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Cryptosporidiosis: Challenges for Chemotherapy to AIDS Subpopulation

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***Abstract*—** *Cryptosporidium* parasites, especially C.*parvum* and C.*hominis* are obligate intracellular apicomplexans protozoan parasite that infect epithelial cells of the small intestine posing life threatening Cryptosporidiosis disease to immunocompromised person and young malnourished children. It is also well known to be a troublesome waterborne pathogen. Pathogen transmission is naturally via contaminated drinking or recreational water supplies by the environmentally resistant and chlorine resistant oocysts. *Cryptosporidium* is classified in category B of bioweapons agents. However nitazoxanide, a nitrothiazole benzamide was approved by FDA Department for treatment of cryptosporidiosis in immunocompetent individuals and Paramomycin is used to treat C.*parvum* infections in animals. *Cryptosporidium* infections starts by ingestion of oocysts with contaminated water or food. Oocysts undergo excystation in gastrointestinal tract where four naked sporozoites are formed which then infect epithelial cell and initiate asexual development. IMPDH an NAD+ enzyme remains a promising drug target for anti-parasitic drugs. *Cryptosporidium* obtains guanine nucleotides via a streamlined pathway that requires IMPDH. Curiously, the gene encoding CpIMPDH seems to have been obtained from a bacteria through lateral gene transfer so that the parasite enzyme is very different from host ortholog. IMPDH is a homotetramer showing square planner symmetry. Inhibition of IMPDH is already used to treat viral infection and could be used to treat parasitic infection as well. IMPDH has been isolated from different sources and 26 crystal structure are deposited in PDB including mammals to apicomplexans and bacteria. There are several FDA approved inhibitors that target IMPDH.

***Keywords*—** *Cryptosporidium Parvum*, Cryptosporidiosis, GMP, IMP, Immunocompromised, Immunocompetent, IMPDH, PVM , XMP

I. INTRODUCTION

Cryptosporidium parasites are protozoan pathogens of increasingly recognized importance in whole world. Nature of it is ubiquitous and zoonotic. Cryptosporidiosis is one of the common human enteric infections in human being as It Occurred in all species of vertebrates and can induce infection with less than 10 oocysts in adult human volunteers. [1]. unlike other intestinal pathogens, *Cryptosporidium* can infect several different hosts. Cryptosporidiosis is a major cause of morbidity in infants and malnutrition in young children in Africa and Asia and is a major cause of waterborne disease in North America and Europe. Infection are self-limiting in immunocompetent individuals but can be chronic and fatal in immunocompromised person [2] Cryptosporidiosis remains a devastating disease in the third world countries, particularly in infants, young children and immunocompromised patients, in whom it is associated with chronic diarrhoea and malnutrition. It is anticipated, however that with the pending completion of the genome sequencing of C.*parvum*, new molecular targets will be identified for a more rational drug design [1].

Study started from 1983 onwards, shows that in the AIDS epidemic, Cryptosporidiosis emerged as a life-threatening disease in this subpopulation [1]. Cryptosporidiosis was reported to occur up to 24% of these patients in the era before highly active antiretroviral therapy (HAART). In patients with HIV/AIDS the severity of the disease varies, depending on the degree of immunosuppression, as reflected by CD4+ counts. In patients with relatively high CD4+ counts (>180 cells/mm^3) the infection may be asymptomatic or results in mild diarrhoea. However, patients with CD4+ counts less than 50 cells/mm^3 can develop persistent or intractable diarrhoea, thus emphasizing the importance of the host immune response in controlling the disease [3].

In addition *Cryptosporidium* oocysts are resistant to commonally used methods of water treatment making this a potential weapon for bioterrism. The potential for intentional contamination of water supplies has led to inclusion of *Cryptosporidium* as a category B priority pathogen for biodefense [3].

In this paper we reviewed *Cryptosporidium* and cryptosporidiosis and IMPDH as an important drug target for treating cryptosporidiosis is carried out.

In section I, general overview of the impact of the disease is given. In section II, brief introduction of *Cryptosporidium* is given. In section III, life cycle of *Cryptosporidium* is explained. How the parasite is transmitted is discussed in section IV. In section V, the role of host immune system against cryptosporidiosis is explained. The structure and reaction mechanism of IMPDH is explained in section VI. In section VII currently available drugs are discussed. And in section VIII, the matter is concluded.

