Review Article

Expression of cysteine proteinases and cystatins in parasites and use of cysteine proteinase inhibitors in parasitic diseases. Part III: 4. Other protozoa

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ABSTRACT

Pathogenesis in intestinal and urogenital protozoan infections is multifactorial, depending mainly on the balance between parasite virulent molecules and host-induced innate immune responses. With exception of primary amoebic meningoencephalitis (PAM), the majority of these infections are asymptomatic or associated with mild manifestations. In spite of that, these protozoa possess several cysteine proteases (CPs) that are considered as important virulence factors. Several studies showed involvement of CPs in diverse processes resulting in severe pathogenesis and virulence in these protozoa such as mucin degradation, cytoadherence, damage of the tight junctions of the inter-epithelial cells, cytoskeleton disruption, as well as evasion of the host immune response. The present review addresses variable molecular mechanisms dealt with by protozoal CPs to invade host tissue and evade host immune response resulting in disease pathogenesis and parasite virulence. In fact, a better understanding of CPs roles in parasite pathogenesis is useful in identification of novel chemotherapeutic targets and/or development of vaccine candidates to reduce transmission of a wide range of communicable diseases as well as prevention of occurrence of high morbidity diseases such as PAM.

Keywords: cysteine proteinases, cystatins, drug target, free living amoeba, intestinal protozoa, *T. vaginalis*.

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Abbreviations: ALA: Amoebic liver abscess; **CP:** Cysteine proteinase; **CPI:** Cysteine proteinase inhibitor; **CYS:** Cystatin; **E-64:** A broad spectrum CPI; **MW:** Molecular weight; **PAM:** Primary amoebic meningoencephalitis; **PCD:** Programmed cell death.

Entamoeba histolytica

A review published in 2013 summarized proteomic and genomic approaches in elucidation of molecular mechanisms modulating virulence and gene expression in *E. histolytica*, in comparison to the non-pathogenic *E. dispar*. It was concluded that virulence variability of *E. histolytica* trophozoites was partly related to genome plasticity and *in vivo* changes in gene expression induced during host invasion. However, exposure to variable stress conditions, e.g. glucose starvation was reported to strongly modulate virulence of *E. histolytica* for severe host tissues invasion and extra-intestinal amoebiasis[1]. To investigate differences in gene expression between *E. histolytica* and non-pathogenic *E. dispar*, a group of investigators from Mexico and Italy utilized liver cut slices from donated organs infected by either species. With other virulence factors, there was increased expression of *E. histolytica* genes encoding *Eh*CP5, *Eh*CP2, *Eh*CP1, and a decreased or lack of expression of *E. dispar* genes encoding *Eh*CP2. In contrast, there was increased expression of the gene encoding IL-10 in *E. dispar*, in comparison to *E. histolytica*[2]. Recently, another review summarized molecular mechanisms provided by *E. histolytica* trophozoites during its life cycle in response to metabolic and oxidative stress conditions. Two types of stress were described: oxidative stress and starvation (glucose, L-cysteine and iron). The reviewers drew a figure showing increased expression of *Eh*CP5, and *Eh*CP7 in iron starvation, as well as *Eh*CP4 in glucose starvation[3].

Another study conducted recently identified an IFN-γ receptor-like protein on the surface of *E. histolytica* trophozoites. Coupling of human IFN-γ to the IFN-γ receptor-like protein on live *E. histolytica* trophozoites significantly increased amoebic CPs (*Eh*CP1, *Eh*CP22, *Eh*CP4, and *Eh*CP5) *in vitro*, with other virulent factors such as amebapore A, cyclo-oxygenase 1, Gal-lectin, and peroxiredoxin in a time-dependent manner. It was concluded that *E. histolytica* trophozoites express a surface functional protein similar to host IFN-γ as an immunoevasion mechanism. This is followed by upregulation of several virulence factors that contribute to severity of amoebic pathogenesis[4].

Based on the two reviews published in USA, seven genes were characterized encoding CPs expression (*Eh*CP 1-7). There was a related highly variable homology between gene sequences of the expressed CPs between pathogenic *E. histolytica* and non-pathogenic *E. dispar*, except in *Eh*CP5 which showed

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unique sequencing only in *E. histolytica*. Amoebic CPs localization and trafficking differ from that in higher eukaryotes, because Golgi complex, endoplasmic reticulum, and lysosomes, the key organelles of protein transport, are rudimentary structures in *Entamoeba* spp. The reviewers proposed existence of acidic vesicles in *E. histolytica* for CPs synthesis, processing, and trafficking[5]. The second review discussed the role played by the three important virulence factors in amoebiasis: 1) surface galactose binding lectin mediating *E. histolytica* cytoadherence to host intestinal cells; 2) amebapores, small peptides capable of forming pores in lipid bilayers to kill host cells; intestinal epithelial, hepatocyte, and host immune cells; and 3) seven CPs for tissue invasion and immunoevasion[6]. It is worth mentioning that amoebic CPs activity is essential for trophozoite survival *in vivo*, not *in vitro* cultures[7].

Out of ~ fifty genes encoding amoebic CPs, surprisingly only four were described under standard axenic culture conditions: *Eh*CP1, *Eh*CP2, *Eh*CP5, and *Eh*CP7. However, among them, only the first three accounted for 90% of amoebic CPs[8]. Later, a study was conducted in Germany investigating amoebic CPs overexpression during amoebic liver abscess (ALA) formation in experimental model; gerbils and mice. Of the approximately fifty genes encoding amoebic CPs, the investigators observed several CPs isotypes that included three CPs families; A, B and C. Under standard axenic culture conditions, only four genes showed expression at high levels; *Eh*cp-a1, *Eh*cp-a2, *Eh*cp-a5, and *Eh*cp-a7. During ALA formation in animal models, there was increased expression of four *Eh*cp genes (*Eh*cp-a3, -a4, -a10, and -c13) in mice and gerbils. While there was increased expression of three *Eh*cp genes (*Eh*cp-a5, -a6, and -a7) in mice, only two genes (*Eh*cp-b8 and -b9) showed increased expression in gerbils. Also, the investigators utilized two cell lines (A and B) derived from the pathogenic *E. histolytica* isolate (HM-1:IMSS). Whereas cell line A is unable to induce ALAs, cell line B is highly pathogenic, characterized by its ability to produce considerable ALAs. Genes that were overexpressed in ALAs formation in gerbils and mice were transfected to cell line A. Results revealed that overexpression of only *Eh*cp-b8, -b9, and -c13 restored the pathogenic phenotype to cause ALAs. Moreover, the investigators reported that *Eh*CP-A1, -A2, and A4 seemed to have no influence on ALAs formation. Variable overexpression of different CPs in different hosts was concluded and variable clinical presentations and pathogenicity in amoebiasis were attributed to multi-copy genes encoding CPs and their role(s) in different outcomes in amoebiasis[9].

Several functions were reported for amoebic CPs; direct contact between trophozoites and enterocytes including cleavage of the intact mucus barrier[10-12], proteolysis of the actin-binding protein villin and disruption of the intestinal epithelium barriers[13], and contribution with Gal/GalNAc lectin (the major *E. histolytica* adhesion factor) in the amoebic adhesion to extracellular matrix. The investigators observed that pretreatment of wild-type amoebae with broad spectrum CP inhibitor (E64), produced a significant decrease in adhesion to laminin and collagen of extracellular matrix[14]. After cellular invasion, other functions were reported; assistance in phagocytosis *via* increased phagosome size and number[15], protein digestion including extracellular matrix and hemoglobin[16], and significant contribution in extra-intestinal amoebiasis, i.e. ALAs[17]. In addition, interference with host immune response including C3 cleavage and degradation of secretory IgA and IgG was also reported[5]. Also, mast cells stimulation to release several pro-inflammatory mediators, i.e. IL-8, IL-13, prostaglandin D2, and tumor necrosis factor-α (TNF-α) was observed during early amoebiasis[18].

