RESEARCH ARTICLE

Genome-wide characterization of folate transporter proteins of eukaryotic pathogens [version 1; peer review: 2 approved with reservations]

Mofolusho Falade ¹, Benson Otarigbo ^{1,2}

¹Cellular Parasitology Programme, Department of Zoology, University of Ibadan, Ibadan, Nigeria

²Department of Biological Science, Edo University, Iyamho, Nigeria

V1 First published: 12 Jan 2017, 6:36
<https://doi.org/10.12688/f1000research.10561.1>

Latest published: 13 Jul 2017, 6:36
<https://doi.org/10.12688/f1000research.10561.2>

Abstract

Background: Medically important pathogens are responsible for the death of millions every year. For many of these pathogens, there are limited options for therapy and resistance to commonly used drugs is fast emerging. The availability of genome sequences of many eukaryotic protozoa is providing important data for understanding parasite biology and identifying new drug and vaccine targets. The folate synthesis and salvage pathway are important for eukaryote pathogen survival and organismal biology and may present new targets for drug discovery.

Methods: We applied a combination of bioinformatics methods to examine the genomes of pathogens in the EupathDB for genes encoding homologues of proteins that mediate folate salvage in a bid to identify and assign putative functions. We also performed phylogenetic comparisons of identified proteins. .

Results: We identified 234 proteins to be involved in folate transport in 63 strains, 28 pathogen species and 12 phyla, 60% of which were identified for the first time. Many of the genomes examined contained genes encoding transporters such as folate-binding protein YgfZ, folate/pteridine transporter, folate/biopterin transporter, reduced folate carrier family protein, folate/methotrexate transporter FT1. The mitochondrion is the predicted location of the majority of the proteins, with 15% possessing signal peptides. Phylogeny computation shows the similarity of the proteins identified.

Conclusion: These findings offer new possibilities for potential drug development targeting folate-salvage proteins in eukaryotic pathogens.

Keywords

Folate transporter, Eukaryotic pathogens, Drug discovery, Putative homologues

Open Peer Review

Approval Status ? ?

	1	2
version 2 (revision) 13 Jul 2017	 view	 view
version 1 12 Jan 2017	 view	 view

1. **Raphael D. Isokpehi**, Bethune-Cookman University, Daytona Beach, USA

2. **Gajinder Singh** , International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India

Any reports and responses or comments on the article can be found at the end of the article.

This article is included in the **Neglected Tropical Diseases** collection.

Corresponding author: Mofolusho Falade (mo.falade@ui.edu.ng)

Competing interests: No competing interests were disclosed.

Grant information: B.O. was supported by a TWAS-CNPq fellowship (FP number: 3240274297)

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2017 Falade M and Otarigho B. This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

How to cite this article: Falade M and Otarigho B. **Genome-wide characterization of folate transporter proteins of eukaryotic pathogens [version 1; peer review: 2 approved with reservations]** F1000Research 2017, **6**:36
<https://doi.org/10.12688/f1000research.10561.1>

First published: 12 Jan 2017, **6**:36 <https://doi.org/10.12688/f1000research.10561.1>

Introduction

A heterogeneous diversity of eukaryotic pathogens is responsible for the most economically important diseases of humans and animals^{1,2}. As a result of underdevelopment, a lack of social infrastructure and insufficient funding of public health facilities, most of these pathogens are endemic to resource-poor countries in sub-Saharan Africa, South-East Asia and South America, where they are responsible for high morbidity and mortality¹⁻³. Of these, parasitic protozoa form a major group, with the apicomplexans and kinetoplastid parasites represented by important members, which cause diseases such as malaria, cryptosporidiosis, toxoplasmosis, babesiosis, leishmaniasis, Human African trypanosomiasis and south American trypanosomiasis or Chagas' disease causing most of the morbidity and mortality^{4,5}. Other important diseases caused by protozoans include giardiasis, amoebic dysentery^{6,7} and trichomoniasis⁸. A vicious cycle of poverty and disease exists for most of these parasites with a high infection and death rate in affected populations⁹⁻¹¹. The appreciable burden of disease caused by these parasites has been aggravated by the lack of a licensed vaccine for most of them¹². Furthermore, current drugs of choice for treatment for many of the parasites have significant side effects, with the added emergence of drug resistant strains¹³⁻¹⁵. Despite the urgent demand for new therapies for control, few drugs have been developed to combat these parasites¹⁶. A major limitation to the development of new drugs is the paucity of new drug targets. There is therefore a need for discovery of novel and alternate potential chemotherapeutic targets that can help in drug development efforts for disease control¹⁶⁻¹⁸. A possible approach to selective antimicrobial chemotherapy has been to exploit the inhibition of unique targets, vital to the pathogen and absent in mammals^{17,18}.

A metabolic pathway that has been exploited considerably for the development of drugs is the folate biosynthetic pathway¹⁹. Antifolate drugs target this pathway and are the most important and successful antimicrobial chemotherapies targeting a range of bacterial and eukaryotic pathogens. While most parasitic protozoa can synthesize folates from simple precursors, such as GTP, p-aminobenzoic acid (pABA) and glutamate, higher animals and humans cannot²⁰. Additionally, a few of these parasites can also salvage folate as nutrient from their host²¹. These folate compounds are important for synthesis of DNA, RNA and membrane lipids and are transported via receptor-mediated or/and carrier-mediated transmembrane proteins; folate transporters²⁰⁻²². Importantly, antifolate chemotherapies that target the biosynthesis and processing of folate cofactors have been effective in the chemotherapy of bacterial and protozoan parasites²¹. More importantly, the folate pathway has also been confirmed as being essential in some eukaryotic pathogens such as *Plasmodium*, trypanosomes and *Leishmania*¹⁹.

In addition to the folate biosynthesis pathway, proteins that mediate transport of useful nutrients such as folic acid have been identified as important chemotherapeutic drug targets^{18,19,23}. Hence, the folate pathway, metabolites and transporters continue to be extensively studied for identification of new enzymes including transporters, which may serve as new drug targets²². Recent estimates have ascribed eight different membrane transporters to eukaryotes²⁴.

Proteins that mediate transportation of folates have been well studied in a few parasites such as *Plasmodium falciparum*, *Trypanosoma brucei*, *Leishmania donovani* and *Leishmania major*^{25,26}. These studies have provided information on mode of action of drugs^{25,27,28} in addition to studies describing mechanisms of parasite drug resistance²⁵⁻³². However, folate transport proteins remain unidentified and uncharacterized in many other eukaryotic pathogens. This is despite the sequencing of the genomes of most eukaryotic pathogens, which has produced a vast wealth of data that could aid in identification of druggable pathogen-specific proteins³³⁻³⁹. It is therefore imperative to search and identify from these parasite genomes additional proteins such as folate transporters that may serve as novel drug targets^{40,41}.

Therefore, in an attempt to identify and characterize targets for novel therapeutics, we report herein an extensive search of folate transporters from pathogen genomes. In addition, we investigated the evolutionary relationship of these transporters in a bid to determine similarities and differences that make them attractive drug targets. The knowledge provided may assist in the design of new antifolates for protozoan parasites.

Methods

Our experiment workflow is depicted in **Figure 1**. We extracted protein sequences of approximately 200 pathogens that mediate transportation or salvage of folates from Eukaryotic Pathogen Genome Database Resources (<http://eupathdb.org/eupathdb/>), and from the literature using a key-word search. We also searched the 200 pathogen genome sequences archived at the Eukaryotic Pathogen Genome Database Resources (<http://eupathdb.org/eupathdb/>). The search was for all proteins that mediate transportation or folate salvage alone or folate salvage and related compounds (such as pteridine, bipterin and methotrexate) together. This database gives public access to most sequenced emerging/re-emerging infectious pathogen genomes⁴². We utilized the word "folate" for search on the gene text and "folic acid" was used to confirm the hits. Hit results containing proteins annotated as folate-binding protein YgfZ, folate/pteridine transporter, folate/bipterin transporter, reduced folate carrier family protein, folate/methotrexate transporter FT1, Folate transporters alone and other folate related proteins were retrieved. The complete list of proteins extracted from Eupathdb is presented in **Dataset 1**⁴³. The folate transporters were classified based on type of transporter, number of transmembrane helix (TMH) and localization (either cell or mitochondrial membrane) of transporter. Gene sequences were obtained in FASTA format for transporter proteins using the sequence download tool on EupathDB (<http://eupathdb.org/eupathdb/>).

