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Sequence Based Information and Phylogenetic Analysis of Dihydrofolate Reductase in *Pneumocystis*

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Abstract

The evolutionary relationships of four sequenced members of the *Pneumocystis* family have been investigated at their protein levels by comparing the protein sequences. The central region of the dihydrofolate protein of *Pneumocystis jiroveci* is highly conserved in amino acid content and arrangement, except for some amino acids. Among the four sequences, although the amino acid composition was not identical, however *P. oryctologi* and *P. jiroveci* were identified to be similar in sequence information showing more than 65 percentages of protein from near the termini to be conserved by the subfamily, while *P. carinii* and *P. murina* were identified to be relative identical by sequence information indicating a divergence of origin within the two groups. Relationships between the *Pneumocystis* genera were evaluated by comparing the protein identity, positives, length, identical sites, molecular weight, and isoelectric point along with the phylogenetic analysis by both pairwise alignment and multiple sequence alignments. The analysis was performed by having *P. jiroveci* and *P. carinii* as a base by using Geneious Pro commercial package version 4.7.5., which revealed that *P. murina* as the most divergent organism from *P. jiroveci* followed by the next most divergent as *P. carinii*, whose sole member infects only rat. However, *P. oryctologi* and *P. jiroveci* shared a common ancestor, and were distinct from *P. murina.*

Keywords: Dihydrofolate reductase (DHFR), *Pneumocystis jiroveci*, Geneious Pro, Sequence information.

Introduction

Twentieth century by most of the parasitologists was dedicated looking for new parasites in the bloodstream, tissues, and feces of normal as well as experimentally infected animals. *Pneumocystis carinii* (PCP) pneumonia, a leading opportunistic infection found among HIV-infected individuals worldwide was first identified by Carlos Chagas in 1909 from lungs of guinea pigs and subsequently by Antonio Carinii in infected rat lungs. It was initially thought to be a trypanosome until, Delanoes in 1912 recognized that this was a new species with a unique tropism to the lung of rat, hence the name *Pneumocystis carinii*. A new binomial nomenclature classified the organism as *Pneumocystis jiroveci* Frankel, which was named in honor of the Czech parasitologist Otto Jirovec who described the microbe in humans [22].

Tremendous increasing World population with the Bacterial pneumonia and *Pneumocystis* pneumonia are the two most common HIV-associated pneumonias [12], although PCP is less common than tuberculosis due to its quick maturation time [14], the prognostic factors that lead to the death of patients with PCP was reported to account for 44% since 2000 in USA, with approximately 1.8% per 100 cases recorded [1]. While in India alone, with the estimated population of 1, 198, 003, 000 almost 18, 795, 363 cases are recorded with HIV infection annually (www.wrongdiagnosis.com), which approximates to 70-80% and perhaps without treatment over 85% of people with HIV eventually develops PCP, hence has become the major killer of people with HIV. Although PCP is now almost in diminutive part of preventable and treatable, still causes death in about 10% of cases [3].

Pneumocystis pneumonia (PCP) is caused by a fungus called *P. jiroveci* which belongs to the Phylum: Ascomycota, Class: Pneumocystidomycetes, Order: Pneumocystidales and Family: Pnuemocystidaceae. *Pneumocystis* contains five major species; *P. oryctologi* from rabbits [5], *P. carinii* and *P. wakefieldiae* from rats, *P. murina* from mice and *P. jiroveci* from humans [10]. Small rRNA subunit of *P. jiroveci* has been established to have a phylogenetic linkage to the fungal kingdom within the ascomycete's fungi and also to that of mammals which was revealed to be distinctly different [6]. It is widely distributed in nature and gets transmitted through air, drinking water and food [22,8]. *Pneumocystis* almost always affects the lungs and in people with HIV sometimes also grows in other parts of the body such as the lymph nodes, bone marrow, spleen, liver and occasionally the eye. Perhaps, People with CD4 cell counts under 200 have the highest risk of developing PCP (www.aidsinfonet.org). When the organism is inhaled, it enters the upper respiratory tract and infects the tiny air sacs called alveoli at the ends of the smaller air tubes (bronchioles) in the lungs, where exchange of oxygen with the blood takes place in the alveoli and therefore transmits the organisms that live in the fluid lining the alveoli.