Recently, a Canadian study presented a figure demonstrating the schematic representation of interaction(s) between amoebic CPs and intestinal macrophage at the intercellular junction. The first interaction was initiated by *Eh*CP5, being expressed on the trophozoite surface, to bind with α5β1 integrin, a receptor at the surface of macrophages. This is followed by activation of inflammasome to secrete IL-1β. Meanwhile, *Eh*CP1 and *Eh*CP4, being localized in intracellular vesicles, are expressed at the intercellular junction with subsequent cleavage of cytoskeletal proteins, e.g. talin, Pyk2 and paxillin. This in turn increased production of the pro-inflammatory cytokine (IL-1β), released from macrophages. The investigators demonstrated that *Eh*CP1 and *Eh*CP4 triggered caspase-6 mediated pathway to degrade the cytoskeletal proteins[19].

Biochemically, Rab proteins possess a GTPase fold that regulates several steps of membrane trafficking including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion[20]. In a study conducted in Japan, the investigators showed that *Eh*CPs transport was regulated by Rab7A small GTPase (*Eh*Rab7A) through specific dynamic molecules termed vacuolar protein sorting-associated protein (Vps26, Vps29, and Vps35)[21]. Two years later, the same group of investigators reported that *Eh*Rab11B also has a central role in CPs secretion and vesicular trafficking. Its overexpression in turn increased the expression of amoebic CPs and their proteolytic activity[22]. Beside Rab7A and Rab11B, the same Japanese investigators identified and characterized a novel class of transmembrane receptors, that specifically bind to different lysosomal hydrolytic enzymes (CPs) and regulates their trafficking in *E. histolytica*. These receptors were termed as the CP-binding protein family (CPBF) that included eleven members. It was reported that CPBF1, with a MW of 110 kDa, regulated *Eh*CP1 and *Eh*CP5 trafficking from the endoplasmic reticulum to lysosomes and enhanced their expression into the

extracellular matrix. Genomic analysis of *E. histolytica* revealed eleven genes encoding CPBF receptors, while *E. dispar* and *E. invadens* possess 13 and 15 CPBF members respectively[23].

Based on epidemiological studies, it was observed that one of the important factors increasing *E. histolytica* CPs expression and their proteolytic activity is the co-association with *Escherichia coli*[24]. Amoebic virulence to intestinal epithelial cells in the presence of pathogenic bacteria was evaluated. Results showed that *E. histolytica* that phagocytosed pathogenic *Escherichia coli* became more virulent, causing more damage to epithelial cells. This was attributed to the inflammatory proteins released by entero-pathogens facilitating amoebic contact to the epithelial cells with CPs expression causing damage[25]. Similar results were also obtained when the investigators observed that entero-pathogenic *Escherichia coli* or nonpathogenic *Entamoeba coli* modified *E. histolytica* virulence causing amoebiasis *in vitro* as well as in experimental models. *In vitro*, there was increased CPs proteolytic activity, shown by zymogram analysis and also increased *Eh*CPs 1, 2, 4, and 5 expression. In addition, there was intense colonic inflammation as well as macroscopic differences in the development of ALAs at early stages of infected hamsters. However, this difference was insignificant. It was concluded that interaction between *E. histolytica* and co-associated pathogens enhanced amoebic CPs expression causing more virulent effects[26].

***Eh*CP5:** The first study proposing *E. histolytica* CP (*Eh*CP5) as an important virulence factor in the pathogenesis of intestinal amoebiasis was conducted in USA. The investigators demonstrated in infected SCID mice (with genetic immunodeficiency of mature B and T lymphocytes), the essential role played by *Eh*CP5 expressed from *E. histolytica* trophozoites to initiate intestinal inflammatory reactions. Besides, its expression on the trophozoite surface, the investigators attributed this function to *Eh*CP5 possession of proteolytic activity preventing production of inflammatory cytokines (IL-1B and IL-8) from infected intestinal epithelium[27]. Over-expression of *Eh*CP5 increased its activity as well as that of the other CPs (*Eh*CP1 and *Eh*CP2), beside its significant increased activity in formation of ALAs[17]. Its essentiality as a virulence factor in ALAs formation was confirmed in a Brazilian study when *Eh*CP5 mRNA transcripts were analyzed in eleven samples isolated from symptomatic patients. Increased transcripts were correlated with the virulence degree determined in hamster liver[28]. For mucin degradation, a Canadian study demonstrated that *Eh*CP5 deficient trophozoites were 60% less potent to degrade colonic mucin compared to wild-type[11]. Similar results were obtained when the specific cleavage sites of mucin were demonstrated[12].

It was observed that pro-domain *Eh*CP5 (PCP5) significantly induced pro-inflammatory cytokine secretion (TNF-α and IL-1β) in human colonic cells. Canadian investigators demonstrated a new mechanism for *E. histolytica* trophozoites for triggering intestinal inflammatory response. It was shown that *Eh*PCP5 used its motif to bind with integrin-mediated adhesion receptors on colonic cells. The latter are important mammalian receptors involved in normal cellular functions and the pathogenesis of inflammation and disease[29]. Moreover, in a review published in France, the role adopted by *Eh*CP5 during hepatic invasion was discussed. When *E. histolytica* trophozoites come in contact with liver sinusoidal endothelial cells (LSEC), several virulence factors are overexpressed. Among them, *Eh*CP5 was discussed in details for its contribution in LSEC retraction and death. The reviewers drew two diagrams to demonstrate the mechanism of entry of *E. histolytica* trophozoites into hepatic sinusoids to escape host immune response. Beside sinusoid blockage by the trophozoites causing obstruction of the blood supply to LSEC and apoptosis, the role played by PCP5 could not be ignored in targeting cell adherence and degradation of extracellular matrix[30].

A recent study conducted in Canada demonstrated that *Eh*CP5 coupled with αvβ3 integrin receptor on the surface of goblet cells, to facilitate mucus production at the site of amoebic invasion. Mucin hypersecretion initiates a signal cascade to activate phosphorylation of protein kinases that contribute with *Eh*CP5 in degradation of colonic mucus layer, the initial step in amoebic pathogenesis. The investigators concluded that *Eh*CP5 is established as a virulence factor in amoebiasis[31].

***Eh*CP7 (*Eh*CP112):** It is characterized as an essential virulence factor in clinical amoebiasis as it contributes with *Eh*ADH112 (adhesin) in the formation of *Eh*CPADH complex, which is an immunogenic, heterodimeric protein involved in cytopathic effect, cytoadherence and phagocytosis. It was reported that *Eh*CPADH complex is located in the plasma membrane and cytoplasmic vacuoles with a MW 124 kDa, encoded by two adjacent genes, *cp112* and *adh112*[32]. It is documented as virulence factor in amoebiasis, as its encoding gene was silenced (RNA interference). Results of evaluation revealed significant reduction in cytolysis and phagocytosis. Accordingly, the investigators recommended use of recombinant *Eh*CP112 in combination with other *E. histolytica* virulence factors as a therapeutic novel drug or vaccine candidate[33]. Because genes encoding *Eh*CP112 and *Eh*ADH are adjacent in the *E. histolytica* genome, an American study demonstrated that the expression of both elements was co-regulated. One knockout gene showed simultaneous downregulation of both elements that produced a significant decrease in trophozoite virulence both *in vitro* and *in vivo*[34]. One year later, Mexican investigators conducted a study to explore the molecular basis of the complex formation utilizing molecular dynamics simulations and docking

calculations. Results revealed that *Eh*ADH showed significant higher binding energy to mature *Eh*CP112 than its proenzyme form[35].

Recently, it was shown that recombinant *Eh*CP112 damaged the tight junctions (TJs) of the inter-epithelial cells. The latter are formed of trans-membrane proteins such as occludin and claudins. The investigators demonstrated *in vitro* and *in vivo* that when *Eh*CP112 reaches intracellular space, it selectively degrades claudin-1 and claudin-2, but not occludin, that was degraded by other trophozoites molecules. It was concluded that *Eh*CP112 contributes with *Eh*ADH, forming *Eh*CPADH virulence complex in TJs disruption causing loss of their defense function, i.e. the cell-cell contact is lost resulting in detachment of intestinal epithelial barrier. The proposed mechanism was presented by a comprehensive figure[36].

