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TKL family kinases in human apicomplexan pathogens

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ABSTRACT

Apicomplexan parasites are the primary causative agents of many human diseases, including malaria, toxoplasmosis, and cryptosporidiosis. These opportunistic pathogens undergo complex life cycles with multiple developmental stages, wherein many key steps are regulated by phosphorylation mechanisms. The genomes of apicomplexan pathogens contain protein kinases from different groups including tyrosine kinase-like (TKL) family proteins. Although information on the role of TKL kinases in apicomplexans is quite limited, recent studies have revealed the important role of this family of proteins in apicomplexan biology. TKL kinases in these protozoan pathogens show unique organization with many novel domains thus making them attractive candidates for drug development. In this mini review, we summarize the current understanding of the role of TKL kinases in human apicomplexan pathogens' (*Toxoplasma gondii, Plasmodium falciparum* and *Cryptosporidium parvum*) biology and pathogenesis.

1. Protein kinase groups in Eukaryotes

Protein kinases play a critical role in signal transduction and regulation of most cellular processes in eukaryotic cells, including gene transcription, translation, cell growth, differentiation and metabolism, and their dysfunction is associated with many diseases [1,2]. Kinases phosphorylate more than 30 % of the cellular proteins by catalyzing the transfer of a phosphoryl group from an ATP molecule to specific hydroxyl groups within the side chain of the target substrate amino acid residues [1,3,4]. Phosphorylation by kinases may cause activation or inhibition of the target substrates, leading to events such as initiation of protein-protein interactions, translocations, and conformational changes [1].

In the human genome, more than 500 protein kinases have been identified, representing approximately 2–3 % of all encoded proteins [3–6]. They are classified as the second largest enzyme family and the fifth largest gene family, after Zinc finger proteins, G-protein coupled receptors, immunoglobulins, and proteases [3]. Among these kinases, about 10 % have been classified as pseudo-kinases, lacking one or more critical conserved residues essential for catalytic activity [6,7].

Protein kinases share a highly conserved catalytic domain consisting of 250–300 amino acids made of two lobes [3,8]. The smaller N-terminal lobe is about 80 amino acids in length, consisting of a five β-strands while the larger C-terminal lobe, made of about 180 amino acids, consists of mainly of α-helices that include motifs essential for recognizing protein substrates [3,9]. The cleft between the two domains forms a pocket for binding ATP molecule and hence is lined with conserved residues required for the catalytic activity [3,6,10].

Protein kinases are broadly categorized into two main superfamilies namely, the eukaryotic protein kinases (ePKs) and the atypical protein kinases (aPKs) [8]. Within the ePKs, the kinases are further classified into nine major groups based on their catalytic domains [10,11]. These include, (1) **AGC** group such as PKA, PKG and PKC, (2) the calcium/calmodulin-dependent protein kinase (**CAMK**) group which includes a family of calcium-dependent protein kinases (CDPKs), (3) casein kinase (**CK1**) group that consists of two major families CK1 and CK2, (4) **CMGC** group including cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases, (5) the **STE** group (related to yeast non-mating or sterile genes), (6) tyrosine kinase (**TK**) group, (7) tyrosine kinase-like (**TKL**) group includes the STKR family of TGF beta serine/threonine kinase receptors, (8) the receptor guanylyl cyclase (**RGC**) group, and (9) the ''**OPK**'' (other protein kinases) group that consists of a mixed collection of kinases that do not align distinctly with the above major groups [8,12].

Apart from the ''typical'' ePKs, more than 32 kinases have been classified as ''atypical protein kinases (aPKs)'', including phosphatidylinositol 3′ kinase (PI3K), DNA-dependent protein kinase (DNA-PK), and members of pyruvate dehydrogenase kinase (PDK) family [8,11,13,14]. These kinases exhibit enzymatic kinase activity but do not share

