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Evaluation of Nelson-Modified Method and ELISA in the Diagnosis of *Cryptosporidium Parvum*

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Abstract

To evaluate the diagnostic sensitivity of two Cryptosporidium diagnostic tests, used by the laboratory of Obstetrics and Gynecology Hospital.Randomly, 1016 samples of feces were collected from children coming to the Obstetrics and Gynecology Hospital (from both sexes under the five ages and from different regions of Governorate) for the period from first January 2012 to the end of January 2013. The feces samples were diagnosed by using modified Ziehl-Neelsen (ZN) staining and compare the results of diagnosis withthe test of ELISA that an immunological method.

The results of the study found that there was a significant difference ($P < 0.05$) between the two diagnostic methodsin the detection of *Cryptosporidiumparvum*.The results also showed that the rates of *Cryptosporidium parvum* infection out of a total of 1016 samples of diarrhea was 11.6%, by using ELISA technique and 7.79% by using ZN stain.The study proved that commercial ELISA more sensitive than ZN as a diagnostic stool of *Cryptosporidium parvum* infection.

Keywords:Ziehl-Neelsen,ELISA,Cryptosporidium parvum.

Introduction

Cryptosporidium parvum, one of the most popular human pathogen that causing infection to the gastrointestinal tract in worldwide, to the children and individuals with AIDS **(Shirley*et al,* 2012).**Cryptosporidium was related to diarrhea at most in children aged, above 24 months**(Sow*et al,* 2016)**.Malnutrition in the children is a result of infected with Cryptosporidiosis therefore;it possesses big public health significance **(Shaposhnik *et al,* 2019).**

In opposite to other parasites in this phylum, like Plasmodium falciparum and *Toxoplasma gondii* the species belong to the Cryptosporidium genus can't be grown in vitro**(Karanis;2018).**In general, the presence of cryptosporidiosis is recognized by detecting the presence of oocysts in the patient's stool .Several methods have been used to diagnose cryptosporidiosis, including the use of the modified Ziehl-Neelsen, immunological methods, genetic methods also possible, take a biopsy of infected

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intestine and make histological examination**(Ahmed*et al,* 2018)**.Modified Ziehl-Neelsen dye was used to detect the presence of coccidian protozoa, especially oocysts of Cryptosporidium species, and it can also be used to detect the presence of *Cyclosporacayetanensis* and *Isospora belli* oocysts**(Pachco*et al,* 2013).**However, the common methods used in hospital laboratories for diagnosis the Cryptosporidium parvum are the modified Ziehl-Neelsen stain , by using the microscope examination , this method requires a high skill to the workers for the purpose of diagnosis**(Tahvildar *et al,* 2014)** . The method of using the commercial kit ELISA , which is the best according to what was recorded by many studies**(Marques*et al,* 2005**)therefore, the aim of our study was to evaluate the efficiency of these two methods that used in the laboratory of Obstetrics and Gynecology Hospital in Ramadi.

Material and method

Collection of samples

After coordinating with the administration of Obstetrics and Gynecology Hospital.This study was done on, 1016 samples of feces that collected from the children under five years suffering from diarrhea and whom attending to the laboratory of the hospital. From different regions of the Governorate for the period from first January 2012 to the end of January 2013.

Diagnosis of *Cryptosporidium parvum*

Modified Ziehl-Neelsen (ZN) staining

On a glass slide thin smears were prepared with one to two drops of specimen and leave until dried. The Fixation step was made with methanol for 3 minutes, alkaline fashion was added on the smears and heated till it evaporate, but not boiled, after five minute smears were washed fully with distilled water. Decolorizationstep was made by 2.5% H2SO4 for one minute after that briefly washed with distilled water. 1% methylene blue for one minute was used as a counter stain, then washing totally and was dried **(Tahvildar *et al,* 2014).** 20 to 30 fields wereexamined by the compoundlight microscopeunder40X objective lens or higher objectives.The oil immersion objective was used to assure the internal morphology**(Nichols*et al,* 2006).**The oocysts were found out and take a photograph of them. To measure *Cryptosporidium parvum*oocysts the Ocular micrometer was used.**Enzyme linked Immunosorbent assay (ELISA)**

