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Comparative analyses and structural insights of new class glutathione transferases in *Cryptosporidium* species

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Cryptosporidiosis, caused by protozoan parasites of the genus *Cryptosporidium*, is estimated to rank as a leading cause in the global burden of neglected zoonotic parasitic diseases. This diarrheal disease is the second leading cause of death in children under 5 years of age. Based on the *C. parvum* transcriptome data, glutathione transferase (GST) has been suggested as a drug target against this pathogen. GSTs are diverse multifunctional proteins involved in cellular defense and detoxification in organisms and help pathogens to alleviate chemical and environmental stress. In this study, we performed genome-wide data mining, identification, classification and in silico structural analysis of GSTs in fifteen *Cryptosporidium* species. The study revealed the presence three GSTs in each of the *Cryptosporidium* species analyzed in the study. Based on the percentage identity and comprehensive comparative phylogenetic analysis, we assigned *Cryptosporidium* species GSTs to three new GST classes, named Vega (ϑ), Gamma (γ) and Psi (ψ). The study also revealed an atypical thioredoxin-like fold in the *C. parvum* GST1 of the Vega class, whereas *C. parvum* GST2 of the Gamma class and *C. melagridis* GST3 of the Psi class has a typical thioredoxin-like fold in the N-terminal region. This study reports the first comparative analysis of GSTs in *Cryptosporidium* species.

Cryptosporidiosis is a zoonotic parasitic disease that is caused by *Cryptosporidium* spp.^{1–3}. This disease is estimated to be among the highest ranking causes in the global burdens of zoonotic parasitic disease, with an estimate of 8.37 million disability-adjusted life years^{2,4}. Recently, large population studies revealed that cryptosporidiosis has become a fast-growing burden to children under the age of 5 years^{5,6}. Moreover, the Global Enteric Multicenter Study (GEMS) showed that *Cryptosporidium* is significantly associated with diarrheal disease among children < 24 months of age in sub-Saharan Africa and South Asia⁵. Similar studies also found *Cryptosporidium* to be the second leading cause of moderate to severe diarrhea in infants after Rotavirus⁶. It is interesting to note that vaccines/treatment are already available or fast being developed for three of four diarrheal pathogens (*Rotavirus*, *Shigella* and heat-stable, enterotoxigenic *Escherichia coli*), the exception being *Cryptosporidium*, highlighting the need to address this disease⁷. Despite the global burden of cryptosporidiosis, to date nitazoxanide (NTZ) is the only treatment available for this disease. NTZ only appears to be effective in patients with a good immune response, whilst having limited efficacy in malnourished children and ineffective in immunocompromised people^{8–10}. The lack of effective treatment for cryptosporidiosis, coupled with the fact that it is now considered the most common cause of human parasitic diarrhea in the world, highlights the need for more research on *Cryptosporidium* to identify new drug targets and thus develop new drugs¹¹.

Cryptosporidiosis is typically characterized by nausea, profuse watery diarrhea, abdominal cramps, vomiting and low-grade fever, which manifest after 14 days and last up to 2.5 months in immune-competent patients^{12,13}.

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These symptoms are usually self-limiting in immune-competent patients; however, in immunocompromised hosts they can be devastating, with the disease manifesting as life-threatening and often becoming extraintestinal¹³. The gastrointestinal infection can spread to other sites, such as the gall bladder, biliary tract, pancreas and pulmonary system. Cryptosporidiosis can be contracted through the fecal–oral route, through contact with infected animals or humans or contaminated food or water¹³.

Of the *Cryptosporidium* species that exist, *C. hominis* and *C. parvum* are responsible for the highest level of clinically relevant infections worldwide³. The remaining species have mild zoonotic properties causing moderate-to-severe diarrhea in humans³. *Cryptosporidium* species are reported to have an efficient defense mechanism that allows it to cope with a wide range of environmental stresses such as changes in temperature, drugs, free radicals, as well as the host's immune responses at various life stages¹². Genome analysis of *C. parvum* revealed that it contains various defense proteins such as glutathione transferase (GST), glutathione peroxidase and superoxide dismutase, which are known for detoxification, signal modulation and aromatic amino acid catabolism¹⁴. The existence of these enzymes may provide *C. parvum* with the abilities to maintain its parasitic lifecycle, enabling it to survive and persist in its host.

