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Research Paper

Spectrum of Infection in Patients with Inflammatory Bowel Disease

MICROBIOLOGY

KEYWORDS : Inflammatory Bowel Disease, Prevalence, Northern India, ELISA, Opportunistic pathogens.

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ABSTRACT

Inflammatory bowel disease (IBD) is common chronic gastrointestinal disorder characterized by various forms of chronic mucosal and trans-mural inflammation of intestine. IBD comprises of two chronic diseases that produce inflammation of the intestine: ulcerative colitis and Crohn's disease. Development of IBD depends on the response to microbial flora which can be dived into two categories: macro pathogens (parasites, helminthes) and micro pathogens (bacteria, viruses).The study was done to determine the spectrum of infection in patients with inflammatory bowel disease.117 stool sample from IBD patients and 101 samples from healthy patients were collected. Macroscopic observation was done followed by microscopy along with culture methods, antigen detection of E. histolytica through ELISA and C. difficile toxin A and b detection by ELISA. Prevalence was high among patients between 30-39 years of age (34%). Average age for onset for the disease was 35.3 years. Overall positivity of microorganisms combining all diagnostic methods was 9 (18%).

INTRODUCTION

Inflammatory bowel disease (IBD) is common chronic gastrointestinal disorder. IBD is heterogeneous group of disorder characterized by various forms of chronic mucosal and trans-mural inflammation of intestine. IBD comprises of two chronic diseases that produce inflammation of the intestine: ulcerative colitis and Crohn's disease. Although they have some common features there are distinct differences between them, both clinical and pathological. IBD is the most common chronic gastrointestinal illness in children and adolescents. Majority persons with IBD have onset of their illness before adulthood.

Current evidence suggests that the patients have genetic defect which modulate the inflammatory outcome in response to offending agents like bacteria, virus or protein in food[1]. Up-regulated immune responsiveness to the intestinal micro biota or specific members there-of may be the central event in the pathogenesis of IBD, whetherclassified as chronic disease or ulcerative colitis. Now it is apparent that infectious agents have major role in etiology and pathogenesis of IBD[2]. They can initiate the onset and relapse of this condition.

Development of IBD depends on the response to microbial flora which can be divided into two categories: macro pathogens (parasites, helminthes) and micro pathogens (bacteria, viruses). Relapse of IBD has also been linked to various bacteria like *Clostridium difficile*, Enterohemorrhagic *Escherichia coli (E.coli)*, Salmonella, Shigella and Campylobacter[3]. *E.coli* strains are among the bacteria of luminal flora suspected of being involved in pathogenesis of IBD. It has been shown that *E.coli* antibody titer is higher in patients with IBD than in controls.

Various parasites like blastocystis, Schistosoma are known to be associated with this condition. In immunodeficient mice, Cryptosporidium was found to be initiating IBD[4]. There has been case reports of IBD associated with Schistosoma mansoni infections[6]. Various parasites like *Strongyloids stercoralis, Anchylostoma duodenale, Cryptosporidium and Entamoeba histolytica* were found in these patients[7].

In developing countries like India, majority of hospitalizations is due to infectious causes. In the light of increasing hospital infections and immune suppressive drugs, the relevance of opportunistic pathogen cannot be ruled out. Therefore this study was planned with an intention to further throw light on this aspect.

MATERIALS AND METHODS

Place of work: This prospective study was carried out in parasitology laboratory of Microbiology in association with the department of Gastroenterology, SGPGIMS, Lucknow, a tertiary care center.

Study period: Stool samples from patients with IBD werecollected during a period of April 2010 to May 2012. Attempt was made to collect 3 consecutive samples from each patient. 117 stool samples from IBD patients were collected. 101 stool samples were collected from healthy controls for comparison. Control population included healthy persons without any gastrointestinal symptoms.

Sample collection: 3 consecutive stool samples from each patient were collected in a clean wide mouthed plastic container avoiding contamination with urine, water disinfectants. These samples were transported to laboratory as soon as possible. A proforma containing details about patients and results were maintained.

Processing: Sample processing was done using following methods:

1. Macroscopic examination:

2. Microscopy[10]:

i. Direct wet smear
ii. Concentration method
iii. Kinyoun and Modified trichrome staining [opportunistic paasitic pathogen].
iv. Calcoflour white staining

3. Culture Methods [10]:

i. Aerobic
ii. Anaerobic (for *C.difficile*)
iii. Microaerophilic (for *Campylobacter spp.*)

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4. Antigen detection by ELISA for *E histolytica*
5. *C. difficile* toxin A and B detection by ELISA

1. Macroscopic Examination:
Stool samples were examined to look for mucus, blood,consistency, texture, presence of whole worm or presence of segments of parasites.

