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Research article

DIFFERENTIAL EFFECT OF PAROMOMYCIN AND NITAZOXANIDE ON CLINICAL ISOLATES OF CRYPTOSPORIDIA *IN VITRO.*

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ABSTRACT: Background: Cryptosporidiosis is one of the most difficult protozoan infections to treat, with only two drugs *i.e.* nitazoxanide and paromomycin known for treatment with variable response in different patients. Human cryptosporidiosis is accounted mainly by *C. hominis* and *C. parvum*. These two species or their subtypes are known to differ in clinical manifestations, and may differ in their response to drugs. So, we planned the study to see the effect of nitazoxanide and paromomycin on different isolates of *Cryptosporidium in vitro*.

Methods: MDCK cell lines were used for *in vitro* growth of parasite and cytotoxicity of drugs to MDCK cells was determined by MTT assay after 3, 12 and 24 hours of drug exposure. Efficacy of non-toxic drug concentrations (<25% cytotoxic) on 12 *Cryptosporidium* isolates (7 *C. hominis* and 5 *C. parvum*) was determined at three different life cycle stages (*in vitro* growth, invasion and oocyst) by quantitative RT-PCR. Unpaired t-test was used to calculate the difference response of *Cryptosporidium* isolates to nitazoxanide and paromomycin.

Results and conclusions: Cytotoxicity of nitazoxanide and paromomycin increased in dose and time dependent manner. After 24 hours of drug exposure, >25% cytotoxic effect was seen with nitazoxanide and paromomycin at concentrations of more than, 25µg/ml and 6mg/ml, respectively. Nitazoxanide was more effective than paromomycin in decreasing *in vitro* growth, invasion inhibition and reducing oocyst viability of *Cryptosporidium* isolates. Drugs effect was higher on growth inhibition followed by invasion inhibition and least in decreasing oocyst viability. Different isolates had variable response to drugs; cumulatively *C. parvum* isolates were more susceptible at particular drug concentrations than *C. hominis* isolates**.**

Keywords: MDCK, q-RT-PCR, oocyst viability, invasion-inhibition, C. hominis, subgenotype.

INTRODUCTION

Cryptosporidiosis has proved to be one of the most difficult protozoan infections to treat. Hundreds of compounds have been tested, but success is evading (Stockdale, H. D. et al.,2008; Cello, J. P. and Day, L. W.,2009). There are only two drugs (nitazoxanide and paromomycin) known for the treatment of cryptosporidiosis; both have varying response in different patients, in terms of stool frequency and oocyst clearance. Paromomycin, though, effective in treating symptomatic cryptosporidial diarrhea, but is less consistent than nitazoxanide and does not always lead to sustained response (Cello, J. P. and Day, L. W.,2009). Nitazoxanide has been approved by US FDA for treatment of cryptosporidiosis in children, adults and teenagers who do not have HIV, but it exhibits moderate clinical efficacy in immunocompetent adults and children and is not effective in patients with advanced AIDS (White, C. A., Jr.,2004; Fox, L. M. and Saravolatz, L. D.,2005; Abubakar, I. et al.,2007). In the latter group, institution of HAART and improvement in CD4 count leads to improvement suggesting the role of host immunity in effectiveness of drugs.

Two *Cryptosporidium* species *C. hominis* and *C. parvum* are predominantly associated with human disease; these 2 parasite species are genetically identical with 95-97% DNA sequence identity and antigenically almost indistinguishable (Sheoran, A. et al.,2012; Widmer, G. and Sullivan, S.,2012). *C. parvum* is considered to infect mammalian species, including humans while *C. hominis* mainly infects humans (Tzipori, S. and Ward, H.,2002; Sheoran, A. et al.,2012; Widmer, G. and Sullivan, S.,2012). Furthermore some studies have reported differences in clinical manifestations between species and in between *C. hominis* subgenotype families (Cama, V. A. et al.,2007; Cama, V. A. et al.,2008; Iqbal, J. et al.,2011).

All the studies related to drug sensitivity assays of *Cryptosporidium* including either chemical- (nitazoxanide, paromomycin, azithromycin, cyclosporine analogues *etc.*) or natural compounds (curcumin, bovine lactoferrin, honey venom phospholipase A2 *etc.*) has been done on *C. parvum* reference isolates (IOWA, Beltsville, GCH1 *etc.*) or isolates from calves (Marshall, R. J. and Flanigan, T. P.,1992; Perkins, M. E. et al.,1998; Theodos, C. M. et al.,1998; You, X. et al.,1998; Giacometti, A. et al.,2000; Siripanth, C. et al.,2004; Chin Lee, S. H. et al.,2008; Downey, A. S. et al.,2008; Shahiduzzaman, M. et al.,2009; Carryn, S. et al.,2012). None of the studies have been done to see the effect of drugs on different isolates of *C. hominis* or its different subtypes. Thus we undertook this study to look for effect of nitazoxanide and paromomycin on subtypes and strains of *C. hominis* and *C. parvum*, *in vitro*. Nitazoxanide was found to be more effective than paromomycin, with maximum effect on growth inhibition followed by invasion inhibition and least on oocyst viability. Detrimental effect of both the drugs was more on *C. parvum* isolates as compared to that on *C. hominis* isolates.

MATERIALS AND METHODS

(A) Samples and processing

The work was approved by the ethics committee of Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh (Reference number 11/5470 dated 31/01/2011). Stool samples were collected from HIV positive patients, children and other immunocompromised patients. *Cryptosporidium* oocysts were detected in dry faecal smears by special staining techniques as described previously (Sharma, P. et al.,2013). Cryptosporidia were genotyped and subgenotyped by 18s rRNA based PCR-RFLP (Xiao, L. et al.,2001) and *Gp*60 based sequence analysis (Alves, M. et al.,2003; Sulaiman, I. M. et al.,2005). *Cryptosporidium* oocysts were purified from stool samples by density gradient centrifugation. Briefly, the stool samples stored at 4°C in 2.5% $K_2Cr_2O_7$ solution, were mixed well, filtered through a 2-layered gauge and subjected to purification by sucrose density gradient centrifugation (Arrowood, M. J.,2002; Arrowood, M. J.,2008). Oocyst viability was determined by trypan blue staining and oocysts were counted under 40× of light Microscope.

