

Immuno-chromatographic Test for Detection of *Cryptosporidium* in Children with Malignancies before & during Chemotherapy in Basra

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ABSTRACT- Immuno-chromatographic tests may do a significant role in the future diagnosis of parasitic diseases because of their speed and simple of use. Acid fast stain technique and their results were compared with ELISA and IC test for the find of *Cryptosporidium* parasite. One hundred and six stool samples were assayed from malignant children aged 2 month to 14 years before and during 6 to 8 weeks of chemotherapy. Using microscopy, 9 sample before chemotherapy and 15 during chemotherapy were diagnosed as positive for *Cryptosporidium*. Results of IC and ELISA tests show 13 positive samples before chemotherapy and 33 during chemotherapy. The sensitivities and specificities of these test were 100% and 95.9% before chemotherapy and 100%, 80.2% during chemotherapy respectively.

KEY WORDS- Cryptosporidiosis, Immuno-chromatographic test (IC), *Cryptosporidium*, ELISA, Acid fast stain technique.

1. INTRODUCTION

Cryptosporidiosis is a parasitic disease caused by an apicomplexan protozoan called the genus *Cryptosporidium*. The famous species *Cryptosporidium parvum* and *Cryptosporidium hominis* that cause infectious in human [1]. *Cryptosporidium* are water-borne, obligate intracellular protozoan parasite [2]. Ernest Edward Tyzzer in 1907 was the first person successively to identify the oocyst of *Cryptosporidium* parasite in mice [3]. The first case of human cryptosporidiosis was reported in 1976 [4]. Human *Cryptosporidium parvum* associated disease is the result of zoonotic transmission of the parasite's infectious stages, the oocysts. The parasite is transmitted via a fecal - oral route and very often via contaminated water and food [5]. Diarrheal caused by *Cryptosporidium* was estimated in immunocompetent individuals between 1% -10% of diarrheal disease worldwide. It is also found in 6% of all patients with AIDS and in 21% of AIDS patients with diarrhea [6]. The life cycle of *Cryptosporidium* is complex [7]. *Cryptosporidium* has a heteroxenous life cycle, fully developed inside the host. The life cycle of *Cryptosporidium* contains a sexual and asexual type. It can be characterized in to six stages: first oocyst excystation and sporozoites formation, replication within intestine of the host, gametes formation, fertilization, oocyst and sporozoites formation. Transmission occurs directly through fecal-oral route and indirectly through water [8]. This study worked on determining the most sensitive and specific method for identifying the parasite to be a uses for diagnostic laboratories.

2. MATERIALS AND METHODS

SUBJECTS AND METHODS

A total of 106 fecal specimens were collected from children with malignant diseases aged 2 months to 14 years before and during (6 to 8 weeks) chemotherapy in Pediatric Oncology

Center at Specialist Basra children's hospital during May to November 2015. Stool samples were collected and examined using Formalin-Ethyl Acetate Sedimentation method, a modified acid fast staining method, Immuno-chromatographic test (IC) and ELISA. Stool samples must be collected in clean containers without any additives. Depending on the test, then these samples either examined directly or stored at 2-8 °C. If stored for more than 3 days, the sample must be frozen at (-20 °C), thus, the sample must be completely thawed out and brought to room temperature before testing begins.

CONCENTRATION OF FECAL SAMPLES

Stool samples were concentrated using the Formalin-Ethyl Acetate Sedimentation technique to achieve a better result in a modified acid fast staining method [9].

STAINING METHOD

Two thin smears were prepared from concentrated materials, dried in room air and fixed, and after stained with Kinyoun's Acid-Fast and Modified Ziehl-Neelsen acid fast staining method, it was examined under the microscope using oil immersion lenses [9]. The oocysts of *Cryptosporidium* spp. were described pink-red Oocysts are 4 to 6 µm in diameter, and four sporozoites may be present internally. The background should be stained uniformly blue [9].