2. Microscopic Examinations:

i. *Direct Wet Mount:*The microscopic examination of stool was carried-out with normal saline as unstained preparation and also with Iodine solution as stained preparation. The unstained preparation was performed for the demonstration of actively motile forms (trophozoites). While, the stained-preparation was carried-out for the demonstration of cysts/ova (infective forms) or dead specimens of trophozoites. Besides these, the stained-preparation was also carried-out for the study of nuclear-characters and glycogen-mass (food-storage sites).

(b) *Concentration Wet Mount:* Formal EtherConcentration Method (Modified from Allen & Ridley, 1970)-Wet Mount, one of the most widely used. It is probably the most useful and convenient of all the concentration procedures, as it will concentrate most of the cysts and ova found in faeces.

*(c) Staining Methods:*The staining procedures used for the diagnosis of opportunistic parasitic pathogens were:

I. Modified Kinyoun's Acid-Fast staining
II. Modified Trichrome staining
III. Calcofluor-White Fluorescent staining

3. Culture-Methods:

(a) *Aerobic-Culture Methods:*To perform aerobic-culture for the selective isolation of any pathogenic aerobic bacteria in the stool-sample,the stool/fecal sample was cultured on plates by Streak-Plate method on three plates:Mac Conkey Agar, DCA (Deoxycholate Citrate Agar), DSRA (D-Sorbitol Rhamanose Agar). Sample was also put in GN (Gram-Negative) Broth for enrichment.Fecal-sample was cultured by Streak-Plate culture (Suface-Plating) method that was routinely employed for the isolation of bacteria in the pure-culture.

(b) *Anaerobic-Culture Method: A*naerobic culture for the selective isolation of *Clostridium difficile* was performed using Cefoxitin Cycloserine fructose agar [CCFA].

(c) *Microaerophilic Culture:*Microaerophilic culture for the selective isolation of *Campylobacter* spp., was performed using Charcoal Cefoperazone Deoxycholate Agar [CCDA].

4. ELISA for Detection of *Entamoeba histolytica* Antigen[11]:
Principle: It uses antibodies as adhesin. The micro-assay wells contain immobilized polyclonal antibody that binds adhesin of *E.histolytica.* During the first incubation, *E. histolytica* antigens present in the stool supernatant are captured by antibodies attached to the wells. In second incubation monoclonal antibody-peroxidase specific for *E.histolytica* adhesion were added. After washings to remove unbound enzyme, a chromogen is added which develops a blue color in presence of enzyme-complex and peroxide. The stop solution ends the reaction and turns the blue color to yellow.

Reagents used:
Test-strips: Micro wells containing anti-*E.histolytica* polyclonal antibodies; 96 test wells in a test strips holder.

Diluent (buffered protein)

*Conjugate :*one bottle containing mouse monoclonal antibody-specific for adhesion coupled to horseradish peroxidase

Positive control: One vial containing 2ml diluted E. histolytica antigen in buffer..

Substrate : One bottle containing Tetramethyl Benzidine (TMB) and peroxide.

Wash concentrate (20X): Two bottles containing 25ml concentrated buffer and surfactant with Thimerosal.

Stop solution: One bottle containing sulfuric acid

Preparation of Wash buffer:
To make Wash buffer, firstly the cap was removed and the wash concentrate was added to 475ml of distilled water and mixed well by inversion shaking.

Preparation of fresh/frozen stool:
The sample was thawed, if needed. Then sufficient diluted Wash buffer was added to make approximately a 1:4dilution (1gm or a pea-sized fecal sample to 3 ml diluted Wash buffer) and mixed well.

Test Procedure:
- Firstly, the required numbers of wells were broken-off (number of samples plus 2 for the controls) and placed in the holder.
- 50 µl conjugate was added in each well.
- 2 drops (approximately 100µl) of negative control was added to the well (1) and 2 drops positive control was added to the well (2).
- 2 drops stool supernatant was added to each test well.
- Incubated at room temperature (15-25°C) for 2 hours and then washed well.
- 2 drops of substrate was added to each well.
- Then incubated for 10 minutes and then washed again.
- Then 1 drop of Stop solution was added to each well.
- Mixed well by gently tapping the sides of the strips holder with index finger.
- Finally the result was read visuallyor at 450/620-650 nm.

Interpretation: (By visually)
Reactive: Any sample that is more yellow than negative control well.

Non-reactive: Any sample that is not more yellow than the negative control well.

