Review Article

Bioinformatics Applications on *Cryptosporidium* Research: A Review

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Abstract

The biology of *Cryptosporidium* has been studied increasingly since it was recognized as a pathogen of humans more than a century. Its recent recognition as a second leading cause of diarrhoea or cryptosporidiosis immunocompromised patients globally has led many researchers to study on this parasite. Many new technologies such as high-throughput omics and bioinformatics tools have been implemented to investigate this zoonotic parasite in a better approach. The aim of this review article is mainly to briefly describe recent applications of structural bioinformatics in order to reveal the potentiality of a suitable therapeutic target in *Cryptosporidium*. This review was written based on the search of cited publications in SCOPUS website with the combination of word '*Cryptosporidium*' with other words like bioinformatics, protein structure, structural biology and homology modeling. The search results then were selected based on the relativeness of updated information needed to be prepared in this review. Several cited publications were used to elaborate the review accordingly despitelimitedreviewupdatesrelatedtoprotein bioinformation of thisparasite. As a conclusion, bioinformatics is a commonly known to be cutting-edge technology that has been recognised for its power to reveal the secret of parasite biology *in silico* including a neglected parasite, *Cryptosporidium*.

Keywords: bioinformatics, cryptosporidiosis, *Cryptosporidium*, *in silico*, parasite.

Bangladesh Journal of Medical Science Vol. 21 No. 01 January'22 Page : 8-18
DOI: https://doi.org/10.3329/bjms.v21i1.56322

Introduction

Cryptosporidium is an enteric parasite that infects across varied hosts including humans and animals which also has been regarded to cause diarrhoea or cryptosporidiosis[1]. Two out of 27 species of *Cryptosporidium* are commonly known to be major causes for human cryptosporidiosis, which are *Cryptosporidium parvum* and *Cryptosporidium hominis*, thereby leading to discover effective ways of combating this human enteric parasite[2]. A recent molecular phylogenetic study has identified that *Cryptosporidium* is more closely related to gregarine parasites rather than coccidian parasites as primarily classified in the last two decades[3]. With respect to this new discovery in terms of its phylogenetic classification, it leads into a new investigation of various aspects on *Cryptosporidium*, including the use of high-throughput bioinformatics and omics technologies for expanding clear cut understanding of this enteric parasite more deeply[4]. To date, there is still a paucity on producing an effective drug for anti-*Cryptosporidium* agent and as the most concern so far, the only US-FDA approved drug is nitazoxanide (NTZ) with moderate efficacy in immunocompetent people rather than the immunocompromised patients[5].

However, genome sequencing shed light to gain insight of advanced research on *Cryptosporidium* in terms of its molecular biology. To date, only three species of *Cryptosporidium* have been fully sequenced of their own genomes including *Cryptosporidium parvum*, *Cryptosporidium hominis* and *Cryptosporidium muris*[6,7]. Particularly, both *Cryptosporidium parvum* and *Cryptosporidium hominis* shared almost similar percentage of sequence identity, million base pairs in size, a similar amount of protein encoding genes, but *Cryptosporidium parvum* has a slight difference in terms of fully assembled genomes with almost 25 percent of its entire assembled genome has been annotated as non-coding genes[7]. In contrast, *Cryptosporidium hominis* contains genome with some gaps and not fully assembled[8]. Nevertheless, most

