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# In silico Identification and Optimization of Natural Inhibitors for Drug Target Sites in Cryptosporidium parvum: A Review

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**Abstract**
Cryptosporidium parvum is the most common enteric protozoan pathogens affecting humans worldwide. Currently approved drugs to treat cryptosporidiosis are ineffective and no vaccines exist against C. parvum. Here, We docked benzoxazole derivatives collected from literature with Cryptosporidium parvum inosine 5′-monophosphate dehydrogenase using AutoDock4.2 tool, which resulted in energy-based descriptors such as Binding Energy, Intermolecular Energy, Internal Energy, Torsional Energy, vdW + Hbond + desolv Energy and electrostatic energy. Molecular dynamics (MD) simulation studies were performed through the NAMD graphical user interface embedded in visual molecular dynamics. After that, we have built quantitative structure activity relationship (QSAR) model using energy-based descriptors yielding correlation coefficient $r^2$ of 0.7948. To assess the predictive performance of QSAR model, different cross-validation procedures were adopted. Our results suggests that ligand-receptor binding interactions for inosine 5′-monophosphate dehydrogenase employing QSAR modeling seems to be a promising approach to design more potent inosine 5′-monophosphate dehydrogenase inhibitors prior to their synthesis.

## 1. Introduction

Cryptosporidium is an enteric parasite that is considered the second greatest cause of diarrhoea and death in children after rotavirus. Currently, 27 species are recognized as valid and of these,

*Cryptosporidium hominis*and *Cryptosporidium parvum*ar e responsible for the majority of infections in humans. Molecular and biological studies indicate that Cryptosporidium is more closely related to gregarine parasites rather than to coccidians. The identification of gregarine-like gamont stages and the ability of Cryptosporidium to complete its life cycle in the absence of host cells further confirm its relationship with gregarines. This opens new avenues into the investigation of pathogenesis, epidemiology, treatment and control of Cryptosporidium. Effective drug treatments and vaccines are not yet available due, in part,

to the technical challenges of working on Cryptosporidiumin the laboratory.

Whole genome sequencing and metabolomics have expanded our understanding of the biochemical requirements of this organism and have identified new drug targets. To effectively combat this important pathogen, increased funding is essential (Ryan U *et al*.,)

Drug development is a challenging field in pharmaceutical industry having two component viz. preclinical and clinical studies. In preclinical studies the drugs are tested in animals *in vivo* and *in vitro*, whereas in clinical studies the tests are conducted in human beings. Pharmacokinetic, bioavailability and bio-equivalence studies are all important components of the drug development process. All these kind of studies require sensitive and high throughput assay methods that are well validated to quantify drugs and metabolites in biological matrix.

Overall drug development is a phase dependent process; each phase requiring a significant investment of time, money and human resources. The stages or rather the processes of drug development are shown in Fig.1.

Cryptosporidium are common causes of morbidity world wind. Nitazoxanide was recently licensed in the United States for treating cryptosporidiasis and is the first product developed for treating giardiasis in more than 20 years. Paromomycin may also be effective in treating cryptosporidiosis in human, but data from a limited number of small clinical trials has been inconclusive.

The world is currently plagued with a global pandemic of human immunodeficiency virus (HIV) infection. HIV infects about 0.5% of the world's population (Anonymous, 2010). HIV-infected individuals in developing countries such as Malaysia are susceptible not only to opportunistic infections but also predisposed to a myriad of enteric pathogens which are endemic in the tropics (Asma *et al.,* 2011). Reports from many regions of the world where HIV/AIDS is endemic have also acknowledged that intestinal parasitism is widespread among these populations (Assefa *et al.,* 2009).

One of the most common opportunistic intestinal parasites is *Cryptosporidium*, a causative agent of cryptosporidiosis, a disease which is commonly found in HIV-infected individual and is currently listed as an AIDS defining illness by US Centers for Disease Control and Prevention (Hunter, 2003). Globally the prevalence rate of *Cryptosporidium* infection may account for 10 to 20% of the cases of diarrhea in HIV-infected patients living in developed countries and as much as 50% in under privileged countries (Navin *et al.,* 1999, Florez *et al.,* 2003).

Cryptosporidiosis is characterized by seasonal anthroponotic transmission of strains typically found in Sub-Saharan Africa (Young *et al.,* 2015). The infection mainly affects young infants, with vomiting and diarrhoea being one of the leading symptoms in *C. parvum* infection. Combining molecular typing and clinical data provides valuable information for physicians and is able to track sources of infections (Eibach D, *et al*., 2015)

Many of the predictions generated *Insilco* by genomics have been validated through functional analysis, including studies of the transcriptome and proteome, and

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led to the identification of essential genes. Knowledge of the latter defines potential targets for new and existing drugs and their specificity can be assessed by comparative genomics with the host or other pathogens. Genomics is also furthering to explore vaccine development by pinpointing potentially antigenic proteins as well as providing better diagnostic tools to detect infection.

