Cellular immune response to *Cryptosporidium parvum* in *Cryptosporidium*-HIV co-infected patients

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Introduction

AIDS - global emergency with far reaching effects

- World

  40 million had HIV infection, December 2006

  (UNAIDS/WHO, 2006)

- India
  - 5.3 million infected in 2005
  - 124995 had reached stage of AIDS - August, 2006

  (NACO, 2006)

- Gastrointestinal tract - major target organ in HIV related opportunistic infections
- *Cryptosporidium* - important enteric pathogen in AIDS & other immunosuppressed patients
- Tops the list out of parasitic infections in HIV patients in India
- Small protozoan parasite, infects digestive tract of a wide range of vertebrate hosts
- First human case – 1976
- Route of infection – Faeco-oral
- Infectious agent – Oocyst (4-6 µm with 4 sporozoites)

- Clinical manifestations – Asymptomiatic to profuse watery diarrhoea
  - Immunocompetent - 3 to 12 days, resolves spontaneoulsy
  - Immunocompromised - >12 weeks, severe, unremitting diarhhoea, refractory to treatment
- Host immune responses
  - prevent initial infection
  - limit its spread
  - facilitate its clearance
- Poorly understood but probably include both B and T lymphocytes mediated processes

CMI - Pathogenesis as well as protection

- Susceptability increases with decreasing CD_4 cell counts
- CD_4 < 140 cells/µl – persistent disease
- Most studies – Animal models
- Differences in clinical manifestations of *Cryptosporidium* in patients with or without HIV
  - Immunocompetent – Self limiting
  - Immunocompromised – Life threatening

Aim & Objectives

The aim of the study was to assess lymphocyte proliferation response to *Cryptosporidium parvum* antigen in HIV/AIDS patients

Objectives

- Detection of *Cryptosporidium parvum* in fecal samples by Ziehl-Neelsen staining, rapid safranine methylene blue staining technique, antigen detection ELISA & PCR in HIV seropositive, HIV seronegative patients with diarrhoea & healthy control subjects
- Assessment of lympho-proliferative response to crude soluble antigen of *Cryptosporidium parvum* in HIV/AIDS patients with cryptosporidiosis & controls

Materials & Methods

Patients & control subjects

Screening (Microscopy, antigen detection ELISA, PCR)

Group A. 200 HIV seropositive

Group B1. 150 HIV seronegative, with history of diarrhoea

Group B2. 50 HIV seronegative, without any history suggestive of cryptosporidiosis

Cellular immune response

Group 1. 11 HIV seropositive *Cryptosporidium* positive*

Group 2. 20 HIV seropositive *Cryptosporidium* negative**

Group 3. 10 HIV seronegative *Cryptosporidium* positive*

- 3A. 4 Post-renal transplantation
- 3B. 6 Immuno-competent

Group 4. 20 HIV seronegative *Cryptosporidium* negative (healthy controls)**

*Positive for *Cryptosporidium* by either Ziehl-Neelsen staining technique or by both antigen detection and PCR

**Negative for *Cryptosporidium* by all the techniques

Examination of stool samples

A. Ziehl-Neelsen

B. Rapid safranine methylene blue

B. Antigen detection ELISA (RIDASCREEN, r-Biofarm, Germany)

C. Nested PCR

DNA extraction – QIAamp Stool Mini Kit (Qiagen)

Cryptosporidium parvum DNA (gift from Dr. Striepen, University of Georgia)

Primary PCR (*Cryptosporidium*)

CF201 – 5'-GGGTTGTATTTATTAGATAAAGAAC-3'

CR201 – 5'-CTTTAAGCACTCTAATTTTCTC-3'

Secondary PCR (*Cryptosporidium parvum*)

CPF 202 – 5'-GACTTTTTGGTTTTGTAATTGGAATG-3'

CPR 202 – 5'-TAAATTATTAACAGAAATCCAACTACGAGC-3'

Gold standard - Either microscopy positive or in face of its negativity positive antigen detection along with PCR

Sensitivity (S)

[Number of true positives/ (Number of true positives + Number of false negatives)] x 100

Specificity (Sp)

[Number of true negatives/ (Number of true negatives + Number of false positives)] x 100

Positive predictive value (PPV)

[Number of true positives/ (Number of true positives + Number of false positives)] x 100

Negative predictive value (NPV)

[Number of true negatives/ (Number of true negatives + Number of false negatives)] x 100

