Evaluation of Recombinant \textit{Plasmodium knowlesi} Merozoite Surface Protein-1\textsubscript{33} for Detection of Human Malaria

Fei Wen Cheong, Yee Ling Lau,\textsuperscript{*} Mun Yik Fong, and Rohela Mahmud

Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Abstract. \textit{Plasmodium knowlesi} is now known as the fifth \textit{Plasmodium} species that can cause human malaria. The \textit{Plasmodium} merozoite surface protein (MSP) has been reported to be potential target for vaccination and diagnosis of malaria. MSP-1\textsubscript{33} has been shown to be immunogenic and its T cell epitopes could mediate cellular immune protection. However, limited studies have focused on \textit{P. knowlesi} MSP-1\textsubscript{33}. In this study, an approximately 28-kDa recombinant \textit{P. knowlesi} MSP-1\textsubscript{33} (pkMSP-1\textsubscript{33}) was expressed by using an \textit{Escherichia coli} system. The purified pkMSP-1\textsubscript{33} reacted with serum samples of patients infected with \textit{P. knowlesi} (31 of 31, 100\%) and non-\textit{P. knowlesi} malaria (27 of 28, 96.43\%) by Western blotting. The pkMSP-1\textsubscript{33} also reacted with \textit{P. knowlesi} (25 of 31, 80.65\%) and non-\textit{P. knowlesi} malaria sera (20 of 28, 71.43\%) in an enzyme-linked immunosorbent assay (ELISA). Most of the non-malarial infection (49 of 52 in by Western blotting and 46 of 52 in the ELISA) and healthy donor serum samples (65 of 65 by Western blotting and ELISA) did not react with recombinant pkMSP-1\textsubscript{33}.

INTRODUCTION

Malaria is one of the most important infectious diseases and it causes high global mortality and morbidity. According to World Malaria Report year 2010, 106 countries or areas still have endemic malaria. Approximately 300 million malaria clinical cases are reported each year, resulting in approximately 1 million deaths. Approximately 2.5 billion persons or 40\% of the world’s populations are at risk.\textsuperscript{1,2} The four known species of human malaria parasites are \textit{Plasmodium falciparum}, \textit{P. malariae}, \textit{P. vivax}, and \textit{P. ovale}. \textit{Plasmodium knowlesi} has recently been recognized as the fifth \textit{Plasmodium} species that can cause malaria in human populations.\textsuperscript{3,4} \textit{Plasmodium knowlesi} is naturally found in long-tailed (\textit{Macaca fascicularis}) and pig-tailed (\textit{M. nemestrina}) macaques. \textit{Plasmodium knowlesi} malaria is widely distributed in counties in Southeast Asia, including Borneo,\textsuperscript{5} Myanmar,\textsuperscript{5} Thailand,\textsuperscript{6} the Philippines,\textsuperscript{7} Singapore,\textsuperscript{8} and Vietnam.\textsuperscript{9,10} High numbers of knowlesi malaria have been reported in Borneo Malaysia states of Sabah and Sarawak.\textsuperscript{3,11} Recently, the infection has been detected in several states in Peninsular Malaysia in rural or suburban areas.\textsuperscript{12}

\textit{Plasmodium knowlesi} has a rapid replication cycle, which may cause hyperparasitemia within a short period. Life-threatening complications such as respiratory distress, abnormal liver function, renal failure, and even death may occur.\textsuperscript{13} \textit{Plasmodium knowlesi} could be misdiagnosed as \textit{P. malariae} or \textit{P. falciparum} by microscopy because early trophozoites of \textit{P. knowlesi} morphologically resemble those of \textit{P. falciparum}, and late and mature trophozoites, schizonts, and gametocytes of \textit{P. knowlesi} are generally indistinguishable from those of \textit{P. malariae}.\textsuperscript{14}

