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Review

Contribution of *Plasmodium* immunomics: potential impact for serological testing and surveillance of malaria

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Abstract

Introduction: *Plasmodium vivax* and *P. knowlesi* account together for a considerable share of the global burden of malaria, along with *P. falciparum.* However, inaccurate diagnosis and undetectable asymptomatic/submicroscopic malaria infections remain very challenging. Blood-stage antigens involved in either invasion of red blood cells or sequestration/cytoadherence of parasitized erythrocytes have been immunomics-characterized, and are vital for the detection of malaria incidence.

Areas covered: We review the recent advances in *Plasmodium* immunomics to discuss serological markers with potential for specific and sensitive diagnosis of malaria. Insights on alternative use of immunomics to assess malaria prevalence are also highlighted. Finally, we provide practical applications of serological markers as diagnostics, with an emphasis on dot immunogold filtration assay which holds promise for malaria diagnosis and epidemiological surveys.

Expert commentary: The approach largely contributes to *P. falciparum* and *P. vivax* research in identifying promising non-orthologous antigens able to detect malaria incidence and to differentiate between past and recent infections. However, further studies to profiling naturally acquired immune responses are expected in order to help discover/validate serological markers of no cross-seroreactivity and guide control interventions. More so, the application of immunomics to knowlesi infections would help validate the recently identified antigens and contribute to the discovery of additional biomarkers of exposure, immunity, or both.

Keywords: Malaria, *Plasmodium*, immunomics, serological markers, serodiagnosis and surveillance, dot immunogold filtration assay.

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1. Introduction

Over the years, *Plasmodium falciparum* (Pf) has long held the record for the morbidity and mortality rates of malaria in populations of tropical and subtropical conditions. But recently, unprecedented reports of severe and complicated malaria caused by *P. vivax* (Pv) [1-4] and *P. knowlesi* (Pk) [5-9] from several regions across the world, have highlighted the growing burden of both vivax and knowlesi malarias. As such, among the five species of *Plasmodium* that regularly infect humans, Pv and Pk together account for a considerable share of the global burden of malaria [6, 10-13], along with Pf [14]; while *P. ovale* (Po) and *P. malariae* (Pm) are known to generally cause a milder form of malaria [15]. The analysis of the complete genome and transcriptome of Pf [16], Pv [17], and Pk [18], revealed a share of 80% of protein-encoding genes which could be identified in the three species. It also provided broad insights into parasite biology, as well as into parasite virulence in the disease severity and transmission patterns. In terms of disease severity, variant surface antigens (VSAs) encoded by a variant gene superfamily including *var* and *kir* (the two major variant gene families within Pk), *var*, *stevor* and *rif* (Pf's three major variant gene families), and *vir* (the major variant gene family within Pv), located on telomeric and subtelomeric regions of the parasite's chromosomes, are the key proteins used by these species to escape host immune system by antigenic variation [14, 19, 20].

The release of the whole-genome sequence of Pf, Pv, and Pk, enabled major advances in malaria post-genomic research. Important differences among gene families found in these three species should be reflected in differences in biology, pathogenicity and clinical features. For example, unlike falciparum, Pv and Pk Duffy-binding proteins (DBPs) use the Duffy blood group antigens as receptors on the surface of red blood cells (RBCs) to selectively invade reticulocytes (young RBCs) [21-23]; even if it was thought that Pv or Pk merozoites are able to interact with Duffy-negative human RBCs, studies showed only normal apical orientation but no invasion since a junction does not take place [21, 24]. In addition, the deletion of gene encoding PkDBP results in the complete inability of Pk merozoites to invade Duffy-positive human RBCs [23], showing that DBP is crucial for invasion during the asexual blood stage of Pv and Pk. More so, parasite sequestration and parasite-mediated rosetting of uninfected RBCs are unique characteristics of a phenomenon central to the pathogenesis of severe falciparum malaria [14]. Pf-infected RBCs together with the parasite's variant surface antigenic ligands – RIFINs and STEVORs, adhere to the vascular endothelium and cause multi-organ failures, making them major factors of virulence and severity of falciparum malaria. Similarly, this phenomenon is also observed with both Pv and Pk VSA families – VIRs and SICAs, respectively. There have been revelations that VIR proteins are expressed on or near the surface of infected RBCs and therefore mediate

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rosetting and cytoadherence of Pv-infected reticulocytes, especially to the ICAM-1 (intracellular adhesion molecule-1) endothelial cell receptor [25-27]. Thus, rosetting is a frequent cytoadhesive phenotype in Pv infections that may contribute to the development of anemia. SICA antigens, encoded by Pk SICA*var* gene family, are also associated with parasite virulence [8]. Infected RBCs with Pk are able to bind to the inducible endothelial receptors – ICAM-1 and VCAM (vascular cell adhesion molecule), although in a variable manner, but none bound to CD36 receptor [28]. This suggests the possibility of cytoadherence to ICAM-1 if this receptor is up-regulated on brain endothelium, but further studies are needed to better understand the pathophysiology of severe knowlesi malaria.

In parasitological terms, Pf maintains high parasite biomass within the host, as opposed to the low parasite burden observed in cases of Pv. Hypnozoites are commonly associated with vivax malaria relapses, which persist in the liver for long durations in dormant forms (latent liver stage) and remain undetectable with current diagnostic tests [29]. *Plasmodium knowlesi* shares a close phylogenetic relationship with Pv [17, 30] and morphological characteristics resembling those of both Pf and Pm [31]. A notable feature of Pk is a similar shape of early trophozoite forms to those of Pf, whereas all other stages recall the aspect of Pm, making the parasitological examinations of Pk difficult. However, Pk differs from Pv by the absence of hypnozoites and its quotidian asexual cycle. The most severe clinical consequence of knowlesi malaria is related to its daily replication cycle and, when not diagnosed timely and countered by an adequate therapy, it may rapidly reach potentially lethal levels of parasitemia [9, 30]. From another point of view, pramaquine-based therapies for the treatment of hypnozoites are contraindicated in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, which causes acute hemolytic anaemia [32]. Hence, without accurate diagnosis for subsequent appropriate and timely treatment of suspected malaria cases, there are high risks of fatalities [6, 8, 9], or drug resistance may occur, as some patients would have not been given the right treatment to their ailments. Therefore, it is vital to improve the current diagnostic tools of malaria to ensure safe deployment of appropriate treatment regimen, as well as to provide a better knowledge on epidemiological assessments to guide control interventions.

The control and elimination strategy of malaria is also fundamentally dependent on sensitive and specific serological testing to treat both blood and liver stages malaria parasites, as well as a need for new surveillance tools which can evaluate epidemic statuses of the infection [33-35]. In the past, the identification of serological markers for malaria surveillance has been hampered by the limited availability of promising approaches [33, 36]. With the advent of immunoproteomics, conventional techniques in immunology such as two-dimensional gel electrophoresis, western blotting, and enzyme-linked immunosorbent assay (ELISA), have given more insights on host immune responses to malaria antigens [37].

