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Review article

Virus-Like Particle Platforms for *Plasmodium knowlesi* Vaccine Development: Current Progress and Future Perspectives

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Abstract: *Plasmodium knowlesi* (*P. knowlesi*) has emerged as a significant zoonotic malaria pathogen in Southeast Asia, necessitating urgent vaccine development efforts. Virus-like particle (VLP) platforms represent a promising approach for malaria vaccine development, offering enhanced immunogenicity through repetitive antigen display and self-adjuvanting properties. While VLP-based vaccines have demonstrated success against other *Plasmodium* species, their application to *P. knowlesi* remains largely unexplored. This review examines the biology and epidemiology of *P. knowlesi*, the principles underlying VLP technology, current VLP-based approaches for malaria antigens, and the specific challenges and opportunities for developing VLP-based vaccines against *P. knowlesi*.

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Introduction

Malaria continues to be a major health problem worldwide, with *Plasmodium falciparum* and *Plasmodium vivax* accounting for most human infections. In recent decades, *Plasmodium knowlesi* (*P. knowlesi*), a simian malaria parasite, has posed a major public health threat in Southeast Asia and is recognized as the fifth human-infecting species of Plasmodium [1, 2]. The zoonotic aspect and the ability to cause serious disease, in addition to the quick replication cycle *of P. knowlesi*, highlight the need for effective preventive interventions [3]. Despite modest progress in malaria vaccine development, new vaccine platforms, such as virus-like particles (VLPs), are being explored to tackle this menace. The delivery of antigens to VLP has various benefits compared to traditional vaccine formats, such as increased immunogenicity due to the repetitive display of the antigen, self-adjuvanting properties, and generation of both humoral and cellular immune responses [4-6]. VLP-based vaccines are effective against *P. falciparum* and *P. vivax* but are still in their early stages of use for *P. knowlesi*. This review will discuss the current status of VLP technology for malaria vaccines and review how these might be adapted to tackle the new challenge of *P. knowlesi* malaria.

Biology and Epidemiology of *P. knowlesi*

P. knowlesi is a simian malaria parasite that naturally occurs in long-tailed and pig-tailed macaques in Southeast Asia. *P. knowlesi* has the shortest asexual replication cycle among human-infecting Plasmodium species (24 hours), which allows for the rapid multiplication of the parasite and may lead to a more serious disease course [7]. The parasite is transmitted by Anopheles mosquitoes, especially in the *Leucosphyrus* group, that cross over to the human population from the macaque reservoir. *P. knowlesi* is epidemiologically the most important malaria species in Malaysian Borneo and has been reported in other Southeast Asian countries such as Thailand, Myanmar,

Indonesia and the Philippines [8]. The zoonotic transmission pattern makes control more difficult, and strategies to eliminate person-to-person transmission alone are not enough. Clinical symptoms may be mild with fever or severe with complications such as respiratory distress and acute kidney injury (AKI), and death, especially in adults. *P. knowlesi* has a rapid life cycle and zoonotic reservoir, requiring vaccine strategies for inducing high and long-lasting immunity against multiple parasite life stages [9].

VLP Technology: Principles and Platforms

Virus-like particles (VLP) are non-replicating, self-assembling nanostructures that have a similar organization and conformation to native viruses but lack viral genetic material and thus cannot infect. The basic premise of the efficacy of VLP is the repeated presentation of antigens in a very organized way, allowing optimal stimulation of B-cell receptors and robust antibody responses. The size of the VLP is typically between 20 and 200 nanometers, which is the ideal size for the cells to uptake and deliver VLP to lymph nodes[10, 11]. The repetitive sequence within VLP has intrinsic adjuvant properties, which activate innate immune pathways and facilitate the formation of germinal centers, resulting in increased antibody affinity maturation and generation of long-lived plasma cells [6, 12]. Figure 1 illustrates the potential of virus-like particles in malaria vaccine development.

