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Original Research Article

Genotyping of cryptosporidium species in cases of irritable bowel syndrome: A case control study from a tertiary care center

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ABSTRACT

Background: Irritable bowel syndrome (IBS) is functional bowel disorder characterized by abdominal pain or discomfort and altered bowel habits in the absence of detectable structural abnormalities. Protozoan cysts are commonly found in the stool samples of patients with IBS and a rising body of evidence suggests a direct causal relationship. Cryptosporidium infection can be a potential candidate for IBS. We studied prevalence, association, and genotype of cryptosporidium at a tertiary care centre.
Materials and Methods: Using a case-control design, a total of 100 cases and 100 controls were selected between 2016 and 2017. Fresh stool samples were collected and processed for microscopic examination. Positive samples were subjected to PCR RFLP for speciation and results analysed for Odd's ratio, relative risk and relationship between variables.
Results: There was a high male preponderance (m: f=9:1). Most patients belonged to the middle age group (34.8-42.4 years). Amongst the clinical subtype IBS with diarrhoea (IBS-D) was the most common subtype (63%). Odd's ratio for having IBS was 57.5 with cryptosporidium with a relative risk of 2.09. Adjusted Odd's ratio for age was 1.014 suggesting a linear correlation (r value=0.07) with age.
Conclusion: Our study shows a significant risk of developing IBS with cryptosporidium infection. The authors recommend studies with larger population samples both within the northern regions of India and across the country to determine prevalence, speciation of cryptosporidium and its relationship to IBS.

1. Introduction

Irritable bowel syndrome (IBS) is a functional bowel disorder characterized by abdominal pain or discomfort and altered bowel habits in the absence of detectable structural abnormalities. It affects 10 to 20% of adults and adolescents globally and is the least understood bowel disease syndrome. Causal factors include a disturbance of brain gut interaction resulting in abnormal central processing causing autonomic and hormonal events. There can be several triggers with psychological disturbances being common. Genetic and environmental factors are also contributory to the disease syndrome.[1]

Protozoan cysts are commonly found in the stool samples of patients with IBS and a rising body of evidence suggests a direct causal relationship. Among the cysts

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described, the presence of Blastocystis, Cryptosporidium and Giardia are higher compared to healthy controls.[2] However, there is high regional variation in the prevalence with Blastocystis Hominis dominating the association with post infectious IBS (PI-IBS) in both developing and developed nations.[3,4] Studies from non-Asian countries describe a high prevalence of Giardia in patients with IBS phenotype.[5,6] Cryptosporidium is usually associated with a self-limiting diarrhoea and is usually not commonly related to IBS. However, most of studies are from the west and limited information is available from the Indian subcontinent.[7] Many species of Cryptosporidium exist that infect humans and also animals. Although *Cryptosporidium parvum* and *Cryptosporidium hominis* (formerly known as *Cryptosporidium parvum* anthroponotic genotype or genotype 1) are the most prevalent species causing disease in humans, infections by *Cryptosporidium felis*, *Cryptosporidium meleagridis*, *Cryptosporidium canis*, and *Cryptosporidium muris* have also been reported.[8]

Cryptosporidium infection has been found in every region of the world except Antarctica and reported prevalence ranges from 7.5% to 30%, affecting mostly children with select communities showing endemicity.[9–12]

Cryptosporidium infection can be a potential candidate for IBS as the symptoms described post-acute cryptosporidiosis are similar to IBS and last for varied durations.[13,14] At our hospital, we observed a trend of finding cryptosporidium in stool samples of retro negative patients reporting with symptoms of IBS identified using Rome IV diagnostic criteria and studied its prevalence, association with IBS and genotype of cryptosporidium associated.

2. Materials and Methods

Using a case-control design, a total of 100 cases (Sample size: Expected proportion exposed in controls is 0.05; odd's ratio 4; confidence level 0.95 and desired power of case control study 0.8, the sample size = 98 cases and 98 controls) of IBS diagnosed using Rome IV criteria and 100 controls from the same population were selected between 2016 and 2017 at our hospital located in Jammu and Kashmir, Northern India.[15]

Inclusion criteria: As per ROME IV diagnostic criterion recurrent abdominal pain on average at least 1 day/week in the last 3 months, associated with two or more of the following criteria:

1. Related to defecation
2. Associated with a change in the frequency of stool
3. Associated with a change in the form (appearance of stool

These criteria should be fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis.