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Recently, genomics, transcriptomics, and proteomics have undoubtedly boosted immunomics for the characterization of potential antigen targets of host immunity, important tools for serological testing. Protein array, an immunomics-based technology have been well documented for its contribution in providing a better understanding of host-pathogen interactions – host humoral immune responses to malaria infections. High-throughput in-fusion cloning (IFC) of target genes, wheat germ cell-free (WGCF) protein expression in *ex-vivo* transcription and translation bilayer reaction systems, probing of proteins in a HTP manner with sera of malaria subjects, are key components that facilitate *Plasmodium* immunomics [38]. The approach has been shown to efficiently screen large amount of human parasite antigens, and has proven to be an innovative and promising tool for antigen discovery, and its effectiveness is unquestionable [36, 39]. *Plasmodium* immunomics has intensively profiled host humoral immune responses to Pf [40-49], while its application in vivax malaria research has slowly advanced [43-46, 48, 50-57]. However, less or nothing is known about immunomics of knowlesi malaria, since the infection has been long considered as benign, and, only few blood stage antigens have been identified very recently using ELISA [58, 59].

In this paper, *Plasmodium* immunomics is reviewed, with an emphasis on the discoveries of promising falciparum and vivax serodiagnostic antigens. Their practical applications as serodiagnostics for clinical/field settings-based screening and epidemiological surveys are also assessed. We conclude with a brief description of the processes involved in the development of dot immunogold filtration assay (DIGFA), a convenient and reliable trans-flow serodiagnostic tool that may leverage these antigens.

2. *Plasmodium* immunomics and characterization of host immune responses

Rapid acquisition of immunity (immunoglobulin (Ig) G) to Pf and Pv has been observed in patients infected with the parasites, and immune responses involve both humoral and cellular components against both pre-erythrocytic and blood-stage antigens [36, 45]. However, although the mechanisms by which hosts respond to *Plasmodium* exposure and acquire immunity remain unclear, naturally acquired immunity is related to host responses to the parasite antigens.

Described as the reactivity of protein targets with antisera from parasite-exposed hosts, where antigen proteins are fixed probes and serum antibodies are targets, immunomics uses protein arrays to screen host sera and detect the magnitude and breadth of antibody responses to proteins regardless of their abundance [36]. In the last ten years, *Plasmodium* immunomics has been more extensively investigated than other human parasite, and the approach has emerged as an excellent application of the parasite's

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genome, in identifying a large repertoire of candidate antigens as serological markers of exposure, immunity, or both [36]. Until recently (August 2018), when searched PubMed database and after unrelated references and reviews or comments were excluded, forty research articles were found to have used this approach to screen blood-stage antigens of both Pf and Pv (unshown data), whereas none have been recorded for knowlesi immunomics. With respect to the source of anti-parasite sera for profiling host humoral immune responses to malaria molecules, serological markers are selected by comparing different cohorts of exposed and unexposed serum samples, where host groups with blood-stage infection in the absence of clinical symptoms are thought to develop acquired clinical immunity compared to symptomatic infections [36, 45]. Therefore, the approach allowed to select for leading biomarkers for serological testing purposes (see Tables 1 and 2). However, the diagnostic utility of many of these candidate antigens needs further validation in more conventional assays.

Helb *et al*. evaluated antibody responses to 856 Pf proteins using sera from 186 sub-Saharan children and identified novel serological markers of malaria exposure and prevalence which informed on an individual's recent or past exposure to the parasite [49]. Humoral responses to three novel Pf serological markers including two *Plasmodium* exported proteins (HYP2 (PF3D7_1002000) and GEXP18 (PF3D7_0402400)), and an exonuclease (PF3D7_1106300), identified as the top three most predictive of days since last infection, accurately classified whether an individual had been infected within the last 30, 90, or 365 days. By contrast, antibody responses to other six Pf antigens including two *Plasmodium* exported proteins (HYP2 (PF3D7_1002000) and PF3D7_0801000), dihydrolipoamide acyltransferase component E2 (PF3D7_1020800), acyl-CoA synthetase (PF3D7_0731600), early transcribed membrane protein 5 (ETRAMP5 (PF3D7_0532100)), and circumsporozoite protein (CSP (PF3D7_0304600), informed on an individual's malaria incidence in the last year (see Table 1).

Recently, Chen *et al.* analysed natural antibody responses to Pv infection by screening the sera of vivax malaria patients (n=15) versus those of uninfected individuals (n=10) against 1936 Pv proteins [50]. They identified a total of 151 highly immunogenic antigens, including five well-characterized antigens of Pv: ETRAMP11 (PVX_090230), apical merozoite protein, (Pv34 (PVP01_0529100)), subtilisin-like protease 1 (SUB1 (PVX_097935)), rhoptry-associated protein 2 (RAP2 (PVP01_1033400)), and merozoite surface protein 4 (MSP4 (PVX_003775)) [44]. Importantly, most of these serological markers are membrane proteins or secreted-types, as they were found to possess a transmembrane domain or a signal peptide. In addition, the authors argued that among the identified antigens (n=151), at least 40 (displaying positive antibody reactions with at least 80% of patient sera used) have serodiagnostic potential for malaria surveillance. Besides, Lu *et al*.

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constructed protein arrays by screening 152 Pv proteins with sera from 22 vivax malaria patients and 10 unexposed individuals. The study revealed 44 potential serological markers, of which rhoptry-associated membrane antigen (RAMA (PVX_087885)), tryptophan-rich antigen (Pv-fam-a (PVX_092995), expressed uniquely in Pv without a homologue in Pf), Phist protein (Pv-fam-b (PVX_093680)), and circumsporozoite-related antigen (EXP1 (PVX_091700)), exhibited positive antibody responses with at least 80% of patient sera used [56]. Additionally, promising Pv serological markers of exposure and immunity have been widely identified recently. The antigens include but not limited to: transmission blocking target antigen a 6-cysteine protein (Pv41 (PVX_000995)); MSP1 (PVX_099980); MSP3 (PVX_097720); MSP7 (PVX_082650, PVX_082700, PVX_082680, and PVX_082655); MSP8 (PVX_097625), MSP10 (PVX_114145); membrane protein pf12 precursor (Pv12 (PVX_113775)); hypothetical conserved proteins (PVX_115450 and PVX_087670); and so on (see Table 2).

Immunomics has been alternatively also used to assess the epidemiology of both falciparum and vivax malarias. Baum *et al*. used the approach to detect exposure to Pf and Pv in both asymptomatic infections and infections with submicroscopic parasite levels in Tak, Thailand. The authors arrayed numbers of 500 Pf and 515 Pv recombinant proteins for antibody response profiles with infected sera, and identified 281 and 177 seropositive antigens of falciparum and vivax, respectively. Importantly, 100% seropositivity to at least 54 antigens have been recorded, with ETRAMP5 (PF3D7_0532100), heat shock protein 70 (PF3D7_0831700), MSP2 (PF3D7_0206800), MSP4 (PF3D7_0207000), plasmodium exported protein (PHISTc (PF3D7_0801000)), and merozoite surface protein (MSA180 (PF3D7_1014100)), the most frequently recognized as antigens of exposure for Pf; while ETRAMP (PVX_090230), major blood stage surface protein Pv200 (MSP1 (PVX_099980)), MSP8 (PVX_097625), MSP10 (PVX_114145), sexual stage antigen s16 (PVX_000930), transmission blocking target antigen (PVX_000995), to mention a few, were the frequently identified serological markers of Pv [44]. All reactive plasma samples probed in these and other immunomic studies to Pf [40-49] and Pv [43-46, 50-57] are evidence of exposure to *Plasmodium* parasites in sample donors, and provide a rich source of promising antigens with potential for serological testing of malaria. Tables 1 and 2 describe both Pf and Pv leading serological markers of exposure, immunity, or both, which have been newly identified or validated using immunomics. These candidate antigens are annoted according to their access number in the PlasmoDB (http://plasmodb.org/plasmo/).