(B) Oocyst production in mice

C. hominis isolates having about 10^3-10^5 viable oocysts/µl were used in the study. *C. parvum* isolates with inadequate number of viable oocysts were amplified in neonatal mice (Swiss Albino). Neonatal pups were kept with their mothers in isolated cages and were infected with single isolate to prevent mixing of strains. All the experiments were carried out in Central Small Facility Animal House and the work was approved by the Institutional Animal Ethics Committee, PGIMER, Chandigarh (Reference number 60/IAEC/346 dated 20/07/2012). For infection of mice, oocysts were washed with normal saline to remove traces of potassium dichromate ($K_2Cr_2O_7$) and inoculated by orogastric route (Arrowood, M. J.,2008). Briefly, 4-8 days old, neonatal mice were infected with 5×10^4 to 5×10^5 oocysts by delivering the inoculum in saline (10µl) to the back of the mouth (using a micropipette and a disposable tip). At 7-8 days post-inoculation, mice were euthanized with chloroform; intestines were removed and placed in 2.5% $K_2Cr_2O_7$. Intestines were homogenized with a glass tissue grinder and *Cryptosporidium* oocysts were detected in intestinal homogenate smears after fluorescent staining. Oocysts from the intestinal homogenates were purified by sucrose density gradient centrifugation (Arrowood, M. J.,2002) and used to inoculate into cell cultures for *in vitro* drug sensitivity study.

(C) Host cell culture:

Madine Darby Canine Kidney (MDCK) cell lines were purchased from National Centre for Cell Science (NCCS), Pune. These were grown in 25 cm^2 culture flasks (Greiner Bio-one, Frickenhausen, Germany) in minimum essential medium (MEM, HiMedia, Mumbai, India) supplemented with 3% L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 1.1% sodium pyruvate (HiMedia, Mumbai, India), penicillin-streptomycin and 10% fetal bovine serum (FBS, HiMedia, Mumbai, India) at 37°C with 5% CO_2. Cell lines were allowed to grow for 48-72 h till the formation of confluent monolayers and the growth medium was changed every 48 h. After the formation of confluent monolayers (examined under inverted phase contrast microscope), cell lines were maintained in maintenance medium (complete MEM supplemented with 2% FBS). The cell lines were then infected with oocysts of *C. hominis* and *C. parvum* isolates as detailed below.

(D) Drugs

Nitazoxanide (NTZ) and paromomycin sulphate (PRM) were obtained from Sigma-Aldrich (Sigma, USA). Stock solution of paromomycin (100mg/ml) was prepared in MEM and that of nitazoxanide (1mg/ml) was dissolved in 100 µl of sterile DMSO with a final volume adjusted to 1000 µl with MEM.

(E) Host cell viability assay:

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the threshold of toxicity of NTZ and PRM for MDCK cell lines. For this, MDCK cells (3×10^4 cells/well) were seeded in 96-well plates and incubated to 90% confluency at 37°C with 5% CO_2. Freshly prepared dilutions of NTZ (100µg/ml to 6µg/ml) and PRM (10mg/ml to 0.25mg/ml) in the growing medium were added to the wells and cultures were further incubated. After 3, 12, and 24 h, post-initiation exposure (freshly prepared medicated medium was replaced at 12 h interval), the cells were washed with 1× sterile PBS, and 100 µl freshly prepared working solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5mg/ml MTT, Sigma, USA) was added for a further incubation period of 3 h. After 3 h MTT solution was removed, and 100 µl of DMSO was added in both treated and non-treated wells to dissolve the converted dye (formazan). After 15 min incubation at 37°C with shaking, optical density was measured spectrophotometrically at 595 nm (620 nm reference wavelength). Cells without NTZ and PRM were taken as a positive control and cells without MTT as negative control. Percent viability was calculated relative to the non-treated control.

(F) Parasite isolates and cell culture infection:

Seven isolates of *C. hominis* subtypes IeA11G3T3 (n=3), IdA15G1 (n=2), IdA15G2, IaA19R3 and 5 isolates of *C. parvum* subtypes IIcA5G3, IIdA15G1 (n=3), and IIeA7G1 were used for *in vitro* drug sensitivity assays (Table1). MDCK cells (2×10^5 or 3×10^4/well) were seeded into 24 or 96-well plates and allowed to grow until they reached 90% confluence at 37°C with 5% CO_2 in growing medium (MEM with 10% FBS). Sucrose gradient purified oocysts stored at 4°C were used for infection of cell lines after disinfection and induction of excystation (Arrowood, M. J.,2002; Arrowood, M. J.,2008). Briefly, $K_2Cr_2O_7$ solution was washed out and oocysts were treated with bleach (sodium hypochlorite, 1:10 dilution) for 10 min at room temperature. Bleach was washed out by centrifugation at 600g for 3 minutes with PBS, and oocysts were resuspended in excystation medium (0.75% sodium taurocholate solution) and incubated for 15 minutes at 37°C. About 5×10^4 or 2×10^3 oocysts were directly excysted on the confluent MDCK cell monolayer in 24 or 96 well plates, respectively and incubated for 3 h at 37°C with 5% CO_2. Unbound parasites were removed by washing with growing medium and the infected monolayers were further maintained in the fresh growing medium for 48 h. Cell culture infection was detected by IIF. Cell culture infection by a dilution series of oocysts (1×10^6 to 10) was used to calculate the standard curve for estimation of infectivity by qRT-PCR.

(G) Effect of Drugs on Parasite Growth in Cell Culture

Indirect immunofluorescent assay (IIF):

Cryptosporidium growth in MDCK cell lines was determined by IIF using ICC protocol (Abcam, www.abcam.com/technical). Briefly, after 48 h incubation, culture media was decanted; monolayers were fixed in methanol, and blocked with 1% BSA in PBST for 30 min. Cell monolayers were incubated with mouse monoclonal antibody to *Cryptosporidium* (BEL0126, Abcam, ab54066, Cambridge, UK) in a humidified chamber overnight at 4°C. Monolayers were washed three times with PBS and incubated with FITC conjugated goat polyclonal secondary antibody to mouse (Abcam, ab97022, Cambridge, UK) for 1 h at room temperature in dark. Monolayers were counterstained with DAPI and the plates were examined under inverted fluorescent microscope equipped with a camera and appropriate filter (350 nm excitation, 450 nm emission for DAPI, and 495 nm excitation and 519 nm emission for FITC).

RNA extraction and qRT-PCR

RNA was extracted from MDCK cells infected with dilution series of oocysts and drug sensitivity assays, using Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) as per manufacturer's instructions for adherent cells. Each RNA sample was quantified by micro-volume spectrophotometer (Nanodrop2000c, Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized from RNA using a Verso cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) and a mixture of *Cryptosporidium* 18s rRNA specific forward primer and random hexamer, as per manufacturer's instructions.

