Virulence factors

Review Article

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ABSTRACT

Parasites developed several strategies for their survival and host tissue invasion. Helminths express potent molecules mainly for immunomodulation, which is why they stay in their hosts for years. Helminths display several mechanisms not only to evade host immune response(s), but also to preserve the host for as long as they could live. In contrast, protozoa evolve several policies primarily for pathogenesis, and invasion. Therefore, variable clinical manifestations are reported in protozoal diseases. Both symptomatic and asymptomatic cases are commonly observed in amoebiasis, giardiasis, trichomoniasis, cryptosporidiosis and toxoplasmosis, while mild, moderate, and severe cases occur in malaria, leishmaniasis, African sleeping sickness and Chagas' disease. This was primarily attributed to strains variability and to a lesser extent, to host immune response(s). With recent evolutionary technology in molecular parasitology and bioinformatics, several molecules are established as virulence factors. These factors encourage researchers and scientists to develop novel drug targets and/or vaccine candidates. The present review aims to highlight, and review virulence strategies adapted by parasites to invade host tissue, enhance its replication and spread, as well as other processes for immunomodulation or immunoevasion of host immune response(s).

Abbreviations: CATH: Cathepsin; **CP:** Cysteine protease; **CPI:** Cysteine protease inhibitor; **CYS:** Cystatin; endogenous CPI; **EMP1:** Erythrocyte membrane protein 1**; EVs:** Extracellular vesicles; **GP:** Glycoprotein; **HSP:** Heat shock protein; **MEROPS:** Proteases database (www.ebi.ac.uk/merops/)**; MP:** Metalloprotease; **PV:** Parasitophorous vacuole; **SP:** Serine protease; **SUB:** Subtilase, subtilisin-like proteases; **VSPs:** Variant surface proteins.

Virulence factors are molecules expressed by pathogens to manipulate disease outcome(s). Disease virulence is defined as direct or indirect reduction in host fitness attributable to pathogen infection. Virulence factors are also defined as influentail factors that contribute together to effect disease severity and duration. Common terms used to evaluate disease virulence are morbidity, mortality, and pathogen burden. Other additional measurements are the host cell damage and the magnitude of both inflammatory and immune responses elicited by the pathogen[1]. From my point of view, contributory factors are accepted in some instances. For example, *Plasmodium* spp. collectively constitute one group of the most virulent parasites and possess only one established virulence factor: the erythrocyte membrane protein 1 (EMP1). Contribution of other factors such as proteases arsenal, and extracellular vesicles (EVs) may influence its virulence. These factors are essentially involved in hemoglobin degradation, and egress and *de novo* invasion cascade that have great influence in malaria complications and severity.

Differences between helminths and protozoa

In helminthic multicellular parasites, virulence factors depend mainly on immunoevasion and modulation of host immune response so as to live longer inside the host, *i.e.* long-term infections. This is achieved by the antigenic adult cuticle or life cycle stages multiplicity[2]. The excretory/secretory (E/S) products released by the majority of helminths showed significant interference with all aspects of host immune responses, starting from initial recognition to end-stage effector mechanisms. Hewitson *et al.*[2] reviewed all antigenic proteins commonly released in E/S products of several prevalent tropical helminthic infections and discussed their mechanism(s) of action. The reviewers observed that most antigens expressed in E/S products, with few exceptions, had an immunomodulatory mechanism of action. However, some cysteine proteases (CPs); cathepsins (CATHs) and legumains, through their proteolytic activities contribute in migration as with the juvenile flukes of *Schistosoma*[3] and *Fasciola* spp.[4]. In spite of that, both CPs are not established as virulence factors to date.

In contrast, virulence factors in unicellular protozoa depend mainly on molecules contributing in survival ability inside phagocytic vesicles for colonization, *i.e.* short-term infections. However, some protozoa avoid host immune response utilizing antigenic variation; GPs in *Leishmania* species[5] and EMP1 in *P. falciparum*[6]. Protozoa, whether intra- or extracellular, utilized various pathways to

communicate within their own populations for several functions including growth promotion, host immune system evasion, disease transmission, and manipulation of micro-environmental stress. Communication is also directed to the host through trafficking transfer of effector molecules to host cells to manipulate host gene expression, and consequently mediate parasite pathogenicity[7].

• **Extracellular vesicles (EVs):** These are nano-scale lipid bilayer membrane-bound structures. They contribute in the trafficking of virulence factors required for parasite nutrition, cytoadherence, host cell migration and invasion, cytotoxicity, and host immune system evasion[7]. Reviewing literature, EVs are classified into exosomes, microvesicles and apoptotic bodies. Exsomes and microvesicles are released with conserved biogenesis and functional roles. For example, exsomes in *G. lamblia*, *T. vaginalis* and pathogenic trypanosomatids are released at the flagellar pocket, whereas they are intracellularly released in apicomplexans as microvesicles[8]. It is worth mentioning that *Plasmodium* EVs include exonemes, micronemes, and mononemes. They are merozoite secretory apical organelles that express in the parasitophorous vacuole (PV) subtilases 1 and 2 (SUB1, SUB2) and rhomboid-1 (ROM1), respectively. Their role in egress and *de novo* invasion cascade will be discussed later[9].

• **Egress cascade:** A wide spectrum of pathogenic bacteria and protozoa adapt several strategies to enter and exit their host with optimum rates of survival, replication, progression through life cycle stages as well as transmission. Pathogen egress is of fundamental importance due to its close association with pathogen spread, transmission and inflammation processes. Accordingly, molecules involved in egress mechanism(s) are considered key steps in transmission and infection, *i.e.*, they are considered indirect virulence factors. In their review Friedrich, and his colleagues[10] listed in a table several molecules involved in egress mechanism(s) in *T. gondii*, and species of *Plasmodium*, *Trypanosoma* and *Leishmania*. Egress strategies are designed to overcome host cellular membranes, cell cytoskeleton, and organelles. Pathogens utilize proteases, lipases, and pore-forming proteins as molecular effectors of active egress. Also, pathogens use molecular mimicry to simulate host cellular cytoskeleton dynamics. For instance, some pathogens such as *T. cruzi* escape PV to replicate in the host cell cytosol. However, the parasite has to control this first egress step to preserve host cell integrity. After replication, a second controlled egress event takes place to release replicates that infect new host cells[10].

In *Plasmodium* spp., egress and *de novo* invasion cascade involves the following steps: 1) while SUB1 is involved in merozoites egress, SUB2 is required for merozoites *de novo* RBCs invasion; 2) degradation of PV membrane; 3) breakdown of both RBC' membrane and cytoskeleton is essentially done by SUB1, SUB2 and ROM1, and 4) serine repeat antigens (SERA5 and SERA6); merozoite surface proteins (MSP1, 6 and 7) released from SUB1, and SUB2 contribute with ROM1 to catalyze the intermembrane cleavage leading to *de novo* RBCs invasion. Plasmepsins, and aspartyl proteases, also established their role in egress and *de novo* cascade, acting as a maturation factor for rhoptery proteins that control SUB1 maturation[9].

• **Ubiquitin-proteasome system (UBS):** The UPS has essential roles in several cellular pathways including those required for parasite biology and virulence, *i.e.* proliferation and cell differentiation, which are the key steps in protozoal colonization inside its host. Turnover of intracellular proteins is carried out by two proteolytic organelles: lysosomes and proteasomes, utilizing their molecules released by UPS[11]. The most commonly reported molecule is 20S, described as a barrel-shaped assembly of 28 protein subunits. For parasite proliferation and differentiation, 20S proteasome degrades its own proteins to oligopeptides (3-15 amino acids), followed by peptide hydrolysis. Therefore, hydrolyzed amino acids are used for biosynthesis of life cycle stages. Muñoz and her colleagues[12] from Chile reviewed roles and functions of 20S in *E. histolytica*, pathogenic trypanosomatids and *T. gondii* validating them as virulence factors and potential drug targets. They also claimed that UPS is not only a degrading machine, but it is also employed as regulatory factor involved in several pathways including cell growth, inflammatory response, and antigen processing[12].

Identification of virulence factors: No doubt that identification of virulence factors would help researchers to discover or develop new or synthetic inhibitors to be used as novel drugs and/or vaccine candidates, utilizing virtual or high-throughput screening. Reviewing literature, two approaches were utilized to identify virulence factors, either comparative transcriptomic analysis between virulent and avirulent isolates or gene knock-out, *i.e.* RNA interference to identify gene function(s).

Mechanisms involved in parasite virulence: Parasites utilized several strategies to establish their persistence in the host, *i.e.* alive (survival) and active (virulent). Reported utilized mechanisms to achieve these tasks include proteolytic activity, antigenic variation, protein folding and mechanical mechanism (Table 1). Proteolytic activity, achieved by several proteases, is the main strategy reported in almost all parasites. Antigenic variation comes next and is most frequently observed in different species of *Leishmania*, *Plasmodium*, and *T. cruzi*. Protein folding achieved by genes encoding heat shock proteins (HSPs) is less frequently reported in few parasites. It is worth mentioning that the mechanical mechanism is only reported in *G. lamblia*.

Table 1. Different mechanisms contributed by virulence factors

Mechanism	Factors	Parasites	Task	Reference
Proteolytic activity	CPs	*E. histolytica*	Cytoadherence & cytotoxicity	[13]
	CPs & MPs	*Leishmania* spp.	Cell invasion	[14]
	MPs	*L. braziliensis*	Immunoevasion	[15]
	SPs	*T. cruzi*	Egress cascade	[10]
	Proteases	*Plasmodium* spp.	Hb degradation & Egress cascade	[16] [9]
Antigenic variation	GPs	*Leishmania* spp.	Immunoevasion	[5]
		T. cruzi		[17]
	EMP1	*Plasmodium* spp.	Cytoadherence & Immunoevasion	[18] [6]
Protein folding	HSPs	*Leishmania* spp.	Stage differentiation	[19]
		T. gondii		[20]
Mechanical	SD & FG	*G. lamblia*	Cytoadherence	[21]

CPs: Cysteine proteases, **EMP1:** Erythrocyte membrane protein, **FG:** Flagella, **GPs:** Glycoproteins, **HSPs:** Heat shock proteins, **MPs:** Metalloproteases (Leishmanolysins), **SD:** Sucking disc, **SPs:** Serine proteases.

It should be considered that virulence may occur, in some instances, due to non-parasitic molecules such as missed diagnosis, ineffective treatment or drug resistance, immunosuppression and associated endosymbiosis. Prior to discussing parasitic virulence factors, two points are to be considered: endosymbiosis and trafficking of virulence factors through cellular membranes.