Fecal supernatant for each specimen was made in a ratio of 1:1 (1 gram of feces totally mixed with identical volume of distilled water). The admixture was centrifuged at 1500 rpm for five min. The supernatant was transported to new tube. To preservative the parasite antigen, 0.2% mentholated was added and then stored till used for diagnosis of Cryptosporidium-specific antigen**(Qader*et al,* 2012).** In this study, to diagnose Cryptosporidium antigen in the feces specimens of children a specialized commercial kit was used to detect the presence of the Cryptosporidium

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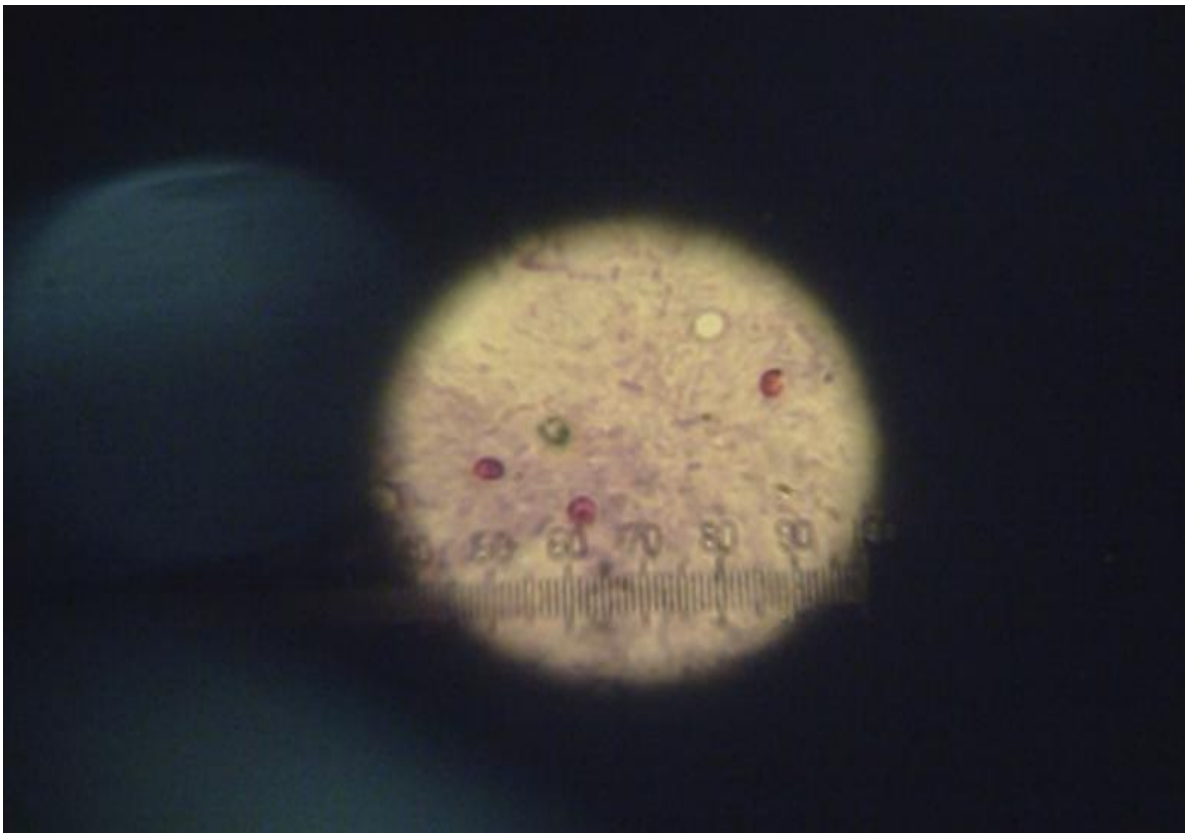
parasite antigen in human faces, and it was supplied by a company **(Carisbad;2016).Statistical analysis**: -Use the chi-square test to compare the efficiency of the immunoassay method (ELISA) and microscopically examined of the feces in the diagnosis of cryptosporidiosis, compare the numbers of oocysts between different patients and a p value of less than 0.05 were considered significant**(Barton.B. & Peat,J. 2014, Khanal ; 2016)**.

Results

Microscopic examination of the oocysts

The microscopic examination of the stool containing the oocysts, after staining by the modified Ziehl-Neelsen stain exhibit that the oocysts are spherical , shining red,**(Khurana*et al,* 2018)** Figure 1.By using the Ocular micrometerthe average diameter of the isolated oocysts from humans were measured6 µm**(Medema *et al,* 1998)**.



