



Evaluation of staining techniques, antigen detection and nested PCR for the diagnosis of cryptosporidiosis in HIV seropositive and seronegative patients

Kirti Kaushik^a, Sumeeta Khurana^a, Ajay Wanchu^b, Nancy Malla^{a,*}

^a Department of Parasitology, Post Graduate Institute of Medical Education & Research, Chandigarh 160012, India

^b Department of Internal Medicine, Post Graduate Institute of Medical Education & Research, Chandigarh 160012, India

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ABSTRACT

The study was designed to determine the efficacy of modified Ziehl–Neelsen (ZN), safranine methylene blue (SM) staining, antigen detection ELISA and a nested PCR assay (specific for *Cryptosporidium parvum*) for detection of *Cryptosporidium* in HIV seropositive and seronegative patients with diarrhoea.

Cryptosporidium was detected in 10 (4.9%), 9 (4.4%), 39 (18.9%) and 27 (13.1%) of 206 HIV seropositive and 7 (4.6%), 6 (3.9%), 21 (13.7%) and 17 (11.1%) of 153 HIV seronegative patients by ZN staining, SM staining, antigen detection ELISA and PCR, respectively. None of the 50 apparently healthy control subjects was found to be infected with *Cryptosporidium* by any of the techniques.

Based on the criteria of 'true positive' samples positive by at least any two techniques out of ZN staining, antigen detection and PCR, sensitivity of ZN and SM staining techniques was 37% and 33.3% in HIV seropositive and 41.2% and 35.3% in seronegative patients, respectively. Sensitivity of antigen detection ELISA was 92.6% and 94.1% in HIV seropositive and seronegative patients, respectively, while sensitivity of PCR was 100% each in HIV seropositive and seronegative patients.

Specificity of all three techniques, i.e. ZN, SM staining and PCR was 100% in both HIV seropositive and seronegative patients while specificity of antigen detection was 92.2% and 96.3% in HIV seropositive and seronegative patients, respectively. The staining techniques were found less sensitive as compared to antigen detection and PCR for detection of *Cryptosporidium* in HIV seropositive patients with CD4 count >200 cells/ μ l.

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1. Introduction

Cryptosporidiosis caused by *Cryptosporidium* has been increasingly reported world-wide both in immunocompetent and immunocompromised individuals causing a spectrum of diseases ranging from asymptomatic carrier state to severe diarrhoea. Infection with this parasite results in severe but self-limiting diarrhoea in immunocompetent and often lethal diarrhoea in immunocompromised individuals, most notably patients with AIDS (Fayer et al., 2000; Chen et al., 2002).

Laboratory diagnosis relies on the recognition of the *Cryptosporidium* oocysts in stool specimen after modified acid-fast staining. Conventional microscopy, however, is time-consuming, tedious and requires experienced microscopists to accurately identify the oocysts. In addition, the detection limit of conventional diagnostic techniques can be as low as 50,000–500,000 oocysts g^{-1} faeces (Weber et al., 1991). Immuno-

logical based detection methods have been developed for use in both clinical and environmental monitoring. However, antigenic variability within clinical isolates of *Cryptosporidium* can result in some infections remaining undetected (Griffin et al., 1992) and antigen detection with varying degrees of sensitivity and specificity of ELISA has been reported (Kehl et al., 1995; Graczyk et al., 1996). In addition, different studies evaluating identical assays have been reported to show diverse sensitivity values. Two separate evaluations (Katanik et al., 2001; Johnston et al., 2003) of the ProSpectT *Cryptosporidium* microplate assay reported sensitivity values of 70% and 100%, respectively. PCR assays have been introduced as a very sensitive and specific method to detect *Cryptosporidium* in environmental and clinical specimens and to define the taxonomic status, as well as the existence of genotypes within *C. parvum* (Higgins et al., 2001). The sensitivity of 97–100% and specificity of 100% has been reported for diagnosis of *Cryptosporidium* by PCR (Morgan et al., 1998; Bialek et al., 2002). However, need of expertise and availability of reagents has hampered its routine use in the diagnosis.

Given the low sensitivity of microscopy and varying sensitivity and specificity of antigen detection ELISA, the present study was

* Corresponding author. Tel.: +91 172 2755166; fax: +91 172 2744401.