IMMUNOCHROMATOGRAPHIC TEST (IC)

Procedure as recommended by RIDA®QUICK *Cryptosporidium* kit instructions.

1. Reagent must be brought at room temperature about (20-25°C).
2. Pipette 1 ml of the extraction buffer diluent into a test tube.
3. Add 100 µl or 50 mg stool sample.
4. mix sample via vortex mixer.

5. Leave stool sample to stabilizer about three minutes.
6. Pull about four drops or 200 µl of the supernatant and put it in round slot of the cassette.
7. Read off results after 5 minutes.

Evaluation

Positive: Red band shows for the test and the Blue band control show together.

Negative: Blue control band appears only

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Enzyme Linked Immunoassay (ELISA) for the detection of *C. parvum* in faecal samples. RIDIASCREEN® test (C 1201). (Art. No.: C 1201. GmbH, Darmstadt, Germany) was used in this study. The test was used for in vitro diagnosis of *Cryptosporidium*. This test is enzyme immunoassay for the qualitative determination of *C. parvum* in faecal samples and performed according to instructions of the kits. Microwell plate and reagents were brought to room temperature (20-25 °C). The washing buffer was diluted with distilled water 1:10, followed by dilution the faecal sample with the sample dilution buffer diluent 1:11. The required micro well strips were placed in the frame followed by addition of 2 drops or 100 µl positive control (control +), negative control (diluent), or sample. After that 2 drops or 100 µl conjugate were added. Incubation was at room temperature (20-25°C) for 60 min followed by Wash for five times with 300 µl diluted wash buffer followed by addition of 2 drops or 100 µl substrate. The faecal sample was incubated again at room temperature (20-25°C) in the dark for 15 min followed by adding 1 drop or 50 µl stop reagent. Finally, photometric measurement was carried out at 450 nm (optional reference wave length > 600 nm).

ASSESSMENT AND INTERPRETATION

CALCULATING THE CUT-OFF

In order to establish the cut-off, 0.15 extinction units are added to the measured extinction for the negative control. (Cut-off = extinction for the negative control + 0.15)

RESULTS

- Positive: if the extinction rate is more than 10 % higher than the calculated cut-off value.
- Marginal: if the extinction rate ranges from 10 % less to 10 % greater than the cut-off value.
- Negative: if extinctions more than 10 % below the calculated cut-off.

STATISTICAL ANALYSIS:

Statistical package of social science (SPSS) version 20 was used to analyze data, Chi-square (X²) test was used to assess the significance of difference between groups and variable, P-value less than 0.05 was considered to be statistically significant.

3. RESULTS

Leukemia, at 58%, was the most common type of malignant cases in the subjects studied. Solid tumors were the second

most prevalent type of malignant cases at 27%, followed by Lymphoma at 15%. The age group most affected were between the ages of 1 - 4 years old, as shown in Table 1. Statistically, the difference between the age groups were significant (X²=4.998, df =6, P <0.05), but the difference between genders did not show significant difference (χ² =0.426, df =2, P>0.05).

Table 1 Distribution of patients according to the gender and age

Type of malignant cases	Age*				Gender**		Total	
	<1	1-4	5-9	10-14	male	female	No	%
Leukemia ¹	4	20	22	15	36	25	61	58%
Lymphoma ²	1	5	5	5	9	7	16	15%
Solid Tumor ³	3	15	7	4	15	14	29	27%
Total	8	40	34	24	60	46	106	100%

* X² =4.998, df =6, P <0.05

** X² = 0.426, df =2, P>0.05

1. Leukemia include: (Acute lymphocytic leukemia, Acute myelocytic leukemia and Chronic myelocytic leukemia).
2. Lymphoma include: (Hodgkin lymphoma and Non-Hodgkin lymphoma).
3. Solid tumor include: (Neuroblastoma, Osteogenic sarcoma, Hepatoblastoma, Adenocarcinoma, Ewing’s sarcoma, Rhabdomyosarcoma and Brain tumor).

