International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 8, Issue 2, 2016, pp.-723-727.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

COMPARISON AND PCR CONFIRMATION OF ENTERIC PROTOZOAL ISOLATES IN AIDS PATIENTS- OPTIMIZING DIAGNOSTIC APPROACH IN DIFFERENT RESOURCE SETTINGS

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Received: February 02, 2016; Revised: February 05, 2016; Accepted: February 06, 2016

Abstract- Opportunistic infections in AIDS patients are a harbinger of death. In this study presence of enteric protozoa (*Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Enterocytozoon bienuesi*) as causative agents of diarrhea in HIV/AIDS patients were detected from their fecal samples. The conventional staining methods used for identification which had been employed earlier were compared with PCR results and different attributes (sensitivity, cost, hands on time, ease of use and batch testing) while performing investigations in the different set ups *viz*. rural, urban government, urban private and hospital cum research/referral centers were prioritized. Testing the samples by PCR gave 100% sensitivity and specificity. Since PCR is costly, a combination of at least two methods be employed in resource limited settings to avoid missing any parasite. This is the first study from India wherein a PCR was employed to confirm the presence of all the three above mentioned protozoa and application of Analytical Hierarchy Process was done.

Keywords- AIDS; diarrhea; sensitivity; PCR; Analytical Hierarchy Process

Citation: Tuli Lekha, et al., (2016) Comparison and PCR Confirmation of Enteric Protozoal Isolates in Aids Patients- Optimizing Diagnostic Approach in Different Resource Settings. Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 8, Issue 2, pp.-723-727.

Introduction

Cryptosporidium parvum, *Microsporidia* spp. and *Cyclospora cayetanensis* have been associated with diarrhea and wasting in AIDS patients and have emerged as important opportunistic pathogens. Although the disease is self-limited in immunocompetent people, in immunocompromised individuals, it often causes chronic or cholera like diarrhea and can be fatal if not properly and timely managed. The immune status of the patient has an important role to play in the intensity and severity of these infections and their incidence in a particular area depends on factors like geographic distribution and seasonal variation [1].

Conventionally, the diagnosis of these protozoa has been carried out by different staining procedures like Giemsa, Haematoxylin, Kinyouns, Ziehl-Neelsen's, fluorescence and immunofluorescence methods. These techniques are not only labor intensive and time consuming but there is also a possibility of getting false positive or negative results due to dependency on the expertise of the reporting person. However, Polymerase Chain Reaction (PCR), which is more sensitive and specific, can minimize this discrepancy in results to a great extent. Thus, many researchers have explored molecular analysis based methods as they have the potential for automation and high output [2,3,4].

A definitive diagnosis precedes an adequate treatment and so is true with the above protozoa as only specific treatment warrants complete cure. Therefore, in this study we identified the protozoa isolated from fecal specimens from AIDS patients. The species identification was done by using PCR and the results were compared with the staining procedures performed earlier. This happens to be the first study from India where molecular methods were employed to identify all the three enteric protozoa (*Cryptosporidium parvum*, *Microsporidia* spp. and *Cyclospora cayetanensis*) together from AIDS patients and compared with the routinely used staining methods. Besides this, we prioritized the different basic attributes while performing investigations in the different set ups *viz*. rural, urban government, urban private and hospital cum research/referral centers.

Materials and Methods

Study set up

The study was conducted in the Microbiology Department of Mahamaya Rajkiya Allopathic Medical College, Ambedkar Nagar, UP, India and Department of Microbiology, IMS (Institute of Medical Sciences), BHU (Banaras Hindu University), Varanasi, India.

Study cases

(a) Inclusion criteria

The stool samples were collected from 600 HIV patients who complained of diarrhea and were investigated for the enteric protozoan as and when they reported.

(b) Exclusion criteria

Subjects who were HIV negative and did not present with the complain of diarrhea were not included in the study.

Controls

The control group in the study were HIV negative family members of the above patients who had diarrhea and came from similar environmental, social and economic background. Stool samples were collected from 200 such people.

Examination of stool samples for enteric protozoa by microscopy and antigen detection

Disposable, wide mouthed, universal containers were used to collect the stool samples avoiding delay in processing and in case of delay they were preserved at 4°C. After direct microscopic examination, samples were concentrated by Modified formol ether technique for identifying *Cryptosporidium parvum* and *Cyclospora cayetanensis* followed by Kinyoun's and Modified safranin staining.

