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Pilot survey of expressed sequence tags (ESTs) from the asexual blood stages of *Plasmodium vivax* in human patients

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Abstract

Background: *Plasmodium vivax* is the most widely distributed human malaria, responsible for 70–80 million clinical cases each year and large socio-economical burdens for countries such as Brazil where it is the most prevalent species. Unfortunately, due to the impossibility of growing this parasite in continuous *in vitro* culture, research on *P. vivax* remains largely neglected.

Methods: A pilot survey of expressed sequence tags (ESTs) from the asexual blood stages of *P. vivax* was performed. To do so, 1,184 clones from a cDNA library constructed with parasites obtained from 10 different human patients in the Brazilian Amazon were sequenced. Sequences were automatically processed to remove contaminants and low quality reads. A total of 806 sequences with an average length of 586 bp met such criteria and their clustering revealed 666 distinct events. The consensus sequence of each cluster and the unique sequences of the singlets were used in similarity searches against different databases that included *P. vivax*, *Plasmodium falciparum*, *Plasmodium yoelii*, *Plasmodium knowlesi*, Apicomplexa and the GenBank non-redundant database. An E-value of $<10^{-30}$ was used to define a significant database match. ESTs were manually assigned a gene ontology (GO) terminology

Results: A total of 769 ESTs could be assigned a putative identity based upon sequence similarity to known proteins in GenBank. Moreover, 292 ESTs were annotated and a GO terminology was assigned to 164 of them.

Conclusion: These are the first ESTs reported for *P. vivax* and, as such, they represent a valuable resource to assist in the annotation of the *P. vivax* genome currently being sequenced. Moreover, since the GC-content of the *P. vivax* genome is strikingly different from that of *P. falciparum*, these ESTs will help in the validation of gene predictions for *P. vivax* and to create a gene index of this malaria parasite.

Background

Plasmodium vivax is the most widely distributed human malaria and responsible for 70–80 million clinical cases each year and large socio-economical burdens for countries such as Brazil and India, where it is the most prevalent species [1]. Unfortunately, due to the problem of maintaining this parasite in continuous *in vitro* culture, the fact that vivax malaria is not as life threatening as falciparum malaria, the low parasitemias associated with natural human infections and the difficulty of adapting field isolates to growth in monkeys, research on *P. vivax* remains largely neglected. Moreover, the strict species-specificity of the naturally acquired antimalarial protective immune responses, makes it unlikely that a vaccine against *Plasmodium falciparum* will be active against *P. vivax*. Together, these data call for a comprehensive research effort to study *P. vivax*.

A genomics approach was used to accelerate gene discovery in *P. vivax* by constructing a library in yeast artificial chromosomes using parasites obtained directly from a human patient [2]. Indeed, sequencing of a 155,771 bp telomeric YAC from this library revealed the existence of a multi-gene family termed *vir* (*P. vivax* variant genes). *vir* genes are most likely involved in immune evasion and represents 15–20% of the total gene content of the parasite assuming a *vir* gene copy number of 600–1000 copies per haploid genome [3]. Further sequencing of a 199,866 bp internal YAC clone from this same library identified 41 genes in conserved synteny with a region of chromosome 3 in *P. falciparum*, but found the YAC sequence to lack orthologs of the *P. falciparum* genes that code for cytoadherence phenotypes within the same region [4].

Large-scale sequence analysis of two mung-bean nuclease-digested genomic DNA libraries: the Pv MBN library from the Belem strain [5] and the Pv MBN library #30 from the Salvador I strain [6], have also accelerated gene discovery in *P. vivax*. Indeed, comparative *in silico* analyses of GSS sequences from these two libraries with GSS and ESTs sequences from libraries of *P. falciparum* and *Plasmodium berghei*, increased by at least 10-fold the number of predicted *P. vivax* genes. Technical problems with extractions of poly(A) mRNA from *P. vivax*, however, have hampered the construction of cDNA libraries of the parasite destined for high-throughput sequencing [6]. Data on ESTs of *P. vivax* are, therefore, needed to validate these gene predictions and to create a gene index of this malaria parasite. Most important, data on ESTs of *P. vivax* will be key to assist in the annotation of the genome of the El Salvador I strain presently sequenced to fivefold coverage by TIGR [7].

The construction of a *P. vivax* cDNA library obtained with parasite material collected directly from 10 different

human patients in the Brazilian Amazon was recently reported [8]. This paper presents a survey of ESTs from this library, which includes similarity analyses, annotations and assignment of gene ontology terminology.

