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DETECTION OF INTESTINAL PROTOZOAN INFECTIONS WITH STRESS ON *BLASTOCYSTIS*, *MICROSPORIDIA* IN EGYPTIAN CHRONIC KIDNEY DISEASE PATIENTS

By

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Abstract

This study assessed intestinal protozoa with stress on *Blastocystis* among patients with CKD and those on hemodialysis (HD) in Nephrology Unit, Medical Research Institute, Alexandria.

The patients were divided into three groups. GI: 50 patients on HD for at least one year, GII: 50 CKD patients not on HD, and GIII: 50 healthy controls. Fresh stool samples were collected in labeled plastic boxes and examined as direct smears after staining with trichrome, Modified Ziehl-Neelsen (MZN) stains and modified trichrome (MT) stains. Besides, stool samples were cultured for *Blastocystis hominis* and Copro-antigen (ICT) was used to detect *E. histolytica /dispar, G. lamblia* and *C. parvum*.

The results showed that the parasitic infections were detected in 79% of the patients; 40% in HD and 39% in CKD patients. The positive samples were (47%) detected by direct wet mount followed by iodine smear (43%) and then trichrome stain (42%). Microsporidiosis was detected in 55 (55%) HD and CKD patients using the MT stain. ICT agreed with microscopic examination in diagnosis of *E.histolytica/ dispar* and *G. lamblia* and showed perfect agreement with MZN in *C. parvum* diagnosis.

Keywords: Chronic kidney disease patients, Intestinal protozoa.

Introduction

Intestinal protozoan infections (IPIs) constitute a significant problem worldwide with more than 58 million cases of IPIs each year, and more than 60% of the world's population being infected with at least one or more intestinal parasites during his/her life time. Numerous pathogenic protozoa inhabited the gastrointestinal tract of humans causing morbidity and mortality worldwide (Kucik *et al,* 2004). Several enteric protozoa were associated with diarrhea and debilitating sickness, especially in immunosuppressed persons (Fletcher *et al,* 2012). Species of *Blastocystis*, *Cyclospora*, *Cryptosporidium*, *Dientamoeba, Entamoeba*, and *Giardia* were the commonest pathogenic ones (McHardy *et al,* 2014).

Despite the continuous improvement in the economic status, standard of living and the environmental sanitation of the Egyptian society, yet parasitic infections continue to be one of the most common public health problems (Mahfouz *et al,* 1997).

Infectious intestinal parasites are transmitted to humans through several ways, including contaminated food and water, inadequately treated sewage/sewage products, livestock and domestic pet handling. Foodborne transmission occurs during the harvesting, handling, and preparation processes, from cross-contamination with soiled implements, animal manure or contaminated water used for food preparation or via the food handlers themselves (Fletcher *et al,* 2012). Waterborne transmission still poses significant risks to human health both in developed and developing countries (Bridge *et al,* 2010). The cysts/ oocysts of several protozoa are highly resistant to conventional water treatment by chlorinetion (Carey *et al,* 2004). Immunodeficiency has deeply changed human-parasite relationships, and promoted the emergence or re-emergence of parasites, indeed, immune protection largely determines parasitic specificity and species barriers. Immune suppression weakens some inhibiting processes. Moreover, it may modify the host-parasite relationship and can promote the emer-

gence of opportunistic infections (Lallo *et al,* 2012). Among immunocompromised group were patients suffering from chronic kidney disease (CKD), which once established, it causes progressive and irreversible loss of kidney function leading to the need for renal replacement therapy. Patients with end stage renal disease (ESRD) undergoing hemodialysis (HD) are individuals with significantly compromised immune system (Gil *et al,* 2013). So, the CKD was accepted as a gained immune deficiency. CKD has negative impacts on neutrophil chemotaxis, phagocytosis, and bactericidal actions as well as T cell function. The problems in maturing of the T lymphocytes increase susceptibility to infections (Meijers *et al,* 2012).