## 2. International & National status

The zoonotic intracellular protozoan parasite *Cryptosporidium* was discovered in mice by Tyzzer in 1907, but did not receive much interest from the scientific community for almost 75 years (Snelling *et al.*, 2007). However, *Cryptosporidium* research interest did intensify significantly in the 1980s due to increasing veterinary attention and the recognition of its impact on human health because of its association with the newly described acquired immunodeficiency syndrome (AIDS) (Casemore *et al.,* 1985). Although research over the last two decades has dramatically increased our knowledge on *Cryptosporidium*, key questions about host parasite interaction, cell-invasion, transmission, life cycle, and epidemiology still remain unclear (Smith *et al*., 2005).

Outbreaks of cryptosporidiosis associated with drinking water have been an emerging problem for the past 20 years (Howe *et al.*, 2002). In the 1990s, cryptosporidiosis became the most common cause of outbreaks associated with public drinking water supplies in the United Kingdom (Percival *et al.*, 2000). Cryptosporidium causes acute self-limiting gastrointestinal disease in healthy individuals. Immunity is slow to develop and the disease can be recurrent and protracted in malnourished children (Khan *et al.,* 2004; Lima *et al.,* 1992; Molbak *et al.*, 1993; Sallon *et al.*, 1988). Malnourished children are not only more susceptible to severe cryptosporidiosis, but the disease itself is an important contributing factor to malnutrition (Molbak *et al.*, 1997). The persistent and resilient nature of the infective oocyst stage in drinking and recreational water poses significant challenges for controlling transmission even in industrialized nations.

Cryptosporidium spp. and Giardia spp. have been detected in a range of host species, including rodents. The aim of this study was to determine the distribution of these pathogens and recognition of the reservoir role of rodents in the maintenance of these pathogens in south-western Poland. Additionally, prelimssinary molecular studies were conducted to elucidate the species and genotypes of Cryptosporidium and Giardia identified in this study. Stool samples (n=266) from *A. agrarius, A. flavicollis* and *M. glareolus*, were subjected for analyses. Values of prevalence were 61.7, 68.3 and 68.1%, respectively, for Cryptosporidium spp. and 41.7, 24.4 and 38.4%, respectively, for Giardia spp. There was a statistically significant correlation between host species and Giardia infection where *A. agrarius* was the species of the highest prevalence. Statistically significant differences were not found for comparisons made for study sites and occurrence of Giardia spp. and Cryptosporidium spp. Due to preliminary nested PCR results, specific amplifications of Cryptosporidium COWP and SSU rRNA genes were obtained for several isolates taken from rodent host species. One isolate recovered from *A. agrarius* (from a semi-aquatic, urban area) was identified as *C. parvum* and revealed 100% similarity with sequences obtained from humans. To the best of the knowledge of the authors, this is the first record of the *C. parvum* zoonotic species from the striped field mouse. Also recorded were the first findings of C. ubiquitum from three small rodent species (Perec-Matysiak A *et al*, 2015)

*Cryptosporidium* infection continues to be a significant health problem in both developed and developing countries (Harp, 2003), where it is recognized as an important cause of diarrhoea in both immunocompromised and immunocompetent people (DuPont *et al.,* 1995; Kjos *et al.,* 2005). Persistent diarrhoea is the leading cause of death in children younger than five years of age in developing countries, where it accounts for 30 to 50 percent of childhood mortality (Ochoa *et al.,* 2004). *Cryptosporidium* is responsible for diarrhoeal diseases that may lead to nutritional deficiencies and significant morbidity and mortality, especially among children in developing countries and patients who have immune defects, e.g. AIDS (Mak, 2004; Huang and White, 2006). Higher prevalence rates also tend to be observed more in rural compared to urban communities (Mak, 2004). *C. parvum* is also an important pathogen in the developed world, where AIDS patients are at risk of severe infection (Carey *et al.*, 2004; Fayer, 2004). The parasite produces spore-like oocysts that are resistant to common methods of water treatment, so *Cryptosporidium* also poses a credible bioterrorism threat (DuPont *et al.,* 1995). The tools to respond to such an incident are woefully inadequate: no vaccines or effective drug treatments are currently available. The damage would be substantial: the economic cost of the 1993 Milwaukee outbreak, where ~400,000 individuals contracted disease, totaled $31.7 million in medical costs and another $64.6 million in productivity losses (Corso *et al.,* 2003). Independent of such bio-terrorism scenarios, effective drugs are urgently needed for the management of cryptosporidiosis in AIDS patients and epidemic outbreaks. In Nepal, a study of acute diarrhoea in 160 children aged five years and below found that *Cryptosporidium* was detected in nine cases (5.6%), and all 50 control children were negative (Shariff *et al.,* 2002).

