Evaluation and comparison of PCR, Coproantigen ELISA and microscopy for diagnosis of *Cryptosporidium* in human diarrhoeic specimens.

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Abstract : Background: *Cryptosporidium* is associated with significant morbidity and mortality, especially among infants and children. Although the modified acid-fast technique is the commonly used stain for its detection, its sensitivity and specificity appeared to be relatively low.**Objective:** *Cryptosporidium* remains largely under diagnosed in current routine diagnostic procedures. The present study aimed to evaluating and comparing the conventional microscopic method with coproantigen ELISA and polymerase chain reaction (PCR) in stool.**Methodology:** Eighty six immunocompromised patients(57 males and 29 females) ranging in age from 6 months to 60 years having acute or chronic diarrhoea were selected from the attendance of the pediatrics, oncology and nephrology clinics in Zagazig University Hospital. Stool samples were collected and each sample immediately divided into three parts. The first part was preserved in 10% formalin then it was subjected to direct saline smear, Formol- ether sedimentation technique staining with Lugol's iodine to identify other intestinal parasites, then staining of smears with Modified Ziehl-Neelsen acid fast stain (MZN)**.** The second part was preserved at -20°C until used for coproantigen ELISA detection. The third part was subjected to DNA extraction. The extracted DNA was stored at -20°C until used for PCR. **Results:** Percentage detection of cryptosporidium was highest by PCR (24.4%) and lowest by microscopy (18.6) while it was (20.9%) by coproantigen ELISA. the sensitivity and specificity of PCR were 100%, the sensitivity and specificity of coproantigen ELISA was 85.7% and 100% respectively compared to PCR. **Conclusion:** Coproantigen ELISA was a simple, rapid, reliable, and standardized for routine diagnosis especially in hospitals and may be useful for large-scale epidemiological studies of cryptosporidiosis, however PCR was more sensitive in detection of cryptosporidium but very expensive so not suitable in routine diagnosis in developing countries .

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1.Introduction:

Cryptosporidiosis represents a major health problem worldwide. It has recently attracted attention as an emerging food borne disease as well as an opportunistic infection in HIV infected individual **(El-Hamsharys *et al.*, 2008).** It is also recognized as a major human waterborne pathogen **(Clancy & Hargy, 2007) In developed countries, massive *Cryptosporidium* food-borne and water-borne outbreaks have been reported, while in developing countries, *Cryptosporidium* is associated with significant morbidity and mortality, especially among infants and children (Xiao & Fayer, 2008).** Diarrhoea caused by parasites account for more than 3.1 million deaths each year among children less than 15 years of age, mostly in developing countries **(Colford *et al.*, 2005).**

Infection with *Cryptosporidium* results in a wide range of manifestations, from asymptomatic infections to severe, life-threatening illness **(Morgan *et al.*, 2002)**. The average incubation period is 7 days. Patients mostly present with watery diarrhea, which can be accompanied by dehydration, weight loss, fever and/or vomiting **(Robertson *et al.*, 2006).** In immunocompetent persons, symptoms are usually short lived (1 to 2 weeks) but in immunocompromised patients the infection is more serious; it can become chronic and sometimes fatal **(Bialek *et al.*, 2002).**

The major routes of transmission include drinking and recreational water (swimming pools). Other routes are through food handlers, animal handlers, day care centre attendants and the faecal-oral route **(Karanis *et al.*, 2007)**.

There are different methods used in detecting *C. parvum* oocysts in clinical and environmental samples, these include microscopic techniques, enzyme immunoassay methods and PCR **(Coupe *et al.*, 2005 and Pelayo *et al.*, 2008).**

Diagnosis of cryptosporidiosis has progressed from histological identification in intestinal biopsies to microscopic examination

of faecal specimens for infective oocysts, enzyme immunoassay (EIA) for parasite antigens and nucleic acid amplification assay as well as use of molecular techniques such as PCR **(Garcia *et al.*, 1997 and Phillip *et al.*, 2008).u**Modified acid-fast staining technique is the commonly used stain for the detection of oocysts in faecal smears, however, the sensitivity and specificity appeared to be rather low, as identification depends on the experience and skills of the microscopist (**Marshall *et al.*, 1997).** The low sensitivity of coprodiagnosis using staining techniques may limit early diagnosis, early treatment and possible prevention of the fulminate life threatening diarrhoea seen in immunocompromiscd patients **(Weber *et al.*, 1991).**

PCR has shown to be sensitive and specific for the detection of *C.parvum* in faecal samples **(Morgan & Thompson, 1995).**

The present study aimed at comparing the conventional diagnostic method using the MZN staining technique to coproantigen ELISA and PCR in diagnosis of cryptosporidiosis.