It is known that cellular immunity plays an important role in defense against parasitic diseases. Suppression of the immune system causes the increase of the pathogenic effects of the parasites and lead to the formation of severe clinical illnesses. CKD patients on HD had other combined conditions as diabetes, hypertension and HCV infection that increase the risk of infection. When the infection risks and related complications of these patients, who are vulnerable are considered, studies towards preventing infections are crucial. Also, it was important to study, factors that increase risk to opportunistic infections, such as *Cryptosporidium, Cyclospora, Isospora belli*, and *Microsporidia,* and other pathogenic intestinal parasites in patients with suppressed immunity (Stark et al 2009).

The work was aimed to detect intestinal protozoa with stress on *Blastocystis* sp.**,** *Microsporidia* sp., using different diagnostic methods among CKD patients and those on HD in the Nephrology Unit, Medical Research Institute, Alexandria.

Materials and methods

A descriptive comparative case-control study was conducted on 150 subjects presenting to nephrology unit, Internal Medicine department and laboratory of parasitology department, Medical Research Institute (MRI). They were divided into three groups: GI: 50 HD patients for at least one year, GII: 50 CKD patients not on HD, and GIII: 50 apparently healthy control. All did not have any anti-parasitic drugs one month prior to the study.

All participants were subjected to: Complete history taking including demographical data and clinical examination.

Stool examination. Fresh samples were divided into three portions: a- First portion for microscopic examination, samples were examined by direct wet saline smear; Formol ethyl acetate concentration method and Lugol's iodine smear (Garcia 2016). Permanent staining of fecal smears from concentrated fecal samples using: Trichrome stain, Modified trichrome stain (MTS), Modified Ziehl-Neelsen (Garcia, 2016), b- Second one for xenic cultivation of *Blastocystis hominis* (Jones, 1946), and c- Third one for copro-antigen detection for *E. histolytica/dispar, G. lamblia* & *C. parvum* using Rida®Quick *Entamoeba/ Giardia/ Cryptosporidium* Combi Test (R-Biopharm AG, Germany) according to the manufactures' instructions.

The protocol of the study was approved by the Research Ethics Committee. All participants were volunteers in the study, after giving their informed written consent.

Statistical analysis: Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level. The results were considered significant if $p \leq 0.05$.

Results

The mean age of patients in GI was 46.16±13.27 years, but it was 43.56±12.66

years in GII and 33.67±11.73 years in GIII. Males constituted about 28 % of GI & 32 % of GII whereas 72 % of GI & 68 % of G II were females, but in GIII, 52% were males & 48% were females. The overall parasitic infections was significantly higher in HD (80%) and (78%) in CKD patients compared to the healthy controls (10 %). No helminthic parasites were found. Using direct wet mount, *B. hominis* was the most frequently detected protozoa in all groups. It was found in a significantly high in HD patients (46%) and the CKD group (36%) compared to control group (8%). There was no significant difference between the studied groups regarding the percentage of *E. histolytica\dispar* and a non-pathogenic one *Entamoeba coli*. *Giardia* and *Cryptosporidium* sp. were detected in HD patients only (4% & 2% respectively). While *Microsporidia* sp. was found in a significantly higher percentage of HD (48%) and CKD (62%) patients compared to the control group (Table 1). Single infection was detected in 65% of infected HD patients and 35% had double infection. While 71.8% of infected CKD patients had single infection and 28.2% had double infection (Tab. 2).

The greatest percentage of protozoan positive samples (47%) was detected by direct wet mount followed by Iodine smear in 43 cases (43%) and Trichrome stain in 42 cases (42%). Concerning *B. hominis*, direct smears and Iodine smears gave higher detection rates (41% and 39% respectively) compared to Trichrome staining which missed 4 positive samples (37%) but this difference didn't reach level of significance. As to the *E. histolytica/dispar*, direct smears and Iodine smears were superior to trichrome stain which missed one positive sample. Iodine smears missed two cases of *E. coli*, which were detected by both direct smears and Trichrome stain. Two *G. lamblia* positive samples were diagnosed by the three parasitological methods (Tab. 3).