The current state of knowledge on Cryptosporidium infectivity, pathogenesis, and transmissibility in light of our contemporary understanding of microbial virulence (Bouzid M*et al*., 2013)

The data available in the literature show that 1.5-3% of Russia's population is carriers of cryptosporidium oocysts. Cryptosporidia are ascertained to be able to cause diarrhea of varying severity in preschool children. However, cryptosporidiosis in the latter is not frequently diagnosed. The urgency of the problem of cryptosporidiosis is also due to the practically ubiquitous prevalence of the causative agent in nature and to the role of this infection in the development of perinatal diseases, as well as complications in immunocompetent patients (infected and uninfected with AIDS virus). Cryptosporidiasis is an HIV-associated infection and of great importance for the diagnosis of AIDS (Turbabina NA, *et al*., 2012)

In India, a study was made by Randhawa *et al.* (2012) in Punjab state, shows that drug combination therapy has a controlling effect against Cryptosporidiosis.

## 3. Drug treatment and novel drug target against Cryptosporidium

Cryptosporidiosis emergence triggered the screening of many compounds for potential anti-cryptosporidial

activity in which the majority were ineffective. The outbreak of cryptosporidiosis which occurred in Milwaukee in 1993 was not only the first significant emergence of Cryptosporidium spp. as a major human pathogen but also a huge waterborne outbreak thickening thousands of people from a major city in North America. Since then, outbreaks of cryptosporidiosis are regularly occurring throughout the world. New drugs against this parasite became consequently urgently needed. Among the most commonly used treatments against cryptosporidiosis are paromomycin, and azithromycin, which are partially effective. Nitazoxanide (NTZ)'s effectiveness was demonstrated in vitro, and in vivo using several animal models and finally in clinical trials. It significantly shortened the duration of diarrhea and decreased mortality in adults and in malnourished children. NTZ is not effective without an appropriate immune response. In AIDS patients, combination therapy restoring immunity along with antimicrobial treatment of Cryptosporidium infection is necessary. Recent investigations focused on the potential of molecular-based immunotherapy against this parasite. Others tested the effects of probiotic bacteria, but were unable to demonstrate eradication of *C. parvum*. New synthetic isoflavone derivatives demonstrated excellent activity against *C. parvum in vitro* and in a gerbil model of infection. Newly synthesized nitro- or non nitro-thiazolide compounds, derived from NTZ, have been recently shown to be at least as effective as NTZ against *C. parvum in vitro* development and are promising new therapeutic agents. (Gargala, 2008)

From the NIH effort, paromomycin and then nitazoxanide emerged as promising candidates for treating cryptosporidiosis (Rossignol, 2010). Paromomycin was the first drug tested in humans for treating cryptosporidial diarrhea. It is an aminoglycoside antibiotic poorly absorbed from the gut epithelium, but apparently it can be absorbed in small quantities across the limiting apical membrane bounding the extracytoplasmic parasite. Its mechanism of action is targeting the bacterial ribosome, where it binds to the A-site and disrupts protein synthesis. In cell culture, it is weakly effective against Cryptosporidium while there was some limited efficacy in some animal model such as the gnotobiotic piglet (Theodos *et al.,* 1998). Paromomycin was the subject of several small and mostly uncontrolled clinical studies and it was suggested that at best based on experimental data and clinical experience, it had a modest activity against Cryptosporidium (Griffiths *et al.*, 1998).