To date, immunomics has not been applied to identify Pk antigens. Two serological markers do existed for Pk: PK66 (apical membrane antigen 1 (PkAMA1 (PKNH_0931500)) [60] and PkSPATR (secreted protein with altered thrombospondin repeat) [61]; but, they

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have a high level of sequence homology with orthologues from other species of *Plasmodium*, particularly in sharing 86% and 85% identity respectively in their sequence of amino acids with Pv. As such, they are not appropriate to identifying species-specific antibody responses, especially in areas where Pk and Pv are co-endemic. Very recently, using *in-silico* data mining tools for designing and expressing Pk recombinant proteins, ELISA has been used to identify and validate a novel panel of Pk biomarkers of serological exposure. These antigens include: PkSERA3 antigens 1 and 2 (serine repeat antigens 1 and 2 (PKNH_0413400)); PkSSP2/TRAP (thrombospondin-related anonymous protein (PKNH_1265400)); and PkTSERA2 antigen 1 (PKNH_0413500), among which the PkSERA3 antigen 2 showed higher antibody responses to sera from individuals of East Malaysia where Pk is endemic [58, 59].

3. *Plasmodium* immunomics for malaria surveillance

Immunomics is also being alternatively used for the detection of *Plasmodium* exposure and prevalence, especially in patients with asymptomatic infections and infections with submicroscopic parasite levels. In other words, it helps extend the seroepidemiologic knowledge of malaria and potentially generates rich epidemiologic surveillance data to guide and evaluate malaria control interventions. Baum and colleagues [44, 48] have assessed the epidemiology of falciparum and vivax malaria in Tak, Thailand, by detecting exposure to Pf and Pv in both asymptomatic and submicroscopic infections. Sample donors, from Tak, were: i) asymptomatic (>90%) and submicroscopic (100%) residents; and ii) clinic patients with submicroscopic parasite levels (~25%). When Pf and Pv recombinant proteins were probed with hundreds of sera from the residents and clinic patients, the authors found that all the parasitemic and non-parasitemic individuals had antibody response, evidence of malaria incidence, with 100% seropositivity to at least 50 protein targets. Therefore, evidence for the prevalence of *Plasmodium* species is higher than that estimated by microscopy, resulting in ongoing transmission at microscopy-undetectable levels, as asymptomatic/submicroscopic infections are not detected and treated.

Meanwhile, in India [46], analysis of serological responses to Pf and Pv have been conducted at three sentinel sites (Raurkela, Nadiad, and Chennai), by probing the parasites' blood-stage molecules with serum samples from both symptomatic and asymptomatic individuals. Indian serum samples were nearly 100% seroreactive to at least 250 Pf and Pv antigens, and have contributed in providing a better understanding of naturally acquired immunity to these species of *Plasmodium* in the region. Similarly, immunomics-based assessments of malaria epidemiology have been carried out in Haiti [47], Uganda [49], and in other parts of the world [45], to mention a few, and have characterized serological

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responses, evidence of parasite exposure and prevalence. The detection of malaria exposure in asymptomatic patients and individuals with submicroscopic parasite levels is vital to argue for targeted surveillance approaches, especially in low-transmission settings, as well as to facilitate discoveries of candidates for serodiagnostic tools to support malaria elimination efforts.

4. Applied value of serological markers for malaria diagnosis and surveillance

4.1 Antigen-based serodiagnostic tests for malaria

Serological assays are similar to microscopy in terms of ease of use and turnaround times. Despite the time required for development and also the persistence of IgG antibodies, serological testing is more sensitive and specific than parasitological examinations when appropriate seromarkers are used. Antibody testing becomes important for: individuals whose blood smears are unable to differentiate the species of *Plasmodium*, or to identify malaria species-specific parasites from other species of parasites (e.g., as *Babesia*); and for malaria patients presenting with low parasitemia and/or asymptomatic infections, proof of a history of exposure to malaria parasites and a source for the transmission of the parasite.

Serodiagnosis of malaria is usually based on the detection of antibodies against blood stage malaria parasites. The detection of malaria antibody could therefore help understand the basis of malaria transmission, even in the absence of more comprehensive information. In recent years, with the rapid development of immunoproteomics, malaria research has been focused on alternative immunoassays to improve tools for the diagnosis of the infection. Blood-stage antigens are promising serological markers of exposure to malaria parasites with potentials to accurately estimate when an individual has been infected with the parasites. With the knowledge that *Plasmodium* proteomics and immunomics have equipped researchers, the detection of malaria antibody has been improved, and becomes vital especially for: screening blood donors; testing an individual (patient) from a malaria endemic area or a patient who has been recently treated for malaria but in whom the diagnosis is questioned; and clinical/field settings-based screening and epidemiological assessments. In Table 3, we discuss serological assays that are commonly used for the detection of antibodies against malaria parasites. These tests mainly include: protein array, immunofluorescence antibody testing (IFA); falcon assay screening test ELISA (FAST ELISA); Dot ELISA; Indirect ELISA; lateral-flow immunochromatographic assays (e.g., as dipsticks); and trans-flow immunogold assays (e.g., as DIGFA).

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Table 3. Antigen-based diagnostic tests for the detection of malaria antibodies.

Method	Principle	Applicability	Merits	Handicaps
Protein array	Ag is coated onto an array slide and probed with individual serum; fluoresce-conjugated anti-human antibody (Ab) is used to detect immune response by an array scanner.	• Costly and not applicable in field conditions; • Clinical/laboratory blood screenings, or epidemiological assessment.	• High-throughput; highly sensitive and specific; qualitative and quantitative. • Small amount of serum samples (1:100 – 1:200).	• Need of laboratory equipment; laborious; • Difficulty of storage and application of fluorescence conjugates in field conditions
IFA	Homologous Ab of *Plasmodium*- infected individual serum forms an antigen (Ag)-Ab complex with Ag fixed on slide; fluorescein-labelled anti-human Ab is used to give an apple green colour.	• Costly and not applicable in field conditions; • Clinical/laboratory blood screenings, or epidemiological assessment.	• Evidence on the extent and degree of endemicity, and on the period of infection; • High sensitivity and specificity.	• Need of fluorescent microscope; time consuming; • Difficulty of storage and application of fluorescence conjugates in field conditions.
FAST ELISA	Molded beads on stick are coated with Ag, then immersed into individual serum, and finally exposed to enzyme-conjugate and substrate.	• Cost-effective and not applicable in field conditions; • Clinical/laboratory blood screenings.	• Sensitive, short assay time (about twenty minutes), and ease operation; • Quantitative sensitivity.	• Need of laboratory equipment; • Difficulty of storage and application of enzyme-conjugates in field conditions.