E-mail addresses: drnancymalla@yahoo.com, kirtipgi@rediffmail.com (N. Malla).

aimed to evaluate two staining techniques (ZN and SM), antigen detection ELISA and a nested PCR assay for the detection of *Cryptosporidium* in HIV seropositive and seronegative patients. Among patients who have AIDS and in whom cryptosporidiosis can be detected early, improvement in immune function with effective antiretroviral therapy can result in dramatic improvement in diarrhoea (Carr et al., 1998; Foudraine et al., 1998). The early detection of *Cryptosporidium* in HIV seropositive patients may further add to the knowledge for clinical management of the disease.

2. Materials and methods

2.1. Patients and controls

Study group included 409 individuals which comprised of 206 HIV seropositive patients, 153 HIV seronegative patients with diarrhoea and 50 HIV seronegative, apparently healthy individuals without any history suggestive of cryptosporidiosis. HIV-seropositivity was defined as positivity based on three different ELISA kits, as per national policy for diagnosis of HIV infection (NACO, 2003). Two hundred and six HIV seropositive patients were selected, based on HIV-seropositivity status attending the immunodeficiency clinic and 153 HIV seronegative patients with diarrhoea were selected randomly from the patients attending the inpatient and outpatient departments of Nehru Hospital attached to Post Graduate Institute of Medical Education and Research, Chandigarh, a tertiary care hospital in North India. Fifty HIV seronegative, apparently healthy individuals without any history suggestive of cryptosporidiosis were included as controls. After obtaining informed consent from each individual, the demographic characters and symptoms (sex, age, history of diarrhoea and any other relevant symptoms) were recorded on preplanned proforma.

2.2. Stool specimens and processing

Fecal samples from 409 subjects were collected in clean, wide mouthed plastic containers. Samples were preserved in 10% buffered-formalin for microscopy and a portion of each sample was stored at -20°C without any preservative for antigen detection and PCR.

2.3. Microscopy

Faecal samples were subjected to formalin-ether sedimentation technique (centrifugation at $500 \times g$ for 10 min) and examined as wet saline and iodine preparation for the detection of protozoa and helminths. For the staining of *Cryptosporidium*, *Isospora* and *Cyclospora*, modified Ziehl–Neelsen (Casemore et al., 1985) and rapid safranin methylene blue (Baxby et al., 1984) staining techniques were performed on concentrated stool specimens.

2.4. Antigen detection

Cryptosporidium antigen was detected by using a commercial ELISA kit for stool samples (RIDASCREEN *Cryptosporidium*, R-Biofarm, Germany) according to manufacturer's instructions. Results were interpreted following the manufacturer's guidelines.

2.5. Polymerase chain reaction

2.5.1. DNA extraction

DNA extraction from the stool samples was done by using QIAmp DNA stool mini-kit (QIAGEN, Hilden, Germany) with minor modifi-

cation, i.e. the samples were boiled for 10 min to disrupt the oocyst walls to release DNA (Bialek et al., 2002).

2.5.2. Nested PCR

A nested PCR targeting the small subunit rRNA gene was performed with the use of earlier reported primers (Zhu et al., 1998). Initial primers CF201 (5'-GGGTGTATTTATTAGATAAAGAAC-3') and CR201 (5'-CTTTAAGCACTATAATTTCTC-3') were specific to the genus *Cryptosporidium*. The nested primers CPF 202 (5'-GACTTTTTGGTTTTGTAATTGGAATG-3') and CPR 202 (5'-TAAATTATTAACAGAAATCCAACCTACGAGC-3') were specific to *C. parvum*. The reaction mixture of the PCR consisted of 25 μl . For the first amplification reaction, the outer primers CF 201 and CR 201 were used. The reaction mixtures were prepared in PCR buffer (50 mM KCL, 20 mM Tris-HCl, 2.5 mM MgCl_2 , pH 8.4) and contained per reaction, 0.1 $\mu\text{g}/\text{ml}$ BSA, 0.4 μM of the respective primers, a 0.2 mM concentration of each of the dNTPs, 1.2 U of recombinant Taq polymerase and 6 μl of the purified DNA. For the second round of amplifications, the reaction mixture was prepared as described above, except that the inner primers, CPF 202 and CPR 202 were used and 3 μl of the amplified product from the first PCR was used as a source of DNA.