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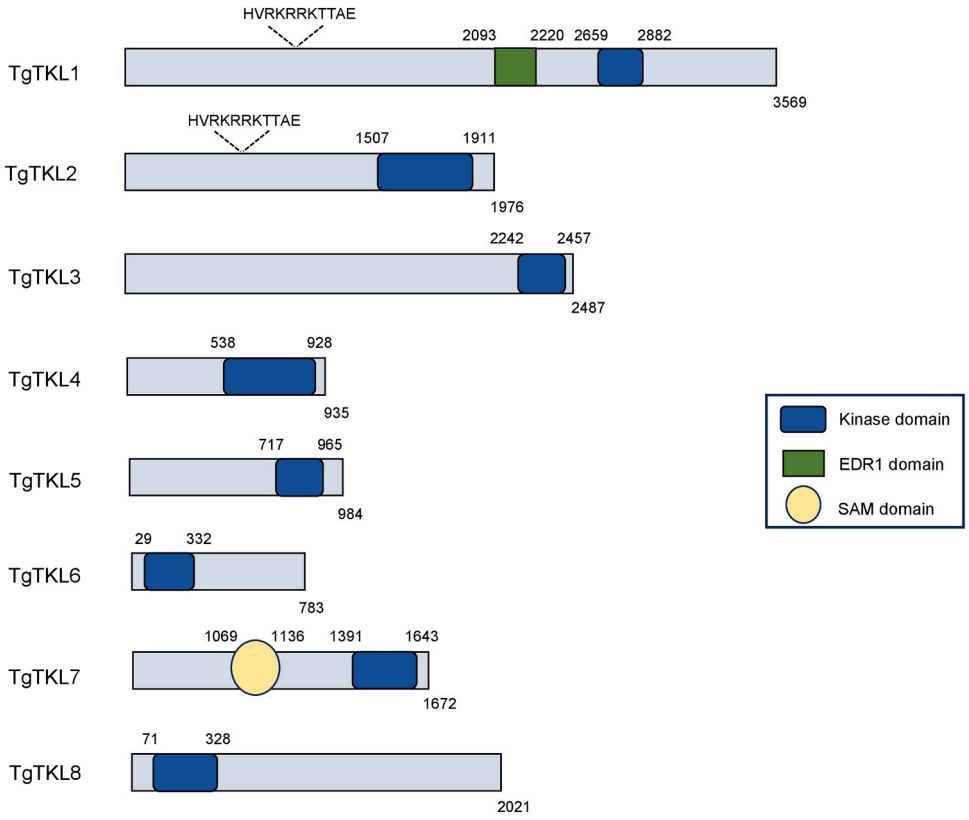

Fig. 1. Schematic representation of the eight TKL kinases in *Toxoplasma*. The locations of different domains and number of amino acids are indicated.

similarities at the primary sequence level [8].

The ePKs have been further classified into three different groups according to the amino acid residues on the target substrate that they phosphorylate, including (1) serine/threonine protein kinases (ST-PKs) which catalyze the phosphorylation of serine and threonine residues, (2) tyrosine protein kinases (TKs) which phosphorylate the tyrosine residues using ATP, and (3) the dual-specificity protein kinases that can phosphorylate both serine/threonine and/or tyrosine residues on their substrates [3,8].

2. TKL kinase family proteins in Apicomplexan parasites

Apicomplexan parasites are obligate intracellular pathogens that cause important diseases in humans and animals [15]. Protein kinases have been shown to play critical roles in apicomplexan biology including parasite motility, invasion, replication, egress, differentiation and virulence [14,16–19]. Since kinases are considered as good targets, many studies have focused on identifying unique and essential members that could be pursued for targeted therapy.

Although a large number of studies have been directed at dissecting the role of different kinase families in apicomplexans, studies aimed at understanding TKL kinase family are gaining traction only in recent years. The TKL family proteins are a group of kinases that share structural similarities with tyrosine kinases, but they are catalytically serine-threonine kinases [8]. Although TKL family proteins are present in various organisms, including metazoans, plants, and protists, interestingly, they are notably absent in fungi [20]. In plants however TKLs constitute one of the largest protein kinase families e.g. seventy percent of the rice kinases belong to the TKL group [21]. Apicomplexan parasites also contain TKL kinase family members and importantly many of these proteins contain domains that are unique and different from their mammalian hosts. Hence, defining the role of TKL kinases offers an excellent opportunity not only to understand their role in parasite biology but also allows for development of novel therapeutic interventions.

Here, in this review, we provide a brief overview of studies on TKL proteins in apicomplexans that cause important human diseases including *Toxoplasma gondii, Plasmodium falciparum, and Cryptosporidium parvum*. Further, we also discuss their organization, known function and potential as targets for pharmacological approaches.