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Dot ELISA	Ag is applied onto a nitrocellulose dot membrane (DM) and incubated with individual serum; enzyme-conjugated Ab and substrate are applied respectively to give a coloured dot readily visualized.	• Affordable and appropriate for low-income settings; • Clinical/field use, or large-scale screenings.	• Specific and sensitive: reduction of the binding of non-specific proteins by DM binding matrix; • Rapid and ease of use; qualitative and quantitative.	• Difficulty of storage and application of enzyme-conjugates in field conditions; • Time consuming
Indirect ELISA	Ag is coated onto the bottom of a 92-well plate and probed with individual serum; conjugated secondary Ab and substrate are used respectively, to give colour change for detection.	• Costly and not reproducible in the field; • Clinical/lab use, or large-scale screenings.	• Qualitative and quantitative; high sensitivity and specificity; • Small amount of serum samples (1:100 – 1:200).	• Need of ELISA microplate reader; laborious; • Difficulty of storage and application of enzyme-conjugates in field conditions.
Immuno-chromatographic assays	Ag is immobilized on the surface on a reaction membrane–strip; serum sample is applied and migrates to a conjugate pad to form a complex which reaches the strip by capillarity flow and gives a colour change for detection.	• Cheap and convenient for in low-income settings; • Clinical/field use, or large-scale screenings;	• Reliable, rapid, and ease of use and result interpretation (both qualitative and quantitative); • Storage at room temperature and no need of specialized or costly equipment.	• Assay time a bit long (about fifteen minutes); • Need of various components (e.g. sample pad, conjugate pad, strip, etc.) for the construction of the device.
DIGFA	Ag is attached on a DM; serum/whole blood sample is	• Cheap and convenient for in low-income	• High specificity and sensitivity: reduction of the binding of non-	• Large volume of samples (up to 20 µl of serum or 40 µl of

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applied, followed by colloidal gold conjugated anti-human Ab to give a red dot visible to the naked eye, indicating the degree of reactivity.	settings; • Clinical/field use, or large-scale screenings;	specific proteins by DM matrix. • Very fast and convenient, ease of use, and qualitative and quantitative.	whole blood).

4.2 Applicability of *Plasmodium* blood-stage antigens for reliable detection of anti-malaria antibodies

Serological testing remains the gold standard for diagnosis in case biologic samples present some limitations or are unavailable. *Plasmodium* proteomics and immunomics have greatly helped improve profiling of antibody responses to identify promising sensitive and specific antigens for serological test purposes, especially in terms of cross-seroreactivity. With respect to Tables 1 and 2, orthologous genes in Pf and Pv have been identified to not sharing cross-reactive antigens reciprocally. For example, antigens encoded by PF3D7_0532100, PF3D7_0207000, and PF3D7_0930300, share orthologs in Pv, but have been shown to be recognized only in Pf infections; similarly, some Pv antigens (encoded by PVX_115450_1o2, PVX_090230_1o2, and PVX_087670_1o1) that have orthologs in Pf have been shown to be recognized in Pv mono-infections [45]. Also, leading non-orthologous species-specific antigens between these two species have been characterized (e.g., as PF3D7_0206800, PF3D7_1036400_e2s2, PVX_092995_2o2, PVX_097720, and PVX_003775_2o2). However, it is expected that further studies in more conventional assay formats validate their applicability. Thus, cross-seroreactivity with antigens that share common epitopes and which usually leads to false-positives and misdiagnosis could be significantly minimized or avoidable, especially in regions where more than one species of *Plasmodium* is endemic. In this context, it is worth noting that Pv-specific reactivity is extremely difficult to distinguish from Pf cross-reactivity in human populations, because individuals in most areas with Pv likely also have prior or co-exposure to Pf or other species. Thus, the inability to manipulate host immune responses and the importance of mono-infections, especially of Pv mono-infections, might mandate additional approaches, where animal models such as non-human primate (NHP) or rodent malaria models may be most appropriate [62, 63]. NHP malarias are promising alternative models for humans, since many of the key human malaria syndromes and research problems have excellent primate models. *Plasmodium cynomolgi* is closely related and mimics the unique biology and pathogenesis of

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P. vivax [64], and the release of the genome sequence of this valuable primate malaria parasite promises for gene expression studies [65, 66]. This species offers opportunities to study the dormant relapsing parasite forms (hypnozoites) and the unique infected RBC features of Pv [67, 68], and help identify and understand the immune response behind host-parasite interactions. More so, the use of humanized rodent parasites carrying introduced genes from human vivax parasite to examining Pv proteins that do not have orthologs in Pf, may improve NHP models for dissecting human immune responses. This is of great importance, since it is possible that human hepatocytes and erythrocytes in rodents will allow the growth of human malaria parasites.

In terms of serological markers which can differentiate between past and currently active *Plasmodium* infections, three Pf antigens (encoded by PF3D7_1002000, PF3D7_0402400, and PF3D7_1106300) have been classified to having the potential to identify with precision whether an individual had been infected with Pf within the last 30, 90, or 365 days; whereas other six (PF3D7_1002000, PF3D7_1020800, PF3D7_0731600, PF3D7_0532100, PF3D7_0801000, and PF3D7_0304600) can accurately predict the incidence of malaria in the previous year [49]. Though it is believed that many of these informative candidate antigens are appropriate for routine serodiagnosis and surveillance to detect malaria incidence and prevalence, but it remains vital to confirm their diagnostic utility. In the following, we discuss the development of an antigen-based serodiagnostic, DIGFA, currently in use by the authors as approach for malaria surveillance (also see Figure 1).

4.2.1 Preparation of recombinant *Plasmodium* antigens for serodiagnosis use

Open reading frames (ORFs) of antigens of interest are selected for polymerase chain reaction (PCR) amplification, then cloned in pET-42a plasmid vector or cloned in pEU-E01-His plasmid vector (CellFree Sciences) using in-fusion gene cloning technique. Constructs of recombinant plasmid (pEU-E01-His/pET-42a$^{target\ gene/ORF}$) carried by competent cells (*Escherichia coli* DH5α or BL21) are then used for protein expression. Wheat germ cell-free (WGCF, (CellFree Sciences)) protein expression technology is known as the unique protein expression system that could efficiently synthesize with high speed and precision malaria proteins from genes that contain large number of A/T sequences [36, 38]. Recombinant antigens are expressed using *E. coli* BL21-based protein expression or using WGCF protein expression system in a bilayer transcription-translation system, with highly purified pEU-E01-His$^{target\ gene/ORF}$ recombinant plasmid [40, 53]. The expression level of crude recombinant antigens is analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), with Coomassie Brilliant Blue R-250 PAGE staining method. Recombinant

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serological markers are purified using an affinity chromatography matrix carrying a His tag (e.g., as Ni-nitrilotriacetic acid agarose column, (Ni-NTA Agarose, (Qiagen)) [54, 69], or His-tagged protein purification resin (Ni-Sepharose column, (GE Healthcare)) [55], and then analysed by Western blot, with Penta • His tag antibody (Qiagen). Purified recombinant antigens are finally quantified using bicinchoninic acid (BCA) protein assay method, in which protein concentrations are determined with reference to standards of a common protein (e.g., as bovine serum albumin, (BSA)).