The reactions were initiated by denaturing the DNA at 95°C for 5 min, followed by annealing of primers at 58.5°C for 2 min with extensions at 72°C for 5 min. Amplification was done in a thermal cycles for 30 (primary amplification) and 20 cycles (secondary amplification). Negative controls consisted of a blank containing all PCR reagents but no DNA. Positive control consisted of *Cryptosporidium parvum* genomic DNA which was a kind gift from Dr. Boris Striepen, University of Georgia. A 50 bp molecular weight marker (MBI Fermentas) and positive and negative controls were tested for each batch run.

2.5.3. Detection of amplified products

The PCR products were analyzed in 2% agarose gel (Sigma-Aldrich, USA), after electrophoresis and staining with ethidium bromide, gels were photographed on UVI-gel documentation system (Uvipro, UK).

2.6. CD4^+ T lymphocyte count

Absolute CD4^+ T lymphocytes were measured by flow cytometry (FACS COUNT, Becton Dickinson, USA) and commercially available monoclonal antibody (Becton Dickinson) in HIV seropositive patients wherever possible.

2.7. Ethical clearance

This study was approved by Institutional Ethics Committee.

2.8. Statistics

For comparative evaluation of diagnostic techniques, sensitivity, specificity, positive predictive value, negative predictive value and diagnostic efficacy of various techniques were determined by standard formulae (Galen and Gambino, 1975). Diagnostic techniques evaluation with respect to age, sex, CD4^+ count and history of diarrhoea was done by Fisher-exact and Mann-Whitney *U* tests.

3. Results

3.1. Demographics

Demographic characteristics of the subjects are shown in Table 1.

Table 1

Summary of demographic characteristics of individuals under study

	HIV seropositive	HIV seronegative	Healthy controls
Number	206	153	50
Sex	Males 140 (68%), females 66 (32%)	Males 101 (66%), females 52 (34%)	Males 25 (50%), females 25 (50%)
Age ^a (years)	33.2 ± 8.2 (range, 21–67)	28.1 ± 16.7 (range, 1.5–65)	27.4 ± 3.0 (range, 23–36)
Diarrhoea	99 (48.1%)	153 (100%)	Nil
Post-transplantation	Nil	23 (15%)	Nil
CD4 count ^a (cells/μl)	274.9 ± 140.7 (range, 2–583)	Not available	Not available

^a Mean ± S.D.**Table 2**

Association between number of patients with diarrhoea and CD4 count

	CD4 count (cells/μl)			
	Total 206 ^a	<200 67 ^a	200–500 115 ^a	>500 24 ^a
No. of patients with diarrhoea (%)	99 (48.1)	50 (74.6)*	42 (36.5)	7 (29.2)
No. of patients without diarrhoea (%)	107 (51.9)	17 (25.4)**	73 (63.5)	17 (70.8)

^a Number studied.

* Vs.

** $p < 0.001$.

On retrospective analysis, CD4 count was available for all 206 HIV seropositive patients. Sixty-seven (32.5%), 115 (55.8%) and 24 (11.7%) patients had CD4 count of <200, 200–500 and >500 cells/μl, respectively. In HIV seropositive patients with CD4 count <200 cells/μl, the number of patients with diarrhoea was significantly higher (74.6%) as compared with patients without diarrhoea (25.3%) ($p < 0.001$) (Table 2).

3.2. Intestinal parasites

Intestinal parasites detected in HIV seropositive and seronegative patients by wet mount, modified ZN and SM staining techniques are shown in Table 3. Fifty-five (26.7%) HIV seropositive and 33 (21.6%) HIV seronegative patients were found positive for intestinal parasites including *Cryptosporidium* (positive by staining techniques, antigen detection and PCR). *Cryptosporidium* was the most common parasite found in both HIV seropositive (19.9%) and HIV seronegative (14.4%) patients by any one or more techniques, while *Isospora* was found in 9 (4.4%) HIV seropositive and none of the HIV seronegative patients.

Out of 206 HIV seropositive patients, 99 (48.1%) and 107 (51.9%) were with and without diarrhoea. Thirty-nine patients with diarrhoea (39.4%) and 16 without diarrhoea (15%) were found positive

for intestinal parasites including *Cryptosporidium*. Positivity for intestinal parasites was significantly higher ($p < 0.001$) in patients with diarrhoea (39.4%) as compared to patients without diarrhoea (15%).

No significant association was found between intestinal parasites positivity and age or sex in HIV seropositive or seronegative patients ($p > 0.05$).