Merozoite surface protein 1 (MSP-1) is a high molecular mass (185–225 kDa) glycoprotein expressed on the surface of blood stage merozoites of \textit{Plasmodium} species.\textsuperscript{15} This molecule is involved in the maturation of merozoite and invasion into erythrocytes, thus making it a leading blood-stage malaria vaccine candidate.\textsuperscript{16} MSP-1 undergoes a two-step processing by proteases, resulting in the production of several fragments. The first processing cleaves the MSP-1 precursor polypeptide into four major fragments, which are held together on the free merozoite surface by noncovalent contacts.\textsuperscript{17,18} The second processing further cleaves one of the fragments (MSP-1\textsubscript{42}) into two fragments with molecular masses of 33 kDa (MSP-1\textsubscript{33}) and 19 kDa (MSP-1\textsubscript{19}). The soluble MSP-1\textsubscript{33}, corresponding to the N-terminal region of MSP-1\textsubscript{42}, sheds from the surface,\textsuperscript{19,20} whereas the membrane-bound MSP-1\textsubscript{19} remains anchored to the merozoite and is carried into the new erythrocyte.\textsuperscript{21}

MSP-1\textsubscript{42} is one of the leading candidates for blood-stage malaria vaccines because it is able to induce cell-mediated and humoral immune responses,\textsuperscript{22} and its proteolytic product MSP-1\textsubscript{33} is responsible for the cellular immune protection.\textsuperscript{23} For example, \textit{P. yoelii} MSP-1\textsubscript{33} induces effector T cells that could assist in protective B cells response and mediate significant antiparasitic activity against \textit{P. yoelii}.\textsuperscript{24,25} \textit{Plasmodium falciparum} MSP-1\textsubscript{33} is naturally immunogenic because antibodies against this antigen could be detected in serum of \textit{P. falciparum}-exposed blood donors.\textsuperscript{26} It was further shown that that T cell response and antibodies against MSP1 were induced in human clinical trials by using \textit{P. falciparum} MSP-1, in which epitopes responsible for cytokine production and memory response development were localized to the 33-kDa region of MSP-1\textsubscript{42}.\textsuperscript{27,28}

Hitherto, most of the studies on MSP-1\textsubscript{33} have focused on \textit{P. falciparum}, \textit{P. vivax}, or \textit{P. yoelii}, but few on \textit{P. knowlesi}. A large amount of \textit{P. knowlesi} MSP-1\textsubscript{33} is required for its biochemical, structural, and immunologic studies. In this study, recombinant MSP-1\textsubscript{33} of \textit{P. knowlesi} was expressed and purified by using an \textit{Escherichia coli} system. The recombinant pkMSP-1\textsubscript{33} was evaluated by using sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), enzyme-linked immunosorbent assay (ELISA), and Western blot assays.

MATERIALS AND METHODS

\textbf{Blood samples and serum collection.} Malaria blood samples were collected from patients > 20 years of age who were admitted to the University of Malaya Medical Center in Kuala Lumpur, Malaysia during July 2008–December 2011.
Patient blood samples infected with malaria were confirmed by microscopic examination, polymerase chain reaction (PCR) based on *Plasmodium* small subunit ribosomal RNA genes, and the BinaxNOW malaria rapid diagnostic test (Inverness Medical International, Stockport, United Kingdom). Non-malarial parasitic infections were confirmed by commercial ELISAs. Patient serum samples were categorized as follows: *P. knowlesi* human malaria; non-*P. knowlesi* human malaria (*P. falciparum, P. vivax, P. ovale*); and non-malarial parasitic infection (filaria, amebiasis, cysticercosis, toxoplasmosis, toxocariasis); and healthy donor.

**DNA extraction and construction of recombinant expression plasmid.** *Plasmodium knowlesi* DNA was extracted from blood sample by using a blood extraction kit (QIAGEN, Hilden, Germany). A primer pair, MSP133_F: 5'-GAGCTCGAGAATCACGTGGCTGCATTCA-3' and MSP133_R: 5'-AGAAGCTCCTACATCTGAGTTTGTACATTTAAC-3' was designed based on the *P. knowlesi* H strain MSP-113 nucleotide sequence (GenBank accession no. XM_002258546). Polymerase chain reaction was performed with an initial denaturing step at 95°C for 4 minutes, followed by 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds. A final elongation step at 72°C for 10 minutes was added to the last cycle. The PCR product was purified by using the QIAquick Gel Extraction Kit (QIAGEN), and the nucleotide sequence of the amplified fragment was confirmed by sequence analysis. The amplified fragment was then cloned into pCR 2.1-TOPO plasmid vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The plasmid was then cloned into pCR 2.1-TOPO plasmid vector using the QIAquick Gel Extraction Kit (QIAGEN), and the nucleotide sequence of the amplified fragment was confirmed by sequence analysis. The amplified fragment was then cloned into pCR 2.1-TOPO plasmid vector using the QIAquick Gel Extraction Kit (QIAGEN), and the nucleotide sequence of the amplified fragment was confirmed by sequence analysis.