Cyclospora cayetanensis was identified by its property to autoflouresce. Detection of *Microsporidia* spp. was done by flourescent staining using Calcoflour White and DAPI (Sigma, USA). Transmission Electron Microscope was used to identify all the three protozoa. To detect *Cryptosporidium parvum* antigen, sandwich ELISA was performed from part of un-concentrated stool samples with the help of a commercially available kit (IVD Research Inc. CA, USA).

Identification of enteric protozoa by PCR

Subsequently, the samples were evaluated for *Cryptosporidium parvum, Cyclospora cayetanensis* and *Enterocytozoon bienuesi* by using PCR. In order to avoid contamination the procedures of DNA isolation, reaction mixture preparation, amplification and analysis were performed in three different areas.

(a) Extraction of DNA

A 200 µl aliquot of the stool samples was made. It was then washed with distilled water and centrifuged at 1400 rpm for 10 minutes. To the pellet 18.6µl Dithiothretol (1M) and 66.6 µl Potassium hydroxide (1M) were added, mixed thoroughly and incubated for 15 minutes at 65ºC. For neutralizing the samples, 8.6 µl of 25% HCl buffered with 160 µl of 2M Tris- HCl (pH 8.3) was used and the mixture was vortexed. To this 1% SDS, 5 µl Proteinase K and 0.4U of chitinase were added and incubated at 55ºC for 2 hours. A 250 µl solution of phenol, chloroform, isoamyl alcohol in the ratio (25:24:1) was added to the above, vortexed and centrifuged at 10,000 rpm for 10 minutes and the aqueous phase was collected. Again equal volume of chloroform and isoamyl alcohol in the ratio 24:1 was added, vortexed, centrifuged for 10 minutes at 10,000 rpm followed by collection of the aqueous phase. Isopropanol (equal volume) was added and kept at room temperature for 5 minutes. Again the step of centrifugation was repeated at 10,000 rpm for 10 minutes. The supernatant was decanted and the pellet was washed with 200 µl of 70% ethanol, centrifuged at 10,000 rpm for 10 minutes. The pellets were dried at 37 ºC for 30 minutes and re-dissolved in 30 µl TE (pH 8).

(b) Primers for PCR and reaction conditions

The previously defined 18s ribosomal DNA genes in *Cryptosporidium parvum, Cyclospora cayetanensis* and *Enterocytozoon bienuesi* were detected by primers (GeNei) [5].

For *Cyclospora cayetanensis*, amplifications were done in 100 ml reaction mixtures which contained 5mM Tris-HCl (pH 9.0), 25 mM KCl, 1mM $MgCl_2$, 100 mM each dATP, dCTP, dGTP, and dTTP, and 0.1 mM each primers F1E (59-TACCCAATGAAAACAGTTT-39) and R2B (59-CAGGAGAAGCCAAGGTAGG-39) and 10 ml of a Taq DNA polymerase (GeNei). Reactions were run in a thermocycler (Eppendorf). The PCR protocol consisted of 5 minutes at 95°C followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing was done at 53°C for 30 seconds, followed by extension at 72°C for 90 seconds and a final extension step at 72°C for 10 min. For the second round, 1 to 5 ml of the first round product was used as template in a reaction volume of 50 ml to which 5 ml of Taq DNA polymerase was added rest all reaction component concentrations were the same as in the first round reaction. The primer pairs used were F3E (59-CCTTCCGCGCTTCGCTGCGT-39) and R4B (59-CGTCTTCAAACCCCCTACTG-39) and the annealing temperature was 60°C.

For *Cryptosporidium parvum*, same reaction conditions were used as for *Cyclospora cayetanensis* except that the primer pairs used were CPB-DIAGF (59-AGCTCGTAGTTGGATTTCTG-39) and CPB-DIAGR (59-TAAGGTGCTGAAGGA GTAAGG-39) and a total of 39 cycles with denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and elongation at 72°C for 1 minute were done.