2.Materials and methods:

Study type: Descriptive analytical study.

Study design: This study was carried out during the period from April 2011 to August 2012, all of the laboratory techniques were performed in Parasitology and Microbiology Departments, Faculty of Medicine, Zagazig University. Eighty six immunocompromised patients (57 males and 29 females) ranging in age from 6 months to 60 years having acute/chronic diarrhoea were selected from the attendants of the pediatrics, oncology and nephrology clinics in Zagazig University Hospital. All the subjects had to fulfill one of the following criteria; children with protein energy malnutrition or diabetes of more than one year, or corticosteroids therapy for more than one year, or malignancy, or end stage renal failure.

Stool samples were collected and immediately divided into three parts. The first part was preserved in 10% formalin and then it was subjected to direct saline smear and staining with Lugol's iodine **(Cheesbrough, 1987)** to identify other intestinal parasites. Formalin ethyl acetate concentration technique was performed followed by staining of smears with MZN stain **(Cheesbrough, 2000)**. The second part was preserved at -20°C until used for Coproantigen ELISA. The third part was subjected to DNA extraction. The extracted DNA was stored at -20°C until used for PCR.

Coproantigen enzyme-linked immunosorbent assay:

Cryptosporidium sp. coproantigen was detected using a commercial ELISA kit for stool samples (RIDASCREEN *Cryptosporidium*, R-Biofarm, Germany) following the manufacturer's recommended procedures. In the RIDASCREEN® *Cryptosporidium* test, specific antibodies are used in a sandwich-type method. Cryptosporidium-specific antibodies against antigens of *Cryptosporidium parvum* are applied to the surface of the well in the microwell plate. A suspension of the stool sample to be tested and the controls are pipetted into the wells of the microwell plate. Next, antibodies conjugated with peroxidase against the antigens of *Cryptosporidium parvum* are added and the plate incubated at room temperature (20 – 25 °C). The negative and positive controls contained in the kits were used. Optical density (OD) of each sample was measured at 450 nm utilizing a micro plate reader (IRE 96, Germany). Samples were considered positive if their extinction is more than 10 % above the calculated cut-off respectively as specified in the formula provided by the manufacturer. After adding the substrate, the attached enzyme changes the color of the previously colorless solution in the wells of the micro well plate to blue if the test is positive. On adding the stop reagent, the color changes from blue to yellow. The extinction is proportional to the concentration of *Cryptosporidium* antigen present in the sample.

DNA extraction and PCR analysis:

Extraction of DNA was conducted using QIA amp DNA stool mini kit (Qiagen, Hilden, Germany).The extracted DNA was amplified by PCR according to described technique **(McLauchlin *et al.*, 2000).** Conventional PCR with new primers (more specific in detection of *Cryptosporidium)* was used to detect *Cryptosporidium* the two primers were used, one forward primer Cry18S-S2, 5'GGTGACTCATAATAACTTTA CGG 3'and one reverse primer Cry18S-As2, 5' ACGCTATTGGAGCTGGAATTAC 3'. The amplification of template DNA was performed in a final volume of 25 uL master mix (containing 100 mmol/L KCL; 40 mmol/L Tris-HCL, pH 8.4;1.6 mmol/L deoxynucleoside triphosphate; iTaq DNA polymerase [50 units/mL], 2 mmol/L Mgcl2) and 10 µL of DNA

sample. The reaction was performed using the Biometra T -gradient thermal cycler. Amplification of each DNA fragment started with an initial denaturation at 94 °C for 3min, followed by 35 cycles of denaturation for one min, annealing at 55 °C for 30 s, and primer extension at 72 °C for 1 min, followed by 10 min at 72 °C. Positive and negative controls were included in each batch of tests. Amplified DNA was analyzed by electrophoresis in 1% agarose gels stained with ethidium bromide and visualized using transilluminator.