**Expression of recombinant pkMSP-113.** A single recombinant BL21 (DE3) pLysS colony was inoculated and propagated overnight in Luria-Bertani broth containing ampicillin (100 µg/mL) and chloramphenicol (35 µg/mL) at 37°C with shaking. The overnight culture was diluted with Luria-Bertani broth to an optical density (OD) of 0.1 at 600 nm, and the culture was allowed to grow to an OD600 of 0.4–0.6. The culture was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 37°C for 3 hours, and the digested target fragment was ligated with expression vector pRSET A (Invitrogen) at 4°C overnight. Transformation into maintenance host *E. coli* TOPO10™ strain was performed with the resulting ligation mixture for propagation and maintenance of the plasmid. Before expression, the plasmid was transformed into expression host *E. coli* BL21 (DE3) pLysS strain.

**Optimization of recombinant pkMSP-113 expression conditions.** Concentration of IPTG used for induction and collection time after inductions were monitored to determine the optimum expression conditions of recombinant pkMSP-113. The IPTG concentrations of 0.1 mM, 0.5 mM, and 1.0 mM were used for induction. Cell culture (1 mL) was collected before induction and every hour after induction up to 5 hours. Cells were harvested and analyzed by SDS-PAGE and Western blotting.

**Quantitation of concentration of recombinant pkMSP-113.** The concentration of the purified recombinant pkMSP-113 was determined by using the Bradford protein assay with bovine serum albumin (BSA) as standard (Bradford Assay Kit; Bio-Rad).
Evaluation of recombinant pkMSP-133 in Western blot assays. The purified recombinant pkMSP-133 was tested by Western blotting using serum samples from patients infected with *P. knowlesi* (n = 31) and non-*P. knowlesi* human Plasmodium species, which include *P. falciparum* (n = 11), *P. vivax* (n = 16), and *P. ovale* (n = 1). In addition, serum samples from patients with non-malarial parasitic infections, which include filariasis (n = 4), amebiasis (n = 16), cysticercosis (n = 12), toxoplasmosis (n = 17), toxocariasis (n = 3) and healthy donors (n = 65) were also tested. Polyvinylidene difluoride membrane strips containing blotted recombinant pkMSP-133, with protein amounts ranging from 70 ng to 350 ng, were incubated with different serum samples, followed by incubation with biotin-labeled goat anti-human IgG, streptavidin–alkaline phosphatase, and nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate substrate.

Evaluation of recombinant pkMSP-133 in ELISA. A checkerboard ELISA was performed to determine the working concentrations of the coating antigen (pkMSP-133) and dilutions of patient serum. Recombinant pkMSP-133 was serially diluted starting from the initial protein concentration of 20 μg/mL by using 0.05 M sodium carbonate buffer, pH 9.6. The diluted antigen was coated on 96-well microtiter plates (TPP, Trasadingen, Switzerland) and incubated overnight at 4°C. The wells were washed three times with phosphate-buffered saline containing 0.1% Tween-20. Blocking buffer (1% BSA in PBS) was added into each well and incubated for two hours at 37°C. The wells were then washed three times. Known positive and negative patient serum samples diluted 1:50, 1:80, 1:100, and 1:200 in 1% BSA/PBS were added respectively into each well and incubated for one hour at 37°C. After samples were washed five times, 1:2,500 diluted peroxidase-labeled goat anti-human IgG, streptavidin–alkaline phosphatase, and nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate substrate.