The results showed that the total infectivity rate of *Cryptosporidium* spp. in children was (31.1%) during chemotherapy in comparison with (12.3%) before chemotherapy as shown in Table 2. The number of positive samples were 33 out of 106 patients during chemotherapy and 13 out of 106 before chemotherapy .Using acid fast stain technique, 9 were positive before chemotherapy and 15 during chemotherapy, while both Immunochromatographic test (IC) and ELISA shows higher positivity rates (13 samples before chemotherapy and 33 during chemotherapy were positive).

Table 2 The positive and negative rate of *Cryptosporidium* diagnosed by acid fast stain technique, IC test and ELISA before and during chemotherapy

Patients group	Staining method				X ²	P value
	Positive		Negative			
	No.	%	No.	%		
Before chemotherapy	9	8.5	97	91.5	1.691	0.139
During chemotherapy	15	14.2	91	85.8		
Patients group	IC test & ELISA				X ²	P value
	Positive		Negative			
	No.	%	No.	%		
Before chemotherapy	13	12.3	93	87.7	11.105	0.001
During chemotherapy	33	31.1	73	68.9		

Table 3 shows the sensitivities and specificities of Immunochromatographic test (IC) and ELISA. Before and during chemotherapy, the sensitivity of both test was 100%, while the specificity before chemotherapy was 95.9% and decreased to 80.2% during chemotherapy for the above tests.

Table 3 Sensitivity and specificity of Immunochromatographic test (IC) and ELISA

Before chemotherapy*				
Test	Result	Staining Method		Total
		No. Patients		
		Positive	Negative	
IC Test	Positive	9	4	13
	Negative	0	93	93
ELISA	Positive	9	4	13
	Negative	0	93	93
During chemotherapy**				
Test	Result	Staining Method		Total
		No. Patients		
		Positive	Negative	
IC Test	Positive	15	18	33
	Negative	0	73	73
ELISA	Positive	15	18	33
	Negative	0	73	73

*Before chemotherapy: Sensitivity = 100%, Specificity = 95.9%

**During chemotherapy: Sensitivity = 100%, Specificity = 80.2%.

In Table 4, among 106 children with malignant diseases, 66 (62.3%) were found to be positive for various species of intestinal parasites before chemotherapy in comparison with 40 (37.7%) during chemotherapy. Statistically, the difference was very highly significant ($X^2 = 15.598$, $df = 2$, $P < 0.05$).

Table 4 Parasitic and *Cryptosporidium* infections among patients with malignant disease before and during chemotherapy.

Patients group	No. examined	Parasitic infections*		<i>Cryptosporidium</i> infections	
		+ve	%	+ve	%
Before chemotherapy	106	66	62.3	13	12.3
During chemotherapy**	106	40	37.7	33	31.1

$X^2 = 15.598$, $df = 2$, $P < 0.05$

*Parasitic infections: including *Cryptosporidium* infection.

**during chemotherapy (6-8 weeks).

4. DISCUSSION

The detection of *Cryptosporidium* antigens in stool specimens using enzyme immunoassays has become an accepted approach to diagnosis and, although it can be used in epidemiological survey for screening multiple specimens simultaneously, being laborious and time consuming make it unpractical as a routine laboratory technique [10]. So, there is need for better diagnostic method that surpasses the limitations of the currently available techniques and not to miss any case of infection. In this context, the Immunochromatographic (IC) test was tried and its performance was assessed in reference to other conventional methods. Immunochromatographic test allows the reliable detection of oocysts in one step, does not require any special skill and is simple to use. Among several authors who performed Antigen detection by immunoassays, Agnamey et al. (2011) concluded that these methods have become a well-established aid to microscopic examination for the diagnosis of cryptosporidiosis [11]. Results also determined high sensitivities and specificities for both Immunochromatographic test and ELISA. A good sensitivities and specificities have been recorded by Agnamey et al. (2011) [11].