Multiple VLP platforms have been developed for vaccine applications. The most advanced platforms include HBsAg-based VLPs, which have been successfully used in the malaria vaccines RTS, S/AS01 and R21/Matrix-M, with the difference that the former contains both CSP–HBsAg fusion protein and unfused HBsAg, while the latter consists only of CSP–HBsAg fusion protein, giving a higher density of CSP on the surface of the VLP [13, 14]. Alternatively, the small surface antigen of duck hepatitis B virus (DHBV) can serve as a platform, as it can form VLP without excess scaffold protein when fused to malaria antigens [15]. The small size (around 22-25 nm), stability, and compatibility with a plug-and-display conjugation system based on SpyTag/SpyCatcher technology have led to the emergence of bacteriophage-derived VLPs, specifically the Acinetobacter phage AP205 capsid [16, 17]. Various malaria antigens, such as apical membrane antigen 1 (AMA1) and merozoite surface proteins, have been displayed using influenza virus-like particle to display them [18, 19]. Plant-based VLP systems can be scaled up and are cost-effective, and VLP systems with antigens like Pfs25 have been shown to provide long-lasting antibody responses [20]. A recent development in VLPs is the emergence of protein nanoparticles that self-assemble from a designed scaffold, which offers the advantages of higher stability and production efficiency. Several factors determine the platform used for VLP production, such as the size and structure of the antigen, the desired immune response profile, the system of production, and the scalability of production [21-23].

VLP-Based Approaches for *P. knowlesi* Antigens

Although *P. knowlesi* has come into recognition as a public health threat, VLP-based vaccine development with target specificity for this parasite is limited. Several protective antigens have been identified in *P. knowlesi*: 74, 66 and 140 kDa merozoite surface proteins (MSPs) [24-26]. Snags that were more recently tested have included apical membrane antigen 1 (AMA1), which proved to be protective in rhesus macaques when administered as a novel adjuvant [27]. Protection from blood stage challenge with *P. knowlesi* was obtained after vaccination using *P. knowlesi* AMA1 formulated in the CoVaccine HT adjuvant and correlated inversely with the onset of the parasite in the blood. Table 1 summarizes various VLP strategies for *P. knowlesi* vaccine development.

The circumsporozoite protein (CSP) is also a good target, since it has been genetically characterized to have B-cell epitopes in the antigenic repeat regions which could be useful for vaccine development [28]. Multiantigen/multistage strategies, which combine two different immunogens with only one of each antigen being a component of the vaccine, have also proven successful, with a subset of macaques immunized with a heterologous prime-boost strategy using CSP, SSP2/TRAP, AMA1, and MSP1 antigens being sterile in a challenge study [29-31]. Immunization with four *P. knowlesi* antigens using a DNA prime/poxvirus boost strategy induced sterile immunity in 2 of 11 monkeys, while 7 of 9 challenged monkeys experienced spontaneous clearance of parasitemia [32]. Vectors expressing *P. knowlesi* antigens have elicited immune responses that delayed the onset of parasitemia and reduced parasite release from the liver by 75-80% [31]. Duffy binding protein alpha (DBPα) and merozoite surface protein-142 (MSP-142) have been recognized as immunogenic antigens, and recombinant proteins have been shown to induce high antibody levels and growth inhibition [33, 34].

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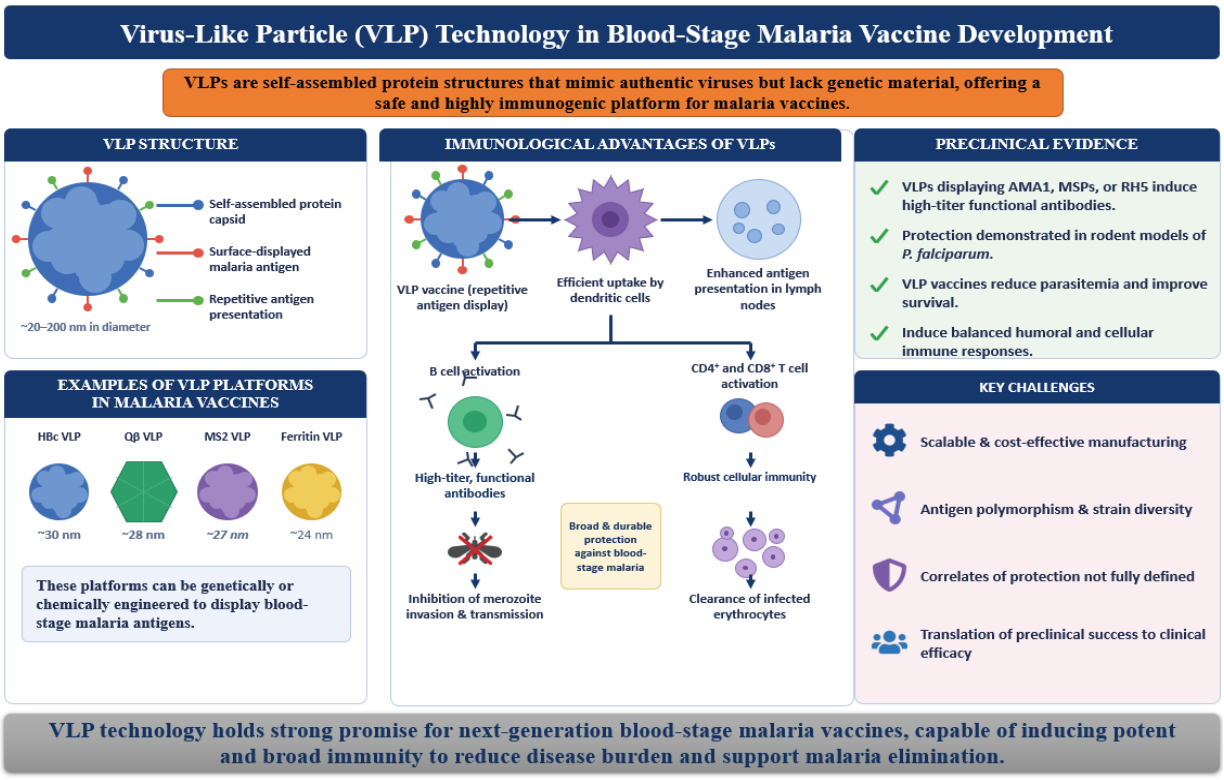