***Eh*CP1:** Being localized in intracellular vesicles, a novel function for *Eh*CP1 in degradation of intestinal antimicrobial peptides (cathelicidins) was reported by an American study. These peptides are essential components of intestinal mammalian innate immune response, including LL-37 in humans and CRAMP (cathelin-related antimicrobial peptide) in mice. The investigators demonstrated *in vivo* and *in vitro* the role of *Eh*CP1 on LL-37 in humans and CRAMP, respectively. Although both cathelicidins were rapidly cleaved by *Eh*CP1, they maintained their inhibitory activity against bacteria. However, *E. histolytica* trophozoites were resistant to killing by both intact and cleaved LL-37 in humans and CRAMP *in vitro*. This report described immunoevasion of host immune response as one of the functions played by amoebic CPs[37].

***Eh*CP2:** Out of the three main *E. histolytica* CPs, a German study succeeded to overexpress only *Eh*CP2 in *E. dispar* trophozoites. No role was proved for *Eh*CP2 in phagocytosis of RBCs and liver abscess formation; however, marked increased destruction of monolayer cells *in vitro* was observed[38]. A similar result was obtained when a Mexican study demonstrated the minimal role played by *Eh*CP2 in experimental acute liver amoebiasis. The investigators localized *Eh*CP2 on the internal and external cell membrane[39].

***Eh*CP4:** Out of the genes encoding amoebic CPs, *Eh*CP4 is the most up-regulated during invasion and colonization in experimental amoebiasis. It was immuno-localized in the cytoplasmic vesicles, nuclear region, and perinuclear endoplasmic reticulum. Mechanisms of nuclear transport are unknown, however, chaperones and cofactors may be involved for co-transportation into the nucleus. It is auto-catalytically activated at acidic pH, however, the highest proteolytic activity was also observed at neutral pH[40].

Regarding the role of amoebic CPs in cyst-trophozoite transformation, expression of genes encoding CPs was analyzed in *E. invadens*. Out of 28 papain-like CPs identified in *E. invadens* genome databases, German researchers investigated expression of eight genes encoding amoebic CPs; CP-A3, -A5, A9, -A11, -B7, -B8, -B9, and -C2. Utilizing Northern blot analysis, no transcripts were detected for *Ei*CP-A9, -B7, -B8 and -C2 in both life cycle stages. While EiCP-A5 transcripts were observed at all times of stage conversion, *Ei*CP-A3 and *Ei*CP-A11 transcripts were trophozoite-specific, and only *Ei*CP-B9 was cyst-specific. In addition, while in immature cysts, *Ei*CP-B9 was immunolocalized near the cyst wall, it was uniformly distributed in mature cysts. The investigators failed to elucidate its role in encystation, but they conducted co-localization studies to refute *Ei*CP-B9 role in autophagy[41]. Later, a Mexican study demonstrated that *Eh*CP-B9 gained its enzymatic activity in slow cyst-trophozoite transformation, and suggested its involvement in the virulence molecular store in amoebic pathogenesis[42].

Calpain: Similar to pathogenic trypanosomatids, calpain-like proteins were described in *E. histolytica* under stress conditions. Mexican scientists observed increased levels of intracellular calcium, shortly after exposure of *E. histolytica* trophozoites to stress conditions *in vitro*. Involvement of a Ca^{2+}-dependent calpain in initiation of programmed cell death (PCD) pathway was strongly suggested. Investigating its expression and activity using real-time quantitative PCR and Western blot assays revealed ~5-fold overexpression of calpain-like gene, 90 min after exposure to stress conditions. Two-fold increase in calpain-like protein with three fold increase of its activity, were detected three and six hours, respectively, after incubation of trophozoites with stress condition. Levels were compared with untreated trophozoites[43]. Later, another group of Mexican scientists immuno-localized calpain like protein in the cytoplasm and near the nucleus. Using Western blot, a 53 kDa band was displayed in a time-dependent induction during PCD. A knocking down gene encoding the calpain-like protein showed 65% decrease in the execution phase of PCD[44].

Applications of amoebic CPs

Diagnosis: Using PCR amplification, a gene encoding CP was utilized to diagnose clinical cases of symptomatic intestinal and amoebic liver abscess with negative results during routine stool examination[45]. Later, an Iranian study developed a single PCR assay utilizing primers with significant homology with nucleotide sequence of *cp5* gene. The developed assay showed simple and rapid detection and differentiation between pathogenic *E. histolytica* and non-pathogenic *E. dispar*[46].

Immunization: Hamsters were immunized by recombinant *Eh*CP112 and *Eh*ADH112 individually and in a combined dose. Results revealed that *Eh*CPADH complex improved the immune response and reduced both the number and the size of ALAs[47].

Cystatins (CYSs)

As previously described, *Plasmodium* spp. and pathogenic trypanosomatids possess one endogenous CP inhibitor (CYS) that belongs to chagasin family[48,49]. Due to lack of a significant identity with CYS classes, it was placed by MEROPS website in a recent classification as clan IX, family I42[50]. However, *E. histolytica* uniquely possesses two isotypes of endogenous CPIs (*Eh*CPI1 and *Eh*CPI2), with distinct biochemical properties and variable difference in their ability to inhibit amoebic CPs. While they equally inhibited *Eh*CP1 and *Eh*CP2, they showed remarkable inhibitory activity for the well-established virulence factor, *Eh*CP5. Interestingly, a homolog of *Eh*ICP2, but not *Eh*ICP1, was detected in *E. dispar* genome. In their study, Sato *et al.*[51] immunolocalized the former in the cytosolic fraction, while the latter was directed from lysosomes to phagosomes during amoebic invasion. Accordingly, the investigators attributed virulence in amoebiasis to the variability in CPI isotypes overexpression[51]. Later, Mexican investigators identified two crystal structures of *Eh*CPI2 with eight β-strands resembling the immunoglobulin fold, similar to the chagasin structure reported in pathogenic trypanosomatids. In contrast to the neutral surface of BE, DE and FG loops in chagasin, both crystal structures had positively charged surfaces of only DE and FG loops. Both crystal structures had conserved BC, DE and FG loops, but they formed a flexible wedge to facilitate blocking of all amoebic CPs[52].

Expression studies using *E. invadens* showed that both endogenous CPIs were detected in both cyst and trophozoite stages. The investigators were able to immunolocalize *Eh*CPI1 in the cytosol, while *Eh*CPI2 was co-localized with *Eh*CPI1 in lysosome-like vesicles. Knocking out or overexpressing of both genes encoding endogenous CPIs revealed no effects on either morphology or viability of the trophozoites. Due to *Eh*CPI2 co-localization in the lysosome-like vesicles, the investigators proposed a role for it, e.g. other tasks different from endogenous CP regulation, i.e. house-keeping processes[53].

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. This result hypothesized that *Eh*Cox-derived prostaglandin E2 acts as a negative regulator of amoebic CPs activity. However, 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. It was concluded that this alteration in CP activity in response to *Eh*cox gene silencing can be a negative feedback response to limit proteolytic activity during amoebic colonization[54].

Free living amoeba

Acanthamoeba species

A cathepsin L-like CP gene was first cloned from *A. culbertsoni* in a Korean study[55]. Later, an *A. healyi* strain was isolated from the brain of a Korean patient with granulomatous amoebic encephalitis. A gene encoding *Ah*CP1 was cloned and sequenced. Utilizing Northern blot analysis, no correlation was observed between its expression and *A. healyi* virulence. In addition, higher gene expression was detected in *A. healyi* isolated from the soil. Accordingly, the investigators denied *Ah*CP1 involvement in virulence and suggested digestion of phagocytosed bacteria or host tissue debris as the main role played by *Acanthamoeba* CPS[56]. Later, another Korean study immuno-localized CP1 in a vesicle-like structure in *A. healyi* and demonstrated its trafficking with a serine proteinase[57]. During encystation, another Korean study identified high expression of a gene encoding CP in the cyst stage compared to the trophozoite. The investigators termed it *cscp*, i.e. cyst specific CP. Recombinant *AC*SCP showed cathepsin B proteolytic activity. During *Acanthamoeba* encystation, the investigators demonstrated its main role in autophagy process after cyst-trophozoite transformation[58]. For *A. castellanii*, several CPs were detected in the crude extract and the investigators suggested their role in *A. castellanii* virulence to cause amoebic keratitis in immunocompetent individuals and granulomatous amoebic encephalitis in immunocompromised patients. In their study, the investigator observed that these amoebic CPs degraded human lactoferrin, transferrin, hemoglobin, and horse spleen ferritin. The obtained results confirmed that amoebic CPs promote iron acquisition from the host[59].

