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Review:

Past achievements, current situation and future challenges for vaccine development against *Cryptosporidium parvum* and *C. hominis* infections

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ABSTRACT

In developing and developed countries, infection with *Cryptosporidium* species is considered as a disease of major health and economic concerns. Among the several identified species, *C. parvum* (zoonotic and anthroponotic agent) and *C. hominis* (anthroponotic agent) are the most important species, inducing diarrhea in humans and calves. Fatality of cryptosporidiosis in AIDS patients and immunocompromized individuals engender the importance of immune response against such disease. As most of parasitic diseases, the vaccine development against *Cryptosporidium* is still problematic because of great complexity of developmental parasitic stages. However, the immuno-prophylactic and therapeutic approaches have exhibited promising results in mouse and cattle models. Both cellular and humoral immunities are required for conferring protective immunity in the tested animal models. Recombinant DNA and protein based on glycoprotein Cp15 and P23 are the predominantly evaluated candidates and the mostly successful vaccine antigens. This review highlighted the previous studies with successful vaccine antigens, summarizes the current challenges and limitations, and suggests solutions for future application in vaccine development against *Cryptosporidium* infection.

Keywords: *Cryptosporidium*; Vaccine; *Cryptosporidium parvum*; *Cryptosporidium hominis*; Immunization

BACKGROUND

Cryptosporidiosis is recognized as an important zoonotic disease which caused by many *Cryptosporidium* species. The disease in susceptible hosts is generally characterized by watery diarrhea, weightloss and anorexia (Chalmer and Katzer, 2013). *Cryptosporidium* was first identified as a cause of diarrhea in calves in 1971 (Panciera et al., 1971), and afterwards it has been recorded as a second leading cause of calve diarrhea worldwide (De Graaf et al., 1999). Additionally, *Cryptosporidium* was reported in human infection case in 1976 (Meisel et al., 1976), and during the early 1980s, it was recognized as the predominant cause of persistent diarrhea in AIDS patients and in children (Current et al., 1983; Sallon et al., 1988). *Cryptosporidium* is reported as a major cause in numerous waterborne outbreaks of diarrhea or abdominal pain of parasitic origin worldwide (Xiao, 2010; Efstratiou et al., 2017). Among numerous

already recognized *Cryptosporidium* species, *C. parvum* which infects primarily cattle and human and *C. hominis* infecting human are acquiring considerable concerns (Rose et al., 2002). There are numerous factors which rendering cryptosporidiosis as one of the most famous parasitic infections worldwide. Among of these factors; lack of effective therapeutic or preventive measures, unavailability of accurate and sensitive diagnostic tools, ineffectual disinfection with chlorine, and high infectivity to human and many other animal species associated with high mortality in immunocompromised patients (Boulter-Bitzer et al., 2007; Checkley et al., 2015; Fereig et al., 2018).

Transmission and Life cycle

As a monoxenous organism, *Cryptosporidium* spp. can complete its life cycle in a single host. The infection occurs via ingestion of food or drinking water contaminated with oocysts or through direct contact with infected humans, animals, or contaminated premises or soil (Rose, 1997). The infective oocysts may be shed extensively in feces of infected animals or humans and are very resistant to many environmental factors such as unsuitable temperatures and humidity (Ramirez et al., 2004). After ingestion, the excystation of oocysts occurs and the sporozoites are liberated to invade the intestinal cells and form trophozoites. Afterwards, some parasites develop to type I meront which liberates merozoites that reproduce a sexually and can reinfect the neighboring cells. Other parasites develop to type II meront which releases merozoites that reproduce sexually by forming micro or macrogamonts which undergo further development resulting in microgametes or macrogametes, respectively. The later stages are responsible for fertilization and formation of zygots which develop to oocysts which induce reinfection of the host or excreted with feces (Current and Garcia, 1991).