Ethical consideration:

Ethical approval was obtained from the Committee of Research, Publications and Ethics of the college of Medicine, Zagazig University, Egypt. All procedures were explained to parents of children and patient in the local language, and written or thumb-printed informed consent was obtained.

Statistical methods:

All data were subjected to statistical analysis using SPSS win statistical package version 11 .Significance was defined as $P < 0.05$. The sensitivity is the probability that the assay will be positive when the infection is present. The specificity is the probability that the assay will be negative when the infection is absent. They were calculated using the following formulas: sensitivity (%) = TP/ (TP+FN) x 100 and specificity (%) = TN/ (TN+FP) x 100 (TP: true positive, FN: false negative, TN: true negative and FP: false positive values). The positive predictive value of a diagnostic test is the proportion of total positive test results that are true positives. The negative predictive value of a diagnostic test is the proportion of total negative results that are true negatives. These were calculated using the following formulas: positive predictive value (%) = TP/ (TP+FP) x 100 and negative predictive value (%) = TN/ (TN+FN) x 100. Accuracy were also calculated as the accuracy%= (TP+ TN)/ all cases examined x 100. Also, each test was evaluated by the multi-attribute evaluation method **(MacPherson & MacQueen ,1993).**

3.Results:

In the present study, based on PCR of 86 stool samples, *Cryptosporidium* was detected in 21 with a detection rate of (24.4%). Using modified acid-fast technique, detection of *Giardia Lamblia* in 23 stool samples. Three samples had mixed infection with *G. lamblia* cyst and *cryptosporidium* oocyst(Table 1).

In comparison to PCR technique, coproantigen ELISA was more sensitive (85.7%) and specific (100%) with (96.5%) accuracy in detection of *Cryptosporidium* in stool samples than MZN (66.6%, 97% and 81.4% respectively)(Tables3,4).

Out of 21 positive stool samples detected by PCR 15(71.4%)showed history of animal contact, and 17(80.9)showed history of residence in rural areas (Table5).

Percentage detection of cryptosporidium was higher in chronic diarrhea than acute diarrhea (Table 6).

In the present study, the multi-attribute evaluation method proved that MZN stain was at the end in ranking as compared to ELISA and PCR, in spite of low cost. ELISA coproantigen were more sensitive(score 4),less time consuming and high score as a batch test. PCR was higher sensitive(score5)but high cost(score1) (Table7).

Table (1): protozoan infections detected by stool examination of 86 diarrheic patients with preparation of saline, iodine and MZN:

Cryptosporidium spp.	16 (18.6%)
Giardia intestinalis	23 (26.7%)
Entamoeba histolytica	5 (5.8%)
Blastocystis hominis	10 (11.6%)

Table (2): Comparison between results of microscopy, Coproantigen ELISA and PCR.

Tests / Cases	Microscopy	Coproantigen ELISA	PCR
+ve cases (No. %)	16 (18.6%)	18 (20.9%)	21 (24.4%)
-ve cases (No. %)	70 (81.3%)	68 (79.06%)	65 (75.5%)

Table (3): Sensitivity, specificity PPV, NPV and accuracy of MZN diagnostic methods compaired to PCR

		PCR (gold standard)		
		Positive	negative	Total
Microscopy	Positive	14	2	16
	Negative	7	63	70
	Total	21	65	86

Sensitivity: 66.6%; Specificity: 97%; Positive predictive value: 87.5%; Negative predictive value 90%; Accuracy: 81.4%

Table (4): Sensitivity, specificity PPV, NPV and accuracy of coproantigen ELISA diagnostic methods compared to PCR.

		PCR (gold standard)		
		Positive	negative	Total
Coproantigen ELISA	Positive	18	0	18
	Negative	3	65	68
	Total	21	65	86

Sensitivity: 85.7% Specificity: 100% Positive predictive value: 100% Negative predictive value 95.5% Accuracy:96.5%

Table(5): distribution of Cryptosporidium spp. Positive cases according to animal contact and Residence.