Endogenous CPI was identified and characterized in *A. castellanii*. Recombinant *Ac*Stefin showed inhibitory potency against various CPs, including human cathepsins B and L. It is worth mentioning that stefins, CYSs type 1, are cytoplasmic proteins with a single domain (~11 kDa) and without disulfide bonds. The investigators observed that *Ac*Stefin showed high expression during cyst-trophozoite transformation. Modulation of CPs proteolytic activity was suggested for the main function of *Ac*Stefin[60].

Naegleria species

Early in 1994, a 30 kDa *N. fowleri* CP, having similar homology with cathepsin, was characterized. American investigators demonstrated its proteolytic activity against extracellular matrix *in vitro*. It also showed cytopathic effect on baby human kidney cells, an adherent cell line culture. In addition, a similar cathepsin was observed in *N. gruberi*, a nonpathogenic species, but unable to tolerate temperatures above 30°C, unlike that of *N. fowleri*[61]. Later, a 37 kDa CP with proteolytic activity against mucin was suggested in a Mexican study. The investigators didn't confirm if it was the previous one or another *N. fowleri* CP. However, they demonstrated that the detected CP evaded host

immune response[62]. In 2014, a Korean study confirmed *N. fowleri* possession of two cathepsins (B and B-like). Both CPs showed proteolytic activities at a maximum pH of 4.5 against several proteins such as human immunoglobulins, collagen, fibronectin, hemoglobin, and albumin. Moreover, there was overexpression of the gene encoding cathepsin B only in the pathogenic trophozoite. Therefore, It was suggested as a virulence factor, involved in the pathogenesis of PAM[63].

To understand the mechanism by which *N. fowleri* trophozoites obtain such nutrients to maintain invasion and cause PAM, a Mexican study was conducted *in vitro*. The investigators utilized total *N. fowleri* crude extracts with conditional medium as control, and conducted zymography assays for hololactoferrin, transferrin, hemoglobin, and ferritin. Results revealed that total crude extract degraded hololactoferrin and transferrin. Performing CPs inhibition assays confirmed their involvement in degradation of iron-binding proteins to promote survival during invasion[64].

A study compared the peptidase pattern between two *N. fowleri* isolates; a highly pathogenic mouse-passaged (strain 1), and a weakly pathogenic culture grown (strain 2), using zymography and activity assays. Results showed no difference, but the crude extract of strain (1) showed a 58 kDa band that disappeared with addition of a broad spectrum CPI (E-64). A similar band was shown in the membrane preparation of strain (2), but not the crude extract. These results suggested that the presence of such a CP in *N. fowleri* membranes may serve to facilitate passage of trophozoites into brain tissues[65].

Due to high morbidity rate of PAM, with maximum 3-7 days survival rate, an American study evaluated the synthetic vinyl sulfone CPI, K11777, and another 33 analogs in treatment of PAM. Only three compounds showed significant inhibitory activity against *N. fowleri in vitro*, with 1.6- to 2.5-fold increase potency over the control drug (miltefosine)[66].

Blastocystis species

Proteinases have an essential role in *Blastocystis* survival and pathogenicity *in vivo* and are documented as virulence factors[67]. Twenty out of twenty-two proteases were identified as CPs, with one serine and one aspartic protease. Genomic analysis revealed that genes encoding CPs represented 96% of *Blastocystis* genome. Among the detected CPs were five legumains and eight cathepsins; three of which were cathepsin B isoforms[68]. Similar results were obtained (22 genes encoding proteinases) when a French study conducted *in silico* genomic analysis of a *Blastocystis* isolate subtype 7. However, the investigators succeeded to identify only two CPs, a cathepsin B and a legumain in the parasite culture supernatant[69]. Later, the same group of investigators conducted a study to characterize the CPs previously identified in the culture supernatants. Results revealed that activation of recombinant legumain proved to be autocatalytic, while proteolytic activity of the recombinant cathepsin B was only observed after co-incubation with legumain. It was also shown that recombinant activated cathepsin B increased human cancer colon (*Caco-2*) cell monolayer permeability which was significantly inhibited by E-64. A role for cathepsin B in increasing intestinal cell permeability was suggested[70].

There is much controversy regarding pathogenicity of *Blastocystis* spp., is it related to its genotype (human subtypes 1-4) or to possession of proteinases? Malaysian reviewers documented several studies that related *Blastocystis* pathogenicity with variable gastrointestinal symptoms to subtypes 1, 2 and 4, while subtype 3 was detected in asymptomatic patients. However, the most reliable virulence factor is the amoebic form that is predominantly detected in microscopic examination of symptomatic stool samples, whatever its subtype. Therefore, the reviewers attributed pathogenicity to CPs expressed by amoebic forms and they documented several studies that showed evidence for their proteolytic activity. They also suggested potent CPIs as novel chemotherapeutic agent for blastocystosis[71].

Two main functions are suggested for CPs; immunomodulation of host immune response and increased permeability of intestinal epithelial cells. Cleavage of human secretory IgA was reported to facilitate evasion of host immune response[72]. It was reported that CPs stimulate mucosal cells to produce IL-8 and granulocyte macrophage colony-stimulating factor. This was followed by increase of pro-inflammatory cells and increased fluid loss due to reorganization of tight junction complex[73]. Another mechanism was previously proposed; IL-8 production was associated with nuclear factor-kB pathway that was activated by CPs[74].

Possession of CPs and its link with *Blastocystis* clinical subtypes was also studied. It was observed that CPs activities in subtype 4 (avian isolates) and subtype 7 (rodent isolates) showed significant variations that were attributed to the differences in their virulence. In addition, the study that was conducted in Singapore observed that avian isolates showed two times higher CPs expression than rodent isolates[75]. In an Egyptian study, the investigators observed a 32 kDa CP that was involved in protein degradation in subtype 3. Accordingly, this CP was documented as a virulence factor[76]. In contrast, a phylogenetic study was conducted in Mexico to analyze genetic polymorphisms of cathepsin B from *Blastocystis*-infected patients with irritable bowel syndrome and from asymptomatic carriers. Phylogenetic reconstruction was conducted and it did not show any difference between cases or

controls or among subtypes. Results suggested that genetic variability of cathepsin B was not related to symptomatology[77].

Giardia lamblia

Cathepsins are the most expressed proteases in *Giardia* genome; 26 genes encoding nine cathepsins B-like, four cathepsins C-like, while the remaining 13 cathepsins were for expression of cathepsins K and L-like[78]. Of these only three cathepsins B-like were essential for life stage transformation process, survival and pathogenicity of giardiasis. Recently CPs 1-3, were termed CP10217, CP14019 and CP16779), respectively[79].

Ward and his colleagues[80] confirmed blockage of *G. lamblia* trophozoite-cyst transformation by addition of CPIs *in vitro*, in a dose-dependent manner. They succeeded to localize a cathepsin B-like in cytoplasmic vesicles that was expressed shortly prior to excystation. Apart from that, the addition of CPIs didn't affect trophozoite or cyst viability, trophozoite motility, or trophozoite replication[80]. Later, utilizing quantitative real-time PCR, an American study succeeded to investigate the expression of all genes encoding CPs detected in *G. lamblia* genome during encystation process. Results revealed that CP14019 was the most highly expressed one in both life cycles with a significant expression during encystation (7 fold up-regulation). Recombinant CP14019 was purified, characterized, and immunolocalized. Results revealed its co-localization with cyst wall protein in encystation-specific vesicles. The investigators demonstrated that CP14019 used its proteolytic activity to degrade cyst wall protein 2 from a 39 kDa to 26-kDa fragment found in the encystation-specific vesicles[81].