Out of 100 examined renal patients stool samples, only one case (1%) had *C. parvum* infection using MZN stain in HD group. While 55 cases in the two groups HD and CKD (24 cases and 31 cases respectively) had *Microsporidia* infection using MTS (Tab. 4).

Microscopic examination of direct wet mount smears detected *B. hominis* in 41% patients (23 HD patients and 18 CKD) and in four apparently healthy controls. On the other hand xenic culture for *B. hominis* using Jones' medium detected only 38% of patients groups, missing three positive cases. Statistical analysis revealed very good agreement between direct smears and xenic culture method (Kappa index = 0.937 *, $p<0.001$) (Table 5).

G. lamblia was detected in two cases by ICT for copro-antigens, while *E. histolytica* and *C. parvum* were detected in only one case each (Tab. 6).

The two positive cases were diagnosed by microscopic examination by iodine and another one case was diagnosed by ICT, and, 97 cases were negative by both techniques. A moderate agreement was detected between both techniques in diagnosing *E. histolytica* (Tab. 7).

One positive case of *C. parvum* was diagnosed by both microscopic examination by MZN stain and ICT. Statistical analysis showed a Kappa index of 1 indicated the perfect agreement between the MZN stain and ICT in diagnosing *C. parvum* infection (Tab. 8).

There were two positive cases of *G. lamblia* detected by both the microscope and ICT. Statistical analysis showed a Kappa index of 1 indicated the perfect agreement between MZN stain and ICT in diagnosing *G. lamblia* infection as well (Tab. 9).

Table 1: Types of protozoan infection detected in groups

Parasitic infection	G\I (n=50)		GII (n=50)		G\III (n=50)		$\chi 2$	P
	No	%	No	%	No	%		

Negative	10	20.0	11	22.0	45	90.0		
Positive	40	80.0	39	78.0	5	10.0	64.448*	<0.001*
Sig. bet. groups	P1=0.806, P2<0.001*, P3<0.001*							
Parasites detected								
G. lamblia	2	4.0	0	0.0	0	0.0	2.682	MCp=0.329
B. hominis	23	46.0	18	36.0	4	8.0	18.476*	<0.001*
Sig. bet. Groups	P1=0.309, P2<0.001*, P3=0.001*							
E. histolyti-ca/dispar	2	4.0	1	2.0	0	0.0	1.858	MCp=0.780
Cryptosporidium spp	1	2.0	0	0.0	0	0.0	1.831	MCp=1.000
E. coli	2	4.0	0	0.0	1	2.0	1.858	MCp=0.779
Microsporidia spp	24	48.0	31	62.0	0	0.0	45.531*	<0.001*
Sig. bet. Groups	P1=0.159, P2<0.001*,P3<0.001*							

$\chi 2$: Chi square test, MC: Monte CarloFE: Fisher Exact, p: p compared 3 groups, p1: p value for comparing between HD and CKD, p2: p compared between HD and Control, p3: p compared between CKD and Control, * significant at $p \leq 0.05$

Table 2: Multiplicity of protozoal infection among infected subjects

Infection status	GI (n=40)		GII (n=39)		GIII (n=5)		$\chi 2$	MCp
	No	%	No	%	No	%		
Single	26	65	28	71.8	5	100	2.282	0.351
Double	14	35	11	28.2	0	0.0		

