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Molecular appraisal of intestinal parasitic infection in transplant recipients

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***Background & objectives*: Diarrhoea is the main clinical manifestation caused by intestinal parasitic infections in patients, with special reference to transplant recipients who require careful consideration to reduce morbidity and mortality. Further, molecular characterization of some important parasites is necessary to delineate the different modes of transmission to consider appropriate management strategies. We undertook this study to investigate the intestinal parasitic infections in transplant recipients with or without diarrhoea, and the genotypes of the isolated parasites were also determined.**

***Methods*: Stool samples from 38 transplant recipients comprising 29 post-renal, two liver and seven bone marrow transplant (BMT) recipients presenting with diarrhoea and 50 transplant recipients (42 post-renal transplant, eight BMT) without diarrhoea were examined for the presence of intestinal parasites by light microscopy using wet mount, modified Ziehl–Neelsen staining for intestinal coccidia and modified trichrome staining for microsporidia. Genotypes of *Cryptosporidium* species were determined by multilocus genotyping using small subunit ribosomal (*SSUrRNA*), *Cryptosporidium* oocyst wall protein (*COWP*) and dihydrofolate reductase (*DHFR*) as the target genes. Assemblage study for *Giardia lamblia* was performed using triose phosphate isomerase (*TPI*) as the target gene. Samples were also screened for bacterial, fungal and viral pathogens.**

***Results*: The parasites that were detected included *Cryptosporidium* species (21%, 8/38), *Cystoisospora* (*Isospora*) *belli* (8%, 3), *Cyclospora cayetanensis* (5%, 2), *G. lamblia* (11%, 4), *Hymenolepis nana* (11%, 4), *Strongyloides stercoralis* (3%, 1) and *Blastocystis hominis* (3%, 1). Multilocus genotyping of *Cryptosporidium* species at *SSUrRNA*, *COWP* and *DHFR* loci could detect four isolates of *C. hominis*; two of *C. parvum,* one of mixed genotype and one could not be genotyped. All the *C. hominis* isolates were detected in adult post-renal transplant (PRT) recipients, whereas the *C. parvum* isolates included a child with BMT and an adult with PRT. *Clostridium difficle*, cytomegalovirus and *Candida albicans* were found in 2, 3 and 2 patients, respectively.**

***Interpretation & conclusions*: In the present study, *C. hominis* was observed as an important parasite causing intestinal infections in transplant recipients. Multilocus genotyping of *Cryptosporidium* species could detect four isolates of *C. hominis*; two of *C. parvum*, one of mixed genotype and one could not be genotyped. Genotyping of *G. lamblia* revealed that assemblage B was most common.**

Key words *Cryptosporidium* - genotypes - loci - multilocus - subtypes - transplant

Infections result due to a shift in the equilibrium between host defence mechanisms and invading microorganism, and the associated infective complications are always a major challenge in transplant recipients. Approximately, two-third of patients experience infection-related complications leading to graft failure following transplantation[1]. The true frequency of intestinal parasitic infections in transplant recipients is often not apparent as many of these patients are asymptomatic[2]. Only five per cent of human pathogenic parasites have been reported to cause a significant illness in transplant recipients[2]. The use of cyclosporine as prophylactic immunosuppressive drug has strong parasiticidal effects, and has remarkably reduced the infections in transplant patients[3]. However, the new immunosuppressive drugs used to prevent graft rejections have resulted in an increase in parasitic infections in these patients. The present study was performed to investigate the frequency of intestinal parasitic infections in transplant recipients with and without diarrhoea. Further, various species and assemblages are known in *Cryptosporidium* and *Giardia lamblia*, respectively, that signify different routes of transmission for causing human infections[4,5]. Hence, as the secondary aim of the present study, molecular characterization of *Cryptosporidium* and *Giardia* isolates was attempted.

