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Genetic diversity of Cryptosporidium isolates from patients in North India

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SUMMARY

Background: Cryptosporidiosis is a significant cause of diarrheal illness in both immunocompetent and immunocompromised populations. *Cryptosporidium* species infect a wide range of hosts including humans. Different species are morphologically indistinguishable, and molecular techniques have become the key to detection and source tracking. The present study was designed to study the genetic diversity of human Cryptosporidium isolates in North India.
Methods: Cryptosporidium oocysts were detected in stool samples by special staining of fecal smears. DNA was extracted with a Qiagen kit and all samples were genotyped by small subunit ribosomal ribonucleic acid (SSU rRNA)-based nested PCR-restriction fragment length polymorphism (RFLP) tool using enzymes SspI and VspI. *Cryptosporidium hominis* and *Cryptosporidium parvum* isolates were subtyped by sequence analysis of the nested PCR amplified gp60 gene.
Results: Fifty-three fecal samples were found to be positive for Cryptosporidium oocysts. RFLP analysis revealed 39 isolates as *C. hominis* and 13 isolates of *C. parvum*; one sample failed amplification. gp60-based sequencing of *C. hominis* and *C. parvum* divided them into eight subgenotype families and 17 subtypes. gp60-based sequencing identified seven cases of mixed infection with *C. hominis* and *C. parvum/Cryptosporidium meleagridis* and showed the presence of *C. meleagridis* in six HIV-positive patients that were indistinguishable in RFLP.
Conclusions: Cryptosporidium isolates obtained in the present study from patients in North India belonged to three species, eight subgenotype families, and 17 subtypes. The existence of many *Cryptosporidium* species, subgenotypes, and subtypes along with mixed infections reveals the complexity of Cryptosporidium transmission; this heterogeneity indicates stable cryptosporidiosis transmission in North India. The results may have further implications in understanding the epidemiology and control of this infection.

1. Introduction

Cryptosporidiosis, caused by Cryptosporidium, a protozoan parasite, is a significant cause of diarrheal illness in both developed and developing countries. Although Cryptosporidium infections have been documented in both immunocompetent and immunocompromised populations, a high prevalence is documented in HIV-positive patients (particularly those with CD4+ count of <200 cells/mm^3), patients with malignancies (including those with hematological malignancies undergoing chemotherapy or bone marrow transplantation), patients with solid organ transplants, and patients on hemodialysis.[1–5] Studies from India have shown varying rates of prevalence ranging from 11% to 33% in HIV patients[6–9] and 39.7% in the rural population.[10] In Chandigarh, 10.8–13.1% of HIV patients have been reported to harbor Cryptosporidium.[11,12]

Humans acquire Cryptosporidium infections through several transmission routes, such as direct contact with infected persons and animals and/or the ingestion of contaminated food and water. To date only eight species have been reported in humans: *C. hominis, C. parvum, C. meleagridis, C. felis, C. canis, C. muris, C. suis*, and *Cryptosporidium* cervine genotype (*C. ubiquitum*).[13,14] Different species/genotypes are morphologically indistinguishable, and molecular techniques have become the key to species identification and source tracking. Genetic typing of several loci, such as those encoding the 18S rRNA, actin, oocyst wall protein, and a 70-kDa heat shock protein, have been used for species identification. The small subunit ribosomal ribonucleic acid (SSU rRNA)-based PCR-restriction fragment length polymorphism (PCR-RFLP) tool is preferred due to the presence of conserved regions with highly polymorphic regions in the gene, allowing the design of primers that can amplify various *Cryptosporidium* species or genotypes and

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differentiate them. Although genotyping tools can distinguish anthroponotic from zoonotic parasites, their use in epidemiologic investigations is limited by the low resolution power of these loci. Several subgenotyping tools including microsatellites and minisatellites have been used in the epidemiologic resolution of *C. hominis* and *C. parvum*. gp60 is a highly polymorphic target similar to microsatellites, with tandem repeats of serine coding trinucleotide at the 5′ end of the gene, and has been used widely for subgenotyping.[15]