Interpretation: (By ELISA Reader)
*(All the wells were read at 450/620-*650 nm)

Reactive: Absorbance reading of 0.500 OD units and above indicates the sample contains E. histolytica antigen.

Non-reactive: Absorbance reading < 0.5 OD units indicates that the sample does not contain detectable levels of E. histolytica antigen.

Validity of Procedure:
For a valid test, the positive control must have an absorbance of at least 0.500 OD units and the negative control must be <0.150 OD units.

5. ELISA for Detection of *Clostridium difficile*Antigen Or Toxin 'A' &'B' [12]:
Principle: During the first incubation, *Clostridium difficile* antigens present in the stool supernatant are captured by antibodies attached to the wells. In second incubation anti- *Clostridium difficile* antibody conjugated to peroxidase was added. After washings to remove unbound enzyme, a chromogen is added which develops a blue color in presence of enzyme-complex and perox-

ide. The stop solution ends the reaction and turns the blue color to yellow.

Reagents Used:
Wash buffer: 20X
Dilution buffer: 1X
Positive control: 2 drops/100µl
Negative control: 2 drops/100µl
Test sample: 2 drops/100µl

Test Procedure:
- Wells for positive control (PC), negative control (NC) and specimens were taken.
- 100 µl of diluted stool was added to appropriate well.
- 2 drops PC and NC were added to the respective wells.
- 1 drop of Enzyme-Conjugate was added to wells.
- Plate sealer was cut to size and pressed firmly onto wells to seal. Incubate the plate for 50min at 35-39°C.
- Wells were washed 4-6 times.
- 2 drops of substrate (100µl) were added to each well.
- Stop solution was added
- Finally the result was read visually or at 450/620-650 nm.

Interpretation: (By visually)
Reactive: Any sample that is more yellow than negative control well.

Non-reactive: Any sample that is not more yellow than the negative control well.

Interpretation: (By ELISA Reader)
(All the wells were read at 450/620-650 nm)

Interpretation:
Positive: OD>0.15
Negative: OD<0.15
Positive Control: OD<2.999
Negative Control: OD<0.15

Limitation of Procedure:
For a valid test, the positive control must have an absorbance of at least 0.5 OD units and the negative control must be <0.15 OD units.

RESULTS
The present study was carried out in the Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), a tertiaryhealth care center at Lucknow. 50 patients of inflammatory bowel disease (IBD) were included in the study. A total number of 117 stool samples were collected from IBD patients. In addition 101 stool samples from 50 healthy controls were also collected for comparison.

IBD PATIENTS
Distribution of cases in relation to age: The patients were divided according to different age groups. Number of patients in different age groups were 1 (2%), 2 (4%), 11 (22%), 18 (36%), 4 (8%), 11 (22%), 3(6%), 0(0%) in 0-9, 10-19, 20-29, 30-39, 40-49, 50-59, 60-69, 70-79 age groups respectively. The mean age of the patients were found to be 38.2 years.

Table 1: age wise distribution of IBD patients (n=50)

S.NO	AGE IN YEARS	NUMBER (%)
1	0-9	1(2%)
2	10-19	2 (4%)
3	20-29	11 (22%)
4	30-39	18 (36%)
5	40-49	4 (8%)
6	50-59	11 (22%)
7	60-69	3 (6%)
8	70-79	0 (0%)

- **Sex-wise distribution of patients : Out of 50 patients 33 (66%) were males and 17(34%) were females**

Table 2: sex-wise distribution IBD (n=50)

S. NO	AGE (IN YEARS)	MALE (%)	FEMALE (%)
1	0-9	0 (0%)	1 (2%)
2	10-19	1 (2%)	1 (2%)
3	20-29	8 (16%)	4 (8%)
4	30-39	14 (28%)	3 (6%)
5	40-49	2 (4%)	2 (4%)
6	50-59	5 (10%)	6 (12%)
7	60-69	3 (6%)	0 (0%)
8	70-79	0 (0%)	0 (0%)
	Total	33 (66%)	17 (34%)

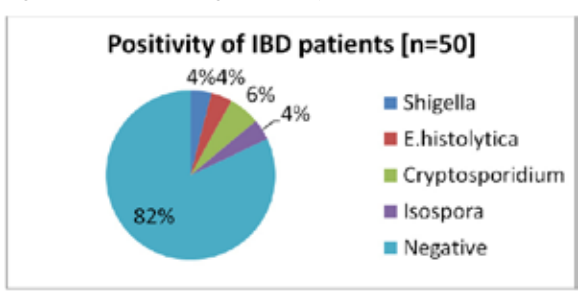
- Characteristics of IBD patients: The mean age of the patients was 38.2 years. Age range was from 8 years to 68 years. The mean age of onset of disease was 35.2 years. The range of age of onset was from 7 years to 62 years.The mean of duration of disease was 3.5 years. The range of duration of disease was 1 year to 6 years. The different types of involvement was proctosigmoiditis 14 (28%), Left-sided colitis 15(30%) and pancolitis 21(42%). 19(38%) patients showed active disease and 31(62%) showed remission of disease.