For qRT-PCR 100 bp region of 18s rRNA of *Cryptosporidium* was amplified on Light Cycler 480 with primers 5' TCA GCT TTA GAC GGT AGG GTA TTG GC 3' and 5' TGT GGT AGC CGT TTC TCA GGC T 3'. *Cryptosporidium* copy number in all drug sensitivity assays was determined by absolute quantification. For absolute quantification standard curve was constructed with cDNA prepared from RNA isolated from MDCK cell lines infected with dilution series of *Cryptosporidium hominis* oocysts (IdA11G2 $1.6\text{x}10^1$, $1.6\text{x}10^2$, $1.6\text{x}10^3$, $1.6\text{x}10^4$, $1.6\text{x}10^5$, $1.6\text{x}10^6$). Copy number of Cryptosporidia in drug treated wells, positive control wells (MDCK cells infected with Cryptosporidia) and negative control wells (uninfected cells) was calculated by importing the external standard curve.

(G i) Effect of drugs on *Cryptosporidium* spp. growth *in vitro* (Shahiduzzaman, M. et al.,2009)

Cryptosporidium growth inhibition test was performed, with working concentrations of NTZ (25 µg/ml - 6 µg/ml) and PRM (6mg/ml - 2mg/ml). After 3 h of excystation, the remaining sporozoites as well as not excysted oocysts and oocyst walls were washed out, and medicated medium was added to the wells. The medicated medium was replaced at 12 h post-initiation of exposure with a fresh preparation of the same concentration of drugs. After 24 h, drugs were removed by washing the cells with growing medium, and the cultures were maintained in the fresh growing medium for another 24 h. At the end of the incubation period, *Cryptosporidium* infection was detected by qRT-PCR. For each *Cryptosporidium* isolate tested for *in vitro* growth inhibition, infected cells without drug was included as positive control and uninfected cells without drugs as negative control.

(Gii) Effect of drugs on invasion-inhibition(Shahiduzzaman, M. et al.,2009)

For invasion inhibition experiments, about 2×10^3 oocysts were incubated on cell culture monolayers (96 well plates) in excystation medium in the presence or absence of drugs for 3 h in 5% CO_2 at 37°C. Medicated medium was removed by washing the monolayers twice with growing medium, and cell cultures were maintained for 48 h in growing medium. After washing of monolayers, qRT-PCR was performed and percent invasion-inhibition was measured in comparison to the non-treated controls.

(Giii) Effect of drugs on oocyst viability(Shahiduzzaman, M. et al.,2009)

To measure the viability of oocysts exposed to drugs, about 1×10^5 oocysts of *C. hominis* and *C. parvum* isolates were incubated at room temperature for 24 h in the presence of drugs. After washing three times with tap water, the size of the inoculum for infection of cell cultures was adjusted to 2×10^3 oocysts/well in counting chambers. In parallel, thermally inactivated (100°C for 15 s) oocysts were used as negative control. The treated oocysts were inoculated into confluent cell monolayers and growth of *Cryptosporidium* was assessed after 48 h by qRT-PCR. The percent oocyst viability was measured in comparison to the non-treated controls.

(H) Statistical analysis: All the statistical analyses and graphs were plotted using GraphPad Prism version 6.03 (GraphPad Software, La Jolla, California, USA). Effect of drugs on in vitro growth inhibition, invasion inhibition and oocyst viability of c. hominis and C. parvum isolates were analysed by unpaired t-test. A *p*-value ≤0.05 was considered significant.

RESULTS

Samples

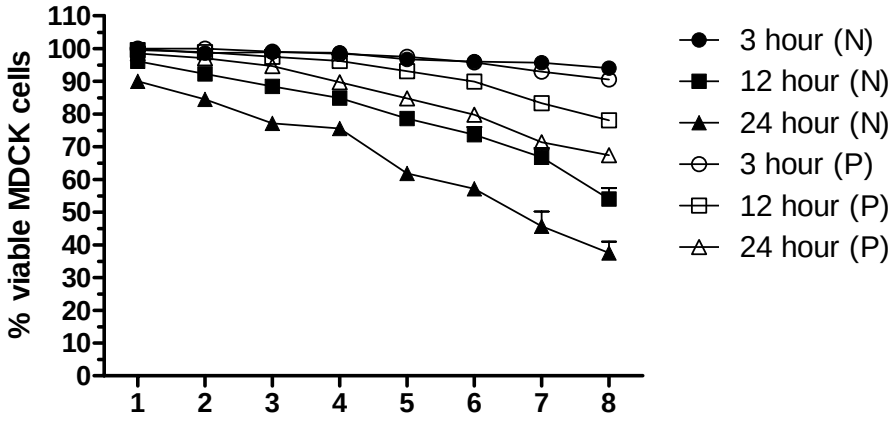
A total of 12 *Cryptosporidium* isolates from stool samples were used in the study which included, 10 isolates from HIV positive patients, one of Child and one of renal transplant patient. These included 7 *C. hominis* and 5 *C. parvum* isolates. (Table1).

Table 1: *Cryptosporidium* isolates used for *in vitro* drug sensitivity assays.

Cryptosporidium subtype	Source	Gene Bank Accession no.
C. hominis IeA11G3T3	HIV	KC813490
C. parvum IIeA7G1	HIV	KC813485
C. hominis IdA15G1	HIV	JF268631
C. parvum IIdA15G1	HIV	KC813489
C. hominis IdA15G1	HIV	KC813481
C. parvum IIcA5G3	HIV	KC813479
C. hominis IeA11G3T3	HIV	JF268639
C. parvum IIdA15G1	Child	KC813478
C. hominis IeA11G3T3	Renal transplant	KC813483
C. parvum IIdA15G1	HIV	KC813484
C. hominis IaA19R3	HIV	KC813480
C. hominis IdA11G2	HIV	KC813482

MDCK cell viability

Cytotoxic effect of nitazoxanide and paromomycin was determined by measuring the reduction of yellow MTT by mitochondrial succinate dehydrogenase to dark purple formazan product. After 3 hours of drug exposure the effect of NTZ (50µg/ml) and PRM (6mg/ml) was nearly equal. While after 12 and 24 hours, NTZ was more cytotoxic than PRM, and this cytotoxic effect increased with increasing individual drug concentrations in a time dependent manner. After 24 hours, at the highest concentrations of paromomycin (10mg/ml), 67% host cells were viable, while at higher concentration of NTZ (80µg/ml), 37.5% MDCK cells were viable. NTZ and PRM concentrations at which more than 75% host cells were viable after 24 hours incubation (upto 25 µg/ml of NTZ and 6mg/ml of PRM), were considered to be non-toxic and used further for drug sensitivity testing of *Cryptosporidium* isolates (Figure1).