Cryptosporidium* infection** / ***Parameter	***Cryptosporidium* infected cases (21)**		*P*
	No.	**%**	
Animal contact:			
Present	**15**	**71.4%**	< 0.001
Absent	**6**	**28.5%**	
Residence :			
Rural	**17**	**80.9%**	< 0.001
Urban	**4**	**19%**	

Table(6): Comparison between results of ELISA and PCR in relation to duration of diarrhoea among *Cryptosporidium* infected cases :

Tests / Diarrhoea	ELISA (NO. %)	PCR (NO. %)
Acute	2(11.2%)	4(19.04)
Chronic	16(88.8%)	17(80.9%)
P	*<0.05	**<0.001

Table (7): Ranking of the diagnostic procedures for *Cryptosporidium spp.*

Ranking of the diagnostic procedures for *Cryptosporidium spp.*					
Techniques	**Ranking for the attributes**				
	Sensitivity	**Time taken**	**Cost**	**Ease of use and Interpretation**	**Batch testing**
Microscopy after formol ether concentration &(MZN)	3	3	4	4	2
ELISA	4	4	2	4	5
PCR	5	1	1	3	5

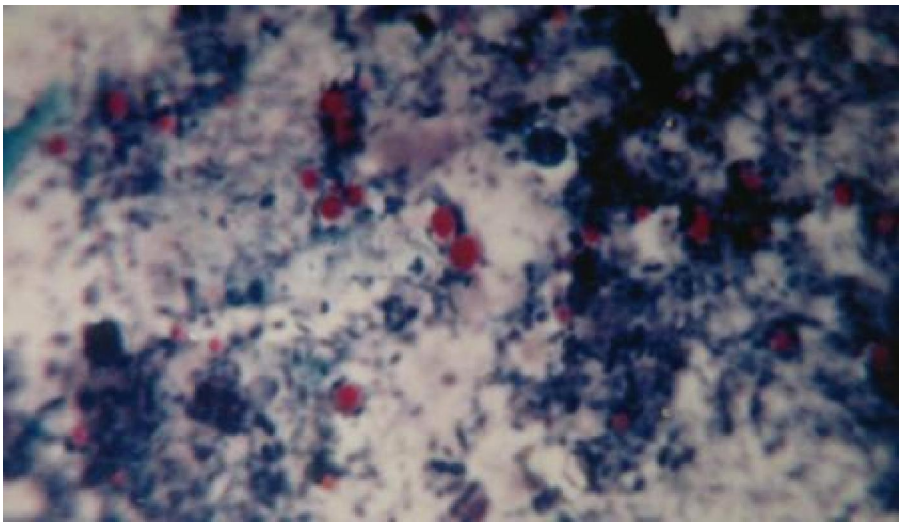

Fig. (1): Stool smear stained by modified Ziehl - Neelsen stain showing *Cryptosporidium* oocysts as bright - rose pink oval or rounded bodies with different degrees of intensity against bluish background (x1000), using formol - ether concentration.