The search for a treatment of cryptosporidiosis began in screening anti-infective drugs including sulfadoxine-pyrimethamine, quinacrine, trimethoprim-sulfamethoxazole, bleomycin, elliptini- um, daunorubicin, pentamidine, alpha-difluoro-methylornithine, diclazuril or N-methylglucamine, none of which was able to prevent or to cure the disease using an immunosuppressed rat model of the infection (Lemeteil *et al.,* 1993). Experimental study of the effects of probiotics, generally L-casei-containing mixtures, on Cryptosporidium infection in neonatal rats showed a trend to a more rapid clearance of parasites in treated animals, but no significant effects on parasite burden, weight gain, mucosal damage or kinetics of mucosal cytokines during infection were observed (Guitard *et al.*, 2006). Several active agents were identified in the rat model including sinefungin (2–10 mg/kg/24 h), lasalocid A (2–10 mg/kg/24 h), met- ronidazole(25–50 mg/kg/24 h), 100 mg/kg/24 h). Sinefungin (10 mg/kg/24 h) and lasalocid A (10 mg/kg/24 h) produced the highest anticryptosporidial effect (Lemeteil *et al.*, 1993). More recently, pyrvinium pamoate, an old anthelminthic which was the treatment of choice of pinworm infections many years ago was found to be also effective against Cryptosporidium in cell culture and in a neonatal mouse model. In cell culture, using an HCT-8 cell line, an IC50of 0.354 lM was calculated compared to 711 lM for paromomycin. In vivo, using a neonatal mouse model, when administered orally three days after infection for 4– 6 consecutive days at doses of 5 and 12.5 mg/kg/ day, it was as effective as paromomycin 100 mg/kg/day. Oocyst shedding was reduced by >90% in animals treated with the low dose (5 mg/kg/day) without significant toxicity. The higher dose (12.5 mg/kg/day) did not improve efficacy and showed some toxicity with three mice dying from treatment. The drug also reduced the level of infection in the intestinal epithelium of treated animals when compared to control animals. These results may be significant since pyrvinium pamoate has been used as anthelminthic for at least four decades. It is safe and well tolerated when given in a single dose or over a few days. It remains to be seen if the drug will be able to be given for prolonged durations as usually needed and sulfadimethoxine (10– in the treatment of cryptosporidiosis in immune-compromised individuals, its bright red color being potentially a problem (Downey *et al.*, 2008). Most of the drugs proposed for cryptosporidiosis were only tested in the laboratory, and therefore, there is no data on their use in treating human disease. Some including spiramycin, clarithromycin, octreotide acetate, atovaquone, letrazuril, and lasalocid were tested in a limited number of patients with AIDS-related cryptosporidiosis and failed to show antidiarrheal or antiparasitic activity (Zardi *et al.*, 2005). Three antibiotics, paromomycin, azithromycin and roxithromycin were the subject of small controlled clinical trials. They generally produced inconsistent results, the number of patients involved as well as the methodology used for these trials hardly qualified them for a potential regulatory approval. Paromomycin and nitazoxanide are the only two drugs which were subjected to well control clinical trials and showed some degree of efficacy.

Paromomycin was the first drug tested in humans for treating cryptosporidial diarrhea. It is an aminoglycoside antibiotic poorly absorbed from the gut epithelium, but apparently it can be absorbed in small quantities across the limiting apical membrane bounding the extracytoplasmic parasite. Its mechanism of action is targeting the bacterial ribosome, where it binds to the A-site and disrupts protein synthesis. In cell culture, it is weakly effective against Cryptosporidium while there was some limited efficacy in some animal model such as the gnotobiotic piglet (Theodos *et al.*, 1998). Paromomycin was the subject of several small and mostly uncontrolled clinical studies and it was suggested that at best based on experimental data and clinical experience, it had a modest activity against Cryptosporidium (Griffiths *et al.*, 1998). Four small uncontrolled clinical trials were conducted in 24, 7, 5 and 6 AIDS patients respectively, a total of 42 subjects with cryptosporidial diarrhea. In these studies, paromomycin was administered orally 500 mg four times a day for two to four weeks. The drug appeared to be beneficial with

36 of the 42 patients showing some degree of response in both the number of diarrheal episodes and oocyst excretion (Bissuel *et al.*, 1994; Fichtenbaum *et al.*, 1993; Clezy *et al.*, 1991; Walllace *et al.*, 1993). These early encouraging results triggered placebocontrolled controlled clinical studies. The first double-blind placebo controlled study was conducted in 10 AIDS patients with cryptosporidial diarrhea who were randomized to receive either paromomycin or a placebo for 14 consecutive days. The number of stools per day and their consistency were recorded, but more interestingly a weekly 24-h stool collection was also carried out allowing the recording of total weight of stool and oocyst excretion. The study showed a statistically significant reduction ($p < .02$) of oocyst excretion in patients receiving paromomycin when compared to those receiving the placebo. Stool frequency also decreased in the treated patients versus controls. The authors concluded that paromomycin treatment resulted in improvement in both clinical and parasitological parameters in AIDS-related cryptosporidiosis (White *et al.*, 1994). The AIDS Clinical Trials Group at NIAID conducted a larger well designed double-blind placebo-controlled study in 34 adult patients with AIDS and CD4 count <150 cells/mm3. Seventeen patients received oral paromomycin 500 mg 4 times a day and 18 received matching placebo for 21 consecutive days. All 35 patients continued the treatment for 21 additional day's with500 mg ofparomomycin 4 times a day. Patients were evaluated on the average of bowel movements per 24 h along with the concurrent need for antidiarrheal agents which should be lower than that used before being included in the trial. No significant difference was observed during the first three weeks of the study between the paromomycin and placebo.J.-F. groups ($p = 0.88$). Three patients in the paromomycin group (17.6%) and 2 patients in the placebo group (14.3%) had a complete response. When complete and partial responses were combined, 8 of 17 (47.1%) patients in the paromomycin group and 5 of 14 (35.7%) in the placebo group showed some degree of response, the difference not being statistically different ($p = .72$).