Table 3: Protozoal infection by different techniques among renal patients

Types of parasite	Direct smear		Iodine smear		Trichome stain		$\chi 2$	P
	No	%	No	%	No	%		
B. hominis	41	41.0	39	39.0	37	37.0	0.336	0.845
Sig. bet.	P1=0.773,P2=0.562,P3=0.771							
E.histolytica/dispar	2	2.0	2	2.0	1	1.0	0.592	MCp=1.000
Sig. bet.	FEp1=1.000, FEp2=1.000,FEp3=1.000							
E. coli	2	2.0	0	0.0	2	2.0	2.080	MCp=0.553
Sig. bet.	FEp1=0.497,FEp2=1.000,FEp3=0.497							
G. lamblia	2	2.0	2	2.0	2	2.0	0.220	MCp=1.000
Sig. bet.	FEp1=1.000, FEp2=1.000,FEp3=1.000							

Table 4: Detection of *Cryptosporidium* sp. and *Microsporida* sp. among renal patients using special stains

Special stain	Positive	
	No	%
Cryptosporidium sp. (M.Z.N)	1	1.0
Microsporidia sp. (M.T.S)	55	55.0

Table (5): Agreement analysis of *B. hominis* detected by direct smear and xenic Culture method

Techniques / *B. hominis*	Direct smear		Xenic culture		$\chi 2$	P
	No	%	No	%		
Negative	59	59.0	62	62.0	88.198*	< 0.001*
Positive	41	41.0	38	38.0		
Total	100	100.0	100	100.0		
Kappa	0.937 (very good)					

Table 6: Distribution of protozoa infection among 100 individuals tested by ICT

	Positive	
	No	%
E. histolytica/dispar	1	1.0
Giardia lamblia	2	2.0
Cryptosporidium sp.	1	1.0

ICT: Immuno-chromatographic test

Table 7: Agreement between microscopic examination (Iodine) and ICT for *E. histolytica/dispar*

Microscopic examination (Iodine)	Immuno-chromatographic test (ICT)		Total
	Positive	Negative	
Positive	0	2	2
Negative	1	97	98
Total	1	99	1

Table 8: Agreement between microscopic examination by MZN stain and ICT for *Cryptosporidium* sp.

M.Z.N stain	ICT		Total
	Positive	Negative	

Positive	1	0	1
Negative	0	99	99
Total	1	99	100

Kappa index = 1, p< 0.001 perfect agreement

Table (9): Agreement between microscopic technique and ICT for detection of *G. lamblia*

	ICT		
Microscope	Positive	Negative	Total
Positive	2	0	2
Negative	0	98	98
Total	2	98	100

,Kappa index = 1, p< 0.001 perfect agreement

Discussion

Protozoa diseases were considered major causes of morbidity and mortality in the developing world. The segment of the population with significant defects in the immune system continues to grow. Chronic diseases that include asplenia, CKD and chronic hepatic disease are considered as immunosuppression status. These patients catch parasitic infections more easily (Abdel-Hafeez *et al,* 2012, Kamki *et al,* 2015, Bora *et al,* 2016).

B. hominis is an obligate anaerobic protozoan found in human and animal's large intestine, and is the most common eukaryotic organism reported in human fecal samples (Zierdt *et al,* 1967). Its diagnose depended on laboratory techniques such as routine direct microscopy of wet preparations or stained with trichrome or iodine stain, the cultivation and immuno-assay methods (Elghareeb *et al,* 2015). *Cryptosporidium, Isospora belli*, and *microsporidia* are intestinal protozoa that cause obligatory intracellular infections. They are transmitted either by stool from person to person or through contaminated water or food by spores or oocysts (Goodgame, 1996).