Health hazards and economic burden

Because of its zoonotic character, worldwide distribution, and wide range of host affection, *C. parvum* is the most common and important *Cryptosporidium* species (Chalmers and Katzer, 2013). In case of farm animals, *C. parvum* constitutes the major cause of clinical cryptosporidiosis in cattle, sheep and goat. In humans, the zoonotic species *C. parvum* and the human adapted species *C. hominis* are the predominantly recognized species in clinical cases of cryptosporidiosis (Morgan-Ryan et al., 2002). These two species are incriminated for more than 90% of human infections worldwide (Chalmers and Giles, 2010). In most cases of infections, *C. parvum* and *C. homins* cause self-limiting watery diarrhea, anorexia and abdominal pain (Klein et al., 2008). In case of complications or in immunocompromized individuals, the disease may trigger serious consequences such as persistence for long time, spreading to several organs; end by death (Denkinger et al., 2008; Mor et al., 2010)..

Profuse watery diarrhea is the major clinical sign induced by *Cryptosporidium* infections and responsible for most of recorded health hazards and economic losses because of the resultant dehydration, electrolyte imbalance and malnourishments. Diarrhea may be occurred by two pathways; malabsorptive way because of destructive intestinal cells and sloughing of villi (Klein et al., 2008), or the secretory way as a result of profuse secretion from intestinal glands and inflammatory response (Clark and Sears, 1996). The economic losses attributed to *Cryptosporidium* infections have never been estimated systematically. However, high losses are expected because of worldwide distribution including the cost of treatment of diarrhea, decreased feed conversion rate,

and also losses due to animal death or even poor carcass quality of infected animals (Sweeny et al., 2011).

Similarly in human, cryptosporidiosis is associated with severe health hazards and also economic losses. Following the Rota virus, infections with *C. parvum* and *C. hominis* are the second leading cause of moderate-to-severe diarrhea in 0–11 month-old infants and the third most common in 12–23 month-old toddlers in sub-Saharan Africa and south Asia (Kotloff et al., 2013). This infection is usually associated with malnutrition, poor growth and impaired cognitive functions in young children in developing countries (Guerrant et al., 1999; Korpe et al., 2016). Moreover, around 202,000 deaths are attributable to cryptosporidiosis among children younger than 2 years old in sub-Saharan Africa, India, Bangladesh, Pakistan, Afghanistan and Nepal (Sow et al., 2016). Not only in developing countries but also in developed countries, cryptosporidiosis is considered a major health concern. Numerous waterborne outbreaks have been recorded in developed countries caused by the contamination with *C. hominis* or *C. parvum* oocysts of drinking (poor treated water resources) or recreational water (swimming pools). Historically, waterborne outbreaks happened in Milwaukee, USA in 1993 was one of the most important ones, in which 403,000 people suffered from watery diarrhea attributable to *Cryptosporidium* spp. infection (Chalmers, 2012; Efstratiou et al., 2017), and many immunocompromised people were died (Hoxie et al., 1997)

Immune response against *Cryptosporidium* species

Innate immunity

Intestinal epithelial cells (IECs) play an important role during *Cryptosporidium* infections. IECs are considered the main target tissues to sporozoites after excystation from infective oocysts. Thus, integrity of these cells is crucial which acts as a natural mechanical and functional barrier to prevent the infection with such organisms (Laurent et al., 2017). In addition to IECs, secreted mucous, cytokines, chemokines and antimicrobial peptides located in intestinal lumen, submucosa and bloodstream are essential in controlling cryptosporidial infection at early stage. IECs express many toll-like receptors (TLR) as TLR2, 4, 5, and 9 which have been reported as essential immune effectors and efficiently modulate host immunity for parasite killing and elimination (Barrier et al., 2006; Costa et al., 2011; O'Hara et al., 2011; Lantier et al., 2014; Perez-Cordon et al., 2014). Following recognition with TLRs, infection with *Cryptosporidium* spp. activates the MyD88 and NF-kB signaling pathway to induce the production of human β-defensin 2 for clearance of parasites (Chen et al., 2005). Up on infection, IECs secrete chemokines and cytokines such as CCL2, CXCL10 and IL-8 which recruits the inflammatory cells to the site of infection and induce subsequent enhancement to adaptive immunity (Laurent et al., 1997; Auray et al., 2007; Pantenburg et al., 2008).