Figure 1. Virus-like particle (VLP) technology for blood-stage malaria vaccine development. VLPs enable repetitive display of malaria antigens, promoting strong antibody and cellular immune responses that inhibit merozoite invasion and clear infected erythrocytes. Preclinical evidence is promising, although manufacturing, antigen diversity, correlates of protection, and clinical translation remain key challenges.

Table1. Comparative assessment of candidate VLP strategies for *P. knowlesi* vaccine development.

Candidate antigen/strategy	Stage and role	Proposed VLP design	Potential advantages	Main limitations	Evidence and supporting references
PkAMA1	Blood-stage merozoite; moving-junction formation and erythrocyte invasion	Display the correctly folded PkAMA1 ectodomain, conserved domains, or selected conformational epitopes on an enveloped VLP or modular capsid	High-density display may enhance invasion-inhibitory antibodies and B-cell activation. PkAMA1 has structurally defined, antibody-accessible inhibitory epitopes	Requires native disulfide bonding and conformational integrity. Polymorphic or immunodominant surface regions may produce strain-biased responses	High-priority *P. knowlesi* target, supported by PkAMA1 structural data and protective *P. berghei* AMA1-VLP studies [19, 35]
PkAMA1-RON2L interface	Blood-stage invasion; AMA1 binds the RON2 loop to form the moving junction	Present a stabilized PkAMA1-RON2L complex, a single-chain fusion, or co-display both components on the same VLP	May focus immunity on a functionally constrained receptor-ligand interface and increase the proportion of neutralizing antibodies	Complex engineering, orientation, antigen density, and preservation of the native interface require careful optimization	Strong structure-based rationale from malaria models; direct *P. knowlesi* VLP testing is still needed [35, 36]