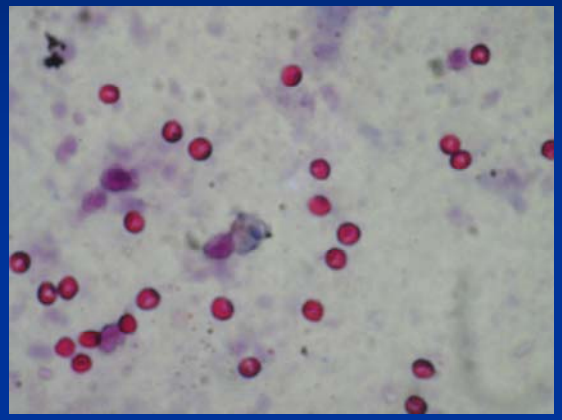
Diagnostic efficacy (DE)

[(Number of true positives + Number of true negatives) / (Number of true positives + Number of true negatives + Number of false positives + Number of false negatives)] x 100

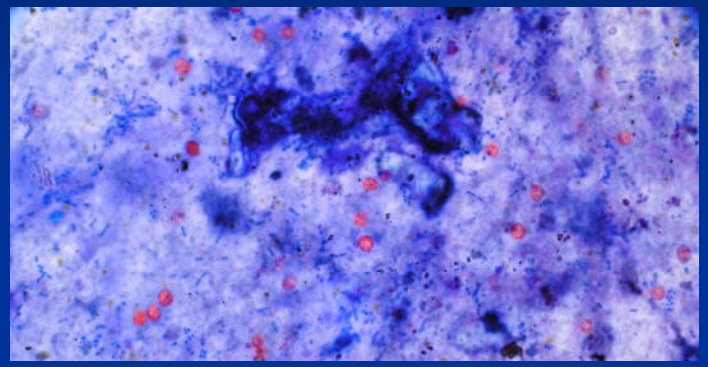
Table 1: Demographic profile of the individuals enrolled in the study

Groups (N)	Mean Age in years (range)	M	F	With diarrhoea	Post transplant patients	CD_4 counts (cells/µl)
A. HIV(206)	33.2 (21 - 67)	140 (68%)	66 (32%)	99 (48%)	Nil	275(2-583)
B1. Non HIV With diarrhoea (153)	28.1 (1.5 - 65)	101 (66%)	52 (34%)	153 (100%)	23 (15%)	-
B2. Healthy control (50)	27.4 (23-36)	25 (50%)	25 (50%)	Nil	Nil	-
Total (409)	30.6 (1.5 to 67)	266 (65%)	143 (35%)	252 (62%)	23 (5.6%)	-

Results



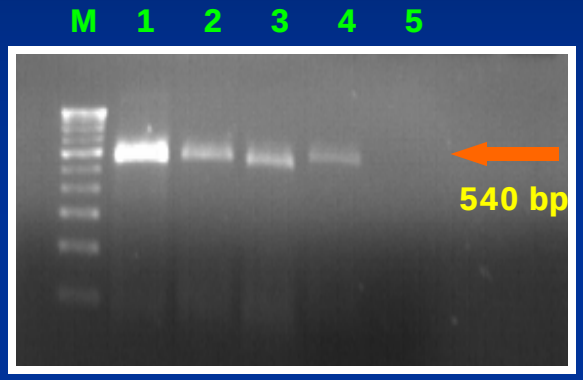
Cryptosporidium
(Ziehl -Neelsen staining)



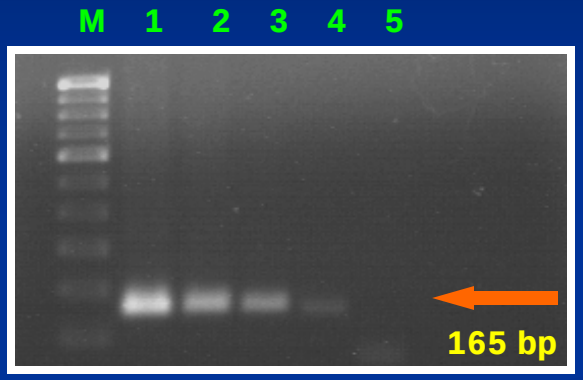
Cryptosporidium **(Rapid safranine methylene blue staining)**

Results

Primary PCR



Secondary PCR



Lane M	= Molecular weight marker(100 bp)
Lane 1	= Positive control
Lane 2,3,4	= DNA samples
Lane 5	= Negative control