To ensure that most of the retrieved proteins had not been previously studied, we performed a literature search on PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/?term=>) and Google Scholar (<https://scholar.google.com>) using the query "folate transporter + Parasite name". The protein sequence information (**Dataset 1**⁴³ and **Table 1**) obtained from literature search was used for a BLAST search on EupathDB (<http://eupathdb.org/eupathdb/>), UniprotDB (<http://www.uniprot.org>) and GeneDB (<http://www.genedb.org/Homepage>). Sequence data were edited on textEdit mac version

Figure 1. Conceptual hierarchical methodology workflow showing the various steps employed in this study to identify, characterize and curate protozoan folate transporters.

and uploaded to Molecular Evolutionary Genetics Analysis (MEGA) platform version 7.0 obtained from <http://www.megasoftware.net>⁴⁴. The 234 sequences were aligned using muscle tools with large alignment (Max iterations = 2) selected while other settings were left at defaults. Evolutionary history was inferred using the Neighbor-Joining method⁴⁵. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was also analysed⁴⁶. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method⁴⁷. While uniform rate and complete deletion was selected for substitution rates and data subset, respectively. Other parameters were at default settings. All positions containing gaps and missing data were eliminated. The newick format of the tree was exported and opened on FigTree 1.4.2 platform downloaded from <http://tree.bio.ed.ac.uk/software/figtree/>⁴⁸. The final tree was constructed using radial tree layout. Additional analysis consisted of sub-phylogenies based on the transporter type. Since folate-binding protein YgfZ, folate/pteridine transporter, folate/biopterin transporter, putative, reduced folate carrier family protein, folate/methotrexate transporter FT1, putative folate transporters alone and others have 10, 25, 132, 2, 7, 49 and 9. So we decided to reconstruct the phylogeny based folate transporter, folate-biopterin transporter after considering the identification number, the species diversity in each category.

Results

A methodological search for folate transporters in all eukaryotic pathogen genomes we examined under EupathDB with validation via GenBank, GeneDB and Uniprot contained a total of 234 proteins (detail features of proteins are presented in [Dataset 1](#)⁴³). We identified these transporters in 28 pathogen species (containing 63 strains) cutting across 12 phyla ([Table 1](#)). The parasites with the highest number of folate transporters are *Phytophthora parasitica* INRA-310, *P. infestans* T30-4 and *Leptomonas pyrrocoris* H10 with 20, 16 and 16 proteins, respectively. While *Aspergillus clavatus* NRRL 1, *A. flavus* NRRL3357, *A. macrogynus* ATCC 38327, *Crithidia fasciculata* strain Cf-C1 and others have one folate transporter protein each ([Table 1](#)). The different proteins identified to be involved in folate salvage or related molecules were folate-binding protein YgfZ, folate/pteridine transporter, folate/biopterin transporter, reduced folate carrier family protein, folate/methotrexate transporter FT1 and folate transporters having a 4%, 11%, 56%, 1%, 3% and 21% identity, respectively. Proteins that did not belong to these groups were classified as others (4%) ([Figure 2A](#)). A good number of the proteins identified had predicted transmembrane helices with a few having none ([Figure 2B](#)). Furthermore, a number of the transporters possess signal peptides ([Dataset 1](#)⁴³), which may be required for targeting to cellular locations. Deciphering the sequence of the targeting signal may indicate its product destination.

Table 1. Pathogen species from which folate transporters were identified. A total of 234 folate transporter proteins from 63 pathogens were identified from the various classes of pathogens. √, Folate transporters found from literature search; X, Folate transporters identified from this study, FTP, Folate Transporter Protein.

S/ N	Species/Strain	No. of FTP	Genera	Family	Order	Class	Phylum/Division	Kingdom	Confirmation Search (References)
1	A. capsulatus G186AR	2	Actinobacillus	Pasteurellaceae	Pasteurellales	Gammaaproteobacteria	Proteobacteria	Bacteria	X
2	A. clavatus NRRL 1	1	Aspergillus	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota	Fungi	√ ⁶⁹
3	A. flavus NRRL3357	1	Aspergillus	Trichocomaceae	Eurotiales	Eurotiomycetes	Ascomycota	Fungi	√ ⁷⁰
4	A. macrogynus ATCC 38327	1	Allomyces	Blastocladiaceae	Blastocladales	Blastocladiomycetes	Blastocladiomycota	Fungi	X
5	C. fasciculata strain Cf-CI	1	Crithidia	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Eukaryota	X
6	C. immitis RS	2	Coccidioides	Onygenaceae	Onygenales	Eurotiomycetes	Ascomycota	Fungi	X
7	C. muris RN66	1	Cryptosporidium	Cryptosporidiidae	Eucoccidiorida	Conoidasida	Apicomplexa	Eukaryota	X
8	C. neoformans var. grubii H99	2	Cryptococcus	Tremellaceae	Tremellales	Tremellomycetes	Basidiomycota	Fungi	X
9	C. velia CCMP2878	11	Chromera	Chromeraceae		Chromerida	Chromerida	Eukaryota	X
10	E. acervulina Houghton	1	Eimeria	Eimeriidae	Eucoccidiorida	Conoidasida	Apicomplexa	Protista	X
11	E. brunetti Houghton	2	Eimeria	Eimeriidae	Eucoccidiorida	Conoidasida	Apicomplexa	Protista	X
12	E. cuniculi GB-M1	1	Encephalitozoon	Unikaryonidae	Microsporidia	Microsporea	Microsporidia	Fungi	√ ^{71,72}
13	E. maxima Weybridge	1	Eimeria	Eimeriidae	Eucoccidiorida	Conoidasida	Apicomplexa	Protista	X
14	E. necatrix Houghton	1	Eimeria	Eimeriidae	Eucoccidiorida	Conoidasida	Apicomplexa	Protista	X
15	E. praecox Houghton	1	Eimeria	Eimeriidae	Eucoccidiorida	Conoidasida	Apicomplexa	Protista	X
16	E. tenella strain Houghton	1	Eimeria	Eimeriidae	Eucoccidiorida	Conoidasida	Apicomplexa	Protista	X

S/ N	Species/Strain	No. of FTP	Genera	Family	Order	Class	Phylum/Division	Kingdom	Confirmation Search (References)
17	<i>F. graminearum</i> PH-1	2	Gibberella	Nectriaceae	Hypocreales	Sordariomycetes	Ascomycota	Animalia	X
18	<i>H. hammondi</i> strain H.H.34	3	Hammondia	Sarcocystidae	Eucoccidiorida	Conoidasida	Apicomplexa	Eukaryota	X
19	<i>L. braziliensis</i> MHOM/BR/75/ M2904	6	Leishmania	Trypanosomatidae	Trypanosomatida	Kinetoplastida	Euglenozoa	Eukaryota	✓ ⁷³
20	<i>L. donovani</i> BPK282A1	10	Leishmania	Trypanosomatidae	Trypanosomatida	Kinetoplastida	Euglenozoa	Eukaryota	✓ ^{74,75}
21	<i>L. infantum</i> JPCM5	9	Leishmania	Trypanosomatidae	Trypanosomatida	Kinetoplastida	Euglenozoa	Eukaryota	✓ ^{76,77}
22	<i>L. major</i> strain Friedlin	8	Leishmania	Trypanosomatidae	Trypanosomatida	Kinetoplastida	Euglenozoa	Eukaryota	✓ ⁷⁸
23	<i>L. mexicana</i> MHOM/GT/2001/ U1103	1	Leishmania	Trypanosomatidae	Trypanosomatida	Kinetoplastida	Euglenozoa	Eukaryota	✓ ⁷²
24	<i>L. pyrrohoris</i> H10	16	Leptomonas	Trypanosomatidae	Trypanosomatida	Kinetoplastida	Euglenozoa	Eukaryota	✓ ⁷⁹
25	<i>L. seymouri</i> ATCC 30220	6	Leptomonas	Trypanosomatidae	Trypanosomatida	Kinetoplastida	Euglenozoa	Eukaryota	✓ ⁸⁰
26	<i>M. daphniae</i> UGP3	1	Mitosporidium	–	–	–	–	–	X
27	<i>N. bombycis</i> CQ1	1	Nosema	Nosematidae	Dissociodihaplophasida	Dihaplophasea	Microsporidia	Fungi	X
28	<i>N. caninum</i> Liverpool	1	Neospora	Sarcocystidae	Eucoccidiorida	Conoidasida	Apicomplexa	Eukaryota	X
29	<i>N. fischeri</i> NRRL 181	1	Neosartorya	Trichocomaceae	Eurotiales	Eurotiomycetidae	Ascomycota	Fungi	X
30	<i>N. fowleri</i> ATCC 30863	2	Naegleria	Vahlkampfiidae	Schizopyrenida	Heterolobosea	Percolozoa	Eukaryota	X
31	<i>P. berghei</i> ANKA	2	Plasmodium	Plasmodiidae	Chromatorida	Aconoidasida	Apicomplexa	Eukaryota	X
32	<i>P. chabaudi</i> chabaudi	2	Plasmodium	Plasmodiidae	Chromatorida	Aconoidasida	Apicomplexa	Eukaryota	X
33	<i>P. falciparum</i> 3D7	2	Plasmodium	Plasmodiidae	Chromatorida	Aconoidasida	Apicomplexa	Eukaryota	✓ ²²