Methods

Web-based resources

Fasta files and results from all analyses including clustering, BLAST similarity searches against the different databases and GO links of all the ESTs are available at <http://malariadb.ime.usp.br/pvivax-ESTs>.

cDNA clones and sequencing

The cDNA library was constructed from mRNA extracted from parasites obtained directly from 10 different human patients from Belem de Pará in the Brazilian Amazon [8]. High quality double-stranded DNA from 1,184 individual bacterial clones from this library was prepared and used as template in sequencing reactions. Single-pass automated sequencing reactions were performed with T3 (forward) primer and the ABI PRISM BigDye terminator cycle sequencing kit version 2.0 (Applied Biosystems). Poor quality sequences were also sequenced with the T7 primer. Samples were resolved and analyzed in an ABI3700 96-capillary DNA sequencer (Applied Biosystems). Bacterial clones from all the ESTs are available on request via the Malaria Research and Reference Reagent Resource Center (MR4) at <http://www.malaria.mr4.org>.

BLAST analysis, Pfam and HMMs

An automated analysis pipeline was constructed to process the reads (E-Gene – a pipeline generation system, A. Gruber & A.M. Durham, manuscript in preparation). The trace files were initially submitted to Phred [9] for base-calling and quality assignment. Then, sequences were sequentially submitted to a quality filter where accepted reads had to present at least 85 bases with a phred quality above 15 in a sliding window of 100 bp. After vector masking using *default* parameters, end trimming and size filtering (reads with <70 bp were discarded), sequences were checked for bacterial, ribosomal and human contamination and filtered when positive. EST clustering was performed by the program CAP3 using default parameters [10]. The average length of the EST clusters was 586 bp and regions of low complexity were masked using the program DUST (Tatusov & Lipman, unpublished; <http://blast.wustl.edu/pub/dust>) and SEG [11] before submission to similarity searches. Sequences were searched against the following databases: 1) PlasmoDB: *P. vivax* (release date: 12/06/2001; <http://plasmodb.org/restricted/GridddPv.shtml>), *P. falciparum* (release date 10/09/2002; <http://plasmodb.org/restricted/GridddPf.shtml>), and *Plasmodium knowlesi* (release date 10/03/2002; <http://plasmodb.org/restricted/GridddPk.shtml>); 2) TIGR: *P. vivax* (release date: 01/15/2003; preliminary

sequence data was obtained from The Institute for Genomic Research through the website at <http://www.tigr.org/tdb/ezk1/pva1>); 3) Sanger Centre: *P. knowlesi* (release date 18/09/02; these sequence data were produced by the Pathogen Sequencing Group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/P_knowlesi/PKN.contigs.18.9.02; 4) GenBank: *Plasmodium yoelii*, Apicomplexa and the non-redundant (nr) databases (release date 11/27/2002; <http://www.ncbi.nlm.nih.gov/>), by using BLAST [12]. Matches with E-value of $<10^{-30}$ for both BLASTN and BLASTX were considered as putative hits. Pfam [13] domains were identified using ESTwise <http://www.no.embnet.org/Programs/SAL/Wise2/>) and each Pfam domain was mapped to Interpro [14] annotation. Hidden Markov Models (HMMs) were built from alignments generated using Clustal [15]. HMMbuild, part of the HMMer package <http://hmm.wustl.edu/>, was used to generate the HMMs and sequences were searched using ESTwise.

Results and Discussion

P. vivax cDNA library

ESTs have proven an invaluable resource for gene discovery [16], including genes of parasitic protozoa [17–19]. Unfortunately, there are no reported ESTs from any life stage of *P. vivax*, the most widely distributed human malaria. This lack of ESTs from *P. vivax* is undoubtedly the result of the difficulties in working with this parasite species which lacks a continuous in vitro culture system and whose parasitemias in natural or experimental monkey infections are low. To partially circumvent this problem, a cDNA library was constructed using parasites obtained from Brazilian human patients during their acute attacks [8]. Several aspects of the construction of this cDNA library are worth mentioning here: 1) priming can and does occur along *Plasmodium* A-T rich messages and therefore some ESTs could represent different segments of *P. vivax* genes, not necessarily cDNAs synthesized from the 3' ends; 2) genes expressed during all asexual blood stages as well as gametocytes should be represented in this library; 3) before library construction, parasite material from all patients was PCR-screened to exclude the possibility of contamination with *P. falciparum* since this malarial species is sympatric with *P. vivax* in the Brazilian Amazon; 4) it was necessary to pool the mRNAs extracted from parasites of 10 different patients for cDNA library construction; 5) in spite of destroying the human red blood cells prior to library construction, most of the mRNAs (> 60%) represented contaminants of human globin genes. Together, this data clearly exemplifies the difficulties of constructing cDNA libraries from the asexual blood stages of *P. vivax* and reinforces the biological value of the ESTs characterized here.

