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Original papers

Presence of intracellular viruses in human *Cryptosporidium* isolates

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ABSTRACT. *Cryptosporidium* is a major cause of diarrheal illness mainly in children and immunocompromised adults. Disease severity ranges from asymptomatic or self-limited gastroenteritis to acute or chronic diarrhoea which may be associated with systemic features. Intracellular viruses that reside in many parasites have been incriminated in pathogenesis of diseases like trichomoniasis, leishmaniasis etc. Thus we attempted to detect and quantitate the intracellular viruses in *Cryptosporidium* isolates and sought to seek a relationship if any, with clinical features. Cryptosporidia in stool samples from immunocompromised patients and children were identified by microscopy and species differentiated by PCR-RFLP of 18s rRNA; further subgenotyped by sequencing of GP60 region. Copy number of dsRNA virus and 18srRNA was calculated in 56 *Cryptosporidium* isolates (39 *C. hominis* and 17 *C. parvum*). Viral copy number per oocyst was calculated as ratio of dsRNA virus copy number to 18s rRNA copy number. Viruses were detected in all the isolates. Mean CSpV/RNA ratio was 0.17±0.4 for *C. hominis* isolates compared with 0.12±0.11 for *C. parvum* isolates, however this difference was not statistically significant. Similarly no association of diarrhoea, vomiting, cough and fever was found with either CSpV copy number or with CSpV/rRNA ratio.

Key words: *Cryptosporidium*, virus, immunocompromised, diarrhoea

Introduction

Cryptosporidium, an apicomplexan protozoan parasite, is a major cause of diarrheal illness mainly in children and immunocompromised adults. Disease severity ranges from asymptomatic or self-limited gastroenteritis to acute or chronic diarrhoea which may be associated with abdominal pain, vomiting, fever and weight loss. Two species namely *C. hominis* and *C. parvum* account for >90% of human cases of cryptosporidiosis [1]. The severity, persistence and ultimate outcome of infection depends upon a number of parasite and host factors many of which are not yet clear. Intracellular viruses have been found in a number of protozoa including *Cryptosporidium*, *Giardia*, *Trichomonas*, *Leishmania*, *Eimeria* and *Babesia* [2,3] but the precise roles of these dsRNA viruses and their encoded products in host-parasite relationships remain largely unknown and evidence is conflicting. Viral density was reported to affect the growth of the *Giardia* parasite, and the presence of dsRNA in *Trichomonas* was associated with phenotypic modifications of the infected cells [2,4–6]. In *Leishmania*, an endoribonuclease activity was demonstrated to be associated with the capsid protein of RNA virus (LRV) [7,8] and high LRV burden in infecting parasites could be a major determinant of disease severity and pathology [9]. These data suggested that extrachromosomal dsRNAs may be directly linked to a change in the host virulence or host-induced pathogenicity and the viral load may be a determinant of disease severity [2]. Bisegmented dsRNA virus (*Cryptosporidium parvum* virus1, CSpV1) associated with human stools containing *Cryptosporidium* oocysts, was

first reported in 1995. CSpV1 is probably associated with persistent, largely avirulent infections of its hosts [10,11] but there are so far no clear examples in which parasite pathogenicity is either positively or negatively modulated by infection with CSpV1 or another CSpV1-like virus [11].

Thus we attempted to detect and quantitate the intracellular viruses in different *Cryptosporidium* isolates and sought to seek a relationship if any, with clinical features of cryptosporidiosis.

Materials and Methods

Faecal samples. Stool samples were collected between years 2009 and 2013 from immuno-compromised (HIV-AIDS and transplant) patients, immunocompetent adults and children attending Nehru Hospital, Postgraduate Institute of Medical Education and Research, Chandigarh, a tertiary care centre in North India and relevant clinical data along with written consent was obtained from these patients. *Cryptosporidium* oocysts were detected by Ziehl-Neelsen staining. Positive stool samples were stored at 4ºC without any preservative for DNA extraction and in 2.5% potassium dichromate for oocyst purification.

***Cryptosporidium* species and subtypes [12].** DNA was extracted from stool samples with QIAamp Stool Mini Kit (Qiagen,Valencia, CA). *Cryptosporidium* species were differentiated by PCR-RFLP analysis of 18s rRNA *C. hominis* and *C. parvum* were further subgenotyped by PCR amplification and sequence analysis of GP60 region. Subgenotyes and subtypes were designated on the basis of sequence differences in non-repeat region of the gene and number of serine coding trinucleotide repeats (TCA, TCG, TCT).