In case of *Enterocytozoon bieneusi* again, the reaction conditions were similar as above with the exception that the primers EBIEF1 (59-GAAACTTGTCCA CTCCTTACG-39) and EBIER (59-CAATGCACCACT CCTGCCATT-39) were used. A total of 35 cycles was carried out with denaturation temperature at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 90 seconds. The PCR products in all the above cases were separated by Agarose gel electrophoresis using 1.5% agarose containing ethidium bromide (0.2 mg/ml). Products were visualized on a UV trans illuminator.

Analytical Hierarchy Process (AHP) was used to determine the optimal tests for the four prevalent diagnostic setups in our country. These being the labs in rural areas, urban government labs, urban private laboratories and the hospitals with research centers.

The parameters under study were sensitivity of the test, cost incurred, infrastructure available, expertise in the immediate vicinity, hands on time and its batch testing ability. First of all the parameters were compared among themselves as per preferences in different laboratory settings. The pairwise comparison of these parameters was done in respect to each other by using the scale as devised by Saaty [6]. Thereafter, the comparison matrices were solved to determine the individual priorities of all the parameters. These calculations were done separately for all the four kind of setups mentioned above and finally tabulated.

Although in this study the enteric protozoa were studied, in some cases the cause of diarrhea might have been bacteria or viruses. Therefore, we made an algorithm for the processing of stool samples in general to identify the microbial cause of diarrhea.

Statistics

The statistical analysis was done by Fisher's exact test and Chi-square test using graph pad software.

Results

The microscopic and serological examination of the 600 fecal samples of the cases showed mainly *Cryptosporidium* spp. (35.83%), *Microsporidia* spp. (21%) and *Cyclospora* spp. (23%). There was a high incidence of *Cryptosporidium* spp. (21%) followed by *Entamoeba histolytica* (2%) in the control group.

In case of *Cryptosporidium* spp. and *Cyclospora* spp. detection direct microscopy was found to be 61.4% and 60.3% sensitive. Whereas, staining methods showed greater sensitivity and specificity. [Table-1]. Safranin staining was found better for visualizing *Cyclospora* spp. whereas, Kinyouns staining for detecting *Cryptosporidium* spp. and. in case of *Enterocytozoon bieunesi* the combination of Calcoflour White and DAPI was found 94.93 % sensitive.

Cryptosporidium parvum antigen detection was done in 600 samples using ELISA kit and an absorbance reading of 0.15 OD units and above indicated presence of *Cryptosporidium* antigen. False negative results were obtained in case of 19 AIDS patients by ELISA.

After using the species specific primers the protozoa were identified as *Cryptosporidium parvum*, *Cyclospora cayetanensis* and *Enterocytozoon bienuesi*. In case of *Cyclospora cayetanensis* a nested PCR performed using F1E and R2B primer pairs for first round reaction gave a 636 base pair product. The next round reaction using F3E and R4B as the primer pairs generated a 294 base pair product. For *Cryptosporidium parvum* CPB-DIAGF and CPB-DIAGR primers gave a 434 base pair product. A 607 base pair product was obtained using EBIEF1 and EBIER primers in case of *Enterocytozoon bienuesi*. However, after employing PCR it was found that among the cases 9 samples of *Cryptosporidium parvum*, which were microscopically negative, showed positive results with PCR. Similarly, 4 samples of *Cyclospora cayetanensis* and 6 samples of *Enterocytozoon bienuesi* which could not be detected by microscopy were found to be positive by PCR. In case of samples from controls, PCR detected *Cryptosporidium parvum* in 2 microscopically negative samples and *Cyclospora cayetanensis* in 1 negative sample.

The application of AHP led to the priority distribution in different setups [Table-2]. In rural settings, cost ranked the highest in the list followed by sensitivity and batch testing was last in the list. In urban government setups cost again became the deciding factor. Batch testing ability for these labs had a comparable weightage to the sensitivity. In urban private setups, sensitivity and cost were found comparable. On the other hand, the private laboratories gave equal weightage to the sensitivity and cost of the tests.

In this study only enteric protozoa were studied in detail however, in general, the stool samples received from AIDS patients should also be subjected to staining and culture for bacteria or fungus simultaneously. Fluorescence microscopy, ELISA or PCR should be employed wherever the facilities are available [Fig-1].

Discussion

With the advent of the AIDS pandemic in the mid 1980s there was rekindling of interest in studying parasitic protozoa owing to increase in food borne disease agents globally. Of all the infectious diseases, protozoal diarrhea is one, which

can be fatal if not treated promptly. Moreover, identification of the infecting species is clinically essential and should be called for every time as different species vary in their response to therapy.