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concern in this review article is the fact that lacking characteristics in this genus, *Cryptosporidium* is eventually leading to the bioinformatics approach as an alternative method of *Cryptosporidium* research[9]. In fact, several characteristics possessed by this genus such as degenerate mitosome without having a mitochondrial genome, less cytochrome-based respiratory chain and lacking *de novo* biosynthetic genes encoding for important biomolecules primarily required by *Cryptosporidium* along with absent mechanism of splicing, especially in post transcriptional process of RNA molecule[7]. Besides both species of this genus, *Cryptosporidium muris* has been sequenced with different characteristics with different characteristics such as the sets of gene report markers for specific function like encoding for the tricarboxylic acid, (TCA) cycle, oxidative phosphorylation and special enzyme involved as generating adenosine triphosphate (ATP) molecule, namely ATP synthase[10]. Unlike both species stated earlier, *Cryptosporidium muris* has mitochondrial structure and related proteins due to both species previously lost their mitochondrial structure after getting diverged from this *Cryptosporidium muris*[11-13]. Consequently, with all these poor characteristics, this parasite is generally scavenging heavily any nutrients from the susceptible host due to loss of important genes which should be presented for generating vital metabolic pathways as predominantly happened in other eukaryotic organisms[14]. There is no recent publication to develop more stable transfection system by which can promote the identification of novel proteins localised in this parasite even though practically. It is difficult due to a complex life cycle of this parasite and more importantly, lacking endogenous promoters that can function effectively for expressing novel proteins[15].

Despite having these difficulties of a complex life cycle and lacking endogenous promoter genes pertaining to this parasite, a tremendous progress in the discovery of anti-*Cryptosporidium* drugs have been attempted until now[16]. However, this progress has been recognised slower compared to other applied research focusing on this parasite. As a result, any effective novel targets are needed for targeting specifically targeted proteins prior to drug development[17]. More recently, drugs such as human 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor has been brought into *in vitro* testing for *Cryptosporidium* treatment and perhaps, can be a potential candidate for *in vivo* *Cryptosporidium* treatment especially in human in future[18]. With respect to anti-*Cryptosporidium* drug discovery, a metabolomics research has been potentially recognised as a powerful tool in order to unravel any novel metabolites and targets prior to production of specific biomarker in diagnostic application even though it is still infancy especially in *Cryptosporidium* research regardless of other eukaryotic organisms that have been previously studied extensively in terms of metabolomics approach[19,20]. Recently, almost 213 enzymes have been identified to be directly related to 540 reactions comprising of 514 intracellular metabolites involved in metabolic biochemical reactions along with transport reactions whereby having 26 metabolites moving across the cell membrane of *Cryptosporidium hominis* as previously studied using *in silico* genome-scale metabolic model[21,22].

Thus, in this review article, *in silico* approaches like bioinformatics tools have been regarded to gain insight on the development of anti-parasitic drugs. Interestingly, upon the establishment of *Cryptosporidium* genomes databases, it is worthwhile to initiate the drug development on specific protein targets by which involve high throughput screening of molecular structures libraries, thereby leading to the discovery of specific drug target candidates based on the determination of three-dimensional structure of the protein target either using X-ray crystallography or nuclear magnetic resonance (NMR)[23]. However, in this review, more concern was given on the protein bioinformatics tools, particularly homology modelling and eventually leading to enhance via molecular docking simulations prior to determination of active site of the targeted molecular protein structure[24,25]. Despite having other methodologies such as *in vitro* and *in vivo* test that could be employed for identification of drug target molecule, *in silico* bioinformatics tools also can be used because of promising preliminary information, starting from protein target identification, then selection of specific example of protein structure retrieved from protein structure databases before submitting to docking simulations[26]. Further, ligand binding affinity to the specific active site of target protein molecule is subsequently evaluated and eventually smaller potential hits of binding affinity will be selected as the best target candidate[27].

Protein Target Selection

It has been reported that 90 structures have been

deposited in the Protein Data Bank (PDB) belong to *Cryptosporidium* species, comprising of 76 protein structures from *Cryptosporidium parvum*, 10 protein structures related to *Cryptosporidium hominis* and 4 protein structures belong to *Cryptosporidium muris*[28-30]. In the case of anti-*Cryptosporidium* drugs, there are several structures of protein targets have been resolved using X-Ray diffraction method[31,27,32]. These protein targets are generally derived from several types of enzymes classification, namely dihydrofolate reductase, lactate dehydrogenase, adjacent gene encoding predicted malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, inosine-5'-monophosphate dehydrogenase, bifunctional dihydrofolate reductase-thymidylate synthase and *S*-adenosylmethionine synthetase[33-39]. So far, the X-Ray resolutions for all protein structures related to both species of *Cryptosporidium* have been resolved mostly between 2.0-2.5 Å (angstrom)[28]. Very few were more than 3.0 angstrom that have been studied previously in three different research groups[40-43]. Out of 77 deposited protein structures of *Cryptosporidium* retrieved from the Protein Data Bank (PDB), only 16 protein structures had X-Ray resolution value less than 2.0 angstrom[44-48].