II. BACKGROUND OF THE TERM *CRYPTOSPORIDIUM*

E. E. Tyzzer, in 1907 described *Cryptosporidium*, a cell associated organism in the gastric mucosa of mice. For several decades, *Cryptosporidium* was thought to be a rare opportunistic animal pathogen. The first case of human cryptosporidiosis in 1996 appeared in a three year old girl from rural area of Tennessee, who suffered severe gastroenteritis for two weeks .Electron microscopic examination of the intestinal mucosa led to the discovery that *Cryptosporidium parvum* was the infectious species in human population also. In The early 1980s the strong association between cases of cryptosporidiosis and immunocompromised individuals brought *Cryptosporidium* to the fore front as a ubiquitous human pathogen [4].

It can be survive most environments for long period of time due to its hardy cysts. C.*parvum* is taxonomically classified as a sporozoa, since its oocysts releases four sporozoites upon excystation. However it differs from related parasite such as Toxoplasma by its Monoxenous life cycle which is primarily completed within the gastrointestinal tract of single host. Here we discuss two major species of *Cryptosporidium* that infect humans. The zoonotic species, C.*parvum* infects animals and humans, whereas the anthroponotic species, *C. hominis* mostly infects humans are major species of *Cryptosporidium*.

Some others species are also found, that are occasionally infect humans. The name of these species are *C. meleagridis, C. muris, C. wrairi, C. saurophilum, C. baileyi, C. andersoni, C. serpentis and C. nasorum* [3].

III. LIFE CYCLE OF CRYPTOSPORIDIUM

Life cycle begins with the ingestion of oocysts by the host. Infections can occur as few as 1 to 100 oocysts are ingested. After the excystation, four naked sporozoites are released in to the intestinal lumen. After that sporozoites invade epithelial cells, particularly in the terminal ileum, and initiate asexual development. Invasion of intestinal epithelial cells takes place through the differentiated apical end of the sporozoites. Sporozoites leis with in a vacuole formed of both host and parasite membranes termed as Parasitophorous vacuole bounded by the Parasitophorous vacuolar membrane (PVM).

Parasitophorous vacuole protrudes out of the host cytoplasm in to the intestinal lumen.

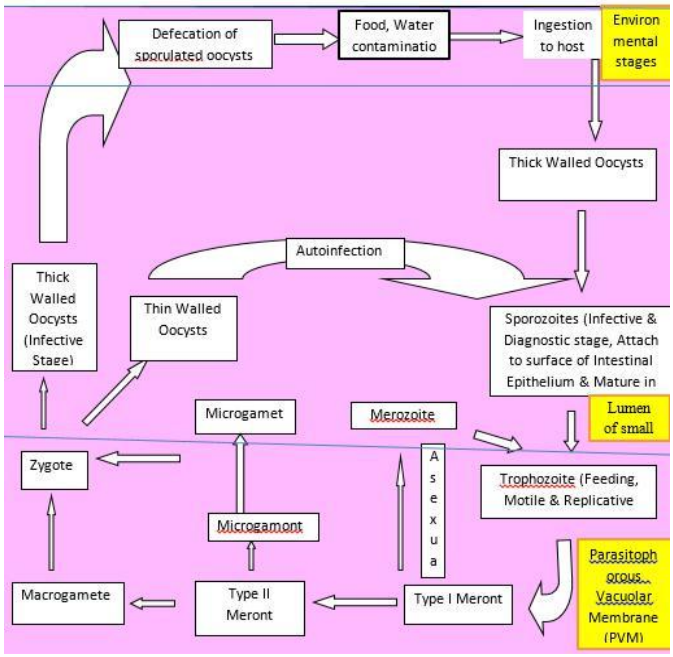

Figure 1. Depictive life cycle of C.*parvum*

This membrane act as portal through which the nutrients from the host enters the parasite [1].

In PVM sporozoites undergo asexual multiplication stage. They become internalized in the gut epithelial cells and undergo two successive generation of merogony. Then eight and four merozoites are released respectively.

The four merozoites released from the second merogony give rise to the sexual developmental stages. The micro and macro gamonts.

The union of microgametes with the macro gamonts releases zygote, which after two asexual division forms oocysts containing four sporozoites still within the PVM [1].

Beside PVM *Cryptosporidium* possess a feeder organelle membrane, which directly separates the cell and parasite cytoplasm [1].