Nitazoxanide is the only drug that has been subjected to a full and well designed development program for cryptosporidiosis under an investigational new drug application (IND) with the United States Food & Drug Administration. The development program included 436 patients enrolled in six clinical trials, five of which were double-blind placebo-controlled studies. One hundred and ninety six (196) patients had cryptosporidial diarrhea associated with AIDS, 146 adults and 50 children. Two hundred and forty (240) patients, 140 adults and 100 children, had no known immune deficiencies. This development program resulted in licensure in the United States for treating cryptosporidiosis in non-immunodeficient adults and children. Results of studies in severely immunodeficient patients with AIDS-related cryptosporidiosis (CD4 counts <50) were somewhat disappointing, while the drug was quite effective in patients with some degree of immunity (CD4 count >50). Clinical testing in AIDS patients was discontinued with the introduction of HAART which essentially eliminated the disease in most parts of the world. Nitazoxanide and its two metabolites, tizoxanide and tizoxanide-glucuronide inhibited the growth of *C. parvum* sporozo-ites and oocysts at concentrations lower than 10 lg/ml (Theodos *et al.*, 1998). They were tested against three stages of the cycle of *C. parvum* in HCT-8 enterocytic cells (Gargala *et al.*, 2000) including the asexual stage, the sexual stage and the completely developed parasite. They were active for up to 46 h when added after sporozoite invasion with IC501.2, 22.6 and 2.2 lg/ml respectively. Inhibitory concentrations on complete parasite development using this methodology were consistent with results obtained by others (Theodos *et al.*, 1998; Giacometti *et al.*, 1999, and Giacometti *et al.*, 2000). In experimentally infected suckling mice, SCID mice, rats, gerbils and piglets, nitazoxanide showed various degrees of efficacy when compared to paromomycin (Theodos *et al.*, 1998; Blagburn *et al.*, 1998; Li *et al.*, 2003; Baishanbo *et al.*, 2006).

In a separate study by Rossignol *et al.* in 1998, Nitazoxanide was used for cryptosporidiosis in AIDS patients. An open-label phase I/II dose range finding study was conducted in 28 patients with chronic AIDS-related cryptosporidiosis in the United States to evaluate safety and efficacy of nitazoxanide administered orally at doses of 500–3000 mg per day. All patients had previously failed other potential treatments for cryptosporidiosis. Fourteen subjects (50%) experienced a significant reduction in oocyst shedding, 10 of whom were free of oocysts in their stools. Eight of 13 subjects (62%) who were treated for at least 12 consecutive weeks had eradicated the parasite as of week 12. The 10 complete parasitological responses occurred at doses of 1500 mg/day ($n = 4$), 2000 mg/day ($n = 5$) and 3000 mg/day ($n = 1$). Each of the 10 subjects (100%) eradicating the parasite showed a clinical re- sponse to treatment, 8 with a complete clinical response and 2 with a partial clinical response. From this study, it was concluded that a dose of at least 1500 mg per day, preferably 2000 mg per day and duration of treatment of 12 weeks is appropriate for eradication of cryptosporidial infection in this type of patient population with low CD4 counts. (Rossignol, personal communication). A double-blind placebo-controlled study carried out in 66 AIDS patients in Mexico compared the efficacy of nitazoxanide administered 500 mg twice daily for 14 days, 1000 mg twice daily for 14 days and a placebo. Based on three negative fecal examinations obtained on days 15, 22 and 29 following the initiation of treatment, 63% (12/19) of the low dose patients and 67% (10/15) of the high dose patients eradicated Cryptosporidium. This compared to only 25% (5/20) of the patients on placebo who did not shed oocysts on day 7 or day 15 following initiation of placebo therapy. The difference between the treated groups and the placebo group was statistically significant ($p = 0.016$ and 0.013). Diarrheal syndrome resolved in approximately 90% of the subjects who eradicated the parasite. A stratification of the patients enrolled in this study based upon baseline CD4 counts revealed an important difference in the responses of the patients. In subjects with CD4 counts above 50, 10 of 14 subjects (71%) treated with 500 mg bid of nitazoxanide for 14 days and 9 of 10 patients (90%) treated with 1000 mg bid for 14 days showed a complete parasitological and clinical response to treatment. These results were statistically sig-nificant ($p < 0.01$) compared to the response of patients with CD4 counts greater than 50 who received placebo. In more severely immunocompromised patients (CD4 counts 650), this study, with a relatively small number of patients and only two weeks of treat- ment, did not show any statistically significant differences between response rates for