2.1. Exclusion criteria

Cases of pain abdomen and diarrhoea with positive stool cultures for bacterial pathogens; medical or surgical cases of steatorrhea, known cases of lactose intolerance or gluten hypersensitivity.

2.2. Control group

Subjects without gastrointestinal symptoms, attending the hospital during the same time for routine examination or for non- gastrointestinal causes.

2.3. Sample collection and processing

Fresh stool samples were collected from patients and controls. Stool samples were processed within 2 hours of collection. Each sample was divided into two portions before processing. One portion was immediately processed for microscopic examination for *Cryptosporidium* and those positive were processed for molecular testing. All stool samples were concentrated using the formalin-ether acetate sedimentation technique (FEA). Modified Kinyoun acid-fast stained smears were prepared from the concentrates. Slides were thoroughly screened (200 to 300 fields) for Cryptosporidium (40X objective and 100X Oil immersion objective), and results were recorded.

2.4. Molecular detection using Polymerase Chain Reaction (PCR)

2.4.1. DNA extraction

The stool samples were frozen with liquid nitrogen for 5 min, then thawed at 75°C for 5 min in a water bath. Sample was vortexed after each cycle of freeze thaw. This freeze-thaw cycle was repeated a total of four times. The DNA was then extracted using QIAampFast Stool DNA Mini Kit. 10μl of eluted DNA was used for PCR.

2.5. Molecular analysis

PCR was done in 25 μl reaction volume consisting of 1.25 μl of each primer, forward and reverse, 12.5 μl of master mix and 10 μl of template DNA targeting 18S rRNA gene. Primers used in this study were 18SFwd (5' AACCTGGTTGATCCTGCCAG 3') and 18SRev (5' TGATCCTTCTGCAGG TTCACCTA 3') to amplify 1,750bp 18S rRNA gene of Cryptosporidium. Cycling conditions of amplification comprised of initial denaturation of 8 min at 94°C then 35 cycles of {94°C (30 s), 68°C (30 s), and 72°C (90 s)}, followed by a 10- min extension step at 72°C.[16] Negative controls containing water in place of DNA and positive controls using synthetic DNA of *Cryptosporidium hominis* were run concurrently.

2.6. RFLP

Cryptosporidium RFLP of 1750 bp amplicon excised by SspI & VspI (Promega Corporation, Madison, Wis.) for 1 h at 37°C. Fragments were separated on agarose, visualized with ethidium bromide, alongside synthetic 18S rRNA of *Cryptosporidium hominis* as control.

2.7. Statistical analysis

Data was analyzed using the statistical software, IBM SPSS statistics version 20. Odd's ratio and relative risk was calculated for exposure (cryptosporidium) and outcome (IBS) and adjusted odd's ratio were calculated using linear correlation i.e., r value to remove the effect of confounding variables. Chi-square test was used to assess association between variables. Significant correlation between two parameters was taken at 95% confidence interval. All statistical tests were two-sided and the threshold for significance was p values < 0.05.

3. Results

3.1. Clinical and demographic profile of cases

100 cases meeting the Rome IV criteria were selected for the study. There was a high male preponderance (m: f=9:1). Most patients belonged to the middle age group (34.8-42.4 years).(Figure 1)

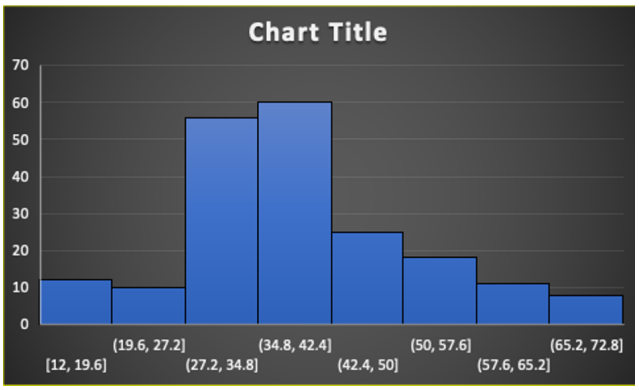

Fig. 1: Histogram showing age distribution among cases with maximum cases among age group 34.8-42.4 years

Mean age of affliction was lower in males as compared to females (Mean age male: 39.79 vs female 43.3 years). Amongst the clinical subtype IBS with diarrhoea (IBS-D) was the most common subtype (63%).(Figure 2)

Family history was positive for similar symptomatology of gastrointestinal disorders in a majority (78%) of cases.