	1	2	3	4	5	6	7	8
Nitazoxanide (µg/ml)	6	10	20	25	40	50	60	80
Paromomycin (mg/ml)	0.25	0.50	1.00	2.00	4.00	6.00	8.00	10.00

Figure 1 Viability of MDCK cells in nitazoxanide (N) and paromomycin sulphate (P) treated monolayers (control=100%) at 3, 12 and 24 hours of drug exposure. Error bar indicates standard deviation of means from duplicate experiments (three replicates in each experiment)

Cell culture infection

Infection of MDCK cell lines with *Cryptosporidium* oocysts was determined by indirect immunofluorescence assay. The indirect immunofluorescence assay was used in initial set up of experiments *i.e.* detection of infection of MDCK cell lines with *Cryptosporidium* oocysts, and detection of cryptosporidia grown in MDCK cells infected with dilution series of oocysts (Figure 2 and 3). Dilution series of oocysts from *C. hominis* subtype isolate IdA11G2 were used for infection of cell lines for the standard curve.

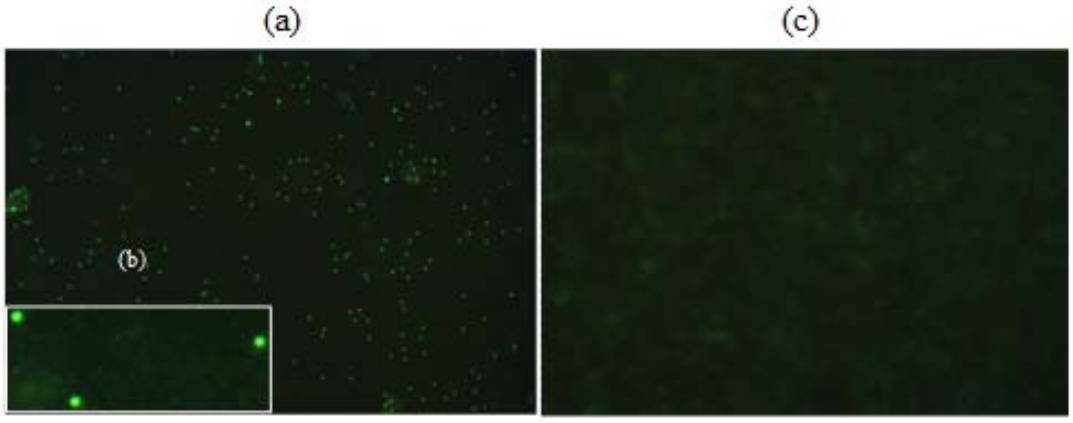


Figure 2 Panel (a) and (b) show *Cryptosporidium* grown in MDCK cell monolayers stained with FITC conjugated monoclonal antibody at low (10×) and high magnification (40×), panel (c) show uninfected monolayers

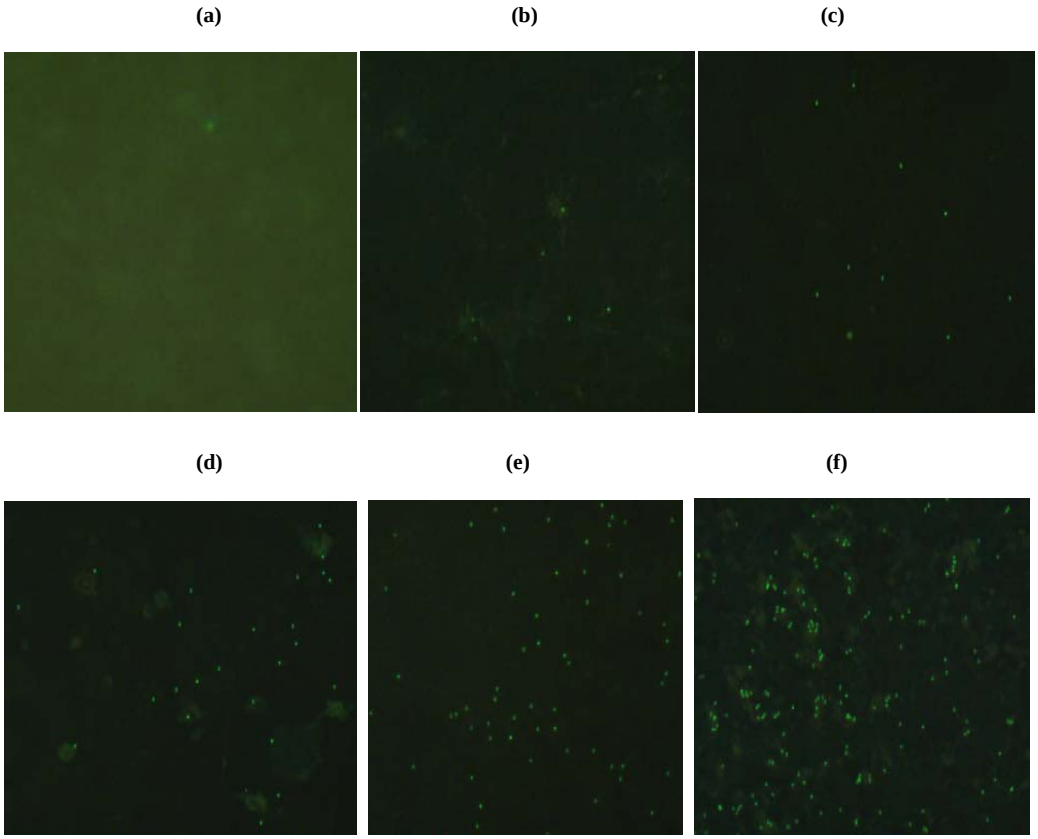


Figure 3 Panels (a)-(f) showing, MDCK cells infected with dilution series of *Cryptosporidium* oocysts stained with FITC conjugated monoclonal antibody (a) 1.6×10^{1}, (b) 1.6×10^{2}, (c) 1.6×10^{3}, (d) 1.6×10^{4}, (e) 1.6×10^{5}, (f) 1.6×10^{6} (Indirect immunofluorescent assay)