Ligand Database Selection

To date , only 76 ligands have been deposited along with specific protein structures belong to both species of *Cryptosporidium*, namely *Cryptosporidium parvum* and *Cryptosporidium hominis* (Table 1). Ligand or simply known as small molecule has been essentially deposited in one of the most commonly used databases, which is PubChem whereby can be a public repository of molecular structure information, particularly in chemical, biological and structural features, thereby can be applied as diagnostic agents[49,32]. This database has been primarily sustained by the National Center for Biotechnological Information (NCBI) and widely used by a scientific community to retrieve available molecular ligand data by which can potentially be used for optimisation of structural profiles of target proteins of interest belong to *Cryptosporidium* via *in silico* approach[50]. PubChem is regarded as one of the virtual screening initiatives that has been linked to other databases such as Entrez comprising of PubMed and PubMed Central which serve as a central repository of ligand libraries[51]. Besides PubChem, another commonly used database which is ZINC has been employed and assigned biologically along with annotation purpose. All molecular ligand molecules can be downloaded in the form of SDF and mol2[52]. In addition, a subset of ligand molecules retrieved from ZINC database can be subsequently used for virtual screening initiatives, particularly on anti-*Cryptosporidium* drug prior to being used later in molecular docking simulations[53].

Molecular Docking Simulation

A new generation of anti-*Cryptosporidium* drugs could be developed that probably influenced by the bioinformatics tools application[54,32]. As stated earlier, with respect to this application of bioinformatics tools, a starting point is regarded as identification of protein target and eventually leading to simulate the newly developed protein structure of a particular specific protein on *Cryptosporidium* using molecular docking simulations (Figure 1)[55,56]. More importantly, the result of molecular docking after processing the protein structure can be a key point for virtual screening on particular protein target of *Cryptosporidium*, mainly for commonly human pathogenic species, *C. parvum* and *C. hominis*. Even though this molecular docking simulation is primarily time-consuming due to high demand on CPU usage, the good side is in return with considerably higher accurate results with the use of specific molecular docking software after a procedure of rigid body and flexible body, along with further analyses of scoring functions[57].

Here, a commonly used method is to combine different scoring functions for improvement of accuracy based on the output of molecular docking result[58-60]. To date, there are several molecular docking programs that have been applied such as DOCK and AUTODOCK[61]. Even though the programs are diverse, the similarity of those related programs is to search for the best fit between two or more ligand molecules to a receptor of protein targetinput coordinates[62]. In addition, all those programs are capable of giving experimental data on docking algorithms whereby the conformations either protein to protein or even protein to ligand is primarily obtained from *in silico* approach. Prior to a comparison of protein structure, the resolved protein structures deposited in the Protein Data Bank (PDB) normally will be used whereby these deposited structures are origin of X-ray crystallography or nuclear magnetic resonance (NMR)-based applications[63].

Molecular docking is somehow used as an alternative to testing different ligands on the same protein target of *Cryptosporidium* if the experimental methodology

is not really applicable in terms of getting an accurate result within a short period of time[64]. However, molecular docking is practically applicable to be established as one of the vital methods in order for virtual screening on anti-*Cryptosporidium* properties towards potential protein target on this neglected parasite, *Cryptosporidium*[65,66]. Moreover, binding affinity evaluation is a must to be coupled with molecular docking for getting an accurate assessment on a virtual screening of the particular protein target. Interestingly, it can possibly be promising in the development of more suitable anti-*Cryptosporidium* drugs in future[67-72].