Among the above-mentioned enzymes, GST is found to be expressed in all stages of the *C. parvum* parasite's life cycle¹⁵, thus making it a promising therapeutic target¹⁶. GSTs have been studied as drug targets against infectious agents and metabolic disorders^{17–19}. GSTs are a diverse group of multifunctional proteins that are distributed ubiquitously in eukaryotes and prokaryotes^{20,21}. These enzymes play an important role in cellular defense and detoxification^{20,22,23}. They catalyze the nucleophilic conjugation of the reduced tripeptide glutathione (GSH) thiol group to the electrophilic substrates to convert them to less harmful, more soluble compounds. Based on the location, the GST superfamily is divided into three sub-families namely, soluble or cytosolic GSTs, mitochondrial GSTs and membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) with the cytosolic GSTs being the most characterized (Table S1). The GSTs are generally divided into classes based on amino acid sequence similarity, with GSTs within each class sharing similar immunological cross-reactivity and specificity towards the electrophilic substrate and sensitivity to inhibitors^{20,24,25}. GSTs within each class typically share as little as 60% amino acid sequence identity; however, some classes can share from as little as 40%^{20,23,26–28}. It is generally accepted that the assignment of different GSTs to specific classes must fall within these limits, with sequences sharing less than 25–30% designated to their own class^{20,23,26–28}. Information on different GST classes found in organisms, their cellular localization and functions are listed in Table S1.

Typical GSTs are dimeric in structure and each monomer is divided into two domains^{20,23}. The N-terminal domain of conical GSTs assumes a topology resembling the thioredoxin fold with a $\beta\alpha\beta$ - $\beta\beta\alpha$ motif. This domain also houses an important conserved region of the active site where a catalytically active Tyr, Ser or Cys is found to interact with the GSH thiol group. The C-terminal domain of typical GSTs is all helical and connected by a short linker sequence called the *cis*-Pro loop with a highly-conserved proline residue in *cis* conformation²³. The active site is comprised of the glutathione binding site (G-site) and the hydrophobic substrate binding site (H-site), located in the N-terminal and C-terminal domain respectively. The G-site exclusively binds glutathione and is highly conserved, whilst the H-site accepts more variability so to accommodate an extensive range of toxic electrophilic substances^{20,23}.

Despite the importance of GSTs, especially as a potential drug target against *Cryptosporidium*¹⁶, to the best of our knowledge, no literature is available to date on *Cryptosporidium* GSTs with regards to their distribution, the GST classes and structural information. Thus, this study is aimed at addressing this research gap. In this study, genome data mining, identification, phylogenetic and structural analysis of GSTs in fifteen *Cryptosporidium* species has been carried out.

Methods

Species and database. *Cryptosporidium* species genomes that are available for public use at the *Cryptosporidium* database or CryptoDB²⁹ (<https://cryptodb.org/cryptodb/app>; release 48 beta, 27 August 2020; accessed on 14 September 2020) and at National Center for Biotechnology information (NCBI)³⁰ (<https://www.ncbi.nlm.nih.gov/datasets/genomes/?txid=5806>; accessed on 14 September 2020) were used in the study. The *Cryptosporidium* pathogens examined in this study include ones from both humans and other mammals (Table 1).

Genome data mining, identification and classification of GSTs. *Cryptosporidium* species genomes available at CryptoDB²⁹ were mined for GSTs. Two different methods followed for GST mining. First, the genomes of *Cryptosporidium* species were mined using the term “glutathione transferase”. Second, the species genomes were blasted with GST proteins from *Homo sapiens* (protein ID: P08263)⁵³ and *C. parvum* Iowa II (protein ID: EAK89476.1)^{14,38}. The BLASTP mined proteins revealed a range of apicomplexan species which were filtered out to show only *Cryptosporidium* species. The hit proteins were then collected and subjected to protein family analysis using the Pfam⁵⁴ and InterPro⁵⁵ programs. The results were analyzed and the hit proteins that were classified as GST by Pfam (PF14497, PF13417 and, PF17172)⁵⁴ and InterPro (IPR036282, IPR004045 and IPR010987)⁵⁵ were selected.

For the collection of more hits, *Cryptosporidium* species genomes available at NCBI database³⁰ was blasted with two GST proteins from *C. andersoni* 30847 (cand_012830 & cand_023790) and from *C. meleagridis* UKMEL1 (CmeUKMEL1_05845) that were collected from CryptoDB²⁹. The hit proteins were screened for GSTs following the method described above.

A final total count was presented by deleting the duplicated GSTs. The selected GSTs were then grouped into different classes or groups based on their percentage identity, following the conventional criterion of less than 25–30% identity being a new class^{20,23,26–28}.