Few studies on the molecular epidemiology have been reported from India, with most of these being from South India; reports from North India are scarce.[8,16–19] There is only one study from North India, which was a multisite study that had Delhi as the northern representative. Moreover, the study was done on children aged <5 years and stool samples were those originally collected for the Indian Rotavirus Strain Surveillance network.[16] They reported SSU and *C. parvum* glycoprotein 40/15 (Cpgp40/15)-based PCR-RFLP, and had done Cpgp40/15-based sequencing in only a few samples (16 from all centers) with ambiguous results for Cpgp40/15-based PCR-RFLP. Studies on the molecular epidemiology of cryptosporidia from North India are lacking. Thus the aim of the present study was to assess the *Cryptosporidium* species and subgenotypes from North Indian isolates.

2. Methods

2.1. Study design

Subjects (n = 1400) attending the outpatient departments of the immunodeficiency clinic, inpatient and outpatient departments of the advanced pediatric center, and the inpatient departments of Nehru Hospital attached to the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, were enrolled in the study based on the following criteria: (1) group A: HIV-positive patients with or without diarrhea (n = 970); (2) group B: transplant patients with or without diarrhea (n = 100); (3) group C: immunocompetent adults with or without diarrhea (n = 200); (4) group D: children with diarrhea (n = 130).

Nehru Hospital attached to PGIMER is a tertiary care hospital, and patients from different areas of North India including Punjab, Haryana, Himachal Pradesh, Uttar Pradesh, and Bihar, attend the hospital for treatment. The study protocol was reviewed and approved by the institutional ethics committee of PGIMER.

2.2. Specimens and processing

Stool samples were collected and screened for Cryptosporidium by staining (Ziehl–Neelsen and auramine 'O'). Aliquots of samples found to be positive for Cryptosporidium by microscopy were stored at −20 °C until further analysis.

2.3. Cryptosporidium genotyping

DNA was isolated from all Cryptosporidium-positive samples by QIAamp Stool Mini Kit in accordance with the manufacturer's instructions (Qiagen Inc., Valencia, CA). Cryptosporidium-positive samples were genotyped by SSU rRNA-based PCR-RFLP tool. This technique amplifies a ~850-bp fragment of SSU rRNA by nested PCR and differentiates *Cryptosporidium* species or genotypes by banding patterns in the restriction analysis of secondary products with restriction enzymes SspI and VspI.[20] For restriction digestion (37 °C for 14 h), 12 μl of secondary product in a 32-μl (total volume) reaction mixture consisting of 2 μl of restriction buffer and 2 U of SspI or 2 U of VspI (Fermentas Life Sciences, Vilnius, Lithuania) was used. Digestion products were fractionated on a 2% agarose gel and visualized by ethidium bromide staining.

2.4. C. hominis *and* C. parvum *subgenotyping and subtyping*

Subgenotyping of *C. hominis* and *C. parvum* was based on sequence analysis of the gp60 gene. This molecular tool amplifies a ~850-bp fragment of the gp60 gene by nested PCR[21] and differentiates subgenotype families and subtypes on the basis of sequence differences in the non-repeat region of the gene and number of serine coding trinucleotide repeats (TCA, TCG, or TCT), respectively. For the samples that failed to be amplified, a smaller fragment (~400 bp) of the gene was amplified using primers AL3531 and AL3533 in a primary PCR and AL3532 and LX0029 in a secondary PCR.[22] Secondary products of expected size (~850 bp and ~400 bp) were purified (QIAamp Gel Extraction Kit) and sequenced in both directions with inner primer pairs. Sequencing was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The categorization into subtype families was done by alignment of gp60 sequences obtained in this study with reference sequences retrieved from GenBank using program ClustalW multiple alignment (BioEdit version 7.0.5.3). Subgenotypes within gp60 allele families were determined on the basis of the number of TCA (A), TCG (G), TCT (T), and 5′-AAA/G ACG GTG GTA AGG-3′ (R) repeats in the microsatellite region, as per the nomenclature described previously.[22]