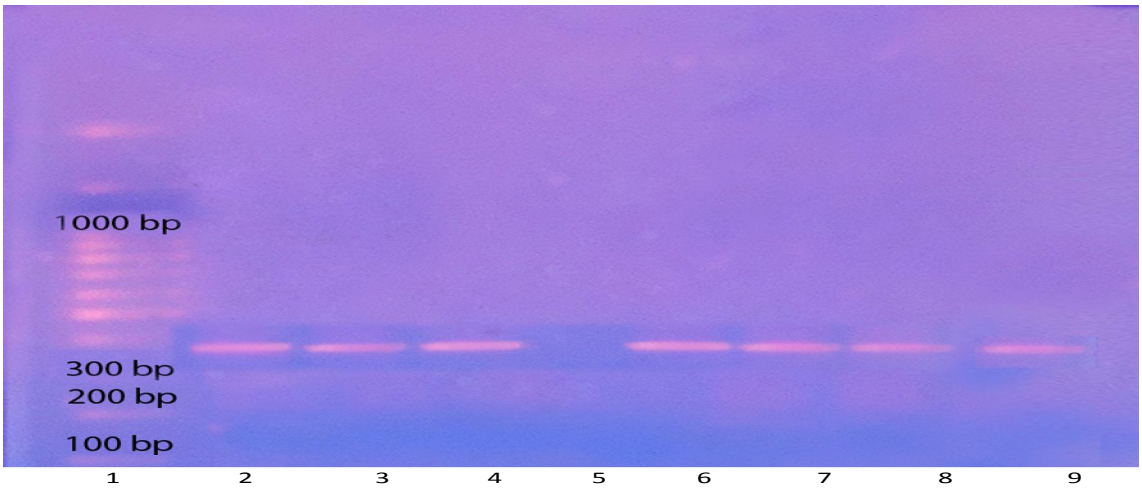

Fig. (2): Agarose gel electrophoresis of PCR product, lane1: DNA ladder (100bp), lane 5 is negative control.the remaining are positive at 347pb.

4.Discussion:

Cryptosporidium parvum is an opportunistic parasite capable of causing gastrointestinal illness in both immunocompetent and immunocompromised patients as well as in children particularly in developing countries **(Oyibo *et al.*, 2011).** Stool microscopic examination, the commonly used method for diagnosis of cryptosporidium, is unreliable in identification of the parasite and relatively time consuming **(Clark, 1999 and Fall *et al.*, 2003)**

Wet mount stained by iodine in the present study, detected other organisms (*Giardia lamblia, E. histolytica* and *Blastocystis hominis*) human cells (epithelial cells, macrophage, polymorphs) or artifacts that could be misdiagnosed as *Cryptosporidium*. In our study, MZN staining has been found to be 97% specific with sensitivity of 66.6% which is in accordance with previous studies where MZN staining has been found to be 98.9-100% specificity with sensitivities ranging from 37-90% **(Morgan *et al.*, 1998 and Tuli *et al.*, 2010).** These finding were supported by **Kaushik *et al.*, 2008** who reported that with MZN staining, difficulties arise due to poor uptake of stain by oocysts sometimes, in discriminating between *Cryptosporidium* oocysts and

other spherical objects of similar size (yeasts) staining dull red.

The need for a test that is easy to perform and cost effective has led to the development of immunoassay techniques like immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) for the diagnosis of Cryptosporidiosis (**Garcia *et al*., 1983)**. Although commercial ELISA kits have been used for the detection of *Cryptosporidium* coproantigens in faecal samples of canines **(Rimhanen-finne *et al.*, 2007and Rinaldi *et al*., 2008)** and bovines (**Srijan *et al*., 2005 and Duranti *et al*., 2009**), they are manufactured mainly for diagnosis of *Cryptosporidiosis* in humans. Oocysts might not be detectable in clinical samples from all cryptosporidiosis cases and in such cases copro-antigen detection assays and PCR-based methods were needed **(Smith, 2008).**

In the current study, ELISA kit is better than microscopic technique in detection of cryptosporidium. It showed higher detection rate (20.9%) than microscopy (18.6%) in patients complaining of diarrhoea, *Cryptosporidium* antigen detection ELISA was 100% specific with a **s**ensitivity of 85.7%. These finding were supported by other studies which reported that, in spite of the limitations of the ELISA technique in the investigation of *Cryptosporidium parvum*, it is still relied on for epidemiological surveys due to higher sensitivity and ease of use compared to microscopy **(Srijan *et al*., 2005).** In addition**,** the existence of other intestinal parasites did not change the ELISA results for cryptosporidial antigens this in agreement with **Smith (2008)**.

The commercially available copro-antigen detection ELISA formats use monoclonal antibodies (mAbs) which recognize different sets of surface epitopes. Cost of test per sample has been reported to be much more than microscopic examination. **Smith (2008)** reported that antigen assays have an advantage of not requiring skills in microscopic identification of organisms and their specificity has been reported to be high.