IECs also produce antimicrobial peptides like β-defensins, which may combat the sporozoites in the intestinal lumen (Carryn et al., 2012). Shortly after infection, the recruited immune effectors cells such as macrophage, dendritic cells and natural killer (NK) cells participate effectively in the scene via secreting IFN-γ to eliminate *C. parvum* infection. IFN-γ is the key mediator in killing several intracellular pathogens including *C. parvum* infection either via innate or adaptive dependent immune pathway (McDonald et al., 2013). IL-12 and IL-18 were reported to control *C. parvum* infection

through the feedback mechanism with IFN-γ (Urban et al., 1996; Choudhry et al., 2012). Nitric oxide is another effector molecule sharing in *C. parvum* elimination and decreases oocyst shedding in chronically infected nude mice via IFN-γ-independent manner (Leitch et al., 1994; Nordone and Gookin, 2010).

Adaptive immunity

Deep understanding of adaptive immune response against *Cryptosporidium* spp. is the key factor for successful vaccine development. The intestinal cells of normal host contain specialized lymphoid tissues which constitute the main line of defense against pathogens (Forchielli and Walker, 2005). As an intracellular pathogen, the cell-mediated immune response is critical for resistance against *Cryptosporidium* spp. infection. T cell subsets are the key immune effector cells playing interactive pathways to achieve the complete resolution of parasite infection. $CD4^+$ T cell is the cornerstone in controlling many intracellular parasites including *Cryptosporidium* species even during innate immune response against acute infection (Fayer and Xiao, 2008). In immunocompromized individual lacking $CD4^+$ T cells, the infection with *Cryptosporidium* spp. is markedly aggravated as evidenced clearly in AIDS patients (Hunter and Nichols, 2002, Rashmi and Ravi Kumar, 2013). In addition, $CD8^+$ T cell is also contributing in protection against infection with *Cryptosporidium* parasites, albeit a lower extent than $CD4^+$ T cell (Kvac et al., 2011). This effect is exerted by cytolytic effect of infected cells.

Similarly to innate immune response, cytokines are crucial in orchestrating the function of adaptive immune response. IL-12 produced from macrophages and dendritic cells and IFN-γ secreted from NK cells are key actors in cell-mediated immunity. Positive feedback among IL-12 and IFN-γ resulted in formation of T helper 1 cell which secrets abundant IFN-γ and IgG2 and promotes differentiation of $CD8^+$ T cell. In another pathway which is not fully understood, IL-4 induces differentiation of a number of $CD4^+$ T cells to Th2 cells which produces additional IL-4, IL-10 and IgG1. IFN-γ has a negative feedback on Th2 cells and similar effect for IL-4 and IL-10 against Th1 (Ludington and Ward, 2015; Lemieux et al., 2017). This effect creates a status of balance between the aforementioned kinds of cells resulting in favorable microenvironment in the host which mostly protects the host cells from pathogen- and inflammation-related damage (Fig. 1).

Regarding the antibody-mediated immunity, several studies have reported their protective role against *Cryptospordium* spp. in mouse, cattle and human models. Although remarkable specific IgG, IgM and IgA antibodies were observed following Cryptosporidial infection, these antibodies could not protect animals against the adverse effect of the disease (Kassa et al., 1991; Allison et al., 2011; Borad et al., 2012). However, immunoprotective effect of antibodies was observed when the newly born animals were passively immunized with already prepared antibodies in colostrum or hyperimmune sera whatever before or shortly after infection (Tatalick and Perryman, 1995; Perryman et al., 1999; Wang et al., 2003; Steele et al., 2013).