2.5. Phylogenetic analysis

To further support the grouping of subtypes, a phylogenetic tree was generated from aligned sequences using one-click mode in the phylogeny.fr server (http://www.phylogeny.fr/). The tree was constructed with sequences obtained in the present study (numbered as PGIMER), one reference sequence corresponding to each Cryptosporidium subtype (GenBank accession number_-species_subtype) and sequences available from Indian studies (GenBank accession number_IN_species). A rooted tree was constructed with *Plasmodium falciparum* MSPI (merozoite surface protein 1, having similar properties to Cryptosporidium 60-kDa glycoprotein Cpgp40/15 or gp60) sequence (GenBank **FJ959104.1**) as an outgroup. All the sequences in FASTA format were pasted in space for analysis in one-click mode. Sequences were aligned with MUSCLE (v3.7), and ambiguous regions (i.e., containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b). The phylogenetic tree was constructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3).

2.6. Nucleotide sequence accession numbers

All the unique gp60 sequences obtained in this study have been submitted to GenBank under accession numbers **HQ241927**–**HQ241932**, **JF268622**–**JF268649**, and **JF495136**–**JF495160**.

3. Results

3.1. Prevalence of Cryptosporidium

Fifty-three of 1400 samples (3.8%) were found to be positive for Cryptosporidium by Ziehl–Neelsen and/or auramine 'O' staining; this included 44/970 HIV patients (4.5%), 3/100 transplant patients (3%), 1/200 immunocompetent adults (0.5%), and 5/130 children (3.8%).

3.2. Cryptosporidium *species/genotypes*

DNA preparations of 52/53 positive samples yielded products of ~850 bp in the nested PCR of the 18s rRNA gene, while one

Table 1
Cryptosporidium species and subgenotype families in the study groups based on SSU rRNA PCR-RFLP and gp60 sequencing

Source	Species (*n*)	Subgenotype families
HIV patients	*C. hominis* (33)[a,b]	Ia, Ib, Id, Ie, If
	C. parvum (10)[b]	IIc, IId, IIe
	C. meleagridis (6)[a]	-
Transplant patients	*C. hominis* (2)	Ie, If
	C. parvum (1)	IIe
Children	*C. hominis* (3)	Ie, If, Id
	C. parvum (2)	IIc, IId
Immunocompetent adults	*C. hominis* (1)	If

SSU rRNA, small subunit ribosomal ribonucleic acid; RFLP, restriction fragment length polymorphism.
[a] Five mixed infections.
[b] Two mixed infections.

(PGIMER32) failed to be amplified. *C. hominis* was the most prevalent species, found in 39 (75%) samples, followed by *C. parvum* in 13 (25%) (Table 1).

3.3. C. hominis *and* C. parvum *subgenotyping and subtyping*

DNA of the 53 samples was also amplified for the gp60 gene. DNAs from 16/53 samples failed to produce the expected PCR product (~850 bp) and were amplified with a new primer set,[22] giving products of ~400 bp. Out of 16 DNA samples (amplified for ~400 bp product), two (PGIMER64, PGIMER65) also failed to give amplification for the small product and eight resulted in multiple products. Multiple products were found in the eight samples as: two products (400 bp, 310 bp) in six samples, three products (420 bp, 380 bp, 320 bp) in one sample, and four products (400 bp, 370 bp, 300 bp, 260 bp) in one sample (PGIMER32).