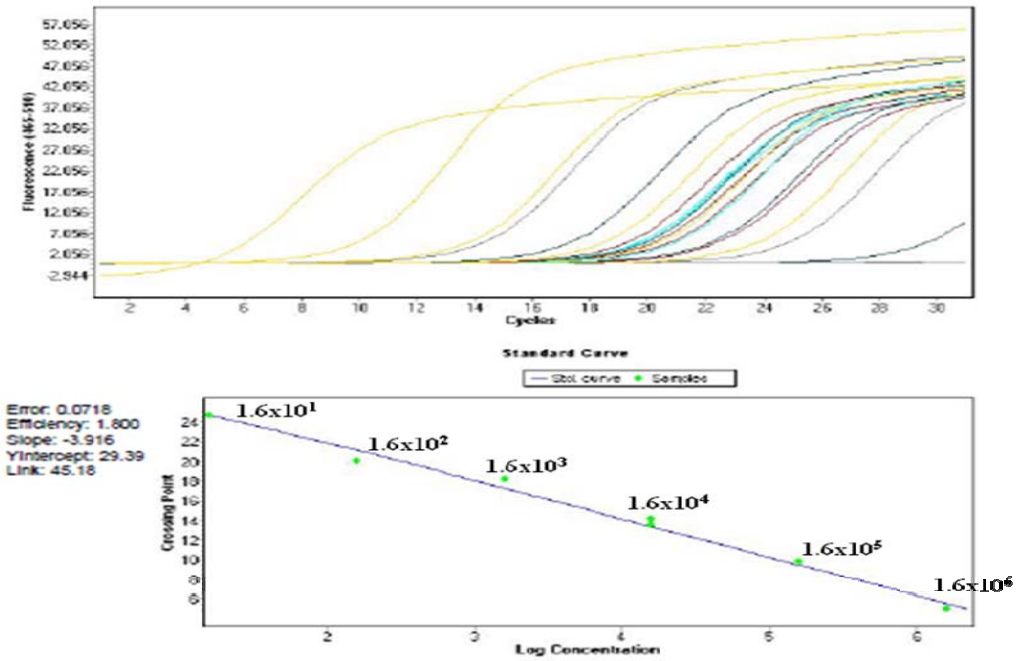


Figure 4 Real time PCR amplification of *Cryptosporidium* 18s rRNA region with cDNA of RNA extract from MDCK cell lines infected with dilution series of *C. hominis* subtype IdA11G2 oocysts showing: Amplification curves (Above) and standard curve (Below)

Standard curve and qRT-PCR

Standard curve was constructed by real-time PCR amplification of cDNA prepared from total RNA extracts of MDCK cell lines infected with dilution series of *Cryptosporidium* oocysts (*Cryptosporidium hominis* IdA11G2) by targeting *Cryptosporidium* 18s rRNA region. For absolute quantification of Cryptosporidia in different drug sensitivity assays, the standard curve was imported and copy number of Cryptosporidia was calculated (Figure 4 and 5).

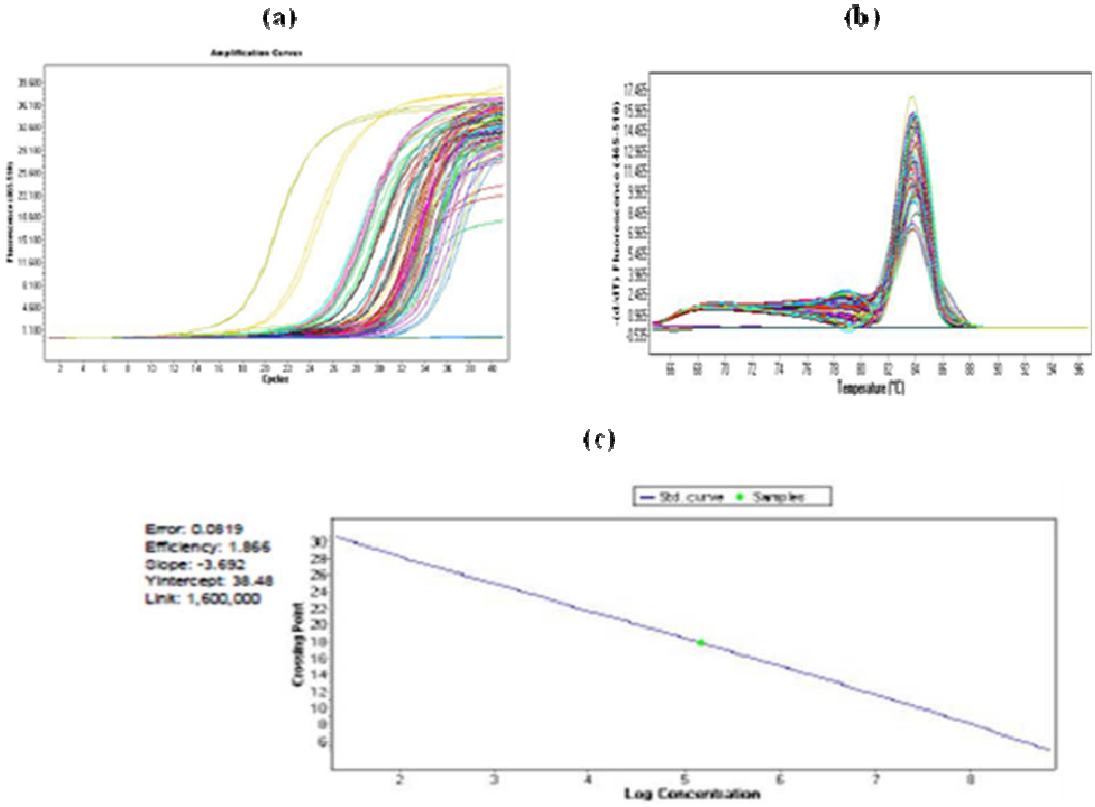


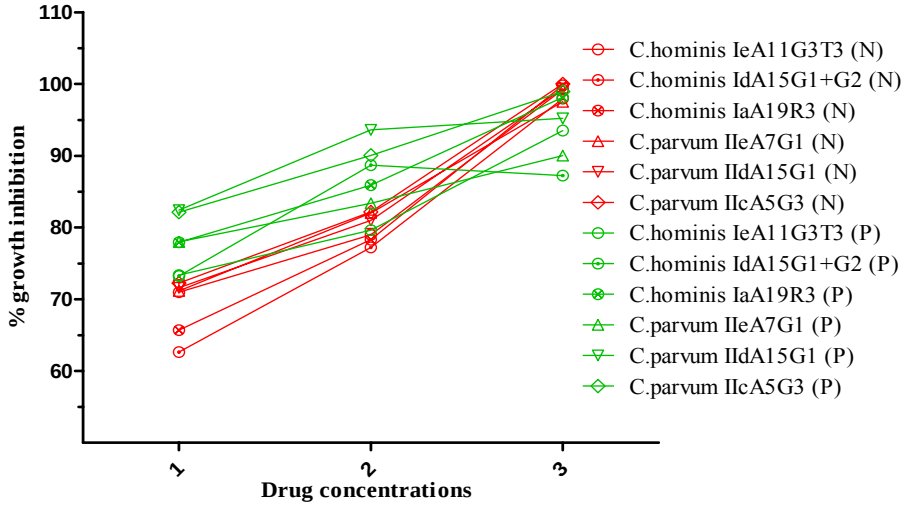
Figure 5 Representative picture showing (a) amplification curves (b) melting peaks and (c) imported standard curve for absolute quantification of Cryptosporidia in drug sensitivity assays