S/ N	Species/Strain	No. of FTP	Genera	Family	Order	Class	Phylum/Division	Kingdom	Confirmation Search (References)
34	<i>P. faicparum</i> IT	2	Plasmodium	Plasmodiidae	Chromatorida	Aconoidasida	Apicomplexa	Eukaryota	✓ ^[22]
35	<i>P. infestans</i> T30-4	16	Phytophthora	Pythiaceae	Peronosporales	Oomycota	Heterokontophyta	Eukaryota	X
36	<i>P. knowlesi</i> strain H	2	Plasmodium	Plasmodiidae	Chromatorida	Aconoidasida	Apicomplexa	Eukaryota	X
37	<i>P. parasitica</i> INRA-310	20	Phytophthora	Pythiaceae	Peronosporales	Oomycetes	Oomycota	Fungi	X
38	<i>P. reichenowi</i> CDC	2	Plasmodium	Plasmodiidae	Chromatorida	Aconoidasida	Apicomplexa	Eukaryota	X
39	<i>P. ultimum</i> DAOM BR144	1	Pythium	Pythiaceae	Pythiales	Oomycetes	Heterokontophyta	Chromalveolata	X
40	<i>P. vivax</i> Sal-1	2	Plasmodium	Plasmodiidae	Chromatorida	Aconoidasida	Apicomplexa	Eukaryota	X
41	<i>P. yoelii</i> yoelii 17X	2	Plasmodium	Plasmodiidae	Chromatorida	Aconoidasida	Apicomplexa	Eukaryota	X
42	<i>P. yoelii</i> yoelii YM	2	Plasmodium	Plasmodiidae	Chromatorida	Aconoidasida	Apicomplexa	Eukaryota	X
43	<i>S. neurona</i> SN3	6	Sarcocystis	Sarcocystidae	Eucoccidiorida	Conoidasida	Apicomplexa	Eukaryota	X
44	<i>S. punctatus</i> DAOM BR117	1	Spizellomyces	Powellomycetaceae	Spizellomycetales	Chytridiomycetes	Chytridiomycota	Fungi	X
45	<i>T. brucei</i> gambiense DAL972	3	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	X
46	<i>T. brucei</i> Lister strain 427	9	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	✓ ^[81]
47	<i>T. brucei</i> TREU927	5	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	✓ ^[81]
48	<i>T. congolense</i> IL3000	2	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	X
49	<i>T. cruzi</i> CL Brener Esmeraldo-like	3	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	✓ ^[82]
50	<i>T. cruzi</i> CL Brener Non- Esmeraldo-like	2	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	✓ ^[82]
51	<i>T. cruzi</i> Dm28c	2	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	✓ ^[82]

S/ N	Species/Strain	No. of FTP	Genera	Family	Order	Class	Phylum/Division	Kingdom	Confirmation Search (References)
52	T. cruzi marinkellei strain B7	6	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	✓ ⁸²
53	T. cruzi Sylvio X10/1	2	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	✓ ⁸²
54	T. equi strain WA	4	Theileria	Theileriidae	Piroplasmida	Aconoidasida	Apicomplexa	Eukaryota	✓ ⁸²
55	T. evansi strain STIB 805	3	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	X
56	T. gondii GT1	3	Toxoplasma	Sarcocystidae	Eucoccidiorida	Conoidasida	Apicomplexa	Eukaryota	✓ ⁸³
57	T. gondii ME49	4	Toxoplasma	Sarcocystidae	Eucoccidiorida	Conoidasida	Apicomplexa	Eukaryota	✓ ⁸³
58	T. gondii VEG	3	Toxoplasma	Sarcocystidae	Eucoccidiorida	Conoidasida	Apicomplexa	Eukaryota	✓ ⁸³
59	T. grayi ANR4	6	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	✓ ⁸⁴
60	T. parva strain Muguga	2	Theileria	Theileriidae	Piroplasmida	Aconoidasida	Apicomplexa	Eukaryota	X
61	T. rangeli SC58	3	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	✓ ⁸⁵
62	T. vivax Y486	1	Trypanosoma	Trypanosomatidae	Trypanosomatida	Kinetoplastea	Euglenozoa	Excavata	X
63	V. brassicaeformis CCMP3155	12	Vitrella	Vitrellaceae	-	-	Chromerida	Eukaryota	X
	Total	234							
							Keys		
							✓	Folate transporters found from literature search	
							X	Folate transporters identified from this study	
							FTP	Folate Transporter Protein	

Our literature search for parasite folate transporters on PubMed and Google Scholar indicated 60% (38 out of 63) of the proteins were identified for the first time as presented in Table 1 and Figure 2C, while 40% have been previously investigated. Besides, the *Leishmania* folate transporters we came across were not found on the EupathDB resource. We thus performed a BLAST search of Kinetoplastida on EupathDB, the returned hits were folate/biopterin

transporter for *L. infantum*. The only *Plasmodium* species with results for proteins that salvage folate was *P. falciparum*. Our study, however, describes for the first time the presence of these transporters in other *Plasmodium* species. There were no transporter proteins deposited in EupathDB for *P. malariae* and *P. ovale*. However, folate transporters I and II were retrieved from our search of GeneDB for *P. malariae* and *P. ovale curtisi*, respectively.

Figure 2. Categorization of proteins identified. This is based on [A] Transporter Type [B] Number of TMMs [C] Novel folate transporters [D] Localization [E] Presence/absence of Signal peptide. TMM, Transmembrane Helix;

Our analysis of folate transporters indicate the presence in *Plasmodium* species of two proteoforms; folate transporter I and II (Dataset 1⁴³). All *Leishmania* species identified possess folate/biopterin transporters and not folate transporters. Trypanosome species have both folate/pteridine and folate transporters; *T. cruzi* Dm28c, *T. cruzi* Sylvio X10/1 and *T. cruzi* CL Brener Esmeraldo-like, *T. cruzi* CL Brener Non-Esmeraldo-like and *T. cruzi* marinkellei strain B7 all have folate/pteridine transporter while *T. brucei* TREU927, *T. brucei* Lister strain 427, *T. brucei* gambiense DAL972, *T. congolense* IL3000 possess folate transporters. Eukaryotic parasites like *Eimeria acervulina* Houghton, *E. brunetti* Houghton, *E. maxima* Weybridge, *E. necatrix* Houghton, *E. praecox* Houghton, *E. tenella* strain Houghton and *Neospora caninum* Liverpool all boast folate/methotrexate transporter FT1. The folate-binding protein YgfZ was found in the fungus, *Allomyces macrogynus* ATCC 38327, the protist *C. fasciculata* strain Cf-Cl, *C. immitis* RS, the feline protozoan, *Hammondia hammondi* strain H.H.34, *Sarcocystis neurona* SN3, *S. punctatus* DAOM BR117, *T. gondii* GT1, *T. gondii* ME49, *T. gondii* VEG and *T. brucei* TREU927. Parasites such as *Microsporidium daphniae* UGP3 and the amoeba *Naegleria fowleri* ATCC 30863 possess the reduced folate carrier family protein (Figure 2D). We observed that 7% of the identified proteins are localized on the mitochondrial membrane of some pathogens such as the fungi *Aspergillus clavatus* NRRL 1, *A. flavus* NRRL3357, *C. immitis* RS, the yeast *Cryptococcus neoformans* var. grubii H99, *Fusarium graminearum* PH-1, *A. capsulatus* G186AR, *Leptomonas pyrrhocoris* H10, the food fungus *Neosartorya fischeri* NRRL 181, *Phytophthora parasitica* INRA-310 and *P. ultimum* DAOM BR144. The remaining proteins are localized on the plasma membrane (Dataset 1⁴³ and Figure 2D).