It is suggested that PVM (Prasitophorus Vacuole Membrane) in *Cryptosporidium* provides only a protective function. While the feeder organelle membrane is the site for nutrient and energy uptake from the host cell. PVM is derived from the host cell membrane. So it shows some of its absorptive and other functional activities [1]. Oocysts is environmentally resistant.
The production and release of these oocysts with in the same host are believed to be the key to autoinfection. The cycle begins a new when these oocysts are ingested by a new host. Transmission is fecal-oral. Directly or indirectly via the contaminated water or food washed or irrigated with fecally contaminated water.

Human and dairy effluents are probably the most important sources of environment. It contains surface water [1]. A major mode of transmission is via contaminated water supplies often resulting in widespread out breaks. Normal immunocompetent host the infection remains localized in the gastrointestinal tract. While the parasites alter the cell membrane to create a niche for itself between the cell membrane and the cell cytoplasm. The unique location of parasite shows enigmatic resistant to chemotherapy.

In immunocompetent person infection are predominantly localized to the jejunum and ileum but in immunocompromised patients infection can extend to other parts of the gastrointestinal tract. Biliary and other organ involvement also occurs in approximately 20% immunocompromised patients. Unfortunately *Cryptosporidium* parasites cannot be cultured continuously in vitro and genetic tools yet do not exist to constrict transgenic reporter parasites that would generally facilitate screening efforts. Tissue culture models of *Cryptosporidium* infection provide an imperfect window to measure drug effects and certainly do not recapitulate the complex environment of the gastrointestinal tract in which it includes a myriad of commensal organism that may influence infection. Several animals model exists that mimic either acute or chronic disease, though these generally require immunosuppression to permit *Cryptosporidium* infection. Due to PVM *Cryptosporidium* is protected from the host immune response and the hostile environment of the gut, while accessing the nutritional and energy reservoirs of the host cells. To kill the parasite antimicrobial agents must first enter the host cell cytoplasm for effectively inactivate intracellular microorganism or kill the host cell containing the intraocular microorganism [1].

IV. PATHOGENESIS OF *CRYPTOSPORIDIUM*

The pathogenic mechanism of *Cryptosporidium* is not clearly understood. The initial host-parasite interactions of attachment, invasion and parasitophorous vacuole formation are complex processes that involve multiple parasite ligands and host receptors. These interactions are best studied in other apicomplexans such as Toxoplasma, Plasmodium. The invasive stage of these microorganism posses specialized secretory organelles collectively known as apical complex.

During initial host parasite interactions these organelles secrete and successively exocytose proteins, which facilitate attachment, invasion and parasitophorous vacuole formation.

In this attachment process many micronemal proteins have adhesive modules that are conserved among apicomplexans parasites [1].

Increasing recognition of *Cryptosporidium* as an emerging human pathogen has led to the identification of surface and or apical complex proteins such as CSL, GP900,p23/27, TRAPC1, GP15, CP15, CP60/15,cp47, gp40/45and gp15/Cp17. These has features in common with those of other apicomplexans. These are implicated in mediating interactions. Many of these proteins have been reviewed previously [1].

Infections may often involve the pyloric region of the gastric mucosa. Parasites forms displace the microvillus border and eventually lead to the loss of the mature surface epithelium. The rapid loss of surface epithelium causes marked shortening and fusion of the villi and lengthening of the crypts due to acceleration of cell division to compensate for the loss of the cells. The combined loss of the microvillus border and villus height diminishes the absorptive intestinal surface and reduces uptake of fluids, electrolytes and nutrients from the gut lumen. The loss of the microvillus border in the proximal small intestine leads, in addition to loss of membrane bound digestive enzymes, whose role in children, in particular is crucial, and contributes to marked maldigestion in addition to malabsorbtion [1]. The disruption of intestinal epithelium which can inhibit sodium chloride absorption. The parasite may promote apoptosis in adjacent epithelial cells while inhibiting apoptosis in the infected cells. Therefore facilitating prolonged survival of the parasites. The incubation period to establish infection can range from one to two weeks.

Two major obstacles that hinder progress in characterization of pathogens are first inability to continuously propagate the parasites in-vitro and second is the inability to cryopreserve the parasites as in the case with majority of microorganism [1].

V. IMMUNE RESPONSES OF HOST AGAINST *CRYPTOSPORIDIUM*

Although the outcome and severity of infection is critically dependent on the immune status of the host. The nature of the immune response in cryptosporidiosis, particularly in humans, is poorly understood. Most investigation have been done in tissue culture models utilizing human cell lines, or on peripheral blood mononuclear cells (PBMCs). In vitro studies on peripheral blood mononuclear cells have limitations. That they may not accurately reflects the intricate dynamics of the mucosal immune system in-vivo. Both Innate and Adaptive immune response play role in marshalling of cryptosporidiosis [3].

