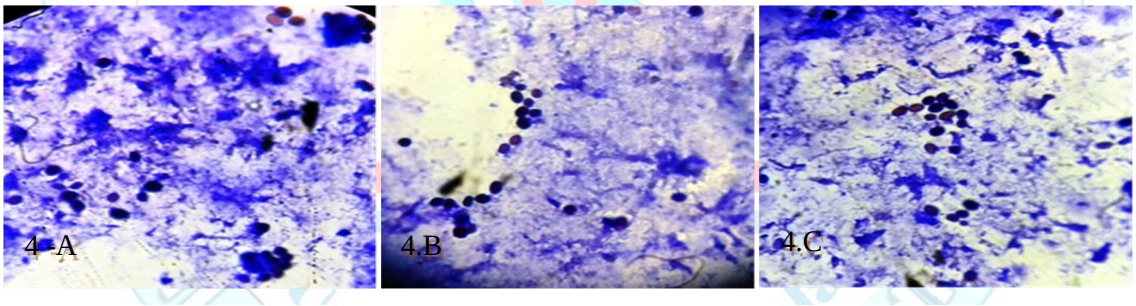

Figure 4. C.parvum oocysts detected in diarrheic stool by Safranin Methelyne Blue staining technique .In calves :A .low score ,B. moderate score, C. heavy score (100x)

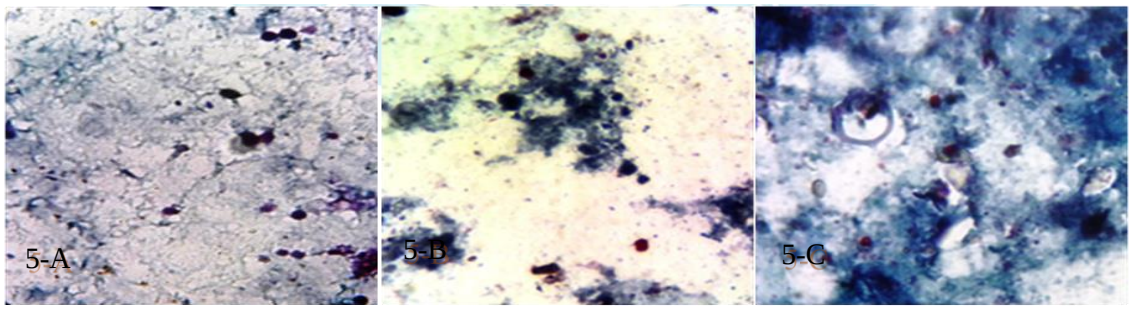

Figure 4.8. *C.parvum* oocysts detected in diarrheic stool by Modified Safranin Methelyne Blue staining In calves :A. low score B. moderate score, C. heavy score (100x)

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Discussion

In developing countries, The laboratory diagnosis of Cryptosporidium infection mostly depends on the microscopic examination of stained fecal smears[1]. 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[35]. In current study, using cold ZN technique, Among calves ,low and moderate score of *C.parvum* oocysts were reported in (24%), (30%) respectively. Heavy score of *C.parvum* oocysts was reported in (12%) . 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[36, 37]. Mucus portion of the fecal sample yielded more number of oocysts [38] .

As both cold and hot ZN staining techniques have similar diagnostic efficacy regarding with a status of calves ,even they differ in their scoring for each case ,this come in line with that reported by [35],stated 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[8].

Regarding Detection of *C.parvum* infection in Calves by concentration using Kinyoun's staining technique, low and moderate score of *C.parvum* oocysts were reported in (25%) while heavy score reported in (24%)of positive cases. *C.parvum* oocysts was detected in (66%) calves suffered from diarrhea .low score of *C.parvum* oocysts were reported in (52 %), moderate score (10%). Heavy score was reported in (4%) . In Kinyoun's staining method, the oocysts appeared as red adjacent to the dark blue background and four sporozoites were visible[8]. A common difficulty with ZN and Kinyoun's staining method is that they cannot distinguish Cryptosporidium oocysts from moulds and yeast [35]. The results of current study agree with that reported by[20] 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 .

Among infected calves low score was reported in (38 %) compared with (30%) in modified protocol of safranine methelyne blue .In the original protocol ,moderate score reported in (16%) compared with (22%) in the modified one . Both original and modified protocol were close in reporting the heavy score of infection ,(12%)V(14%) .

In safranine methelyne blue staining technique; oocysts appeared as orange-pink bodies and the sporozoites within the oocysts stained somewhat darker. Yeast, bacteria, fungal spores and other fecal debris took the counter stain methylene blue in the safranine methelyne blue staining technique staining method. Thus, the method has got the benefit over other methods in differentiating oocysts from yeasts and molds [39].

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In current study, significant correlation regarding the detection and scoring of *C.parvum* oocysts among calves was reported between ZN cold and ZN hot staining procedure ,with obvious differences in scores which come in contrary with previous report by[40] ,who found significant difference in rate of detection between them without utilizing of any scoring system. Current result reveal good agreement between ZN hot staining procedure and ZN cold for diagnosis of *C.parvum* oocysts in calves (kappa =0.780 , p value= 0.000), .This come in line with [40],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 which facilitate the penetration of the stain to the oocysts wall.

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[1]. 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) [41]

In current study significant difference in scores was reported between stains and significant correlation regarding the detection of *C.parvum* oocysts among calves was reported between Kinyoun's and ZN hot staining procedure (P value=0.000). moderate agreement between Kinyoun's and ZN hot staining procedure (kappa =0. 586) in calves 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[23] .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[42] . 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[23].

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 calves was reported between SMB , modified SMB and ZN hot staining procedure with 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 differences 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[20, 43]

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 calves . According to good agreement between ZN hot and(ZN cold ; SMB , Modified SMB) ,these stains can be used as first line alternative to hot ZN stain in diagnostic laboratory .While Kinyoun's staining technique used as a second alternatives due to moderate agreement with ZN hot staining for diagnosis of *C.parvum* oocysts in calves which represent good alternatives in rural areas and low income countries

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