Approximately 15% (34/234) of the folate transporters identified possess signal peptides (Figure 2E) with the trypanosomes with the most signal peptides. Deductions can be made of the probable destination within the cell of any transporter by its signal peptide sequence; thus, further work may seek to decipher the sequence of the targeting signal to determine its localization. The proteins identified all have transmembrane helices with the exception of the alveolate *Chromera velia* CCMP2878, apicomplexan *P. berghei* ANKA, *S. neurona* SN3, the kinetoplastid *T. brucei* TREU927, *T. grayi* ANR4 and protist *Vitrella brassicaformis* CCMP3155 with Gene ID's Cvel_17766, PBANKA_0713700, SN3_01500005, Tb927.8.6480, Tgr.2739.1000 and Vbra_15327, respectively (Dataset 1⁴³).

The phylogenetic tree (Figure 3) shows the evolutionary position, history and relationship of all the folate transporters identified in this work. The type of transporter or species/strain was used for constructing phylogenetic trees, with the 234 proteins identified forming two clades, a major and minor. The major clade lacked a sub-clade, while the minor clade possessed a sub-clade. All proteins identified were distributed between the two major clades; except for folate/methotrexate transporter and mitochondrial folate transporter, with the latter present on the major clade and the former on the minor clade exclusively. All the species are represented on both clades, however, *V. brassicaformis* CCMP3155, *Plasmodium* species, *A. clavatus* NRRL, *A. flavus* NRRL3357, *A. macrogynus* ATCC 38327, *C. fasciculata* strain Cf-Cl, *C. immitis* RS, *C. immitis*

RS, *C. muris* RN66, *C. neoformans* var. grubii H99, *C. neoformans* var. grubii H99, *Leishmania* species, *N. bombycis* CQ1, *N. caninum* Liverpool, *F. graminearum* PH-1 and *H. hammondi* strain H.H.34 are exclusively on the major clade. There are some parasites that were identified once, as shown in Dataset 1⁴³; these are mostly in the large clade. Some of these pathogens include *P. ultimum* DAOM BR144, which has mitochondrial folate transporter/carrier proteins similar to *Homo sapiens*, *E. cuniculi* GB-M1, which has proteins similar to folate transporter, and *S. punctatus* DAOM BR117, which has folate-binding protein YgfZ. These were the only proteins of the aforementioned species identified in this work. However, *M. daphniae* UGP3, which had reduced folate carrier domain containing protein, was the only parasite that was found in the small clade. Improving on our phylogenetic analysis, we performed a sub-phylogenetic reconstruction (Figure 4–Figure 6) based on the substrate type of the transport proteins. After phylogenetic analysis each sub-phylogeny show a clear characterization except for folate-biopterin transporters (Figure 5), which fell in a different clade save for *Leptomonas* species and *C. velia*.

Dataset 1. Complete list of proteins extracted from Eupathdb and literature search, including their properties

<http://dx.doi.org/10.5256/f1000research.10561.d148742>

These data are available in a .xlsx file.

Discussion

Folate transporters are important proteins involved in the salvage of folate, cofactors and related molecules in eukaryotic pathogens important for metabolism and survival in their respective hosts²¹. We identified proteins that could mediate the salvage of folates into cells and/or mitochondria from eukaryotic pathogen genomes in EupathDB. Many of these proteins are involved in folate biosynthesis or transport and are present in many of the eukaryotic pathogens we queried. In this study, 234 genes encoding homologues of folate salvaging proteins were identified in the genome of 64 strains, representing 28 species of eukaryotic pathogens. Some of the pathogens include *P. falciparum* 3D7 and IT, *P. knowlesi* H, *P. berghei* ANKA, *P. chabaudi* chabaudi, *T. brucei* Lister 427, *T. brucei* TREU927, *T. brucei* gambiense DAL972, *Encephalitozoon cuniculi* GB-M1. The pathogens range from bacteria through to fungi, intracellular parasites such as *Plasmodium* and *leishmania* species, to extracellular parasites such as trypanosome species. This suggests a widespread presence of the proteins cutting across a range of pathogens that infect humans and animals.

A few of the proteins we identified have been previously identified and characterized in parasites such as *Plasmodium falciparum*^{22,30}, Trypanosome species²⁶, *Leishmania* species and *Toxoplasma gondii*⁴⁹. It has been estimated that over half of the drugs currently on the market target integral membrane proteins of which membrane transporters are a part, but unfortunately, these transporters have not been adequately explored as drug targets⁵⁰. Folate transporters therefore represent attractive drug targets for treatment of infectious diseases. Thus their identification from other eukaryotic pathogens could open a window for novel chemotherapeutics for disease control^{51,52}.

Figure 3. Phylogenetic tree showing relatedness of folate transporters compared across all studied pathogen species. Tree was constructed using Neighbor-Joining method (Bootstrap test was a 1000 replicates).

Figure 6. Phylogenetic tree showing relatedness of proteins involved in only folate-binding protein YgfZ transport across all studied pathogen species. Tree was constructed using Neighbor-Joining method (Bootstrap test was a 1000 replicates).

In *Plasmodium* two folate transporters have been identified, namely *PfFT1* and *PfFT2*. These transporters have been shown to mediate the salvage of folate derivatives and precursors in *P. falciparum* and proposed blocking of their salvage activities may improve the antimalarial efficacy of several classes of antimalarial drugs. In our work we identified folate transporters for other plasmodial species, which, like *P. falciparum*, may also be chemotherapeutic targets. Transport of folate in higher eukaryotes is made possible by a high affinity folate-biopterin transporters FBT or BT1 family^{22,30}. In the trypanosomes and related kinetoplastids, a member of these transporters, the folate biopterin transporter (FBT) family of proteins was identified in *Leishmania*²⁸. It is thought that MFS proteins are related to the FBT. These proteins have been characterized in a few protozoa and cyanobacteria⁵³. Results from our study describing the presence of these transporters across several phyla corroborate results other researches, establishing the conservation of folate transport function among FBT family proteins from species from plants and protists^{22,53}.

Malaria parasites encode transporters belonging to the organo anion transporter (OAT) folate-biopterin transporter (FBT), glycoside-pentoside-hexuronide: cation symporter (GPH), families, which are closely related to the major facilitator super-family of membrane proteins⁵⁴. The inhibition of these transporters by blockers of organic anion transporters such as probenecid has been implicated in sensitization of *Plasmodium* resistant parasites to antifolates^{55,56}. Thus, in *Plasmodium* chemotherapy, identification of folate transporters could lead to screening for compounds that interfere with folate transport and salvage for antimalarial chemotherapy^{22,30}. We identified several types of folate transporters that have been described and functionally characterized in *Leishmania* with some implicated in the import of the antifolate methotrexate^{57,58}. Thus far, only protozoan transporters in *Plasmodium*, *Leishmania*, and *Trypanosoma brucei* have been characterized and these are known to mediate the uptake of the vitamins folate and/or biopterin^{22,59,60}. Thus in parasites species of medical importance folate transporter proteins may provide new targets for therapy.

We also identified folate salvaging proteins from fungi such as *Coccidioides immitis* and *A. clavatus*, fungi found in soil^{61–63}, vegetable⁶¹ and waters in tropical and subtropical areas⁶⁴. These fungi are known to occasionally become pathogenic and act as opportunistic pathogens for animals and man⁶³. Coccidioidomycosis caused by *C. immitis* in association with AIDS has been known to be a fatal disease⁶⁵. Treatment of acute and chronic infections with antifungals such as amphotericin B have not been adequate, hence folate transporters may present new targets in these group of pathogens. Identification was also made on pathogens such as *C. fasciculata* that parasitize several species of insects including mosquitoes and has been widely used to test new therapeutic strategies against parasitic infections⁶⁶. As a model organism, folate

transporters identified in *C. fasciculata* may be useful in research for developing new drugs in medically important Kinetoplasts as has been shown for other targets in this protozoan parasite⁶⁷.

We noticed that *P. parasitica* INRA-310 and *L. pyrrhocoris* H10 had the highest number of folate transporters identified. Their utility as model fungal (*P. parasitica*) and monoxenous kinetoplast may provide models instrumental for developing new antifolates for fungal and protozoan diseases. The relatedness of these proteins across the different pathogens shows that there are two major phylogenetically distinct clades in the eukaryotic pathogens examined. The clustering of these proteins suggests that these transport proteins have highly conserved regions often required for basic cellular function or stability^{68–85}. Thus, antifolate chemotherapeutic drugs that are effective against one pathogen might have some effect on others.