Material & Methods

This cross-sectional study was conducted at the department of Microbiology, All India Institute of Medical Sciences, New Delhi, a tertiary care referral and teaching hospital located in north India, from July 2011 to June 2013. The study protocol was approved by the Institutional Ethics Committee. Informed written consent was also obtained from all the patients included in the study. Three consecutive stool specimens were collected for three consecutive days from 38 transplant patients with acute, persistent and chronic diarrhoea and 50 transplant patients without diarrhoea who fulfilled the inclusion and exclusion criteria. Information pertaining to age, gender and history of patient's illness was obtained from each patient using a validated structured questionnaire on receiving the sample. A total of 106 patients were sent from the inpatients (n=68) and outpatient (n=38) departments of the hospital. Diarrhoea was defined as passage of at least three unformed stools in a day and further classified into acute, persistent and chronic diarrhoea, if the diarrhoeal episode was <14 days, between 14 and 29 days and for >30 days duration, respectively[6]. Patients with food allergy and those who had either used probiotics in the previous three weeks or antiparasitic drugs due to some ailments in the past three months were excluded from the study. Patients who did not comply with the procedures involved in the study were summarily excluded from the study. The stool samples were subjected to microscopic examination using both direct and formal-ether concentration method for the detection of ova, larvae, trophozoites and cysts of intestinal parasites[7]. In addition, modified Ziehl–Neelsen staining technique[7] and modified trichrome staining[8] were performed for the detection of intestinal coccidia (*Cryptosporidium* species, *Cyclospora cayetanensis* and *Cystoisospora belli*) and microsporidia, with special reference to *Enterocytozoon bieneusi*, respectively. The clinical specimens were also screened for bacterial, fungal and viral pathogens using appropriate microbiological diagnostic methods.

For the molecular characterization of intestinal coccidia (*Cryptosporidium* spp., *C. cayetanensis* and *C. belli*), *E. bieneusi* and *G. lamblia*, genomic DNA was extracted from all the clinical specimens using QiaAmp mini stool kit (Qiagen, USA) as per manufacturer's protocol. Polymerase chain reaction (PCR) assay was carried out using *18S rRNA* as the target gene for the detection of *Cryptosporidium* species[9], *C. cayetanensis*[9], *C. belli*[10] and *E. bieneusi*[11], using previously described primers and PCR conditions. Genus-specific primers were used for *Cryptosporidium* and species-specific primers for *C. cayetanensis*, *C. belli*, *E. bieneusi* and *Giardia* species. Genotyping of *Cryptosporidium* species at multiple loci was performed by PCR-restriction fragment length polymorphism (RFLP) assay. Small subunit ribosomal RNA (*SSUrRNA*), *Cryptosporidium* oocyst wall protein (*COWP*), dihydrofolate reductase (*DHFR*) using previously described primers and PCR conditions[12-14] were used for genotyping, and RFLP was performed using restriction enzymes *Ssp*I and *Ase*I (New England Biolabs, USA) for *SSUrRNA* gene (Fig. 1) and *Rsa*I (New England Biolabs, USA) for *COWP* gene[12,13] (Fig. 2). *Cryptosporidium* glycoprotein (*Cpgp40/15*) locus was amplified using previously described primers and PCR conditions for subgenotyping *Cryptosporidium* species and RFLP was done using *Rsa*I (New England Biolabs) restriction enzyme[15] (Fig. 3). It is known that *C. cayetanensis* and *C. belli* are the only known species responsible for human infection, and genotyping was performed by PCR-RFLP assay using *18S rRNA* for

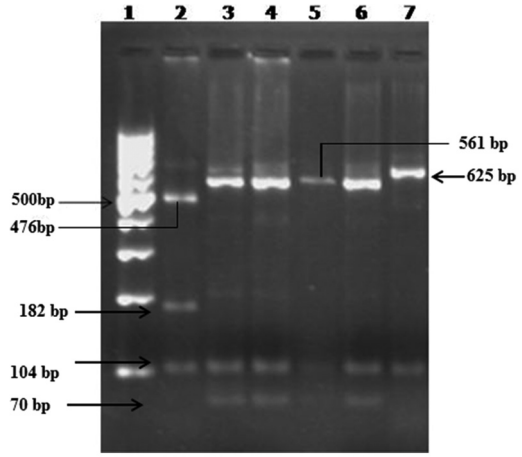

Fig. 1. Restriction fragment length polymorphism using *Ase*I RE for *SSUrRNA* gene. Lane 1-100 bp DNA ladder, lane 2 - *Cryptosporidium felis* control (*Ase*I-104, 182, 476 bp), lanes 3, 4 - *C. parvum* (monkey genotype - *Ase*I-70, 104, 559 bp), lanes 5, 6 - *C. hominis*, lane 7 - *C. parvum* (bovine genotype-*Ase*I-104, 625 bp).

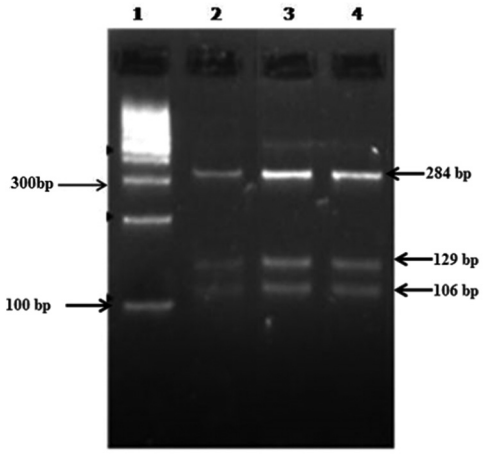

Fig. 2. Restriction fragment length polymorphism assay using *Rsa*I RE for *Cryptosporidium* oocyst wall protein gene. Lane 1 - 100 bp DNA ladder, lanes 2, 3, 4 - *Cryptosporidium hominis* isolates (*Rsa*I - 284, 129, 106 bp).