PkMSP1 fragments	Merozoite surface; contributes to erythrocyte recognition and invasion	Display conserved PkMSP1 fragments, particularly MSP1-42 or MSP1-19, on non-enveloped capsid VLP or enveloped VLP	Repetitive presentation may improve antibody titre, opsonization, complement fixation, and functional invasion blockade	Fragment selection and folding are critical; not all MSP1 antibodies are inhibitory. Antigen processing may alter protective epitopes	Moderate biological rationale; related Plasmodium MSP VLP have generated protective immunity, but direct PkMSP1-VLP evidence is limited[37, 38]
PkDBPα region II	Merozoite ligand; binds the Duffy antigen receptor for chemokines during erythrocyte invasion	Display stabilized PkDBPα-II or conserved receptor-binding epitopes on Qbeta, AP205, hepatitis B core, or other modular VLP	Could induce antibodies that directly prevent receptor engagement; multivalent display may enhance otherwise subdominant conserved epitopes	Sequence diversity and positive selection may reduce breadth. Native cysteine-rich folding and receptor-binding conformation must be preserved	Promising *P. knowlesi*-specific invasion target; supported by functional and population-genetic studies [39, 40]
PkCSP	Sporozoite surface; mediates pre-erythrocytic infection and hepatocyte invasion	Display central repeat motifs together with conserved N- or C-terminal regions on hepatitis B surface antigen, Qbeta, or AP205 VLP	Dense repeat presentation may induce high-titre antibodies that immobilize sporozoites and block liver infection; strong precedent exists for particle-based CSP vaccines	Repeat and C-terminal variation may affect strain coverage. Protective epitopes and optimal antigen ratios require definition	Strong platform precedent but limited direct PkCSP-VLP evaluation. PkCSP diversity data should guide construct selection [41, 42]
PkAMA1 plus microneme antigen	Blood-stage invasion; combines complementary micronemal ligands involved in attachment and entry	Co-display PkAMA1 with a selected *P. knowlesi* microneme-associated antigen on the same influenza-derived or other enveloped VLP	Simultaneous targeting of complementary invasion steps may broaden protection and reduce escape from a single-antigen response	Antigen competition, unequal incorporation, immune dominance, and more demanding manufacturing controls	Cross-species proof of concept: combined *P. berghei* AMA1-MIC VLP reduced parasitaemia and prolonged survival in mice [43, 44]
Mosaic blood-stage VLP	Targets multiple invasion pathways using PkAMA1, PkMSP1, and PkDBPalpha-II	Use a mosaic VLP displaying multiple antigens or formulate a mixture of antigen-specific VLP with controlled antigen doses	Broader blockade of erythrocyte invasion and reduced dependence on a single antigen or allele	Complex analytical characterization, antigen-density control, immune interference, and increased manufacturing cost	Conceptual strategy supported by the independent biological relevance of each component; direct comparative studies are required [35, 38, 42]
Multistage PkCSP-PkAMA1 vaccine	Targets both pre-erythrocytic sporozoites and pathogenic blood-stage merozoites	Co-display PkCSP and PkAMA1, combine separate antigen-specific VLP, or use a heterologous prime-boost regimen	Could reduce establishment of infection and provide a second layer of control if parasites reach the blood stage	Balancing responses to two lifecycle stages is challenging; suitable challenge models and stage-specific correlates of protection are needed	Strategically attractive, based on strong antigen biology and established CSP/AMA1 vaccine concepts, but currently experimental for *P. knowlesi* [19, 35, 41, 45]

Challenges in *P. knowlesi* VLP Vaccine Development

Development of a VLP-based vaccine against *P. knowlesi* faces several challenges. Antigenic variation is a major challenge for vaccines, as reported for the 140 kDa merozoite surface antigen of malaria, where immunized monkeys were found to have parasites with novel variant antigens within weeks of challenge [46]. This requires a choice of antigen, with a preference for conserved epitopes, or using multiple variant forms in VLP vaccines. To address the problem of antigenic diversity, the mosaic and cocktail VLP approaches have been developed for *P. falciparum* PfEMP1 variants [47].

Secondly, an optimal antigen selection and combination for VLP vaccines against *P. knowlesi* is yet to be defined. Multistage, multiantigen strategies have been investigated in traditional vaccine formats and appear promising [29, 30], but will require special attention for compatibility of antigens, VLP loading capacity, and immune interference when implementing such approaches in VLP vaccine formats. In addition to this, the zoonotic transmission of *P. knowlesi* makes vaccine development difficult because vaccines need to be developed that will induce immunity against both species of parasites, in human and macaque populations. Another technical issue with the production of VLPs and antigen conjugation needs to be overcome. The *P. knowlesi* antigens may require some optimization before they can be expressed or may not fold properly when fused to the VLP scaffolds. Although flexible, the plug-and-display SpyTag/SpyCatcher system needs optimization for every combination of antigen-VLP [48].

Moreover, the unavailability of an appropriate animal model for testing vaccines against *P. knowlesi* also poses a challenge. Rhesus macaques have been heavily used for vaccine studies [12, 29, 32], but throughput is limited by cost and ethical concerns. Another serious concern arises due to *P. knowlesi* fast replication cycle; vaccines must be able to generate very strong and quick immunity to prevent the exponential growth of the parasite. This could require higher antibody levels than would be required for other Plasmodium species. Lastly, there are no clear regulatory and clinical development routes for *P. knowlesi* vaccines; the disease is geographically limited, which may hinder commercial interest.