Table-1 *Comparison of the Diagnostic Methods for the identification of the enteric protozoa*

	Sensitivity	Specificity	PPV	NPV
	Cryptosporidium spp.			
Direct microscopy	61.4	93.25	83.54	81.22
After concentration	71.63	95.84	90.59	85.81
Kinyoun's	87.91	97.14	94.5	93.5
Safranin	80.47	98.18	96.11	90
TEM	95.81	100	100	97.72
ELISA	91.16	98.44	97.03	95.23
PCR	100	100	100	100
	Microsporidia spp.			
Calcoflour White	93.48	97.45	91.49	98.04
Calcoflour White + DAPI	94.93	98.48	94.93	98.48
TEM	95.65	100	100	98.72
PCR	100	100	100	100
	Cyclospora spp.			
Direct microscopy	60.3	97	84.44	90.2
After concentration	74.6	97.9	90.38	93.55
Kinyoun's	82.54	98.1	92.04	95.48
Safranin	87.3	98.73	94.83	96.69
Autofluorescence	96.03	100	100	98.96
TEM	96.81	100	100	99.16
PCR	100	100	100	100

ELISA-Enzyme Linked Immunosorbant Assay, PCR- Polymerase Chain Reaction, TEM – Transmission Electron Microscopy

Table-2 *Prioritization of attributes in different laboratory settings*

Attributes	Rural C.I.=0.04	Urban Govt. C.I.=0.07	Urban Private C.I.=0.1	Hospital with Research Centre C.I.=0.11
Sensitivity (.34)	0.18	0.19	0.24	0.31
Cost (.18)	0.52	0.36	0.24	0.21
Hands on Time (.08)	0.09	0.14	0.15	0.16
Expertise (.18)	0.13	0.14	0.20	0.17
Batch testing (.05)	0.07	0.17	0.16	0.15

Consistency Index (C.I.) is a measure to indicate consistency of judgments

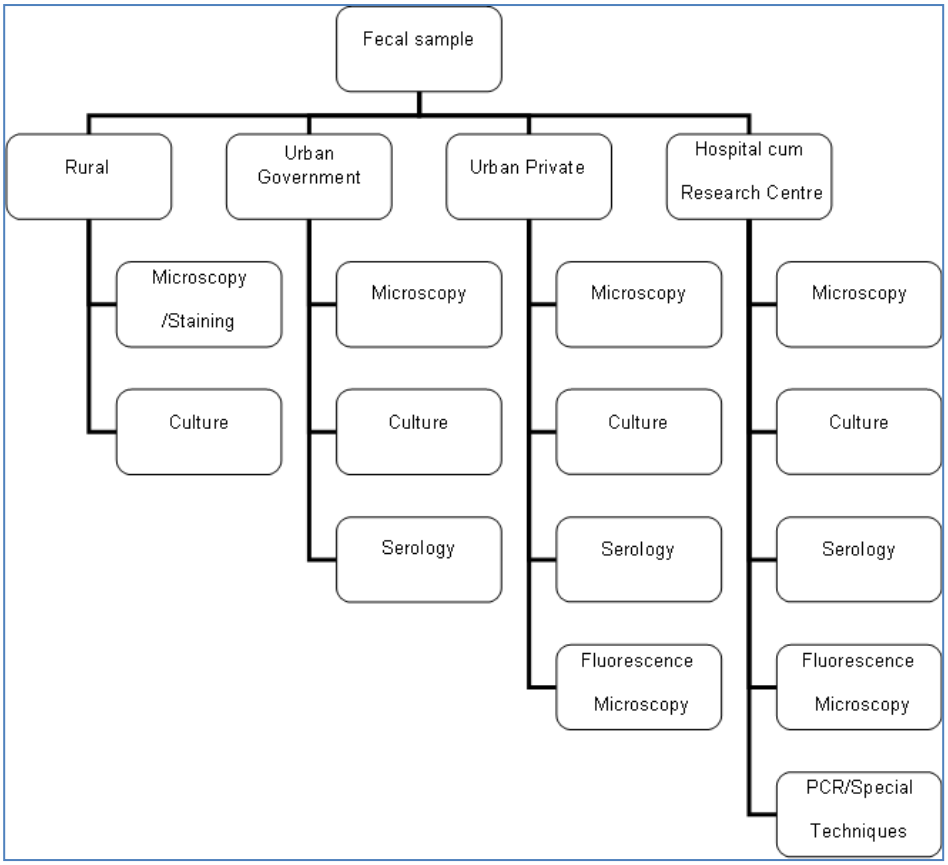

Fig-1 Algorithm for processing stool samples collected from AIDS patients in different resource settings