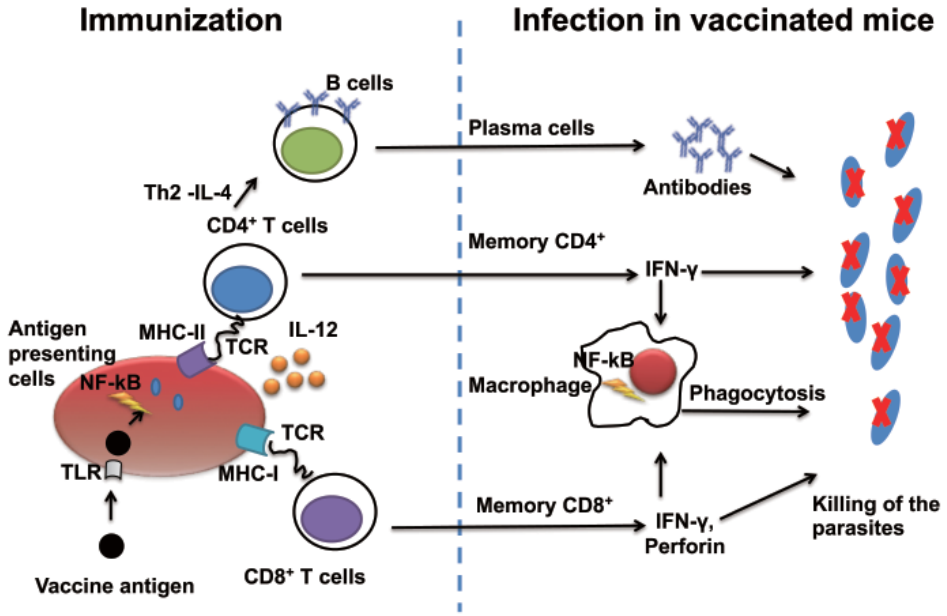

Fig. 1. A diagram showing the prospects for induction of protective immunity using vaccine antigen. In the case of successful immunization, vaccine antigen is processed by professional antigen presenting cells (APCs) such as macrophages or dendritic cells. Efficient pathways are incorporated in this process including pathogen recognition receptors (TLR) and transduction pathways (NF-kB). Activated APCs enhance the MHC class I and MHC class II which in turn elicits $CD8^+$ and $CD4^+$ cells, respectively. APCs also release effector cytokine IL-12 that is considered crucial for the production of IFN-γ. IFN-γ is the triggering cytokine for cell-mediated immunity. APCs also assisted in the production of IL-4 which is essential for the induction of humoral immunity. Eventually, the efficient vaccine antigen will enhance the production of specific antibodies, memory B-cells and memory T cells ($CD4^+$ and $CD8^+$). After infection with *Cryptosporidium,* APCs identify the parasite-derived molecules. This mechanism leads to rapid clearance of the parasite via adaptive or memory-related immune mechanisms, in addition to innate immune responses.

Vaccination against *C. parvum* and *C. hominis*

Past and current achievements

Many vaccination studies have revealed successful vaccine antigens and approaches against *Cryptosporidium* spp., albeit the remarkable limited number if compared to other related protozoan parasites. Hopefully, this review and other similar studies will represent helpful information and insights for researchers for further future attempts to reach the optimal goal. In Table 1, we illustrated the antigen type, vaccination procedures and obtained results. Adequate immunogenicity (reactivity with effector immune cells and molecules) and antigenicity (ability to generate specific antibodies) are essential properties for vaccine antigen possessing protective efficacy against cryptosporidiosis.