All the distinct PCR products for small as well as large products were gel-extracted and sequenced. PCR amplicons for the gp60 gene succeeded in sequencing reactions for all samples; however for PGIMER32, out of the four products, only one PCR product of 300 bp succeeded in the sequencing reaction and was found to be *C. meleagridis*. Mixed infections of *Cryptosporidium* species/subgenotypes/subtypes were found in seven samples and all were from HIV-seropositive patients. In five samples *C. hominis* was found with *C. meleagridis*, in one sample *C. hominis* was found with *C. parvum*, while in one sample two *C. hominis* subgenotypes were present with *C. parvum* (Tables 1 and 2).

3.4. C. hominis *and* C. parvum *subgenotype families and subtypes*

Phylogenetic analysis of sequences revealed that *C. hominis* and *C. parvum* isolates belonged to eight subgenotype families. Most of the *C. hominis* isolates ($n = 33$) belonged to subgenotype families Ie ($n = 13$), Ia ($n = 12$), and Id ($n = 8$). Subgenotype families Ie and Id had two subtypes, subgenotype family Ia had six subtypes, while Ib and If had one subtype only. Both anthroponotic (IIc and IIe) and zoonotic (IId) subtypes of *C. parvum* were isolated. Two subtypes of IId subgenotype family were IIdA14G1 and IIdA15G1, while in IIc and IIe only one kind of subtype was found. Concurrent infection of *C. hominis* subgenotype families Ia and Ie was found with *C. parvum* subgenotype family IIc in one sample, while in another sample *C. hominis* subgenotype Ia was found with *C. parvum* subgenotype IIe. In four samples *C. hominis* subgenotype Ie was found with *C. meleagridis*, while in one sample *C. hominis* subgenotype Id was present with *C. meleagridis* (Table 2 and Figure 1).

4. Discussion

This is the first study to genetically characterize *Cryptosporidium spp* from immunocompromised patients, children, and immunocompetent adults in North India by SSU rRNA-based PCR-RFLP and to subtype *C. hominis* and *C. parvum* by microsatellite-based sequence analysis of the gp60 gene. Studies from South India have been based on multilocus genotyping of Cpgp40/15 by PCR-RFLP in HIV-seropositive patients, showing five *C. hominis* (Ia, Ib, Ic, Id, and If) and three *C. parvum* subtypes (IIa, IIb, and IIc),[18] and multilocus sequence typing in children which identified four *C. hominis* subtypes (Ia, Ib, Id, and Ie) but no sample was identified as *C. parvum*.[23] gp40/15 sequence-based subtyping has been done in children in a multisite study including Delhi. However, they were subtyped mainly by Cpgp40/15-based PCR-RFLP and sequencing was done in only 16 samples (from all three centers, viz. Christian Medical College, Vellore, South India, Child Jesus Hospital, Trichy, South India, and St. Stephen's Hospital, Delhi, North India), which had ambiguous results for the Cpgp40/15-based PCR-RFLP.[16]

On RFLP analysis *C. hominis* was the most commonly identified species (75%). This is in concordance with previous studies from developing nations[24] and South Indian studies in children,[8,16,17,19] adults,[19] and HIV-infected individuals.[18] In the present study zoonotic species *C. parvum* was identified in 25% of the children and immunocompromised patients, but in none of the immunocompetent adults. The prevalence of *C. parvum* (25%) was similar to that reported from South India.[8,16,17,19] Earlier studies from South India have documented the presence of other zoonotic species of *Cryptosporidium*, i.e., *C. felis, C. muris*, and *C. meleagridis*, by RFLP,[17,18] but in the present study these species were not found by SSU rRNA-based PCR-RFLP.