An Update on Structural Bioinformatics of Cryptosporidium Research

Cryptosporidium is primarily categorised in the protozoan phylum Apicomplexa which is responsible for cryptosporidiosis, which has remarkably elevated rates of infection in relatively low economic nations of the world[73]. The advancement of vaccine and drug development has been improved by the production of purified *Cryptosporidium* recombinant proteins in order to combat cryptosporidiosis[4].

So far, there is no vaccine or wholly effective drug treatment available for combating *Cryptosporidium* infection despite having an existing treatment such as nitazoxanide and paromomycin that are said to be effective based on clinical data, particularly for those of immunocompromised patients[74]. In other words, the need for new drug-lead discovery is a must and can be considered to be urgently highlighted in *Cryptosporidium* research[75]. Until now, limited successful outcome has been reported for expressing heterologously of *Cryptosporidium* proteins and also its crystallization approach[70]. In an attempt to discover for the effectiveness of crystallised proteins based on a standardized genome-scale methodology, a large number of targeted *Cryptosporidium* genes were selected for the distinct cellular classes, together with chosen orthologues from two *Cryptosporidium* species which are *C. parvum* and *C. hominis*[5].

All target proteins that have been studied extensively for years especially on *Cryptosporidium* research are enzymes that have different properties with the specific anti-*Cryptosporidium* designed-drug ligand[70]. One of the studied targeted proteins is triosephosphate isomerase by which predominantly exists in all organisms that perform glycolysis[48]. Triosephosphate isomerase is commonly known to be involved in the formation of glyceraldehyde 3-phosphate from dihydroxyacetone phosphate, thereby ensuring the maximum production of ATP molecule per glucose molecule[48]. On the other hand, one of the enzymes primarily involved in the glycolytic pathway, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) also has been studied in *Cryptosporidium* by which demonstrate the plasticity of the substrate binding site from the ternary complex of *C. parvum* GAPDH with other GAPDH complexes as well[36]. In addition, the active site of GAPDH can accommodate the substrate in multiple conformations at multiple locations during the initial encounter[36].

Besides triosephosphate isomerase and GAPDH enzymes, both *Cryptosporidium parvum* and *Cryptosporidium hominis* generally contain a group of calcium-dependent protein kinases (CDPKs), which have pivotal roles in the calcium-signaling pathway. This key enzyme of that pathway essentially can be found in plants, ciliates and apicomplexan parasites like *Cryptosporidium* but not in humans or fungi[46]. Furthermore, this enzyme is regarded to have a capability on blocking of an early stage of *C. parvum* invasion of HCT-8 host cells and essentially comprising of a calmodulin-dependent kinase (CaMK)-like kinase domain regulated by a calcium-binding domain in the C terminus[46]. On top of that, *C. parvum* has been reported to have inosine 5′-monophosphate dehydrogenase (IMPDH) by which involves in a streamlined pathway for synthesizing guanine nucleotides from host adenosine[76]. This enzyme is recognised as a promising target that has remarkable potential for treating *Cryptosporidium* infections[77]. Essentially, *Cryptosporidium* relies on this enzyme in order to obtain guanine nucleotides prior to its dependency on glycolytic pathway for energy production[76]. In contrast, the proliferation process of *Cryptosporidium* will be shut down if this enzyme is inhibited[76]. The previous study has been published about this enzyme structure for optimising the selectivity of several inhibitors that can fit effectively to it[78]. More importantly, effective inhibition process will successfully turn on for suppressing the function of this enzyme, thereby might be a potential target for producing anti-*Cryptosporidium* drugs in future[77].

Lactase dehydrogenase is also an enzyme that has gained attention to unravel the anti-*Cryptosporidium* target for this parasite due to the fact this enzyme is known to be a major regulator of glycolytic reaction[79]. Interestingly, there was a study of this

enzyme that subjected to characterize with high resolution for crystallization that ends up with the apo-enzyme and four ternary complexes[79]. More importantly, *C. parvum* lactate dehydrogenase is considerably different from those in the human counterpart in terms of the active site loop and the antigenic loop[79]. However, structural characteristics and enzymatic properties of this enzyme from *C. parvum* are essentially similar to its counterpart from other related parasites[79]. With respect to its common ancestry, this enzyme has a closely related genetic relatedness with malate dehydrogenase from the structural comparison between both enzymes[79].