C. cayetanensis[16] and direct sequencing for *C. belli* to study genetic heterogeneity[17], if any. Assemblage study for *G. lamblia* was performed using PCR-RFLP where triose phosphate isomerase (TPI) was used as a target gene and *Rsa*I as a restriction enzyme[4]. All the patients with cryptosporidiosis received intravenous fluid replacement and were treated with nitazoxanide for 10-21 days. In conjunction with this regimen, dosage of immunosuppressive drugs was also reduced. The patients with *Cyclospora* and *Cystoisospora* (*Isospora*) infection were treated with albendazole and co-trimoxazole, respectively, for a minimum of 10 days. The patients infected with *Giardia* and other helminths also received standard therapy.

Statistical analysis: The statistical analysis was performed using STATA version 11.2 for Windows (Stata Corp LP, Texas, USA). All values were expressed as mean ± standard deviation for continuous variables and percentages for categorical variables. Categorical variables were compared using Pearson's Chi-square test or Fisher's exact test and continuous variables were assessed by Wilcoxon rank-sum (Mann–Whitney U) test.

Results

A total of 38 transplant recipients comprising 30 adults (21 males) and eight children (6 males) were included in the study with a mean age of 29 ± 14.1 yr. These included 29 post-renal transplant (PRT), two liver and seven bone marrow transplant (BMT) recipients. Of these 38 patients, seven, 11 and 20 patients had episodes of acute, persistent and chronic diarrhoea, respectively. Diarrhoeal episodes in 14 patients could be attributed to the immunosuppressive treatment that included eight patients who received prednisolone and the remaining six received mycophenolic acid. Twenty five diarrhoeal episodes were caused by infectious agents comprising bacterial (*Clostridium difficle*, n = 2), cytomegalovirus infection (n = 3), *Candida albicans* in two and parasites (pathogenic) in 19 cases. Parasitic infections were presented as either single parasite or multiple parasites. The parasites (n = 23) that were detected in these patients by light microscopy included *Cryptosporidium* species (21%, 8/38), *C. belli* (8%, 3), *C. cayetanensis* (5%, 2), *G. lamblia* (11%, 4) *Blastocystis hominis* (3%, 1), *Hymenolepis nana* (11%, 4) and *Strongyloides stercoralis* (3%, 1). No *E. bieneusi* could be detected in any of the samples. Multiple parasites were observed in only five adult PRT recipients, of whom two were co-infected with *Cryptosporidium* species with *B. hominis* and *G. lamblia* and two patients with *H. nana* co-infected with *C. belli* and *G. lamblia* each. Only one patient had co-infection of *G. lamblia* and *Endolimax nana*. All intestinal protozoa (*Cryptosporidium* spp., *C. cayetanensis, C. belli* and *G. lamblia*) positive by light microscopy were also positive by their respective PCR assays.

In comparison, 12 (24%) intestinal parasites including five pathogenic (*G. lamblia* = 3, *H. nana* = 2) and seven non-pathogenic protozoa were detected

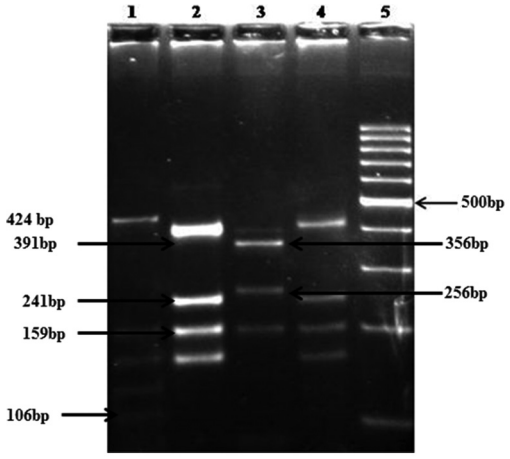

Fig. 3. Restriction fragment length polymorphism assay using *Rsa*I RE for *Cpgp40*/15 gene. Lane 1 - Ie isolates (424, 159, 134, 129, 106 bp), lanes 2, 4 - Ia (391, 241, 159, 143 bp), lane 3 - IIc (356, 256, 143 bp), lane 5 - 100 bp DNA ladder.

by light microscopy in 12 of the total 50 transplant recipients without diarrhoea (28 adult males). No coccidia or microsporidia could be detected in this group either by light microscopy or PCR assays.