3.3. Comparative evaluation of techniques for *Cryptosporidium* detection

Cryptosporidium was detected in 41 HIV seropositive and 22 HIV seronegative patients by any one or more techniques. Antigen detection ELISA showed highest positivity [39 (18.9%) in HIV seropositive and 21 (13.7%) in HIV seronegative] followed by PCR. Twenty-seven (13.1%) HIV seropositive and 17 (11.1%) HIV seronegative patients with diarrhoea were found positive for *Cryptosporidium parvum* by both primary (*Cryptosporidium* specific) and secondary (*C. parvum* specific) PCR, while only 10 (4.9%) and 9 (4.4%) HIV seropositive and 7 (4.6%) and 6 (3.9%) HIV seronegative patients were found positive for *Cryptosporidium* by modified ZN and SM staining techniques, respectively (Table 5).

Table 3Intestinal parasitic infestation (including *Cryptosporidium* by staining techniques, antigen detection and PCR) in HIV seropositive and seronegative patients as seen by microscopy

Parasite	HIV seropositive (n = 206)	HIV seronegative with diarrhoea (n = 153)	Healthy controls (n = 50)	Total (n = 409)
<i>Giardia</i>	4 (1.9%)	6 (3.9%)	Nil	10 (2.4%)
<i>Cryptosporidium</i> ^a	41 (19.9%)	22 (14.4%)	Nil	63 (15.4%)
<i>Isospora</i>	9 (4.4%)	Nil	Nil	9 (2.2%)
<i>Entamoeba histolytica/E. dispar</i>	3 (1.5%)	2 (1.3%)	Nil	5 (1.2%)
Hook worm	1 (0.5%)	2 (1.3%)	Nil	3 (0.7%)
<i>Cyclospora</i>	1 (0.5%)	1 (0.7%)	Nil	2 (0.5%)
<i>Hymenolepis nana</i>	Nil	1 (0.7%)	Nil	1 (0.2%)
Total	55 ^b (26.7%)	33 ^c (21.6%)	Nil	88 (21.5%)

^a Positive by one or more techniques (ZN, SM, antigen detection and PCR).^b Four patients with dual infection.^c One patient with dual infection.

Table 4*Cryptosporidium* positivity by any one or more techniques

ZN ^a	SM ^b	Antigen detection ELISA	PCR	HIV seropositive (n = 206)	HIV seronegative with diarrhoea (n = 153)
+	+	+	+	7 (3.4)	6 (4)
+	+	—	+	2 (1.0)	Nil
+	—	+	+	1 (0.5)	Nil
—	—	+	—	14 (6.8)	5 (3.3)
—	—	+	+	17 (8.3)	10 (6.5)
+	—	—	+	Nil	1 (0.7)
—	—	—	—	165 (80)	131 (85.6)

^a Modified Ziehl–Neelsen staining.^b Safranin methylene blue staining.

Number of samples positive for *Cryptosporidium* by one or more techniques is shown in Table 4. Seven (3.4%) and 6 (4%) patients were found positive by all the four techniques in HIV seropositive and seronegative patients, respectively, while, 14 (6.8%) HIV seropositive and 5 (3.3%) HIV seronegative patients were found

positive by only antigen detection. None of the patient was found positive by only staining techniques (ZN or SM) or by only PCR in both HIV seropositive and seronegative groups. All patients found positive by SM were also found positive by ZN staining, while 1 patient each in HIV seropositive and seronegative

Table 5

Comparative analysis of modified Ziehl–Neelsen (ZN), safranin methylene blue (SM) staining, antigen detection and PCR for detection of *Cryptosporidium* in HIV seropositive patients (n = 206) and HIV seronegative patients with diarrhoea (n = 153) based on five different criteria (a–e) of 'true positive'