Conclusion

In summary, we have identified and classified 234 proteins after an extensive search of pathogens genome in eukaryotic pathogen resource databases, though experimental studies will be required to confirm the expression and function of these proteins in parasites. Our results show that these proteins that mediate the transportation folate are widely distributed in different pathogen species examined in various phyla. The identification of folate salvage proteins in diverse eukaryotes extend the evolutionary diversity of these proteins and suggests they might offer new possibilities for potential drug development targeting folate-salvaging routes in eukaryotic pathogens.

Data availability

Dataset 1: Complete list of proteins extracted from Eupthadb and literature search, including their properties. These data are available in a .xlsx file. Doi, [10.5256/f1000research.10561.d148742](https://doi.org/10.5256/f1000research.10561.d148742)⁴³

Author contributions

M.O.F. and B.O. Conceptualized and Designed the study. M.O.F. and B.O. structured methodology. B.O. performed analysis. M.O.F. and B.O. wrote manuscript.

Competing interests

No competing interests were disclosed.

Grant information

B.O. was supported by a TWAS-CNPq fellowship (FP number: 3240274297).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Fadiel A, Isokpehi RD, Stambouli N, *et al.*: **Protozoan parasite aquaporins**. *Expert Rev Proteomic*. 2009; **6**(2): 199–211.
[PubMed Abstract](#) | [Publisher Full Text](#)
2. Prole DL, Taylor CW: **Identification and analysis of putative homologues of mechanosensitive channels in pathogenic protozoa**. *PLoS One*. 2013; **8**(6): e66068.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
3. Wiser M: **Protozoa and human disease**. *Garland Science*. 2010.
[Reference Source](#)
4. Turrens JF: **Oxidative stress and antioxidant defenses: a target for the treatment of diseases caused by parasitic protozoa**. *Mol Aspects Med*. 2004; **25**(1–2): 211–20.
[PubMed Abstract](#) | [Publisher Full Text](#)
5. Shiadeh MN, Niyiyati M, Fallahi S, *et al.*: **Human parasitic protozoan infection to infertility: a systematic review**. *Parasitol Res*. 2016; **115**(2): 469–77.
[PubMed Abstract](#) | [Publisher Full Text](#)
6. Khanum H, Kadir R, Arju T, *et al.*: **Detection of Entamoeba histolytica, Giardia lamblia and Cryptosporidium sp. Infection among diarrheal patients**. *Bangladesh J Zoology*. 2015; **43**(1): 1–7.
[Publisher Full Text](#)
7. Herman ML, Surawicz CM: **Intestinal Parasites**. In: *Textbook of Pediatric Gastroenterology, Hepatology and Nutrition*. Springer International Publishing. 2016; 185–193.
[Publisher Full Text](#)
8. Ton Nu PA, Nguyen VQ, Cao NT, *et al.*: **Prevalence of Trichomonas vaginalis infection in symptomatic and asymptomatic women in Central Vietnam**. *J Infect Dev Ctries*. 2015; **9**(06): 655–60.
[PubMed Abstract](#) | [Publisher Full Text](#)
9. Garcia LS, Bruckner DA: **Diagnostic medical parasitology**. Washington, DC. 2001; 131–5.
10. Macpherson CN: **Human behaviour and the epidemiology of parasitic zoonoses**. *Int J Parasitol*. 2005; **35**(11–12): 1319–31.
[PubMed Abstract](#) | [Publisher Full Text](#)
11. Andrews KT, Fisher G, Skinner-Adams TS: **Drug repurposing and human parasitic protozoan diseases**. *Int J Parasitol Drugs Drug Resis*. 2014; **4**(2): 95–111.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
12. Ouattara A, Laurens MB: **Vaccines against malaria**. *Clin Infect Dis*. 2015; **60**(6): 930–6.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
13. Monzote L, Siddiq A: **Drug development to protozoan diseases**. *Open Med Chem J*. 2011; **5**(1): 1–3.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
14. Petersen I, Eastman R, Lanzer M: **Drug-resistant malaria: molecular mechanisms and implications for public health**. *FEBS Lett*. 2011; **585**(11): 1551–62.
[PubMed Abstract](#) | [Publisher Full Text](#)
15. Castillo E, Dea-Ayuela MA, Bolás-Fernández F, *et al.*: **The kinetoplastid chemotherapy revisited: current drugs, recent advances and future perspectives**. *Curr Med Chem*. 2010; **17**(33): 4027–51.
[PubMed Abstract](#) | [Publisher Full Text](#)
16. Dias DA, Urban S, Roessner U: **A historical overview of natural products in drug discovery**. *Metabolites*. 2012; **2**(2): 303–36.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
17. Croft SL: **The current status of antiparasite chemotherapy**. *Parasitology*. 1997; **114**(Suppl): S3–15.
[PubMed Abstract](#)
18. Pink R, Hudson A, Mouriès MA, *et al.*: **Opportunities and challenges in antiparasitic drug discovery**. *Nat Rev Drug Discov*. 2005; **4**(9): 727–40.
[PubMed Abstract](#) | [Publisher Full Text](#)
19. Paiardini A, Fiascarelli A, Rinaldo S, *et al.*: **Screening and in vitro testing of antifolate inhibitors of human cytosolic serine hydroxymethyltransferase**. *ChemMedChem*. 2015; **10**(3): 490–7.
[PubMed Abstract](#) | [Publisher Full Text](#)
20. Nzila A, Ward SA, Marsh K, *et al.*: **Comparative folate metabolism in humans and malaria parasites (part I): pointers for malaria treatment from cancer chemotherapy**. *Trends Parasitol*. 2005; **21**(6): 292–8.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
21. Nzila A, Ward SA, Marsh K, *et al.*: **Comparative folate metabolism in humans and malaria parasites (part II): activities as yet untargeted or specific to Plasmodium**. *Trends Parasitol*. 2005; **21**(7): 334–9.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
22. Salcedo-Sora JE, Ward SA: **The folate metabolic network of Falciparum malaria**. *Mol Biochem Parasitol*. 2013; **188**(1): 51–62.
[PubMed Abstract](#) | [Publisher Full Text](#)
23. Costi MP, Ferrari S: **Update on antifolate drugs targets**. *Curr Drug Targets*. 2001; **2**(2): 135–66.
[PubMed Abstract](#) | [Publisher Full Text](#)
24. Zhao R, Diop-Bove N, Visentin M, *et al.*: **Mechanisms of membrane transport of folates into cells and across epithelia**. *Annu Rev Nutr*. 2011; **31**: 177–201.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
25. Müller IB, Hyde JE: **Antimalarial drugs: modes of action and mechanisms of parasite resistance**. *Future Microbiol*. 2010; **5**(12): 1857–73.
[PubMed Abstract](#) | [Publisher Full Text](#)
26. Emmer BT, Nakayasu ES, Souther C, *et al.*: **Global analysis of protein palmitoylation in African trypanosomes**. *Eukaryot Cell*. 2011; **10**(3): 455–63.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
27. Müller IB, Hyde JE: **Folate metabolism in human malaria parasites—75 years on**. *Mol Biochem Parasitol*. 2013; **188**(1): 63–77.
[PubMed Abstract](#) | [Publisher Full Text](#)
28. Vickers TJ, Beverley SM: **Folate metabolic pathways in Leishmania**. *Essays Biochem*. 2011; **51**: 63–80.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
29. Borst P, Ouellette M: **New mechanisms of drug resistance in parasitic protozoa**. *Annu Rev Microbiol*. 1995; **49**(1): 427–60.
[PubMed Abstract](#) | [Publisher Full Text](#)
30. Wang P, Brobey RK, Horii T, *et al.*: **Utilization of exogenous folate in the human malaria parasite Plasmodium falciparum and its critical role in antifolate drug synergy**. *Mol Microbiol*. 1999; **32**(6): 1254–62.
[PubMed Abstract](#) | [Publisher Full Text](#)
31. Ouellette M, Légaré D, Papadopoulou B: **Multidrug resistance and ABC transporters in parasitic protozoa**. *J Mol Microbiol Biotechnol*. 2001; **3**(2): 201–6.
[PubMed Abstract](#)
32. Stein WD, Sanchez CP, Lanzer M: **Virulence and drug resistance in malaria parasites**. *Trends Parasitol*. 2009; **25**(10): 441–3.
[PubMed Abstract](#) | [Publisher Full Text](#)
33. Agüero F, Al-Lazikani B, Aslett M, *et al.*: **Genomic-scale prioritization of drug targets: the TDR Targets database**. *Nat Rev Drug Discov*. 2008; **7**(11): 900–7.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
34. Franzén O, Jerlström-Hultqvist J, Castro E, *et al.*: **Draft genome sequencing of giardia intestinalis assemblage B isolate GS: is human giardiasis caused by two different species?** *PLoS Pathog*. 2009; **5**(8): e1000560.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
35. Butt AM, Nasrullah I, Tahir S, *et al.*: **Comparative genomics analysis of Mycobacterium ulcerans for the identification of putative essential genes and therapeutic candidates**. *PLoS One*. 2012; **7**(8): e43080.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
36. Tiffin N, Adie E, Turner F, *et al.*: **Computational disease gene identification: a concert of methods prioritizes type 2 diabetes and obesity candidate genes**. *Nucleic Acids Res*. 2006; **34**(10): 3067–81.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
37. Götz S, García-Gómez JM, Terol J, *et al.*: **High-throughput functional annotation and data mining with the Blast2GO suite**. *Nucleic Acids Res*. 2008; **36**(10): 3420–35.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
38. Nikolskaya T, Bugrim A, Nikolsky Y, *et al.*: **Methods for identification of novel protein drug targets and biomarkers utilizing functional networks**. United States patent US 8000949 B2; 2011.
[Reference Source](#)
39. Panwar B, Menon R, Eksi R, *et al.*: **Genome-wide Functional Annotation of Human Protein-coding Splice Variants Using Multiple Instance Learning**. *J Proteome Res*. 2016; **15**(6): 1747–53.
[PubMed Abstract](#) | [Publisher Full Text](#)
40. Antony AC: **Folate receptors**. *Annu Rev Nutr*. 1996; **16**(1): 501–21.
[PubMed Abstract](#) | [Publisher Full Text](#)
41. Girardin F: **Membrane transporter proteins: a challenge for CNS drug development**. *Dialogues Clin Neurosci*. 2006; **8**(3): 311–21.
[PubMed Abstract](#) | [Free Full Text](#)
42. Aurrecoechea C, Heiges M, Wang H, *et al.*: **ApiDB: integrated resources for the apicomplexan bioinformatics resource center**. *Nucleic Acids Res*. 2007; **35**(Database issue): D427–30.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
43. Falade M, Otárhigo B: **Dataset 1 in: Genome-wide characterization of folate transporter proteins of eukaryotic pathogens**. *F1000Research*. 2017.
[Data Source](#)
44. Saitou N, Nei M: **The neighbor-joining method: a new method for reconstructing phylogenetic trees**. *Mol Biol Evol*. 1987; **4**(4): 406–25.
[PubMed Abstract](#)
45. Sanderson MJ: **Confidence limits on phylogenies: the bootstrap revisited**. *Cladistics*. 1989; **5**(2): 113–29.
[Publisher Full Text](#)
46. Nei M, Kumar S: **Molecular evolution and phylogenetics**. Oxford university press; 2000.
[Reference Source](#)
47. Rambaut A: **FigTree 1.4.2 software**. Institute of Evolutionary Biology, Univ. Edinburgh.
48. Massimine KM, Doan LT, Atreya CA, *et al.*: **Toxoplasma gondii is capable of exogenous folate transport. A likely expansion of the BT1 family of transmembrane proteins**. *Mol Biochem Parasitol*. 2005; **144**(1): 44–54.
[PubMed Abstract](#) | [Publisher Full Text](#)
49. Swaan PW: **Membrane Transport Proteins and Drug Transport**. *Burger's*