Innate Immune Response

Chief Player of innate immune response are the Toll-Like receptors, Antimicrobial peptides, Mannose binding lectin, Chemokines, Cytokines, Prostaglandins and Substance P.

Toll-Like Receptors (TLRS)

Toll-like receptors (TLRs) are a family of cell membrane associated molecules that play an important role in mediating resistance to a wide array of pathogens by recognizing specific pathogen associated molecular patterns (PAMPs) C.*parvum* induced recruitment of TLR2 and TLR4 to the site of infection leading to activation of downstream effectors human cholangiocyte model in–vitro. Although TLR ligands for *Cryptosporidium* have not been identified [3].

Antimicrobial Peptides (AMPS)

These are one of the components of the intestinal mucosal barriers. AMPs are small polypeptides (<100 amino acids) that have antimicrobial and immunomodulatory properties and are evolutionary conserved effectors of the innate immune system include α-and β– defensins and cathelicidins. In-vitro studies in human intestinal epithelial cell models have shown that C.*parvum* infection initially down regulates AMPs production, perhaps thus facilitating parasites survival [3].

Mannose Binding Lectin (MBL)

It is another highly conserved component of the innate immune system. It is a collagenous lectin found in serum that bind to specific carbohydrate residues on a variety of infectious organism including *Cryptosporidium*. Upon binding, MBL activates the lectin complement pathway in an antibody– dependent manner via mannose-binding lectin–associated serine proteases (MASPs), thereby promoting opsonization and phagocytosis. MBL activation is thought to result in formation of a membrane attack complex that may diminish parasite viability in addition to promoting opsonization and phagocytosis. Cryptosporidiosis in AIDS causes low serum MBL level. Low serum levels may also be related to malnutrition or protein losses in the gut [3].

Chemokines

These are a family of small 8-10 KDa protein molecules that are produced by epithelial cells. They exert their effects by interacting with G-protein–linked Trans membrane chemokine receptors and function as chemo attractants for inflammatory cells by inducing chemotaxis and activating leucocytes. They are divided in to two major groups, CC chemokines with adjacent cysteine residues, and CXC chemokines with cysteine group separated by an amino acid. The CC chemokine is ligand (CCL)-5 is a potent chemoattracnt that is unregulated in a human intestinal epithelial cell model of C.*parvum* infection. The CXCchemokine CXCL-10 (IFN-γ inducible protein 10), which recruits IFN-γ producing T-cells was expressed in higher levels in jejunal biopsies of AIDS patients with cryptosporidiosis compared with uninfected control patients or normal. Additional studies are required to confirm the role of specific chemokines in human cryptosporidiosis [3].

Cytokines

These are proteins that play a key role in modulation of both innate and adaptive immune responses. In human intestinal epithelial cells IFN-γ directly prevents the parasites from invading host cells, most likely by activation of the JAK/STAT signalling pathway following binding to IFN-γ receptors on intestinal epithelial cells, as well as by modification of intracellular Fe+2 concentrations. A recent studies suggested that C.*parvum* infection may down regulate IFN-γ by suppression of STAT1-α signalling, thus facilitating invasion. Again additional studies are essential to define the role of specific cytokines in human cryptosporidiosis [3].

Prostaglandins

These are potent lipid molecules that exert their effects via a wide array of receptors. C.*parvum* infection of human intestinal epithelial cells in-vitro resulted in activation of prostaglandins H synthase 2 expression and increased production of Prostaglandins (PG)-E2 and F2-α.Although prostaglandins may contribute to the pathogenesis of secretory diarrhoea by altering chloride uptake and fluid secretion ,they may also up regulate mucin production from epithelial cells, which can protect the host intestinal mucosa from being infected with C.*parvum* by interfering with attachment of the parasite.

Substance P is a neuropeptide that is located in the gastrointestinal tract .It can cause chloride ion secretion and play a role in secretory diarrhoea. Jejunal biopsies from AIDS patients with cryptosporidiosis showed increased expression of substance P mRNA and protein with diarrheal symptoms. However the exact role of prostaglandins and substance P need to be further defined [3].