In the present work, the majority of participants were older than 40 years (66% GI & 68% GII) and about two thirds of them were females (70%). The overall percentage of protozoa detected in patients was 79% {HD (40%) and CKD patients (39%)} in the following frequency:55 % *Microsporidia* spp, 41 % *B. hominis*, 3% *E. histolytica*, 2% for each of the *G. lamblia* and *E. coli* and 1% *Cryptosporidium* spp., without helminthes. Of 79 infected patients, single protozoan was in 54 patients (68%) and double protozoa were in 25 (31.6%). Regarding controls; four individuals were infected with *B. hominis* and one with the non-pathogenic *E. coli*. The results agreed with Shehata *et al* (2019) who found a significantly higher prevalence rate of intestinal parasitoses among HD patients compared to apparently healthy controls (52.5% vs. 12.0%, respectively), and without helminthes. The parasites among patients were *Cryptosporidium* sp. (32.5%), *B. hominis* (24.2%) and microsporidia (11.7%). Ali *et al.* (2000) in HD patients and controls with diarrhea detected 33.3% protozoa in patients & 5% in controls. *C. parvum* was found in 15% and *Microsporidia* in 8.3% of patients. Kulik *et al.* (2008) reported that *Blastocystis* sp. in 21% of HD patients, in mixed or single infections, while *Cryptosporidium* sp. and *E. coli* were 4.7% for each one. The difference in prevalence may be due to differences in population demographics, behavior, nutritional status, educational level, socioeconomic and seasonal factors, as well as diagnostic methods (Al-Hindi *et al,* 2008).

In the present study, some protozoa positive samples were missed by trichrome staining. The positive samples (47%) were detected by direct wet mount followed by iodine smear (43%) and then trichrome stain (42%). It was hypothesized that wet mount in physiological saline always been the mainstay of any initial laboratory examination to detect motile trophozoites, but difficult to distinguish non-motile amoebae from macrophages or polymorphonuclear leucocytes. Also, the larger and more ma-

ture cyst forms of *E. coli* and *E. histolytica* were not well stained by trichrome but detected by concentration (Shetty *et al,* 1988). However, Shoaib *et al.* (2002) found that Trichrome stain of fecal smears was better in diagnosis of protozoa as compared to the conventional wet mount. They added that morphological organism features were noticeable against the pale background making the visibility of protozoa more prominent and increased its sensitivity. Darabian *et al.* (2016) showed that trichrome staining was more sensitive than both direct smear and formalin ethyl acetate sedimentation techniques in the detection of *B. hominis* in stool samples of patients with the irritable bowel syndrome (IBS). However, an obvious disadvantage is the tedious protocol of Trichrome staining which requiresd an hour fixation and a total time of about two hours to complete. Also, appropriate fixation periods coupled with sufficient washing steps are significant in obtaining a well-stained nucleus; this may require the preparation of a number of slides for each stool sample. Repeated use of acid alcohol in de-staining trichrome stain decreased its efficiency and required a longer time although a better alternative was fresh solution and needed skills of an experienced microscopist (Tan *et al,* 2010).

In the present study, microsporidiosis was in 55 cases (55%) in both HD & CKD, by modified Trichrome stain. El-Nadi *et al*. (2004) reported that fecal samples from HD patients and cultured on modified agar plate, & subjected to formol-ether and sucrose sugar concentration method then MZN acid-fast staining, showed *C. parvum, C. cayetanensis, Isospora belli* and *Microsporidia* in 48%, 12%, 4% and 2% respectively.

Light microscopy proved to be simple, easy, least expensive method to detect the *Blastocypstis* spp. in feces (Garcia, 2016).

The present study showed an agreement between direct smears and xenic culture method for *B. hominis*. Kukoschke *et al.* (1990) did not find any difference between microscopy and cultures methods. They used a highly nutritive biphasic medium for isolation, causing an overgrowth of bacteria, resulting in low isolation rate of *Blastocystis*. But, culture may allow preferential growth of specific strains while eliminating others false negative cases. The Jones' medium proved good for xenic culture of *Blastocystis* spp. and several factors affected detection of *Blastocystis* spp. via culture method and various available media (Leelayoova *et al,* 2002; Stensvold *et al,* 2007).

Termmathurapoj *et al* (2004) found that in vitro cultivation was the 'gold standard' for *B. hominis*. Yakoob *et al.* (2010) reported that *B. hominis* was positive by stool microscopy in 49%, by culture in 53% and by PCR positive in 44%of cases.