Medicinal Chemistry and Drug Discovery. 2003.

[Publisher Full Text](#)

50. Mansour TE: **Chemotherapeutic targets in parasites: contemporary strategies**. Cambridge University Press; 2002.
[Reference Source](#)
51. Alam A, Goyal M, Iqbal MS, *et al.*: **Novel antimalarial drug targets: hope for new antimalarial drugs**. *Expert Rev Clin Pharmacol*. 2009; 2(5): 469–89.
[PubMed Abstract](#) | [Publisher Full Text](#)
52. Dean P, Major P, Nakjang S, *et al.*: **Transport proteins of parasitic protists and their role in nutrient salvage**. *Front Plant Sci*. 2014; 5: 153.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
53. Saier MH Jr, Beatty JT, Goffeau A, *et al.*: **The major facilitator superfamily**. *J Mol Microbiol Biotechnol*. 1999; 1(2): 257–79.
[PubMed Abstract](#)
54. Sowunmi A, Fehintola FA, Adediji AA, *et al.*: **Open randomized study of pyrimethamine-sulphadoxine vs. pyrimethamine-sulphadoxine plus probenecid for the treatment of uncomplicated *Plasmodium falciparum* malaria in children**. *Trop Med Int Health*. 2004; 9(5): 606–14.
[PubMed Abstract](#) | [Publisher Full Text](#)
55. Nzila A, Mberu E, Bray P, *et al.*: **Chemosensitization of *Plasmodium falciparum* by probenecid in vitro**. *Antimicrob Agents Chemother*. 2003; 47(7): 2108–12.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
56. Richard D, Kündig C, Ouellette M: **A new type of high affinity folic acid transporter in the protozoan parasite *Leishmania* and deletion of its gene in methotrexate-resistant cells**. *J Biol Chem*. 2002; 277(33): 29460–7.
[PubMed Abstract](#) | [Publisher Full Text](#)
57. Richard D, Leprohon P, Drummelsmith J, *et al.*: **Growth phase regulation of the main folate transporter of *Leishmania infantum* and its role in methotrexate resistance**. *J Biol Chem*. 2004; 279(52): 54494–501.
[PubMed Abstract](#) | [Publisher Full Text](#)
58. Kündig C, Haimour A, Légaré D, *et al.*: **Increased transport of pteridines compensates for mutations in the high affinity folate transporter and contributes to methotrexate resistance in the protozoan parasite *Leishmania tarentolae***. *EMBO J*. 1999; 18(9): 2342–51.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
59. Gottesdiener KM: **A new VSG expression site-associated gene (ESAG) in the promoter region of *Trypanosoma brucei* encodes a protein with 10 potential transmembrane domains**. *Mol Biochem Parasitol*. 1994; 63(1): 143–51.
[PubMed Abstract](#) | [Publisher Full Text](#)
60. Stewart RA, Meyer KF: **Isolation of *Coccidioides immitis* (Stiles) from the soil**. *Exp Biol Med*. 1932; 29(8): 937–8.
[Publisher Full Text](#)
61. Greene DR, Koenig G, Fisher MC, *et al.*: **Soil isolation and molecular identification of *Coccidioides immitis***. *Mycologia*. 2000; 92(3): 406–10.
[Publisher Full Text](#)
62. Litvinseva AP, Marsden-Haug N, Hurst S, *et al.*: **Valley fever: finding new places for an old disease: *Coccidioides immitis* found in Washington State soil associated with recent human infection**. *Clin Infect Dis*. 2015; 60(1): e1–3.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
63. Hajji M, Kanoun S, Nasri M, *et al.*: **Purification and characterization of an alkaline serine-protease produced by a new isolated *Aspergillus clavatus* ES1**. *Process Biochem*. 2007; 42(5): 791–7.
[Publisher Full Text](#)
64. Rodríguez-Cerdeira C, Arenas R, Moreno-Coutiño G, *et al.*: **Systemic fungal infections in patients with human immunodeficiency virus**. *Actas Dermosifiliogr*. 2014; 105(1): 5–17.
[PubMed Abstract](#) | [Publisher Full Text](#)
65. Awadelkarim FM, Hunter KJ, Kirby GC, *et al.*: ***Crithidia fasciculata* as feeder cells for malaria parasites**. *Exp Parasitol*. 1995; 80(1): 98–106.
[PubMed Abstract](#) | [Publisher Full Text](#)
66. Krungkrai J, Cerami A, Henderson GB: **Pyrimidine biosynthesis in parasitic protozoa: purification of a monofunctional dihydroorotase from *Plasmodium berghei* and *Crithidia fasciculata***. *Biochemistry*. 1990; 29(26): 6270–5.
[PubMed Abstract](#) | [Publisher Full Text](#)
67. Eyal E, Najmanovich R, McConkey BJ, *et al.*: **Importance of solvent accessibility and contact surfaces in modeling side-chain conformations in proteins**. *J Comput Chem*. 2004; 25(5): 712–24.
[PubMed Abstract](#) | [Publisher Full Text](#)
68. Fedorova ND, Khaldi N, Joardar VS, *et al.*: **Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus***. *PLoS Genet*. 2008; 4(4): e1000046.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
69. Nierman WC, Yu J, Fedorova-Abrams ND, *et al.*: **Genome sequence of *Aspergillus flavus* NRRL 3357, a strain that causes aflatoxin contamination of food and feed**. *Genome Announc*. 2015; 3(2): e00168–15.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
70. Katinka MD, Duprat S, Cornillot E, *et al.*: **Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi***. *Nature*. 2001; 414(6862): 450–3.
[PubMed Abstract](#) | [Publisher Full Text](#)
71. Rogers MB, Hilley JD, Dickens NJ, *et al.*: **Chromosome and gene copy number variation allow major structural change between species and strains of *Leishmania***. *Genome Res*. 2011; 21(12): 2129–42.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
72. Selman M, Sak B, Kváč M, *et al.*: **Extremely reduced levels of heterozygosity in the vertebrate pathogen *Encephalitozoon cuniculi***. *Eukaryot Cell*. 2013; 12(4): 496–502.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
73. Kaur K, Coons T, Emmett K, *et al.*: **Methotrexate-resistant *Leishmania donovani* genetically deficient in the folate-methotrexate transporter**. *J Biol Chem*. 1988; 263(15): 7020–8.
[PubMed Abstract](#)
74. Beck JT, Ullman B: **Affinity labeling of the folate-methotrexate transporter from *Leishmania donovani***. *Biochemistry*. 1989; 28(17): 6931–7.
[PubMed Abstract](#) | [Publisher Full Text](#)
75. Richard D, Leprohon P, Drummelsmith J, *et al.*: **Growth phase regulation of the main folate transporter of *Leishmania infantum* and its role in methotrexate resistance**. *J Biol Chem*. 2004; 279(52): 54494–501.
[PubMed Abstract](#) | [Publisher Full Text](#)
76. Ouameur AA, Girard I, Légaré D, *et al.*: **Functional analysis and complex gene rearrangements of the folate/biopterin transporter (FBT) gene family in the protozoan parasite *Leishmania***. *Mol Biochem Parasitol*. 2008; 162(2): 155–64.
[PubMed Abstract](#) | [Publisher Full Text](#)
77. Ubeda JM, Légaré D, Raymond F, *et al.*: **Modulation of gene expression in drug resistant *Leishmania* is associated with gene amplification, gene deletion and chromosome aneuploidy**. *Genome Biol*. 2008; 9(7): R115.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
78. Flegontov P, Butenko A, Firsov S, *et al.*: **Genome of *Leptomonas pyrrhocoris*: a high-quality reference for monoxenous trypanosomatids and new insights into evolution of *Leishmania***. *Sci Rep*. 2016; 6: 23704.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
79. Kraeva N, Butenko A, Hlaváčková J, *et al.*: ***Leptomonas seymouri*: Adaptations to the Dixerous Life Cycle Analyzed by Genome Sequencing, Transcriptome Profiling and Co-infection with *Leishmania donovani***. *PLoS Pathog*. 2015; 11(8): e1005127.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
80. El-Sayed NM, Myler PJ, Blandin G, *et al.*: **Comparative genomics of trypanosomatid parasitic protozoa**. *Science*. 2005; 309(5733): 404–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
81. El-Sayed NM, Myler PJ, Bartholomeu DC, *et al.*: **The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease**. *Science*. 2005; 309(5733): 409–15.
[PubMed Abstract](#) | [Publisher Full Text](#)
82. Massimine KM, Doan LT, Atreya CA, *et al.*: ***Toxoplasma gondii* is capable of exogenous folate transport. A likely expansion of the BT1 family of transmembrane proteins**. *Mol Biochem Parasitol*. 2005; 144(1): 44–54.
[PubMed Abstract](#) | [Publisher Full Text](#)
83. Kelly S, Ivens A, Manna PT, *et al.*: **A draft genome for the African crocodilian trypanosome *Trypanosoma grayi***. *Sci Data*. 2014; 1: 140024.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
84. Stoco PH, Wagner G, Talavera-Lopez C, *et al.*: **Genome of the avirulent human-infective trypanosome—*Trypanosoma rangeli***. *PLoS Negl Trop Dis*. 2014; 8(9): e3176.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
85. Kumar S, Stecher G, Tamura K: **MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets**. *Mol Biol Evol*. 2016; 33(7): 1870–4.
[PubMed Abstract](#) | [Publisher Full Text](#)