RESULTS

Cloning and expression of recombinant pkMSP-133. Primers were designed to amplify the *P. knowlesi* MSP-133 gene from genomic DNA by PCR, which had an expected size of 705 basepairs. The gene was cloned into expression vector pRSET A and transformed into BL21 (DE3) pLysS host cells. The recombinant clone showed high expression of a protein with an approximate size of 28 kDa (Figure 1), which was absent in the non-recombinant plasmid control.

For determination of optimal expression time and conditions, a time-course experiment was conducted. The recombinant pkMSP-133 could be expressed by induction with all three concentrations of IPTG tested (0.1 mM, 0.5 mM and 1.0 mM). Expression of pkMSP-133 could be observed as early as one hour after induction with IPTG. However, the quantity of protein expressed at one hour after induction was lower compared with the quantity of protein expressed at two hours after induction. Thus, we concluded that recombinant pkMSP-133 could be expressed optimally after two hours of induction with 0.1 mM IPTG at 37°C with constant shaking (250 rpm).

Purification of recombinant pkMSP-133. Purification of recombinant pkMSP-133 was performed by using the MagneHis (Promega) system and ProBond™ (Invitrogen) purification systems (Figure 1). For larger-scale purification,
the OD₆₀₀ of culture after two hours of IPTG induction was 0.756, which contained 3.78 × 10⁶ cells/mL. Lysozyme (8 mg) was used during native cell lysis preparation, and 8 mL of 6 M guanidinium lysis buffer (pH 7.8) was used during hybrid cell lysis preparation. The concentration of the purified pkMSP-1₃₃ was then measured by using the Bradford assay. The concentration of purified pkMSP-1₃₃ was 0.8 mg/mL.

**Western blot analysis of recombinant pkMSP-1₃₃ with human serum samples.** The purified pkMSP-1₃₃ was evaluated by Western blotting using serum samples from persons with Plasmodium knowlesi human malaria, non–Plasmodium knowlesi human malaria, non-malarial parasitic infections, and healthy donors (Figure 2). The recombinant pkMSP-1₃₃ detected all Plasmodium knowlesi infections and showed 100% sensitivity (31 of 31) for Plasmodium knowlesi infections. Results indicated that 96.43% of the 28 samples of Plasmodium vivax, P. falciparum, and P. ovale (non–Plasmodium knowlesi malarial infections) reacted with recombinant pkMSP-1₃₃. The specificity was of Western blotting was 97.44% (114 of 117).

**ELISA of recombinant pkMSP-1₃₃ with human serum samples.** Based on results from of a checkerboard ELISA with protein concentration of 5, 10, and 20 μg/mL, no significant difference was observed between concentrations of 10 μg/mL and 20 μg/mL. Serum samples used in Western blot assays were used in ELISAs with 10 μg/mL of recombinant pkMSP-1₃₃ coated on each well and a 1:80 dilution of serum samples. The sensitivity of the recombinant pkMSP-1₃₃ for Plasmodium knowlesi infection and non–Plasmodium knowlesi malarial infection was 80.65% (25 of 31) and 71.43% (20 of 28), respectively, and the overall sensitivity for malarial detection was 76.27% (45 of 59). Six samples of persons with non-Plasmodium vivax parasitic infections and healthy donor serum samples reacted with recombinant pkMSP-1₃₃, which indicated a specificity of 94.87% (111 of 117). Results of detection of recombinant pkMSP-1₃₃ with serum from persons infected with Plasmodium knowlesi and other parasitic species in Western blot assays and ELISA are shown in Table 1. Sensitivity and specificity of Western blot assays and ELISA using recombinant pkMSP-1₃₃ are shown in Table 2.

**DISCUSSION**

Plasmodium knowlesi replicates and completes its blood stage cycle in 24 hours, the shortest of all human Plasmodium species. Thus, it can lead to quotidian fever and rapid hyperparasitemia, which is potentially severe and fatal if not treated. A previous study showed that protective T cell responses can be induced by epitopes within the dimorphic regions of MSP-1₃₃, instead of MSP-1₄₂, in Plasmodium falciparum because of the low prevalence of T cell responses to MSP-1₄₂. The availability of Plasmodium knowlesi MSP-1₃₃ protein is a prerequisite for its biochemical and immunologic characterization. Thus, in this study, Plasmodium knowlesi MSP-1₃₃ was chosen as the target protein for recombinant expression in an E. coli system.