subjects who received nitazoxanide versus placebo (Rossignol *et al.*, 1998). A second double-blind placebo-controlled study was subse- quently carried out in Thailand where nitazoxanide was adminis- tered in 50 adult AIDS patients with cryptosporidial diarrhea and CD4 count <50. They received two nitazoxanide 500 mg tablets (1000 mg nitazoxanide) or matching placebo twice daily for 8 weeks. Symptom resolution occurred in 7 patients (32%) in the nitazoxanide group compared to 1 (5%) in the placebo group ($p = 0.0497$). Parasite eradication occurred in 2 patients in the nita- zoxanide group compared to none in the placebo group (not significant) (Rossignol, personal communication). The efficacy and safety of nitazoxanide suspension as a treatment of cryptosporidiosis in immunocompromised and immunodeficient children was studied Hospital in Lusaka, Zambia. Children with cryptosporidial diarrhea were admitted to the hospital and randomized to receive nitazoxanide (100 mg twice daily orally for 3 days) or placebo. The trial was stratified by HIV serology. Fifty HIV seropositive and fifty HIV seronegative children were recruited for the study, and 96 with cryptosporidiosis confirmed at randomization were analyzed. Of these 96 children, 92 (96%) were malnourished. In HIV seronegative children, 14 (56%) of 25 receiving nitazoxanide and 5 (23%) of 22 receiving placebo ($p = 0.037$) had no symptoms of cryptosporid- iosis 4 days after the end of treatment (studyday 7). Cryptosporidium was eradicated from stool in 13 (52%) of 25 children receiving nitazoxanide compared to 3 (14%) of 22 receiving placebo ($p = 0.007$). Four children (18%) of 22 in the placebo group died over 8 days of observation compared to none of 25 in the nitazoxanide group ($p = 0.041$). HIV seropositive children did not benefit, but after a further 3 days of open-label treatment, 77% appeared to have responded. The data suggested that HIV-infected children may benefit from longer courses of therapy (Amadi *et al.*, 2002)

A randomized double-blind study was carried out in the Nile Delta of Egypt in non-immunodeficient adults and children with cryptosporidial diarrhea. Four days after the end of a 3-day course of treatment, resolution of diarrhea was observed in 80% (39/49) of the patients receiving nitazoxanide versus 41% (20/49) of the patients receiving the placebo ($p < 0.0001$). *C. parvum* was eradicated 48 J.-F. based on two post-treatment negative fecal examinations in 67% (39/49) of the patients receiving nitazoxanide versus 22% (11/50) of the patients receiving a placebo ($p < 0.0001$) (Rossignol *et al.*, 2001a). A second randomized double-blind study in non-immunodeficient adults and adolescents with cryptosporidial diarrhea produced similar results. Four days after the end of a 3-daycourseoftreatment, symptom resolution was observed in 96% (27/28) of the patients receiving nitazoxanide tablets, 87% (27/31)of the patients receiving nitazoxanide suspension and 41% (11/27) of the patients receiving the placebo tablets ($p < 0.0001$). The proportions of patients with two negative stool examinations for Cryptosporidium four to seven days following treatment were 26/28(93%) for the nitazoxanide tablets, 28/31 (90%) for the nitazoxanide suspension, and 10/27 (37%) for the placebo tablets ($p < 0.0001$) (Rossignol *et al.*, 2006).

Antiretroviral therapy the use of highly active antiretroviral therapy (HAART) in patients with AIDS has dramatically reduced the prevalence of infection with Cryptosporidium and the length and severity of its clinical course. This effect has been attributed to the recovery of the host immunity as demonstrated in other cases of cryptosporidiosis associated with other causes of immunodeficiency such as primary immunodeficiency, organ transplantation, cancer, diabetes and malnutrition for which antiretroviral therapy is not indicated. Some studies using protease inhibitors such as ritonavir, saquinavir, and indinavir claim a drastic reduction of Cryptosporidium infection both in vivo and in vitro (Mele *et al.*, 2003; Hommer *et al.*, 2003). Whether or not aspartyl proteases could have some important function is not known as there are no reports of its pres- ence in Cryptosporidium.

