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Research Article

DETECTION OF CRYPTOSPORIDIUM PARVUM IN HIV/AIDS PATIENTS BY USING VARIOUS METHODS A DESCRIPTIVE CROSS-SECTIONAL STUDY

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ABSTRACT

Background: Cryptosporidium parvum is one of the most important enteric opportunistic parasitic infections in immunocompromised patients. Cryptosporidium parvum is a coccidian protozoal parasite that inhabits the brush border of enterocytes, damages the epithelial cells and causes diarrhoeal disease. It causes severe and prolonged diarrhoea in HIV sero-positive/AIDS patients with CD4counts 200 cells/cumm. Early detection of cryptosporidium will enable the clinician in effective management of the disease. Various techniques based on different principles are available for the diagnosis of cryptosporidiosis. **Aims and Objectives:** This study was aimed to determine the incidence of cryptosporidial infection in HIV sero- positive/AIDS patients both with diarrhoea as well as without diarrhoea, to evaluate various methods of detection of *C.parvum* and correlate the CD4 counts with the incidence of cryptosporidiosis. Materials and methods Stool samples were collected from 110 HIV positive patients presenting with and without diarrhoea at RIMS, Raichur after obtaining informed consent. Each stool sample was divided into four parts and subjected to modified Ziehl Neelsen staining method, immunofluorescent microscopy, ELISA. **Results:** Out of 110 cases studied, 65 patients presented with diarrhoea and the remaining 45 were without diarrhoea. The major group affected was 31- 40 years with mean age 34.4 years. Male preponderance was seen. Out of 110 patients, 80 (73%) patients had CD4 count less than 200 cells/cumm. Maximum positivity was detected by ELISA i.e. 95.4% followed by Immunofluorescent Microscopy 92.6%, Modified ZN staining 77.3%. **Conclusion:** Our study highlights the importance of routine examination of stool samples for cryptosporidium oocysts in all HIV sero-positive /AIDS patients, irrespective of gastrointestinal symptoms. ELISA was found to be the most reliable method for diagnosis.

INTRODUCTION

Cryptosporidiosis due to *Cryptosporidium parvum* is an important zoonotic disease distributed world-wide. The description of *Cryptosporidium parvum* was 1streported in 1907 in the gastric crypts of laboratory mouse by Edward Tyzzer. This disease is now well documented inhuman beings, especially among immunocompromised individuals[1].

In 1981Michael Gottlieb and his colleagues at Los Angeles reported abroad range of opportunistic enteric parasites responsible for gastrointestinal infections like cryptosporidiosis, in patients with severe immunosuppression[2].*Cryptosporidium parvum* causes rigorous and protracted diarrhoea and is considered as one of the most important enteric opportunistic infection in AIDS[2]. *Cryptosporidium parvum* is a coccidian protozoal parasite that inhabits the brush border of enterocytes, damages the epithelial cells and causes diarrhoeal disease[2].

World health Organization (WHO) in 2004 considered cryptosporidiosis as the most ignored disease mainly in developing countries. This is attributed to the scarcity and non-availability of proper laboratory facilities. Cryptosporidiosis can cause severe mortality in immunocompromised (HIV seropositive/AIDS) and malnourished individuals mainly in countries which are underdeveloped[3].

The immune conditionis a key factor in determining the severity of cryptosporidiosis[4].Eighty percent of AIDS patients with cryptosporidiosis have CD4 count below 200cells/cumm[5].Different studies have revealed various prevalence rates in varied geographical locations[6]. In India, prevalence rate of cryptosporidiosis is 80%[7].

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Due to higher incidence of HIV sero-positivity /AIDS in our area, i.e., Raichur District of Karnataka, it is important to know the opportunistic parasitic diseases like cryptosporidiosis and its correlation with the immune status of the patient.
Early detection of cryptosporidium will enable the clinician in effective management of the disease.Various techniques based on different principles are available for the diagnosis of cryptosporidiosis. There is a need toevaluate these methods for optimal benefit of the patients.

AIMS ANDOBJECTIVES

- To determine the incidence of cryptosporidiosis in HIV sero-positive/AIDS patients.
- To evaluate various methods of detection of cryptosporidiosis.

MATERIALS AND METHODS

Type of Study: The present study undertaken is a cross sectional descriptive study.

Source of Data: The study group consisted of inpatients and out patients of Government Medical College (RIMS), Raichur.

During of study: One year (from May 2018 toin May 2019) Inclusion criteria: All HIVsero-positive/AIDS patients with and without diarrhoea were included in the study.

Exclusion criteria: HIV sero-negative patientswere excluded.

Data Collection Procedure

Ethical consideration Approval taken from Government Medical college(RIMS). Before including in the study, informed consent was obtained from each participant.