3.2. Stool examination and molecular typing

In 54 cases, stool samples were positive for oocysts of cryptosporidium by modified ZN stain. While in the control

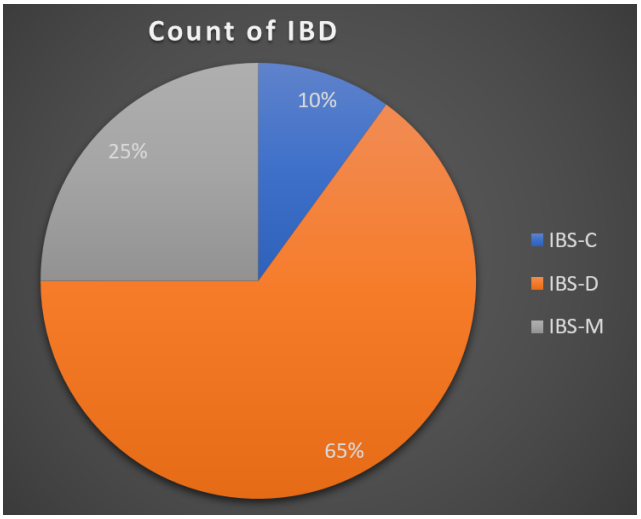

Fig. 2: Pie chart depicting the distribution of types of IBD among patients

group only two samples were positive. (n=100 for both groups) which was statistically significant (P<0.01).

These were then processed for PCR-RFLP. All 54 samples were positive for 18S rRNA (amplicon size1750 bp) of Cryptosporidium. (Figure 3). RFLP of 1750 bp amplicon by VspI enzyme (AT-TAAT) revealed restriction fragments of 825, 925 bp (Figure 4) in 34 samples as well as in positive control of *Cryptosporidium hominis*. There was no variation in fragment lengths of positive samples by RFLP. Those samples, which didn't have any restriction sites for VspI were incubated with SspI resulting in varying fragment lengths (350bp, 150bp, 100bp & <100bp length) (Figure 5). These fragment lengths were suggestive of species other than *Cryptosporidium hominis* and require further sequencing to establish speciation.

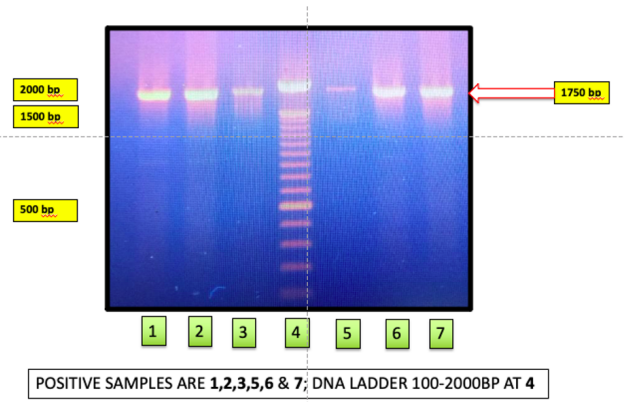

Fig. 3: PCR: Cryptosporidium 18s rRNA

Odd's ratio for having IBS was 57.5 with cryptosporidium with a relative risk of 2.09. Adjusted Odd's ratio for age was 1.014 suggesting a linear correlation (r