Species and isolates	Host range	Reference(s)
<i>Cryptosporidium andersoni</i> isolate 30847	Cattle, sheep, bactrian camel, gerbil	31
<i>Cryptosporidium hominis</i> isolate TU502_2012	Humans, monkeys, macaque, kangaroo, calf and piglets	32,33
<i>Cryptosporidium hominis</i> isolate 30976	Humans, monkeys, macaque and kangaroo	33,34
<i>Cryptosporidium hominis</i> TU502	Humans, monkeys macaque, kangaroo, calf and piglets	33,35
<i>Cryptosporidium hominis</i> UdeA01	Humans, monkeys, macaque, kangaroo	36,37
<i>Cryptosporidium meleagridis</i> strain UKMEL1	Human, turkey, chicken, bobwhite quail, dog	32,37
<i>Cryptosporidium parvum</i> Iowa II	Humans, cattle, sheep, pigs, deer and mice	14,37–39
<i>Cryptosporidium tyzzeri</i> isolate UGA55	Domestic mice	40
<i>Cryptosporidium ubiquitum</i> isolate 39726	Deer, sheep, goat, squirrel, mice and beavers	31,41
<i>Cryptosporidium muris</i> RN66	Mice and cats	42,43
<i>Cryptosporidium baileyi</i> strain TAMU-09Q1	Chickens and black-headed full, quails, ostriches and ducks	37,44,45
<i>Cryptosporidium viatorum</i> isolate UKVIA1	Humans and rats	46,47
<i>Cryptosporidium</i> sp. chipmunk LX-2015	Mice, squirrels, chipmunks	41,48,49
<i>Cryptosporidium ryanae</i> isolate 45019	Cattle	50
<i>Cryptosporidium bovis</i> isolate 42482	Sheep and cattle	51,52

Table 1. *Cryptosporidium* species used in the study and their major host specificity.

Analysis of homology. The percentage identity between GSTs was deduced using Clustal Omega⁵⁶. The full-length GSTs were subjected to Clustal analysis which produced the percentage identity amongst each of the proteins as matrix identity results. These results were laid out in an Excel spreadsheet where the results were analyzed to identify the percentage identity between GSTs.

Collection of different GST classes' protein sequences. For comparative analysis, GST protein sequences belonging to different GST classes were collected using multiple methods to build a library for phylogenetic analysis. On the European Molecular Biology Laboratory (EMBL) site⁵⁷, GSTs sequences that are placed under the GST superfamily (IPR040079) were retrieved. The GST classes namely CLIC (IPR002946), Alpha (IPR003080), Mu class (IPR003081), Pi (IPR003082), Omega (IPR005442), Zeta (IPR005955) and Sigma (IPR003083) were collected under EMBL. More sequences were obtained through text search using the UniProt protein knowledge base⁵⁸. A specific GST class was searched on the site and the hits obtained were further verified using Pfam⁵⁴ and InterPro⁵⁵ to ensure uniformity with the GSTs collected from the EMBL site⁵⁷. The remaining GSTs that were not in the databases were retrieved from published articles.

The *Cryptosporidium* species GST sequences along with protein sequences of different GST classes used in the phylogenetic analysis are presented in Supplementary Dataset 1.

Phylogenetic analysis. The GST sequences in supplementary dataset 1 were used to make a phylogenetic tree for inferring their evolutionary relationship. First, all the GST protein sequences were aligned by MAFFT v6.864 embedded on the Trex-online server⁵⁹. Then, the alignment was automatically submitted to the server for inferring the tree with different models and the optimized tree was selected. Finally, the tree was submitted to iTOL for viewing and annotation⁶⁰. Thioredoxin from *Oryctolagus cuniculus* (protein ID: P08628) was used as an outgroup.

For the construction of the phylogenetic tree of the *Cryptosporidium* GST proteins, the protein sequences were aligned using MUSCLE software⁶¹ embedded in MEGA7⁶². The evolutionary history was inferred by using the maximum likelihood method with 100 bootstrap replication based on the JTT matrix-based model⁶³. Evolutionary analyses were conducted in MEGA7.

Cellular localization and transmembrane helices prediction. Cellular localization of GSTs was predicted using the Bologna Unified Subcellular Component Annotator (BUSCA)⁶⁴. BUSCA is the latest, accurate program available for the prediction of proteins' subcellular localization; it integrates different computational tools such as identifying signal and transit peptides (DeepSig and TP-pred3), GPI-anchors (PredGPI) and transmembrane domains (ENSEMBLE3.0 and BetAware) with tools for discriminating subcellular localization of both globular and membrane proteins (BaCelLo, MemLocian and SChloro)⁶⁴. The outcomes of these different programs were processed and integrated to predict subcellular localization of both eukaryotic and bacterial proteins⁶⁴. Prediction of transmembrane helices in GSTs was done using TMHMM Server v. 2.0⁶⁵. This program is well known for its high degree of accuracy in the prediction of transmembrane helices and discrimination between soluble and membrane proteins.

Template identification. To construct 3D models of proteins, reference protein structures previously solved by crystallization or Nuclear Magnetic Resonance are needed. These would serve to simulate not only the fold of a protein but also a full atom model to build. These proteins are referred to as templates. Either single or multiple templates can be used in constructing the 3D model of a protein⁶⁶. In this study, three different web servers, namely NCBI BLAST (v2.10.1)⁶⁷, i-TASSER (v5.1)⁶⁸ and PHYRE (v2.0)⁶⁹, were consulted to identify the

most suitable templates for GST proteins. Based on the highest percentage identity and sequence coverage, the best templates were selected for modeling each GST protein. In cases where the templates had the same percentage identity and sequence coverage, we selected the template with the highest resolution for modelling.