The most common signs and symptoms include fever, dry cough, fast heartbeat with trouble in breathing and occasionally pain or tightness in the chest that leads to weight loss, malaise and diarrhea (http://www.aidsmap.com/cms1032624.asp). Doctors use a number of different tests to diagnose PCP, such as chest X-rays and measurements of the amount of oxygen in the blood. However, a variety of other infections can cause identical symptoms, and the only way to diagnose PCP

definitively is to look for the *Pneumocystis* organisms themselves that can be detected in sputum (spit and mucus) or fluid recovered from the lung (http://www.aidsmap.com/cms1032624.asp).

Although, *Pneumocystis* has been isolated from monkeys, rats, mice, ferrets, sloths, dogs, cats, sheeps, marmosets and voles with the exception of human [22], research has been hampered by the inability to culture the organism under *In vitro* condition. Perhaps structurally it has been characterized by examining the tissue from the host only, which revealed the presence of cysts or tropic forms [12,8,13,25]. The maturation cycle of *P. jiroveci* has two major forms such as trophozoite and cyst (sporozoite). Dihydrofolate reductase (DHFR) an enzyme encoded by DHFR gene reduces dihydrofolic acid to tetrahydrofolic acid that is required for growth and maturation of sporozoites in *Pneumocystis*. It constitutes 798 BP fragment of DNA encoding DHFR gene [11]. However, many efforts are underway to control the disease caused by *P. jiroveci* and infact the data derived from scientists across world to inhibit dihydrofolate reductase by mutating the gene of dihydrofolate reductase also draws our attention [21,16]. Hence, has provided a major in the control of its abundant growth and also investigating its mechanism of developing resistant to the available drugs viz., Tri-methoprim, Sulfa methoxazole, Dapsone and Pentamidine that leads to many side effects.

In this perspective, an attempt was made to characterize DHFR enzyme responsible for the growth of *P. jiroveci* by both genomic and proteomic approaches, since, the literary survey indicated a lacuna in the structural characterization and its evolutionary relationships.

Methodology

Protein sequence characterization

DHFR sequences of four species (Q96V99- *P. murina*, Q548M5- *P. carinii*, Q96V94- *P. oryctologi* and Q9UUP5- *P. jiroveci*) were obtained from Swiss-Prot database (http://www.expasy.ch/sprot/) and analyzed for its physical and chemical sequence information using Prot-Param (http://www.expasy.ch/tools/protparam.html) with default parameters, which included the molecular weight, theoretical pI, atomic composition, extinction coefficient, half-life time, instability index, aliphatic index and grand average of hydropathicity (GRAVY). Further, the secondary structural characterization was predicted using the online software's like GOR4, HNN and SOPMA (http://www.expasy.ch/tools/) which calculated the amino acid composition of helixes, beta strands, turns and coils.

Phylogenetic analysis

Dihydrofolate reductase sequence of four species were aligned using commercial software Geneious Pro version 4.7.5 to predict the similarity score and identify the evolutionary relationship by pairwise and multiple sequence alignments.

Pairwise alignment

Pairwise sequence alignment method was used to find the best-matching of two query

sequences by Global and Local alignments through Needleman-Wunsch algorithm which aligned the entire length of all query sequences (http://www.biorecipes.com/DynProgBasic/solution.html). Pairwise alignment was done by having *P. jiroveci* and *P. carinii* respectively as a target to identify the conserved region among the four sequences.

Multiple alignments

Multiple sequence alignment incorporated more than two sequences at a time to identify the conserved sequence regions across a group of sequences hypothesized to be evolutionarily related. Such conserved sequence motifs in conjunction with structural information located the catalytic active sites of enzymes that enabled to reveal information on single and multiple gap penalties in the alignment view to identify the node distances of the four species.