In vitro growth inhibition test

NTZ and PRM inhibited the *in vitro* growth of *Cryptosporidium* isolates to varying extents ranging from 67%-99%. Growth inhibition was dose dependent, with the maximum growth inhibition *i.e.* 98.8%-99% and 91.1%-97.9%, at higher concentrations of NTZ and PRM, respectively. Percent growth inhibition of *C. hominis* and *C. parvum* isolates at higher concentration of NTZ (25µg/ml) was more than that of PRM (6mg/ml), but this difference was not statistically significant. The growth inhibitory effect of 2 and 4 mg/ml of PRM on *C. parvum* isolates (76.8% and 88.2%) was significantly higher as compared to *C. hominis* isolates (67% and 82.4%). At 6 mg/ml the growth inhibition of *C. parvum* (97.9%), was not significantly higher than *C. hominis* (91%). Similar trend was observed with nitazoxanide *i.e.* at lower concentrations (6 and 10µg/ml) growth of *C. parvum* (71.6% and 81%) isolates was inhibited to a greater extent than *C. hominis* isolates (66.6% and 78%), while at the higher concentration, effect on growth inhibition was almost similar (Figure 6, Table 2).

In vitro invasion inhibition test:

Both the drugs were effective in inhibiting the invasion process of *Cryptosporidium* sporozoites. The invasion inhibition effect of NTZ and PRM (range, 80-98% and 74-95%, respectively) increased with an increase in drug concentrations reaching 97-98% and 92-95%, at the highest concentration. Highest concentration of NTZ (25µg/ml) was more effective than that of PRM (6mg/ml) and this effect was significantly higher on *C. hominis* isolates (*p* value 0.009). Invasion inhibition effect of 2, 4 and 6 mg/ml of PRM was higher on *C. parvum* isolates (81.5, 90.9 and 94.9%, respectively) than *C. hominis* isolates (73.96, 85.7 and 91.5%) but this difference was statistically significant at 2 mg/ml. In case of NTZ, invasion inhibition of *C. parvum* isolates was slightly lower than that of *C. hominis* isolates at low concentrations (6 and 10µg/ml) but this difference faded away at highest concentration (Figure7, Table 3).

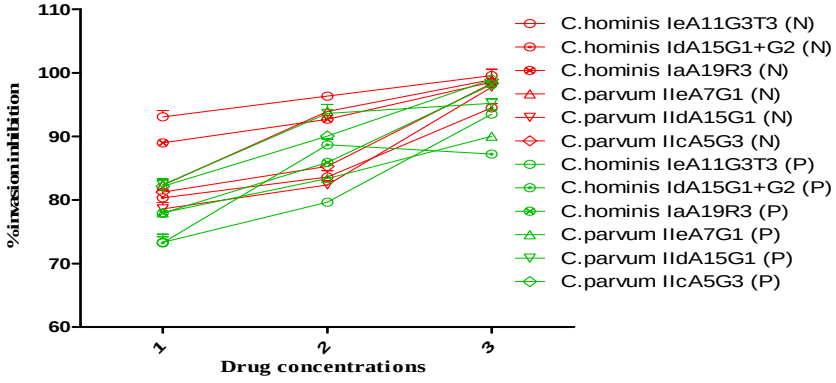


	1	2	3
Nitazoxanide (µg/ml)	6	10	25
Paromomycin (mg/ml)	2	4	6

Figure 6 Effect of nitazoxanide (N) and paromomycin sulfate (P) on *in vitro* growth inhibition of *C. hominis* and *C. parvum* isolates. Data presented as mean value of percent growth inhibition of all isolates in respective subgenotype families

Table 2: Differences in *in vitro* growth inhibition of *C. hominis* and *C. parvum* isolates at different concentrations of drugs (unpaired t-test).

	C. hominis (n=7)	*C. parvum* (n=5)	
P2mg/ml	67	76.8	0.0005*
P4mg/ml	82.4	88.2	0.0004*
P6mg/ml (a)	91.1	97.9	0.12
N6µg/ml	66.64	71.63	0.03
N10µg/ml	78	81	0.0002*
N25µg/ml (b)	98.8	99	0.5
a v/s b *p*-value	0.04	0.16	



	1	2	3
Nitazoxanide (mg/ml)	6	10	25
Paromomycin (mg/ml)	2	4	6

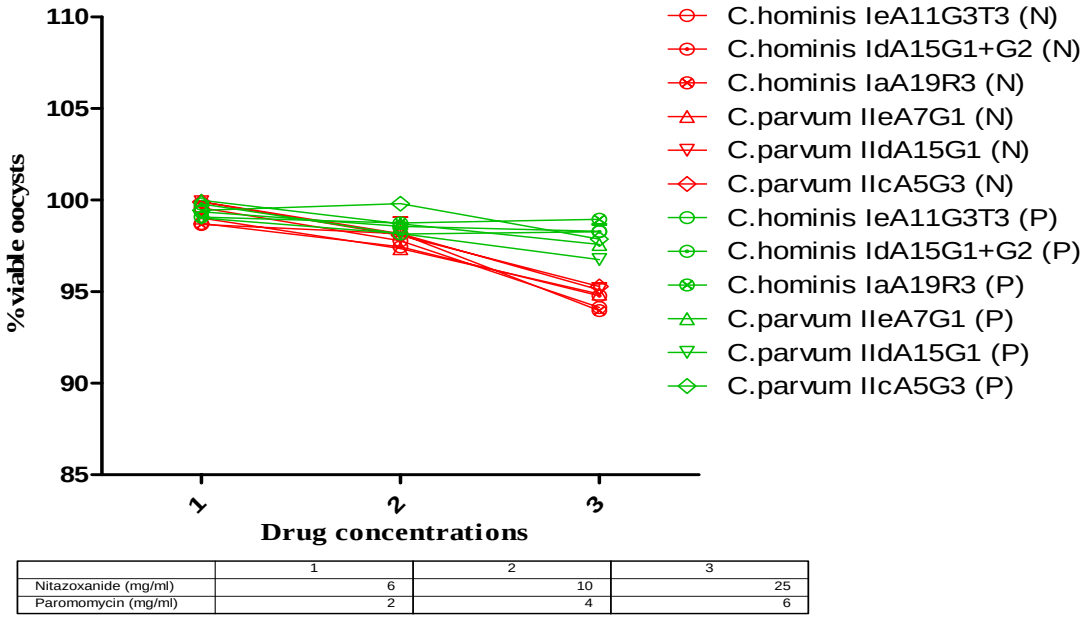
Figure 7 Effect of nitazoxanide (N) and paromomycin sulfate (P) on *in vitro* invasion inhibition of *C. hominis* and *C. parvum* isolates. Data presented as mean value of percent invasion inhibition of all isolates in respective subgenotype families