Endosymbiosis: There is much controversy over the contribution of intestinal enteropathogenic *Escherichia coli* and *E. histolytica* virulence. Incubation of *E. coli* in *E. histolytica* cultures can decrease[22] or increase[23] its virulence. A recent study showed that either enteropathogenic *E. coli* or nonpathogenic *Entamoeba coli* modified *E. histolytica* virulence causing amoebiasis in cell line culture as well as in experimental models due to increased proteolytic activity of expressed *Eh*CPs 1, 2, 4, and 5[24]. On the other hand, Burgess and her colleagues[25] focused in their review on the contribution of intestinal nonpathogenic microbiota not only on intestinal protozoa, but also on extra-intestinal protozoa as *Plasmodium* spp. and *T. vaginalis*. The reviewers attributed decreased virulence in intestinal protozoa to decreased parasite cytoadherence at the mucosal sites. However, intestinal microbiota may alter systemic immunity by alteration of granulopoiesis and/or adaptive immunity and by increasing virulence in non-intestinal protozoa. In addition, the reviewers tabulated commonly reported intestinal nonpathogenic microbiota associated with *E. histolytica*, *G. lamblia*, *T. vaginalis*. *P. falciparum* and species of *Cryptosporidium* and *Blastocystis*. Interestingly, it was concluded that treatment using microbiota may provide a cost-effective prophylactic strategy for intestinal protozoal infections[25].

Treatment of filarial patients with tetracycline was suggested to cause worm sterility in symbiotically associated filarial worms and *Wolbachia*. It was shown that recombinant *Wolbachia* surface protein predisposed to host immunoevasion, increasing disease pathogenicity and virulence[26]. Additionally, the role of *T. vaginalis* virus (TVV) in the parasite virulence was demonstrated in several studies; as induction of various phenotypic changes[27] and contribution in parasite cytoadherence[28,29]. Similar studies were also conducted for *Leishmania* RNA virus[30] and *G. lamblia* virus[31].

Palmitoyl acyltransferases (PATs) family: Protein S-palmitoylation is a dynamic biochemical process in which the fatty acid palmitate is covalently linked to cysteine, or less frequently serine and threonine residues on proteins utilizing palmitoyl acyltransferase (PAT). Then, it can be removed by lysosomal palmitoyl-protein thioesterase (PPT) or cytosolic acyl-protein thioesterase (APT). These residues of proteins are commonly found in protozoal membrane proteins. Therefore, palmitoylation plays a significant role in proteins trafficking between membrane compartments, and it is usually a reversible reaction. Dynamic palmitoylation facilitates targeting proteins to specific intracellular compartments through trafficking pathways, proteins cycling between membranes, as well as protein stability. In their review, Brown and his colleagues[32] claimed that large families of PATs were identified in some eukaryotic parasites; *T. brucei* (*Tb*DHHC 1–11), *P. falciparum* (*Pf*DHHC 1–12), *T. gondii* (*Tg*DHHC 1–18), and *C. parvum* (*Cp*DHHC 1–10). The reviewers discussed their roles in diverse aspects of pathogenesis, such as life cycle stages differentiation, biogenesis of secretory organelles, and targeting virulence factors to the plasma membrane. In addition, it was observed that apicomplexan PATs are mainly localized in the Golgi apparatus and endoplasmic reticulum, however, few are found in the plasma or nuclear membranes. Several PATs in *P. falciparum* and *T. gondii* are localized in unique apicomplexan organelles, rhoptries, essential organelles for gliding motility and are also involved in secretion of factors necessary for parasite invasion[32].

Commonly reported established virulence factors
I. Proteases

Proteases degrade proteins and polypeptides by cleavage of their peptide bonds. According to their catalytic site, they are classified into five major classes: cysteine, serine, threonine metallo- and aspartyl proteases. To prevent unwanted own digestion, parasites synthesize proteases as zymogens containing a prodomain for regulatory function and a mature domain for catalytic proteolytic activity. The prodomain also acts as an endogenous inhibitor of the mature enzyme. Meanwhile, to activate the mature enzyme, it is necessary to remove the prodomain which is achieved by different biochemical modes. They are crucial for a variety of biological processes in all creatures ranging from lower microorganisms to the higher mammals. Proteases in all intracellular protozoa utilize their proteolytic activity for acquisition of nutrients, biosynthesis and differentiation of life cycle stages, apoptosis and autophagy, migration and host cell invasion, egress and no novo invasion cascade, as well as immunomodulation of host protective responses[33]. *Plasmodium* spp. express several proteases including six cysteine (two falcipains; two serine-repeat antigens; SERA 5 and 6, and two dipeptidyl aminopeptidases, DPAPs), four aspartyl (plasmepsins), and one of each of the other proteases; metallo- (aminopeptidase P), serine (ROM1), and threonine (HslV). Besides, there are three subtilisin-like proteases; SUBs 1-3[9].

1. Cysteine proteases (CPs)

They are involved in essential functions, *e.g.* obtaining nutrients by hemoglobin degradation, processing surface proteins, extracellular matrix turnover, parasite invasion, contributing in parasite cascade, as well as antigen presentation and immunoevasion. Degradation of host proteins is primarily used as parasite nutrients for life cycle stages synthesis. In *Plasmodium* spp., falcipains degrade host hemoglobin into heme and globin, and the latter is hydrolyzed to free amino acids essential for parasitic stages synthesis[16]. Proteolytic activity is also utilized to degrade cellular membrane contributing in egress cascade[10].

According to MEROPS (the proteases database), protozoal CPs are classified into several clans that include families, and CATHs that are mainly expressed in all parasites. Calpains, leugumains and metacaspases are also frequently reported[34]. It is worth mentioning that CATHs reported in *E. histolytica*, free living amoeba, *Blastocystis* spp., *G. lamblia*, *T. vaginalis* and *Leishmania* spp. are established virulence factors (Table 2).

• **E. histolytica:** Proteolytic activity of CATHs is directed to the actin-bundling protein villin, causing disruption of the intestinal epithelium barriers[35] and host cellular invasion by *E. histolytica*. CATHs degrade mucus barrier[36], as well as contribute with Gal/GalNAc lectin, a major adhesion factor, in amoebic adhesion to extracellular matrix[37].

• **Free living amoeba:** A Korean study showed *N. fowleri* possession of two CATHs (B and B-like); however, overexpression of the gene encoding CATH B was observed only in the pathogenic trophozoite. Gene knock-out confirmed its significant contribution in pathogenesis and virulence of primary amoebic meningoencephalitis[38].

• **Blastocystis spp.:** Comparison of the protease activity in three isolates; asymptomatic, symptomatic with and without amoebic forms, revealed significant positive correlation with the percentage of amoebic forms. Investigators attributed *Blastocystis* virulence and pathogenicity to CPs heavily expressed from amoebic forms during parasite establishment through increasing adherence to the intestinal epithelial cells[43]. Later, CATH B, as virulence factor, was exclusively expressed from the amoebic forms[44].

• **G. lamblia:** CATHs are the most expressed proteases in *Giardia* genome. Among them, only three B-like CATHs were potentially essential for excystation. Giardipain-1 (CP10217) was established as virulence factor due to its influence on cell membrane; causing gaps along cell-cell junctions, and contributing in cellular damage and apoptosis[39].

• **T. vaginalis:** Reviews published during the last decade documented only CPs as established virulence factors in *T. vaginalis*[40-41] and *T. gallinae*[45]. Roles played by *T. vaginalis* CPs in virulence were tabulated including mucus layer invasion, cytoadherence, cytotoxicity,

Table 2. Cysteine proteases reported as established virulence factors.

Parasites	CPs*	Task	Reference
E. histolytica	CP 2	Cellular junction damage	[35]
	CP 5	Mucus degradation	[36]
	CP 7	Cytoadherence	[37]
N. fowleri	CP B	Invasion	[38]
G. lamblia	Giardipain 1	Cellular junction damage	[39]
T. vaginalis	Legumain-1	Cytoadherence	[40]
	CP 65	Cytotoxicity	[41]
Leishmania spp.	CPs (A-C)	Invasion	[14]
Cryptosporidium spp.	Cryptopain	Host cell invasion & egress	[42]

* All CPs are cathepsins (Clan CA, family C1) except for legumain-1 (Clan CD, family C13)

cytoskeleton disruption of red blood cells and hemolysis, evasion of host immune response through immunoglobulins degradation, and apoptosis[41].

• **Leishmania spp.:** Brazilian reviewers tabulated the proteolytic activity of CATHs (CP A-C), as virulence factors, expressed by different *Leishmania* spp. on the mammalian host. The majority of the reported activities were directed to cytokines and host immune responses, and few were directed to parasite invasion[14].

• *Cryptosporidium*-specific virulence factors are not definitely characterized due to difficulty in employing *in vitro* cultures for genetic knock out techniques. In spite of that, cryptopain was established as virulence factor[42].

2. Serine proteases (SPs)

• **Pathogenic trypanosomatids:** Two SPs were reported in trypanosomatids: oligopeptidase B (OPB) and prolyl oligopeptidase (POP), both of which are members of clan SC, family S9A. A group of Brazilian and French scientists published two reviews. The first reviewed the structural and functional catalytic characterization of both members expressed by the pathogenic trypanosomatids, *T. cruzi*, *T. brucei* and *Leishmania* spp., and their participation in disease virulence. They also suggested the mechanisms of action for both members. They postulated that the proteolytic activity of POPs is directed to degrade human collagens (types I and IV), the major proteins of extracellular matrix (ECM) and basement membrane, respectively. Thus, it enhances parasite migration through ECM, contributes in parasite dissemination in the blood and lymphatic system and finally facilitates cell invasion. On the other hand, OPBs increases cellular proliferation of amastigotes inside the infected macrophage. Accordingly, the reviewers discussed several trials for development of novel drugs for treatment of Chagas' disease, African sleeping sickness and visceral leishmaniasis[46]. In the second review, authors claimed that three OPBs were expressed from some genera of pathogenic trypanosomatids. Due to negative report of their expression in any mammalian genomes, the reviewers suggested OPBs as important virulence factors and recommended them as potential drug targets in trypanosomatids[47].

• **Leishmania spp.:** Establishment of OPBs as virulence factors in all *Leishmania* species is controversial. Upregulation of OPB was observed during *Leishmania* differentiation to amastigotes, with a relationship between OPB expression and *Leishmania* ability to remain undetected in macrophage infections[48]. Proteomic analysis of *L. amazonensis* amastigotes isolated from wild type mice and mice lacking T cells enabled the investigators to identify two potential virulence factors; OPB and HSP70[49]. However, the results of a British study suggested that *L. major* OPB itself is not an important virulence factor but rather acts in conjunction with other factors. These results

indicated functional differences between species of *Trypanosoa* and *Leishmania* in their interaction with the mammalian host[50]. In an Indian study, the investigators immunolocalized a SP (115 Kd) in the flagellar pocket of *L. donovani* amastigotes and metacyclic stages of promastigotes. Using flow cytometry and confocal immunofluorescence analysis, they observed decreased expression from attenuated strains compared to virulent ones. Also, the investigators demonstrated its ability to degrade extracellular matrix. Accordingly, it was suggested that the expressed SP played major roles(s) in the pathogenesis of visceral leishmaniasis and was validated as virulence factor and potential drug target. It is worth mentioning that the investigators did not identify the expressed SP as OPB[51].