Of all the organisms isolated among the cases, *Cryptosporidium parvum* (35.83%) was the one most commonly found followed by *Enterocytozoon bienuesi* (21%) and *Cyclospora cayetanensis* (23%) in this study. The observed incidence of these organisms in AIDS patients was significantly higher ($p< 0.001$, Fischers exact test) compared to that of the controls. A similar study showed prevalence rates of 10.8% *Cryptosporidium parvum*, 3.3% *Cyclospora cayetanensis* and 2.5% *Enterocytozoon bieneusi* [7]. Lower parasitic incidence in the other studies could be due to less sensitive procedures involved for identification. In a study from Southern India conducted by Ballal, fewer *Cryptosporidium* spp. (9%) were isolated [8]. The pattern of incidence of parasitic infection was found a little different in Mumbai where Joshi et al reported *Isospora belli* to be 17% followed by 8.5% *Cryptosporidium parvum* [9]. These could be due to the variation in the geographic distribution of these protozoa [1]. A study conducted in Gautemala showed prevalence of 2.1% *Cyclospora* infection and 1.2% *Cryptosporidium* spp. [10].

The above mentioned protozoa were identified by the conventional staining methods. However, they could detect the protozoa only up to the genus level. Therefore, PCR was employed for the species identification. In the past two decades many studies have been conducted to evaluate new molecular diagnostic tools in order to improve the detection rate, especially for *Cryptosporidium* spp. in clinical samples [11]. The only studies on molecular characterization of *Cryptosporidium parvum* carried out in India have focussed on infection in children [12,13]. Another study by Nagamani et al again focussed on *Cryptosporidium parvum* alone [14]. However, fewer studies have been carried out for *Cyclospora* spp. and *Enterocytozoon* spp. From India there is only one study reported for *Enterocytozoon bienuesi* [15]. Ours is the first study from India to employ molecular methods for detection of *Cyclospora cayetanensis.* Polymerase chain reaction (PCR) ranks the highest, among the molecular techniques. The DNA extraction method from stool samples should be efficient enough to liberate the parasitic DNA from spores or oocysts and at the same time prevent the DNA adsorption to stool constituents and remove PCR inhibitors. In our study we used the phenol-chloroform method for dna isolation and found it very effective. To disrupt the α-chitin in the inner endospore layer of the spore wall of *Enterocytozoon bienuesi*, chitinase was used in this study as used by Muller et al [16]. Carnevale et al also found the phenol-chloroform extraction method very useful [17]. In another study, Muller et al found good results with the QIAmp tissue kit [16] and Da Silva et al found the modified Fast DNA kit to be better than the Laureth 12-glass bead disruption method [18]. However, different studies conducted using filter paper specimens showed facilitation in collection, transport, storage and maintenance of stable DNA during long periods [19,20,21]. Subrungruang et al also found Flinders technology associates (FTA) filter paper method to be very useful while performing their experiments with *Enterocytozoon bieneusi* [22] as it is an extraction free method and the template is filter-based impregnated with denaturants, chelating agents, and a free-radical trap because of which, when the organisms come in contact with the FTA filter paper, lysis of the organisms occurs and the DNA gets trapped in the matrix. In case of *Cyclospora*, oocysts are difficult to crack as they are covered with a thick layer of carbohydrates, which, probably interfere with PCR, and it is very difficult to isolate the DNA during purification. The presence of some kind of matrix as in the case of FTA filters is effective. In our study, we used the primers to detect the previously defined regions of the 18S ribosomal DNA gene in *Cryptosporidium parvum*, *Enterocytozoon bieneusi* and *Cyclospora cayetanensis*. The ribosomal RNA sequences have proven to be useful as diagnostic tools for these protozoa. The EBIEF1/EBIER1 primer pairs used by us have been used by da Silva et al in 1996 who tested and found them highly specific for *Enterocytozoon bieneusi* on comparison with 13 other *Microsporidia* spp. [23]. In another study however, a different set of primers compared to ours were used for identifying *Cyclospora cayetanensis* and were found highly specific in comparison to rDNA sequences of related coccidian [24].