Table 1. Types, procedures and protective effect of previous successful vaccine antigens against *C. parvum* and *C. hominis*

Vaccine antigen	Parasite spp., Animal model and Route of immunization	Effect	Reference
Lyophilized *C. parvum* oocysts	- *C. parvum* - Cattle - Oral	Reduce duration of diarrhea and oocysts shedding	Harp and Goff, 1995
Cp15 DNA vaccine model	- *C. parvum* - Goat - Intranasal	Reduce oocysts number and period of shedding	Sagodira et al.,1999
A recombinant protein (rC7) incorporated in monophosphoryl lipid A trehalose dimycolate adjuvant	- *C. parvum* - Cattle - Subcutaneous	Reduce oocysts number	Perryman et al.,1999
Cp15/60 DNA vaccine model	- *C. parvum* - BALB/c mice - Intramuscular	Reduce oocysts number	He et al., 2004
Cp15-P23	- *C. parvum* - BALB/c mice - Unidentified route	Prevent the infection	Hong-Xuan et al., 2005
Cp23 DNA vaccine model	- *C. parvum* - Interleukin-12 (IL-12) knockout (KO) mice - Subcutaneous	Reduce oocysts number	Ehigiator et al., 2007
Cp12, Cp21 DNA vaccine model	- *C. parvum* - BALB/c mice - Intramuscular and Intranasal	Reduce oocysts number	Yu et al., 2010
Cp15, p23 DNA vaccine model	- *C. parvum* - C57BL/6 mice - Intramuscular	Reduce oocysts number	Wang et al., 2010
Cp15-23 Freund's adjuvant	- *C. parvum* - BALB/c mice - Subcutaneous	Reduce oocysts number	Liu et al., 2010
Acidic ribosomal protein P2 (CpP2) DNA vaccine model	- *C. parvum* - C57BL/6 mice - Subcutaneous	Reduce oocysts number	Benitez et al., 2011
AB protein of *C. andersoni* Vector-based vaccine	- *C. parvum* - BALB/c mice - Intramuscular	Reduce oocysts number	Zheng et al., 2011
Cp15 Vector-based vaccine (*Salmonella* serovar Typhi CVD 908-htr A vector followed by rCp15 protein in Freund's adjuvant)	- *C. hominis* - C57BL/6 mice - Intranasal for vector-based vaccine and intraperitoneal for rCp15 + adjuvant	Reduce oocysts number	Roche et al., 2013
rP23+ Freund's adjuvant	- *C. parvum* - Cattle - Subcutaneous	Reduce oocysts number and delay onset of shedding	Askari et al., 2016

Several previous studies have reported that Cp15 and P23 are crucial molecules for host-parasite interaction, and as the most promising antigens for vaccination and diagnostic studies (Boulter-Bitzer et al., 2007; Checkley et al., 2015). Cp15 is a glycoprotein, which is indispensable for the parasite motility, attachment, and invasion of host epithelial cells (Reperant et al., 1994). Specific antibodies to Cp15 were found in serum and colostrum of cattle, and in serum of mouse and rats infected with *C. parvum* (Jenkins et al., 1993; Jenkins et al., 1999). Similarly, P23 is a glycoprotein contributing in sporozoite gliding and locomotion (Enriquez and Riggs, 1998), specific antibodies were reported in human and cattle (Arrowood et al., 1991). Additionally, CpP23 is

considered the mostly used antigen for serodiagnosis of *C. parvum*-specific antibodies in field animal samples, peculiarly in cattle (Wyatt and Perryman, 2000; Bannai et al., 2006; Wang et al., 2009; Fereig et al., 2016; Fereig et al., 2018, Ichikawa-Seki et al., 2018, Masatani et al., 2018).

In the same context, the current review reported the success of Cp15 and P23 as a vaccine antigen that protect efficiently against adverse effect of *C. parvum* and *C. homins* infection. Immunization with Cp15 as DNA or protein vaccine could protect against *C. parvum* and *C. homins* infection in goat and different mouse strains as recorded in reduced oocysts shedding in feces. In a similar way, the vaccination with P23 protected the cattle and mouse models against challenge infection with *C. parvum* oocysts. Expectedly, the combination with both antigens (Cp15+P23) also exerted a remarkable protective efficacy against *C. parvum* infection when used in BALB/c mice model (Boulter-Bitzer et al., 2007; Checkley et al., 2015). Accordingly, the current review has reported the adequacy of Cp15 and P23 as potential vaccine antigens using different approaches and animal models, and their usefulness for further use at experimental or field trials. More details on antigen types, vaccination procedures, protective indices and reference are summarized in Table 1.