Future Perspectives

The future of *P. knowlesi* VLP vaccine development lies in strategically leveraging lessons learned from VLP vaccines targeting other *Plasmodium* species. Several promising directions merit exploration. The first and most obvious opportunity is to use the already successful VLP platform developed for *P. falciparum* and *P. vivax* vaccines for the display of *P. knowlesi* antigens. Given the genetic characterization of PkCSP and the predicted antigenicity of the PkCSP repeat regions, the R21 Matrix M vaccine platform using HBsAg VLP to display CSP could be easily adapted to display *P. knowlesi* CSP [28]. Likewise, the DHBV-based VLP system, which has been able to present the *P. falciparum* CSP, was adaptable to present antigens of the pre-erythrocytic stage of the *P. knowlesi* life cycle.

Secondly, bacteriophage-derived VLPs, especially those with SpyTag/SpyCatcher conjugation, have excellent versatility for displaying multiple *P. knowlesi* antigens [16, 17]. This platform has worked well for transmission-blocking antigens and could be modified to include antigens of the blood stage (e.g., AMA1, MSP-142 or DBPalpha). Additionally, multivalent VLP vaccines combining multiple *P. knowlesi* antigens simultaneously are a very promising strategy. A multistage and multiantigen approach led to high levels of protection in conventional vaccine formats and could therefore be used to synergistically enhance the immunogenicity of the antigens displayed on VLP scaffolds. The strategy might be adapted to VLP displays in which complementary antigens are presented in highly ordered and dense arrays that could yield expanded immune responses and additive or synergistic protection. There are many considerations in the choice of antigens, relative antigen density, and immunodominance; however, combining multiple antigens does not necessarily result in better vaccine efficacy [29, 49]. Several influenza VLP-based antigen co-display platforms have been successfully developed for displaying multiple malaria antigens [43].

Furthermore, epitope-based VLP vaccines could avoid antigenic variation problems by targeting conserved, functionally important epitopes. As seen with *P. falciparum* antigens, epitope-targeting-based VLPs could be applied to *P. knowlesi* antigens, but a vaccine designed to target the chosen CSP epitopes in *P. knowlesi* has yet to be developed. The structure of *P. knowlesi* AMA1 [50] serves as a basis for rationally selecting epitopes. In addition, new VLP platforms are continually being developed that are more stable.

Protein nanoparticles derived using computational design have demonstrated similar immunogenicity to VLPs derived from phages, and higher production yields [4]. Moreover, there may be opportunities to optimize protective efficacy through the use of a combination of VLP vaccines with other vaccination modalities. VLP vaccines combined with viral vectors are promising for malaria by heterologous prime-boost strategies[51].

Lastly, VLP vaccines targeting sexual-stage antigens could play a role in the control *of P. knowlesi* via zoonotic transmission. The success of vaccines that display Pfs47 and Pfs25 (VLP) for transmission blocking offers a blueprint for the development of similar vaccines [16, 20]. Finally, the use of *P. knowlesi* as a model system for developing *P. vivax* vaccines [9, 52] opens the door to parallel development. Controlled human infection models with *P. knowlesi* might enable early-stage vaccine efficacy testing [53].

Conclusion

Virus-like particle platforms represent a highly promising yet underexplored approach for developing vaccines against *P. knowlesi* malaria. The proven efficacy of VLP-based vaccines in the context of other Plasmodium species and the large number of well-characterized protective *P. knowlesi* antigens supports the development of VLP vaccines. A range of key antigens known to be immunogenic or protective, such as AMA1, CSP, MSP-142, and DBPalpha, are immunogenic and protective targets, and their use on the VLP scaffold may greatly improve their immunogenicity and efficacy. There are several VLP platforms available, such as HBsAg, DHBV, AP205 bacteriophage, and various types of computationally designed nanoparticles. Rational vaccine design and novel strategies for preclinical evaluation are needed to overcome the hurdles of antigenic variation, optimal antigen selection, zoonotic transmission dynamics, and limited animal models. VLP vaccines carrying several conserved epitopes from various stages of the parasite are particularly interesting as they may protect multiple stages of the parasite over time. *P. knowlesi's* short replication period and high disease severity highlight the need for vaccine development. Effective vaccines for *P. knowlesi* are likely to be available within the decade, with strategic investment in proven VLP platforms and innovative approaches like epitope-based vaccines and transmission-blocking vaccines. In conclusion, VLP vaccines could be vital to help control this new zoonotic malaria threat alongside vector control and treatment measures to curb the impact of *P. knowlesi* malaria in Southeast Asia.

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