The development of molecular tools including PCR to detect DNA of *Cryptosporidium* oocyst in stool has led to major advances in accurate diagnosis during recent years. This in turn has assisted clinical diagnosis and appropriate therapy .In this study conventional PCR with new primers (more specific in detection of *Cryptosporidium)* was used to detect *Cryptosporidium* and as a result *Cryptosporidium* was detected in 24.4% of examined stool samples. Although all samples detected by microscopy were also diagnosed by PCR but PCR was the only method able to detect 3 positive cases. The sensitivity of the PCR method is about 20 oocysts in 1 mL of stool sample **(Lindergard *et al.,* 2003)**

In our study, the PCR method proved to be more effective in determining the stool samples. The primers used in our study work well and have high sensitivity and selectivity, thus preventing unwanted amplification products from being obtained. On the contrary **Amar *et al.,* 2004** found less positivity by PCR as opposed to microscopy. The discrepancies between the present and other previous result may be due to differences in the primers used.

The majority of the cases diagnosed with *Cryptosporidium* spp. in our study lived and bred animals in rural areas (Lack of hygiene and poor living conditions, direct contact with farm animals where cryptosporidiosis has a high prevalence, and oocyst-contaminated food and water account for the spread of the infection. This is in agreement with **Selma & Umit (2011)** who reported that the reason for the spread of the parasite, especially in rural areas, is contaminated water. Because the water consumed by *Cryptosporidium spp*. infected patients from cities was not analyzed, the spread of the parasite remains unexplained and was attributed to possible food contamination.

In the present study a higher detection of *Cryptosporidium* in patient with chronic diarrhoea 88.8% by ELISA coproantigen and 80.9% by PCR. This is result may be due to most of patients in hospitals were immunodeficient. These finding were supported by **Huang *et al*. (2004)** who reported that immunodeficient individuals develop severe, chronic diarrhoea that rarely can lead to extra intestinal cryptosporidiosis.

In the present study, the multi-attribute evaluation method proved that MZN stain was at the end in ranking as compared to ELISA and PCR. In spite of low cost, more time in preparation, staining, reading and interpretation of the smear. Additionally, it is requires considerable expertise to identify the oocysts and not to confuse it with other ingredients of stool like yeast spores. In addition, it is not enable to bulk processing.

In case of ELISA, high score was found as it required less time to be easily prepared and interpreted with no technical expertise. Its cost is relatively higher than staining but it is enable to bulk processing which in accord with **Elgun & Koltas 2011.**

Although PCR ranked higher than MZN, its wide use is blocked by its relative high cost, tendency

to be time consuming, and its high technology equipments, these data agreed with **Paul *et al.* 2009** who proved that PCR is not an option for a particular laboratory setting, especially in developing countries.

In conclusion the commercially available coproantigen ELISA test is easy to perform, sensitive and cost effective so it should be included for diagnosis of the disease in all hospitals especially in developing countries. Unlike other methods used in the present study, PCR proved to be a more sensitive for detection of *Cryptosporidium* in fecal samples. However, it is expensive and needs technical skills so, application of PCR –based methods in routine diagnosis, especially in developing countries is difficult.

Author contribution:

Elsettawy MA and Fathi GM,proposed the research idea,shared in writing and reviewing, shared in the study design, practical work and writing manuscript.