Results

Table2: No of patients positive for *Cryptosporidium* by one or more techniques

	Groups	N	No of positives (%)
A	HIV seropositive	206	41(20)
B1	HIV seronegative with diarrhoea	153	22 (14.4)
B2	Healthy controls	50	Nil
	Total	409	63 (15.4)

Results

Table 3: Comparative analysis of Ziehl-Neelsen staining, rapid safranine methylene blue staining, antigen detection ELISA & PCR for detection of *Cryptosporidium parvum*

A. HIV seropositive patients (n=206)

Techniques	Positive samples (%)	S (%)	Sp (%)	PPV (%)	NPV (%)	DE (%)
ZN	10 (5%)	36	100	100	91	91
RSM	09 (4.4%)	33.3	100	100	91	91
Ag detection ELISA	39 (19%)	93	92	64	99	92
PCR	27 (13%)	100	100	100	100	100

B. HIV seronegative patients with diarrhoea (n=153)

Techniques	Positive samples (%)	S (%)	Sp (%)	PPV (%)	NPV (%)	DE (%)
ZN	07 (4.6%)	41	100	100	93	93.5
RSM	06 (4%)	35	100	100	92.5	93
Ag detection ELISA	21 (14 %)	94	96	76	96	96
PCR	17 (11%)	100	100	100	100	100

Conclusions

- *Cryptosporidium parvum* was detected in 20 % & 14 % in HIV seropositive & HIV seronegative patients, respectively
- Sensitivity

  PCR > Ag detection ELISA > Microscopy – for detection of *Cryptosporidium* in HIV seropositive and HIV seronegative patients
- This observation is in agreement with reports from London [Pedraza-Diaz et al, 2001, Mc Lauchlin et al, 1999] and New York [Zhu et al, 1998] and in disagreement with report from California [Mayer and Palmer, 1996] whereby low sensitivity of PCR reported

Objective 2

Cellular immune response

Materials and Methods

Preparation of *Cryptosporidium parvum* crude soluble antigen

Cryptosporidium parvum oocysts (Iowa strain, NIH AIDS research and reference reagent program), freeze-thawed, sonicated, protein estimation by Lowry's method

Lymphocyte Proliferation Assay

PBMCs separated & cultured in RPMI-1640 medium (supplemented with 10% FCS & antibiotics) in presence of *C. parvum* crude antigen (2 µg / ml) & PHA (5 µg / ml) at 37^0C in 10% CO_2

↓

1µCi (^{3}H) thymidine added after 48 hrs, followed by overnight incubation

↓

Pellet washed twice with normal saline

↓

Addition of 1 ml TCA (10%) followed by incubation at 37^0C for 2 hrs

↓

Pellet washed with methanol, kept for drying overnight,

cells harvested with help of solubilizer & placed in scintillation fluid & counted on Scintillation counter

Counting of cells

SI (Stimulation index) = Counts per minute in stimulated culture/counts per minute in un-stimulated culture

Stimulation Index

PHA >20	Significant
CCA >2	Significant

Comparison of mean CPM and SI – Mann-Whitney test

Results

Table 4: Demographic profile of the individuals studied for LPA

Groups (N)	Mean Age in years (range)	M	F	With diarrhoea	Post transplant patients	CD4 counts (cells / µl)
I (11) HIV + Crypto +	34.1 (25-46)	7	11	5	Nil	182.5 (46-379)
I (20) HIV + Crypto -	34.2 (25-64)	15	05	9	Nil	198.6 (30-583)
III (10) HIV - Crypto +	26.2 (3.5-46)	6	4	10	4	-
IV (20) HIV - Crypto - (normal healthy)	26.9 (23-35)	10	10	Nil	Nil	-
Total (61)	30.5 (3.5-64)	38	30	24	4	-

Results

Table 5: No. of subjects with significant proliferation in response to *Cryptosporidium* (CCA) and PHA

Groups	N	Number of subjects with significant proliferation	
		CCA(SI>2)	PHA(SI>20)
1 ($HIV^+Crypto^+$)	11	9 (82%)	3 (27%)
2 ($HIV^+Crypto^-$)	20	3 (15%)	11 (55%)
3 ($HIV^-Crypto^+$)	10	10 (100%)	3 (30%)
4 ($HIV^-Crypto^-$)	20	4 (20%)	20 (100%)
Total	61	26 (42.6%)	37 (61%)