4.2. 2 Assessment of the sensitivity and specificity of recombinant serological markers

In order to consider any *Plasmodium* biomarker for diagnostic utility, there is vital to evaluate its sensitivity and specificity through a combined serum/blood screening strategy. Evaluation and validation tests not only guide the sensitivity and specificity of target candidates, but also aid prioritize suitable biomarkers for the development of serodiagnostics. These tests include: assessment of anti-*Plasmodium* IgG antibodies in sera from a large repertoire of both infected and normal individuals using antigen-based conventional assays (e.g., as ELISA formats and dipsticks,); and comparison with standard methods (e.g., as microscopic examination of blood smears, nested PCR amplification for the detection of the small subunit ribosomal RNA (ssrRNA) gene of human *Plasmodium* species, or both).

In terms of promising serological markers of diagnostic utility for Pv, PvMSP1 (PVX_099980_1o1) is under consideration and is currently being used to assess Pv malaria transmission in areas where vivax endemicity is low, with both sensitivity and specificity revealed to be > 92% (unpublished data). Therefore, the development of a user-friendly serological test for the detection of *Plasmodium* IgG antibodies becomes necessary for control programmes, especially to monitor malaria transmission in areas where the disease is approaching the elimination stage.

4.2.3 DIGFA, a promising antigen-based serodiagnostic test for malaria surveillance

DIGFA is a trans-flow serodiagnostic test that uses serum or whole blood applied to protein antigen attached on a nitrocellulose membrane, followed by colloidal gold conjugated antibody to give a red colour spot. As similar rapid diagnostic tests such as lateral-flow immunochromatographic assays, the test uses essentially same technology with a simple procedure and no special training required, making it have practical value in resource-poor community settings for clinical diagnosis or community mass screening surveys. The assay would provide several advantages over more conventional assay formats such as ELISA

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which needs a minimum of laboratory equipment, or Dot/FAST ELISA which has also limitations, since enzyme-conjugates are difficult to store and are unlikely to be applied in field conditions (also see Table 3). Both lateral-flow immunochromatographic assays and DIGFA are qualitative and quantitative (interpretation of result at naked eye or using a quantitative rapid tester reader). However, the later gives a reliable diagnostic result in record time (only within a very few minutes) with no significant differences observed between serum or heparinised blood; whereas most of lateral-flow assays need about fifteen minutes, or IFA and ELISA which are time-consuming. More so, the results from DIGFA can be preserved for control at a later time (see Figure 1). DIGFA is also reliable to achieve improved diagnostic accuracy, since it showed high sensitivity and specificity of Pv MSP1 (PVX_099980_1o1) to be 94.1% and 97.5% compared to those in ELISA, which were as high as 92.2% and 95.6% respectively (unpublished data). In addition, the specificity and sensitivity of soluble egg antigen of *Schistosoma japonicum* in DIGFA have showed similar sensitivity and specificity in ELISA formats, with no statistical differences [70]. The limitation of DIGFA is that its uses relatively large sample volumes (10-20 uL of serum or 20-40 uL of whole blood), compared to ELISA and array formats (1/100-1/200 dilutions of serum), or immuno-chromatographic lateral-flow assays (5 uL of whole blood).

In the following, we describe the procedure for DIGFA that we developed according to [70] with slight modifications. Briefly, the solid phase antigen membrane of the dot is prepared by coating purified recombinant antigen (protein concentration 0.25 mg/ml) onto a nitrocellulose membrane (pore size 0.45μm, (Millipore, Co.)), which is then preserved at 4 ˚C. Colloid gold-labelled antibody is obtained by adding purified anti-human IgG (Sigma) to colloidal gold solution (made by sodium citrate reduction of hydrogen tetrachloroaurate ($HAuCl_4$, pH 8.2)) until it becomes red, then adjusted with polyethylene glycol to a final concentration of 0.5 mg/ml. The serodiagnostic is further assembled in dedicated plastic frame (~30 mm × 25 mm), consisting of a base and a tightly fitting lid with a 10 mm diameter well in the middle (see Figure 1). Under the well, the micropore solid phase antigen membrane (diameter 25 mm) is positioned on top of a water-absorbing pad. Tris hydrochloride buffer (20mM Tris–HCl, pH 8.2)) is used as washing buffer (WB).

First, 50 μl of WB is put in the well of the device, then 20 μl of serum or 40 μl of heparinised blood is added until completely infiltrated. Thereafter, another 50 μL of WB is added, followed by 50 μL of anti-human IgG probe labelled-colloidal gold, and finally 50 μL of WB. Each reagent is added after the previous is totally absorbed (generally less than one minute per step). The result is observed and recorded immediately after the last WB has filtrated, and the whole assay usually take four to five minutes. The result can be read with the naked eye comparing the resulting colour with that of both positive and negative controls

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(see Figure 1), or by quantitative rapid test reader dedicated for DIGFA (e.g., as Gold Colloidal Quantitative Reader). The red colour as a spot from the colloidal gold conjugate indicates that the individual serum being tested contains antibodies that are reacting with the antigen used. The intensity of the coloration indicates the degree of immune combination, which reflects in general the level of seroreactivity in a serum sample. Cut-off is defined as follows: (i) qualitatively by eye (the degree of positive red colour is subjective and judged between "+" and "++++" according to the colour-darkness level); or (ii) quantitatively with rapid test reader which is affordable and is likely to be used in resource poor settings.

5. Expert commentary

Despite successful efforts in the control of malaria, severe and complicated malaria caused by infection with Pf, Pv, and Pk, continue to increase the global burden of the disease. In addition, ongoing transmission at parasite levels undetectable by microscopy makes arduous the research agenda for the eradication of malaria.

Microscopy is the ''gold standard'' for the diagnosis of malaria parasites. However, the technique is insensitive to detect low-level parasitemia and non-blood-stage malaria parasites, as such, causes gross underestimation of parasite prevalence in areas where most infections are subpatent. The low-level of Pf in blood stream as well as Pv dormant forms in the liver (hypnozoites) which remain asymptomatic until relapse ensues, serve as carriers of infection, potentially contributing to malaria transmission within populations. Additionally, Pk shares close morphological characteristics with both Pf and Pm, making it frequently and easily misidentified when microscopic examinations are performed. It is worth noting that malaria transmission will not be interrupted if asymptomatic/submicroscopic infections are not detected and treated, and importantly if the current microscopic examination continues to fail in distinguishing Pk from other species of *Plasmodium*. Molecular detection is used to discriminate species of *Plasmodium* by targeting the parasite's 18S rRNA gene. PCR methods, either as nested PCR or real-time PCR (qPCR), are likely to consistently detect infections at parasite densities as lower as 0.1 parasites/μL, making them sufficiently sensitive for detecting all carriers. The sensitivity of PCR-based tests for identifying low level parasitemia has been shown to be improved by performing qPCR using DNA from high blood volumes (up to 1 ml) with a limit of detection (LoD) of 0.022 parasites/μL [71]. However, the tests are only available in reference laboratories at a cost both financially and in turnaround time, and are unlikely to be consistently used in resource-limited settings where malaria is common. Malaria rapid diagnostic tests (mRDTs), using monoclonal antibodies directed against the histidine-rich-protein 2 (HRP2) specific for Pf, or species-specific parasite lactate dehydrogenase (pLDH), have been proposed as

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possible aid to microscopy to help detect malaria parasites. However, mixed infections of Pk with either Pf or Pv could not be properly identified [72, 73]. In addition, high proportion of false-negative results are observed for PfHRP2/3 when mRDT is used, as individuals are being infected with a mutant of Pf (deletion of *hrp2/3* gene within the parasite) [74, 75]. Therefore, the integration of the information gained from the 'omics' is necessary to dive deeper into the realms of host-parasite interactions.