Since multiple protozoa infections coexised in the same sample, there was a need for improved diagnostic procedures, as rapid immunoassay test for *E. histolytica/ dispar, Giardia lamblia* and *Cryptosporidium* spp. The triage parasite panel was a qualitative stool enzyme immunoassay capable of detecting any of these parasites in fresh or frozen human fecal specimens (Goni *et al,* 2012). In the present results, triple ICT copro-antigen were as followed for *E. histolytica* antigen detected in 1% of cases, *G. lamblia* in 2 %, while *Cryptosporidium* antigen in 1%. There was an agreement between ICT and the microscopic technique stressed on \reliability to diagnose *G. lamblia* and *C. parvum* and a moderate agreement was between ICT and microscopic technique to diagnose *E. histolytica*. Swierczewski *et al.* (2012) compared the results of 266 stool samples to the triage parasites panel, sensitivity and specificity for *E. histolytica*/dispar were 100% & 100%, for *G. lamblia*: 100% & 100% and *C. parvum*: 70% & 100%, without cross reactivity with other stool parasites. This agreed with Garcia *et al.* (2000).

Goni *et al.* (2012) considered microscopy as gold standard and PCR as reference technique to differentiate between *E. histo-*

lytica and *E. dispar*, although both have limitations, agreement with microscopy and PCR was over 90%, but agreement between microscopy and ICT for *E. histolytica* was 76.3%, due to the microscopic inability to differentiate *E. histolytica* from nonpathogenic *E. dispar* (Goni *et al,* 2012). The ICT advantages were being simple, rapid, completed in less than 15 minutes, used with either fresh or frozen unfixed stool, easily read, and interpreted as compared to ova examination, without cross reactivity with other intestinal parasites (coated with monoclonal antibodies).

Conclusion

Microsporidia was the most common protozoa detected among renal patients followed by *B. hominis*. ICT can replace the staining methods in diagnosis of *E. histolytica/dispar, G. lamblia,* and *C. parvum.*

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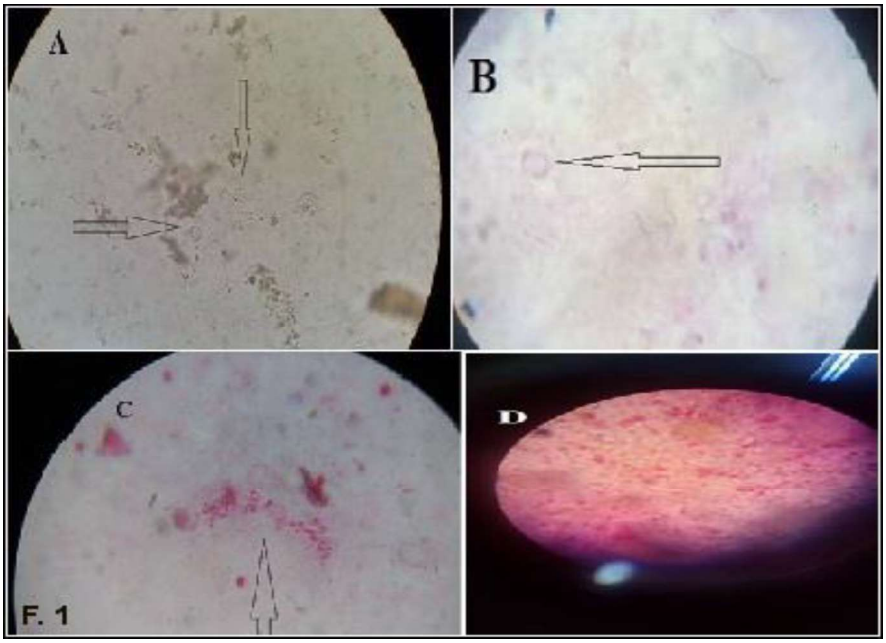
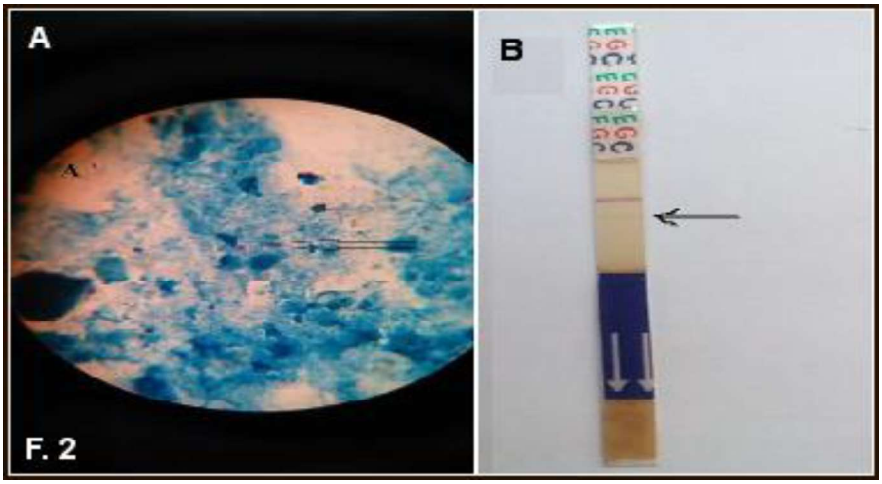
Explanation of figures