• **Free living amoeba:** To clarify the possible roles played by proteases in the pathogenesis of amoebic keratitis, Korean investigators purified and characterized a 33 kDa SP. Only SP specific inhibitors caused its inhibition. Its identified physical features included optimum pH (8.5) and temperature (55°C). Signification proteolytic activity was observed to degrade collagen type I, a major collagen in the corneal stroma. The investigators suggested its vital roles in the pathogenesis of amoebic keratitis including corneal tissue invasion, immune evasion, and nutrient uptake[52]. The same investigators compared the specific activity and cytopathic effects of the purified SP from several *Acanthamoeba* strains with different degrees of virulence. Results of *in vitro* cultures with human corneal epithelial (HCE) cells confirmed its potentiality as virulence factor. The most virulent strain exhibited the highest cytopathic effects on HCE cells in comparison with other evaluated strains[53].

3. Metalloproteases (MPs)

• **Leishmania spp.:** Early in this century, only one MP was reported and established as virulence factor in *Leishmania* spp.; the major surface protein (MSP), also was known as gp63. The gp63, that belongs to the family M8, is abundantly expressed on the surface of *Leishmania* spp. and other trypanosomatids[54]. It was reported that its biological activity has a role in protecting *Leishmania* against host enzymes either in the vector midgut or the mammalian macrophages. During macrophage infection, gp63 was observed to modulate cytokines in the mammalian host' immune system[55]. Cellular localization showed that gp63 is mainly located in the flagellar pocket of the parasite[56]. In MEROPS, the term leishmanolysins replaces MPs in *Leishmania* spp.[34].

Genes encoding leishmanolysins correspond to 52% of protease genes in *L. braziliensis*[57]. In an attempt to investigate the rational of gene multiplicity and significance in *L. braziliensis*, Brazilian investigators succeeded to identify three subgroups of leishmanolysins based on sequencing analysis. The heterogeneity identified in 29 leishmanolysins

sequences led the investigators to hypothesize that this variation allows efficient proteolysis of a broader spectrum of substrates. In addition, they suggested that this variation may influence the leishmanolysins orientation along the surface membrane, for immunoevasion[15].

- **E. histolytica:** Two homologues of leishmanolysin, the metallo-surface proteases (MSPs) in *Leishmania* spp. were detected in *Entamoeba* species. However, one of them (*Eh*MSP-1) is absent in the non-pathogenic *E. dispar* suggesting its role in *E. histolytica* pathogenesis and virulence. Localization of *Eh*MSP-1 was affilaited to trophozoites surface, and regulation of *E. histolytica* adherence to host epithelial cells was suggested. Also, its depletion was associated with decreased trophozoites motility and decreased phagocytosis as well as tissue invasion. Similar to *Leishmania* spp., *Eh*MSP-1 was established as a virulence factor and validated as a novel drug target. The investigators recommended further studies to elucidate mechanism of action of *Eh*MSP-1 to influence parasite pathogenicity and virulence[58].

- **T. vaginalis:** The immuno-dominant variable surface antigen in *T. vaginalis*, gp63-like MP, was reported to be involved in cell adhesion *in vitro*[59]. Additionally, the same group of Mexican investigators presented two studies. The first identified a 50 kDa MP; aminopeptidase P, that belongs to clan MG, family M24 as a virulence factor, and observed that *Tv*MP50 expression correlated significantly with cytotoxic effects on the prostatic DU145 cell monolayers. These effects were inhibited by addition of specific MP inhibitors[60]. In their second study, they investigated its three-dimensional structure and suggested a hypothesis to develop a specific inhibitor as novel drug for treatment of trichomoniasis[61].

- **Plasmodium spp.:** Site-2 proteases (S2Ps) belong to a distinct family of polytopic membrane MPs (M50). Members of this family perform an essential intramembrane proteolytic function through activation of membrane-bound transcription factors. Two decades ago, S2Ps were identified as processing proteases for sterol regulation in human[62]. Then subsequently, a similar mechanism was described in all pathogens except for viruses and some bacteria with small sized genome[63]. In a study investigating the role of *Plasmodium* S2P in parasite virulence, the researchers observed significant virulence attenuation in *s2p* gene mutant parasites. Moreover, localization studies revealed that S2P was in close proximity to the nucleus in all invasive stages[64].

II. Endogenous protease inhibitors
1. Cysteine protease inhibitors (Cystatins)

They are reversible, tight-binding CPs inhibitors which are endogenously expressed to regulate CPs expression, thus protecting parasite proteins from unwanted CPs proteolytic activity. Thy are divided into three major categories according to their MW and presence of disulfide bridge; the 1st (type I) includes those with no disulfide bridge and with mean MW 11 kDa, type II includes those with two bridges and mean MW 14 18 kDa, while type III are glycoproteins (GPs) with high MW (60 to 120 kDa)[65].

- **Helminths:** Lengthy persistence of the majority of helminths in their host, was partially attributed to parasite' possession of immunomodulatory molecules, and CYS is one of them. Therefore, CYSs are suggested as virulence factors for their regulatory role because they are reversible, tightly binding CPs inhibitors. Onchocystatin (*Ov*7), the endogenous CP inhibitor released in E/S products of *O. volvulus*, was the only established virulence factor in helminths[66].

- **Protozoa:** Some protozoa such as *E. histolytica*, and species of *Cryptosporidium*, *Plasmodium* and pathogenic trypanosomatids possess CYSs that belong to chagasin family. Due to lack of a significant identity with this CYS class, it was placed by MEROPS website in a recent classification as clan IX, family I42[34]. Interestingly, *E. histolytica* uniquely possesses two isotypes of endogenous CPIs (*Eh*CPI1 and *Eh*CPI2). While both equally regulate CPs expression, *Eh*CPI2 showed remarkable inhibitory activity for the well-established virulence factor, *Eh*CP5[67]. Later, Mexican investigators identified two crystal structures of *Eh*CPI2 with eight β-strands resembling the immunoglobulin fold[68]. Recently, a Canadian study identified an important endogenous regulator for amoebic CPs expression and virulence. The investigators observed that knocking down the gene encoding *E. histolytica* cyclooxygenase-derived prostaglandin E (*Eh*Cox) produced significant increased expression of CPs without altering amoebic CP gene transcripts. Since CPs expression was not observed at the transcriptional level, the investigators suggested the possibility of post-translational modification of CPs overexpression and higher proteolytic activity[69].

2. Serine protease inhibitors (SPIs)
- **Leishmania spp.:** *L. major* parasites possess three orthologues of the *Escherichia coli* serine peptidase (SP) inhibitor; ecotin. Although *L. major* has several SPs belonging to six families, no genes encoding SPs from the S1A family of clan PA were detected in *L. major* genome. It was suggested that these endogenous ecotin orthologues were expressed to regulate expression of *Leishmania* SPs, and as well to inhibit host SPs, necessary for macrophage resistance to *Leishmania* infection. Accordingly, they were termed inhibitor of S1As (ISPs). Additionally, SPI1 was expressed in the vector, whereas SPI2 expression was detected in in all life cycle stages, with a higher expression in metacyclic promastigotes and amastigotes, the causative stages for infection initiation and persistence in the mammalian macrophages. In contrast, SPI3 expression was not detected in either stage[70].

To characterize SPI2 role in initiation and establishment of murine macrophage infection,

a group of British and Brazilian investigators conducted two studies on mutant promastigotes deficient for both genes and concluded that the obtained results are primarily due to SPI2. The first study showed that mutant metacyclic promastigotes enhanced infectivity due to increased parasite uptake by cultured macrophage *in vitro*, *i.e.* no host SPs. While *in vivo*, mutant parasites were more infective in early stages, and became similar to wild type parasites later. It was concluded that inhibition of host SPs by SPI2 is critically essential to initiate and establish intracellular adaptation of *L. major* for living in host macrophages[71]. In the second study, the investigators identified the molecular mechanism(s) mediated by SPI2 to regulate and establish the intracellular development of *L. major* amastigotes inside the infected macrophages. Results revealed that most mutant parasites were killed *in vivo*, while the remaining alive ones showed delayed intracellular development. It was observed that SPI2 played an essential role in controlling the activity of neutrophil elastase (NE) and preventing the activation of toll-like receptor 4 (TLR4) mediated responses by *L. major*. Additionally, the investigators showed that neutrophil elastase regulated macrophage infection *in vivo*. The obtained data supported the hypothesis that SPIs prevented the activation of TLR4-NE signaling cascade during early parasite-macrophage contact, leading to down-regulation of parasite phagocytosis. It was concluded that SPI2 deletion increased parasite killing and decreased its infectivity and virulence[72].

• ***T. gondii:*** It was observed that *T. gondii* SP inhibitor 1 (*Tg*PI1) was the highest expressed protease inhibitor in tachyzoites. Two isoforms were detected; TgPI1α and β, and they were secreted into the PV and transferred to the host cell cytoplasm. Mutant tachyzoites showed normal replication, with enhanced differentiation *in vitro*, as well as increased parasite burden during acute infection *in vivo*. Two mechanisms were suggested for increased virulence and bradyzoite differentiation; regulation of parasite SPs and inhibition of host SPs[73].

III. Heat shock proteins (HSPs)

These are stress chaperon proteins expressed on exposure to a range of environmental stress conditions including increased temperature, oxygen deprivation, pH extremes and nutrient deprivation. They are classified into four categories according to their molecular weight and functions. The HSP10-HSP60 system is involved in classical protein folding, and the HSP40-HSP70 system stabilizes peptides in a linear, unfolded state and delivers them to the first system. The HSP90 family members are involved in mediating the folding of specialized proteins such as steroid receptors and protein kinases. The large HSP100 and those with MWs higher than 100 are engaged in thermal tolerance, disaggregation and unfolding of aggregated proteins

for enzymatic digestion[74]. The main function of HSPs is correction of the folding or functional conformation of cell proteins (under stress). They also elicit humoral/ and or cellular host immune response (potential vaccine candidates), and contribute in parasite survival, growth, and life cycle stages differentiation as well as pathogenicity and parasite virulence (potential drug targets)[75].

There is a significant association between HSP70 expression and *T. gondii* virulence[20], and *E. tenella* virulent strains[76]. Gene encoding *Leishmania* HSP100 was established as virulence factor since gene replacement mutants (strains lacking this gene) became avirulent in mice[19].

IV. Glycoproteins (GPs)

• ***Leishmania* spp.:** Surface GPs are established virulence factors in bacteria, fungi and pathogenic trypanosomatids. The dense glycocalyx membrane on the surface of *Leishmania* promastigotes is composed of lipid-anchored-GPs and polysaccharides. All pathogenic trypanosomatids contain a highly conserved glycosylphosphatidylinositol (GPI)-anchor motif. *Leishmania* spp. utilized different GPIs to be conjugated in the surface membrane complex such as lipophosphoglycan (LPG), glycosylinositol phospholipids (GIPLs), proteophosphoglycans (PPGs) and gp63.

The composition of the *Leishmania* glycocalyx membrane dynamically changes during stage transformation. This strategy of immunoevasion is importantly utilized by *Leishmania* promastigotes because their surface constitutes the first interface with host immune response(s). It was reported that LPGs represent the most abundant promastigote-specific surface glycoconjugates. Due to LPGs clear implication in multiple activities for the sake of parasite survival and virulence, they received much attention than other GPs. Additionally, LPG-mutant strains of *L. major* showed attenuated virulence during infections of murine macrophages. Genetic approaches to elucidate LPGs function on the host immune response were reviewed. Interference with pro-inflammatory cells *via* binding with toll-like receptors 2 and 4 on macrophages and natural killer cells, respectively, was suggested[5].