Among strategies that are currently being used to overcome the numerous challenges in *Plasmodium* research, immunomics is regarded as the leading approach that helps understand host immune responses and enable the characterization of potential serological markers in a HTP manner [40-42, 49-52, 56]. The approach is also alternatively being used to extend the seroepidemiologic knowledge of malaria [44, 46-48]. With respect to the studies conducted worldwide, immunomics has been proven to be an excellent application of malaria genome. It provides general information about receptor-ligand interactions, and have been helpful in profiling clear antibody responses and identifying large amounts of blood-stage immunodominant antigens that other approaches were not able to achieve [36]. Serological markers for diagnostic utility are selected by comparing immunoreactivities to serum samples from case-control cohorts.

It is believed that *Plasmodium* species-specific genes may give rise to species-specific antigens, and the need of biomarkers with potentials for routine diagnosis and surveillance, is vital. However, malaria parasites are likely to share epitopes for antibody recognition, as the majority of genes in Pf and Pv are orthologs of each other, as well as Pk which shares a close phylogenetic relationship with Pv. Inevitably, this may not keep from antigenic cross-reactivity, making arduous the interpretation of antibody recognition of malaria proteins, especially in areas where there is coincident exposure to both Pf and Pv, or Pv and Pk. For example, antigenic cross-reactivity has been attributed to Ugandan, Malawian, and Zambian Pf mono-exposed individual samples, where in addition to high reactivity to Pf antigens, robust reactivity to a subset of Pv antigens has been also observed. Reciprocally, in some areas of Peru where populations are known for Pv mono-infections, cross-reactivity has been reported to a subset of Pf antigens [45]. Orthologous or non-orthologous genes in Pf and Pv have been revealed to not share cross-reactive antigens reciprocally, and are therefore believed for diagnosis and surveillance purposes in areas where Pf or Pv is endemic, or both. However, the diagnostic utility of most of the candidate antigens listed in this review need to be validated in more conventional assay formats. The importance of mono-infections in animal models (e.g., as NHP and humanized rodent malaria models) would greatly help overcome the bottleneck in the difficulty to assess immune responses of Pv mono-infections in individuals. In short, several malaria antigens have shown the highest

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overall seroreactivity and are considered as leading serological markers of exposure. These antigens include: Pf MSP2, LSA1, ETRAMP5, MSP4, MSP1, MSP10, PfHR2, ETRAMP14, HYP2, PfACS5, and LSA3, for Pf; and Pv-FAM-a, MSP3, MSP4, Pv conserved hypothetical proteins (PVX_115450_1o2, PVX_087670_1o1, and PVX_090095_101), ETRAMP11, FAD-dependent glycerol-3-phosphate dehydrogenase, T-complex protein 1, MSP1, and Pv12, for Pv. In addition, antibody responses to falciparum antigens have instructed on individuals exposure in providing accurate estimates of the incidence to Pf. Such predictive serological markers of both Pv and Pk are also needed to inform on an individual's recent or past incidence with Pv/Pk malaria.

Asymptomatic individuals may not be protected from malaria parasite infection, but they may possess immunity against symptomatic disease. Pf blood-stage antigens associated with clinical immunity have been recognized with greater seroreactivity in asymptomatic malaria compared to symptomatic infection. These antigens include: exported proteins to the infected RBCs during the intraerythrocytic stages of the development of Pf (e.g., as PfEMP1 family, PTP5, HSP70, RESA, ETRAMP, HSP70, GEXP18, and RIFIN family); or invasion-related proteins such as AMA1, SYN6, MSP2, MSP4, MSP10, MSP11, and SERA4 (see Table 1). By contrast, promising serological markers of transmission of Pv (e.g., as PVX_119445_1o1, PVX_090095_1o1, and PVX_095220_1o1), could identify individuals lacking adequate immunity, and are therefore helpful in monitoring acquired immunity in populations, which, below a critical level may put populations to high risk of fatalities.

Antibodies play key role in the acquisition of malaria protective immunity: low antibody levels targeting blood-stage antigens are predictive of *Plasmodium* infection, and with increasing exposure, antibody levels also increase and clinical acquired immunity develops to reach a critical limit. We estimate that leading serological markers identified by the screening of sera from microscopy-diagnosed individuals, would be appropriate for routine serodiagnosis purposes; whereas those identified by the screening of sera from both asymptomatic and submicroscopic infections, promise for surveillance purposes in low transmission settings, but, their diagnostic utility remain to be validated. More so, there is urgent need to prioritize and pursue studies that will help identify novel serological markers of Pk infection and validate the list of the four (PkSERA3 antigens 1 and 2, *Pk*SSP2/TRAP, PKNH_1265400, and PkTSERA2 antigen 1) that have been recently discovered using ELISA. We believe that the information provided in this review will further equip and strengthen the knowledge of researchers that are interested in working on malaria, because it explicitly addresses the impact of *Plasmodium* immunomics on malaria research.

In clinical practice, serological testing cannot be applied for diagnosing acute malaria [76], because from the initial infection to the host humoral immune responses, the production

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of anti-*Plasmodium* IgG antibodies may take one to two weeks after the initial infection, and may only persist for three to six months [77, 78]. In addition, commercially approved rapid and convenient antibody detection tests for malaria diagnosis is almost non-existent. On the one hand, these limitations are due to experimental results which have been too variable not only because of the type of antigen preparations used (e.g. crude, recombinant purified, etc.), but also the use of non-standardized test procedures. On the other hand, cross-seroreactivity with antigens that share common epitopes leads to false-positives and misdiagnosis, especially in regions where more than one species of *Plasmodium* is endemic. To a lesser extent but nonetheless important is the inability of immunodiagnostic tests to differentiate between an individual's recent and past incidence with malaria infections. Furthermore, antibody detection tests cannot be used in malaria infections that do not develop a significant antibody response. Therefore, there is still a need to improve the current serodiagnosis approaches available. Since the advent of *Plasmodium* immunomics, there is a new hope to improve existing serodiagnostic tools, because the approach effectively helps characterize serological markers of species-specificity to accurately provide the time when an individual had incidence with malaria parasites. Finally, DIGFA is proposed for being the promising antibody detection test for routine diagnosis and surveillance of malaria, and its fastness and convenience are unquestionable.