BLAST analyses

Out of 1,184 clones fully sequenced from both ends, a total of 806 were high quality *P. vivax* ESTs with an average length of 586 bp, which allowed robust similarity analyses. Clustering of the 806 ESTs with CAP3 revealed that they correspond to 571 singlets and 95 contigs representing 666 cluster events. The majority of the contigs (91.48%) comprised 2–3 sequences and the largest one contained 16 sequences. The complete set of 666 cluster events was used in similarity searches against different databases using BLAST (Figure 1). An E-value of $<10^{-30}$ for both BLASTN and BLASTX was used to define a significant database match. Considering this E-value, 641 cluster events were identified by similarity, whereas 25 did not meet this criterion and were considered unidentified. As expected, the vast majority of the cluster events were similar to *P. vivax* and more events matched entries in the databases of *P. knowlesi* than those of *P. falciparum* or *P. yoelii*, even though the genomes of the latter two have been completely sequenced [20,21]. These data are in agreement with a closer phylogenetic relationship between *P. vivax* and monkey malaria parasites than to *P. falciparum* or rodent malaria parasites [22]. Interestingly, 127 ESTs (corresponding to 103 cluster events) were identified in all plasmodia, probably representing ancestral genes involved in essential functions during the asexual blood stages. Fasta files and results from all analyses are available as supplementary material at the Malaria Data-Bank of the University of São Paulo http://malariadb.ime.usp.br/Pvivax_ESTs.

Annotations

For annotations, a BLASTX search was initially performed against the *P. falciparum* genome databases and, for all positive matches with an E-value of $<10^{-30}$, the same annotations were adopted as reported by the international *P. falciparum* consortium and deposited at PlasmoDB (an international consortium was established in 1996 to sequence the genome of the human malaria parasite *Plasmodium falciparum*, strain 3D7. The sequencing centers involved in this research program are: The Institute for Genomic Research in collaboration with the US Naval Medical Research Center, The Sanger Institute, and the Stanford Genome Technology Center at Stanford University. To facilitate the access and divulgation of genomic information, the malaria sequencing consortium established a centralized database, PlasmoDB, <http://www.plasmodb.org>). Using this procedure, 231 ESTs were annotated. A BLASTX search of the remaining ESTs was performed against *P. knowlesi* databases; positive matches with an E-value of $<10^{-30}$ were blasted again against the *P. falciparum* genome and for those entries with an E-value of $<10^{-30}$, the falciparum annotations were adopted (presently, there are no annotations available from the genome of *P. knowlesi*). Fifty-six new ESTs were annotated. This