Techniques	HIV status	Patients positive (%)	Sensitivity (%)	Specificity (%)	Positive predictive (%)	pre-value	Negative pre-dictive value (%)	Diagnostic efficacy (%)	Agreement with 'gold standard' (kappa value)
ZN	Positive	10 (4.9%)	37 ^a	100 ^a	100 ^a		90.7 ^a	91.1 ^a	50.6 ^a
			100 ^b	98.8 ^b	80 ^b		100 ^b	98.9 ^b	88.4 ^b
			100 ^c	98.8 ^c	80 ^c		100 ^c	98.9 ^c	88.4 ^c
			100 ^d	100 ^d	100 ^d		100 ^d	100 ^d	100 ^d
			32 ^e	98.8 ^e	80 ^e		90.7 ^e	90.1 ^e	41.7 ^e
	Negative	07 (4.6%)	41.2 ^a	100 ^a	100 ^a		92.9 ^a	93.2 ^a	55.4 ^a
			100 ^b	99.2 ^b	85.7 ^b		100 ^b	99.3 ^b	92 ^b
			100 ^c	99.2 ^c	85.7 ^c		100 ^c	99.3 ^c	92 ^c
			100 ^d	100 ^d	100 ^d		100 ^d	100 ^d	100 ^d
			37.5 ^e	99.2 ^e	85.7 ^e		92.9 ^e	92.6 ^e	48.9 ^e
SM	Positive	09 (4.4%)	33.3 ^a	100 ^a	100 ^a		90.2 ^a	90.6 ^a	46.5 ^a
			87.5 ^b	98.8 ^b	77.8 ^b		99.4 ^b	98.3 ^b	81.6 ^b
			87.5 ^c	98.8 ^c	77.8 ^c		99.4 ^c	98.3 ^c	81.6 ^c
			90 ^d	100 ^d	100 ^d		99.4 ^d	99.4 ^d	94.5 ^d
			28 ^e	98.8 ^e	77.8 ^e		90.2 ^e	89.6 ^e	37.1 ^e
	Negative	06 (3.9%)	35.3 ^a	100 ^a	100 ^a		92.3 ^a	92.6 ^a	49.2 ^a
			100 ^b	100 ^b	100 ^b		100 ^b	100 ^b	100 ^b
			100 ^c	100 ^c	100 ^c		100 ^c	100 ^c	100 ^c
			85.7 ^d	100 ^d	100 ^d		99.2 ^d	99.3 ^d	92 ^d
			37.5 ^e	100 ^e	100 ^e		92.9 ^e	93.2 ^e	51.8 ^e
Antigen detection ELISA	Positive	39 (18.9%)	92.6 ^a	92.2 ^a	64.1 ^a		98.8 ^a	92.2 ^a	71.3 ^a
			100 ^b	84.2 ^b	20.5 ^b		100 ^b	84.8 ^b	29.5 ^b
			100 ^c	84.2 ^c	20.5 ^c		100 ^c	84.8 ^c	29.5 ^c
			80 ^d	84.2 ^d	20.5 ^d		98.8 ^d	83.9 ^d	27 ^d
			100 ^e	92.2 ^e	64.1 ^e		100 ^e	93.1 ^e	74.3 ^e
	Negative	21 (13.7%)	94.1 ^a	96.3 ^a	76.2 ^a		99.2 ^a	96.1 ^a	82 ^a
			100 ^b	89.7 ^b	28.6 ^b		100 ^b	90.1 ^b	40.8 ^b
			100 ^c	89.7 ^c	28.6 ^c		100 ^c	90.1 ^c	40.8 ^c
			85.7 ^d	89.7 ^d	28.6 ^d		99.2 ^d	89.5 ^d	38.6 ^d
			100 ^e	96.3 ^e	76.2 ^e		100 ^e	96.7 ^e	84.7 ^e
PCR	Positive	27 (13.1%)	100 ^a	100 ^a	100 ^a		100 ^a	100 ^a	100 ^a
			100 ^b	89.7 ^b	29.6 ^b		100 ^b	90.1 ^b	42.3 ^b
			100 ^c	89.7 ^c	29.6 ^c		100 ^c	90.1 ^c	42.3 ^c
			100 ^d	90.7 ^d	37.0 ^d		100 ^d	91.1 ^d	50.6 ^d
			100 ^e	98.8 ^e	92.6 ^e		100 ^e	99 ^e	95.6 ^e
	Negative	17 (11.1%)	100 ^a	100 ^a	100 ^a		100 ^a	100 ^a	100 ^a
			100 ^b	92.3 ^b	35.3 ^b		100 ^b	92.6 ^b	49.2 ^b
			100 ^c	92.3 ^c	35.3 ^c		100 ^c	92.6 ^c	49.2 ^c
			100 ^d	92.9 ^d	25.9 ^d		100 ^d	93.2 ^d	55.4 ^d
			100 ^e	99.2 ^e	94.1 ^e		100 ^e	99.3 ^e	96.6 ^e

^a Positive by at least any two techniques out of ZN staining, antigen detection and PCR.^b Positive by all three (ZN staining, antigen detection and PCR).^c Positive by ZN staining and antigen detection.^d Positive by ZN staining and PCR.^e Positive by antigen detection and PCR.

group was found positive by ZN and negative by SM staining (Table 4).