6. Five-year view

During the next few years, it will become mandatory to increasingly assessing surveillance of malaria, paying also great attention to the epidemiological evolution affected by the three species of *Plasmodium*, especially *knowlesi* malaria which is expected to worsen according to the time elapsed. We expect that Pk immunomics would have been investigated to characterize more broadly new serological markers for diagnostic utility, since the high risk of severity and morbidity to human Pk infections have been increased recently. Also, we look forward to the successful completion and validation of the specificity and sensitivity of the panel of Pf/Pv/Pk biomarkers of exposure that promise for diagnostic purposes. Meanwhile, we encourage malaria researchers to carry out investigations to identify the most informative serological markers of the other species of *Plasmodium* that infect humans such as malariae and/or ovale parasites which are also co-endemic and express cross-reactive antigens to Pf and/or Pv. In the future, we expect that more researchers would engage in studies for population surveillance using immunomics, as well as for the development of rapid, convenient, and reliable antigen-based serodiagnostics for routine screening and epidemiological assessments of malaria. As such, rich epidemiologic surveillance data could

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be generated to provide key information on malaria transmission for control programmes, especially when parasite rates are low.

7. Key issues

• Blood-stage antigens of Pf, Pv, and Pk, are involved in either invasion of RBCs or sequestration/cytoadherence of parasitized erythrocytes, and evade host immune responses.

• Pv and Pk account together for a considerable share of the global morbidity and mortality of malaria, along with Pf.

• Pk presents with high parasitemia and easily fails to distinguish microscopically from both Pf and Pm due to their very similar appearance.

• Asymptomatic and submicroscopic parasite level infections, as well as infections with Pv hypnozoites, serve as carriers of transmission within populations.

• Although PCR-based tests can discriminate species of *Plasmodium* and are very sensitive for detecting all carriers, they are time consuming and are unlikely to be consistently used in resource-limited settings where malaria is common.

• Serological testing is the gold standard when biologic samples are unavailable, and when species-specific mRDTs that use monoclonal antibodies directed against antigens fail.

• Serological tests are not appropriate to diagnose severe malaria infections as a reasonable level of anti-malaria antibody production takes place over a period of time after infection.

• *Plasmodium* immunomics has contributed to the characterization of serological markers that promise for diagnostic utility, and to the generation of rich epidemiologic knowledge to guide control interventions.

• Highly sensitive species-specific and non-orthologous Pf and Pv antigens have been immunomics-discovered or validated, as well as promising biomarkers of exposure to differentiating between past and recent infections.

• Less or nothing is known about Pk immunomics. The use of immunomics is therefore expected to characterize additional serological markers of Pk and to validate the four leading blood-stage antigens that have been recently discovered.

• The development of rapid, convenient, and reliable antigen-based serodiagnostics will greatly help detect both symptomatic and asymptomatic, or submicroscopic carriers.

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• Among the current serological tests for malaria, DIGFA holds promise for fast clinical diagnosis as well as for epidemiological surveys in large-scale application.

• As on top of being fast, easy, and highly sensitive and specific, DIGFA does not require any specific instrument and training, and the reagent is stable at 4 ˚C for at least six months.

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Due to limits on the number of references, it was not possible to mention all existing and relevant papers

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Legend for Tables and Figures

Table 1: Top serological markers of *P. falciparum.*

*****Antigen candidates that displayed most frequent positive antibody responses with serum samples tested.

ᵠ Serodiagnostic antigens that were most associated with asymptomatic and submicroscopic malaria infections.

‡ PfEMP1 IDs: PF3D7_0200100_e1, PF3D7_0420700_e2s1, PF3D7_0617400_s4, PF3D7_0711700_e1, PF3D7_0800200_e1, PF3D7_1300300_e1, PF3D7_1041300_CIDR1, PF3D7_0425800_CIDR1, PF3D7_1300300_CIDR1, PF3D7_0800300_e2s1, PF3D7_1300300_s2, PF3D7_0800200_e2s1, PF3D7_0421100_e1

Table 2: Top serological markers of *P. vivax.*

*****Antigen candidates that displayed most frequent positive antibody responses with serum samples tested.

ᵠ Serodiagnostic antigens that were most associated with asymptomatic and submicroscopic malaria infections.

Table 3. Antigen-based **diagnostic tests** for the detection of malaria antibodies.

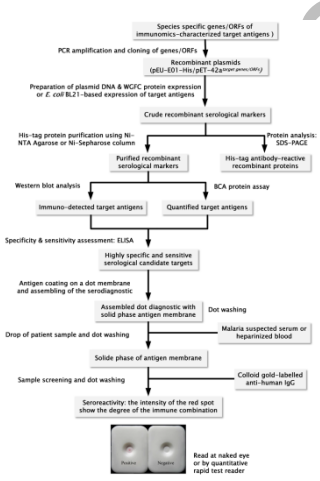

Figure 1: Schematic depiction of the processes involved in the development of dot immunogold filtration assay (DIGFA).

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Table 1: Top serological markers of *P. falciparum*.

Protein ID	Target description	Type	References
PF3D7_0532100*[φ]	Early transcribed membrane protein 5 (ETRAMP5)	Secreted	[41, 44-49]
PF3D7_0206800*[φ]	Merozoite surface protein 2 (MSP2)	Membranous	[40, 41, 44-49]
PF3D7_0207000_1o2*[φ]	Merozoite surface protein 4 (MSP4)	Membranous	[40-44, 46]
PF3D7_0801000_e2s2*[φ]	Plasmodium exported protein (PHISTc), unknown function	Membranous	[42, 44, 46-49]
PF3D7_1014100_s1*[φ]	Merozoite surface protein MSA180, putative	Secreted	[40-42, 44, 48]
PF3D7_1033200_1[φ]	Early transcribed membrane protein 10.2 (ETRAMP10.2)	Secreted	[42, 44]
PF3D7_0220000_2[φ]	Liver stage antigen 3 (LSA3)	Membranous	[41, 42, 44-46, 48]
PF3D7_1300300, CIDR1[φ]	Erythrocyte membrane protein 1, PfEMP1	Membranous	[42, 44, 46-48]
PF3D7_0930300_s1/s2*[φ]	Merozoite surface protein 1 (MSP1)	Secreted	[40-42, 44, 45, 47, 48]
PF3D7_1335100	Merozoite surface protein 7 (MSP7)	Secreted	[41, 42, 44, 48]
PF3D7_1002100_2o2*[φ]	EMP1-traffcking protein (PTP5), Pf70	Membranous	[42, 44, 46-49]
Multiple IDs[‡]*[φ]	Erythrocyte membrane protein 1, PfEMP1 (VAR)	Membranous	[42, 44, 46, 62]
PF3D7_1007700_e1s2*[φ]	Transcription factor with AP2 domain(s) (ApiAP2)	Membranous	[46, 47]
PF3D7_0422100*[φ]	Transmembrane emp24 domain containing protein, putative	Secreted	[44, 46, 47]
PF3D7_1133400*[φ]	Apical membrane antigen 1 (AMA1)	Secreted	[40, 41, 44, 46]
PF3D7_0530100_2o2*[φ]	SNARE protein, putative (SYN6)	Membranous	[44, 46]
PF3D7_0620400_1o1*[φ]	Merozoite surface protein 10 (MSP10)	Secreted	[40, 42, 44-48]
PF3D7_1036000_1o1[φ]	Merozoite surface protein 11 (MSP11)	Secreted	[42, 44, 46, 48]
PF3D7_1335300_1o1[φ]	Reticulocyte binding protein 2 homologue b (RH2b)	Membranous	[42, 44-46, 48]
PF3D7_1452000_s1[φ]	Rhoptry neck protein 2 (RON2)	Secreted	[42, 44, 46, 48]
PF3D7_0207700_2o4[φ]	Serine repeat antigen 4 (SERA4)	Secreted	[44, 46, 48]
PF3D7_0800300_e2s1[φ]	Erythrocyte membrane protein 1, PfEMP1 (VAR)	Membranous	[42, 44, 46, 49]
PF3D7_0102200_e2s2[φ]	Ring infected erythrocyte surface antigen (RESA)	Membranous	[44, 46, 48]
PF3D7_0702400_e1s1[φ]	Small exported membrane protein 1 (SEMP1)	Membranous	[44, 46, 48]
PF3D7_0831700_2o2[φ]	heat shock protein 70, putative (HSP70-x)	Secreted	[44, 46, 48]
PF3D7_0402400_2o2[φ]	Exported protein, unknown function (GEXP18)	Membranous	[44, 46, 48, 49]
PF3D7_0823300*[φ]	Histone acetyltransferase GCN5 (GCN5)	Membranous	[44, 46, 48]
PF3D7_1401400_1o1*[φ]	Early transcribed membrane protein 14.1 (ETRAMP14)	Secreted	[40, 42-44, 46, 48]
PF3D7_1149200_2[φ]	Ring infected erythrocyte surface antigen	Membranous	[44, 46, 48]
PF3D7_0933900_4o4[φ]	Conserved protein, unknown function	Membranous	[44, 46, 48]
PF3D7_1036400_e2s2/1o2*[φ]	Liver stage antigen 1 (LSA1)	Secreted	[42, 44, 45, 47, 48]
PF3D7_1410400_e1s1[φ]	Rhoptry-associated protein 1 (RAP1)	Secreted	[44, 47, 48]
PF3D7_1334800[φ]	MSP7-like protein (MSRP2)	Secreted	[40, 44, 47, 48]
PF3D7_0900200_e2s1[φ]	Rifn (RIF)	Membranous	[45, 47, 48]
PF3D7_1002000_2s2*[φ]	Plasmodium exported protein, HYP2	Membranous	[44, 46, 48, 49]
PF3D7_1106300_2o2*[φ]	Exonuclease, putative	Secreted	[44, 46, 49]
PF3D7_1020800*[φ]	Dihydrolipoamide acyltransferase component E2	Secreted	[49]
PF3D7_0731600_1s1*[φ]	Acyl-CoA synthetase (ACS5), PfACS5	Secreted	[44, 46, 48, 49]
PF3D7_0304600*[φ]	Circumsporozoite (CS) protein	Secreted	[46, 49]