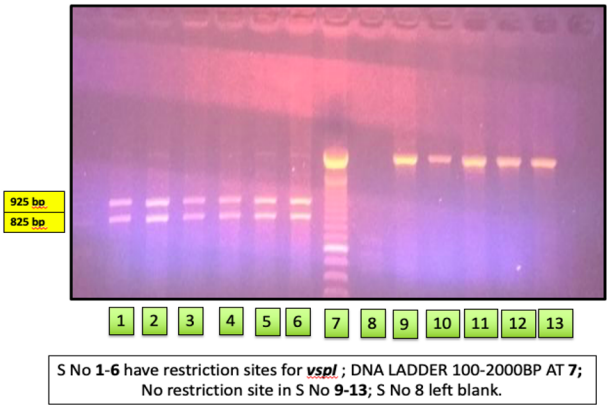

Fig. 4: PCR-RFLP: Cryptosporidium 18s rRNA vspl

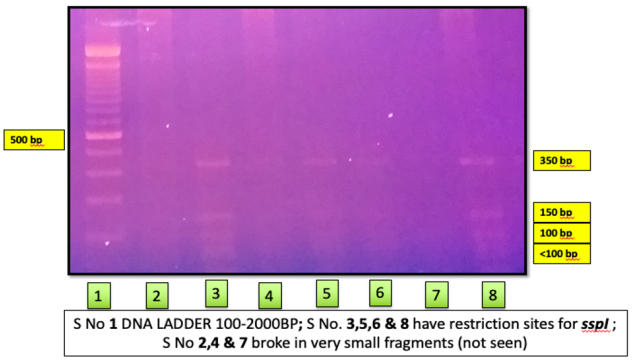

Fig. 5: PCR-RFLP: Cryptosporidium 18s rRNA sspl

value=0.07) with age. (Table 1)

Table 1: Odd's ratio and relative risk of IBS with Cryptosporidium infection

	Cases with IBS	Controls	Total
Cryptosporidium Positive	54	02	56
Cryptosporidium Negative	46	98	144
Total	100	100	200

No significant association was found between gender and IBS (P=0.2) or its sub-types (P=0.92). (Table 2)

4. Discussion

Our study shows a significant risk of developing IBS with cryptosporidium infection (Odd's ratio = 57.5, RR =2.09). Such a strong correlation with cryptosporidium has not been reported in literature though presence of protozoa has been well documented. We report a high prevalence of 54% in patients with IBS which is significantly higher compared to controls (2%) (P<0.01). Compared to similar studies by Jadallah et al.,[2] we not only report a much higher prevalence (54% vs 9.2%), but also a higher prevalence of the IBS-D phenotype seen in our population. The association has been attributed to jejunal hypersensitivity following or during cryptosporidium infection.[17] In addition the study shows a much higher male preponderance which is attributable to the male dominant clientele in service hospitals and the bias was not foreseen during study design.

Pathogenic mechanisms of gut involvement by Cryptosporidium incude infection of intestinal epithelial cells leading to activation of nuclear factor kappa B (NF-κB), which then activates several target genes, including genes for anti- apoptotic molecules such as osteoprotegerin. These anti-apoptotic molecules are responsible for the parasite to form and release merozoites before cell death. However, this activation also leads to upregulation of a pro-inflammatory cascade. Many of these effects are mediated by upregulation or downregulation of micro-RNAs, including let-7, miR-27b, and miR- 98. Murine models, human intestinal xenografts in severe combined immunodeficiency (SCID) mice, and both biopsy specimens and stool studies from human infection demonstrate increased expression of proinflammatory cytokines and mediators of inflammation.[18]

We have also employed sensitive molecular techniques such as PCR and RFLP which are superior to ELISA and antigen based tests in detection, though the gold standard remains sequencing of the amplified product. PCR RFLP is easier and better suited to epidemiological studies. Such techniques have been formerly employed with success by Xiangeng Leng et al. to sub speciate cryptosporidium and Kimbell et al. in various animal models.[16,19] Compared to PCR/RFLP, sequencing though more informative in terms of tangible results is prohibitive in terms of cost and logistics involved in establishment of infrastructure.

We recommend studies with larger population samples both within the northern regions of India as well a multicentric approach across the country to determine prevalence of protozoan infections especially cryptosporidium which appears to be a major causative agent in causing IBS. Sensitive techniques such as PCR and RFLP seem to be suited to epidemiological purpose and speciation and can provide valuable data in diagnosis and management.

5. Limitations of the Study

The method of PCR-RFLP is lengthy and the results need to be confirmed by sequencing. However, sequencing despite being the gold standard remains an expensive option and not suitable from an epidemiological perspective.

The study was limited to hospital population of a specific region in northern India. To generalise our findings similar studies with larger populations over different geographic regions would yield better association and their findings will further strengthen our study.

Table 2: Association of IBS with gender

Gender			IBD		Total	P value
			Absent	Present		
sex	F	Count	17	10	27	
	M	Count	83	90	173	0.2
Total		Count	100	100	200	

6. Source of Funding

None.

7. Conflicts of Interest

There are no conflicts of interest to declare.

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