Protein sequence alignment for modeling. T-COFFEE webserver⁷⁰ was used for aligning the GST proteins and the template sequences. The aligned files were downloaded in FASTA format and modified to generate files to be used for protein modelling⁷¹.

Protein modeling, optimization and validation. The MODELLER v9.21 program⁷¹ was used to build GST models. Multiple structures were produced by Modeller 9.21. The model with the best DOPE assessment was selected as the output structure to be used. The structures modeled were viewed using PyMOL⁷². The model for each GST was then subjected to evaluation for stereochemistry and energetic quality at the Structural Analysis and Verification Server (<http://servicesn.mbi.ucla.edu/SAVES/>) and ProsaII (<https://prosa.services.came.sbg.ac.at/>)⁷³. Based on the validation results, the protein models were then refined on the GalaxyWeb Refiner server⁷⁴. After refinement, the models were again subjected to evaluation and validation using programs such as ERRAT⁷⁵, Verify3D⁷⁶, PROCHECK^{77,78}, and RAMPAGE⁷⁹ and ProsaII⁷³.

Results and discussion

Two different sizes of GSTs present in *Cryptosporidium* species. Genome data mining of 15 *Cryptosporidium* species revealed the presence of 3 GST genes in each of the species genomes (Table 2). The presence of more than one GST gene is common in eukaryotic species²³. Among 45 GSTs, 30 were found to have the characteristic GST motifs^{20,27}, such as the N-terminal domain, which houses the G site, and C terminal domain, which determines the substrate specificity (H-site) (Table 2 and Fig. S1). The remaining 15 GSTs have one of the characteristics GST motifs indicating either these sequences are diverse or fragmented or not properly annotated (Table 2). These GSTs were considered incomplete and were not included for further analysis unless indicated. Future genome editing and better gene prediction programs will help in getting the complete sequences for these GSTs and possibly predicting characteristic N- and C-terminal motifs. In total, 30 GSTs were taken for further analysis. Analysis of GST protein sizes revealed the presence of two different lengths of GSTs in *Cryptosporidium* species (Table 2). One type of GST protein is shorter in size with amino acids ranging between 157 and 268, and another type of GST protein is longer in size, with amino acids ranging between 373 and 466 (Table 2). GSTs from *Cryptosporidium* species seem to be the longest in amino acid length, as most of the GSTs reported in other organisms to date are 200–250 amino acids in length²³. Furthermore, it can be noted that the addition in length is found only on the outer N- and C-terminal regions, with the center of the protein containing the GST-superfamily domains (Table 2). In order to assess whether *Cryptosporidium* species GST proteins are indeed properly annotated gene products, we further analyzed the gene structure. Interestingly, all the longer GSTs had a single exon, thus no introns, but shorter GSTs were the products of 1–4 exons (Table 2). This could be indicative of shorter GSTs being prone to having multiple isoforms owing to gene shuffling. Due to presence of these multiple introns, the production of more diverse short GSTs can be expected compared to longer GSTs⁸⁰.

***Cryptosporidium* species GSTs are cytosolic in nature.** Most of the GSTs identified in organisms are cytosolic in nature, with the exception of GSTs belonging to the classes MAPEG and Kappa (mitochondrial) (Table S1). In order to identify the cellular localization, we subjected *Cryptosporidium* species GST protein sequences to the TMHMM Server v. 2.0 for the prediction of transmembrane helices in their structure⁶⁵ and the BUSCA server⁶⁴ for identifying possible localization in a cell. TMHMM prediction revealed that none of the *Cryptosporidium* species GSTs had transmembrane helices, indicating they were soluble and thus possibly cytosolic (Table S2). To authenticate our results, we also subjected 395 GSTs belonging to 17 different classes to TMHMM prediction (Table S3). The TMHMM predicted the presence of no transmembrane helices in previously designated cytosolic GSTs, whereas transmembrane helices were predicted for previously designated microsomal GSTs (Table S3). This indicated that the TMHMM results on the prediction of no transmembrane helices in *Cryptosporidium* species GSTs were in agreement with previous annotations. Furthermore, BUSCA indicated that all 30 *Cryptosporidium* species GSTs were cytosolic (Table S4). Based on these *in silico* results, we concluded that the 30 *Cryptosporidium* species GSTs were cytosolic in nature.