*statistically significant difference at α=0.05, P: paromomycin, N: nitazoxanide

Table 3: Differences in invasion inhibition of *C. hominis* and *C. parvum* isolates at different concentrations of drugs (unpaired t-test).

	***C. hominis* (n=7)**	***C. parvum* (n=5)**	
P2mg/ml	73.96	81.5	7.97e-5*
P4mg/ml	85.73	90.9	0.05
P6mg/ml (a)	91.5	94.9	0.2
N6µg/ml	87.04	79.9	0.04
N10µg/ml	88.31	85.3	0.5
N25µg/ml (b)	97.43	98.1	0.7
a v/s b *p*-value	0.009*	0.1	

* Statistically significant difference at α=0.05, P- paromomycin, N-nitazoxanide.



	1	2	3
Nitazoxanide (mg/ml)	6	10	25
Paromomycin (mg/ml)	2	4	6

Figure 8 Effect of nitazoxanide (N) and paromomycin sulfate (P) on oocyst viability of *C. hominis* and *C. parvum* isolates. Data presented as mean value of percent viable occysts of all isolates in respective subgenotype families

Table 4: Differences in oocyst viability of *C. hominis* and *C. parvum* isolates at different concentrations of drugs (t-test).

	***C. hominis* (n=7)**	***C. parvum* (n=5)**	
P2mg/ml	99.5	99.3	0.4
P4mg/ml	98.42	98.6	0.5
P6mg/ml (a)	98.4	97.14	0.001*
N6µg/ml	99.08	99.73	0.1
N10µg/ml	97.8	97.98	0.5
N25µg/ml (b)	94.39	95.09	0.02
a v/s b *p*-value	3.18e-9*	4.45e-5*	

*Statistically significant difference at α=0.05, P- paromomycin, N-nitazoxanide.

In vitro oocyst viability test:

NTZ and PRM were slightly effective in decreasing oocyst viability of *Cryptosporidium* isolates. Though there was a decrease in oocyst viability with increasing drug concentrations, but it was very less (range, 99.7-94.4% and 99.5-97%, respectively). Even at the highest concentration of NTZ (25µg/ml) and PRM (6mg/ml) most of the *Cryptosporidium* oocysts were viable and able to grow *in vitro* (94.4-95% and 97-98.4%, respectively). Despite of very less effect, highest concentration NTZ was more effective in decreasing oocyst viability of *C. hominis* and *C. parvum* isolates, than that of PRM. The oocyst viability of *C. hominis* and *C. parvum* isolates was decreased to nearly the same extent at all the concentrations of both the drugs, except 6mg/ml PRM which was having a significantly higher adverse effect on *C. parvum* isolates than *C. hominis* isolates (Figure 8, Table 4).

DISCUSSION

Considering the major health impact of cryptosporidia on immunocompromised patients, children, and general population in outbreak situations, and lack of specific drugs; reliable methods for testing anticryptosporidial drugs or inactivation measures are urgently required. More than, 200 chemotherapeutic agents have been tested against *Cryptosporidium*, but none of them were able to clear the parasite from the host. Two drugs *i.e.* nitazoxanide and paromomycin are currently used for the treatment of cryptosporidiosis. Nitazoxanide is the only drug which is FDA approved for the treatment of cryptosporidiosis in children and adults who do not have HIV (Fox, L. M. and Saravolatz, L. D.,2005; Pantenburg, B. et al.,2009). There is no drug that is very effective in eliminating cryptosporidia from hosts and treatment in HIV positive individuals, mainly relies on restoration of CD4+ count by antiretroviral therapy. There may be differences in drug sensitivity of *Cryptosporidium* species or subtypes. All the earlier studies have performed the drug sensitivity on either reference *C. parvum* isolates or isolates from calves. In the present study, we used different subgenotype isolates of *C. hominis* and *C. parvum* from humans and looked for their sensitivity to, nitazoxanide and paromomycin.