Table 3: Characteristics of patients (IBD)

VARIABLE	MEAN	RANGE
Age (years)	38.2	8-68
Age at onset(years)	35.3	7-62
Duration of disease(years)	3.5	1-6
Sex(male/female)	33(66%)	17(34%)
Proctosigmoiditis	14(28%)	
Left-sided colitis	15(30%)	
Pancolitis	21(42%)	
Active	19(38%)	
Remission	31(76%)	

- **Positivity of microorganisms in IBD patients:** Overall positivity of microorganisms combining all diagnostic methods was 9 (18%). Different microorganisms found were: Cryptosporidium 3 (6%), Isospora 2 (4%). *Entamoeba histolytica* found were 2 (4%) in number. *Shigella flexneri* was isolated in culture from 2 patients (4%). Antigen ELISA for *Entamoeba histolytica* was positive in 2 patients.

Fig 1: Chart showing positivity of IBD patients (n=50)



- **Comparison of direct microscopy and concentration method:** All samples were first examined by direct microscopy and after concentration method. 3 samples were positive for *E.histolytica* in direct microscopy. Total positivity bydirect microscopy was 3(6%). All samples which were positive in direct microscopy were also positive in microscopy after concentration. After concentration 3 samples were positive for *Entamoebahistolytica* which were not seen in direct microscopy. Total positivity after concentration was 6 (5%). 4 samples were positive for Cryptosporidium. 2 samples were positive for Isopsora. 1 sample

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was positive for both Cryptosporidium and Isospora in Kinyoun's staining and calcoflour white staining.

Table 4: Comparison of direct microscopy and microscopy after concentration

Microscopy	Direct (n=117)		After concentration (n=117)			
	NS	Iodine	NS	Iodine	K	CW
Positive	Entamoeba (3)	Entamoeba (3)	Entamoeba (6)	Entamoeba (6)	Crypto (4) + Isospora(2) +Crypto and Isospora (1)	Crypto (4) + Isospora(2) +Crypto and Isospora (1)
Total	3 (2%)	3 (2%)	6 (5%)	6 (5%)	7 (6%)	7 (6%)
Negative	114 (98%)	114 (98%)	111 (95%)	111 (95%)	110 (94%)	110 (94%)

- **Comparison of direct microscopy and antigen detection for *Entamoeba histolytica* in IBD patients:** 1 *Entamoeba histolytica* wasdetected in direct microscopy. 2 were positive for antigen ELISA for *E.histolytica*.

Table 5: Comparison of ELISA for antigen and direct microscopy

ELISA FOR ANTIGEN	DIRECT MICROSCOPY (N=117)	
	POSITIVE	NEGATIVE
Positive	1	1
Negative	0	115

HEALTHY CONTROLS

- **Distribution of cases in relation to age:** The controlswere divided according to different age groups. Number of patients in different age groups were 0 (0%), 2(4%),6(12%), 30(60%),12(24%),0(0%),0(0%), 0(0%) in 0-9,10-19,20-29,30-39,40-49,50-59,60-69,70-79 age groups respectively.

Table 6: age wise distribution of healthy controls (n=50)

AGE IN YEARS	NUMBER (%)
0-9	0 (0%)
10-19	2 (4%)
20-29	6 (12%)
30-39	30 (60%)
40-49	12 (24%)
50-59	0 (0%)
60-69	0 (0%)
70-79	0 (0%)
TOTAL	50

- **Sex-wise distribution of controls:** Total of 50 controlswas taken. Out of this 48(96%) were males and 2(4%) were females.

Table 7: sex-wise distribution (n=50)

S. NO	AGE (IN YEARS)	MALE (%)	FEMALE (%)
1	0-9	0	0
2	10-19	0	2(4%)
3	20-29	6(12%)	0
4	30-39	30(60%)	0
5	40-49	12(24%)	0
6	50-59	0(0%)	0
7	60-69	0(0%)	0
8	70-79	0(0%)	0
	TOTAL	48(96%)	2(4%)

- **Positivity of microorganisms in healthy controls:** Giardia was found in 2(4%) of patients.