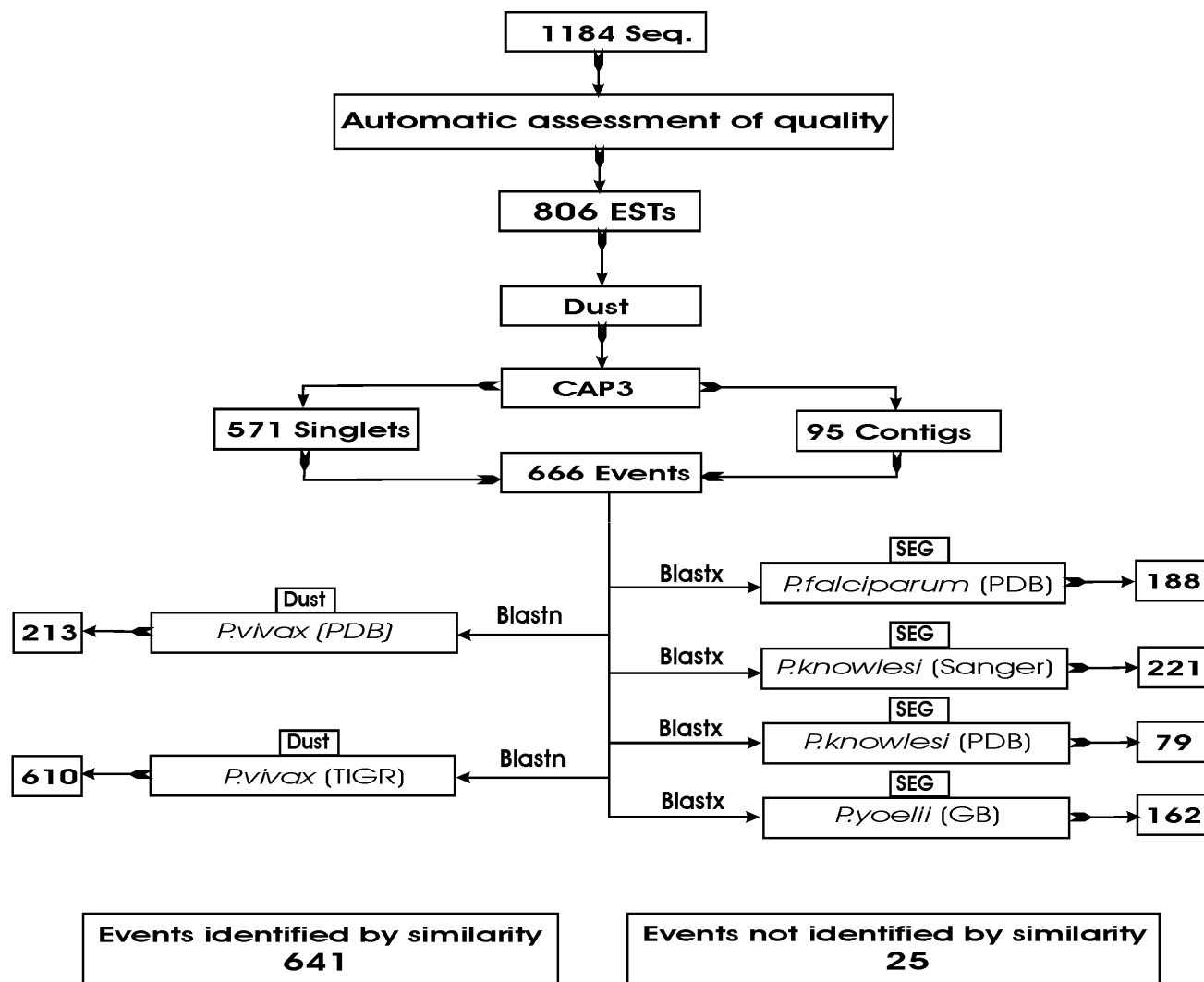


Figure 1
Overview of the pipeline used in the ESTs identification process. Sequences were automatically assessed for quality and removal of contaminants. BLAST similarity searches against PlasmoDB (PDB), GenBank (GB), and TIGR (preliminary sequence data was obtained from The Institute for Genomic Research through the website at <http://www.tigr.org>) data bases were performed after assembling the sequences with CAP3 [10] and masking regions of low complexity with the SEG [11] or DUST (Tatusov & Lipman, unpublished; <http://blast.wustl.edu/pub/dust>) programs. An E-value of 10^{-30} for both BLASTN and BLASTX defined a significant database match.

same procedure was used for the remaining ESTs against *P. yoelii*, Apicomplexa and GenBank nr databases. These latter BLAST searches did not provide additional annotation information (data not shown).

Two other programmes were used to assist in annotations. Firstly, Pfam [13] domains were identified using ESTwise and each Pfam domain was mapped to Interpro annotations. Out of sixty-two ESTs matching to ESTwise, five had

not been identified by BLAST analysis and adopted the Interpro annotations. Secondly, the HMMs performed to predict *vir* genes were unsuccessful. Thus, using three different approaches to assist in annotations, BLAST, Pfam, and HMMS, we were able to confidently annotate 292 ESTs of which 127 were annotated as hypothetical proteins in *P. falciparum*. These results reinforce the value of these ESTs to assist in the future annotation of the *P. vivax* genome where most experimental data is missing.