For the purpose of detecting the rate of oocysts shedding by patients attended to the Obstetrics and Gynecology Hospital, ten samples were randomly selected from ten patients, and two slides were prepared from each sample and the oocysts were counted in 100 microliters of the sample, and it was found that there were significant differences between the number of oocysts shedding by the patients, at a rate of 1528 per mltable 1.

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Table 1. The total number of oocysts in the prepared slices.

prepared slices	Number of total oocysts on slide in100 µl	Number of total oocysts on slide in 1ml
First slide	112	1120
Second slide	145	1450
Third slide	177	1770
Fourth slide	134	1340
Fifth slide	189	1890
Sixth slide	109	1090
Seventh slide	178	1780
Eighth slide	146	1460
Ninth slide	156	1560
Tenth slide	182	1820
Average	152.8	1528

There were significant differences in the numbers of oocyst between these slides (X2 =48.67) at (P< 0.05).

The results of our current study showed that the diagnosis of the cryptosporidium parasite by the ELISA method is more efficient than the method of staining with Ziehl-Neelsen stain table 2. This study included examining 1016 stool samples collected from children under the age of five and smears were prepared for each stool sample to check for the presence of parasitic oocysts.

Enzyme linked immunosorbent assay (ELISA)

The results of the ELISA test were that (118) individuals had Cryptosporidiosis (infected percentage 11.61%) and 898 negative fecal samples (uninfected percentage 88.4%), while the results of the staining method with the modified Ziehl-Neelsen stain found that (81) individuals had cryptosporidiosis with a percentage of infections (7.97) and 935 fecal samples are negative with a percentage(92.03) table 2.

The statistical analysis indicates that there were significant differences between the two diagnostic methods with a significant level (p <0.05) figure 2.

Table 2. Number of tests (infected and uninfected) for samples isolated from Obstetrics and Gynecology Hospital in Ramadi, by using two type of diagnosis

No. of diagnosis samples	Method of diagnosis	Positive	% of infection	Negative	% non infection
1016	Ziehl-Neelsen	81	7.97%	935	92.03%
	ELIZA technique	118	11.6%	898	88.4%

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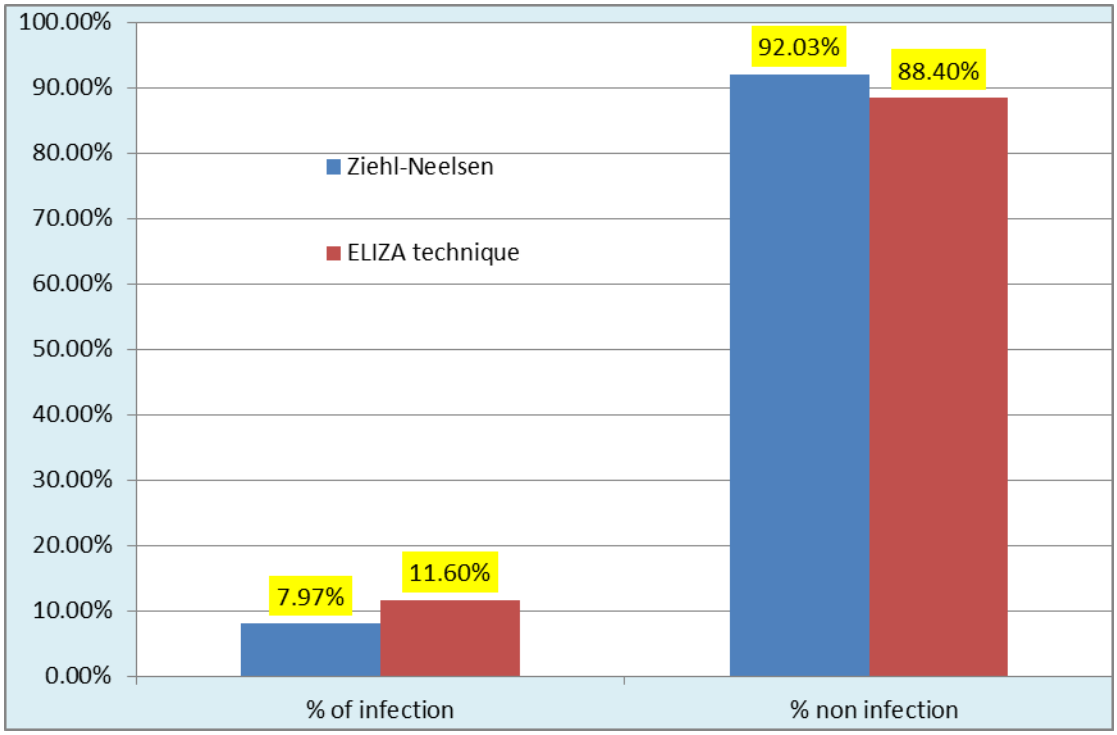

Figure 2. There was significant difference between ELISA and modified Ziehl-Neelsen method (X^2= 6.87) at (P< 0.05).