2.1. TKL kinases in Toxoplasma gondii

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate intracellular protozoan parasite that affects nearly one-third of the human population worldwide [22]. Humans become infected following the oral ingestion of bradyzoites in contaminated raw or undercooked meat, or upon the ingestion of sporulated oocysts in food or water contaminated with felines' feces [23,24]. Vertical transmission also occurs via the placental spread of tachyzoites from an infected mother to the fetus, causing congenital toxoplasmosis. Following infection with *Toxoplasma*, the tachyzoites rapidly spread to various organs in the host causing acute infection. Under the control of the host immune response, the tachyzoites evade the immune system and subsequently differentiate into the slow replicating bradyzoite cysts in the brain and skeletal muscles that persist for the lifetime of the host [25]. In immunocompromised individuals or patients undergoing immunosuppressive therapies, chronic toxoplasmosis may reactivate causing fatal encephalitis and other serious conditions that may cause death [25]. In pregnant women, infection causes miscarriage, while in newborn children, blindness and cognitive impairments can occur [22].

The drugs currently used for treating toxoplasmosis include sulfadiazine and pyrimethamine. However, these drugs have severe side-effects, and more importantly do not treat chronic form of the disease. Hence there is a critical need to develop new therapeutic strategies for effective clinical management of toxoplasmosis [26,27]. In *Toxoplasma,* kinases have been shown to play a critical role in parasite motility, invasion, replication, and egress processes and promote the parasite

survival within the host [19,27]. Accordingly, targeting kinases that are important for parasite biology will be key in developing new drugs and this is very well manifested with calcium dependent protein kinase (CDPK) inhibitors that are currently being explored as anti-*Toxoplasma* agents [16,28].

In *Toxoplasma* there are about 159 kinases, that constitutes nearly 2 % of its genome. Among these, 108 are classified as true kinases, while 51 are designated as pseudo-kinases based on KEDD signature motif analysis [29]. The catalytically active kinases of *Toxoplasma* include enzymes that belong to several of the ePK groups including AGC, CAMK, CK1, CMGC, STE and TKL kinases. Intriguingly, the TK and RGC groups of kinases are notably absent within *Toxoplasma*. Furthermore, *Toxoplasma* presents an expanded group of coccidian-specific secreted rhoptry kinases (ROPK), that have been demonstrated to play a role in parasite virulence [17,29].

The *Toxoplasma* genome contains a total of eight TKL genes (Fig. 1). A recently conducted clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based genome-wide gene disruption screen identified about 200 parasite genes important for *Toxoplasma* growth *in vitro* [30]. More specifically, in this study, the fitness conferring genes were identified with a negative phenotypic score while those genes that were dispensable for parasite propagation were awarded with positive phenotypic scores. The eight TKLs in *Toxoplasma* are thus named based on their CRISPR/Cas9 phenotypic scores with TgTKL1 having the lowest phenotypic number. Endogenous tagging of the six TKLs predicted to be important for parasite propagation revealed that the proteins localize to different compartments in the parasite. Both TgTKL1 (TgGT1_301270) and TgTKL2 (TgGT1_234970) contain an NLS motif and accordingly localize to the parasite nucleus. The next two proteins, TgTKL3 (TgGT1_253860) and TgTKL4 (TGGT1_237210) localize to the parasite cytoplasm. TgTKL5 (TGGT1_209050) is found in the inner membrane complex while TgTKL6 (TGGT1_236240) appears to be a trans-Golgi Network (TGN) protein in *Toxoplasma* [31].

TgTKL1, a nuclear kinase in *Toxoplasma* is an interesting kinase with unique organization of domains. The kinase contains an Enhanced drug resistance 1 domain (EDR1) in addition to the kinase domain present in the C-terminal region. In plants, EDR1 domain-containing kinases, such as the *Arabidopsis* kinases EDR1 and CTR1, have been shown to play a role in stress response pathways, including ethylene-mediated signaling. Importantly, EDR1 domain-containing kinases are found only in plants and protozoa, making them attractive candidates for drug development [31].