Conclusions

SI in response to CCA (*Cryptosporidium*)

- Significant response was found in more no. of *Cryptosporidium* infected [Gp I & III] as compared to *Cryptosporidium* un-infected [Gp II & IV] individuals ($p<0.05$)
- Significant response was found in more no. of HIV seronegative *Cryptosporidium* positive as compared to HIV seropositive *Cryptosporidium* positive patients, however the difference is not statistically significant ($p>0.05$)

Conclusions

SI in response to PHA

- Significant response was found in more no. of normal healthy individuals as compared to other groups ($p<0.05$)
- Significant response was found in more no. of HIV seronegative *Cryptosporidium* positive as compared to HIV seropositive *Cryptosporidium* positive patients, however the difference is not statistically significant ($p>0.05$)

Table 6: (^{3}H) Thymidine incorporation (stimulation index) following culture of lymphocytes stimulated with PHA & *C. parvum* crude soluble antigen (CCA)

Groups	PHA	CCA	Control
	SI (SD)	SI (SD)	SI
I (n=11)	18.0 (8.8)	4.4 (3.8)	1
II (n=20)	18.3 (9.9)	1.4 (0.6)	1
III (n=10)	16.3 (4.4)	6.6 (3.7)	1
IIIA (n=04)	12.8 (2.5)	3.1(0.5)	1
IIIB (n=06)	18.6 (3.8)	9 (2.8)	1
IV (n=20)	25.1 (4.0)	1.4 (0.5)	1
p I Vs II	NS	<0.001	NS
p I Vs III	NS	NS	NS
P I Vs IIIA	NS	NS	NS
P I Vs IIIB	NS	0.03	NS
P IIIA Vs IIIB	0.03	0.01	NS
p I Vs IV	0.001	0.002	NS
p II Vs III	NS	<0.0001	NS
P II Vs IIIA	NS	0.003	NS
P II Vs IIIB	NS	<0.0001	NS
p II Vs IV	<0.05	NS	NS
p III Vs IV	<0.0001	<0.0001	NS
p IIIA Vs IV	0.002	0.022	NS
p IIIB Vs IV	0.003	<0.0001	NS

I = HIV seropositive *Cryptosporidium* positive, II = HIV seropositive *Cryptosporidium* negative
III = HIV seronegative *Cryptosporidium* positive, IV = HIV seronegative *Cryptosporidium* negative

Conclusions

Proliferation in response to specific antigen (CCA)

- No significant difference was observed in mean SI observed in HIV seropositive *Cryptosporidium* positive (Gp I) as compared to HIV seronegative *Cryptosporidium* positive (Gp III) patients
- On further analysis of Gp III, mean SI was found significantly lower in HIV seropositive *Cryptosporidium* positive (Gp I, p=0.03) and HIV seronegative *Cryptosporidium* positive (Gp III A, p=0.01) patients who had renal transplantation when compared to HIV seronegative *Cryptosporidium* positive immunocompetent patients (Gp III B)
- This observation is in agreement with the only earlier one report available from Italy [Morales et al, 1999] which showed that proliferation in response to *Cryptosporidium* was significantly different in HIV seropositve and seronegative patients infected with *Cryptosporidium*
- Mean SI significantly higher in *Cryptosporidium* infected (Gp I & III) when compared to *Cryptosporidium* un-infected (Gp II & IV) individuals (p=0.001)

Conclusions

Proliferation in response to non-specific antigen (PHA)

- Mean SI significantly higher in normal healthy (Gp IV) when compared to other (Gp I, II & III) individuals (p=0.01)
- No significant difference in mean SI observed in HIV seropositive *Cryptosporidium* positive (Gp I) as compared to HIV seronegative *Cryptosporidium* positive (Gp III) patients (p>0.05)

Summary

- The study suggests that *Cryptosporidium parvum* induces significant *in-vitro* lympho-proliferative response in sensitized HIV seropositive and HIV seronegative individuals
- Proliferation was significantly higher in *Cryptosporidium* infected, immunocompetent patients when compared to *Cryptosporidium* infected, immunocompromised patients (post-transplantation and HIV seropositive).
- The study suggests that immune status of the host does appear to play significant role in modulating proliferative responses to *Cryptosporidium* antigen. However, more studies in this regard are desired to confirm the findings.

Acknowledgement

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Thank You