Oocyst purification and RNA extraction from *Cryptosporidium* isolates [13]. *Cryptosporidium* oocysts were purified from stool samples preserved in 2.5% $K_2Cr_2O_7$ by sucrose density gradient centrifugation [14]. RNA was extracted from about 10^5 oocysts suspended in 100µl of PBS containing 2U RNase inhibitor and subjected to 3 cycles of freezing and thawing at -70ºC and 50ºC to lyse the oocysts. The lysate was subjected to RNA extraction with QIAamp Viral RNA Mini kit (Qiagen,Valencia, CA) according to manufacturer's instructions. RNA was treated with 2.5 U DNase (Qiagen,Valencia, CA), by incubation at room temperature for 15 minutes followed by heat inactivation of DNase at 70ºC for 15 min. RNA was stored at -20ºC until used as a template for reverse transcriptase (RT)-PCR.

cDNA synthesis and real time PCR. cDNA synthesis was accompanied by RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Vilnius, Lithuania) with random hexamers. The dsRNA virus levels were compared between different isolates using quantitative real time PCR. To control for slight differences in oocyst numbers, the dsRNA real time PCR signal was normalised to *Cryptosporidium* 18s rRNA signal. The qPCR was performed using a Light Cycler 480 quantitative instrument (Roche Diagnostics, Mannheim, Germany) and SYBR green mix. In brief, each 20µl reaction contained 10µl of 2X SYBR green mix, 6µl of water, 1µl of each primer (10µM forward and reverse dsRNA primers or 10µM forward and reverse 18s rRNA primers) and 2µl of cDNA template. Primers for viral dsRNA were as described by Jenkins et al., [13]. Primers for *Cryptosporidium* 18s rRNA were designed using Primer-blast (http://www.ncbi.nlm.nih.gov/tools /primer-blast/). These primers, CSF 5'-TCA GCT TTA GAC GGT AGG GTA TTG GC-3' and CS R 5'-TGT GGT AGC CGT TTC TCA GGC T-3'amplified a region of 100bp. Amplification of dsRNA was accomplished by initial denaturation at 95ºC for 3 minutes followed by 45 amplification cycles at 94ºC for 20s, 55ºC for 45s and 72ºC for 1 minute. *Cryptosporidium* 18s rRNA was amplified by initial denaturation at 95ºC for 10s followed by 45 amplification cycles at 95ºC for 10s, 62ºC for 20s and 72ºC for 20s. Melting was performed by increasing the temperature in 0.2ºC increments starting at 55ºC (dsRNA virus) or 62ºC (18srRNA) until the temperature reached 97ºC.

Cloning, sequencing, and standard curves for absolute quantification. About 173bp and 100bp regions of *Cryptosporidium*-dsRNA virus and 18s rRNA, respectively were amplified by conventional PCR, and amplicons were cloned into pGEM-Easy vector (Promega, Madison, WI, USA). Briefly, PCR products were purified by gel extraction kit (Qiagen Inc., Valencia, CA) and 2µl of purified product was used as insert for ligation into plasmid vector. Chemically competent DH5α cells were transformed with ligation mixture and transformed cells were selected on LB agar plate containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactopyranoside (IPTG) as per manufacturer's instructions (Promega, Madison, WI, USA). White

Table 1. ds RNA virus copy number in samples with single *Cryptosporidium* species/subgenotype infection and mixed *Cryptosporidium* species/subgenotype infections

Species (n)	Subgenotype family (n)	ds RNA virus copy number
C. hominis (39)	Ia (13)	150, 150, 151, 171, 197, 223, 314, 314, 335, 595, 966, 1150, 3920
	Ib (1)	266
	Id (9)	121, 139, 145, 184, 200, 229, 323, 359, 1150
	Ie (11)	82, 110, 152, 206, 210, 239, 241, 312, 410, 412, 8170
	If (5)	95, 137, 145, 220, 300
C. parvum (17)	IIc (6)	42, 139, 220, 312, 312, 506
	IId (8)	86, 150, 203, 212, 212, 258, 385, 392
	IIe (3)	258, 258, 418
C. hominis +	Ie (4), Id (1)	206, 214, 239, 241, 555
C. meleagridis (5)		
C. hominis	Ie + Ia + IIc	925
C. parvum (2)	Ia + IIe	303

colonies were selected and subcultured on Luria broth (LB) agar plates at 37ºC overnight. Isolated colonies were then grown in 5ml LB broth at 37ºC with continuous shaking at 150 rpm (New Brunswick Scientific Co., Inc., USA). Plasmid was extracted with High-Speed Plasmid Mini Kit (Geneaid Biotech Ltd, Sijhih City, Taiwan) following manufacturer's instructions. Standard curve was prepared with serial dilutions of plasmid template (http://www6.appliedbiosystems.com /support/tutorials/pdf/quant_pcr.pdf). Serial dilutions were run in triplicate and standard curves were generated using LightCycler® 480 system (Roche Diagnostics Brussels, Belgium). External standard curves were imported for absolute quantification of dsRNA virus and 18s rRNA. PCR amplicons of