Recombinant protein has some advantages over native protein. Expression of recombinant protein is rapid, simple, and convenient. Difficulties in long-term in vitro cultivation of Plasmodium knowlesi have led to a limited supply of live Plasmodium knowlesi. Thus, sustainable recovery of sufficient quantities of pure native Plasmodium knowlesi MSP-1₃₃ is a limiting factor. The E. coli expression system was used chosen in this study because of its safety, simplicity, known genetic properties, and efficiency. Foreign DNA can easily be transformed into E. coli with well-established genetic manipulation methods. Also, the E. coli system can express large amounts of protein within a few hours because its growth rate is high. Large amounts of native, non-glycosylated recombinant pkMSP-1₃₃ can be

**Figure 2.** Detection of recombinant Plasmodium knowlesi merozoite surface protein-1₃₃ (pkMSP-1₃₃) with patient serum samples infected with Plasmodium knowlesi and other parasite species. Each Western blot strip, which contained 70 ng of the purified recombinant pkMSP-1₃₃, was tested with serum from each category. Selected samples from each category are shown. Lane 1, Bio-Rad (Hercules, CA) Prestained Broad Range Protein Marker; lanes 2 and 3, serum of patients infected with Plasmodium knowlesi; lane 4, serum of a patient infected with Plasmodium falciparum; lane 5, serum of a patient infected with Plasmodium vivax; lanes 6–10, serum of patients infected with non-malarial parasitic diseases (6 = filariasis, 7 = amebiasis, 8 = toxoplasmosis, 9 = cysticercosis, 10 = toxocariasis), lane 11, healthy donor serum, which served as a negative control.

<table>
<thead>
<tr>
<th>Human serum group</th>
<th>Western Blot</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. serum samples tested</td>
<td>No. positive</td>
</tr>
<tr>
<td>P. knowlesi</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>Non–P. knowlesi human malaria</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>P. falciparum</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>P. ovale</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Non-malarial parasitic infection</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Filarisasis</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Amebiasis</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Cysticercosis</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Toxoplasmosis</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Healthy donor</td>
<td>65</td>
<td>0</td>
</tr>
</tbody>
</table>

*pkMSP-1₃₃ = recombinant Plasmodium knowlesi merozoite surface protein-1₃₃, ELISA = enzyme-linked immunosorbent assay.

**Table 2**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Plasmodium knowlesi infection</th>
<th>Non–P. knowlesi malarial infection</th>
<th>Overall</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western blot</td>
<td>100.00</td>
<td>96.43</td>
<td>98.31</td>
<td>97.44</td>
</tr>
<tr>
<td>ELISA</td>
<td>80.65</td>
<td>71.43</td>
<td>76.27</td>
<td>94.87</td>
</tr>
</tbody>
</table>

*ELISA = enzyme-linked immunosorbent assay; pkMSP-1₃₃ = recombinant Plasmodium knowlesi merozoite surface protein-1₃₃.
produced with this efficient and inexpensive system compared with other expression systems using virus, yeast, or mammalian cells. Soluble and stable recombinant pkMSP-1-33 could be easily expressed and obtained by induction with 0.1 mM IPTG and constant shaking (250 rpm) for two hours at 37°C. For larger-scale protein purification, ProBond® (Invitrogen) native and hybrid conditions were chosen to preserve the protein structure. In the hybrid condition, the protein lysate and column were prepared under denaturing conditions and native buffers were used during the washing and elution steps to refold the protein.