The loss of TgTKL1 leads to significant defects in *Toxoplasma* growth *in vitro*. Studies also suggested that it is mainly the *Toxoplasma* invasion into host cells that is significantly compromised in the absence of TgTKL1. Further dissection revealed that loss of TgTKL1 leads to dysregulation of global gene expression in the parasite and many genes related to invasion and virulence are down-regulated in *Toxoplasma* lacking TgTKL1. Specifically, TgSUB1, a protease that has been shown to be essential for processing of secreted micronemal proteins is down-regulated in TgTKL1 null mutants and this leads to defects in microneme processing and host-cell attachment during invasion process. More importantly loss of TgTKL1 results in complete loss of virulence in the animal model suggesting that TgTKL1 is an essential virulence factor. Further inoculation of TgTKL1 null mutants results in generation of protective immune response in mice suggesting that TgTKL1 knockout parasites could be used as live vaccine candidate in the future [31,32]. Since TgTKL1 is a nuclear kinase that regulates gene expression in *Toxoplasma*, it is feasible that substrate candidates for this protein are parasite transcription factors. And currently, efforts are underway to identify substrates of TgTKL1 and the findings from this study should divulge if this hypothesis is indeed true.

TgTKL4 is another interesting member of the TKL family in *Toxoplasma* as it shows cell-cycle dependent expression during endodyogeny. The kinase is expressed only during S and M/C phases and is absent in G1 stage of the cell division. Interestingly, although the protein is predominantly cytoplasmic during S phase, in the late M/C phases, some fraction of the protein associates with the *Toxoplasma* cytoskeleton as evidenced through colocalization with inner membrane complex (IMC) region. The loss of TgTKL4 leads to significant defect in *Toxoplasma* growth that is mainly due to slow replication and impaired invasion. Additionally, TgTKL4 is also an important for *Toxoplasma* pathogenesis *in vivo* as lack of TgTKL4 results in significant attenuation of parasite virulence in the mouse model. Intriguingly, the TgTKL4 null mutants show defects in morphology not only during cell division but also in extracellular stages as well. Specifically, in contrast to slender and long appearance of wildtype parasites, TgTKL4 knockout parasites are misshaped with short and rounded appearance. Further, studies using expansion microscopy revealed that loss of TgTKL4 results in defect in arrangement of subpellicular microtubules that have been previously shown to be critical determinants of *Toxoplasma* morphology. Specifically, the changes seen in TgTKL4 null mutants include an increased space between the subpellicular microtubules, a significant decrease in their length and also loss of spiral arrangement seen normally in wildtype parasites. Importantly, quantitative phospho-proteome analysis indicated that absence of TgTKL4 results in decreased phosphorylation of many cytoskeletal proteins suggesting that a few of these could be putative substrates of this kinase [33].

2.2. TKL kinases in Plasmodium falciparum

Malaria is a serious health problem affecting approximately about 40 % of the human population worldwide. It is caused by the intracellular protozoan parasite that belongs to the genus *Plasmodium*. *Plasmodium* parasites are transmitted to humans through the bites of infected female *Anopheles* mosquitoes. When infected mosquitoes take a bloodmeal, the sporozoites are injected during that process. The sporozoites are the infective motile stage and these migrate from the bite site to the liver where they multiply in hepatocytes. Inside the hepatocytes, through exo-erythrocytic schizogony a sporozoite is able to generate thousands of merozoites. The merozoites are then released into the bloodstream and invade erythrocytes, undergo schizogony, and egress resulting in massive destruction of red blood cells. A fraction of merozoites within the erythrocytes differentiate into gametocytes, which are infective to the mosquito. Once ingested by the insect, the gametocytes develop into male and female gametes and fuse into a zygote in the mosquito midgut. The zygote develops into an ookinete that crosses the midgut and develops into an oocyst. Further sporogony leads to formation of sporozoites that migrate to the salivary glands of the mosquito and gain entry into a new host when the insect takes a bloodmeal [34–36].

Plasmodium infection has many clinical forms that range from asymptomatic to lethal depending on the host immunity and the stage of infection. Amongst the five known species of *Plasmodium* that infect humans, *Plasmodium falciparum* is responsible for the most severe form of malaria. It is widespread in many tropical and subtropical regions, including Africa, Southeast Asia, South America, and Western Pacific. Although available treatments can totally cure malaria infection, the recent rise of drug-resistant malaria parasites imposes an urgent need to develop new therapeutic strategies and identify new targets for antimalarial treatments [34].