Adaptive Immune Response

The critical role of cell-mediated immune responses in protection and resolution of cryptosporidiosis has been well established in both murine models and human studies.

T-cell immune responses play a critical role in resolution of cryptosporidial infection, predominantly via CD4+ cells and IFN- γ mediated pathways. IFN- γ may play a role in T-cell memory responses [3].

Humoral Immunity & Antibody Responses

Several studies have reported serum antibody responses to cryptosporidial antigens following infection. A number of antigens, including gp900, p23/27, gp40 and gp15/17, that mediate *Cryptosporidium* host-parasites interactions induce antibody and or cell-mediated immune responses in humans .Human volunteer studies show that those with pre-exiting anticryptosporidial antibodies are protected from diarrheal symptoms but not infection [3].

VI. IMPDH: STRUCTURE AND REACTION MECHANISM

IMPDH (IMPDH. E.C. 1.1.1.205 and CAS number 9028-93-7) is a ubiquitous enzyme found in species from bacteria to mammals and it appears that there is a high degree of sequence conservation between several IMPDH proteins. IMPDH belongs to One $(\beta/\alpha)_8$ barrel protein superfamily along with guanosine monophosphate reductase (GMPR), which catalyze similar reaction with apposing metabolic consequences

.Inosine 5' monophosphate also serves as a substrate for the two step biosynthesis of adenosine 5' monophosphate (AMP)

[8].

It is considered as an important target in the quest for drugs in the immunosuppressive, antiviral, antibacterial, anticancer and anticryptosporidial therapeutic areas [5].

In human two isoforms of IMPDH have been identified, designated as type I and type II. Each contain 514 AA and they share 84% sequence identity. IMPDH present in bacteria and protozoa share 30%-40% sequence identity with the human enzyme. Phylogenetic analysis recommend that *C.parvum* IMPDH was obtained from a bacterial source by lateral gene transfer Regardless of species native IMPDH exist as homotetramer with subunit molecular masses in 56-58 kDa, which is the active form of IMPDH [6]. Each monomer is consist of two domains, a major catalytic domain and a smaller flanking domain. Only 301 out of 400 residues are visible in the most structured monomer. The disordered region include catalytically important residues 214-222,299-333 and 380-400 as well as residues 92-122, which are not required for enzymatic activity. Residues 214-226, It include the catalytic cys219, are observed in most of the monomers, as are the parts of the active site flap (residues302-330) containing the characteristics ArgTyrmotif. Lastly the SerGlyGly linker is visible in all monomers. This structure has been deposited in PDB (3FFS) [7]. In CpIMPDH the 394 residues catalytic domain forms an eight standard parallel α/β barrel core with attached N-, C-, terminal sections. The active site (residues 302-330) containing the characteristics ArgTyr motif is formed by the loops between the strands of the barrel and thus is bound by one face of the barrel, as well as an 18- residues loop (residues 325-342) and 54 residues flap (residues 398-451) inserted on the barrel face. The 12 residues flanking domain (residues 113-232) lies adjacent to the catalytic domain inserted between the α2 helix and β3 sheet of the barrel. The function of the flanking domain in IMPDH is unknown as it is not required for activity but contains two copies of a cystathione β synthasase like domain [8]. The IMPDH active site consists of a long cleft in the barrel face located close to the monomer-monomer interface. The cleft has IMP and NAD-binding subregions.Binding of IMP places the inosine base close to the catalytic residue Cys 331.This 5residue forms part of a mobile loop that covers a portion of the IMP binding subsite. This geometry allows formation of the covalent adduct between the Cys 331 thiol group and the C2 carbon on the Inosine base. The NAD cofactor binds in the continuation of the active site cleft adjacent to the IMP subsite. In this position, the NAD nicotinamide ring stacks with its B-face against the inosine base, insuring efficient stereochemical hydride transfer between the two moieties. The Nicotinamide phosphate and adenosine moieties lie in the remainder of the active site cleft. Both the substrate and the cofactor are anchored in the active site by a series of polar interactions involving conserved residues on the walls of the active site cleft and the residues on the active site loop. In addition, a highly mobile 50-residues flap covers portions of both the IMP and NAD sites, and also forms interactions with the substrate and cofactor [8]. Inosine5, monophosphate dehydrogenase catalyzes the irreversible Nicotinamide adenine dinucleotide (NAD) dependent oxidation of inosine 5' monophosphate (IMP) to xanthosine 5' monophosphate (XMP) with concomitant reduction of NAD+, which is first committed and rate limiting step in de-novo synthesis of guanosine nucleotide biosynthesis. XMP is subsequently converted to GMP by the action of GMP synthetase (GMPS) [5].In these reaction IMPDH catalyzes a hydride transfer reaction involving a Nicotinamide cofactor, with formation of the covalent intermediate E-XMP. Hydrolysis of this adduct produces XMP which is converted to GMP by the action of GMP synthase enzyme thus IMPDH catalyzes two distinct chemical transformations. In the first step, cys319 attacks C2 of IMP and hydride (H-) is transferred to NAD+ to produce the covalent intermediate E-XMP. This step is probably passes through a tetrahedral