Leukemia is the most common type reported in our study. It was (61/106) about (58%) and in this study most of patients belong to the age group (1-4) years (40/106) about (38%) in Table 1, similar result was observed by different studies [12-14]. Interpretation of this result is due to the younger age groups, or may be due to the reason in this age group because of the influence of other diseases which lead to a change in the immune system and weakness in resistance to disease [15, 16]. In our research also showed in relation to gender of the patients, males were more affected than females with malignant diseases and the highest rate in leukemia, The cause of infections in the proportion of males is higher than females may be due to the activity and the movement of males

over females, and in other research remained unjustified or reason for the existence of gene shows why males more susceptible to infection [17].

Cryptosporidium was found in (12.3%) in patients with malignant diseases before chemotherapy compared with (31.1%) in the same groups patient during chemotherapy in Table 2. These results are different to some studies, as in Basra city which found *Cryptosporidiosis* is about 9.9% and 8% as reported by Mahdi et al. (2002) [18] and Mahdi et al. (1997) [19], while in Baghdad 14.78% was reported by Al-Warid et al., (2012) [20]. Different rates of infection were reported in other locations (3.4% in Kuwait [21]; 4.8% in Egypt [22]; 4.2% in Iran [23]; 10.4% in Ethiopia [24]; 12.6% in German [25] and 3.8% in India [26]. Explanation the differences and disparities in rates of *Cryptosporidium* infection among children of cancer patients may be attributed to that the patients who are exposed to chemotherapy will result in weaknesses in both their cells and their immune system and so the parasite can penetrate body [27-29], also *Cryptosporidiosis* is considered to be one of the most serious opportunistic infections that complicates cancer [30], in addition to their rapid route transmission as it is fecal-oral and person to person transmission [31]. *Cryptosporidium* is one of the diseases transmitted by animals to humans and vice versa [31, 32], that may be attributed to their high prevalence.

In this study also find the presence of other intestinal parasites were decreased during chemotherapy than before chemotherapy, because of giving treatment provided to the children. This significant reflect two major changing reduction in the all-percent increasing in *Cryptosporidium* infection shown in Table 4. These results are differently to some studies, as in Basra was found (49.5%) in different types of malignancy [22], in Baghdad was found parasitic infections about (71.4%) [34]. In Egypt found parasitic intestinal about (12.9%) [35], Botero et al. in 2003 reported about (32.9%) of intestinal parasites reported found in leukemia patients [36]. In Iran Zabolinejad et al. found 35.9% [37].

This study found a good correlation between the Immunochromatographic (IC) test and ELISA when compared with staining method giving sensitivities of (100%) and specificities (95.9%) before chemotherapy, while Immunochromatographic test (IC) and ELISA giving sensitivities of (100%) and specificities of (80.2%) during chemotherapy. This result shows similarity to study in UK [38]. Also in the same table (3) was the emergence of positive cases in the examination IC test and ELISA and negative in the staining method for the same patients. This result is due to

the number of oocysts, which take during the examination where in the way staining method need oocysts about 500,000 oocysts per ml of stool [39], but in ELISA needed about 500 oocysts per ml in stool [40] and in IC needed about 1000 oocysts per ml in stool [41]. So observed proportion of positive cases in Immunochromatographic test and ELISA is more than the proportion observed in staining method because in staining method needed high number of oocysts, also it needs to be versed examiner to detect oocysts due to the small size of the oocysts by microscopic examination [42].

In conclusion, We can conclude that Immunochromatographic test is specific, sensitive and easy to use test for diagnosis of *Cryptosporidium* oocysts in stools.

To our knowledge, this is the first study in IRAQ that uses RIDA® Quick *Cryptosporidium* test to detect *Cryptosporidium* infection in humans and compare their sensitivity and specificity with that of standard staining method used with in malignant children before and during chemotherapy.

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