Cryptosporidium also has been targeted with different protein targets such as Thymidylate synthase (TS) and dihydrofolate reductase (DHFR) which both have bifunctional enzymatic properties[38,80]. Both enzymes are necessary for biosynthesizing folate pathway and particularly, both have been regarded as well-established function in drug targets on cancer, bacterial infection and malaria[38]. Based on this possibility of having anti-*Cryptosporidium* target, the structural basis of DHFR can be extensively studied for antifolate resistance[80]. For instance, antifolate candidate that can be designed as thymidylate synthase inhibitor to *C. hominis* is 2-amino-4-oxo-5-substituted pyrrolo[2,3-d]pyrimidines[43]. More importantly, if this enzyme from *Cryptosporidium* has been compared with its counterpart from human type enzyme, it reveals the difference in terms of active site that can possibly be used prior to selectively-species inhibitor design[44]. It has been stated that *C. hominis* for the combination between TS and DHFR could exhibit catalytic functionality at a high rate at least greater 10-fold difference[81]. Both enzymes can unusually initiate high rate of catalysis due to two non-conserved residues that facilitate this enzyme at the folate tail-binding region[41]. With respect to the development of potential anti-*Cryptosporidium* target to this enzyme, both non-conserved TS and DHFR residues can be mutated that eventually give negative regulation prior to multiple steps in its catalytic cycle[41]. Thus, TS-DHFR enzyme belongs to *C. hominis* could be a potential target for the design of anti-*Cryptosporidium* agent for those affected people with weak immunity like AIDS-related immunocompromised patients[82]. Interestingly, TS-DHFR enzyme from C. *hominis* has unique feature of a homodimer which its monomer has a crossover helix that interact with adjacent cleft on the other active site within this enzyme[41,82]. In addition, homodimer state of this enzyme is primarily absent in its counterpart form that available in human-type enzyme[83]. Thus, it might have gained insight into the development of selective allosteric inhibitors that are specific to this enzyme in order to affect its stability and catalytic activity via site-directed mutagenesis. Additionally, the approach of site-directed mutagenesis can be applied on crossover α- helix domain or other mutational analysis to this enzyme based on the principle of helical protein interactions[41].

One of the breakthrough findings on the discovery of potential anti-*Cryptosporidium* target is via kinomic research that involved in the elucidation of a set of kinome derived from *Cryptosporidium* genome[49,84]. The kinome analysis generally related to structural and biochemical studies of CDPK and MAP kinase families. It is important prior to design of promising drug target on the same studied enzyme of different parasites[85]. Based on its essential role, MAP kinase is pyruvate kinase which plays a vital role in glucose metabolism that generally acts as a key regulator of glycolytic reaction[49]. However, *Cryptosporidium* is different from other parasites especially for this enzyme that essentially showed no allosteric property when the effector molecules like phospho sugar present. In addition, MAP kinase is allosterically regulated by other effector molecules due to its structural features that have tetrameric nature, thereby leading to structural changes that happened in other pyruvate kinases in different organisms[84]. Fundamentally, this distinct feature is predominantly present in Cryptosporidium MAP kinase which is its active site is open, unlike other structures of different parasites that normally show partially closed active site or fully closed state[85]. The other pyruvate kinase also show an active site containing sulphate ion that primarily occupied by a phosphate molecule of effector molecules such as phospho sugar[84], while the MAP kinase derived from *Cryptosporidium* is different due to having disulfide bonds linking to two monomeric cysteine residues from short and long helix[49]. Thus, both residues are potentially unique and can be a possible target for anti-*Cryptosporidium* drugs. However, the disulphide bond intact within MAP kinase enzyme still remained unclear[85].