Other GPs contributing in immunoevasion strategy by *Leishmania* spp. were summarized by Brazilian reviewers. Both GIPLs and PPGs contribute in amastigotes survival inside macrophages, but with two different mechanisms: *via* inhibition of nitric oxide synthase and protein kinase C, or *via* binding to macrophage receptors, respectively. Another difference was reported, while GIPLs remain highly expressed throughout the parasite life cycle stages, PPGs are only expressed in promastigotes, and detected in amastigotes as free, not membrane-conjugated. On the other hand, gp63 is one of the major GPI-anchored glycosylated proteins, present

on the promastigote plasma membrane, and its function and role as virulence factor was previously discussed as a MP[14].

- **T. cruzi:** Surface membrane GPs are critical factors against host immune response(s) in *T. cruzi*. It was demonstrated that the membrane was predominantly covered by mucin-like molecules, and GIPLs were predominantly involved in cellular invasion and immunosuppression of host immune response(s). Accordingly, they were established as virulence factors in Chagas' disease[17].

- **E. histolytica:** It was reported that the trophozoite surface is covered by LPGs-PPGs, GPI-anchored molecules with a peptide moiety. Pathogenic *E. histolytica* trophozoites were reported to possess LPGs-PPGs that significantly differ in structure from non-pathogenic commensal *E. dispar*[77]. Monoclonal antibodies raised against amoebic LPG-PPG antibodies prevented trophozoites adherence to the intestinal epithelium *in vivo*[78].

- **Cryptosporidium spp.:** Several GPs were suggested as contributory virulence factors[79] and will be reviewed later.

V. Other established virulence factors

1. E. histolytica: Other than proteases (CPs and MSP), and GPs, virulence factors in *E. histolytica* include amebapores; Gal/GalNAc lectin, and lysine- and glutamic acid-rich protein (KERP1).

- **Amebapores:** Since ~30 years, amebapores were discovered as critical effector molecules for *E. histolytica*-mediated cytolysis. They are a family of three 77-amino acid peptides capable of forming pores in lipid bilayers, causing ion channels disturbance and subsequent death of intestinal epithelial cells, hepatocytes, and host defense cells. They are found within cytoplasmic granules of *E. histolytica* and may exocytose to the target cell membrane on contact[80]. In a review published early in this century, it was reported that necrotized intestinal epithelial cells are capable of releasing IL-1α, stimulating cytokine production from non-infected cells. Also, there was evidence that amebapores cause induction of apoptotic pathway in an animal model of amebic liver abscess[13].

- **Specific surface lectin:** The primary molecule implicated in the adhesive process is a galactose/N-acetyl-galactosamine-specific lectin (Gal/GalNAc lectin). It consists of heavy subunit of a carbohydrate binding domain and a small cytoplasmic tail involved in signaling by the lectin. This specific surface lectin proved to have an important physiological role in the adhesion process, and subsequent cellular invasion[81]. It was demonstrated that Gal/GalNAc lectin facilitates *E. histolytica* adherence to host intestinal cells and contributes in increasing amebic resistance to complement. Meanwhile, it has a role in cytokine production due to TNF-α production from macrophages exposed to amoebic surface Gal/GalNAc lectin. It also contains a region with antigenic

cross-reactivity with CD59, a membrane inhibitor of the C5b-9 attack complex in human RBCs. It was suggested that this region confers trophozoite resistance against lysis by the membrane attack complex[13].

- **Lysine- glutamic acid-rich protein (KERP1):** A French group of investigators conducted three studies to investigate the role of lysine-glutamic acid rich proteins (KERPs) as proposed essential factors in contact-dependent cytolysis of the host intestinal epithelial cells caused by *E. histolytica*. Beside the well-identified molecules; Gal/GalNAc lectin and amebapores, new proteins rich in lysine and glutamic acid; KERP1 and KERP2 were identified as unique surface adhesion factors invading the brush border of human enterocytes *in vitro*. However, only KERP2 was identified in the non-pathogenic *E. dispar*, and PCR also showed absence of the gene encoding KERP1. *In vitro* studies demonstrated KERP1 binding to the epithelial cell surface, and its location in the vesicles on the plasma membrane of the cultured trophozoites[82]. In the second study, the investigators observed highly significant KERP1 expression during amoebic liver abscesses development in experimentally infected mice. Accordingly, KERP1 is suggested to have two roles as virulence factor: cytoadherence and development of hepatic abscess[83].

To characterize KERP1 structure and function, the investigators utilized bioinformatics analysis to show the 3-D-structural modeling of KERP1. Results revealed that KERP1 possesses a trimeric α-helical rich core with three domains and high degree of structural disorder. Only the central domain was demonstrated to be involved in cytoadherence[84].

2. Free living amoeba: In *Acanthamoeba* keratitis, *Acanthamoeba* trophozoites express on their surface a 400 kDa protein, mannose binding protein (MBP), to mediate adhesion to the host corneal epithelial cells. The MBP is a transmembrane protein with characteristics of a typical cell surface receptor. If activated, it causes upregulation of a contact-dependent MP and several contact-independent SPs. Through their proteolytic activity, they produce cytopathic effects including direct cytolysis, phagocytosis and apoptosis[85]. Another protein, laminin binding protein (LBP), was observed contributing in corneal adherence and facilitating corneal invasion[86]. Later, a Malaysian study was conducted to compare *Acanthamoeba* MMP and LPB expression as virulent factors for ten *Acanthamoeba* axenic isolates. Results revealed that highly virulent isolates expressed higher levels of both proteins that were associated by increased corneal cytopathic effects. Because several previous publications established MMP[85,87,88] and LBP[89] as virulence factors, the investigators denied matrix MP as virulence factor. Also, they proposed that analysis of the genes encoding MBP and LBP will be useful in differentiation between virulent and avirulent *Acanthamoeba* strains[90].

3. *G. lamblia*

- **Mechanical factors:** Trophozoites possess several complex cytoskeletal elements involved in several vital process including motility, chromosome segregation, organelle transport, cell shape maintenance and stage transformation. In their review, Ankarklev *et al*.[91] discussed two virulence factors identified to date: mechanical using flagella and adhesive disc, and antigenic variation using variant surface proteins (VSPs). Flagellar motility removes intestinal mucus to facilitate adhesion, then the ventral adhesive disc contributes with surface lectins to maintain trophozoite' attachment to the host intestinal endothelium. Antigenic utilization is utilized to avoid host immune response for the parasite survival[91].

  Later, an American study demonstrated the structure of *G. lamblia* cytoskeleton complex. Beside four pairs of flagella and a ventral disc, the complex includes parallel microtubules and micro-ribbons with associated proteins. The investigators also described trophozoite' attachment in steps showing formation of a seal between the attached surface and the disc. Another suggested role of flagellar motility was the creation and maintenance of a negative pressure underneath the disc. They observed conformational changes in disc elements (microtubules and micro-ribbons) that are essential for surface attachment. Transfer of the associated proteins *via* the microtubules and micro-ribbons to host was suggested to establish trophozoites adhesion and initiation of the infection[21].

- **Tenascins:** Using proteomic analyses of *G. lamblia* assemblages A and B and the supernatants derived from their culture, British investigators succeeded to identify virulence factors that belong to four groups: CATH B, high-cysteine membrane proteins (HCMPs), VSPs family, and a new class termed "tenascins". The latter are a highly conserved variable group of proteins secreted by trophozoites. Additionally, the investigators observed abundant extracellular nuclease. Based on the obtained results, the investigators suggested tenascins role and influence in *Giardia* pathogenesis and virulence. When conditions favor trophozoites growth, extracellular nuclease degrades the outer layer of the intestinal mucus to give access for CATH B' proteolytic activity. Further degradation of the protective mucous barrier is followed by disruption of intestinal intracellular junctions. Finally, tenascins utilize epidermal growth factor (EGF) receptor, present at the surface of intestinal cells, and causes its ligation to prevent repair of damaged cellular junctions. Interestingly, sequence analysis of tenascins proved that they have EGF repeats to enable their acting as ligands for EGF receptors. The investigators recommended further studies to validate and/or confirm this proposed mechanism of pathogenesis and virulence. It was reported that HCMPs are a new group of proteins with few associated functional studies. It was suggested that HCMPs may protect trophozoites against proteolysis and oxidative damage[92].

4. *Leishmania* spp.

- **A2 protein:** It was observed that A2 protein is highly expressed in *L. donovani* amastigotes and plays essential roles for intracellular survival of amastigotes in the visceral organs of experimentally infected mice. A Canadian study evidenced its nature as a stress protein capable of binding with endoplasmic reticulum HSP70 to enable amastigotes to survive following differentiation. In addition, the investigators detected variable A2 expression observed within an amastigote population when promastigotes were induced to differentiate into axenic amastigotes. These results confirmed the hypothesis that A2 contributes in helping *L. donovani* survive stress conditions associated with infected feverish visceral organs[93].

- **Internalin A protein:** Indian investigators succeeded in detecting surface proteins in *L. donovani* that are similar to internalin-A (Inl-A) protein of Listeria monocytogenes. It was reported that expression of Inl-A -like proteins represents a new class virulence factor in *Leishmania* spp. The investigators suggested that Inl-A -like proteins may have essential roles in cell invasion by interacting with host cell receptors, *e.g.* E-cadherin on the cell membrane to facilitate host cell invasion[94].

- **KMP-11:** Kinetoplastid membrane protein-11 (KMP-11), is a protein associated with the membrane surface, as well as expressed in the flagellar pocket, and intracellular vesicles of promastigotes and amastigotes of *Leishmania* spp. Higher expression was observed in amastigotes' surface during transformation to metacyclic promastigotes. Brazilian investigators established KMP-11 as virulence factor in *L. amazonensis* and validated its use as a vaccine candidate for visceral leishmaniasis[95].

5. *P. falciparum:*

Virulence of *P. falciparum* is significantly linked with parasite ability to express VSPs in a dynamic exclusive pattern and evading host antibodies. This is achieved by the major VSP, *P. falciparum* EMP1 which is responsible for cytoadherence of infected RBCs to the vascular endothelium. This is not strange if *Pf*EMP1 is encoded by a family of ~ 60 var genes[96]. Maurer clefts, membranous structures elaborated in RBC cytoplasm, sort proteins trafficking to the RBC membrane, and it was reported that they contribute in *Pf*EMP1 trafficking[97]. One year later, Australian investigators demonstrated that *Pf*EMP1 is exported across the parasite plasma membrane and PV membrane to be inserted into the RBC membrane. It is worth mentioning that *Pf*EMP1 binds with different host receptors, *e.g.* CD36 as a parasite strategy for immunoevasion, and subsequent RBC cytoadherence. It was shown that endogenous *Pf*EMP1 was trafficked as a complex rather than in vesicles, and Maurer clefts represent an important intermediate compartment[98].