Results and Discussion

Primary Sequence Information

The sequence length of *P. carinii* and *P. jiroveci* was found to be the same (206 BP) and was observed to be larger than other two species which contained 186 BP (Table 1). The molecular weight of *P. carinii* was found to be larger (23.88 KD) than the other three species of *Pneumocystis*, which accounted to 23.40 KD in *P. jiroveci*, 21.40 KD in *P. murina* and 21.11 KD in *P. oryctologi* respectively. However, its atomic composition was also found to be higher with amount of nitrogen (292 atoms) and sulfur (9 atoms) in *P. jiroveci*, which could attribute be creating problem in human body, because nitrogen domination strengthens cell wall thickness and thereby posing difficulty in breaking them open during disruption by the antibiotics [23]. The co-efficient value was estimated to be high in *P. carinii* (43430 %) which probably could aid in protein folding and binding affinity by the pathogen [2] therefore leading to the severity of pathogenesis in rats. Since, the Instability index is a measure of stable proteins, the value of less than 40 could indicate its ability to be at stable form under invitro condition [7]. The GRAVY value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids divided by the number of residues in the sequence.

Table 1 : Prot-Param analysis of Dihydrofolate reductase protein sequences of *Pneumocystis.*

S.N	**Name**	**Value**			
		P.jiroveci	***P.carinii***	***P.murina***	***P.oryctolagi***
1.	Number of amino acids	**206**	**206**	186	186
2.	Molecular weight (Daltons)	23409.0	**23883.5**	21401.7	21115.5
3.	Theoretical pI	9.14	9.19	8.74	**9.64**
4.	Total number of negatively charged residues (Asp + Glu)	21	**24**	21	15

5.	Total number of positively charged residues (Arg + Lys)		25	**29**	24	23
6.	Atomic composition	Carbon (C)	1049	**1086**	973	948
		Hydrogen (H)	1666	**1688**	1515	1501
		Nitrogen (N)	**292**	288	259	269
		Oxygen (O)	297	**305**	271	260
		Sulfur (S)	**9**	7	7	**9**
7.	Total number of atoms		3313	**3374**	3025	2987
8.	Instability index		**37.12**	21.36	36.30	25.55
9.	Protein		**stable**			
10.	Ext. coefficient		39085	**43430**	36565	28085
11.	Grand average of hydropathicity *(GRAVY)*		**-0.100**	-0.341	-0.187	-0.168
12.	Estimated half-life	Mammalian reticulocytes, in vitro	**30**	**30**	4.4	4.4
		Yeast, in vivo	>20			
		E. coli, in vivo	>10			

Secondary structure prediction

The structural elements of the predicted secondary structures, revealed (Table 2) the highest value (38.35%) of alpha helix in *P. carinii* through HNN, while the least value of 18.28% was observed in *P. oryctologi* through GOR4 analysis. However SOPMA gave a clear pattern, which indicated 8% difference in the alpha helix pattern for *P. carinii* compared to GOR4 analysis. Moreover, the extended strand was revealed to be higher (29.57%) in GOR4 and lower (12.37%) in SOPMA for *P. oryctologi*. Perhaps, the variation of strand percentage was very high (17%). The Random coils represented a pattern dissimilar to the above analysis, which exhibited high and low values of (60.75%) and (46.60%) respectively through HNN predict in *P. oryctologi* and *P. jiroveci* respectively. Beta turn was observed only through SOPMA which was found to be more in *P. murina* confirming the stability of protein by folding.

Table 2 : Secondary structure prediction of the DHFR protein sequences of *Pneumocystis*.