None of the 50 healthy controls was found positive for *Cryptosporidium* by any of the technique.

3.4. Sensitivity, specificity, positive predictive value, negative predictive value, diagnostic efficacy and inter-rate agreement of the techniques

These were assessed to define the best possible criteria as 'true positive' for evaluating the techniques, based on following five different criteria (Table 5).

- Positive by at least any two out of ZN staining, antigen detection and PCR.
- Positive by all three (ZN staining, antigen detection and PCR).
- Positive by ZN staining and antigen detection.
- Positive by ZN staining and PCR.
- Positive by antigen detection and PCR.

Criteria 'a' (positivity by at least any two out of ZN staining, antigen detection and PCR) was selected as the best possible criteria as 'true positive' for further analysis and discussion.

On comparison based on gold standard criteria 'a', sensitivity of ZN and SM staining techniques was 37% and 33.3% in HIV seropositive and 41.2% and 35.3% in HIV seronegative patients, respectively. Sensitivity of antigen detection ELISA was 92.6% and 94.1% in HIV seropositive and seronegative patients, respectively, while sensitivity of PCR was 100% each in HIV seropositive and seronegative patients. Specificity of both the staining techniques and PCR was 100% each in both HIV seropositive and seronegative patients, while specificity of antigen detection was 92.2% and 96.3% in HIV seropositive and seronegative patients, respectively. No significant difference was observed in sensitivity, specificity, positive and negative predictive value, diagnostic efficacy and inter-rate agreement of the techniques for detection of *Cryptosporidium* between HIV seropositive and HIV seronegative patients (Table 5).

3.5. Correlation between *Cryptosporidium* positivity and other factors

- Age and sex: No significant association was found between *Cryptosporidium* positivity by any one or more techniques and age or sex in HIV seropositive or seronegative patients with diarrhoea ($p > 0.05$).
- Diarrhoea: Out of 206 HIV seropositive patients, 99 (48%) and 107 (51.9%) patients were with and without history of diarrhoea and number of *Cryptosporidium* positive patients by any one or more technique was 25 (25.2%) and 16 (14.9%) in patients with or without diarrhoea. The number of *Cryptosporidium* positive patients with diarrhoea was not significantly different as compared to number of *Cryptosporidium* positive patients without diarrhoea ($p > 0.05$).
- CD4 count: Out of 67 HIV seropositive patients with CD4 count < 200 cells/ μ l, *Cryptosporidium* was detected in 16 (23.9%), 12 (17.9%), 7 (10.4%) and 6 (9%) patients by antigen detection ELISA, PCR, modified ZN and SM staining technique, respectively. Out of 115 patients with CD4 count 200–500 cells/ μ l, *Cryptosporidium* was detected in 21 (18.3%) and 13 (11.3%) by antigen detection ELISA and PCR, respectively, while it was detected in 3 (2.6%) patients each by ZN and SM staining technique. Out of 24 patients with CD4 count > 500 cells/ μ l, *Cryptosporidium* was detected in 2 (8.3%) patients each by antigen detection ELISA and PCR whereas none was found positive for *Cryptosporidium* by modified ZN and SM staining. *Cryp-*

tosporidium was found in significantly higher ($p < 0.05$) number of patients with CD4 count < 200 as compared to > 200 cells/ μ l by ZN and SM staining techniques.

- Post-transplantation patients: Out of 153 HIV seronegative patients with diarrhoea, 23 (15%) had undergone kidney transplantation. Out of these 23, 4 (17.4%) patients were found positive for *Cryptosporidium*, whereas out of remaining 130 patients, 18 (13.8%) were found positive for *Cryptosporidium* by any one or more techniques ($p > 0.05$).

4. Discussion

Studies comparing four techniques, i.e. modified ZN staining, SM staining, antigen detection ELISA and PCR for the detection of *Cryptosporidiosis* in HIV seropositive or seronegative individuals are scarce. The present study was an attempt to evaluate these four diagnostic techniques on large number of HIV seropositive and seronegative individuals so as to find out the best possible diagnostic marker for the diagnosis of *cryptosporidiosis*.