***Cryptosporidium* species GSTs belongs to new classes.** Phylogenetic analysis of *Cryptosporidium* species GSTs revealed that the 30 GSTs could be grouped into three different groups (Fig. 1). The shorter GSTs were grouped together (Group 1) and so were the longer GSTs (group 2). Interestingly, despite the short amino acid length, four GSTs diverged from these two groups (Group 3) (Fig. 1). Analysis of the amino acid percentage identity among *Cryptosporidium* species GSTs further confirmed that they indeed belonged to three different groups. Group 1 GSTs shared an amino acid percentage identity of 54–100%, whereas groups 2 and 3 shared identities of 48–100% and 42–71%, respectively. Group 3 GSTs had 13–21% identity with Group 2 GSTs and 14–22% identity to Group 1 GSTs. The percentage identity between Groups 1 and 2 was 17–25%. This indicates that all three groups of *Cryptosporidium* species GSTs indeed belonged to three different classes as the percentage identity between these groups was below 25–30%, qualifying them to be their own class^{20,23,26–28}.

Although the above results clearly indicated that *Cryptosporidium* species GSTs belong to three different groups, it was still not clear whether they fell under one of the GST classes described in the literature (Table S1). Thus, the comprehensive phylogenetic analysis of proteins belonging to 17 known GST classes and *Cryptosporidium* species GSTs was carried out (Fig. 2). Phylogenetic analysis revealed that *Cryptosporidium* species GSTs did not align with any of the 17 pre-existing GST classes and formed three new groups (Fig. 2). This clearly

Species	Total number of GSTs	GST number	Protein ID	Protein size (no of amino acids)	Characteristic GST motifs location		Gene structure (no. of exons)
					N terminal	C terminal	
<i>Cryptosporidium andersoni</i> isolate 30847	3	GST1	cand_012830 ^a	197	12–97	95–195	3 exons
		GST2	cand_023790 ^a	466	67–149	166–319	1 exon
		GST3	OII73498.1 ^b	260	–	124–235	1 exon
<i>Cryptosporidium hominis</i> isolate TU502_2012	3	GST1	ChTU502y2012_407g2365 ^a	186	1–62	64–186	2 exons
		GST2	ChTU502y2012_421g0615 ^a	428	69–151	146–315	1 exon
		GST3	ChTU502y2012_303g0055/OLQ15919.1 ^a	268	–	153–236	1 exon
<i>Cryptosporidium hominis</i> isolate 30976	3	GST1	GY17_00002363 ^a	186	1–62	60–183	2 exons
		GST2	GY17_00000733 ^a	428	69–151	146–315	1 exon
		GST3	PPS94453.1 ^b	268	–	152–236	1 exon
<i>Cryptosporidium hominis</i> TU502	3	GST1	XP_667744.1 ^b	161	1–62	64–161	1 exon
		GST2	Chro.80347 ^a	428	69–151	146–315	1 exon
		GST3	XP_666781.1 ^b	268	–	154–236	1 exon
<i>Cryptosporidium hominis</i> UdeA01	3	GST1	CUV07467.1 ^b	161	1–62	64–161	1 exon
		GST2	CHUDEA8_2970 ^a	428	69–151	146–315	1 exon
		GST3	CUV04748.1 ^b	268	–	154–236	1 exon
<i>Cryptosporidium meleagridis</i> strain UKMEL1	3	GST1	CmeUKMEL1_03350 ^a	193	9–94	96–193	3 exons
		GST2	CmeUKMEL1_14570 ^a	428	69–151	146–315	1 exon
		GST3	CmeUKMEL1_05845 ^a	268	31–118	101–243	1 exon
<i>Cryptosporidium parvum</i> Iowa II	3	GST1	cgd7_4780 ^a	186	1–62	60–183	2 exons
		GST2	cgd8_2970 ^a	429	69–151	146–315	1 exon
		GST3	cgd2_3730 ^a	268	–	156–236	1 exon
<i>Cryptosporidium tyzzeri</i> isolate UGA55	3	GST1	CTYZ_00001095 ^a	186	1–62	60–186	2 exons
		GST2	CTYZ_00000322 ^a	429	69–151	146–315	1 exon
		GST3	TRY52903.1 ^b	268	–	153–236	1 exon
<i>Cryptosporidium ubiquitum</i> isolate 39726	3	GST1	cubi_03151 ^a	213	1–89	91–213	4 exons
		GST2	cubi_03523 ^a	428	69–151	146–315	1 exon
		GST3	XP_028873506.1 ^b	266	–	159–235	1 exon
<i>Cryptosporidium muris</i> RN66	3	GST1	XP_002141168.1 ^b	160	1–60	58–158	2 exons
		GST2	XP_002140043.1 ^b	466	–	211–312	1 exon
		GST3	XP_002142877.1 ^b	260	–	164–233	1 exon
<i>Cryptosporidium baileyi</i> strain TAMU–09Q1	3	GST1	JIBL01000090.1 ^b	156	1–57	59–156	1 exon
		GST2	JIBL01000106.1 ^b	390	36–118	113–275	1 exon
		GST3	JIBL01000138.1 ^b	236	1–87	69–223	1 exon
<i>Cryptosporidium viatorum</i> isolate UKVIA1	3	GST1	QZWW01000010.1 ^b	161	1–62	64–161	1 exon
		GST2	QZWW01000018.1 ^b	428	69–151	146–315	1 exon
		GST3	QZWW01000026.1 ^b	249	–	134–217	1 exon
<i>Cryptosporidium</i> sp. chipmunk LX–2015	3	GST1	JXRN01000042.1 ^b	205	1–106	108–205	1 exon
		GST2	JXRN01000009.1 ^b	425	69–151	–	1 exon
		GST3	JXRN01000023.1 ^b	250	–	135–217	1 exon
<i>Cryptosporidium ryanae</i> isolate 45,019	3	GST1	VHLK01000064.1 ^b	166	–	37–154	1 exon
		GST2	VHLK01000046.1 ^b	373	36–118	113–274	1 exon
		GST3	VHLK01000056.1 ^b	230	1–85	89–221	1 exon
<i>Cryptosporidium bovis</i> isolate 42,482	3	GST1	VHIT01000033.1 ^b	147	–	30–142	1 exon
		GST2	VHIT01000012.1 ^b	376	21–103	98–264	1 exon
		GST3	VHIT01000028.1 ^b	227	1–85	98–221	1 exon