Adhesion of *G. lamblia* trophozoites to epithelial intestinal cells using the sucking disc and flagellar movements represents the first step in the pathogenesis of giardiasis. Therefore, Mexican investigators conducted a study to investigate the contribution of CPs expression in trophozoites cytoadherence. In a short communication study, trophozoites were cultured *in vitro* in IEC6 epithelial cell monolayers in the presence or absence of different protease inhibitors, including E-64, and other serine and aspartic proteases. Cytoadherence assays of the supernatants identified three CPs with MW of 35, 38 and 95 kDa. Several mechanisms were suggested for the CPs role in cytoadherence; association with an adhesin molecules or exposure of putative receptors on the membrane of either epithelial cells or trophozoites, as suggested for *E. histolytica* and *T. vaginalis*, respectively. Another mechanism was suggested; mucin degradation, thus facilitating cytoadherence. Further studies were recommended to identify the precise mechanism[82].

Giardiasis can occur concurrently with other pro-inflammatory GIT pathogens, e.g. *E. histolytica*, *Blastocystis* spp. and *Cryptosporidium* spp., as well as *Helicobacter pylori, Salmonella* and rotaviruses. Canadian investigators investigated the ability of *G. lamblia* to modulate host's pro-inflammatory responses for the sake of *Giardia* survival, virulence and pathogenesis. In other words, does co-infection increase severity of giardiasis? does *Giardia* CPs have a role? *In vitro* and *ex vivo* studies in *Caco-2* monolayers, were conducted using small intestinal mucosal biopsies from Crohn's disease. Results revealed that cathepsin B CPs expressed from *G. lamblia* trophozoites degraded IL-8 (CXCL8) secreted from host intestinal epithelial cells in response to exposure to pathogen-derived pro-inflammatory stimuli[83]. *Giardia* CPs also attenuated granulocyte tissue infiltration due to decreased expression of several cytokines associated with bacterial toxin-induced colitis[84].

In a recent study conducted in Mexico, the investigators succeeded to identify and characterize a cathepsin B-like, termed giardipain-1, as a virulence factor in giardiasis. Monoclonal antibodies raised against *G. lamblia* surface components (MAb1G3) reacted with a 25 kDa protein that showed expression on trophozoite surface and flagella. Sequence analysis revealed that giardipain-1 has homologous similarity with cathepsin B-like, and its proteolytic activity was inhibited by addition of E-64. An *in vitro* study on cell culture showed cell membrane blebbing, appearance of pore-like regions and gaps along cell-cell junctions, i.e. initial steps in apoptotic process. In contrast, giardipain-1 gene knockout significantly decreased cellular damage *in vitro*. Accordingly, further studies were recommended to validate giardipain-1 as a novel drug target for treatment of metronidazole-resistant giardiasis[85].

Another mechanism was suggested for the role of *Giardia* cathepsins expression at the surface of the intestinal epithelial cells, that is inhibition of goblet cells and depletion of human mucin-2. Experimentally infected mice showed a thinner mucous layer and demonstrated differential mucin gene expression (muc2 → muc5ac). There are 20 types of mucin, but it is well known that muc2 is the main component of small and large intestine, whereas muc5ac is only found in the respiratory tract and stomach. *In vitro* incubation of human LS174T goblet-like cells showed mucus depletion shortly after incubation with *G. lamblia* trophozoites. In addition, E-64 abolished mucus degradation and mucin depletion[86].

Recently, two studies were conducted to characterize the three main expressed cathepsins B like (CP14019, CP16160 and CP16779). The same group of investigators aimed to investigate their role during interaction with intestinal epithelial cells *in vitro*. In the first study, CPs were immunolocalized to the endoplasmic reticulum and cytoplasmic vesicle-like structures. The study demonstrated that recombinant

CPs were more active in acidic environment (pH 5.5-6) with high affinity for fluorogenic substrates. Proteolytic activity on cleavage of proteins (claudin-1 and -4, occludin, β-catenin and E-cadherin) of the apical junctional complex of the intestinal epithelial cells was also demonstrated. Several cytokines were degraded during *Giardia*-host cell interactions. In addition a figure was presented showing mechanism(s) that allow *Giardia* trophozoites to pass intestinal epithelial barrier to induce giardiasis[79]. In the second study, the investigators demonstrated that three CPs cleaved immunoglobulins, e.g. IgA and IgG and defensins (α-HD6 and β-HD1) were possible targets for protease activity *in vitro*. It is worth mentioning that defensins are host defense peptides produced by innate immune response cells. They are characterized as small cysteine-rich cationic proteins. They include molecules displaying either direct anti-microbial activity, immune signaling activities, or both. Accordingly, *Giardia* CPs contribute in cleavage of several components of the human mucosal defense machinery[87].

G. lamblia is categorized into eight distinct genetic assemblages (A-H), however, only assemblages A and B genotypes are infective to humans[88]. Cathepsin expression was observed in a strain-dependent manner. Enhanced cathepsin expression occurred on incubation of *Giardia* trophozoites (assemblage A) with human enterocytes, but not by assemblage B. With the virulent genotype, Canadian investigators observed degradation of human intestinal epithelial monolayers and severe affections in the intestinal epithelial cytoskeletal protein villin. These observations were inhibited on addition of E-64. Accordingly *Giardia* cathepsins expression was proposed as an important virulence factor and novel drug target[89].

Cysteine protease inhibitor (CPI)

E-64 was suggested for treatment of giardiasis in an Egyptian study. The investigators proved both *in vitro* and *in vivo* that *Giardia* CPs principally contributed to cyst-trophozoite transformation. The *in vitro* study revealed failure of 90% *G. lamblia* cysts incubated with E-64 to excyst, while only 10% showed partial excystation (5%) or no excystation (5%). In contrast, cysts incubated without E-64 addition showed 90% excystation. The *in vivo* study also showed similar significant results regarding cysts output in infected mice treated with E-64 compared to non-treated control group. Histopathological examination correlated positively with counting cysts output, i.e. only grade I was observed in the treated group, while all other severe grades were found in infected non treated group[90].

Cystatin (CYS)

Recently, *G. lamblia* endogenous CPI (type 1 CYS), with cytoplasmic localization, showed significant inhibitory potency against three *G. lamblia* CPs (CP14019, CP16160 and CP16779), but it was a weak inhibitor of human cathepsin B[91].

Trichomonas vaginalis

Utilizing tandem mass spectrometry, Brazilian investigators identified and characterized eight *T. vaginalis* CPs in high and low virulent isolates, with quantitative and qualitative differences between isolates. Seven belonged to clan CA, family C1 (cathepsin L-like), while the remaining CP belonged to clan CD, family C13, asparaginyl endopeptidase-like CP (legumain). Toxicity to *HeLa* cells *in vitro* showed significant results by high and low virulent isolates (98.3% versus 31%, respectively). Addition of a specific papain-like CPI significantly decreased cytotoxicity to 21.7% and 0.8%, respectively. It was concluded that *T. vaginalis* cathepsins L-like CPs play an essential role in pathogenicity and virulence of trichomoniasis[92]. Later, a study reported that up to 156 CPs were analyzed from *T. vaginalis* genome sequence. However, only a few were identified and characterized, and their proteolytic activity was recognized as necessary for parasite recognition and adhesion to the host vaginal epithelial cells[93]. In a review article published in 2014, Hernández and her colleagues[94] claimed that trichomoniasis is characterized by broad clinical manifestations, and the exact mechanism(s) for the pathogenic process have not been clearly elucidated to date. However, cytoadherence to the vaginal epithelial cells remains the first critical step for subsequent pathogenesis. The reviewers presented in details CPs involvement in several pathogenic processes such as invasion of the mucous layer, contribution with surface adhesins for cytoadherence, cytotoxicity and apoptosis of vaginal epithelial cells, cytoskeleton disruption of red blood cells and hemolysis, and evasion of host immune response *via* degradation of immunoglobulins[94].