NAME	SPECIES	GOR 4	HNN	SOPMA
Alpha helix	*P.jiroveci*	21.84%	30.10%	29.61%
	P.carinii	20.39%	**38.35%**	25.24%
	P.murina	26.34%	27.96%	28.49%
	P.oryctolagi	**18.28%**	18.82%	24.73%
Extended strand	*P.jiroveci*	27.67%	23.30%	15.05%
	P.carinii	28.16%	14.08%	16.50%
	P.murina	25.27%	22.04%	12.90%

	P.oryctolagi	**29.57%**	20.43%	**12.37%**
Beta turn	*P.jiroveci*	0.00%		4.85%
	P.carinii			5.34%
	P.murina			**5.38%**
	P.oryctolagi			3.76%
Random coil	*P.jiroveci*	50.49%	**46.60%**	50.49%
	P.carinii	51.46%	47.57%	52.91%
	P.murina	48.39%	50.00%	53.23%
	P.oryctolagi	52.15%	**60.75%**	59.14%

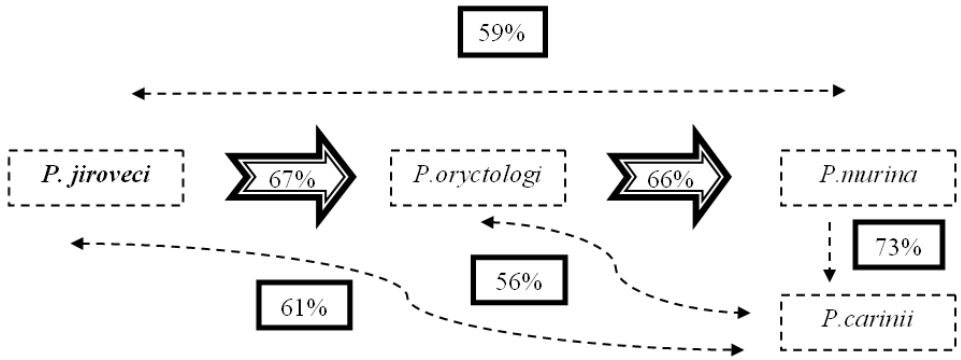
¶ GOR 4 and HNN does not identify beta turn.

Phylogenetic relationships

One to one comparison was performed by Geneious Pro align to compare the evolution among the dihydrofolate reductase protein sequences. It was observed that any species of *Pneumocystis,* when compared with the other three species exhibited similarity of not less than 61%. One species is too divergent to be compared to others, because only this organism that causes human PCP is now named *P. jiroveci* [18].

Pairwise alignments

The pairwise alignment done having *P. jiroveci* as a query to compare with other three species in the order of *P. oryctologi, P. murina* and *P. carinii* revealed 67%, 59% & 61% of relativeness respectively. The same analysis, having *P. carinii* as a query and comparing with the other three species the order of *P. murina, P. jiroveci* and *P. oryctologi* revealed 73%, 61% & 56% of relativity. Based on these analyses, it is inferred that there is a close relationships between *P. jiroveci* and *P. oryctologi* & *P. carinii* and *P. murina*, however, their common ancestor was derived through multiple sequence alignment (Fig.1 & 2).



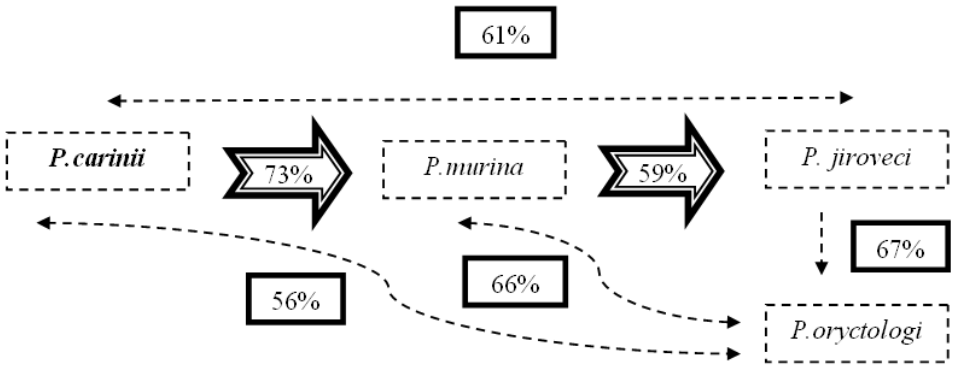


Fig. 1 Phylogenetic Analysis of different species of *Pneumocystis.* (a) Represents the relationship among the species based on *Pneumocystis jiroveci* through global alignment of Geneious Pro. (b) Represents the relationship among the species based on *Pneumocystis carinii* through global alignment of Geneious Pro.