References

1. **Amar, C.F.L.; Dear, P.H.; McLauchlin, J. (2004):** Detection and identification by real time PCR/RFLP analyses of *Cryptosporidium* species from human faeces. Let Appl Microbiol; 38: 217-22.
2. **Bialek, A.J.; Ives, N.J.; Gazzard, B.G. and Easterbrook, P.J. (2002):** The changing pattern of AIDS–defining illness with the introduction of (HAART) in a London clinic. Journal Infection 42(2):134–139.
3. **Chessbrough, M. (1987):** Medical laboratory Manual for Tropical countries, 2nd ed., English Language Book society/ Tropical Health Technology/ Butter- Worths, PP: 548- 77.
4. **Chessbrough, M. (2000):** District laboratory practice in tropical countries, part I, Cambridge University press., Low. Pric. Editions, PP: 196-207.
5. **Clancy, J.L.and Hargy, T.M; (2007):** Waterborne: drinking water. Fayer R, Xiao L, eds. Cryptosporidium and Cryptosporidiosis. Second edition. Boca Raton, FL: CRC Press, 305–333.
6. **Clark, D.P.(1999):** New insights into human cryptosporidiosis. Clin. Microbiol. Rev. 12:554–63.
7. **Colford, J.S.; Wade, T.; Wright, C.; Charles, S. and Eisenberg J. (2005):** A pilot randomized, controlled trial of an in-home drinking water intervention among HIV positive persons J Water&Health 3(2):173-184.
8. **Coupe, S.; Sarfati, C.; Hamame, S.and Derouin F, (2005):**Detection of Cryptosporidium and identification to the species level by nested PCR and restriction fragment length polymorphism. J. Clin. Microbiol. 43: 1017
9. **Duranti, A.; Caccio, S. M; Pozio, E.; Diegidio, A.; Decurtis, M.; Battisti., A. and Scaramozzino, P. (2009) :** Risk Factors Associated with *Cryptosporidium parvum* Infection in Cattle. Zoonoses Public. Health. **56**: 176–182.
10. **El-Hamshary,E.M.; El-Sayed,H.F.; Hussein,E.M.; Rayan,H.Z. Rasha, H.and Soliman,R.H.(2008):**Comparison of Polymerase Chain Reaction, Immunochromatographic Assay and Staining Techniques in Diagnosis of Cryptosporidiosis. P.U.J.1(2):77-86.
11. **Fall,A.; Thompson, R.C.; Hobbs, R.P. and Morgan-Ryan, U.M. (2003):** Morphology is not a reliable tool for delineating species within *Cryptosporidium*. J. Parasitol. 89: 399–402.
12. **Flgun, G. and Koltas, I. S. (2011):** Investigation of *Cryptosporidium spp.* antigen by ELISA method in stool specimens obtained from patients with diarrhea. Parasitol. Res., 108:395–397.
13. **Garcia, L. S; Bruckner, D. A; Brewer, T. C. and Shimizu, R. Y. (1983):** Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens. *J. Clin. Microbiol.* **18**:185–190.
14. **Garcia, L.S.; yesShimizu, R.Y.; Bruncker, S.R. (1997):** Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of *Giardia lamblia* and *Cryptosporidium parvum* in human fecalspecimens. J. Clin. Microbiol. 35: 1526–1529.
15. **Huang, D.B.; Chappell, C.; Okhuysen, P.C.(2004):** Cryptosporidiosis in children. Semin. Pediatr. Infect. Dis. 15(4):253-9
16. **Karanis, P.; Kourent, C. and Smith, H. (2007):** Waterborne transmission of Protozoan parasites: a world wide review of outbreaks and lessons learnt .J Water Health 5: 1-38.
17. **Kaushik, K.; Khurana, S.; Wanchu, A. and Malla, N.(2008):** Evaluation of staining techniques, antigen detection and nested PCR for the diagnosis of cryptosporidiosis in HIV seropositive and seronegative patients. Acta. Trop. 107:1-7
18. **Lindergard, G.; Nydam, D.V.; Wade, S.E.; Schaaf, S.L.and Mohammed, H.O. (2003):** A novel multiplex polymerase chain reaction approach for detection of four human infective *Cryptosporidium* isolates: *Cryptosporidium parvum*, types H and C, *Cryptosporidium*canis, and *Cryptosporidium* felis in fecal and soil samples. J Vet Diagn Invest 15: 262-67.
19. **MacPherson, D.and MacQueen, R. (1993):** Cryptosporidiosis multi-attribute evaluation of six diagnostic methods. J. Clin. Microbioi. 31: 198-202.
20. **Marshall, N.; Naumovitz, S.; Ortega, Y. *et al.* (1997):** Water borne protozoanpathogens.Clin. Microbiol. Rev. 10: 67-85.