Processing of samples

Macroscopic examination: The stool samples were observed forcolour, odour, PH & consistency. The presence of blood or mucous, live worms or segments of the worms was also noted. Each sample was divided into 4 portions. • First portion wasused to prepare the smear and stained by Modified ZiehlNeelsen staining technique (MZN). • Second portion was used to prepare the smear, fixed with ethanol and stored at room temperature for Immunofluorescent microscopy technique(IFT). • Third portion was preserved in formalin at room temperature for processing by Enzyme-Linked ImmunoSorbent Assay (ELISA).

Modified Ziehl-Neelsen staining

Procedure

After the heat fixation of the prepared faecal smear, following staining procedure will be employed.

- Cold carbolfuchsin for 10-15 minutes.
- Rinse with tap water.
- Decolorize with 3%-6% suphuric acid i.e.H_2So_4 rocking the slide until color stops leaching from material.
- Rinse with tap water.
- Counterstain with 1% Methelyene blue for 30seconds- 1 minute.
- Dry the slide, mount the smear and examine under microscope with oil immersion objective.

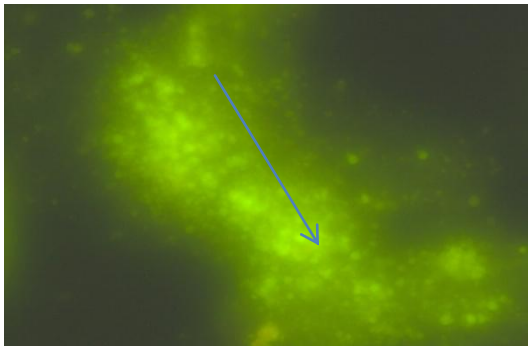
Result: Total 110 samples examined for Microscopy, 85 samples were found positive by Modified ZeihlNeelsen staining (MZN).

Immunofluorescent Staining (IFT)

Procedure

- The stool sample is smeared on the glass slide and fixed with ethanol
- The smear is fixed with pre-cooled ethanol for 5 minutes
- Then rinse the slide with PBS with wash bottle or dropper bottle.
- Then block the slide with 5% H_2O_2 for 10 minute
- Briefly immerse the slide in jar of PBS for 2minutes
- Then apply diluted primary antibody (1:500 dilution) for 40 minutes on the slide
- Then wash the slide with PBS for 2-3 minutes
- Then apply diluted secondary antibody (1:400 dilution) for 30 minutes on the slide
- Again immerse the slide in PBS for 2-3 minutes
- Then observe in the fluorescent microscope
- Can be mounted in 90% glycerol in PBS with cover slip
- Primary antibody: Goat/IgG Polyclonal antibody - FITC antibody - Oocysts purified from bovine faces (Unconjugated).
- Secondary antibody: Anti- Goat IgG (Whole molecule) - FITC antibody produced in rabbit affinity isolated antibody, buffered aqueous solution

Result: Total 110 samples were subjected to Immuno-fluorescent microscopy, of which 102 samples were found positive.



Oocysts of cryptosporidium parvum

Enzyme linked Immuno-sorbent Assay (ELISA)

Procedure

- Break required wells needed from the microtitre plate and two control wells (positive and negative) place in the holder.
- Using the Micropipette add 100ul of positive control and negative control into wells.
- Add 50ul of Dilution buffer to each sample well (Don't add dilution buffer to control wells).
- Add 50ul of stool sample to each sample well and incubate for 60 minutes at room temperature ($15\text{-}25^0$) . Then wash and slap the wells to remove excess of buffer.
- Add 2 drops of Enzyme conjugate to each well at room temperature ($15\text{-}25^0$)
- Again incubate for 30 minutes
- Then wash and slap the wells to remove excess of buffer.
- Add two drops of chromogen to each well and incubate for 10 minutes.

- Add two drops of stop solution to each well and mix the wells by tapping to side to side.
- Read the reaction after adding stop solution and read the results within 5 minutes visually or ELISA plate reader. Microwells containing the anti-cryptosporidium antibodies.
- Enzyme conjugate used diluted cryptosporidium antibodies conjugated to horseradish peroxidase Kit used is DRG International, USA.(EIA-3467)

Result: Total 110 samples were subjected to ELISA, 105 samples were found positive

RESULTS

This study was conducted on a total of 110 stool specimens obtained from HIV infected patients admitted to RIMS Hospital, Raichur.

Study groups Out of 110 cases studied 65 patients presented with diarrhoea and the remaining 45 were without diarrhoea.