The *P. falciparum* kinome comprises over 80 potential protein kinases, including 65 ePKs, 5 aPKs, and 19 FIKKs (Apicomplexan specific ePK-related kinase family FIKK (Phe (F) – Ile (I) – Lys (K) – Lys (K)). Notably, *P. falciparum* genome lacks the TK and STE kinase related groups. Among the active protein kinases, several have been shown to be essential for parasite survival. Moreover, many *Plasmodium* kinases lack human orthologs or are totally absent in mammalian hosts, indicating that the regulatory mechanisms and functions of these kinases differ from their human counterparts. Hence, the identification of malaria kinases that play essential roles in the parasite's growth and pathogenesis could pave the way for developing therapeutic interventions to combat the deadly infection [18,37,38].

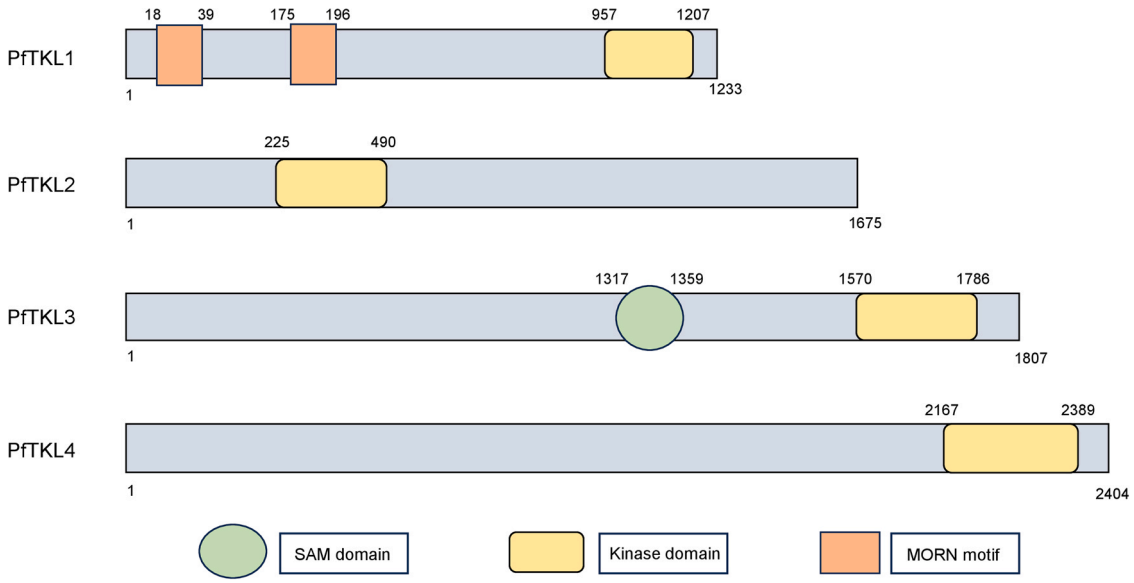

Fig. 2. Schematic representation of the four TKL proteins in *Plasmodium falciparum*. The locations of different domains and number of amino acids are indicated.

The *P. falciparum* genome contains four TKL members namely, PfTKL1 (PF3D7_0211700), PfTKL2 (PF3D7_1121300), PfTKL3 (PF3D7_1349300) and PfTKL4 (PF3D7_0623800) (Fig. 2). Of these kinases, only PfTKL3 has been subjected to functional analysis and these studies have shown that the protein is indeed essential for asexual division in human erythrocytes [39]. However, little is known about the other PfTKL members including their molecular mechanisms and role in pathogenesis.

PfTKL1 is about 1233 amino acids in length, with its kinase domain located towards the end of the C-terminus. This kinase contains two conserved Membrane Occupation and Recognition Nexus (MORN) motifs, each 21 amino acids in length, located towards the N-terminal region. PfTKL2 is 1675 amino acids in length and interestingly is the only PfTKL member in which the kinase domain is located within first half of the protein. PfTKL4 is the largest member of TKL family in *Plasmodium falciparum* with 2404 amino acids and the kinase domain is at the C-terminus. The bioinformatic analysis of PfTKL2 and PfTKL4 however did not reveal any recognizable domains in addition to the kinase domain [39].