Open Peer Review

Current Peer Review Status: ? ?

Version 1

Reviewer Report 06 March 2017

<https://doi.org/10.5256/f1000research.11380.r20419>

© 2017 Singh G. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Gajinder Singh

Molecular Medicine Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, Delhi, India

Below are my major concerns:

1. The abstract does not provides an adequate summary of the article.

The authors have claimed much higher scope of their work than actually reported. While their work is restricted to folate transporters, they have claimed to work on whole folate pathway. In the Abstract: "We applied a combination of bioinformatics methods to examine the genomes of pathogens in the EupathDB for genes encoding homologues of proteins that mediate folate salvage in a bid to identify and assign putative functions." "These findings offer new possibilities for potential drugConclusion: development targeting folate-salvage proteins."

2. No proper justification for the work is provided.

a) While folate pathway is well established as drug target, the authors have only identified folate transporters. So please give examples of drugs (with names) which are known to target folate transporters in any organism. If no sufficient information is provided, the usefulness of the work is severely reduced.

b) How many of folate transporters are essential in species where essentiality data is available such as *P. berghei* and *T. gondii*?

3. The methodology to identify transporters is not comprehensive.

Since authors have used key-word searches to identify folate transporters, they are likely to miss many transporters not labelled as such. An appropriate methodology will incorporate profile (such as HMM) based searches. Thus the number of transporters identified by authors is most likely to be an underestimate.

4. The methodology is not clear.

Authors write that "we utilized the word "folate" for search on the gene text and "folic acid" was used to confirm the hits", then how only transporters were retrieved?
The Figure 1 is confusing. Where is BLAST used here?

5. The manuscript is written very poorly with so many scientific and grammatical mistakes that it is very difficult for the reader to follow the manuscript. Below are some examples.

a) "The mitochondrion is the predicted location of the majority of the proteins, with 15% possessing signal peptides." - how can mitochondria be majority location if only 15% have signal peptides and even less with mitochondrial signal peptide? Shouldn't the majority then be cytoplasmic?

b) "We identified 234 proteins to be involve in folate transport".

c) "Since folate-binding protein YgfZ, folate/ pteridine transporter, folate/biopterin transporter, putative, reduced folate carrier family protein, folate/methotrexate transporter FT1, putative folate transporters alone and others have 10, 25, 132, 2, 7, 49 and 9." What are these numbers?

d) "So we decided to reconstruct the phylogeny based folate transporter, folate-biopterin transporter after considering the identification number, the species diversity in each category."

e) "The different proteins identified to be involved in folate salvage or related molecules were folatebinding protein YgfZ, folate/pteridine transporter, folate/biopterin transporter, reduced folate carrier family protein, folate/methotrexate transporter FT1 and folate transporters having a 4%, 11%, 56%, 1%, 3% and 21% identity, respectively." What does this statement mean?

f) Does Table 1 really need to be 4 page long?

g) "The only Plasmodium species with results for proteins that salvage folate was P. falciparum"

h) "However, folate transporters I and II were retrieved from our search of GeneDB for P. malariae and P. ovale curtisi, respectively." What are these transporter classes?

i) "Some of these pathogens include P. ultimum DAOM BR144, which has mitochondrial folate transporter/carrier proteins similar to Homo sapiens, E. cuniculi GB-M1, which has proteins similar to folate transporter, and S. punctatus DAOM BR117, which has folate-binding protein YgfZ."

j) "After phylogenetic analysis each sub-phylogeny show a clear characterization except for folate-biopterin transporters"

h) "In this study, 234 genes encoding homologues of folate salvaging proteins were identified in the genome of 64 strains, representing 28 species of eukaryotic pathogens. Some of the pathogens include *P. falciparum* 3D7 and IT, *P. knowlesi* H, *P. berghei* ANKA, *P. chabaudi chabaudi*, *T. brucei* Lister 427, *T. brucei* TREU927, *T. brucei gambiense* DAL972, *Encephalitozoon cuniculi* GB-M1. The pathogens range from bacteria through to fungi, intracellular parasites such as *Plasmodium* and *Leishmania* species, to extracellular parasites such as trypanosome species" Which bacteria was included in the study?

i) "It has been estimated that over half of the drugs currently on the market target integral membrane proteins of which membrane transporters are a part, but unfortunately, these transporters have not been adequately explored as drug targets. Folate transporters therefore represent attractive drug targets for treatment of infectious diseases." Please tell us how many drugs are available in the market which target folate transporters, which is a more relevant statistic with respect to this study.

j) "In the trypanosomes and related kinetoplastids, a member of these transporters, the folate biopterin transporter (FBT) family of proteins was identified in *Leishmania*."

k) "It is thought that MFS proteins are related to the FBT." What is MFS?

l) "Results from our study describing the presence of these transporters across several phyla corroborate results of other researches, establishing the conservation of folate transport function among FBT family proteins from species from plants and protists".

m) "The clustering of these proteins suggests that these transport proteins have highly conserved regions often required for basic cellular function or stability". The clustering does not suggest that these transport proteins have highly conserved regions often required for basic cellular function or stability.

n) " We also performed phylogenetic comparisons of identified proteins. .".