C. hominis subgenotypes were from Ie (13), Ia (12), Id (8), If (5), and Ib (1) subgenotype families in order of frequency. These five subgenotypes have been reported from children in an earlier multisite study in India, however as most of the results were based

Table 2
gp60 sequence-based subgenotypes and subtypes

Species	Subgenotype family	Total isolates	Subtype
C. hominis	Ia	12	IaA18R3[a], IaA19R3, IaA21R3, IaA26R3, IaA27R3, IaA29G1T3R3[b]
	Ib	1	IbA9G3
	Id	8	IdA14G1, IdA15G1[1c], IdA16G1
	Ie	13	IeA11G3T2[1c], IeA11G3T3[3a,c]
	If	5	IfA13G1
C. parvum	IIc	6	IIcA5G3[a]
	IId	5	IIdA14G1, IIdA15G1
	IIe	3	IIeA7G1[b]
C. meleagridis		6	

[a] Triple infection.
[b] Double infection.
[c] With *C. meleagridis*.

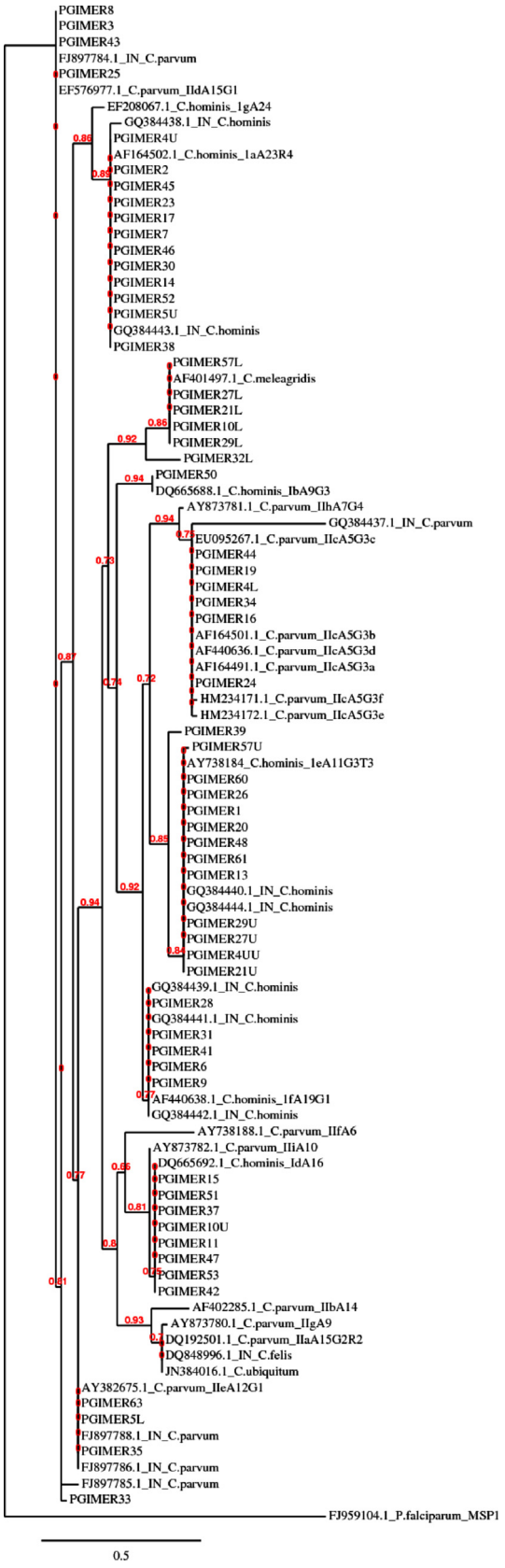

Figure 1. Phylogenetic relationship among North Indian isolates with sequences retrieved from GenBank corresponding to reference sequences of *C. meleagridis* and *C. hominis*, *C. parvum* subgenotype/subtypes, and sequences obtained in Indian studies ('IN' with GenBank accession number stands for Indian isolates). Sequences obtained in the present study have numeric values starting with PGIMER and all are available in GenBank. Values on tree branches are branch support values.