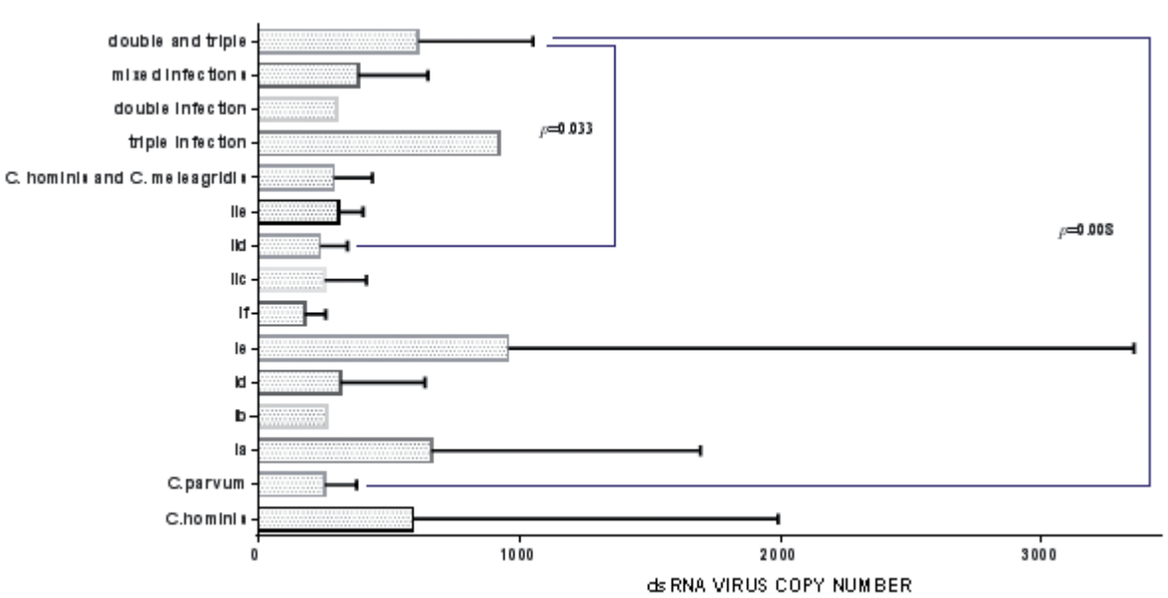

Fig. 1. Column graph showing dsRNA virus copy number in different *Cryptosporidium* isolates
Explanations: double and triple: mixed infections of *C. hominis* and *C. parvum* isolates (n=2); mixed infections: all the mixed infection cases (n=7); double infection: double infection of *C. hominis* and *C. parvum* isolate (n=1); *C. hominis* and *C. meleagridis*: mixed infections of these two species (n=5)

dsRNA virus each from *C. hominis* and *C. parvum* isolates were sequenced and sequences were aligned with reference sequences to find any genotypic variation.

Statistical analysis. Copy number of dsRNA virus and 18srRNA was calculated in different isolates. Viral copy number per oocyst was calculated as ratio of dsRNA virus copy number to 18s rRNA copy number. Mean value of dsRNA copy number and mean dsRNA virus/18s rRNA (CSpV/rRNA) ratios between *Cryptosporidium* species or subtypes were compared in an unpaired *t*-test. Association of CSpV copy number or CSpV/rRNA ratio with diarrhoea, vomiting, fever or any one of these symptoms was analysed by Mann-Whitney U test. All the statistical analyses were done using SPSS version 16.0 (SPSS Inc., Chicago, Illinois, USA).

Results

Cryptosporidium species and subtypes

A total of 63 samples of 35 males and 28 females including 5 children were microscopically positive for *Cryptosporidium* oocysts. These were characterised as 46 *C. hominis* and 17 *C. parvum* by 18s rDNA based restriction analysis. Sequence analysis of GP60 gene revealed 7 cases of mixed infection, 5 of *C. meleagridis* with *C. hominis* subtypes, one as triple infection of two *C. hominis* subtypes with one *C. parvum* subtype, and one as double infection of *C. hominis* and *C. parvum* subtypes. There were 5 subgenotypes (Ia, Ib, Id, Ie, If) with 16 different subtypes and 3 subgenotypes (IIc, IId, IIe) with 4 different subtypes for *C. hominis* and *C. parvum,* respectively. All the unique GP60 sequences obtained in this study have been submitted to Genbank under accession numbers HQ241927-HQ241932, JF268622-JF268649, and JF495136-JF485160.

Clinical features

Clinical data was recorded as diarrhoea, vomiting, fever, cough or any of the symptoms. Clinical symptoms were reported in 60.9% (28/46) *C. hominis* infected patients with diarrhoea in 27, vomiting in 2, fever in 10 and cough in 3 while 64.7% (11/17) of *C. parvum* infected patients had symptoms with diarrhoea in 10, vomiting in none, fever in 3 and cough in 2 patients.