Suggested virulence factors
1. *E. histolytica*
• **Anti-oxidant molecules:** Proteomic analysis between two *E. histolytica* strains, virulent (HM-1:IMSS) and avirulent (Rahman), identified two molecules with anti-oxidative properties; peroxiredoxin and superoxide dismutase (SOD). Both molecules showed significant high expression (five-fold) in the virulent strain. Transgenic Rahman trophozoites with peroxiredoxin overexpression rendered them more resistant to killing by H2O2 *in vitro*. *In vivo* studies showed their capability to cause higher levels of colonic inflammation. It was suggested that high levels of peroxiredoxin, and SOD expressed in virulent strains protect trophozoites against host oxidative defense[99].

• **Prostaglandin E2:** Canadian investigators demonstrated that *E. histolytica* trophozoites synthesized prostaglandin E2 (PGE2) that induced IL-8 release by colonic epithelial cells. It was hypothesized that increased production of IL-8, as a potent chemoattractant and activator of pro-inflammatory cells, initiated the onset of amoebic colitis. The investigators also showed the role of PGE2 in IL-8 production; *via* activation of prostaglandin E2 receptor 4 (EP4) in a contact-independent manner. Therefore, *E. histolytica* PGE2 was proposed as amoebic virulence factor and EP4 inhibitors were suggested as novel drugs in treatment of amoebic colitis[100].

• **Alcohol dehydrogenase 3:** Proteomic analysis of *E. histolytica* and *E. dispar*, revealed significant higher expression of alcohol dehydrogenase 3 (*Eh*ADH3) on *E. histolytica* trophozoite surface. Although there was also significant higher *Eh*ADH3 expression in the virulent strain (HM-1:IMSS) than in the reduced virulent strain (Rahman), the obtained results didn't show a link between *Eh*ADH3 expression neither with severity of amoebic colitis, nor with occurrence of hepatic amoebic abscess in experimentally infected mice. Also, there was no evidence that EhADH3 contributed in trophozoites cytoadherence. Accordingly, the investigators suggested that there was no direct link between *Eh*ADH3 and pathogenicity, and virulence in *E. histolytica*[101].

• **Transmembrane kinases (TMKs):** They have an essential role in several cellular processes in almost all eukaryotic cells. They contain two domains: an extracellular transmembrane domain and an intracellular serine/threonine or tyrosine kinase domain. *E. histolytica* TMKs are grouped into six distinct families, and genomic analysis revealed 35 members of B1 family involved in cellular proliferation. In an Indian study, the investigators immunolocalized *Eh*TMKB1-9 in trophozoites surface membrane, and cell lines expressing *Eh*TMKB1-9 without its kinase domain and showed altered trophozoites survival and growth. In addition, *Eh*TMK B1-9 expression was induced *via* a lipid-dependent signaling pathway to contribute in endocytosis and cytotoxicity. Accordingly, the investigators suggested the essential role played by *Eh*TMKB1-9 in *E. histolytica* pathogenicity and virulence[102].

• **Adhesin-CP complex:** It was reported that *Eh*CPADH112, a complex formed by the combination of *Eh*CP7 and *E. histolytica* adhesin (*Eh*ADH112), is involved in cytoadherence, phagocytosis, and cytotoxicity of human intestinal epithelia cells[103]. Later, it was found that *E. histolytica* ADH112 possesses a protein domain-like (Pro1) which is involved in trafficking of proteins required for the biogenesis of multi-vesicular bodies (MVBs). Mutant trophozoites in the gene encoding *Eh*ADH112 showed diminished rates of phagocytosis. The investigators characterized *Eh*ADH112 as a novel member of Bro1 domain-containing protein present at cellular surface and endosomal compartments, to contribute in MVBs pathway during phagocytosis[104].

• **Occludin-like protein:** Utilizing antibodies highly specific to human occludin, Canadian investigators detected an occludin-like protein on *E. histolytica* trophozoites surface membrane. It was suggested that this molecule might contribute in displacement of the mucosal epithelial tight junction interactions resulting in epithelial disruption. Virulence factor was proposed for *E. histolytica* occludin due to its influence in cellular invasion and pathogenesis of amoebic colitis[105].

• **Genes encoding carbohydrate metabolism:** A comparison study was conducted to detect gene expression changes (transcriptomes) between two *E. histolytica* strains: virulent (HM1:IMSS) and less virulent (Rahman) in culture and during contact with the human colon. In HM1:IMSS strain, there was upregulation of genes encoding glycolytic enzymes and carbohydrate catabolism enzymes. Gene knock-out studies decreased mucus degradation and subsequently prevented invasion of human epithelial cells. The investigators suggested a potential role of carbohydrate metabolism in cellular invasion by virulent *E. histolytica*. They attributed their results to the high expression of enzymes necessary to exploit carbohydrate resources derived from the human mucus[106].

2. Free living amoeba
In a Swiss study, the investigators utilized genomic, transcriptomic and proteomic approaches to identify 22 proteins with potential involvement in the pathogenicity of primary amoebic meningoencephalitis caused by *N. fowleri*. Functional characterization of these proteins suggested that *N. fowleri* virulence factors were mainly localized in its membrane vesicles. Possible involvement of actin-dependent processes with intracellular trafficking *via* these vesicles was suggested. The investigators attributed *N. fowleri* virulence to re-organization of trophozoite cytoskeleton that mainly depends on actin filaments. It is worth mentioning that phagocytosis in the pathogenic free-living amoeba is an actin-dependent process[107].

3. *G. lamblia*

In a Mexican study, the investigators suggested 9B10A protein, one of the VSPs family, as a conditional virulence factor. It is reported that members of VSPs are excretory/secretory products and are involved in antigenic variation, as a protective strategy against host immune response. *In vitro* studies demonstrated cytotoxic damages, and cell wall detachment of IEC-6 and MDCK cell monolayers[108].

4. *Leishmania* spp.

Proteomic analysis of *L. amazonensis* showed that prolonged (5 months) *in vitro* cultures of stationary promastigotes induced alterations in their protein content (either increase or decrease) leading to subsequent reduction in their infectivity, *in vitro* or *in vivo*. A group of American and Brazilian investigators identified several altered molecules and conducted a computational screening. They succeeded in identifying one protein, termed small myristoylated protein-3 (SMP-3), and it was considered as a virulence factor. It was observed that SMP-3 showed low structural homology to human proteins. Therefore, recombinant SMP-3 was investigated for potential protection against visceral leishmaniasis[109].

5. *P. falciparum*

- **Variant surface antigens (VSAs):** In 2011, genes encoding two new families of VSAs in *P. falciparum*, STEVOR and RIFIN were sequenced[110]. Expression of STEVOR was observed in the invasive merozoite and as well as on the surface of the infected RBCs. The investigators suggested that STEVOR, being on RBC surfaces, enable schizont-infected RBCs to bind with non-infected RBCs, forming rosettes. Utilizing this strategy, STEVOR was suggested as potential virulence factor as it contributes in protection of released merozoites from host antibodies[111].

- **Merozoite surface protein 2 (MSP2):** It was suggested as a virulence factor in *P. falciparum*, due to its involvement in RBC invasion, and was considered as a potential vaccine candidate. Therefore, effects of genetic *msp2* polymorphism on *P. falciparum* virulence was investigated. Genetic comparison was conducted on msp2 sequences of single *P. falciparum* clone isolated from patients with uncomplicated malaria, and severe malaria with and without cerebral disease. Interestingly, two loci polymorphisms (K allele at codon 17 of block 4 in 3D7 family, and the N allele at codon 8 of block 2 in FC27 family) were associated with severe and cerebral malaria, respectively. Accordingly, *msp2* was proposed as a genetic virulence marker[112].

6. *T. gondii*

Hunter and Sibley[113] reviewed genetic differences between the most widely accepted *T. gondii* lineages; types I, II and III. They hypothesized that different strategies exhibited by the parasite to evade host immune responses and assure parasite survival would help in identification of contributory virulence factors. Rhoptry kinases (ROP18, ROP16, ROP5, and ROP15) were identified as effector mediators to identify parasite virulence. The ROPs are protein kinases, expressed by rhoptry organelles into the host cell where they exhibit their function through PV membrane. Variant transcriptome mapping of these ROPs with their effects on host were tabulated. When ROPs diversity was linked to the immunological studies, the reviewers observed reduced IL-12 production and reduced parasite clearance in high virulent type I. Enhanced parasite clearance was observed in the other types. However, high IL-12 was observed in the intermediate virulence type II, and lower IL-12 production was found in low virulence type III. The reviewers concluded that virulence differences are associated with ROP18 and ROP5[113]. In contrast, a study conducted recently revealed no associated link between ROP protein allelic profile (ROP18/ROP5) and virulence. Genotyping utilizing ROP loci revealed that highly virulent Caribbean isolates showed different ROP18/ROP5 allelic profile in comparison to Brazilian virulent isolates. However, avirulent Caribbean isolates showed similar ROP18/ROP5 pattern as avirulent European isolates[114].

7. *Cryptosporidium* spp.

Genomic studies of *C. parvum* and *C. hominis* revealed characterization of over 25 putative virulence factors involved in a variety of issues related to adhesion, proliferation, locomotion, and cellular invasion. Bouzid and her colleagues[79], in their review, tabulated the previously reported *Cryptosporidium* virulence factors and discussed their proposed functions and roles in virulence of cryptosporidiosis. They claimed that the majority of these factors were identified in experimentally *C. parvum*-infected mice. They classified potential virulence factors to three categories: adhesion and locomotion, cellular invasion and damage, and intracellular proliferation and survival. Besides, other factors such as an aminopeptidase (MP) and a SP were reported to contribute in excystation, and HSP70 and HSP90 in protection against stress conditions[79].

Adherence and locomotion factors include seven molecules; CSL, GP900, GP15/40, P23, P30, TRAP-C1, and MIC1. The circumsporozoite-like (CSL) GP is associated with the apical complex of sporozoites and merozoites, whereas GP900 is a large mucin-like GP located at the surface of invasive merozoites and sporozoites during gliding motility. The complex GP15/40, a sporozoite and merozoite cell surface protein, was described in two species (*Cp*GP40/15 and *Ch*GP40/15): Both GPs play a role in motility and cytoadherence. The sporozoite surface proteins (P23 and P30) are released during the initial stages of infection. Thrombospondin-related adhesive protein *Cryptosporidium* 1 (TRAP-*C*1) is one of the micronemal proteins, localized on the apical pole of sporozoites. It was reported to be also involved in gliding motility. Another TRAP was identified at the apical complex of

sporozoites and merozoites, *Cp*MIC1 that was shown to be essential for gliding motility and host cell attachment. Six factors are potentially involved in cellular invasion and damages: two *Cryptosporidium* antigens (*Cp*a135 and *Cp*2), Muc, SUB, phospholipase, and hemolysin (H4). Both antigens as well as *Cp*Muc, small molecule with mucin sequence, were expressed throughout the cellular invasion. A *C. parvum* subtilisin-like SP (*Cp*SUB) was described to be responsible for the processing of GP40/15. On the other hand, phospholipase, and H4 are suggested for disruption of vacuolar membranes and contribution in egress cascade[79].