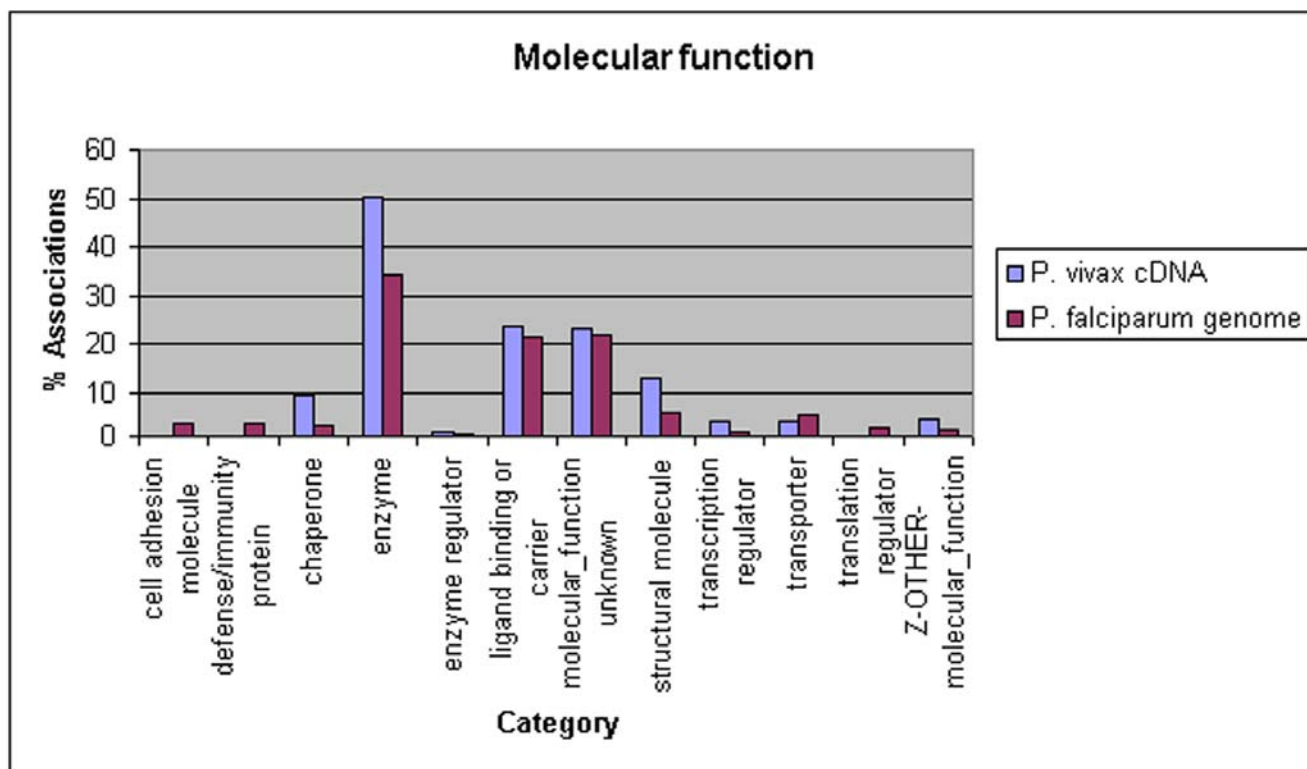


Figure 2
Gene Ontology classification of the P. vivax ESTs Classification of *P. vivax* ESTs according to "Molecular Function" of the GO system [21]. Figures on "Biological Process" and "Cellular Component" can be obtained from http://malariaadb.ime.usp.br/Pvivax_ESTs.

Assignment of GO terms and comparison with P. falciparum

The gene ontology terminology is a controlled vocabulary to describe each product in terms of its molecular function, biological process or cellular [23] and which has been expanded to include specialized and parasite-specific terms [24]. We assigned GO terms manually to 164 ESTs and these results were compared with the annotations from the *P. falciparum* genome project (Figure 2 and supplementary figures at http://malariaadb.ime.usp.br/Pvivax_ESTs). The remaining ESTs did not have sufficient protein similarity to justify GO terminology. During the asexual blood stages, malaria parasites undergo several rounds of invasion, growth, differentiation and mitotic replication. Accordingly, biological processes were mostly related to metabolism such as glycolysis, haem digestion, and ubiquitin-dependent proteasome degradation. Furthermore, this data is in agreement with recent expression and proteome analyses of the asexual blood stages of *P. falciparum* [25–27]. Interestingly, ESTs representing molecules involved in cell adhesion and antigenic variation

were absent in *P. vivax*. Unlike *P. falciparum*, *P. vivax* does not cytoadhere and thus the absence of cell adhesion molecules was not unexpected. In fact, absence of genes coding for cytoadherent molecules had been previously documented from the complete sequence of a 199,866 bp genome region of *P. vivax* [4]. In contrast, the lack of immune evasion molecules within the *P. vivax* ESTs was striking, since *P. vivax* contains the *vir* multi-gene family with circa 600–1000 copies per haploid genome likely involved in antigenic variation [3].