Viral copy number in different isolates

Viral copy number in different *Cryptosporidium* isolates was determined by qRT-PCR and expressed as *Cryptosporidium* dsRNA virus/18s rRNA ratio (CSpV/RNA). The dsRNA virus copy number in *Cryptosporidium* isolates from 56 sample with single infection and 7 samples with mixed infections is given in Table 1. The mean value of ds RNA virus copy number in *Cryptosporidium* isolates from single infection (n=56) cases was 492 (range, 42–8170), and that in *C. hominis* (n=39) isolates was higher (594.7; range, 82–8170) as compared to *C. parvum* (n=17) isolates (256.65; range, 42–506), but this difference was not statistically significant (*p* value, 0.326). Similarly no difference in mean value of dsRNA virus copy number in *C. hominis* and *C. parvum* subgenotype

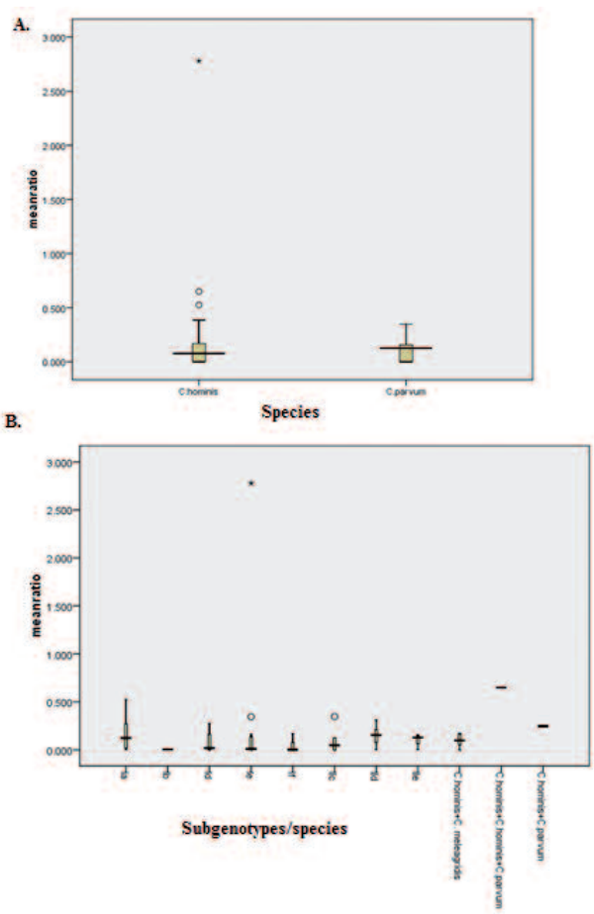

Fig. 2. Box plots showing mean dsRNA virus/18s RNA ratio in *C. parvum* or *C. hominis* (A) and *C. hominis* and *C. parvum* subgenotypes and in mixed infection cases (B)

Explanations: the median (central line), 25% and 75% quartile ranges (IQR/box length) and upper and lower limits (bars) are shown. Outliers (more than 1.5 times the IQR) are represented by circles and extreme values (more than 3 times the IQR) are shown by asterisks

Table 2.Viral copy number and CSpV/rRNA mean ratio (dsRNA virus/oocyst) in *Cryptosporidium* species and subtypes as determined by t-test for equality of means

Species/subgenotype	Viral copy number (mean±SD) *p* value	CSpV/rRNA mean ratio (mean ± SD) *p* value
C. hominis (n=39)	594.7 ± 1396	0.17 ± 0.4
C. parvum (n=17)	256.7 ± 121.9 (0.326)	0.12 ± 0.11 (0.665)
Ia (n=13)	664.3 ± 1030	0.159 ± 0.16
Ib (n=1)	266	0.004
Id (n=9)	316.7 ± 322.9	0.09 ± 0.105
Ie (n=11)	958.5 ± 2394.17	0.317 ± 0.82
If (n=5)	179.5 ± 81.12	0.05 ± 0.07
IIc (n=6)	255.2 ± 161	.095 ± 0.135
IId (n=8)	237.3 ± 106	0.15 ± 0.102
IIe (n=3)	311 ± 92	0 0.096 ± 0.08
C. hominis + *C. meleagridis* (n=5)	291 ± 148	0.098 ± 0.07
Triple infection (n=1)	925	0.649
Double infection (n=1)	303	0.247
Mixed infections (n=7)	383.3 ± 267.9	0.198 ± 0.215
Double and triple (n=2)	614 ± 439.8	0.448 ± 0.284

Explanations: double and triple: mixed infections of *C. hominis* and *C. parvum* isolates (n=2); mixed infections: all the mixed infection cases (n=7); double infection: double infection of *C. hominis* and *C. parvum* isolate (n=1); *C. hominis* + *C. meleagridis*: mixed infections of these two species (n=5)

solates was found. The mean value of dsRNA copy number in double and triple infection cases of *C. hominis* and *C. parvum* isolates (614±439.8) was significantly higher than viral copy number in *C. parvum* isolates (256.7±121.9) and its subgenotype family IId isolates (237.3±106) (Table 2 and Fig. 1).