Table 2. Glutathione transferase (GST) analysis in *Cryptosporidium* species. The GST number in column 2 is an indication of the number of GSTs that a specific species possesses. Whilst the number on column 3 indicates the group the protein belongs to (based on the percentage identity)^{20,23,26–28}. ^aProtein ID from CryptoDatabase. ^bProtein ID from NCBI database. –, characteristic GST domain not identified.

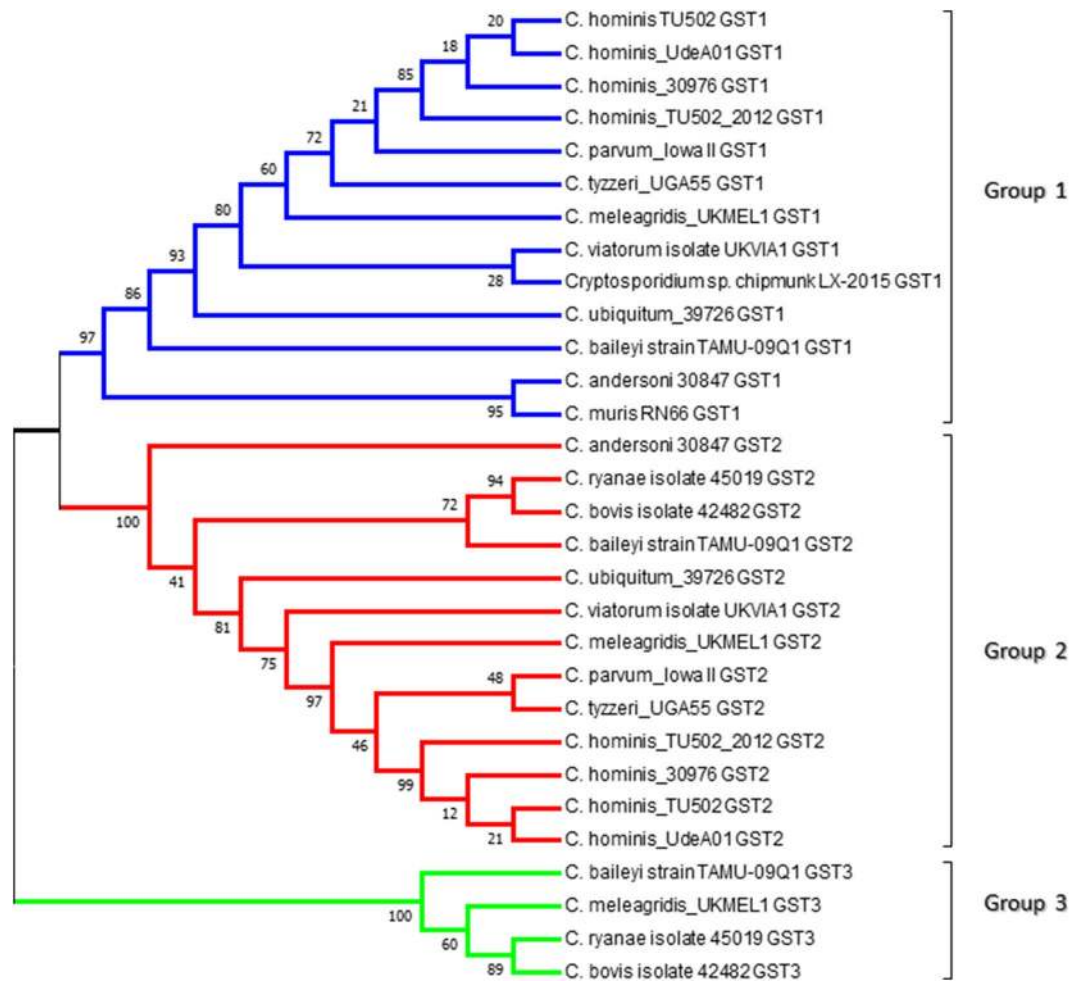


Figure 1. Phylogenetic analysis of glutathione transferase (GST) proteins from *Cryptosporidium* species. The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model⁶³. Evolutionary analyses were conducted in MEGA7⁶². The percentage of trees (bootstrap value) in which the associated taxa clustered together is shown next to the branches.

indicates that *Cryptosporidium* species GSTs belong to three different new GST classes. Thus, we named groups 1, 2 and 3 of *Cryptosporidium* GSTs Vega (δ), Gamma (γ) and Psi (ψ), respectively. A point to be noted is that all the GST proteins aligned together as per their GST class on the phylogenetic tree, indicating our phylogenetic analysis is correct and thus we conclude that *Cryptosporidium* species GSTs indeed belong to new GST classes.

***Cryptosporidium parvum* GST1 of Vega class has atypical thioredoxin-like fold.** Identification of three new GST classes in *Cryptosporidium* species in this study necessitated examination of the structural aspects of these new classes to see if any deviations or novel folds might be present, compared to the canonical structure of GSTs^{20,27}. Analysis of the primary structure revealed that all *Cryptosporidium* species GSTs have N- and C-terminal regions characteristic of GSTs that usually contain a G-site and H-site^{20,27}, respectively (Table 2 and Fig. S1). All GSTs have the highly conserved proline amino acid residue (Fig. S1) that is part of the *cis*-Pro loop responsible for connecting the N- and C-terminal regions in order to maintain the GST structural integrity⁸¹. It was observed from Fig. S1 that Psi class GSTs have a Tyr residue in the N-terminal domain in close proximity to the expected active site Tyr. The same was observed with the Vega class GSTs with the expectation of *C. muris* and *C. baileyi*. Vega and Psi GSTs have a few tyrosine residues in the N-terminal region, but they are not at a position that is considered part of an active site^{20,27} (Fig. S1). Similarly, the majority of the Gamma class GSTs consist of an active site Tyr residue with the exception *C. andersoni*, *C. baileyi*, *C. ryanae* and *C. bovis* species. In these species, Phe replaces the active site Tyr residue. Mutagenesis studies have shown that the presence of Phe at the supposed position of the active site Tyr significantly reduces the catalytic activity. This highlights the critical role played by the active site Tyr in the catalytic activity of GST^{82,83}. The effect of these mutations in the context of *Cryptosporidium* GSTs is yet to be studied.

Multiple sequence alignments of Vega and Gamma GSTs revealed that amino acids in the N- and C-terminal regions of these GSTs are highly conserved (Fig. S1). For this reason, we selected *C. parvum* GSTs 1 and 2