**3.1 Other drugs**

Azithromycin and Roxithromycin are two antibiotic agents were the subject of limited clinical investigations in the treatment of cryptosporidial diarrhea in AIDS. Azithromycin was found to be weakly effective against *C. parvum*, azithromycin at a concentration of 8 mg/L only showed a 63.4% reduction of the parasite development in a cell line culture assay (Giacometti *et al.*, 1999). In the limited number of patients involved in the some studies, short or prolonged duration of treatment did not produce a reduction of the oocyst shedding but some transient moderate reduction of diarrhea was observed most likely due to the cyclical nature of the disease (Kadappu *et al.*, 2002; Dionisio *et al.*, 1998). Only one open-label trial was carried out using roxithromycin. Uip *et al.* (1998) treated 26 patients with AIDS at various stages of disease. Fifteen patients were considered cured (68.2%), 6 improved (27.3%) while one failed. Given the variable and cyclical nature of the disease and the lack of a control group, these findings are considered anecdotal at best.

**3.2 Drug combination therapy**

A recent study was made by Randhawa *et al.* (2012) in Punjab state, shows that drug combination therapy has a controlling effect against Cryptosporidiosis. The study described the outbreak of cryptosporidiosis in neonatal cross bred cattle calves ageing 1-2 months in an organized dairy farm. The protozoan infection was confirmed by identifying bright red oocysts of Cryptosporidium spp. in the faecal samples after staining with modified acid Fast Zeihl-Neelsen stain. Metronidazole and furazolidone combination was able to induce clinically and parasitological recovery. This is believed to be the first report on the successful use of this drug combination against cryptosporidiosis.

Patients with Tac and mycophenolate mofetil combination therapy had a significantly high risk of Cryptosporidium infection. Cryptosporidial infection may require prolonged nitazoxanide therapy, either alone or in combination, with or without reduction in immunosuppression. (Bhadauria *et al.*, 2015)

## 4. Advancements in Vaccine development

*Cryptosporidium parvum* is an important human pathogen and potential bioterrorism agent. No vaccines exist against *C. parvum*, the drugs currently approved to treat cryptosporidiosis are ineffective, and drug discovery is challenging because the parasite cannot be maintained continuously in cell culture.

There is currently no vaccines exist, and the available drugs are inadequate in treatment for cryptosporidiosis. The more widely used drugs paromomycin and azithromycin are unreliable and the efficacy of nitazoxanide, which recently received FDA approval, is dependent upon a robust immune response (Amadi *et al.*,

2009). The options in particular for the treatment of chronic AIDS-related cryptosporidiosis are severely limited (Rossignol, 2009) and there is an overall urgent need for new chemotherapy.

## 5. Identification of lead compounds against cryptosporidiosis

tudy made by Umejiego *et al.* (2008), targeted a prokaryotic protein in a eukaryotic pathogen for the identification of lead compounds against cryptosporidiosis. Mining the sequence of the *C. parvum* genome has revealed that the only route to guanine nucleotides is via inosine-5'-monophosphate dehydrogenase (IMPDH). Moreover, phylogenetic analysis suggests that the IMPDH gene was obtained from bacteria by lateral gene transfer. They have studied the unexpected evolutionary divergence of parasite and host enzymes by designing a high-throughput screen to target the most diverged portion of the IMPDH active site and identified four parasite-selective IMPDH inhibitors that display antiparasitic activity with greater potency than paromomycin, the current gold standard for anticryptosporidial activity.

Antibody therapy the close relationship between Cryptosporidium infection and host immune response led to investigations of antibody therapy. Some investigators hypothesized that immunoglobulins in bovine colostrum immunized against particular pathogens may help pro- tect against some specific pathogens such as Cryptosporidium spp. unfortunately, bovine colostrum supplements vary widely in terms of their specific constituents. Results obtained from bovine colostrum antibody therapy are mostly contradictory, and no well designed controlled clinical studies were published in the litera- ture. Riggs *et al.* in 2002, in a work regarding neutralizing monoclo- nal antibodies (MAbs) hypothesized that targeting the apical complex and surface antigens CSL, GP25-200, and P23 could pas- sively immunize against cryptosporidiosis. MAbs were evaluated for therapeutic efficacy against persistent infection in adult gamma interferon-depleted SCID mice. The results indicated that anti-CSL MAb 3E2 had highly significant efficacy in reducing, but not eliminating, persistent Cryptosporidium infection.