For *Cyclospora* identification Safranin technique was found better compared to Kinyoun's staining which was, on the other hand found better for *Cryptosporidium parvum* detection. Kehl et al in 1995 reported Kinyouns staining to be 96% sensitive and 99% specific for *Cryptosporidium* spp. Detection [25]. In spite of some individual propensities of the two staining techniques for different protozoan, they had better diagnostic ability than the unstained smear examination (Fishers exact test, $p<0.05$). While comparing the different methods for the identification of the enteric protozoa we found PCR to be impeccable, with a sensitivity and specificity of 100% in identifying all the three organisms. Morgan et al in 1998 compared the conventional acid-fast staining procedure and reported it to be 83.7% sensitive and 98.9% specific compared to PCR [26]. In our study, in the case of *Cryptosporidium parvum*, ELISA and combined staining techniques were found comparable. Jayalakshmi et al also found ELISA to be reliable and less subjective test for diagnosing and screening large number of specimens routinely [27]. For the detection of *Cyclospora cayetanensis*, autofluorescence ranked second after PCR and was comparable to the staining methods employed together. Although, the staining methods are easy, practical and provide a stained slide that can be archived but they are not as sensitive or specific as PCR which is easier to interpret and adaptable to batch analysis. However, PCR set up is more expensive [28].

Though, PCR is undoubtedly the best method available but it is unaffordable by all diagnostic setups. Thus, optimization of the available techniques according to the available resources is the need of the hour. So, different health setups in our country were compared to see for the variation in the preferences and feasibility of the available methods. This was based upon the priorities of different setups for various individual parameters like sensitivity of the test, cost incurred, infrastructure available, expertise in the immediate vicinity, hands on time and the batch testing ability of the tests. We found that the labs in rural setups had facilities for routine and staining microscopic examination only. In rural settings cost had the maximum priority because of the poor economic status of the people and thus inability to pay for expensive tests. All the more batch testing ability was rated to be of least significance because of the less sample load at this level. In Urban government set up again cost got maximum weightage as the tests are at subsidized rates in this setting increasing the economic burden of the government. In Urban private settings sensitivity was given more importance with an equal weightage to the cost, followed by expertise. The reason may be that these laboratories aim at higher yields. Hospital cum research centers gave highest priority to sensitivity of the tests which was due to easy availability of funds and expertise. Thus, the algorithm given by us holds good not only for fecal samples but any samples received for detecting opportunistic infections and if followed will lead to a substantial advance in management of public health.

Conclusion

Thus with the better availability of resources a gradual increase was observed in the importance of sensitivity. The main cause of diarrhea among these patients from rural areas is the practice of unhygienic toilet habits so, the maximum disease load is actually present at the level of rural settings. Therefore a combination of funds, faster and reliable techniques and expertise are needed at this very level to curb the further spread of the disease. The education level of the patients being very low, counselling and also free distribution of toiletries is needed to put a further check on the infections which are mainly acquired and transmitted due to lack of sanitary habits. This would help in minimizing the mortality rates. For optimizing the diagnostic yield in these low resource settings, staining techniques should be advocated and practiced as it has comparable sensitivity to ELISA in case of *Cryptosporidium parvum* and to that of autoflourescence detection in case of *Cyclospora cayetanensis*. In the laboratories equipped with flourescent microscope, autoflouresence detection for *Cyclospora cayetanensis* and Calcoflour staining for *Microsporidia* will serve the purpose as effectively as PCR for these two organisms. However well equipped labs must always aim at getting highest and accurate yield. Thus there is a need of appointing not only good counsellors but also efficient Microbiologists in resource limited settings.

Conflicts of interest: none declared

Acknowledgements

We would like to thank Dr. Shyam Sundar and Dr. Gajendra Singh for their

guidance, Dr. Joan Shields for providing with the control strains of the protozoa and Dr. Madhu Yashpal for providing assistance in Electron Microscopy.

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