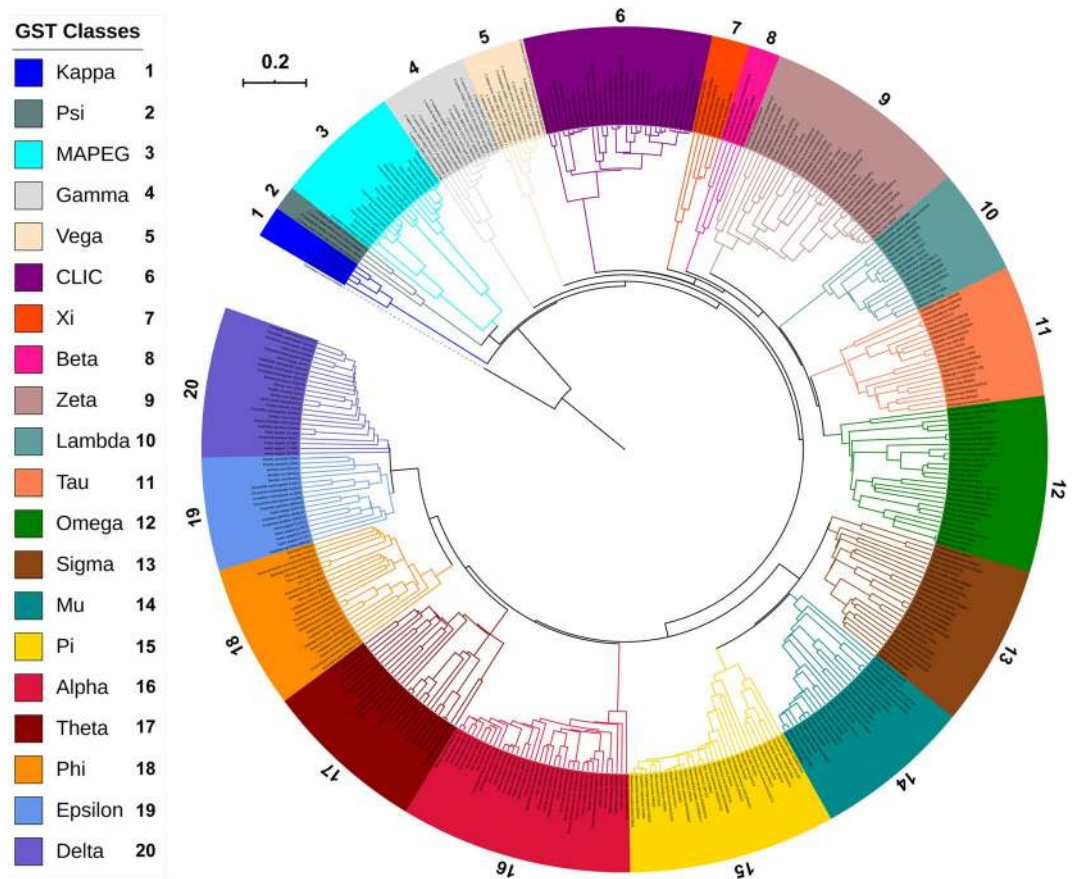


Figure 2. Phylogenetic tree of the glutathione transferases (GSTs) protein sequences of *Cryptosporidium* species with GSTs from 17 different GST classes. Thioredoxin from *Oryctolagus cuniculus* (protein ID: P08628) is used as an outgroup. Three new GST classes reported in this study from *Cryptosporidium* species named Vega, Gamma and Psi are also shown in the tree. A high-resolution phylogenetic tree is provided in Supplementary Dataset 2.

(CpGST1 and CpGST2) as representative of the Vega and Gamma GST classes for structural analysis along with *C. meleagridis* UKMEL1 GST3 (CmGST3) for the Psi class. Structural analysis of the three GSTs was carried out using in silico homology modeling. The structural analysis was aimed at assessing only the secondary structural elements that are characteristic of GST proteins^{20,27}. These GST models are not aimed to assess the binding affinities or the residues involved in binding to different ligands. In order to build 3D models we performed a template search at three different webpages, namely NCBI⁶⁷, PHYRE⁶⁹ and I-TASSER⁶⁸. The templates found were of low sequence identity but had relatively good coverage (Table S5). This was expected, since these GSTs are new. We then proceeded to build 3D models using a multiple template method, as this approach is known to improve the quality of homology models⁸⁴. We built 3D models for all three GSTs, attempting single and multiple templates, while also using different combinations of the available templates listed in Table S5. The best 3D models with good quality closest to the templates were chosen for the structural analysis.