Discussion

Microscopic examination of oocysts isolated from humans

The modified Ziehl-Neelsen stain was depended upon to test and define the form and measurements of the oocysts isolated from the infected children with the *Cryptosporidiumparvum*, as the oocysts appeared spherical, red in color, and the average size was 6 micrometers, figure 1 this result similar to the result of **(Borowski*et al,* 2010)**that the average size of oocyst is five to seven µm and close to the results of **(Khurana*et al,* 2018,Fayer*et al,* 2000)**that refer to the size of oocysts ranging from three to eight µm.Oocysts of C. parvum are small, most oocysts measure 4-6 mm, appear nearly spherical **(Fayer*et al,* 2000).**Another study by Al-Hashimi **(Al-Hashimi; 2000)** indicated appears as red spherules against the pale green background on the slide. The grad of the stain possessed by thesingle oocyst differsand can be confounded, with several structures like fecal debris, cells of yeast, and spores of bacteria which also stain red but ,are small in size if comparative with oocysts **(Khurana*et al,* 2018).**

The current study used the ELISA test and the modified Ziehl-Neelsen stain to detect the presence of *Cryptosporidium parvum* oocyst in the stool. The results indicated that the ELISA test was the most efficient in detecting the presence of the parasite. This result is in agreement with results of **((Qader*et al,* 2012).**

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, Valdez*et al,* 1997) that the ELISA method is highly efficient and easy to use, but at the same time the ELISA diagnoses method may not be sensitive enough to diagnosis the presence of oocysts when they were in low concentrations. But the advantages of ELISA test were usefulfor examining large numbers of specimens in a short period of time, also does not rely on the skills of microscopy **(Shirley*et al,* 2012)**these results consistent with **(Qader*et al,* 2012).** That found 5.49% positive cases of Cryptosporisiasis when tested by ZN stain and 10.07% positive cases when tested by ELISA. The current study suggests significant dispersal of cryptosporidiosis through the population of the study region identical results were noticeably in other developing countries **(Valdez*et al,* 1997)**.

The difference in the examination results between the ELISA method and the direct examination with Ziehl-Neelsen stain can be explained on the basis that the microscopic examination only identifies healthy cysts that can be seen in a light microscope, and in the event that the cysts are broken in such models, the result of the examination will be negative because the cysts cannot be seen. In contrast, the ELISA test detects the presence of antigens in the solution, including the antigens in the intact and destroyed egg sacs. Therefore, ELISA testing can be considered to be more efficient and faster for many models,while the Ziehl-Neelsen stainshowed a lower efficiency in diagnosis, these results were inconsistent with**(Al-Hashimi; 2002,Rahi*et al,*2013)**.The speed, simplicity, reliability, quality and sensitivity of the immunoassay ELISA test make it one of the best tests used in the routine laboratory examination of the Cryptosporidium parivum , as well as it can be used in large-scale in epidemiological studies of Cryptosporisiasis**(Jayalakshmi*et al,*2008)**.

There were significant differences between the numbers of oocysts shedding by the patients whom infected with *Cryptosporidiumparvum*, at a rate of 1528 per ml, while one of the studies indicated that the rate of oocysts shedding10^5 to 10^7 oocysts/g **(Rahi*et al,* 2013).**The cause for the differences in the number of oocysts shedding by the patient is the amount of the dose that was exposed to it, which caused the infection(**Zambriski*et al,* 2013**).Where the study showed that the calves that shed fewer oocysts are the calves that were exposed to the lowest dose, which confirms that the number of oocysts is inversely proportional to the amount of the oocysts dose that causes the infection. .

ConclusionThe immunological ELISA test is an easy, fast and dependable test that is qualitative and sensitive and is used for routine screening and may be helpful for the great -scale epidemiological studies of cryptosporidiosis .There were significant differences between the numbers of oocysts shedding by the patients that infected with *cryptosporidiosis* disease .Our study proposes significant spread of cryptosporidiosis through the population for the studied city.

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