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Table 2: Top serological markers of *P. vivax*.

Protein ID	Target description	Type	References
PVP01_1033400 ᵠ	Rhoptry-associated protein 2 (RAP2)	Secreted	[44, 50]
PVP01_0529100 ᵠ	Apical merozoite protein (Pv34)	Secreted	[44, 50]
PVX_097935_2o2 ᵠ	Subtilisin-like protease 1 (SUB1), putative	Secreted	[44, 50]
PVX_119445_1o1*	FAD-dependent glycerol-3-phosphate dehydrogenase, putative	Secreted	[50]
PVX_115450_1o2*	Hypothetical protein, conserved	Membranous	[43-45, 48, 50, 52]
PVX_090095_1o1*	Hypothetical protein, conserved	Membranous	[50]
PVX_090230_1o2* ᵠ	Early transcribed membrane protein (ETRAMP)	Secreted	[44-46, 50, 52]
PVX_095220_1o1*	T-complex protein 1, epsilon subunit, putative	Membranous	[50]
PVX_003565_1o1 ᵠ	Early transcribed membrane protein (ETRAMP)	Secreted	[44, 48, 50, 52, 53]
PVX_003840_2o4*	Serine-repeat antigen 3 (SERA3)	Secreted	[44, 50]
PVX_087680*	Chitinase	Secreted	[44, 50]
PVX_001945_1o1 ᵠ	Deoxyribose-phosphate aldolase, putative	Secreted	[44, 50, 52]
PVX_087885*	Rhoptry-associated membrane antigen (RAMA), putative	Secreted	[56]
PVX_092995_2o2*	Tryptophan-rich antigen (Pv-fam-a)	Secreted	[44, 45, 52, 56]
PVX_093680	Phist protein (Pv-fam-b)	Membranous	[56]
PVX_091700	Circumsporozoite-related antigen (EXP1), putative	Secreted	[56]
PVX_114145_1o1* ᵠ	Merozoite surface protein 10 (MSP10), putative	Secreted	[44, 46, 52, 53, 63]
PVX_099980_1o1* ᵠ	Pv200 (Merozoite surface protein 1, MSP1)	Secreted	[44, 46, 48, 53, 54, 57]
PVX_097625_1o1* ᵠ	Merozoite surface protein 8 (MSP8), putative	Secreted	[44, 46, 48, 51-53]
PVX_000930_1o1* ᵠ	Sexual stage antigen s16, putative	Secreted	[44, 52]
PVX_000995_1o1* ᵠ	Transmission blocking target antigen (6-cysteine protein Pv41)	Secreted	[44, 48, 53]
PVX_099315_2o2* ᵠ	Heat shock protein 70 (HSP70), putative	Secreted	[44, 52]
PVX_118705_1o1* ᵠ	Hypothetical, predicted Pf homolog liver stage antigen 3	Membranous	[44, 46, 52]
PVX_097730_1o1* ᵠ	Hypothetical protein, conserved	Secreted	[44, 46, 52]
PVX_110935_1o1* ᵠ	Hypothetical protein, conserved	Secreted	[44, 46, 52]
PVX_082650_1o1*	Merozoite surface protein 7 (MSP7)	Secreted	[44, 52]
PVX_092275_1o1*	Apical merozoite antigen 1 (AMA1)	Membranous	[52, 53]
PVX_119355_1o1 ᵠ	Circumsporozoite (CS) protein precursor, putative	Secreted	[44, 51, 52]
PVX_094805_1o1 ᵠ	DNA repair protein RAD23, putative	Membranous	[43, 44, 48, 50, 52, 64]
PVX_086200_1o1 ᵠ	Hypothetical protein, conserved	Secreted	[44, 52]
PVX_100835_2o2 ᵠ	Hypothetical protein, conserved	Secreted	[44, 52]
PVX_082700_1o1 ᵠ	Merozoite surface protein 7 (MSP7)	Secreted	[44, 52]
PVX_082680_1o1 ᵠ	Merozoite surface protein 7 (MSP7), putative	Secreted	[44, 48, 52, 53]
PVX_097720* ᵠ	Merozoite surface protein 3 alpha (MSP3a)	Secreted	[44, 45, 48]
PVX_081550_1o1 ᵠ	StAR-related lipid transfer protein, putative	Secreted	[44, 48, 52, 53]
PVX_113775_1o1*	Membrane protein pf12 precursor (Pv12), putative	Secreted	[44, 48, 53, 55]
PVX_082655_1o1	Merozoite surface protein 7 (MSP7), putative	Secreted	[44, 48, 52, 53]
PVX_087670_1o1*	Hypothetical protein, conserved	Membranous	[45, 48, 52]
PVX_003775_2o2* ᵠ	Merozoite surface protein 4 (MSP4), putative	Secreted	[43-45, 48, 50, 52]

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