Another possible target protein that can be manipulated to design for anti-*Cryptosporidium* drug is an enzyme, namely as tryptophanyl-tRNA synthetase (TrpRS)[86]. Essentially, this enzyme is regarded to be present in the genome of *Cryptosporidium* parvum contains a

single gene encoding for TrpRS protein that eventually turns into functional form after post-translational modification processes[86]. In particular, this gene encodes for an N-terminal domain of conserved core domain for this translated protein that originally derived from this TrpRS gene available in *Cryptosporidium parvum* genome[86]. From the perspective of sequence analysis, TrpRS enzyme has an extra domain which is recognizably conserved among several apicomplexan parasites including *Cryptosporidium* species[86]. However, TrpRS enzyme of *Cryptosporidium parvum* can remain to charge tRNA actively even though the extra domain will be shortened[86]. While In some other eukaryotes, the core function of TrpRS enzyme is different in modification activities either through the addition of extra domain or variant isoform expression[86]. Generally, this enzyme is necessary for activating and attaching specific amino acid like tryptophan towards a cognate tRNA molecule prior to being used later in the process of protein synthesis[86].

Conclusion

Cryptosporidium genome projects are generally a rich source of biological information pertaining to *Cryptosporidium* that could be used primarily in identification of potential protein targets by which can be beneficial for promising anti-*Cryptosporidium* drugs. The genomics advancement has significantly gained our understanding of *Cryptosporidium* biology. The main focus on *Cryptosporidium* has been continually recognised to reveal potential new drug targets due to its limited availability of suitable anti-*Cryptosporidium* drugs from past to present. Bioinformatics tools can be used to elucidate three-dimensional structure after identification of protein target prior to subsequent steps such as binding pocket identification, ligand molecule selection and eventually virtual screening on molecular docking. Prior to this molecular docking, further evaluation is very crucial to be assessed for determination of the best ligand binding affinity towards a given protein binding site of the studied protein structure of *Cryptosporidium*. Once the best docking results selected, the desired one can show best fit between ligand molecules that can potentially become target protein of interest in order to design as a potential drug target for this parasite. However, based on the recent studies of structural bioinformatics research regarding this parasite, most experimental work has employed the application of X-ray crystallography or diffraction for determining the accurate three-dimensional structure of all studied enzymes so far. Interestingly, some of those enzymes are regarded to be potentially protein target of interest for the anti-*Cryptosporidium* drug in future. One of the main goals of this review has been to provide insights on the direction that this new currently growing field continually has contributed to the elucidation of potential anti-*Cryptosporidium* targets.

Acknowledgment:

This research was supported by University of Karabük (UNIKA), Karabük Üniversitesi Demir Çelik Kampüsü, Karabük, Turkey.

Conflict of Interest

We declare that we have no conflict of interest.