Mean dsRNA virus/18s rRNA (CSpV/RNA) ratio in different isolates

The ratio of mean values of ds RNA copy number and 18s rRNA copy in different isolates is represented by box-plots (Fig. 2). The median value for *C. hominis* and *C. parvum* isolates was 0.02 and

Table 3. Association of viral copy number and CSpV/rRNA mean ratio with diarrhoea, vomiting, cough, fever or any of the symptoms as determined by Mann-Whitney U test

Clinical features	Viral copy number Mann-Whitney U (*p* value)	CSpV/rRNA mean ratio Mann-Whitney U (*p* value)
Symptoms	354.5 (0.108)	383 (0.229)
Diarrhea	403.5 (0.279)	399.5 (0.255)
Vomiting	31.5 (0.279)	46 (0.59)
Cough	122.5 (0.578)	131.5 (0.739)
Fever	216.5 (0.065)	235.5 (0.128)

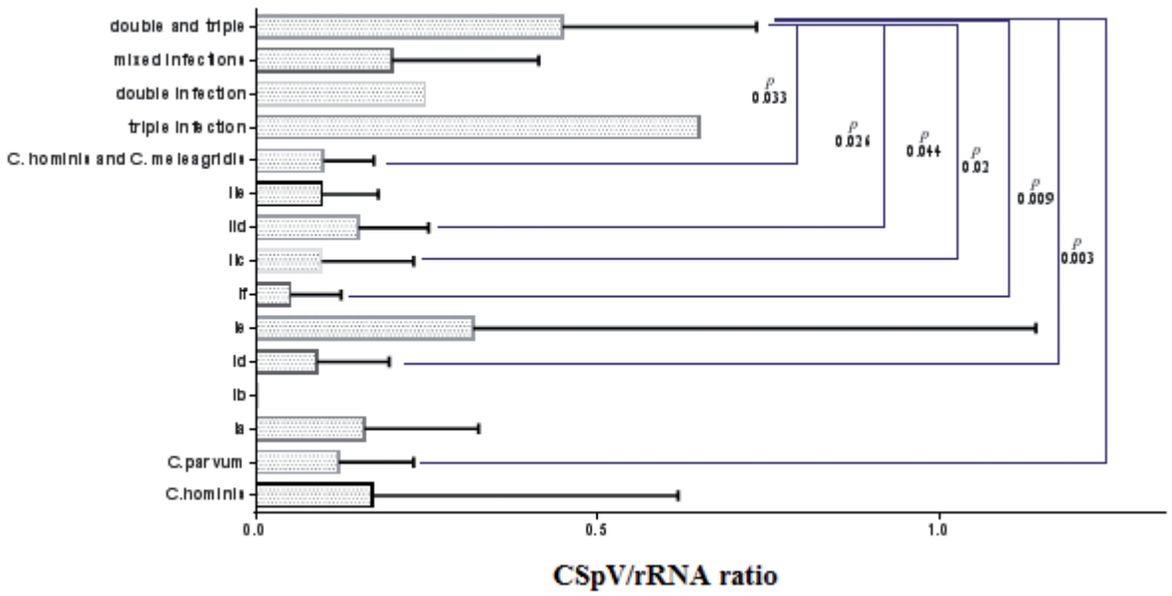

Fig. 3. Column graph showing the mean dsRNa virus/18srRNA ratio (virus copy number/oocyst) in different *Cryptosporidium* isolates

Explanations: double and triple: mixed infections of *C. hominis* and *C. parvum* isolates (n=2); mixed infections: all the mixed infection cases (n=7); double infection: double infection of *C. hominis* and *C. parvum* isolate (n=1); *C. hominis* and *C. meleagridis*: mixed infections of these two species (n=5)

0.125, respectively. The median values of CSpV/RNA ratio in *C. hominis* and *C. parvum* isolates, as well as their subgenotypes and also in mixed infections of *C. meleagridis* with *C. hominis* subgenotypes, double infection of *C. hominis* with *C. parvum* subgenotype, are not significantly different. But in triple infection mean ratio is significantly higher than all others subgenotypes and mixed infections (p value < 0.05) except *C. hominis* subtype Ie which may be due to outliers and extreme values in Ie isolates.

Mean CSpV/RNA ratio was 0.17±0.4 for *C. hominis* isolates compared with 0.12±0.11 for *C. parvum* isolates, however this difference was not statistically significant. Mean CSpV/RNA ratio in *C. hominis* subtypes Ia, Ib, Id, Ie, If was 0.16±0.16, 0.004, 0.09±0.1, 0.32±0.8, 0.05±0.7, respectively while in *C. parvum* subtypes IIc, IId, IIe, it was 0.1±0.1, 0.2±0.1, 0.1±0.08 respectively. Mean Cspv/rRNA ratio for oocysts purified from 5 mixed infections of *C. hominis* subtype with *C. meleagridis* was 0.098±0.07, while it was 0.649 and 0.247 in samples with 3 and 2 *Cryptosporidium* subtypes, respectively (Table 2).