Cathepsins

***Tv*CP65:** Early in this century, a CP with a MW of 65 kDa was immunolocalized on the plasma membrane and in the cytoplasm of *T. vaginalis*. *In vitro* addition of E-64 reduced trophozoites cytoadherence to *HeLa* cell monolayers. It was reported that *Tv*CP65 proteolytic activity was optimal at pH 5.5, and degraded collagen IV and fibronectin, but not hemoglobin. In addition, sera and vaginal smears showed positive precipitation assays indicating possession of anti-*Tv*CP65 antibodies. It was concluded that *Tv*CP65, a surface CP, is involved in cytoadherence and cytotoxicity, and accordingly was suggested as a virulence factor in trichomoniasis[95]. Later, two studies were conducted by the same Mexican investigators. In the first, they investigated the role of iron on *Tv*CP65 cytoadherence. Cytotoxicity studies demonstrated lower CP65 expression as well as cytoadherence in higher iron concentrations. Western blot and PCR studies also showed reduction in *Tv*CP65 expression and transcripts in iron-rich cultures, compared with those of parasites grown in

normal and iron-depleted media. It was concluded that iron significantly down-regulated *Tv*CP65 proteolytic activity, and cytoadherence *in vitro*[96]. In contrast, polyamine depletion down regulated *Tv*CP65 expression and cytoadherence. It is worth mentioning that polyamines are organic compounds with more than two amino groups, and inhibition of its biosynthesis, retards or stops cell growth[97].

***Tv*CP39:** Another CP with a MW of 39 kDa was detected in the vaginal secretions from females complaining of symptomatic trichomoniasis. *In vitro* studies showed positive tissue specific binding with *HeLa* epithelial cells, vaginal epithelial cells, and human prostatic cancer cells, but not *Caco* cell line. Moreover, it showed proteolytic degradation of extracellular matrix proteins such as collagens (I, III, IV, and V), human fibronectin, hemoglobin as well as immunoglobulins A and G. It showed its proteolytic activity at a wide pH range (3.6-9.0), that was inhibited by E-64[98]. Similar to *Tv*CP65, putrescine (a polyamine) biosynthesis was reported to regulate the proteolytic activity of *Tv*CP39. Trophozoites treated by DAB, an inhibitor of putrescine biosynthesis, showed low level expression of *Tv*CP39 as demonstrated by reduction of~80% of its transcripts assayed by quantitative reverse transcription PCR. The investigators observed that exogenous addition of putrescine restored *Tv*CP39 transcripts, indicating its role in *Tv*CP39 mRNA stability. In addition, in DAB-treated experiments, *Tv*CP39 was localized in the cytoplasm and trophozoite membrane, however on addition of putrescine, it was re-localized in the nucleus. It was concluded that putrescine not only regulates *Tv*CP39 expression, proteolytic activity, and cytotoxicity effects, but also regulates its cellular localization[99].

***Tv*CP30:** In 2007, two studies were conducted in India. The first study investigated the role of CP30 in *T. vaginalis* cytoadherence in symptomatic and asymptomatic isolates (each 20 isolates). It was reported that *Tv*CP30 was detected in all fresh isolated samples, but with higher expression in symptomatic isolates. In long-term cultures, *Tv*CP30 was detected in all symptomatic isolates and only in 70% of the asymptomatic. In addition, symptomatic isolates demonstrated significantly higher cytoadherence to vaginal epithelial cells *in vitro*, compared to asymptomatic ones. Cytoadherence was significantly inhibited by CP30 specific hyperimmune serum. These results confirmed that *Tv*CP30 expression is consistently associated with disease pathogenicity and parasite virulence[100]. Therefore, the investigators hypothesized the possible use of *Tv*CP30 as diagnostic marker to differentiate between symptomatic and asymptomatic clinical patients. It was reported that *Tv*CP30 was identified in both samples (serum and vaginal washes) of all symptomatic and the majority of asymptomatic women. However, significant detection of anti-CP30 antibodies in the symptomatic women suggested its use as a diagnostic tool. Contribution of other factors with *Tv*CP30 in pathogenesis and virulence of trichomoniasis was concluded[101].

The role of *Tv*CP30 in apoptosis was investigated. American investigators demonstrated that the expressed CP isolated from the soluble fraction of *T. vaginalis* induced apoptosis in human vaginal epithelial cells *in vitro*. Apoptosis was inhibited by E-64. Interestingly, SDS-PAGE analysis of the CP fraction showed triplet bands at 30 kDa (*Tv*CP30) and two closely associated bands (23.6 and 23.8 kDa), corresponding to *Tv*CP2 and *Tv*CP4, respectively[102]. Later, the same group of investigators observed that apoptosis induced by *Tv*CP30 was significantly decreased by iron high concentration in culture medium, while iron low concentration increased CP30 expression and apoptosis[103]. In addition, it was concluded that vaginal epithelial cells apoptosis induced by *Tv*CP30 might contribute in death of multiple mucosal immune cell types, one of the mechanisms to evade host immune response[104].

***Tv*CP2:** A recent study conducted in Mexico investigated the effect of glucose restriction, as a nutritional stress condition, on *Tv*CP2 proteolytic activity. As previously reported, *Tv*CP2 was found to induce cellular damage of human vaginal epithelial cells. Conducting cytotoxicity assays, results revealed that glucose-restriction enhanced *HeLa* cell monolayers destruction (~95%) compared to trophozoites in culture media with high glucose concentration. Trophozoites pretreatment with anti-recombinant *Tv*CP2 polyclonal antibodies significantly reduced induction of *HeLa* cells apoptosis in a concentration-dependent manner. In addition, the investigators localized *Tv*CP2 expression in vesicles close to trophozoite plasma membrane[105].

***Tv*CP4:** The role of *Tv*CP4 (34 kDa MW) in *T. vaginalis* virulence was investigated by raising polyclonal antibodies against recombinant *Tv*CP4. Utilizing western blot, and mass spectrometry, the investigators demonstrated that polyclonal antibodies reacted with three protein spots (24, 22 and 21 kDa). It was reported that *Tv*CP4 was localized in cytoplasmic vesicles, lysosomes, and on the trophozoite surface. Trophozoites grown in iron-rich media showed higher *Tv*CP4 expression, compared with normal and iron-depleted media. In addition, anti-r-*Tv*CP4monoclonal antibodies protected human RBCs from lysis *in vitro*[106]. Because the same Mexican group of investigators observed that *Tv*CP4 contains a "prepro" fragment, they conducted a second study to identify its role in *Tv*CP4 proteolytic activity. Interestingly, recombinant *Tv*CP4 prepro region inhibited the proteolytic activity of *T. vaginalis* CPs with MW of 97, 65, 39, and 30 kDa. Using mass spectrometry, inhibited CPs were identified as *Tv*CP2, *Tv*CP4, *Tv*CP4-like, and *Tv*CP39. In addition, hemolysis of human RBCs was significantly reduced when trophozoites were pretreated with recombinant

*Tv*CP4 prepro region, in a concentration dependent manner[107].

***Tv*CP12:** A single copy gene encoding the sixth *T. vaginalis* CP was identified and characterized. It was found that *Tv*CP12 has a MW of ~34 kDa and was localized in cytoplasmic vesicles. Gene sequencing showed 70–73% homology with other CPs of the papain family. Quantitative reverse transcription PCR studies showed its only expression in low concentration of iron in culture media, and with very low transcript level in high iron concentrations[108].

***Tv*CP60:** Being an important nutrient, *T. vaginalis* trophozoites evolve several mechanisms to obtain iron from specific iron-binding and iron-containing proteins such as lactoferrin and hemoglobin, respectively. The ability of *Tv*CP60 proteolytic activity to degrade human immunoglobulins and hemoglobin was demonstrated before the 20th century[109].