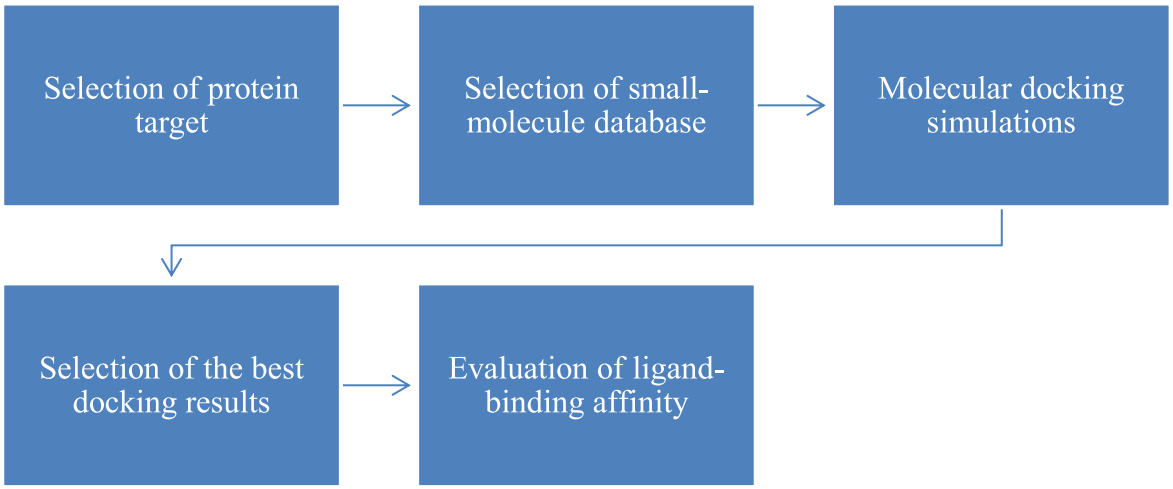

Figure 1: *In-silico* drug design steps of anti-cryptosporidial drugs using bioinformatics tools. After the assembly of full-length sequences selected from expressed sequences tag library, that particular sequence was subjected to be a protein target prior to its identification using the contigs and singletons. Protein sequence was then searched in BLASTp program against the Protein Data Bank database (PDB) that related to crystal structures of homologous proteins to the protein target of query protein sequences. This homologous reference protein structures that had been crystallised can be used as template for protein homology modeling. This model of protein structure can be employed for virtual high-throughput experiments like molecular docking using established docking software. For docking experiments, small molecule like a ligand structure has to be converted into its three-dimensional form before proceed to assessment of model quality based on the suitability of ligand and catalytic site of protein structure (enzymes) belong to this parasite, *Cryptosporidium* in binding affinity.

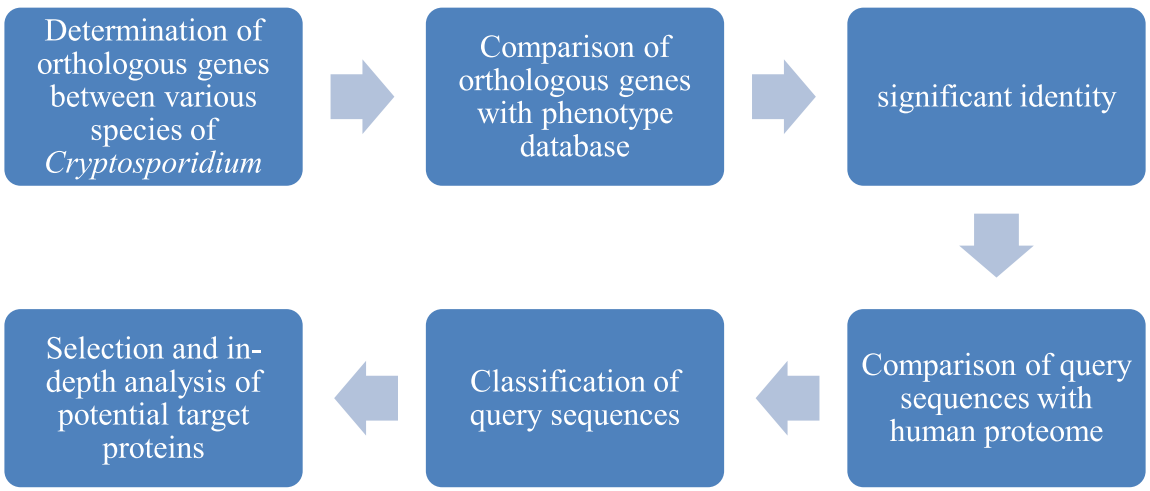

Fig. 2. Orthologous proteins of various species from *Cryptosporidium* could be determined for exhibiting a potential candidate of protein targets that might happen in vivo. The orthologous genes were then compared with a phenotype database for checking if the protein target is vital for main function of this parasite even it is commonly known for its streamline metabolic pathway. Next, human proteome dataset was subjected to be compared with query sequences of orthologous proteins, particularly potential protein target. Hence, conservational degree of the protein targets was elucidated and unravelled with proteins of host organisms prior to detail characterization of selected protein targets. Later, the characterised target of potential protein was subjected for further in-depth analysis especially in a bioinformatics studies.

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