Animal infection models are the gold standard for drug efficacy testing, however they are expensive, time-consuming, laborious and of ethical concern. *In vitro* infection models *i.e.,* cell culture based assays, are partially suited to replace animal experiments (Armson, A. et al.,1999; Rochelle, P. A. et al.,2002). HCT-8 cell lines are believed to represent ideal cell lines, however for immunofluorescence assays MDCK cells are preferred due to their growth peculiarities (Arrowood, M. J.,2008). Human patients tend to provide small and variable quantities of infectious material, which remain viable for shorter time in $K_2Cr_2O_7$ solution (6-12 months). So, isolates need to be passaged through animals, usually calves or mice for *C. parvum*, and piglets or gerbils for *C. hominis* (Tzipori, S. and Widmer, G.,2008). In case of *C. hominis* use of germ free (gnotobiotic) animals is recommended, as in natural systems contamination with *C. parvum* may occur. But, germ free animals are costly to maintain, requiring sophisticated laboratory. So, we did not propagate *C. hominis* isolates and used only 7 fresh isolates having sufficient number of viable oocysts to conduct *in vitro* experiments. We propagated 5 *C. parvum* isolates with viable oocysts in neonatal mouse models (Swiss Albino). Several detection formats such as microscopy, immunoassays and various molecular biological methods targeting parasite nucleic acids are available (Arrowood, M. J.,2008). We used the MDCK cell lines for the culture of parasite and indirect immunofluorescence assay followed by fluorescence microscopy for detection of the parasite. But, due to some limitations of immunofluorescent assays (time consuming, cumbersome and tedious procedure, high cost and limited quantity of primary antibodies and less sensitivity), we shifted to the molecular detection formats. We quantified, the copy number of cryptosporidia in infected MDCK cell lines by quantitative reverse-transcriptase real time PCR (q-RT-PCR) targeting *Cryptosporidium* 18s rRNA. Varying concentrations of NTZ and PRM were tested for their cytotoxicity to MDCK cell lines, and cytotoxicity levels of drugs were found to increase in parallel to increasing drug concentrations and incubation times. NTZ and PRM were cytotoxic at concentrations more than 25µg/ml and 6 mg/ml, respectively. Earlier studies on MDBK cell lines have shown toxicity of –1.7%, 74.1%, and –25%, at 2mg/ml PRM, 100 and 10µg/ml of NTZ, respectively (Theodos, C. M. et al.,1998). While other study had shown 36% and 39% cytotoxicity of 25 and 50µg/ml NTZ to HCT8 cell lines (Gargala, G. et al.,2000). Another study using A549 cell lines had shown cytotoxicity to be practically absent (-8.9 to 11.2%) at all concentrations (0.5-8µg/ml) of NTZ (Giacometti, A. et al.,2000). The effect of non-toxic concentrations of drugs were determined on different isolates of *Cryptosporidium* at three life cycle stages, important in infection of host cells. First was *in vitro* growth inhibition, in which the efficacy of drugs in inhibiting the *in vitro* development of parasite was determined by allowing the *Cryptosporidium* sporozoites to enter the host cells (presumed to be completed by 3 h) and then exposing to drugs for 24 h Second was effect on invasion (first and important step involving several proteins in infection of host cells), in which the excysted sporozoites were allowed to invade the host cells for 3 h, in the presence of drugs and then cultures were maintained in drug-free media. Third was the effect on oocysts (environmentally hardy and infectious stage of parasite), in which oocysts were incubated with drugs for 24 h, after which drugs were washed out and oocysts were used for infection of host cells.

Nitazoxanide was more effective drug at all three stages of parasite than PRM. NTZ is a nitrothiazolyl-salicylamide derivative, which is known for its activity against both intestinal protozoa and helminths (Fox, L. M. and Saravolatz, L. D.,2005). NTZ is known to inhibit pyruvate-ferredoxin oxidoreductase (PFOR), which is an enzyme involved in anaerobic energy metabolism in the amitochondriate eukaryotic human parasites (*T. vaginalis*, *E. histolytica, G. Intestinalis*, including *C. parvum*) and anaerobic bacteria (Rossignol, J. F. et al.,2001; Sisson, G. et al.,2002), but the interference with PFOR may not be the only pathway of antiprotozoal activity (Fox, L. M. and Saravolatz, L. D.,2005). In earlier studies related to *in vitro* efficacy of NTZ, authors had incubated *Cryptosporidium* strains from AIDS patients with A-549 cells (Giacometti, A. et al.,2000) or MDBK cells (Theodos, C. M. et al.,1998) and exposed to drugs for 48 hours, either after allowing the sporozoites to invade or including the drug exposure during invasion also, have shown 56.1% growth inhibition of *C. parvum* with 8µg/ml NTZ or 93% growth inhibition with 10µg/ml NTZ, respectively. Earlier studies using real time PCR analysed the effect of NTZ on growth inhibition of *C. parvum* isolates (IOWA and KSU1) and found that growth was almost completely inhibited at 12.5 µg/ml or higher concentrations of NTZ (Cai, X. et al.,2005). In the present study, we have analysed the effect of NTZ on growth inhibition, invasion inhibition and oocyst viability and those at the highest concentration of NTZ (25µg/ml) were, 98.8%, 97.4%, 94.4% for *C. hominis* and 99.1%, 98.1%, 95.1% for *C. parvum*, respectively. Effect of NTZ on growth inhibition of both *C. hominis* and *C. parvum* isolates was slightly more than on invasion inhibition, but it was less effective in decreasing oocyst viability (5-6%, decrease). An earlier study has shown the negligible effect of NTZ and PRM on oocyst viability of *C. parvum* isolate (Castro-Hermida, J. A. et al.,2004).

PRM, an oral nonabsorbable aminoglycoside has been used for treatment of cryptosporidiosis in adults and children (Hashmey, R. et al.,1997; Stockdale, H. D. et al.,2008). It causes misreading of mRNA and synthesis of proteins containing abnormal amino acid sequences. Most of the earlier studies related to drug sensitivity of *Cryptosporidium* isolates had looked for the effect of PRM on growth inhibition of *C. parvum* strains (IOWA, KSU-1), while others have reported the effect on oocysts or both invasion and growth inhibition, with effective concentrations ranging from 69.5µM-711µM, or percent inhibition varying from 60-70% (200µg/ml), 82% (2mg/ml), 86% (5mg/ml), ~95% (810µg/ml) (Marshall, R. J. and Flanigan, T. P.,1992; Perkins, M. E. et al.,1998; Theodos, C. M. et al.,1998; You, X. et al.,1998; Kayser, O. et al.,2002; Siripanth, C. et al.,2004; Cai, X. et al.,2005; Downey, A. S. et al.,2008). In the present study we have reported that 6mg/ml of PRM resulted in growth inhibition, invasion inhibition and oocyst viability of *C. hominis* and *C. parvum* isolates by 91.1%, 91.5%, 98.4% and 97.9%, 94.9%, 97.1%, respectively. More than 90% growth inhibition with 6 mg/ml PRM reported in the present study is close to that reported by Marshall and Flanigan (Marshall, R. J. and Flanigan, T. P.,1992) *i.e.* 86% with 5mg/ml PRM. The decrease in oocyst viability was also significantly lower than NTZ. Earlier studies by Castro-Hermida and co-authors (Castro-Hermida, J. A. et al.,2004) have also reported the negligible effect of PRM on oocyst viability and slightly more effect of NTZ on oocyst viability (determined by dye exclusion assay) as compared to paromomycin.

In conclusion NTZ was more effective than PRM, at all three stages of parasite (*in vitro* growth inhibition, invasion inhibition and oocyst viability). Drugs were more effective in growth inhibition followed by invasion inhibition and less effective in decreasing oocysts viability. Different isolates exhibited a variable response to drugs and at all the concentrations of drugs, adverse effect on *C. parvum* isolates was more than *C. hominis* isolates.

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Conflicts of interest: All authors have nothing to declare.

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