In the present study, highest positivity was shown by antigen detection (18.9% in HIV seropositive and 13.7% in HIV seronegative) followed by PCR (13.1% in HIV seropositive and 11.1% in HIV seronegative), ZN staining (4.9% in HIV seropositive and 4.6% in HIV seronegative) and SM staining (4.4% in HIV seropositive and 3.9% in HIV seronegative).

Earlier reports from India using ZN staining indicated *Cryptosporidium* in 8.5% from Manipal (Ballal et al., 1999), 4.6% from Mumbai (Joshi et al., 2002), 12% from Chennai (Kumar et al., 2002) and 10% from Chandigarh (Mohandas et al., 2002). Reports from Australia (Stark et al., 2007), Malaysia (Oguntibeju, 2006), South Italy (Brandonisio et al., 1999), Zambia (Chintu et al., 1995) and France (Cotte et al., 1993) indicated *Cryptosporidium* in 2.2%, 50%, 21.5%, 14% and 37.3% HIV seropositive patients, respectively, by ZN staining.

In our study, *Cryptosporidium* was detected in 7 (4.6%) HIV seronegative patients by ZN staining while earlier reports from India reported 13% (Malla et al., 1987) and 1.4% in children and 0.06% in adults from Chandigarh (Sethi et al., 1999), 5.6% from Kolkata (Pal et al., 1989) and 1.5% from Pondicherry (Parija et al., 2003). *Cryptosporidium* has been reported in 6%, 6.8% and 0.6% from Zambia (Chintu et al., 1995), Nepal (Sherchand and Shreshtha, 1996) and Australia (Stark et al., 2007), respectively, in HIV seronegative patients.

ZN and SM staining showed a sensitivity of 37% and 33.3% in HIV seropositive and 41.2% and 35.3% in HIV seronegative patients, respectively, in the present study. Earlier study (Morgan et al., 1998) has reported a sensitivity of 83.7% for microscopy; however, HIV status of the patients was not mentioned.

In the present study, *Cryptosporidium* antigen detection ELISA (RIDASCREEN) showed 92.6% and 94.1% sensitivity in HIV seropositive and HIV seronegative patients, respectively, which is in agreement with an earlier study whereby sensitivity, specificity, positive and negative predictive value of 82.4%, 100%, 100% and 98.5%, respectively, has been reported by the use of similar kit (Weitzel et al., 2007). Variable sensitivity (66.3–100%) of antigen detection ELISA has been reported from other parts of world (Chapman et al., 1990; Ungar, 1990; Newman et al., 1993; Rosenblatt and Sloan, 1993; Baveja, 1998; Bialek et al., 2002; Garcia et al., 2003).

In the present study, PCR showed highest sensitivity (100%) in both HIV seropositive and seronegative patients, which is in accordance with earlier reports from India (Muthusamy et al., 2006) and other parts of world (Morgan et al., 1998; Zhu et al., 1998; Pedraza-Díaz et al., 2001). Bialek et al. (2002) reported sensitivity

of PCR (96.5%) similar to ELISA and direct fluorescence assay, in HIV infected and other patients (HIV status not mentioned). Another study while comparing PCR and microscopy, reported sensitivity of PCR and microscopy as 100% and 83.7%, respectively (Morgan et al., 1998). Ubiquitous PCR inhibitors in faecal specimens have been found to cause significant problems, therefore, in this study, a commercial kit (Qiagen stool mini kit) was used that guarantees reproducibility and simplicity. Further, proper disruption of oocysts is essential, and therefore samples were boiled to disrupt the oocysts wall (Bialek et al., 2002).

Both ZN and SM staining techniques showed a specificity of 100% in both HIV seropositive and HIV seronegative patients. Earlier study (Morgan et al., 1998) has reported a specificity of 98.9% for microscopy. In the present study, specificity of antigen detection ELISA was 92.2% in HIV seropositive and 96.3% in seronegative patients, respectively, which is in accordance with the earlier reports indicating ELISA specificity ranging from 92 to 100% (Chapman et al., 1990; Ungar, 1990; Newman et al., 1993; Rosenblatt and Sloan, 1993; Baveja, 1998; Garcia et al., 2003). In the present study, PCR revealed 100% specificity in both HIV seropositive and seronegative patients which is similar to earlier study (Morgan et al., 1998) indicating 100% specificity. However, in nested PCR, there is greater chance of contamination due to use of highly concentrated amplicon during nested PCR, specially in a clinical microbiology laboratory handling infectious diseases. There is need to take extreme precaution to avoid contamination and therefore, we used 5 negative controls with every PCR to check the contamination.