Multiple sequence alignment

Multiple sequence alignment (MSA) was performed to identify the sequence similarity among the species and to evolve their relationships with one another by descending from a common ancestor as observed from Fig. 2 a & b.

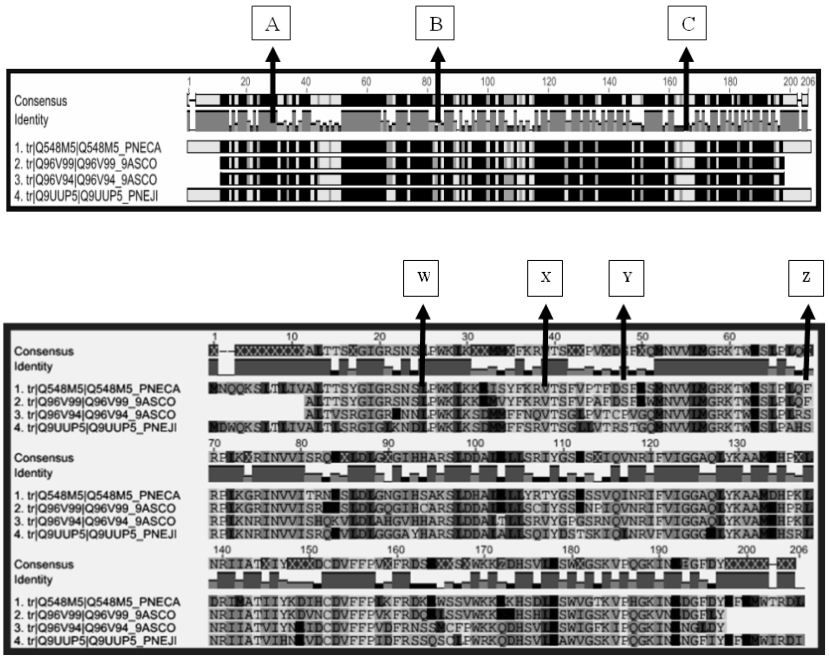


Fig. 2 Geneious Pro Multiple Sequence Alignment for species of *Pneumocystis*. (a) Geneious Pro confers the best relationship among species of *Pneumocystis* as highlighted by the alphabets. (b) Amino acid pattern analysis of multiple sequences.

The consensus layer illustrates the evolutionary relationship among the input sequences which revealed the amount of conservation at consensus sites as indicated by the black and grey shadings. (W - 100% similar; X - 80 to 100% similar; Y - 60 to 80% similar and Z - less than 60% similar. Identity layer demonstrate with letter codes support represented A for identity, B for single gaps and C for multiple gap penalties).

The consensus layer represented with most abundant amino acid residues at each position of the alignment were therefore involved in the important structural and functional role of DHFR protein. The identity layer which represented the arrangement conserved sequences at each column revealed the occurrence of leucine to be 11.2% in *P. jiroveci,* 9.2% in *P. carinii,* 9.7% in both *P. murina* and *P. oryctologi* respectively, indicating the fact that higher level in *P. jiroveci,* could led to other toxicity ill effects such as urine attributing to Maple Syrup Urine Disease (MSUD) to patients with pneumonia [4]. Consensus and identity layers of all the four species showed 100% of leucine similarity at 12 positions (13, 56, 72, 86, 88, 98, 102, 104, 105, 128, 138, 177) and methionine at 5 positions (52, 57, 133, 178, 192) respectively.