21. **McLauchlin, J.; Amar, C.; Pedraza-Diaz, S.and Nichols, G.L.(2000):** Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. In 1705 fecal samples from humans and 105 fecal samples from livestock animals. Journal of Clinical Microbiology 38:3984-90.
22. **Morgan, U. and Thompson, R.(1995):** PCR detection of *Cryptosporidium:*the way forward? Parasitol. Today. 14(6):241-5.
23. **Morgan, U.M; Pallant, L.; Dwyer, B.W.; Forbes, D.A.; Rich, G.and Thompson, R.C. (1998):** Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: Clinical trial. J Clin Microbiol .36:995-8.
24. **Morgan, U.R.; Weber, L.; Xiao, I.; Sulaiman, R.C.; Thompson, W.N.; Lab, A.; Moore, A. and Deplazes, P. (2002):** Molecular characterization of Cryptosporidium isolates obtained from human immunodeficiency virus-infected individuals living in Switzerland, Kenya, and the United States.J. Clin. Microbiol. 38: 1180-1183.
25. **Oyibo, W.A.; Okangba, C. C1; Obi, R.K2.; Nwanebu, F.C2 and Ojuromi, (2011):** Diagnosis of intestinal cryptosporidiosis in Africa: Prospects and Challenges. Journal of Applied Biosciences 40: 2659 – 2667.
26. **Paul, D.; Chandra, A.K.; Tewari, P.S.; Banerjee, D.D.; Ray, R.; Boral, J.R. and Rao,S. (2009):** Comparative evaluation and economic assessment of coprological diagnostic methods and PCR for detection of *Cryptosporidium spp*. in bovines. Veterinary Parasitology. 164 : 291–295
27. **Pelayo, L.; Nunez, F. A.; Rojas, L.; Wilke, H.; Furuseth, H.E.; Mulder, B.; Cyerde, B. and yesRobertson, L. (2008):** Molecular and Epidemiological investigation of cryptosporidiosis in Cuban children. Annals of Tropical Medicine and Parasitology. 102 (8): 659-669.
28. **Phillip, J.L; Rebecca, C.L.; Robinson, M.; yes Okhuysen, P.C. and yes Chappell, L. C. (2008):** *Cryptosporidium muris* in Texas cainin population. Am J. Trop Med. Hyg 78 (6): 917-921.
29. **Rihmanen - Finne , R ; Enema, R. K. ; Kolehmainen, J; Toropainen, P. and Hanninen, M.L. (2007):** Evaluation of immunofluorescence microscopy and enzymelinked immunosorbent assay in detection of *Cryptosporidium* and *Giardia* infections in asymptomatic dogs. Vet. Parasitol. **145**: 345–348.
30. **Rinaldi, L.; Maurelli, M.P; Musella, V; Veneziano, V.; Carbone, S.; Disarno, A.; Paone, M. and Cringoli, G. (2008):** *Giardia* and *Cryptosporidium* in canine faecal samples contaminating an urban area. Res. Vet. Sci. 84(3):413 – 415.
31. **Robertson, L.J.; Hermansin, L.and Gjerde, B.K. (2006):** Occurrence of Cryptosporidium oocyst Giardia cyst in sewage in Norway. Appl. Env. Microbiol. 72: 5297-5303.
32. **Selma, U.and Umit, A.(2011):** Detection and genotyping of *Cryptosporidium* spp. in diarrheic stools by PCR/RFLP analyses. Turk J Med Sci., 41 (6): 1029-1036.
33. **Smith, H.(2008):**Diagnostics.In: Fayer R, Xiao L, editors. *Cryptosporidium* and cryptosporidiosis. New York: CRC Press; p. 173-208.
34. **Srijan, A.; Wongstitwilairoong, B.; Pitarangsi, C.; Serichantalergs, O.; Fukuda, C.D.; Bodhidatta, L. and Mason, C.J. (2005):** Reevaluation of commercially available enzyme linked immunosorbent assay for the detection of *Giardia lamblia* and *Cryptosporidium* spp. from stool specimens. *Southeastern Asian J. Trop. Med. Publ.Health.* **36**: 26–29.
35. **Tuli, L.; Singh, D.K.; Gulati, A.K.; Sundar, S.and Mohapatra, T.M.(2010)**: A multiattribute utility evaluation of different methods for the detection of enteric protozoa causing diarrhea in AIDS patients. BMC Microbiol .10:11.
36. **Weber, R.; Bryan, R. and Juranek, D.(1991):** Improved stool concentration procedures for detection of *Cryptosporidium* oocysts in fecal specimens. J. Clin. Microbiol. 30(11): 2869-73.
37. **Xiao, L.and Fayer, R.(2008):** Molecular characterization of species and genotypes of *Cryptosporidiutn* and *Giardia* and assessment of zoonotic transmission. Int. J. Parasitol. 38 (11): 1239-55.