Purified recombinant pkMSP-1-33 was analyzed and probed with serum from patients infected with *P. knowlesi* and other parasite species. A total of 176 serum samples were used to test the purified recombinant pkMSP-1-33 in Western blot assays and ELISA. The sensitivity and specificity of Western blot assays were relatively higher than that of the ELISA. This discrepancy might be caused by borderline activity of patient serum samples in the ELISA. In a study comparing ELISA and Western blotting for detection of antibody against human papillomavirus type 16 E7, Suchankova and others reported positive results in an ELISA but negative results by Western blotting, and they suggested that this result was apparently associated with the borderline activity of serum samples, for which some of OD values were just below or above the cutoff value. A similar situation occurred in our study. Some serum samples from persons infected with *P. knowlesi* and from persons infected with non- *P. knowlesi* Plasmodium species had OD values just below the cutoff value, and serum samples from persons infected with non-*Plasmodium* parasites had OD values just above the cutoff value. However, there are several advantages of the ELISA over Western blot assays. An optimized ELISA requires lower volumes of serum for each reaction compared with Western blot assays, and this factor could be crucial for small volume samples. Also, the ELISA can quantitatively determine the antibody titer for serum, as the OD value of the sera directly resembles the titer of antibody.

The reactivity of recombinant pkMSP-1-33 with serum from persons with non-*P. knowlesi* human malaria could be explained by cross-reactivity caused by their high identities and similarities in MSP-1-33 DNA and amino acid sequences (Table 3). Valderrama-Aguirre and others showed that antibodies generated in mice against *P. vivax* MSP-1 fragment known as Pv200L recognized and cross-reacted with *P. falciparum* recombinant MSP-1 P/190L fragment, which is the homologous region to Pv200L, indicating the sharing of B cell epitopes by these two MSP-1 proteins. The *P. vivax* MSP-142 (pvMSP-142) protein, which shows 71% homology with *P. cynomolgi* MSP-142 (pcMSP-142), is able to induce similar level of protection during *P. cynomolgi* challenge in rhesus monkeys because of the immunologic and structural relatedness of these proteins. Antibodies against several antigens of *P. cynomolgi* and *P. vivax* have been shown to cross-react in different studies. Thus, *P. knowlesi* MSP-1-33 which has amino acid similarity of 82%, 60%, and 70% with that of *P. vivax*, *P. falciparum* and *P. ovale*, respectively, could be sharing some common epitopes with these human *Plasmodium* species.

Another explanation for the reactivity of recombinant pkMSP-1-33 with serum from patients with non- *P. knowlesi* human malaria and non-*Plasmodium* parasitic infections is the possibility of previous exposures or infections with *P. knowlesi*. Wipasa and others have demonstrated that antibodies against malaria infection were stably maintained over periods of more than five years after the last known malaria infection. Thus, antibodies against *P. knowlesi* could still be detected by Western blotting or ELISA after many years of acute infection. It was noted that one of malaria serum samples did not react with the recombinant pkMSP-1-33. Although the reason for this observation is unclear, it was likely that the serum sample was collected before the production of antibodies against the malaria parasite.

Based on these results, pkMSP-1-33 may be useful in general serodiagnosis of malarial infections, such as determination of prevalence and endemicity of malarial infection in a population and blood donor screening. It will be particularly useful in seroepidemiologic screening in human and macaque populations in malaria-endemic areas such as Sabah and Sarawak, Malaysia.

In conclusion, recombinant pkMSP-1-33 was successfully expressed by using the *E. coli* system under optimal conditions. Large amounts of protein could be obtained to facilitate work on the application of *P. knowlesi* MSP-1-33 in immunodiagnosis and vaccination designs for malarial infections. There is a possibility that pkMSP-1-33 could cross-react with other *Plasmodium* species. Therefore, further evaluation with immune serum samples against MSP-142 and/or MSP-1-33 of non-*P. knowlesi* *Plasmodium* species should be performed.

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**REFERENCES**


| *Plasmodium knowlesi* nucleotide and amino acid identities and similarities compared with other human *Plasmodium* species* |
|---|---|---|---|
|  | Nucleotide identities (%) | Amino acid identities (%) | Amino acid similarities (%) |
| *P. vivax* | 74 | 70 | 82 |
| *P. falciparum* | 48 | 41 | 60 |
| *P. malariae* | 55 | 48 | 71 |
| *P. ovale* | 58 | 49 | 70 |

* Amino acid similarity was calculated based on their characteristics, which include charge and hydrophobicity.