Similar to mean value of dsRNA copy number, the mean value of dsRNA copy number per oocyst (mean Cspv/rRNA ratio) in *C. hominis* isolates was higher than *C. parvum* isolates with no statistical significance. The mean value of dsRNA copy number per oocyst in *C. hominis* and *C. parvum* subgenotype isolates was not significantly different. But, the mean Cspv/rRNA ratio in double and triple infection cases of *C. hominis* and *C. parvum* subgenotypes (0.448±0.284) was significantly higher than that in *C. parvum* isolates (0.12±0.11), *C. hominis* subgenotype family Id (0.09±0.105) and If (0.05±0.07) isolates, *C. parvum* subgenotype family IIc (0.095±0.135) and IId (0.15±0.102) isolates, and *C. hominis* and *C. meleagridis* mixed infection (0.098±0.07) cases (Table 2 and Fig. 3).

Association of dsRNA virus with cryptosporidiosis

The viral copy number and mean CSpV/RNA ratio in patients with symptoms was 419.9±100 and 0.128±0.02 (Mean±SE) with median values of 258 and 0.113 respectively while in those without symptoms was 577.6±332 and 0.212±0.115 (Mean±SE) with median values of 213 and 0.0485 and this difference was statistically not significant (Fig. 4). Similarly no association of diarrhoea, vomiting, cough and fever was found with either CSpV copy number or with CSpV/rRNA ratio (Table 3).

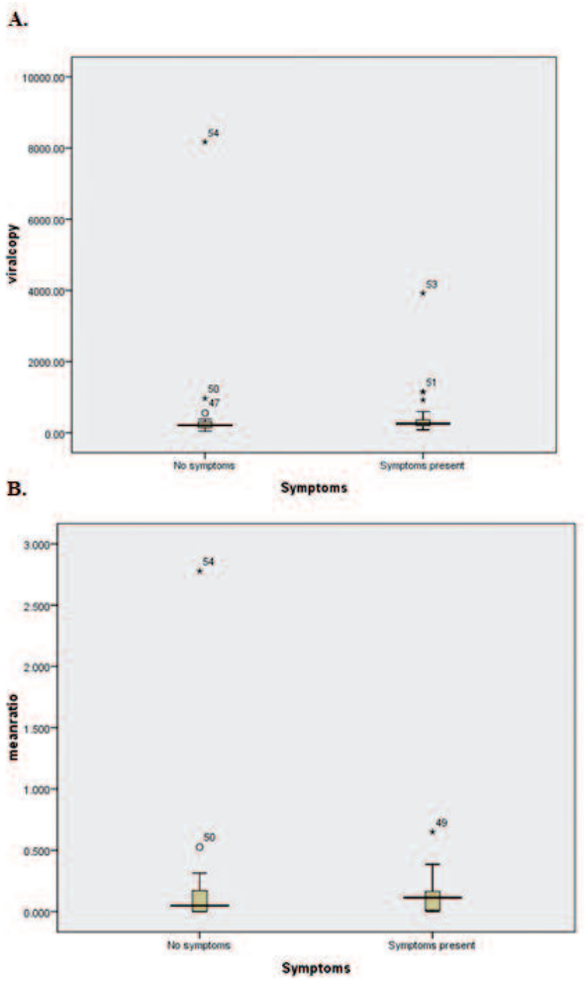

Fig. 4. Box plots showing the viral copy number (A) and CSpV/rRNA ratio (B) in patients infected with *Cryptosporidium* who are with any of the symptoms or are without any symptoms

Explanations: the median (central line), 25% and 75% quartile ranges (IQR/box length) and upper and lower limits (bars) are shown. Outliers (more than 1.5 times the IQR) are represented by circles and extreme values (more than 3 times the IQR) are shown by asterisks

Discussion

Cryptosporidiosis is a diarrheal illness of both immunocompetent and immunocompromised patients. It is characterised by significant heterogeneity in its clinical presentation such as pathogenicity, sequelae of infection etc. Host immunity though clearly plays an important role in terms of CD4+ count but the role of parasite factors is not clear yet.