Fig. 3 Geneious Pro View of Phylogenetic Tree.
R- Root, N- Nodes, B- Branches, S- Species and C- Clades.
Q548M5 - S1- *Pneumocystis carinii,*
Q96V99 - S2- *Pneumocystis murina,*
Q9UUP5 - S3- *Pneumocystis jiroveci,*
Q96V94 - S4- *Pneumocystis oryctologi.*

The four species arrived from the root measures (R) branched out to 3 with the distances of 0.1356 and 0.0739 for B1 and B2 respectively. Perhaps, third branch (B3) was further diversified to 2 sub branches namely B3A and B3B respectively. These three branches were distanced from the branch length (floating point values). Species was identified as B1S1 (*P. carinii)*, B2S2 (*P. murina)*, B3S3 (*P. jiroveci)* and B3S4 (*P. oryctologi)*, hence S1 and S2 were examined as the first clade (C1) while S3 and

S4 occurred as the second clade (C2). However, C1 was connected from the first node (N1) and therefore was found very near to the root while C2 connected from the second node (N2) was very extensive than C1. Based on these results, it is conferred that C1S2B2 that represented *P. murina* to remain very near to the node of the root were farther away from the node. While the other 3 species such as C1S1B1 (*P. carinii),* C2S4B3B (*P. oryctologi)* and C2S3B3A (*P. jiroveci)* respectively. Since, Neighbor joining algorithm, which is a very fast and best method for tree construction was employed (Fig. 3), it constructed the tree by performing the bootstrapping process 100 times to select the best position of node distance to exhibit 100% accuracy.

Sequence comparison of four DHFR species of *Pneumocystis* identified the different conserved regions in all species. The primary and secondary structure characterization revealed the two elements viz., nitrogen and sulfur to play an important role of strengthening the cell wall of *Pneumocystis.* Nitrogen is a constituent of amino acids in proteins. Sulfur containing amino acids (cysteine and methionine) each plays a distinguished role in the formation of protein structure and increase the life time of *Pneumocystis,* hence, highly extending the persistence of these organisms for a long time in host and deliberately dominating in the environment when it thrives [23]. Higher positive charge residues in *P. jiroveci*, as characterized from primary sequence analysis revealed the hyper activity of dihydrofolate reductase in *Pneumocystis*. The Instability index provided an estimate of the stability of protein, whose value if exceeds 40 is considered to be unstable [7], however, analysis revealed all species to lie below the range confirming the stability of the protein. Currently, Secondary structure analysis confirmed that DHFR is also a complex protein, due to the fact that native aggressive fold configuration depends both on sequence and the amino acids interaction of the protein [17]. Perhaps, the function of the amino acid composition with respect to methionine, cysteine and leucine are believed to play a major role in the structural configuration of the protein and ligand binding activities [24] of the protein. In fact, leucine (L) was found to serve as the major carbon source for sterol biosynthesis in *Pneumocystis* species that is obtained from the host. It is remarkable to note that this particular leucine cannot be synthesized in mammals (human, animals) except plants and microorganisms and however is responsible for the growth of muscle protein [15].

Although, data deciphering the origin of the most extensively studied *Pneumocystis* was unclear [19,20], the current analysis was able to achieve at it. The phylogenetic relationships of four protein sequences by the Neighbor joining method highlighted the conserved regions among the four sequences; it showed two clusters: the first included the genus of *P. murina* and *P. carinii,* which shared a common ancestor, while the second included the genus *P. oryctologi* and *P. jiroveci* respectively. All the analysis declared a close relationship between the clusters, however both extended from a common ancestor, indicating the order of *P. murina, P. carinii, P. oryctologi* and *P. jiroveci* respectively, which has already been evidenced by Keely *et al.,* (2000) [9], according to whom, the sequence of *P. jiroveci* diverged from each other was classified as separate genera. This overall investigation found some targets like methionine, cysteine, and leucine residues with sulfur and nitrogen elements to inhibit the function of DHFR protein. Since, currently there are no best

drug to control these targets in *Pneumocystis*, leaves behind an interesting arena in the future to investigate on the proteome analysis and drug designing for *Pneumocystis.*

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[34] Pentamidine (http://www.nlm.nih.gov/medlineplus/druginfo/meds/a601208.html);
[35] Pneumocystis Genome Project (http://pgp.cchmc.org);
[36] Prot-Param (http://www.expasy.ch/tools/protparam.html);
[37] Swiss-Prot (http://www.expasy.ch/sprot/);
[38] TMP-SMX (http://www.drugs.com/pdr/trimethoprimsulfamethoxazole.html).