Here, we present the combination of templates that gave CpGST1, CpGST2 and CmGST3 models. The templates used to model CpGST1 were a *Bombyx mori* Sigma class GST (3VPQ-A)⁸⁵ that had 94% coverage and 26% identity and a *Penaeus vannamei* Mu class (5AN1-A)⁸⁶ with 98% coverage and 23% identity (Fig. 3 and Table S5). For CpGST2 the templates were both from *Homo sapiens* Alpha class (1K3Y-B)⁸⁷ and Pi class (19GS-A)⁸⁸, with sequence identity at 21%, coverage at 94% and 22% identity and 84% coverage (Fig. 4 Table S5), respectively. The CmGST3 templates used were from *Caenorhabditis elegans* Pi class GST (1ZL9-A) (<https://www.rcsb.org/structure/1ZL9>) with 94% coverage and 21% identity and a *Homo sapiens* Alpha class (1K3Y-B)⁸⁷ with 98% coverage and 22% identity (Fig. 5 and Table S5).

For each GST, 20 models were built using the MODELLER v9.21 program⁷¹. The best model evaluated by DOPE score was selected and subjected to structural quality analysis. The selected model for each GST was then refined on the GalaxyWeb Refiner server⁷⁴ and further subjected to structural quality evaluation using different programs such as ERRAT⁷⁵, Verify3D⁷⁶, PROCHECK^{77,78}, RAMPAGE⁷⁹ and ProsaII⁷³. The overall quality of the models was assessed by the combination of these programs' values and by comparing these with the templates' structural evaluation scores (Tables S6 and S7). The models generated for CpGST1 and CpGST2 were found to be of good quality, as different structural validation programs indicated that the quality of the model structures was close to the quality of the template structures (Tables S6 and S7). The model generated for CmGST3 had

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Author contributions

K.S., I.A. and T.K. designed and conceptualized the study. K.S. and T.K. provided funding for the study. All authors are involved in generation, analysis and interpretation of data. All authors reviewed and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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