CONCLUDING REMARKS

1. Parasite virulence factors are contributory expressed molecules, involved in changing disease severity and/or duration for the parasite' sake. A different strategy was proposed for multicellular (helminths) and unicellular (protozoa) parasites; immunoevasion utilizing E/S products for long term infections, and survival and colonization *via* EVs for short term infections, respectively. Additionally, egress and *de novo* invasion strategy are usually adapted in intracellular protozoa.

2. Several mechanisms to increase parasite virulence are utilized such as proteolytic activity by several proteases, surface antigenic variation by VSPs, protein folding through HSPs, and mechanically which is observed only in *G. lamblia*.

3. *E. histolytica* is characterized by possession of several established virulence factors. Besides amebapores, Gal/GalNAc lectin and CPs (CPs 2. 5 and 7), trophozoites possess two surface molecules: a leishmanolysin homologue (*Eh*MSP-1), and GPI-anchored molecules for cytoadherence. The lysine- and glutamic acid-rich protein (KERP1) is also established as virulence factor for cytoadherence and development of hepatic abscess. Additionally, *E. histolytica* uniquely possesses two chagasins (*Eh*CPI1 and *Eh*CPI2), and the latter is established as virulence factors due to its significant regulation of *Eh*CP5.

4. Also, several factors are also suggested as putative virulence factors in *E. histolytica* such as peroxiredoxin, superoxide dismutase, prostaglandin E2, alcohol dehydrogenase 3, transmembrane kinase B1-9, the adhesin-CP complex (*Eh*CPADH112), the occludin-like protein and genes encoding carbohydrate metabolism.

5. Similarly, *Leishmania* spp. have several virulence factors; seven proteases; [three CPs (A-C), three MPs (leishmanolysins 1-3), and one SP (POP)], and four GPI-anchored molecules (LPGs, GIPLs, PPGs and KMP-11). Besides, *Leishmania* spp. possess one endogenous serine protease inhibitor (SPI2), and two important proteins (A2 and Inl-A) for parasite survival in stress conditions, and to facilitate host cell invasion, respectively. Additionally, gene encoding *Leishmania* HSP100 is also established.

6. It is also established that GPI-anchored surface molecules (LPGs, GIPLs and PPGs) and two SPs (OPB and POP) are the main virulence factors in *Trypanosoma* spp. They are involved in immunoevasion, and degradation of extracellular matrix to enhance cellular invasion.

7. Virulence of *P. falciparum* is significantly linked with *Pf*EMP1 due to its dynamic antigenic variation. Several molecules contribute in virulence of *Plasmodium* spp. such as membrane MPs (site-2 proteases; S2Ps), and chagasin. It is worth mentioning that two important surface proteins are reported as putative virulence factors: STEVOR, a member of VSAs family, and polymorphism in the gene encoding merozoite surface protein 2 (MSP2).

8. The cytoskeletal complex constitutes the main strategy developed by *G. lamblia* trophozoites. The complex includes a ventral disc, four pairs of flagella, and microtubules and micro-ribbons with associated VSPs. Besides, there are three virulence factors: giardipain-1 (CP10217), tenascins and high-cysteine membrane proteins (HCMPs). The latter factors contribute together in cellular invasion after the mechanical adherence adapted by cytoskeletal complex.

9. Proteases are the only virulence factors reported in *T. vaginalis*: two CPs (CP65 and legumain-1) and two MPs (gp63-like MP and aminopeptidase P). They are involved in cytoadherence, cytotoxicity, and degradation of host immunoglobulins.

10. While *N. fowleri* trophozoites possess only CATH B, there is much controversy linking matrix SP to virulence in *Acanthamoeba* spp. However, two surface molecules are established as virulence factors: mannose binding (MBP), and laminin binding (LBP) proteins.

11. In *T. gondii*, endogenous SP inhibitor 1 (*Tg*PI1) and HSP70 are established as virulence factors. While the *Tg*PI1 is highly expressed in tachyzoites; the latter is linked with survival and growth under stress conditions. However, genes encoding rhoptry kinases are identified as contributory factors in pathogenicity and virulence of toxoplasmosis.

12. Only one CP is established as a virulence factor for *Blastocystis* and *Cryptosporidium* spp. The first possesses CATH B that proved to be expressed from the amoebic forms, while the latter expresses cryptopain. However, several putative factors are suggested for *Cryptosporidium* spp. virulence such as three GPs (CSL, GP900, and GP15/40),

two sporozoite surface proteins (P23 and P30), two thrombospondin-related adhesive proteins (TRAP-C1, and MIC1), two antigens (*Cp*a135 and *Cp*2), a phospholipase, and hemolysin (H4).

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REFERENCES

1. Casadevall A, Pirofski L. Host-pathogen interactions: the attributes of virulence. J Infect Dis 2001; 184:337-344.

2. White RR, Artavanis-Tsakonas K. How helminths use excretory secretory fractions to modulate dendritic cells. Virulence. 2012; 3(7):668-677.

3. Skelly PJ, Shoemaker CB. *Schistosoma mansoni* proteases *Sm*31 (cathepsin B) and *Sm*32 (legumain) are expressed in the cecum and protonephridia of cercariae. J Parasitol 2001; 87(5):1218-1221.

4. Dalton JP, Neill SO, Stack C, Collins P, Walshe A, Sekiya M *et al*. *Fasciola hepatica* cathepsin L-like proteases: biology, function, and potential in the development of first generation liver fluke vaccines. Int J Parasitol 2003; 33(11):1173-1181.

5. Forestier CL, Gao Q, Boons GJ. *Leishmania* lipophosphoglycan: how to establish structure-activity relationships for this highly complex and multifunctional glycoconjugate? Front Cell Infect Microbiol 2014; 4:193.

6. Sampaio NG, Eriksson EM, Schofield L. *Plasmodium falciparum* PfEMP1 modulates monocyte/macrophage transcription factor activation and cytokine and chemokine responses. Infect Immun 2017; 86(1). DOI: e00447-17.

7. Khosravi M, Mirsamadi ES, Mirjalali H, Zali MR. Isolation and functions of extracellular vesicles derived from parasites: The promise of a new era in immunotherapy, vaccination, and diagnosis. Int J Nanomedicine 2020; 15: 2957-2969.

8. Wu Z, Wang L, Li J, Wang L, Wu Z, Sun X. Extracellular vesicle-mediated communication within host-parasite interactions. Front Immunol 2018; 9: 3066.

9. Mishra M, Singh V, Singh S. Structural insights into key *Plasmodium* proteases as therapeutic drug targets. Front Microbiol 2019; 10: 394.

10. Friedrich N, Hagedorn M, Soldati-Favre D, Soldati T. Prison break: pathogens' strategies to egress from host cells. Microbiol Mol Biol Rev 2012; 76(4):707-720.

11. Tanaka K. The proteasome: from basic mechanisms to emerging roles. Keio J Med 2013; 62(1):1–12.

12. Muñoz C, San Francisco J, Gutiérrez B, González J. Role of the ubiquitin-proteasome systems in the biology and virulence of protozoan parasites. Biomed Res Int 2015; 2015:141526.

13. Stanley SL Jr, Reed SL. Microbes and microbial toxins: paradigms for microbial-mucosal interactions. VI. *Entamoeba histolytica*: parasite-host interactions. Am J Physiol Gastrointest Liver Physiol 2001; 280(6):G1049-54.

14. Silva-Almeida M, Pereira BA, Ribeiro-Guimarães ML, Alves CR. Proteases as virulence factors in *Leishmania* spp. infection in mammals. Parasit Vectors 2012; 5:160.

15. Sutter A, Antunes D, Silva-Almeida M, de Souza Costa MG, Caffarena ER. Structural insights into leishmanolysins encoded on chromosome 10 of *Leishmania* (*Viannia*) *braziliensis*. Mem Inst Oswaldo Cruz 2017; 112(9): 617-625.

16. Pandey KC, Wang SX, Sijwali PS, Lau AL, McKerrow JH, Rosenthal PJ. The *Plasmodium falciparum* cysteine protease falcipain-2 captures its substrate, hemoglobin, *via* a unique motif. Proc Natl Acad Sci USA 2005;102(26):9138-9143.

17. Flávia-Nardy A, Freire-de-Lima CG, Morrot A. Immune evasion strategies of *Trypanosoma cruzi*. J Immunol Res 2015; 2015:178947.

18. Rug M, Cyrklaff M, Mikkonen A, Lemgruber L, Kuelzer S, Sanchez CP, *et al*. Export of virulence proteins by malaria-infected erythrocytes involves remodeling of host actin cytoskeleton. Blood 2014; 124(23):3459-3468.

19. Reiling L, Chrobak M, Schmetz C Clos J. Overexpression of a single *Leishmania major* gene enhances parasite infectivity *in vivo* and *in vitro*. Mol Microbiol; 2010, 76(5):1175-90.

20. Dobbin CA, Smith NC, Johnson AM. Heat shock protein 70 is a potential virulence factor in murine *Toxoplasma* infection *via* immunomodulation of host NF-kappa B and nitric oxide. J Immunol 2002; 169(2):958-965.

21. Schwartz CL, Heumann JM, Dawson SC, Hoenger A. A detailed, hierarchical study of *Giardia lamblia*'s ventral disc reveals novel microtubule-associated protein complexes. PLoS One 2012;7(9): e43783.

22. Padilla-Vaca F, Ankri S, Bracha R, Koole LA, Mirelman D. Down regulation of *Entamoeba histolytica* virulence by monoxenic cultivation with *Escherichia coli* O55 is related to a decrease in expression of the light (35-kilodalton) subunit of the Gal/GalNAc lectin. Infect Immun 1999; 67: 2096-2102.

23. Galván-Moroyoqui JM, Del Carmen Domínguez-Robles M, Franco E, Meza I. 2008. The interplay between *Entamoeba* and enteropathogenic bacteria modulates epithelial cell damage. PLoS Negl Trop Dis 2: e266.

24. Fernández-López LA, Gil-Becerril K, Galindo-Gómez S, Estrada-García T, Ximénez C, Leon-Coria A, *et al*. *Entamoeba histolytica* interaction with enteropathogenic *Escherichia coli* increases parasite virulence and inflammation in amoebiasis. Infect Immun 2019; 87(12): e00279-19.

25. Burgess SL, Gilchrist CA, Lynn TC, Petri, Jr WA. Parasitic protozoa and interactions with the host intestinal microbiota. Infect Immun 2017; 85(8): e00101-17.

26. Shiny C, Krushna NSA, Haripriya K, Babu S, Elango S, Manokaran G, *et al*. Recombinant *Wolbachia* surface protein (WSP)-induced T cell responses in *Wuchereria bancrofti* infections. Parasitol Res 2012; 110(2): 787-797.

27. Wang A, Wang CC, Alderete JF. *Trichomonas vaginalis* phenotypic variation occurs only among trichomonads

infected with the double-stranded RNA virus. J Exp Med 1987; 166:142-150.