The present study indicated that the number of HIV seropositive patients with history of diarrhoea was significantly higher in patients with CD4 count <200 cells/ μ l. Significant association between positivity for intestinal parasites and history of diarrhoea was observed in HIV seropositive patients as positivity for intestinal parasites was significantly higher ($p < 0.001$) in HIV seropositive patients with diarrhoea (39.4%) as compared to that in patients without diarrhoea (15%).

In our study, number of *Cryptosporidium* positive patients by any one or more techniques was 25 (25.2%) and 16 (14.9%) in HIV seropositive patients with or without diarrhoea, respectively. The number of *Cryptosporidium* positive patients with diarrhoea was not significantly different as compared to number of *Cryptosporidium* positive patients without diarrhoea ($p > 0.05$). In the earlier study from our institute (Mohandas et al., 2002), *Cryptosporidium* was reported in significantly higher number (22.6%) of HIV seropositive patients with diarrhoea as compared to 0.5% in patients without diarrhoea by ZN staining and from Vellore, India (Mukhopadhyay et al., 1999) *Cryptosporidium* positivity was observed in 9.8% and 6% HIV seropositive patients with and without diarrhoea, respectively, by SM staining whereas in a recent study from Vellore (Muthusamy et al., 2006) *Cryptosporidium* was reported in significantly higher number (25.2%) of HIV seropositive patients with diarrhoea as compared to 4.7% in patients without diarrhoea. Thus, the studies, in general indicate higher number of *Cryptosporidium* positive patients in HIV seropositive than HIV seronegative and in patients with diarrhoea as compared to without diarrhoea. However, more recently, asymptomatic cryptosporidiosis is also being increasingly reported. In the present study, 14.9% asymptomatic HIV seropositive patients without diarrhoea were positive for *Cryptosporidium* infection. In support of this, the report from Tanzania indicates cryptosporidiosis in 16.7% in asymptomatic as compared to 18% in symptomatic HIV-infected patients (Hout Eric et al., 2005) and from Bolivia (Esteban et al., 1998) and Korea (Yu et al., 2004) indicated cryptosporidiosis in 8–32% in healthy individuals. *Cryptosporidium* was detected in 4.6% in diarrhoeic cases and 1.2% in

non-diarrhoea cases in Children from Kolkata, India (Das et al., 2006).

In the present study, comparative analysis of four techniques revealed that *Cryptosporidium* could be detected by ZN and SM staining only in 10.4% and 9%, respectively, in HIV seropositive patients with CD4 count <200 cells/ μ l and even less in patients (2.6% each) with count 200–500 cells/ μ l and in none of the patients with count >500 cells/ μ l, while antigen detection ELISA and PCR could detect *Cryptosporidium* in 18.3% and 11.3% patients with CD4 count between 200 and 500 cells/ μ l, respectively, and both the techniques also detected *Cryptosporidium* in 8.3% patients with CD4 count above 500 cells/ μ l, thereby suggesting that by the most commonly used modified ZN and SM staining techniques it may not be possible to detect *Cryptosporidium* in HIV seropositive patients, if CD4 count is greater than 200 cells/ μ l.

In conclusion, to the best of our knowledge this is the first study comparing four techniques to detect *Cryptosporidium*, simultaneously on large number of HIV seropositive and HIV seronegative subjects. The staining techniques (ZN and SM) were less sensitive for detection of *Cryptosporidium* in HIV seropositive patients with CD4 count >200 cells/ μ l and this suggests that cases of cryptosporidiosis may be missed in patients who have CD4 count above 200 cells/ μ l, if only ZN or SM staining technique is employed. PCR was found 100% sensitive and specific. However, availability of reagents and equipment required for DNA extraction and PCR in diagnostic laboratories in the developing countries may hamper its routine use. Moreover, there is need for proper DNA extraction and removal of PCR inhibitors in addition to extreme precaution required to avoid contamination while doing PCR. Antigen detection ELISA is a simple diagnostic test and the study suggests that it can be used in place of less sensitive staining techniques for better diagnostic yield. In developing countries, where facilities for PCR do not exist at various peripheral diagnostic laboratories, it may eliminate some of the skills needed in performing staining procedures and recognizing morphology of the small *Cryptosporidium* oocysts.

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