PfTKL3 is 1807 amino acids in length, with its kinase domain towards the C-terminal region. In addition to the kinase domain PfTKL3 contains several accessory domains in the N-terminal part of the protein. The accessory domains of PfTKL3 include: (1) a putative PEXEL motif (*Plasmodium* Export Element) that mediates trafficking to host-erythrocytes, (2) a potential 14–3–3 mode II binding motif, (3) two putative MORN motifs (MORN motifs are predicted to be important for membrane interactions), along with (4) a putative sterile α-motif (SAM) domain. PfTKL3 is expressed in all stages of the asexual cycle as well as in gametocytes and sporozoites. Further among the asexual stages, the protein shows highest expression in schizonts followed by trophozoites and least expression in ring stages [39]. IFA analysis revealed that PfTKL3 shows dynamic localization in schizont and merozoite stages, the protein predominantly localizes to the sub-pellicular membrane complex in gametocytes. More importantly *in vitro* kinase assay using recombinant kinase domain confirmed that PfTKL3 is indeed a true kinase. Although the gene locus of PfTKL3 is amenable to genetic manipulation, viable parasites with loss of PfTKL3 expression could not be obtained suggesting that the protein is essential for asexual propagation of *Plasmodium*. Further mutational analysis of the SAM domain suggested that this domain is involved not only in regulating the PfTKL3 kinase function but also mediates protein oligomerization [39]. These findings underscore the importance of PfTKL3 in mediating cellular signaling pathways, highlighting its critical role as a regulator of developmental biology in *Plasmodium*. And since the domain structure of PfTKL3 is quite unique and the protein appears to be essential for merozoite division, PfTKL3 is considered as a potential drug target in *Plasmodium* parasites.

2.3. TKL kinases in Cryptosporidium

Cryptosporidiosis is another worldwide infection caused by the apicomplexan parasite belonging to the genus of Cryptosporidium [40]. *Cryptosporidium spp* infect many vertebrate species, including humans, causing acute gastroenteritis, abdominal pain, and diarrhea. While around 30 *Cryptosporidium* species have been identified, only two, *Cryptosporidium parvum* and *Cryptosporidium hominis*, are commonly found in humans. Individuals with weakened immune systems or young children are particularly susceptible to this infection. Transmission of *Cryptosporidium* primarily occurs through the fecal–oral route, typically by ingesting viable oocysts present in food or water contaminated with animal and/or human's feces. Moreover, *Cryptosporidium* has emerged as the most common cause of recreational water-related outbreaks on a global scale [40–43].

Cryptosporidiosis infection is initiated by the ingestion of oocysts, and the oocyst wall breaks down in the upper intestinal tract to release four infective sporozoites. Following excystation, the motile sporozoites bind to receptors on the surface of the intestinal epithelial cells and reside within the parasitophorous vacuoles (PV). Inside the PV, the parasites undergo asexual reproduction (schizogony) and sexual or gametogenic reproduction to produce microgamonts and macrogamonts. Upon fertilization of the microgamonts by the microgametes, two types of oocysts are produced: (1) the thin-walled oocysts involved in autoinfection, and (2) the thick-walled oocyst protected by a resistant wall, typically excreted from the host to the environment, thus allowing direct fecal-oral transmission. The pathology in cryptosporidiosis is primarily due to destruction of intestinal epithelial lining when parasites egress from infected cells. To date, there is no vaccine to prevent *Cryptosporidium* infection in humans or animals. Moreover, nitazoxanide is the sole approved drug, but its efficacy is limited, especially in immunocompromised patients and malnourished children [41,42,44,45]. Hence, there is an urgent need to develop new drugs to effectively treat patients, particularly those at the highest risk for infection.