The eight *Cryptosporidium* isolates were subjected to multilocus genotyping at *SSUrRNA*, *COWP* and *DHFR* gene and *Cpgp40/15* loci. Of these seven isolates of *Cryptosporidium*, four could be genotyped as *C. hominis*, two as *C. parvum*, one as mixed genotype and one could not be genotyped. All the *C. hominis* isolates were detected in adult PRT recipients, whereas the *C. parvum* isolates were detected in a child with BMT and an adult with PRT. Symptoms included prolonged diarrhoea, fever, abdominal pain and weight loss. The distribution of subtypes was determined at *Cpgp40/15* locus. Two Ia and one each of Ie and If subtypes were observed in *C. hominis* isolates and IId and IIc among *C. parvum* isolates. The isolate with mixed genotype had IIa subtype. The subtype Ia of *C. hominis* was associated with abdominal pain and anaemia. Three different fragments of 140, 106 and 48 bp were obtained after restriction digestion of PCR products from both the isolates of *C. cayetanensis*. All the four *G. lamblia* parasites were of assemblage B. The stool samples from all the patients with parasitic infections were re-examined after four weeks of receiving treatment. All these patients recovered eventually from the disease and had no recurrence during the six-month follow up.

Discussion

Organ transplant recipients may acquire parasitic infection in three different ways such as (*i*) transmission with the graft, (*ii*) *de novo* infection, or (*iii*) reactivation of latent infection as a consequence of immunosuppression. Most of the infections due to reactivation are extraintestinal. Relapse of cryptosporidiosis in immunocompromised patients even after treatment can occur, suggesting that the infection may remain in the latent stage; however, no significant evidence is available on the role of reactivation of latent infection of other intestinal protozoan parasites. The reactivation of latent infection of *C. felis* in cats by administration of prednisolone has been reported[18]. In the present study, the frequency of intestinal parasitic infections was relatively higher in transplant recipients with diarrhoea (66%, 23/38) as compared to transplant recipients without diarrhoea (24%, 12/50) (P<0.01). The most common parasite identified in the present study was *Cryptosporidium* species (21%). The global prevalence of cryptosporidial infection in transplant recipients has been reported as 18.8-34.8 per cent[19]. A study on renal transplant recipients in India identified cryptosporidial diarrhoea in 16.6 per cent of cases[20]. Similarly, *Cryptosporidium* spp., has been identified in 1.7-11 per cent of the patients undergoing allogeneic BMT[21]. Cryptosporidiosis has also been reported in children with liver transplantation[22]. In contrast, in a study carried out on renal transplant recipients in Brazil, *S. stercoralis* (11/16) was the most common helminthic infection[23].

In developing countries including India, *C. hominis* is the most common luminal coccidial species responsible for a majority of human infections in immunocompromised patients[24]. In the present study, *C. hominis* was the predominant *Cryptosporidium* spp., and it was also associated with prolonged duration of diarrhoea than infection with *C. parvum*. *C. parvum* has been reported in a bone marrow recipient in Iran[25]. Detection of both *C. hominis* and *C. parvum* in our study highlights the possible mode of transmission of this parasite. The zoonotic transmission of *Cryptosporidium* infection cannot be excluded for transplant recipients undergoing immunosuppressive therapy based on exposure to socio-economic settings. While in the present study, subtype Ia of *C. hominis* was associated with abdominal pain and anaemia, an association of subtype Ia with older age was observed in the study by Ajjampur *et al*[26].

Infections with *C. cayetanensis* and *C. belli* are less common than *Cryptosporidium* species and have occasionally been reported in renal transplant recipients[27]. A single case of liver transplant with

isosporiasis has also been documented[28]. Genotyping of *G. lamblia* was performed to investigate whether different genotypes and/or intraspecies variations within the genotypes had any effect on the clinical symptomatology. Assemblage A, based on PCR-RFLP of *tpi* gene of *G. lamblia,* has been reported as the most common genotype associated with giardiasis, whereas assemblage B has been predominantly found in children[29]. Most of our patients had assemblage B. *B. hominis* that had long been considered non-pathogenic, has also been incriminated as a diarrhoeagenic agent and has been reported in transplant recipients[30].

In conclusion, transplant recipients undergoing immunosuppressive therapy are a major risk group for acquiring intestinal parasitic infections. Improving the level of knowledge about covert parasitic infections and relevant risk factors will have obvious influence on withdrawing the infection rate among this population and would significantly reduce the morbidity. With the accumulated data, it is of further importance to associate genotypes and subgenotypes with special reference to *Cryptosporidium* spp., with the clinical outcome of the disease.

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Conflicts of Interest: None.

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