Presence of dsRNA viruses belonging to virus family Partitiviridae has been known in *Cryptosporidium* species. Similar kind of intracellular viruses of family Totiviridae are known in other parasites, but there are conflicting reports of association of viral presence or numbers with disease pathology or symptomatology. In some studies, presence of virus in *Trichomonas vaginalis* (TVV) was correlated with expression of some virulence factors and presence of particular symptoms and signs in infected patients [15], but in other studies TVV was found in fresh isolates from both symptomatic and asymptomatic women [16] and TVV infection was not associated with clinical signs as presence of discharge, dysuria, genital pruritus, genital irritation or odour [17]. *Leishmania* RNA virus exists within many species of *Leishmania* as a stable infection [9] and only parasites with high levels of LRV exacerbated disease severity i.e. progression of cutaneous to mucocutaneous and disseminated leishmaniasis [18,19]. In *Giardia lamblia* higher dsRNA virus density (200,000-500,000 per trophozoite) have been shown to stop parasite adherence and its growth [20] and long dsRNA have been shown to downregulate specific genes in *Giardia lamblia* [21]. Infections of the *Cryptosporidium* host cells appear to be persistent and largely avirulent [10]. Although *Cryptosporidium* species are pathogens of humans and other vertebrates, there are so far no well-established examples in which parasite pathogenicity is either positively or negatively modulated by *Cryptosporidium* virus infection [11].

In the present study we have looked for the viral presence in clinical isolates, quantified the viral load and then statistically analysed for association of viral load with clinical symptomatology. dsRNA viruses were detected in all clinical isolates from North India. This is in concordance with reports from other parts of world where the presence of intracellular viruses have been shown in *C. parvum* genotypes I and II (*C. hominis* and *C. parvum*) [22] and also in *C. meleagridis* [23]. In our study all the isolates were either *C. hominis* or *C. parvum* and *C. meleagridis* was found only as concurrent infection with *C. hominis* [12].

The copy number of dsRNA virus in triple infection (925) cases of *C. hominis* and *C. parvum* subgenotypes was higher than all other subgenotypes of single infections cases with few exceptions, and *C. hominis* + *C. meleagridis* mixed infection cases (p value < 0.05). Similarly median value of dsRNA copy number per oocyst in triple infection of *C. hominis* and *C. parvum* subgenotypes was significantly higher than *C. hominis* and *C. meleagridis* mixed infections as well all other *C.*

hominis and *C. parvum* subgenotype isolates from single infection cases except subgenotype Ie isolates. dsRNA copy number and mean CSpV/rRNA ratio was also higher in *C. hominis* and *C. parvum* double infection case as compared to mean values in mixed infections of *C. meleagridis* with *C. hominis* subgenotype isolates. This may be due to non-amplification of dsRNA viruses from *C. meleagridis* isolates which in turn may be due to variability in primer binding regions, attributable to variability in dsRNA virus sequence. This is supported by earlier studies where they had identified one mismatch in *C. meleagridis* sequence at both primer-annealing regions (using different set of primers) and diversity of 86% from *C. hominis* and *C. parvum* [24]. This is the first study to quantify dsRNA copy number in different *C. hominis* and *C. parvum* clinical isolates and analysing at subgenotype level.

We did not find any association of viral copy number with clinical manifestations which is in contrast to Jenkins *et al.* [13] who have shown association of higher viral load in *C. parvum* Iowa isolate with more oocyst production in calves when compared to *C. parvum* Beltsville isolate, however its relationship with severity of disease in calves was not sought. No association of *Cryptosporidium* virus with clinical symptomatology is supported by similarity of this virus with other members of family patitiviridae. Members of this virus family are known to be associated with persistent, largely avirulent infections of hosts [22,25,26].

Acknowledgements

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References

[1] Bouzid M., Hunter P.R., Chalmers R.M., Tyler K.M. 2013. *Cryptosporidium* pathogenicity and virulence. *Clinical Microbiology Reviews* 26:115-134.

[2] Drakulovski P., Carcy B., Moubri K., Carret C., Depoix D., Schetters T.P., Gorenflot A. 2003. Antibodies raised against Bcvir15, an extrachromosomal double-stranded RNA-encoded protein from *Babesia canis*, inhibit the *in vitro* growth of the parasite. *Infection and Immunity* 71:1056-1067.

[3] Wang A.L., Wang C.C. 1991. Viruses of parasitic protozoa. *Parasitology Today* 7:76-80.

[4] Khoshnan A., Alderete J.F. 1995. Characterization of double-stranded RNA satellites associated with the *Trichomonas vaginalis* virus. *Journal of Virology* 69:6892-6897.

[5] Provenzano D., Khoshnan A., Alderete J.F. 1997. Involvement of dsRNA virus in the protein composition and growth kinetics of host *Trichomonas vaginalis*. *Archives of Virology* 142:939-952.

[6] Wang A., Wang C.C., Alderete J.F. 1987. *Trichomonas vaginalis* phenotypic variation occurs only among trichomonads infected with the double-stranded RNA virus. *Journal of Experimental Medicine* 166:142-150.

[7] Macbeth K.J., Patterson J.L. 1995. Single-site cleavage in the 5'-untranslated region of *Leishmania* virus RNA is mediated by the viral capsid protein. *Proceedings of the National Academy of Sciences of the United States of America* 92:8994-8998.

[8] Macbeth K.J., Patterson J.L. 1998. Overview of the *Leishmania* virus endoribonuclease and functions of other endoribonucleases affecting viral gene expression. *Journal of Experimental Zoology* 282:254-260.