Legumains

It was claimed that a total of six genes encoding CPs were identified in *T. vaginalis*. In their study, the investigators identified six CPs, four belonged to cathepsin-like family, while the other two belonged to legumain-like family (*Tv*LEGU-1 and *Tv*EGU-2). Both CPs belong to asparaginyl endopeptidase sub-family of the family C13, clan CD, with MW of 42.8 and 47.2 kDa. In addition, they were cloned and sequenced and showed ~ 40% identity at the amino acid level[110]. Later, ten genes encoding legumain-like CPs were reported in *T. vaginalis* genome[111]. In 2013, a study to identify, characterize, and determine *Tv*LEGU-1function was conducted. It was demonstrated as a surface CP, also localized in lysosomes and Golgi complex. Its release was found in the vaginal secretion during trichomoniasis, suggesting its role as a potential biomarker. It showed its proteolytic activity on *HeLa* cells *in vitro* suggesting its essential role in cytoadherence. Anti-r*Tv*LEGU-1 antibodies and aza-peptidyl Michael acceptor reduced cytoadherence up to 45% and 80%, respectively. It also showed highest expression in iron rich trophozoites. According to their results, *Tv*LEGU-1 was proposed as a virulence factor, and novel drug target[112].

Application

Diagnosis: A proteomic analysis of *T. vaginalis* protein extracts was performed by Ramón-Luing *et al.*[113] to identify potential markers for diagnosis. Nine reactive spots were identified around 30-kDa region, seven cathepsins (*Tv*CP1, *Tv*CP2, *Tv*CP3, *Tv*CP4, *Tv*CP4-like, *Tv*CP12 and *Tv*CPT), and two legumains (*Tv*LEGU-1 and *Tv*LEGU-2). Only three genes encoding *Tv*CP4, *Tv*CPT, and *Tv*LEGU-1 were cloned and sequenced. Purified recombinant CPs were recognized in patient sera. However, the investigators claimed that some CPs could be potential biomarkers for serodiagnosis of trichomoniasis, but they didn't report their results[113]. One year later, the same group of investigators utilized involvement of *Tv*CP39 in cytotoxicity and evaluated its use as one of the surface glycosylated antigen in diagnosis of trichomoniasis. Results revealed that *Tv*CP39 was an immunogenic antigen found in vaginal secretions. Utilizing Western blot, anti-recombinant *Tv*CP39 antibodies reacted with parasitic surface antigen (*Tv*CP39) present in vaginal smears from patients with trichomoniasis. Accordingly, it was concluded that *Tv*CP39 could be a useful diagnostic marker[114].

Cystatins (CYSs)

In two Mexican studies, the investigators identified and characterized genes encoding endogenous CYS-like CPIs. It was reported that *T. vaginalis* trophozoites possess three trichocystatins (TC 1-3). The first study cloned and expressed *tvicp-2* gene, and prepared specific polyclonal antibodies against the purified recombinant *Tv*TC-2. Results revealed that it was identified in *T. vaginalis* active degradome in association with *Tv*CP39 that was reported to be cytotoxic to vaginal epithelial cells. Its MW was 10kDa (type I, stefins) and was co-localized in the cytoplasm and lysosomes with *Tv*CP39. Except for legumains, it showed regulatory inhibitory activity against all *T. vaginalis* CPs. Trophozoites treated with recombinant *Tv*TC-2, in concentration dependent manner, showed significant decreased cytotoxic effects on *HeLa* cell monolayers *in vitro*[115]. The second study identified and characterized *Tv*TC-3. Interestingly, it was found that *Tv*TC-3 has two putative glycosylation sites (type II, CYS), but without disulfide bridge and with small MW (type I, stefins). It was localized in Golgi complex, cytoplasm, large vesicles, as well as the plasma membrane. It was concluded that *Tv*TC-3 has essential roles in regulating expression of *T. vaginalis*[116].

***Cryptosporidium* species**

Twenty CPs belonging to clan CA, family C1 were listed in *C. parvum*[50]. However, only cryptopain 1, expressed in sporozoites, was reported in several publications facilitating host cell invasion, degrading nutritive host proteins, and building up parasite proteins required for life cycle transitions[117-119]. Cryptopain 1 is registered in the CryptoDB database as cgd6_4880, while the other cathepsins L-like, termed cryptopains 2 and 3, are registered as cgd3_680 and cgd7_2850, respectively[120]. On the other hand, five genes encoding cathepsin L-like CPs, were identified in the *C. parvum* genome[121].

In an American study, a proteinase of 24 kDa was characterized as metallo-dependent CP on the surface of *C. parvum* sporozoites. Proteolytic activity was observed against azocasein, casein, bovine serum albumin, and gelatin at a pH of 6.5-7.0. Enzyme activity was inhibited with E-64 addition, but neither with serine, nor aspartate protease inhibitors. Using monospecific antibodies, sporozoite CP was not detected on oocyst surface[122]. One year later, another American study detected a similar CP in *C. parvum*

homogenate that was prepared from partially excysted oocysts. Its proteolytic activity against azocasein was at high peak one hour after incubation, but significantly decreased later. There was significant inhibition of its enzyme activity with serine protease inhibitors as well as E-64. However, there was no complete inhibition with a single inhibitor. Even so, combined inhibitors inhibited 95% of its activity. In addition, serine inhibitors, but not E-64, significantly inhibited oocyst excystation. It was concluded that *C. parvum* possesses both proteinases, however, serine proteases are mainly involved with excystation[117].

Later, three Korean studies were conducted by the same group of investigators. In the first report, the investigators identified and biochemically characterized the gene encoding cryptopain-1, the main *C. parvum* CP (*Cp*CP-1). Recombinant *Cp*CP-1 showed typical biochemical properties of cathepsin L-like enzymes. Western blot studies observed its expression in *C. parvum* sporozoites with proteolytic activity against collagen and fibronectin, but not globular proteins, suggesting its role in host cell invasion and/or parasite egress[119]. The second study identified and characterized cryptostatin, the endogenous CPI of *C. parvum*. It was reported that cryptostatin showed low sequence identity to chagasin-family ICPs, as well as with 8 β-strands resembling immunoglobulin fold. Over a wide pH range, recombinant cryptostatin showed inhibitory potency against human cathepsins B and L, and *Cp*CP-1. Due to diffuse localization of cryptostatin within oocysts and meronts, a role of host cell invasion was suggested[123]. The third study discovered the gene encoding *C. parvum* otubain (*Cp*OTU), a new CPs family. Recombinant *Cp*OTU showed similar proteolytic activity at neutral pH with an unusual C-terminal extension, essential for its activity. It was reported that the unusual C-terminal extension showed negative homology with other OTUs previously identified in human, mouse, and *Drosophila*. The investigators observed that its expression peaked in oocysts, suggesting its role in excystation[124].

It was reported that *Cryptosporidium* sporozoites are unable to deeply penetrate host cells due to deposition of extra-cytosolic electron-dense actin layer formed by host calpain during the initial invasion. In an American study, the role played by calpastatin, an endogenous CPI, was demonstrated. The investigators suggested that activation of host calpain during initial invasion was modulated or regulated by calpastatin overexpression from the apical organelles, similar to all apicomplexan protozoa[125].

In a trial to develop a novel drug for treatment of cryptosporidiosis, a study was conducted to investigate the inhibitory potency of K11777, a vinyl sulfone CPI, on *C. parvum*. Both *in vitro* and *in vivo* studies were performed in human gastrointestinal cell lines, and C57BL/6 γ-interferon receptor knockout mouse model, respectively. The investigators used this animal model because it is highly susceptible to cryptosporidiosis. The *in vitro* study demonstrated significant *C. parvum* death at comparable doses or lower than that of the drug control (paromomycin). Mice treated with an oral or intraperitoneal dose of K11777 (210 mg/kg of body weight/day) for 10 days showed only minimal inflammation with no epithelial changes. In addition, the mice became free of *C. parvum* for three weeks after treatment. Furthermore, homology modelling, knocking studies and inhibitor competition assays demonstrated K11777 efficient binding with cryptopain 1[126].