- **Comparison of direct microscopy and concentration method:** 5(5%) samples were positive for Giardia in direct microscopy. Same 5 (5%) samples were positive after concentration

Table 8: Comparison of direct microscopy and microscopy after concentration

Microscopy	Direct (n=101)		After concentration(n=101)				
	NS	Iodine	NS	Iodine	K	Au	KW
Positive	Giardia (5)	Giardia (5)	Giardia (5)	Giardia (5)	0(0%)	0(0%)	0(0%)
Total	5(5%)	5(5%)	5(5%)	5(5%)	0(0%)	0(0%)	0(0%)
Negative	96(95%)	96(95%)	96(95%)	96(95%)	0(0%)	0(0%)	0(0%)

Table 9: Comparison of positivity of IBD and control

POSITIVITY	IBD(n=50)	CONTROL(n=50)
E.histolytica	2	0
H.nana	0	0
Shigella	2	0
Cryptosporidium	3	0
Isospora	2	0
Cyclospora	0	0
Giardia	0	2

DISCUSSION

IBD is generally a disease of youngpeople because it most commonly develops between the ages of 10 to 30 years[9]. However, a second smaller peak of developing disease is seen between the age-group of 50-60 years. Similar findings were seen in our study. Our study shows that the patients in the age-group between 20-40 years were highly affected (58%). A second peak was seen in 50-60 years (22%). Mean age of IBD patients was found to be 38.2 years. This was similar to findings of Banerjee D et al[7] where mean age of the patients was found to be 35.8 years and Sood A et al [8] where the mean age was 31 years. A Malaysian study also reported similar findings (mean age 31 years)[13].

In our study of 50 IBD patients, 33(66%) were males and 17(34%) were females. So the ratio was approximately 2:1. A similar finding of male preponderance has been quoted by many workers. They found male and female ratio of 2:1[7,14]. Sood A et al[8] reported the ratio to be 1.04:1. Still the males were affected slightly more.

Inour study31 (62%) of patients showed remission of the disease. 19(38%) of patients showed active disease. These findings are in accordance to the literature that showed patients of IBD typically show chronic, relapsing –remitting course. Pancolitis is the dominant type of involvement as suggested by a study (38.8%) as compared to proctosigmoiditis (26.5%) and Left-sided colitis(34.7%). Similar findings were noted in our study where pancolitis was the dominant type of involvement(42%).

5% of patients admitted for IBD, was found to be positive for *C.difficile*[15]. It is now a major concern in hospitalized patients. Mayer et al[15] in a study of 54 IBD patients isolated 10 *Clostridium difficile* and 2 *Campylobacter jejuni*. However no *Clostridium difficile* or *Campylobacter jejuni* were isolated in our study. Furthermore, ELISA for clostridium toxin was negative in our patients. *So C.difficile* may not have a major role in IBD in our setting.

Being endemic for Entamoeba infection, Indian population is at increased risk of IBD. In our study, 4% IBD patients were found to be positive for *E.histolytica* as compared to 0% in controls. These findings are close to that reported by Prokopowicz et al [16] who showed *E.histolytica* prevalence of 4.85% in 103 IBD patients. However Ustun S reported higher positivity of E.histolytica infection (10%) in IBD cases as compared to 1.9% of controls [5]. Small sample size in our study could be one of the reasons for discrepancy. Still positivity of infection was found to be more in IBD than controls.

Cryptosporidium parvum is another protozoan parasite which causes gastroenteritis and diarrhea worldwide. It produces severe diarrhea in immunocompromised patients. It can induce acute relapse in patients with pre-existing IBD. Recent study from India has reported Cryptosporidium infection to be 2% in 49 patients of IBD[7]. In our study, Cryptosporidium was found in 3(6%) patient. One patient also had Isospora co-infection which may have increased the severity of the condition. These opportunistic infections may have major role especially in IBD patients who develop the disease after hospital admissions.

Anotherparasite which was isolated from IBD patients in our study was giardia. Although Giardia produces signs and symptoms different from that of IBD, the immunological mechanisms triggered by this parasite may have a role in IBD. Similar findings were found by Toruner.M et al[17].

CONCLUSION

On the basis of the observation in the present study, following conclusions can be drawn:

1. Most IBD patients were middle aged persons. Males are affected more than females.
2. Opportunistic pathogens like Cryptosporidium and Isospora may be found in patients with IBD.
3. Concentration methods are helpful in isolating more infectious agents from stool samples.
4. Bacteria like Shigella, *Clostridium difficile*;parasitic agents like *H.nana*, Giardia,and *Entamoeba histolytica* are associated with IBD.

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