[9] Zangger H., Ronet C., Desponds C., Kuhlmann F.M., Robinson J., Hartley M.A., Prevel F., Castiglioni P., Pratlong F., Bastien P., Muller N., Parmentier L., Saravia N.G., Beverley S.M., Fasel N. 2013. Detection of *Leishmania* RNA virus in *Leishmania* parasites. *PLoS Neglected Tropical Diseases* 7:e2006.

[10] Khramtsov N.V., Upton S.J. 2000. Association of RNA polymerase complexes of the parasitic protozoan *Cryptosporidium parvum* with virus-like particles: heterogeneous system. *Journal of Virology* 74:5788-5795.

[11] Nibert M.L., Woods K.M., Upton S.J., Ghabrial S.A. 2009. Cryspovirus: a new genus of protozoan viruses in the family Partitiviridae. *Archives of Virology* 154:1959-1965.

[12] Sharma P., Sharma A., Sehgal R., Malla N., Khurana S. 2013. Genetic diversity of *Cryptosporidium* isolates from patients in North India. *International Journal of Infectious Diseases* 17:e601-605.

[13] Jenkins M.C., Higgins J., Abrahante J.E., Kniel K.E., O'brien C., Trout J., Lancto C.A., Abrahamsen M.S., Fayer R. 2008. Fecundity of *Cryptosporidium parvum* is correlated with intracellular levels of the viral symbiont CPV. *International Journal for Parasitology* 38:1051-1055.

[14] Arrowood M.J. 2002. *In vitro* cultivation of cryptosporidium species. *Clinical Microbiology Reviews* 15:390-400.

[15] Fraga J., Rojas L., Sariego I., Fernandez-Calienes A., Nunez F.A. 2007. Double-stranded RNA viral infection of *Trichomonas vaginalis* and association with clinical presentation. *Acta Protozoologica* 46:93-98.

[16] Malla N., Kaul P., Sehgal R., Gupta I. 2011. The

presence of dsRNA virus in *Trichomonas vaginalis* isolates from symptomatic and asymptomatic Indian women and its correlation with in vitro metronidazole sensitivity. *Indian Journal of Medical Microbiology* 29:152-157.

[17] Wendel K.A., Rompalo A.M., Erbelding E.J., Chang T.H., Alderete J.F. 2002. Double-stranded RNA viral infection of *Trichomonas vaginalis* infecting patients attending a sexually transmitted diseases clinic. *Journal of Infectious Diseases* 186:558-561.

[18] Ives A., Ronet C., Prevel F., Ruzzante G., Fuertes-Marraco S., Schutz F., Zangger H., Revaz-Breton M., Lye L.F., Hickerson S.M., Beverley S.M., Acha-Orbea H., Launois P., Fasel N., Masina S. 2011. *Leishmania* RNA virus controls the severity of mucocutaneous leishmaniasis. *Science* 331:775-778.

[19] Ronet C., Beverley S.M., Fasel N. 2011. Muco-cutaneous leishmaniasis in the New World: the ultimate subversion. *Virulence* 2:547-552.

[20] Miller R.L., Wang A.L., Wang C.C. 1988. Identification of *Giardia lamblia* isolates susceptible and resistant to infection by the double-stranded RNA virus. *Experimental Parasitology* 66:118-123.

[21] Rivero M.R., Kulakova L., Touz M.C. 2010. Long double-stranded RNA produces specific gene downregulation in *Giardia lamblia*. *Journal of Parasitology* 96:815-819.

[22] Khramtsov N.V., Chung P.A., Dykstra C.C., Griffiths J.K., Morgan U.M., Arrowood M.J., Upton S.J. 2000. Presence of double-stranded RNAs in human and calf isolates of *Cryptosporidium parvum*. *Journal of Parasitology* 86:275-282.

[23] Leoni F., Gallimore C.I., Green J., Mclauchlin J. 2003. Molecular epidemiological analysis of *Cryptosporidium* isolates from humans and animals by using a heteroduplex mobility assay and nucleic acid sequencing based on a small double-stranded RNA element. *Journal of Clinical Microbiology* 41:981-992.

[24] Leoni F., Amar C., Nichols G., Pedraza-Diaz S., McLauchlin J. 2006. Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhoea in England between 1985 and 2000. *Journal of Medical Microbiology* 55:703-707.

[25] Ghabrial S.A., Buck K.W., Hillman B.I., Milne R.G. 2005. Partitiviridae. In: *Virus taxonomy: eighth report of the International Committee on Taxonomy of Viruses*. (Eds. C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball). Elsevier-Academic Press, San Diego, California, USA: 581-590.

[26] Ghabrial S.A., Ochoa W.F., Baker T.S., Nibert M.L. 2008. Partitiviruses: General Features. In: *Encyclopedia of virology*. (Eds. B.W.J. Mahy, M.H.V. van Regenmortel). Elsevier-Academic Press: 68-75.

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