28. Fraga J, Rojas L, Sariego I, Nuñez FA. Species typing of Cuban *Trichomonas vaginalis* virus by RT-PCR, and association of TVV-2 with high parasite adhesion levels and high pathogenicity in patients. Arch Virol 2012; 157:1789-1795.

29. Fraga J, Rojas L, Sariego I, Fernández-Calienes A. Characterization of specific RAPD markers of virulence in *Trichomonas vaginalis* isolates. Iran J Parasitol 2015;10(3):448-456.

30. Hartley MA, Ronet C, Zangger H, Beverley SM, Fasel N. *Leishmania* RNA virus: when the host pays the toll. Front Cell Infect Microbiol 2012; 2:99.

31. Janssen MEW, Takagi Y, Parent KN, Cardone G, Nibert ML, Baker TS. Three-dimensional structure of a protozoal double-stranded RNA virus that infects the enteric pathogen *Giardia lamblia*. J Virol 2015; 89(2):1182-1194.

32. Brown RW, Sharma AI, Engman DM. Dynamic protein S-palmitoylation mediates parasite life cycle progression and diverse mechanisms of virulence. Crit Rev Biochem Mol Biol 2017; 52(2):145-162.

33. Verma S, Dixit R, Pandey KC. Cysteine proteases: modes of activation and future prospects as pharmacological targets, Front Pharmacol 2016; 7:107.

34. Rawlings ND, Barrett AJ, Thomas PD, Huang X, Bateman A, Finn RD. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. Nucleic Acids Res 2018; 46, D624-D632.

35. Lauwaet T, Oliveira MJ, Callewaert B, De Bruyne G, Mareel M, Leroy A. Protease inhibitors TPCK and TLCK prevent *Entamoeba histolytica* induced disturbance of tight junctions and microvilli in enteric cell layers *in vitro*. Int J Parasitol 2004; 34(7):785-794.

36. Freitas MA, Fernandes HC, Calixto VC, Martins AS, Silva EF, Pesquero JL, *et al*. *Entamoeba histolytica*: cysteine protease activity and virulence. Focus on cysteine protease 5 expression levels. Exp Parasitol 2009; 122(4):306-309.

37. Ocádiz-Ruiz R, Fonseca W, Martínez MB, Ocádiz-Quintanar R, Orozco E, Rodríguez MA. Effect of the silencing of the EhCP112 gene on the *in vitro* virulence of *Entamoeba histolytica*. Parasit Vectors 2013; 6(1):248.

38. Lee J, Kim JH, Sohn HJ, Yang HJ, Na BK, Chwae YJ, *et al*. Novel cathepsin B and cathepsin B-like cysteine protease of Naegleria fowleri excretory-secretory proteins and their biochemical properties. Parasitol Res 2014; 113(8):2765-2776.

39. Ortega-Pierres G, Argüello-García R, Laredo-Cisneros MS, Fonseca-Linán R, Gómez-Mondragón M, Inzunza-Arroyo R, *et al*. Giardipain-1, a protease secreted by *Giardia* duodenalis trophozoites, causes junctional, barrier and apoptotic damage in epithelial cell monolayers. Int J Parasitol 2018; 48(8):621-639.

40. Rendón-Gandarilla FJ, Ramón-LuingLde L, Ortega-López J, Rosa de Andrade I, Benchimol M, Arroyo R. The

*Tv*LEGU-1, a legumain-like cysteine protease, plays a key role in *Trichomonas vaginalis* cytoadherence. Biomed Res Int 2013; 2013:561979.

41. Hernández HM, Marcet R, Sarracent J. Biological roles of cysteine proteases in the pathogenesis of *Trichomonas vaginalis*. Parasite 2014; 21: 54.

42. Na BK, Kang JM, Cheun HI, Cho SH, Moon SU, Kim TS, *et al*. Cryptopain-1, a cysteine protease of *Cryptosporidium parvum*, does not require the pro-domain for folding. Parasitology 2009; 136(2):149-157.

43. Rajamanikam A, Govind SK. Amoebic forms of *Blastocystis* spp.: evidence for a pathogenic role. Parasit Vectors 2013 11; 6(1):295.

44. Kumarasamy V, Anbazhagan D, Subramaniyan V, Vellasamy S. *Blastocystis* sp., parasite associated with gastrointestinal disorders: An overview of its pathogenesis, immune modulation and therapeutic strategies. Curr Pharm Des 2018; 24(27):3172-3175.

45. Martínez-Herrero MDC, Garijo-Toledo MM, González F, Bilic I, Liebhart D, Ganas P, *et al*. Membrane associated proteins of two *Trichomonas gallinae* clones vary with the virulence. PLoS One. 2019; 14(10): e0224032.

46. Bastos IM, Motta FN, Grellier P, Santana JM. Parasite prolyl oligopeptidases and the challenge of designing chemotherapeuticals for Chagas disease, leishmaniasis and African trypanosomiasis. Curr Med Chem 2013; 20(25):3103-3115.

47. Motta FN, Azevedo CDS, Neves BP, Araújo CN, Grellier P, Santana JM, *et al*. Oligopeptidase B, a missing enzyme in mammals and a potential drug target for trypanosomatid diseases. Biochimie 2019; 167:207-216.

48. Swenerton RK, Zhang S, Sajid M, Medzihradszky KF, Craik CS, Kelly BL, *et al*. The oligopeptidase B of *Leishmania* regulates parasite enolase and immune evasion. J Biol Chem 2011; 286(1):429-440.

49. Teixeira PC, Velasquez LG, Lepique AP, de Rezende E, Bonatto JM, Barcinski MA, *et al*. Regulation of *Leishmania* (*L.*) *amazonensis* protein expression by host T cell dependent responses: differential expression of oligopeptidase B, tryparedoxin peroxidase and HSP70 isoforms in amastigotes isolated from BALB/c and BALB/c nude mice. PLoS Negl Trop Dis 2015; 9(2):e0003411.

50. Munday JC, McLuskey K, Brown E, Coombs GH, Mottram JC. Oligopeptidase B deficient mutants of *Leishmania major*. Mol Biochem Parasitol 2011; 175(1):49-57.

51. Choudhury R, Das P, Bhaumik SK, De T, Chakraborti T. *In situ* immunolocalization and stage-dependent expression of a secretory serine protease in *Leishmania donovani* and its role as a vaccine candidate. Clin Vaccine Immunol 2010; 17(4):660-667.

52. Kim HK, Ha YR, Yu HS, Kong HH, Chung DI. Purification and characterization of a 33 kDa serine protease from *Acanthamoeba* lugdunensis KA/E2 isolated from a Korean keratitis patient. Korean J Parasitol 2003; 41(4):189-196.

53. Kim WT, Kong HH, Ha YR, Hong YC, Jeong HJ, Yu HS, *et al*. Comparison of specific activity and cytopathic effects of purified 33 kDa serine protease from *Acanthamoeba* strains with different degree of virulence. Korean J Parasitol 2006; 44(4):321-330.

54. Yao C, Donelson JE, Wilson ME. The major surface protease (MSP or gp63) of *Leishmania* sp. Biosynthesis, regulation of expression, and function. Mol Biochem Parasitol 2003; 132(1):1-16.

55. Thiakaki M, Kolli B, Chang KP, Soteriadou K. Down-regulation of gp63 level in *Leishmania amazonensis* promastigotes reduces their infectivity in BALB/c mice. Microbes Infect 2006;8(6):1455-1463.

56. Cuervo P, Santos AL, Alves CR, Menezes GC, Silva BA, Britto C, *et al*. Cellular localization and expression of gp63 homologous metalloproteases in *Leishmania* (*Viannia*) *braziliensis* strains. Acta Trop 2008;106(3):143-148.

57. Silva-Almeida M, Souza-Silva F, Pereira BA, Ribeiro-Guimarães ML, Alves CR. Overview of the organization of protease genes in the genome of *Leishmania* spp. Parasit Vectors 2014; 7:387.

58. Teixeira JE, Sateriale A, Bessoff KE, Huston CD. Control of *Entamoeba histolytica* adherence involves *Eh*MSP-1, a M8 family surface metalloprotease with homology to leishmanolysin. Infect Immun 2012; 80(6): 2165-2176.

59. Castro HC, Abreu PA, Geraldo RB, Martins RC, dos Santos R, Loureiro NI, *et al*. Looking at the proteases from a simple perspective. J Mol Recog 2011; 24(2), 165-181.

60. Puente-Rivera J, Villalpando JL, Villalobos-Osnaya A, Vázquez-Carrillo LI, León-Ávila G, Ponce-Regalado MD, *et al*. The 50 kDa metalloproteinase *Tv*MP50 is a zinc-mediated *Trichomonas vaginalis* virulence factor. Mol Biochem Parasitol 2017; 217:32-41.

61. Arreola R, Villalpando JL, Puente-Rivera J, Morales-Montor J, Rudiño-Piñera E, Alvarez-Sánchez ME. *Trichomonas vaginalis* metalloproteinase *Tv*MP50 is a monomeric aminopeptidase P-like enzyme. Mol Biotechnol 2018; 60(8):563-575.

62. Rawson RB, Zelenski NG, Nijhawan D, Ye J, Sakai J, Hasan MT, *et al*. Complementation cloning of S2P, a gene encoding a putative metalloprotease required for intramembrane cleavage of SREBPs. Mol Cell 1997; 1(1):47-57.

63. Kinch LN, Ginalski K, Grishin NV. Site-2 protease regulated intramembrane proteolysis: sequence homologs suggest an ancient signaling cascade. Protein Sci 2006; 15(1):84-93.

64. Koussis K, Goulielmaki E, Chalari A, Withers-Martinez C, Siden-Kiamos I, Matuschewski K, *et al*. Targeted deletion of a *Plasmodium* site-2 protease impairs life cycle progression in the mammalian host. PLoS One. 2017; 12(1): e0170260.

65. Abrahamson M. Cystatins. Methods Enzymol 1994; 244:685–700.

66. Schönemeyer A, Lucius R, Sonnenburg B, Brattig N, Sabat R, Schilling K *et al*. Modulation of human T cell responses and macrophage functions by onchocystatin, a secreted protein of the filarial nematode *Onchocerca volvulus*. J Immunol 2001; 167:3207–3215.

67. Sato D, Nakada-Tsukui K, Okada M, Nozaki T. Two cysteine protease inhibitors, *Eh*ICP1 and 2, localized in distinct compartments, negatively regulate secretion in *Entamoeba histolytica*. FEBS Lett 2006; 580(22):5306-5312.

68. Casados-Vázquez LE, Lara-González S, Brieba LG. Crystal structure of the cysteine protease inhibitor 2 from *Entamoeba histolytica*: functional convergence of a common protein fold. Gene 2011; 471(1-2):45-52.

69. Shahi P, Moreau F, Chadee K. *Entamoeba histolytica* cyclooxygenase-like protein regulates cysteine protease expression and virulence. Front Cell Infect Microbiol 2018; 8:447.

70. Ivens AC, Peacock CS, Worthey EA, Murphy L, Aggarwal G, Berriman M, *et al*. The genome of the kinetoplastid parasite, *Leishmania major*. Science 2005; 309:436-442.