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 27 February 2017

<https://doi.org/10.5256/f1000research.11380.r20535>

© 2017 Isokpehi R. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Raphael D. Isokpehi

College of Science, Engineering and Mathematics (CSEM) , Bethune-Cookman University , Daytona Beach, FL, USA

Summary of Referee's Report

The manuscript presents a strong justification for research on folate transporter proteins as drug targets for diseases caused by eukaryotic pathogens including the malaria parasite. The manuscript reports a data curation effort that involves the use of the Eukaryotic Pathogens Database (EuPathDB) Resource. Several novel results to guide future research are included such as (i) a list of 234 folate transporter proteins from 63 eukaryotic microbes including eukaryotic pathogens; and (ii) phylogenetic trees of relatedness of the protein sequences. The authors observed the clustering of the protein sequences that indicate the possibility that antifolate drugs could be effective for multiple eukaryotic pathogens.

Major concerns are:

- (i) The need for a clearer description of the workflow for the construction of the protein list.
- (ii) There is inadequate support for the statement that "60% of the proteins were identified for the first time".
- (iii) Confusion between retrieval and identification of protein sequences. The workflow diagram indicates retrieval of sequences but narrative text describes identification in multiple sections. The potential drug targeting categorization of the retrieved protein sequences is a key contribution of the research study.

Some minor concerns include (i) typographic errors such as spelling of abbreviations (e.g. EuPathDB, PubMed and UniProt); (ii) classification of *A. (Ajellomyces) capsulatus* G186AR as a bacteria; and (iii) need to quantify the statistics associated with observations (Examples: in abstract "Many of the genomes"; in discussion: "A few of the proteins").

Title and Abstract:

Is the title appropriate for the content of the article?

The manuscript title is "Genome-wide characterization of folate transporter proteins of eukaryotic pathogens". "Genome-wide characterization" does not effectively describe the accomplishments of the research reported. The manuscript in the conclusion section (Page 15) states "we have identified and classified 234 proteins...". The workflow (Figure 1) provides categorization of proteins by features such as cellular location, presence of signal peptide and number of transmembrane helices. Figure 2 has the title "Categorization of proteins identified". A suggested revised title is "**Categorization of potential drug targeting folate transporter proteins from eukaryotic pathogens**". The "potential drug targeting" is obtained from the conclusion section.

Does the abstract represent a suitable summary of the work?

There are sentences in the abstract that should be revised to accurately represent a suitable summary of the research performed. Comments/suggestions are provided below.

1. Quantify the observations described as many. For example "genome sequences of many":
How many?
2. "eukaryotic protozoa" > "eukaryotic microbes". The term "eukaryotic microbes" encompasses pathogens and non-pathogens (e.g. *Chromera velia* and *Vitrella brassicaformis*¹).

3. "important data" > "critical biological information"
4. "pathway are important for" > "pathways are necessary for"
5. "Methods: We applied a combination of bioinformatics methods to examine the genomes of pathogens in the EupathDB for genes encoding homologues of proteins that mediate folate salvage in a bid to identify and assign putative functions." > "Methods: We developed automated search strategies in the Eukaryotic Pathogen Database Resources (EuPathDB) to construct a protein list and retrieve protein sequences of folate transporters encoded in the genomes of 200 eukaryotic microbes. The folate transporters were categorized according to features including mitochondrial localization, number of transmembrane helix, and protein sequence relatedness.
6. Provide key result(s) of the protein list retrieval and phylogenetic comparison of the retrieved proteins. For example, We constructed a list of 234 folate transporter proteins associated with 63 eukaryotic microbes including ??? algae, ??? fungi and ??? protozoa. Seven percent of the proteins were predicted to localize on the mitochondrial membrane. Phylogenetic tree revealed major (??? proteins) and minor (??? Proteins) clades. All the folate transporter sequences from the malaria parasite, *Plasmodium*, belonged to the major clade.
7. "The mitochondrion is the predicted location of the majority of the proteins". This statement is not supported by Figure 2D, where 7% of the protein sequences are labelled as Mitochondrial folate transporters.

Article content:

Have the design, methods and analysis of the results from the study been explained and are they appropriate for the topic being studied?

Design and Methods:

1. Figure 1 presents a conceptual hierarchical methodology. The rectangle labelled "Protein names/ sequences verification" has arrows to PubMed, UniProt, Membranetransporters.org, NCBI, GeneDB, Google Scholar and Phylogenetic analyses. It appears that the integrated results from the search strategies in the databases provided the input for the phylogenetic analyses. Please clarify.
2. Which step of the workflow resulted in the list of 234 proteins?
3. How many proteins were retrieved from the initial search using EuPathDB?
4. Protein Features Retrieved rectangle: Was the retrieval of protein features performed on only the 234 proteins?
5. There is adequate explanation of the methods for phylogenetic analyses. Please provide the Newick format phylogenetic tree as a supplementary dataset.

Analysis of the Results:

1. Table 1 is a major curation effort presented in the manuscript.
 - a. The title of Table 1 should be updated to "Eukaryotic microbes from which folate

transporters were identified". The list includes non-pathogens.

b. The content of the table (especially the Kingdom entries) should be checked for accuracy. The Kingdom column could be updated to Eukaryotic Microbe Group with entries as algae, protozoa or fungi.

c. The column entries for *A. capsulatus* G186AR (mislabelled as bacteria) should be updated as the organism name is for a fungus (genus *Ajellomyces*). This update will also affect the Phylogenetic Tree (Figure 3). The node labelled *Actinobacillus* clusters with *Ajellomyces macrogynus*.

d. An updated Table 1 should be presented as Dataset 2 in a spreadsheet file. This would enable secondary data analysis by other researchers.

e. A new Table 1 could consist of columns for Eukaryotic Microbe Group, Genera of Eukaryotic Microbe, List of Species/Strain and Number of Folate Transport Proteins. This will provide reader with an overview of how the 234 proteins is distributed into the genera of the eukaryotic microbes.

f. References listed for confirmation searches. Page 8, Paragraph 1, Sentence 1: "Our literature search for parasite folate transporters on PubMed and Google Scholar indicated 60% (38 out of 63) of the proteins were identified for the first time as presented in Table 1. Comment: Among the references included in Table 1, only eight references (22, 73 to 77 and 82) on the basis of the article title provide experimental assessments of the folate transporters. Reference 85 is a reference for MEGA7 software. In the sentence "proteins" should be eukaryotic microbes. The proportion of eukaryotic microbes whose folate transporter(s) have been previously investigated with functional assays should be revised.

2. Figure 2. Categorization of proteins identified.

Authors should consider representing Figure 2A and 2B as bar graphs. Figures 2C, 2D and 2E have only two categories that can be described in the Results section.

3. Discussion

a. "genomes of 64 strains". Table 1 has 63 eukaryotic microbes.

b. Discuss *Chromera velia* and *Vitrella brassicaformis* as organismal systems for investigating folate transporter function. See Woo et al.¹

4. Conclusion

Consider revising "In summary, we have identified and classified 234 proteins..." to "In summary, we have retrieved information on 234 folate transporter proteins from the Eukaryotic Pathogen Database (EuPathDB) resources. The folate transporter proteins were categorized into potential drug targeting features including mitochondrial localization, number of transmembrane helix, and protein sequence relatedness."

Data (if applicable):

Has enough information been provided to be able to replicate the experiment? Are the data in a usable format/structure and have all the data been provided?

1. Table 1 needs to be revised and converted to a Dataset.

2. Please provide the Newick format phylogenetic tree as a supplementary dataset.

3. The Gene Identifiers [Gene ID] in Dataset 1 can be used to retrieve the protein sequences from EuPathDB.

References

1. Woo YH, Ansari H, Otto TD, Klinger CM, et al.: Chromerid genomes reveal the evolutionary path from photosynthetic algae to obligate intracellular parasites. *Elife*. 2015; **4**: e06974 [PubMed Abstract](#) | [Publisher Full Text](#)

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research