on Cpgp40/15 RFLP, sequencing was done in only 16 samples for which the subtype details were given.[16] Most of the Ie subtypes were IeA11G3T3 (12/13), which was the most commonly identified subtype in developing countries including India.[16,24] Another Ie subtype was IeA11G3T2. Under subgenotype family Ia, six subtypes were found which differed in the number of trinucleotide serine coding repeats, but all had a constant number (3) of 15-bp 5′-AAA/G ACG GTG GTA AGG-3′ repeat (R3). In an earlier study from South India, four Ia subtypes were found by multilocus sequence typing (MLST),[23] and in another multisite study in children, two subtypes IaA18 and IaA19 were reported.[16] In the present study, three subtypes for subgenotype Id were observed, while for subgenotypes If and Ib only one subtype was found. In the multisite study in children they also reported one and the same If subtype (IfA11G3), but for Ib and Id subtype details were not given. *C. hominis* subtype IbA9G3 is commonly seen in Australia,[25] India,[23] Kenya,[15] and Malawi,[26] while in the present study only one isolate of this subtype was found. The existence of many subtypes within *C. hominis* subgenotype families Ia, Id, and Ie supports the complexity of Cryptosporidium transmission, as reported in earlier studies from other developing countries.[23,25,27]

C. parvum anthroponotic subgenotypes IIc and IIe were most common, along with zoonotic subgenotype IId. In developing countries, the IIc subgenotype family causes most human infections, and IIe subgenotype in addition to IIc is seen in Kenya and Malawi.[15,25–29] *C. parvum* subgenotype family IId has been reported from Portugal.[30,31] In the multisite study from India, four *C. parvum* subtypes (IIc, IId, IIm, and IIn) were reported, but subgenotype IIe was not reported, while in the present study new subtypes IIm and IIn were not reported. In the present study, there were two *C. parvum* IId subtype alleles (IIdA14G1 and IIdA15G1), while for IIc and IIe only one subtype was seen. In the earlier multisite study from India, one subtype of IIc (IIcA5G3), as well as IId (IIdA15G1) was reported.

Subgenotyping of *C. hominis* and *C. parvum* by gp60-based sequencing revealed mixed infections of *Cryptosporidium* species/subgenotypes/subtypes in seven samples. In one sample with three concurrent infections, *C. hominis* subtypes IaA18R3 and IeA11G3T3 were found with *C. parvum* subtype IIcA5G3, while in another sample *C. hominis* subtype IaA29G1T3R3 was found with *C. parvum* subtype IIeA7G1. Mixed infections of *C. hominis* and *C. parvum* have been reported in several patients in Switzerland, Scotland, the UK, and USA,[32–36] and South India.[19] In a multisite study of Indian children, RFLP patterns showed a mixed infection with Ia and If in one child from Delhi.[16] In the present study, five cases of coinfection of *C. meleagridis* with *C. hominis* subtype families Id ($n = 1$) and Ie ($n = 4$) was also found. Two cases of mixed infection of *C. hominis* and *C. meleagridis* has also been reported from children in Kolkata by multilocus sequence analysis.[23] In the present study mixed infections were not found by restriction analysis and this observation is supported by earlier studies that have shown only 31–74% success of the SSU rRNA-based PCR-RFLP tool in detecting concurrent infections.[37] As is true for all genus-specific tools, SSU rRNA-based PCR-RFLP can detect and differentiate a wide range of *Cryptosporidium* species or genotypes, but its use in detecting mixed infections is compromised by preferential amplification of dominant species or genotypes in the samples.[15,38] Thus, the use of genus-specific primers in combination with species-specific primers can help to resolve the issue of mixed infections.

In conclusion, Cryptosporidium isolates obtained in the present study from patients in North India belonged to three species, eight subgenotype families, and 17 subtypes. The existence of many *Cryptosporidium* species, subgenotypes, and subtypes along with mixed infections of species/subgenotypes/subtypes reveals the complexity of Cryptosporidium transmission

and this heterogeneity indicates stable cryptosporidiosis transmission in North India. The results may have further implications in understanding the epidemiology and control of this infection.

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Conflict of interest: None of the authors involved in the study have any competing interest.

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