In another aspect, passive immunization has shown as a useful trend in protecting animal models (cattle and mouse) against *Cryptosporidium*. The passive transfer of anti-*Cryptosporidium* antibody via colostrum or hyperimmune sera from previously infected animals has reduced the number and duration of oocyst shedding and alleviated severity of infection. In addition, the administration of hyperimmune bovine colostrum for the infected humans has also been evaluated, with variable successes (Riggs, 2002; Boulter-Bitzer et al., 2007).

Future challenges, expectations and recommendations

In view of problematic situation of the control strategy of *Cryptosporidium* spp. infection, further studies are required to develop potent vaccines including screening novel candidates and applying more effective technologies and approaches. To date, there is no FDA-approved vaccine against *Cryptosporidium* spp. infection. There are several reasons for unavailability of potent vaccine against cryptosporidiosis in animals or humans. Immature *in vitro* culturing system of different parasitic stages is the major limiting point in this aspect. The greater complexity of life cycle and developmental stages of this parasite compared to bacteria and viruses is another point. Thus, as a result of the above-mentioned reasons, available information about the development, pathogenesis, and specific immune responses are extremely ambiguous and our understanding is greatly low. This critical situation necessitates the applying of more studies that focus on basic peculiarities of *Cryptosporidium* spp. such as parasite categorization, host susceptibility, invasion requirements, and pathogenesis as preliminary steps for establishing effective control strategies including vaccine development.

Concerning the vaccine development, special attention should be directed to the interaction of *Cryptosporidium* spp. with host immunity. The key players of vaccine-related immunity as cellular and humoral immunities should be investigated thoroughly. As highly expressed receptors in IECs and relevant defense cells, TLRs are considered as essential actors that should be extensively evaluated in vaccine development against *Cryptosporidium* spp. TLR ligands have been previously investigated as adjuvants in

multiple vaccine trials, with inducing marked improvement in the protective efficacy. In *Cryptosporidium* infected-mice, the administration of TLR agonists induced strong immune response and enhanced the parasite clearance, suggesting their feasibility as effective vaccine adjuvants (Barrier et al., 2006; Lantier et al., 2014). Stimulation of professional phagocytic cells such as macrophages and dendritic cells is a promising approach in vaccination trials. Such cells are programmed automatically to uptake the vaccine antigens which undergo subsequent presentation and processing to deal with following immunological interface.

Even with low practicability and incomplete protection triggered with the induction of antibody-based immunity against *Cryptosporidium* spp. infection, the mechanism of this pathway should be elucidated more deeply. This approach has already proved its feasibility in protection against *Cryptosporidium* spp. (Riggs, 2002; Boulter-Bitzer et al., 2007). The limited success of this phenomenon can be improved and exploited in animal model as shown in cattle, newly born calves receiving colostrum containing specific antibody to *Cryptosporidium* from previously infected dams have shown milder signs than those unfed calves. However, the restricted experimentations on human hinder the evaluation of this effect as occurred in animal models. At all events, this information corroborates the feeding of newly born animals or human babies with colostrum of dams or mothers, respectively. Unfortunately, the high cost and risk of using biological stuffs (serum or colostrum) containing specific antibodies to *Cryptosporidium* may prevent the large scale use of this approach especially in humans.

In conclusion, this review highlighted the defective situation of vaccine development against *Cryptosporidium* infection and intensifies the need to find novel vaccine candidates and potent technologies. Although promising results reported in vaccine studies, vaccine development against *Cryptosporidium* spp. is still distant than those recorded in other intestinal invading protozoan parasites such as *T. gondii* and *N. caninum*. These studies are primarily focused on a limited number of *Cryptosporidium*-derived antigens; mostly Cp15 and P23. Moreover, the shedding of oocysts in the tested animal models was mostly estimated as a solely protective index in all the screened studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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