Fig. 1: Parasites detected by microscopic examination: (A) *B.hominis* by Direct smear (40x) (B) *B.hominis* by Trichrome stain (100x); (C, D) *Microsporidia* by modified Trichrome stain (MTS) (100x).

Fig. 2: (A) *Cryptosporidium* by microscopic examination (MZN stain)(100x), (B) *Cryptosporidium* by ICT strip

Fig. 3: (A) *G.lamblia* by microscopic examination (iodine)(40x), (B) *G.lamblia* by ICT strip


A
B
C
D
F. 1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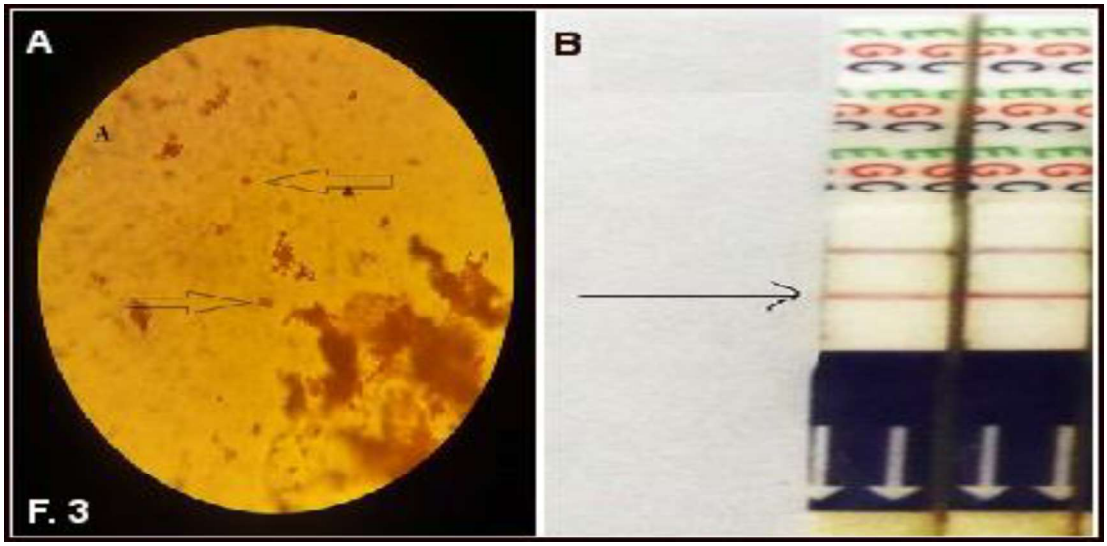
A
B
F. 3