Eimeria species

In a trial to identify essential proteases in *E. tenella*, the related genome database was screened. Forty genes encoding proteases were identified, among them only sixteen were determined to encode CPs. The investigators carried out semi-quantitative PCR studies to determine which stage expresses a specific protease gene. Similar to other apicomplexans, 13 versus 17 genes were highly expressed or confined to the sexual gametocytes, and asexual merozoites, respectively. These results addressed the essential role played by proteinases, with specific emphasis on cathepsins, in asexual *E. tenella* developmental stages, involved in host cell invasion[127]. Because CPs are established as major virulence factors in *E. tenella*, a group of French and Australian investigators identified five genes encoding cathepsins; one of each cathepsin L (*Et*CPL) and B (*Et*CPB) and three cathepsin Cs (*Et*CPC 1-3). The study focused on *Et*CPB, later termed as eimeripain, that showed sequence homology with human cathepsin B (43%), as well as toxopain-1 (53%) expressed from *T. gondii*. It was reported that eimeripain was expressed only in the fully sporulated oocysts, whereas *Et*CPL and two cathepsin Cs (*Et*CPCs 2 and 3) were highly expressed in unsporulated oocysts. Further studies were recommended to elucidate eimeripain role in sporulation[128]. Because deletion of the gene encoding eimeripain was lethal, a group of British and German scientists proposed eimeripain as a new drug target in treatment of avian and livestock coccidiosis. Molecular modeling studies revealed significant homology difference from host enzymes suggesting its use as a suitable drug target. Utilizing high throughput screening, recombinant eimeripain was screened against several compounds, and three lead inhibitors including compounds containing nitrile, thiosemicarbazone, and oxazolone were identified. The investigators proposed the latter compounds as novel CPIs in treatment of coccidiosis[129]. Later, a study conducted in Japan investigated eimeripain expression during asexual and sexual developmental stages of *E. tenella in vivo*. In asexual stages, pro-mature CP was observed only in the early immature schizonts (specifically second generation), whereas the mature form significantly was detected in the middle-sized immature schizonts. Although both forms disappeared on maturation of schizonts, they were significantly expressed again in the

third generation of schizonts. Regarding sexual stages, both forms were found localized in the cytoplasm of micro- and macro-gametocytes and zygotes, however, decreased levels of expression were noticed on oocyst formation. The obtained results validated eimeripain as a novel, effective anti-coccidian drug and/or vaccine candidate[130].

The full sequence of a cathepsin-L-like CP in *E. tenella* (*Et*CATH-L) was characterized and its expression profiles were analyzed in different developmental stages. It showed 47 and 49% identity with that of *T. gondii* and *E. acervulina*, respectively. Results of reverse transcript PCR showed its highest expression at the initial phase of sporulation. Chickens immunized with recombinant *Et*CATH-L showed significant reduction of weight loss, with relative decrease in oocyst production compared to control chickens. It was concluded that *Et*CATH-L is an effective immunogen, and future studies were recommended to enhance its potential use as a vaccine candidate[131].

CONCLUDING REMARKS

1. *E. histolytica* trophozoites express several CPs encoded by ~ fifty genes, but only four, *Eh*CP1, *Eh*CP2, *Eh*CP5, and *Eh*CP7, are highly expressed in culture with identified cellular localizations. Whereas *Eh*CP1 is present in intracellular vesicles, *Eh*CP2 localizes in the internal and external cell membranes, and *Eh*CP5 is on the cell surface. On the other hand, *Eh*CP7 binds with an adhesin molecule (*Eh*ADH112) forming *Eh*CPADH complex that is located in the plasma membrane and cytoplasmic vacuoles.

2. Several functions were reported for amoebic CPs including cleavage of the intact mucus barrier and intestinal epithelium barriers (almost all CPs), contribution with adhesion factor (adhesin) for cytoadherence (*Eh*CP7), phagocytosis (cytotoxicity) (*Eh*CP7 and *Eh*CP2), significant contribution in extra-intestinal amoebiasis (*Eh*CP5 and *Eh*CP2), as well as C3 cleavage and degradation of secretory IgA and IgG (almost all CPs). *Eh*CP-B9 is involved in slow cyst-trophozoite transformation.

3. *A. castellanii* possesses several CPs that serve as virulence factors causing keratitis and granulomatous encephalitis. They have proteolytic activity against human lactoferrin, transferrin, hemoglobin, and ferritin. On the other hand, *N. fowleri* has only two cathepsins with proteolytic activity against human immunoglobulins, collagen, fibronectin, hemoglobin, and albumin. In addition, they are involved in evasion of host immune response.

4. *Blastocystis* has eight cathepsins, out of which are three isoforms of cathepsin B and five legumains. Several studies documented high CPs expression in amoebic forms that correlate to virulent *Blastocystis* isolated from symptomatic patients. Two main functions are assigned; increased permeability of intestinal epithelial cells and immunomodulation of host immune response.

5. In *G. lamblia*, out of several genes encoding cathepsins B, C, K and L, only three B-like cathepsins showed essential roles in life stage transformation process; survival and sucking disc cytoadherence. They are known as *Gl*CP 1-3. It is worth mentioning that *Gl*CP 1 was known as giardipain-1. Recently they are termed as CP10217, CP14019 and CP16779.

6. Recent studies demonstrated several functions for *Giardia* CPs including inhibition of goblet cells and depletion of human mucin-2, cleavage of proteins of the apical junctional complex of the intestinal epithelial cells, and cleavage of IgA, IgG as well as defensins (host defense peptides) produced by innate immune response cells.

7. *T. vaginalis* trophozoites possess nine CPs; seven cathepsins L-like (*Tv*CP65, *Tv*CP30, *Tv*CP39, *Tv*CP2, *Tv*CP4, *Tv*CP12, *Tv*CP60) and two legumains (*Tv*LEGU-1 and *Tv*LEGU-2). Cathepsins L like contribute in degradation of the mucus layer, cytoadherence (mainly *Tv*CP65), cytotoxicity and apoptosis of the vaginal epithelial cells (mainly *Tv*CP30), RBCs cytoskeleton disruption and hemolysis (*Tv*CP4), as well as evasion of host immune response (*Tv*CP39 and *Tv*CP66) *via* host immunoglobulins cleavage.

8. Because *Tv*CP30 is highly expressed in symptomatic isolates, and *Tv*CP39 is a surface glycosylated immunogenic antigen detected in vaginal secretions, both are used as diagnostic markers. Due to its role in cytoadherence, *Tv*LEGU-1 is established as virulence factor, and suggested as a novel drug target.

9. Three cathepsins L-like are identified in *C. parvum*, termed cryptopain 1-3. Their functions include facilitating host cell invasion, degrading nutritive host proteins, and building up parasite proteins required for life cycle transitions

10. CPs are established as major virulence factors in *E. tenella*, there are five genes encoding cathepsins; one of each cathepsin L (*Et*CPL) and B (*Et*CPB) and three cathepsin Cs (*Et*CPC 1-3). Cathepsin B like is also termed eimeripain and is highly expressed only in the fully sporulated oocysts. Because knockdown gene encoding eimeripain is lethal, it is proposed as a novel drug target in treatment of avian and livestock coccidiosis.

11. Several factors are incriminated in increased CPs expression in *E. histolytica*, *Blastocystis* spp., *G. lamblia*, *T. vaginalis* and *Cryptosporidium* spp. These include association with microbiota and co-association with *Escherichia coli*, as well as glucose and iron starvation.

12. Endogenous CPIs belonging to chagasin (clan IX, family I42) are only identified in *E. histolytica* and *C. parvum*, with two chagasin isotypes in the first. Regarding other protozoa in the present review, endogenous CPIs in *A. castellanii* and *G. lamblia* are identified and characterized as stefins CYS type 1. *T. vaginalis* trophozoites possess three trichocystatins (TC 1-3). However, only *Tv*TC-2 and *Tv*TC-3 were purified and characterized. The first is type I, stefins, while the second has two putative glycosylation sites (type II, CYS), but without disulfide bridge and with small MW (type I, stefin).

13. Vinyl sulfone CPI, K11777, and similar analogs proved to be novel drugs for treatment of PAM and cryptosporidiosis, whereas E-64 is successfully used in prevention of excystation and treatment of giardiasis.

Conflict of interest: There is no conflict of interest.

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Errata

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Molecular genetic characterization of human *Cryptosporidium* isolates and their respective demographic, environmental and clinical manifestations in Egyptian diarrheic patients

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