intermediate which facilitates the hydrolysis of E-XMP complex. The catalytic water molecule forms hydrogen bond to Thr321 from the same loop that contains the catalytic Cys319, as well as the two residues of the flap, Arg418 and Tyr419.in this step Arg418 act as the base that activates water by extracting a proton from catalytic water. Proton transfer is rate limiting and almost completed in the transition state. Further studies moreover revealed the existence of another pathway for the activation of water in which Thr321 activates water through a proton relay with Glu431.Thr321 pathway activates water at low pH, while the Arg 418 pathway dominate at high pH The hydrolysis of E-XMP produces XMP and NADH [9].

Inhibition of IMPDH effects various cell processes like cell proliferation, cell propagation and reduction in guanine nucleotide which results in defective formation of RNA and DNA biosynthesis. Furthermore, this inhibition affects efficient cell propagation, cell signalling and also depletes energy source [8].

VII. CURRENTLY AVAILABLE CHEMOTHERAPEUTIC AGENTS

Nitazoxanide, a nitrothiazole benzamide derivative was approved for use in treating *Cryptosporidium* infection in children aged 1-11 Egypt and Zambia but at present nitazoxanide is not licensed for use in treating cryptosporidiosis in immunodefecient person. Nitazoxanide has demonstrated in vitro activity against C. *parvum* and G. intenstinalis in humans with healthy immune system. It has been shown to inhibit the growths of sporozoites of C.*parvum* on its own and has also demonstrated combined in –vitro activity with both azithromycin and rifampin, suppressing growth of C.*parvum* by 83.9% and 79.8% respectively, compared with 56.1% when used alone. Gastrointestinal side effects of nitazoxanide treatment is generally well tolerated and no significant adverse effects have been noted in human trials [10].

Dicyclic and Tricyclic Diaminopyrimidine derivatives are the diaminopyrimidine inhibitors of dihydrofolate reductase (DHFR) common example of these are trimethoprim, pyrimethamine, trimetrexate, and piritrexim. These are used in the prophylaxis and treatment of *Cryptosporidium* infections in patients whose immune system are impaired as a result of human immunodeficiency virus infection or immunosuppressive chemotherapy [11].

Mycophenolate mofetil **(**MPA) is a selective, reversible, noncompetitive inhibitor of eukaryotic inosine monophosphate dehydrogenase [12].

VIII. CONCLUSION

In this paper, an extensive study of *Cryptosporidium* and cryptosporidiosis has been discussed from currently available literatures. Despite numerous investigation going on Cryptosporidiosis remain a devasting disease for immunocompromised person. However different various types of drugs have been created to cure Cryptosporidiosis in immunocompromised patients but these drug are not fully effective against cryptosporidiosis, they are only recommended as a substitute of effective drug therapy. There are various targets (against which drug has to be synthesized) present in *C. parvum*, but the most promising emerging target is IMPDH, as it has been transferred to this protozoan via some bacteria through lateral gene transfer. This paper would be helpful for more effective drug discovery for cryptosporidiosis.

IX. FUTURE PROSPECTIVE

Investigation of genetic polymorphism in Toll-like receptors and MBL genes and susceptibility to cryptosporidiosis in vulnerable populations. Investigation of the role of antimicrobial peptides in infected humans. Investigation of systematic and mucosal cell-mediated and humoral responses to putative protective antigens in longitudinal birth cohort studies in vulnerable human populations such as malnourished children in developing countries. Further studies in humans to determine whether antibody responses are themselves protective, or if they are just markers of cellular immune responses. Investigation of immune responses to glycotopes of putative protective antigens. Use of genomic and proteomic approaches to identify additional putative protective antigens. Development of targeted prophylactic and therapeutic immune–based strategies [3].

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