The *C. parvum* kinome contains 73 potential protein kinases with intact catalytic triads, categorized into distinct groups such as AGC,

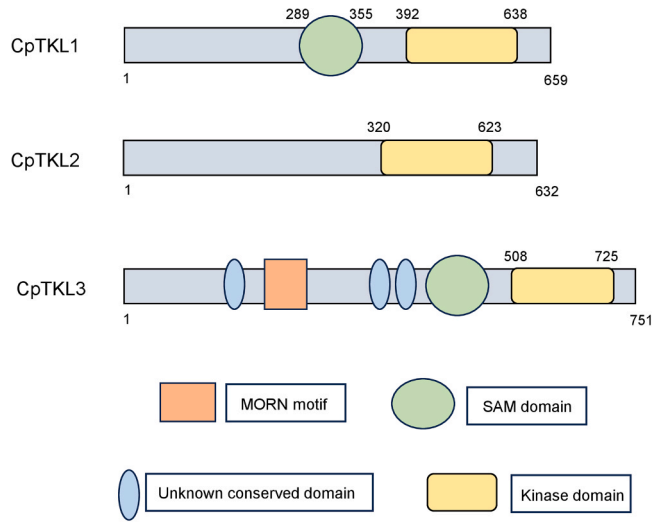

Fig. 3. Schematic representation of the three TKL proteins in *Cryptosporidium parvum*. The locations of different domains and number of amino acids are indicated.

CaMK, CK1, CMGC, TKL, atypical, and OPK [46]. Similar to *P.falciparum*, *C.parvum* notably lacks the STE and TK groups of kinases [46]. *Cryptosporidium* genome contains three TKL enzymes and these include CpTKL1 (cgd8_2430), CpTKL2 (cgd3_2900) and CpTKL3 (cgd3_4310) [46] (Fig. 3).

In *Cryptosporidium*, CpTKL1 and CpTKL2, measure about 659 and 632 amino acids and these contain a kinase domain at the end of the C-terminus. Additionally, CpTKL1 contains a SAM domain of 66 amino acids of in the center of the protein. CpTKL3 is made of 751 amino acids with its kinase domain located towards the C-terminal region. The CpTKL3 kinase is an ortholog of PfTKL3 in *Plasmodium* and hence contains accessory domains in the N-terminal region of the protein including a MORN domain and a SAM domain [47]. Although CpTKL3 and PfTKL3 share about 30 % sequence identity, CpTKL3 lacks PEXEL and 14_3_3 mode II binding motifs, upstream of the SAM domain found in PfTKL3 [46].

The significance and the need for TKL kinase research in *Cryptosporidium* was highlighted by the findings of a recent study that performed high-throughput screening of kinase library against this gut pathogen [48]. The study screened a total of 473 known kinase inhibitors to identify compounds that are effective against *Cryptosporidium* [48]. The findings revealed 11 compounds that inhibit parasite growth and interestingly three of these small molecules are known to target TK group of kinases [48]. Since *Cryptosporidium* genome lacks any TK enzymes in its arsenal, it is hypothesized that these compounds could be targeting TK related TKL kinases in this parasite. Since molecular genetic tools for dissecting gene functions of *Cryptosporidium* are now available [49], TKLs in *Cryptosporidium* are expected to receive increased attention in the coming years.

3. Conclusions and future perspectives

Protein kinases play critical roles in regulating the developmental stages of parasites across their life cycle. Since kinase activity could be nullified using chemical inhibitors, they present promising targets for the development of novel antiparasitic agents. To date, only a limited number of TKL kinases have been studied in Apicomplexan species. Some of the underlying reasons could be that many of the TKL members are essential genes and in some apicomplexan members molecular genetic tools have become available quite recently. Hopefully more research would be directed on understanding the role of TKL proteins and their significance in parasitic infections in the coming decade. Since apicomplexan TKLs are evolutionarily distant and have distinct structural organization, they are promising avenue for developing selective agents without affecting the host cellular functions. Ideally, systematic characterization of the parasite kinome typically involves the identification of genes important and/or essential for pathogenesis. Subsequent determination of protein localization is crucial to provide a deeper understanding of their functionalities and regulatory mechanisms, through a diverse array of knockout and knock-down approaches. Additionally, identification of the interactome using advanced methodologies such as quantitative phosphoproteomics, thio-phosphorylation and proximity-based labelling systems is crucial for understanding the molecular functions and kinase-mediated signaling pathways. This comprehensive approach against TKL kinases will lay the groundwork for development of targeted drug therapies against important apicomplexan parasites that pose significant risk to human health.

Declaration of Competing Interest

None

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