## 6. Genomics and drug target discovery of *Cryptosporidium parvum*

The genomes of all three fully-sequenced members of the *C. parvum* complex contain ~4.4 (+/– 0.1) Mb and harbour ~4,000 genes that are predicted to encode proteins and 50 genes for stable ribonucleic acid (RNA) species. In the case of *C. parvum*, bioinformatics analysis resulted in the attribution of precise functions to ~40% of the 4,000 genes. Some functional information was inferred for a further 20%, but nothing was learned about the remaining 40%. When functional information was available, it often enabled investigators to identify potential drug targets on the basis of their proposed biological role or their similarity to known bacterial drug targets. However, now that more *C. parvum* sequences are becoming available, it is possible to establish which genes are generally found in mycobacteria or restricted to a given species. The functions encoded by these genes, if essential, could represent novel targets for chemotherapy that are exceptionally specific. Their widespread conservation is certainly of biological significance and some of these genes were subsequently found to play critical roles. Thus, they represent novel targets for new broad spectrum antibiotics. Similar screens of the genome sequences of other pathogens can be undertaken to enhance specificity. Among the many attractive features of highly specific drugs, are the avoidance of transferable drug resistance mechanisms, such as those that have plagued certain broad spectrum antibiotics, and the reduction of unwanted side-effects, like the indiscriminate destruction of the bowel flora. (Ryan and Hijjawi, 2015)

Use of cryptosporidium genomes has helped to identify promising therapeutic targets, and drugs are in development, but methods to assess the efficacy in vitro and in animals are not well standardised. Partial immunity after exposure suggests the potential for successful vaccines, and several are in development; however, surrogates of protection are not well defined. Improved methods for propagation and genetic manipulation of the organism would be significant advances. ( Checkley *et al*., 2014)

Although uncommon, cryptosporidial infection should be suspected in patients with hematological malignancies who have persistent diarrhea. Stool examination with the modified acid-fast Kenyoun stain establishes the diagnosis in the majority of cases. Antiparasitic treatment is effective in controlling the infection (Tandon *et al*., 2014)

Several different approaches are available to determine which genes of *C. parvum* are essential and thus worthy of further investigation as targets for drug development. These include gene knockouts, transcript analysis and definition of the proteome. (Sponseller *et al.,* 2014). Having identified potential candidates, it is important to demonstrate that the genes are expressed, particularly during infection, and here transcriptomics and proteomics offer great promise by allowing global analyses to be undertaken. All of these approaches are considerably facilitated by the availability of the complete genome sequence.

## 7. Conclusions

### 7.1 Screening drug targets

If the corresponding protein has an assayable function, kinase activity for example, it can be used as the basis of an *in vitro* screen to identify inhibitors of the enriched or purified enzyme. The advantage of this approach is that it can generally be automated or converted to high-throughput format to facilitate screening of large or complex libraries of synthetic compounds or natural products. However, whole organism screens, involving recombinant *C. parvum* with reporter activity, are often considered preferable as they avoid drug permeability problems. Once an active pharmacophore has been uncovered, numerous analogues can be synthesized or identified in combinatorial libraries to isolate more active derivatives. (Zhou *et al*., 2014) Their potency can also be evaluated using reporter assays or biochemical techniques, such as transcriptome or proteome analysis. In this way, genes that are co-regulated can be uncovered whose products may also serve as potential drug targets in turn, as they often act concertedly in the same metabolic process.(Iannotti *et al*., 2014) Identification of the target enables large amounts of the corresponding protein to be produced by genetic engineering for further studies. Knowledge of the three-dimensional structure of known or potential drug targets is also highly desirable for drug development purposes and can be obtained through structural biology. (Zhou *et al*., 2014)

### 7.2 Impact of proposed research in academic/industry

This research will lead to the identification of novel drug target sites and active sites in *Cryptosporidium parvum* for the development of drug designing. A new database for drug target sites in *Cryptosporidium parvum* will also be aiding to develop for further analysis of wet lab data. The proposed research will lead to be a milestone in the field of drug discovery for Cryptosporidiosis diseases.

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Genomic data information [71.]

[72.] All genomic data will be retrieved from the NCBI complete genome repository.

[73.] NCBI complete genomes (ftp://ftp.ncbi.nih.gov/genomes/)

EPD - The Eukaryotic Promoter Database.

[74.]

[75.] PromoSer - Human, Mouse and Rat promoter extraction service.

[76.] Human genome: http://arep.med.harvard.edu/labgc/adnan/humanupstreamsequences/

[77.] http://www.pht.uni-koeln.de

[78.] (http://www.genome.ad.jp/kegg/kegg2.html