vir genes

vir genes are highly variable and expressed during the asexual blood stages [3]. Moreover, recent data has demonstrated that *vir* genes are expressed in the trophozoite and schizont stages of individual parasites (Fernandez-Becerra and del Portillo, unpublished). Thus, ESTs corresponding to *vir* genes should have been represented in this cDNA library and yet BLAST analysis failed to identify any single one corresponding to *vir* genes. This result is most likely due to the variant nature of *vir* genes and the

E-value threshold of $<10^{-30}$ used throughout this work to define a positive match. Indeed, a BLASTX search of all the *vir* genes described in IVD10 [3] was performed and found that, excluding the E-value of each *vir* BLAST hit to itself, most E-values ranged from 10^{-10} to 9.0. Moreover, manual inspection of BLASTX and ESTwise alignments to previously described *vir* peptide sequences suggests that two ESTs are *vir* transcripts: PVBE06F08.E which is most similar to *vir11* (ESTwise BITS score 95 BLASTX E-value $1.5e-29$) and PVBE12B11.E which is most similar to *vir35*, (ESTwise BITS score 34.54 and BLASTX E-value $5.7e-14$). Accordingly, many of the unidentified ESTs from this cDNA library might correspond to highly variant regions of *vir* genes not identified by BLAST analysis. Alternatively, *vir* messages were not abundant and/or unstable precluding their representation in this cDNA library. Worth of mentioning, an attempt to predict ESTs corresponding to *vir* genes by making HMMs for all the *vir* genes described in the telomere YAC IVD10 was also unsuccessful.

AT-content

The genome of *P. vivax* is remarkably different from that of *P. falciparum* in that it is composed of two major isochores with different GC-contents [22]. Moreover, analysis of the GC-content of two *P. vivax* YAC clones revealed that the AT-content augments towards the telomeres and that genes within the subtelomeric regions are AT-rich (>70%) [3,4]. Interestingly, analysis of the different ESTs from this work revealed an average GC-content of 46.48% for the whole set of ESTs, displaying a large extent of variation ranging from ~20% to 65%. It is, therefore, tempting to speculate that *P. vivax* genes will have genes with varying GC-content depending on the genome region where they reside. Those within internal genome regions will have genes that are GC-rich and mostly related to house-keeping functions whereas those within subtelomeric regions will be mostly AT-rich variant *vir* genes involved in immune evasion. Indeed, the putative *vir* gene sequences [3] and PVBE12B11.E and PVBE06F08.E have a GC-content of 25.1% and 25.9% respectively.

Conclusions

Research on *Plasmodium vivax* has been largely neglected due to the problems of *in vitro* culture of this malaria parasite. Genomics-based approaches including the construction of a YAC library and sequences from it [2-4], the generation of GSS sequences from other genomic libraries [5,6], and the present effort of TIGR in finishing the complete genome sequence of the *P. vivax* El Salvador I strain [7], all are accelerating gene discovery of this human malaria. Presently however, there are no expressed sequence tags available for *P. vivax* in any public databases. This pilot survey of ESTs from the parasite asexual blood stages obtained directly from human patients thus

represents a valuable resource to validate gene predictions, to create a gene index for this malaria parasite and to help in the annotation of the *P. vivax* genome. Indeed, BLASTN analysis using the recently released *P. vivax* database of TIGR <http://www.tigr.org/tdb/e2k1/pva1> scored an E-value of $<10^{-30}$ in 736/806 ESTs of which 404 are *P. vivax*-specific. Moreover, it was possible to confidently annotate 292 ESTs of which 105 already had been predicted [6] and assigned GO terminology to 164. Most important, as these ESTs represent parasite genes expressed during the stages responsible for the pathology associated with vivax malaria, sequence comparisons with the data from the *P. vivax* genome should assist in identifying SNPs for genetic mapping and population diversity studies [28,29].

Author's contributions

EFM performed all BLAST analyses, manually annotated and assigned GO terminology to the ESTs and prepared all web-pages available at http://maliariadb.ime.usp.br/Pvivax_ESTs. CFB screened the cDNA library for human contaminants. CFB and AMBNM coordinated all the sequencing. ALM wrote perl programs to facilitate the analysis of data. AD and AG developed an automated pipeline to process the reads and AG facilitated the process of annotations and contributed significantly to writing the manuscript. NH suggested the process for annotations, made GO comparisons between *P. vivax* and the *P. falciparum* genome and made HMMs to predict *vir* genes. HAP conceived the study, coordinated all aspects of this work and drafted the manuscript.

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