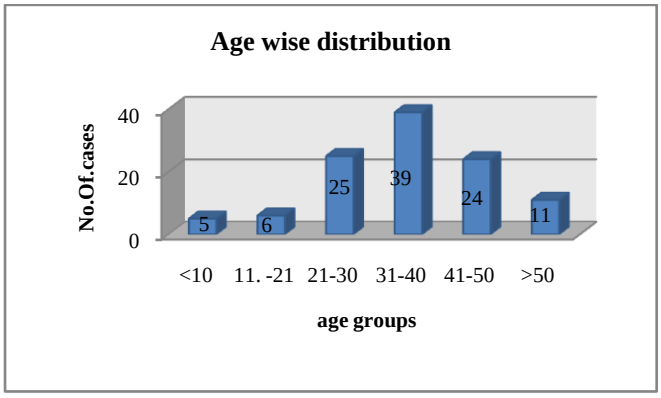
Table 1 Study groups

Study group	Description	No. of individual
I	HIV seropositive /AIDS patients with diarrhoea	65 (59%)
II	HIV seropositive /AIDS patients without diarrhoea	45 (41%)
Total	-	110 (100%)

Diarrhoeal vs. Non diarrhoeal is $\chi 2 = 3$, P- value

Age wise distribution of study individuals

Maximum number of cases were in age group of 31-40 years.Least number of cases was found in the age groups 0-10 years & 11-20 years. Mean number of cases in the study is 34.45 in age group 31-40 years



Sex distribution in the study group

Out of 110 cases Of HIV/AIDS studied the male predominance was observed 61(55%) followed by female population 49 (45%).Male to Female ratio is 10:6.

DISCUSSION

Incidence

The present study depicts a high incidence of cryptosporidiosis i.e. 95.4% amongst HIV sero-positive / AIDS cases in Raichur District of Karnataka. One of the studies by A. Singh from Manipalincidence of cryptosporidiosis was 42.9% quite lower than our study[8] and another study by Anand from Manipur[9] it was 94.4% which is equal to my study. Globally the incidence rate of cryptosporidiosis is 60.3%[10].

Age and sex distribution

In the present study, amongst 110 HIV sero-positive / AIDS cases, 61(55%) were males and 49 (45%) were females. Male predominance was observed (Male to Female ratio was 10:6. These findings are in accordance with the reports of Darji et al (2013) at Gujarat (males-61% and females-39%), Paudyal et al (2013) at Kathmandu (males-66% and females-34%)and A.Singh et al (2003) at Manipal(males-97% and females-3%) [118,,12,,]Males are more prone to develop HIV infection as compared to females due to increased mobility, work-related issues and similar findings have been reported in other studies conducted by S.V. Kulkarin et al13 and S. Gupta etal[14] .

Detection of cryptosporidiosis by various methods Detection of cryptosporidium oocysts

Microscopic technique There is an increasing demand for diagnostic testing of cryptosporidium parvum, with a priority being placed on obtaining diagnostic results in an efficient and timely manner. Cryptosporidiosis is diagnosed by microscopic techniques by demonstrating cryptosporidium oocysts in stool samples. Oocysts size ranges from 4.5- 5 microns and shape is spherical or slightly oval[15] .

In the present study, positivity of cryptosporidial infection by Modified Ziehl- Neelsen staining was 77.3% which is closer to the results of two studiesi.eDarji's et al at Gujarat(74%), Ibrahim R AlyShalash et al at Egypt (72.2%) and results of two other studies were lower than our study i.eAmadi et al at Zambia (50%) and Blanco et al at Guniea (56.6%)[15,16,17, 18] . SoumendraNathMaity et al from Hyderabad found highest incidence (100%) amongst all studies[19]Demonstration of characteristic oocysts with Modified ZiehlNeelsen stain is the common method to detect cryptosporidiosis. Howeverthis method is laborious and less sensitive (Bialeketal 2002)[20].
Oocysts of cryptosporidium are very small and can easily be missed in faecal samples as artifacts. They can also be easily confused with other oocysts. This staining method does not distinguish oocysts of cryptosporidial species. Also the red and pink stains taken by stool debris add to the confusion. It is a disadvantage as compared to other methods. The advantage of this method is that it isinexpensive and adoptable method, for this reason all underdeveloped and developing countries still depend on this method (Connelly et al)[21].

Detection of Cryptosporidium parvum by various methods

Method	Total cases	Positive	%	P< 0.005	Sensitivity	Specificity
Modified ZN acid fast staining	110	85	77.3	P<0.0001**	94%	100%
ELISA	110	105	95.4	P<0.0001**	98%	100%
Immunoflourescent Microscopy	110	102	92.6	P<0.0001**	96.2%	100%

CONCLUSION

- This study enhanced the awareness of cryptosporidiosis among HIV sero-positive / AIDS patients in Raichur District of Karnataka.
- Our study highlights the importance of routine screening for cryptosporidiosis in all HIV sero-positive/AIDS patients, irrespective of gastrointestinal symptoms.
- ELISA can be considered the most reliable method for the detection of cryptosporidial oocysts in fecal specimens.

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