71. Eschenlauer SC, Faria MS, Morrison LS, Bland N, Ribeiro-Gomes FL, DosReis GA, *et al*. Influence of parasite encoded inhibitors of serine peptidases in early infection of macrophages with *Leishmania major*. Cell. Microbiol. 2009; 11, 106-120.

72. Faria MS, Reis FC, Azevedo-Pereira RL, Morrison LS, Mottram JC, Lima AP. *Leishmania* inhibitor of serine peptidase 2 prevents TLR4 activation by neutrophil elastase promoting parasite survival in murine macrophages. J. Immunol 2011; 186, 411-422.

73. Pszenny V, Davis PH, Zhou XW, Hunter CA, Carruthers VB, Roos DS. Targeted disruption of *Toxoplasma gondii* serine protease inhibitor 1 increases bradyzoite cyst formation *in vitro* and parasite tissue burden in mice. Infect Immun 2012; 80(3):1156-1165.

74. Shonhai A, Maier AG, Przyborski JM, Blatch GL. Intracellular protozoan parasites of humans: the role of molecular chaperones in development and pathogenesis. Protein Pept Lett 2011; 18(2):143-157.

75. Bolhassani A, Agi E. Heat shock proteins in infection. Clin Chim Acta 2019; 498: 90-100.

76. Del Cacho E, Gallego M, Lee SH, Lillehoj HS, Quilez J, Lillehoj EP, *et al*. Induction of protective immunity against *Eimeria tenella* infection using antigen-loaded dendritic cells (DC) and DC-derived exosomes. Vaccine 2011; 29(21):3818-3825.

77. Moody-Haupt S, Patterson JH, Mirelman D, McConville MJ. The major surface antigens of *Entamoeba histolytica* trophozoites are GPI-anchored proteophosphoglycans. J Mol Biol 2000; 297: 409-420.

78. Zhang Z, Duchêne M, Stanley SL Jr. A monoclonal antibody to the amebic lipophosphoglycan-proteophosphoglycan antigens can prevent disease in human intestinal xenografts infected with *Entamoeba histolytica*. Infect Immun 2002; 70(10):5873-5876.

79. Bouzid M, Hunter PR, Chalmers RM, Tyler KM. *Cryptosporidium* pathogenicity and virulence. Clin Microbiol Rev 2013; 26(1):115-134.

80. Leippe ME, Sebastian E, Schoenberger OL, Horstmann RD, Muller-Eberhard HJ. Pore-forming peptide of pathogenic *Entamoeba histolytica*. Proc Natl Acad Sci USA 1991; 88(17):7659-7663.

81. Vines RR, Ramakrishnan G, Roger JB, Lockhart LA, Mann BJ, Petri WA. Regulation of adherence and virulence by the *Entamoeba histolytica* lectin cytoplasmic domain, which contains a B2 integrin motif. Mol Biol Cell 1998; 9(8):2069-2079.

82. Seigneur M, Mounier J, Prevost MC, Guillén N. A lysine- and glutamic acid-rich protein, KERP1, from *Entamoeba histolytica* binds to human enterocytes. Cell Microbiol 2005; 7(4):569-579.

83. Santi-Rocca J, Weber C, Guigon G, Sismeiro O, Coppée JY, Guillén N. The lysine- and glutamic acid-rich protein KERP1 plays a role in *Entamoeba histolytica* liver abscess pathogenesis. Cell Microbiol 2008; 10(1):202-217.

84. Perdomo D, Baron B, Domínguez AR, Raynal B, England P, Guillén N. The α-helical regions of KERP1 are important in *Entamoeba histolytica* adherence to human cells. Sci Rep 2013; 3: 1171.

85. Panjwani N. Pathogenesis of *Acanthamoeba* keratitis. Ocul Surf 2010; 8(2): 70–79.

86. Rocha-Azevedo BD, Jamerson M, Cabral GA, Marciano-Cabral F. *Acanthamoeba culbertsoni*: analysis of amoebic adhesion and invasion on extracellular matrix components collagen I and laminin-1. Exp Parasitol 2010; 126(1):79-84.

87. Imbert-Bouyer S, Merlaud A, Imbert C, Daniault G, Rodier M-H. A mannose binding protein is involved in the adherence of *Acanthamoeba* species to inert surfaces. FEMS Microbiol Lett 2004; 238(1):207-211.

88. Alizadeh H, Li H, Neelam S, Niederkorn JY. Modulation of corneal and stromal matrix metalloproteinase by the mannose-induced *Acanthamoeba* cytolytic protein. Exp Eye Res 2008; 87(3):286-291.

89. Hong Y-C, Lee W-M, Kong H-H, Jeong H-J, Chung D-I. Molecular cloning and characterization of a cDNA encoding a laminin-binding protein (AhLBP) from *Acanthamoeba healyi*. Exp Parasitol 2004; 106(3):95-102.

90. Ng SL, Nordin A, Abd Ghafar N, Suboh Y, Ab Rahim N, Chua KH. *Acanthamoeba*-mediated cytopathic effect correlates with MBP and *Ah*LBP mRNA expression. Parasit Vectors 2017; 10(1):625.

91. Ankarklev J, Jerlstrom-Hultqvist J, Ringqvist E, Troell K, Svard SG. Behind the smile: cell biology and disease mechanisms of *Giardia* species. Nat Rev Microbiol 2010; 8(6):413-422.

92. Dubourg A, Xia D, Winpenny JP, Al Naimi S, Bouzid M, Sexton DW, *et al*. *Giardia* secretome highlights secreted tenascins as a key component of pathogenesis. Gigascience 2018; 7(3):1-13.

93. McCall LI, Matlashewski G. Localization and induction of the A2 virulence factor in *Leishmania*: Evidence that A2 is a stress response protein. Mol Microbiol 2010; 77(2):518-530.

94. Mukherjee I, Chakraborty A, Chakrabarti S. Identification of internalin-A-like virulent proteins in *Leishmania donovani*. Parasit Vectors 2016; 9(1):557.

95. de Mendonça SCF, Cysne-Finkelstein L, Matos DC. Kinetoplastid membrane protein-11 as a vaccine candidate and a virulence factor in *Leishmania*. Front Immunol 2015; 6: 524.

96. Kim K. Malaria var gene expression: keeping up with the neighbors. Cell Host Microbe 2012; 11(1):1-2.

97. Kriek N, Tilley L, Horrocks P, Pinches R, Elford BC, Ferguson DJP, *et al*. Characterization of the pathway for transport of the cytoadherence-mediating protein, *Pf*EMP1, to the host cell surface in malaria parasite-infected erythrocytes. Mol Microbiol 2003; 50: 1215-1227.

98. Knuepfer E, Rug M, Klonis N, Tilley L, Cowman AF. Trafficking of the major virulence factor to the surface of transfected *P. falciparum*-infected erythrocytes. Blood 2005; 105(10):4078-4087.

99. Davis PH, Zhang X, Guo J, Townsend RR, Stanley SL Jr. Comparative proteomic analysis of two *Entamoeba histolytica* strains with different virulence phenotypes identifies peroxiredoxin as an important component of amoebic virulence. Mol Microbiol 2006; 61(6):1523-1532.

100. Dey I, Chadee K. Prostaglandin E2 produced by *Entamoeba histolytica* binds to EP4 receptors and stimulates interleukin-8 production in human colonic cells. Infect Immun 2008; 76(11):5158-5163.

101. Davis PH, Chen M, Zhang X, Clark CG, Townsend RR, Stanley SL Jr. Proteomic comparison of *Entamoeba histolytica* and *Entamoeba dispar* and the role of *E. histolytica* alcohol dehydrogenase 3 in virulence. PLoS Negl Trop Dis 2009; 3(4):e415.

102. Shrimal S, Bhattacharya S, Bhattacharya A. Serum-dependent selective expression of EhTMKB1-9, a member of *Entamoeba histolytica* B1 family of transmembrane kinases. PLoS Path 2010; 6: e1000929.

103. Garcia-Rivera G, Rodriguez MA, Ocadiz R, Martinez-Lopez MC, Arroyo R, Gonzalez-Robles A, *et al*. *Entamoeba histolytica*: A novel cysteine protease and an adhesin form the 112 kDa surface protein. Mol Microbiol 1999; 33:556–568.

104. Bañuelos C, García-Rivera G, López-Reyes I, Mendoza L, González-Robles A, Herranz S, *et al*. EhADH112 is a Bro1 domain-containing protein involved in the *Entamoeba histolytica* multivesicular bodies pathway. J Biomed Biotechnol 2012; 2012: 657942.

105. Goplen M, Lejeune M, Cornick S, Moreau F, Chadee K. *Entamoeba histolytica* contains an occludin-like protein that can alter colonic epithelial barrier function. PLoS One 2013; 8(9):e73339.

106. Thibeaux R, Weber C, Hon CC, Dillies MA, Avé P, Coppée JY, *et al*. Identification of the virulence landscape essential for *Entamoeba histolytica* invasion of the human colon. PLoS Pathog 2013; 9(12):e1003824.

107. Zysset-Burri DC, Müller N, Beuret C, Heller M, Schürch N, Gottstein B, *et al*. Genome-wide identification of pathogenicity factors of the free-living amoeba *Naegleria fowleri*. BMC Genomics 2014; 15:496.

108. Cabrera-Licona A, Solano-González E, Fonseca-Liñán R, Bazán-Tejeda ML, Raúl Argüello-García, Bermúdez-Cruz RM, *et al*. Expression and secretion of the

Giardia duodenalis variant surface protein 9B10A by transfected trophozoites causes damage to epithelial cell monolayers mediated by protease activity. Exp Parasitol 2017; 179:49-64.

109. Oliveira MP, Martins VT, Santos TTO, Lage DP, Ramos FF, Salles BCS, *et al*. Small myristoylated protein-3, identified as a potential virulence factor in *Leishmania amazonensis*, proves to be a protective antigen against visceral leishmaniasis. Int J Mol Sci 2018; 19(1).

110. Joannin N, Kallberg Y, Wahlgren M, Persson B. RSpred, a set of hidden Markov models to detect and classify the RIFIN and STEVOR proteins of *Plasmodium falciparum*. BMC Genomics 2011; 12:119.

111. Niang M, Bei AK, Madnani KG, Pelly S, Dankwa S, Kanjee U, *et al*. STEVOR is a *Plasmodium falciparum* erythrocyte binding protein that mediates merozoite invasion and rosetting. Cell Host Microbe 2014; 16(1):81-93.

112. Chaorattanakawee S, Nuchnoi P, Hananantachai H, Tumkosit U, Saunders D, Naka I, *et al*. Sequence variation in *Plasmodium falciparum* merozoite surface protein-2 is associated with virulence causing severe and cerebral malaria. PLoS One 2018; 13(1):e0190418.

113. Hunter CA, Sibley LD. Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. Nat Rev Microbiol 2012; 10(11):766-778.

114. Hamilton CM, Black L, Oliveira S, Burrells A, Bartley PM, Melo RPB, *et al*. Comparative virulence of Caribbean, Brazilian and European isolates of *Toxoplasma gondii*. Parasit Vectors 2019; 12(1):104.