INTEGRATED MALARIA DIAGNOSTICS AND DRUG RESISTANCE MONITORING: A MULTIPLEXED NUCLEIC ACID BASED LATERAL FLOW APPROACH

INTRODUCTION

Background

Malaria remains a significant global health challenge, particularly in regions with a high prevalence of the disease. According to the World Health Organization (WHO), an estimated 241 million cases and 627,000 deaths occurred in 2020 (WHO, 2020), with most of the cases concentrated in sub-Saharan Africa. Despite extensive efforts to control malaria, challenges persist in accurately diagnosing the disease and real time monitoring emerging drug resistance.

Traditional methods such as microscopy and malaria Rapid Diagnostic Tests (mRDTs) have been the cornerstone of malaria diagnosis. Microscopy has been the gold standard for malaria diagnosis whereas mRDTs have been widely used for point of care diagnosis of malaria as recommended by WHO on global malaria program (WHO, 2021). In rare cases standard conventional PCR and real time PCR have been used to detect malaria though mostly the latter have been reserved for research use.

However, these methods have many limitations. Microscopy requires highly skilled personnels, well established infrastructure and it may lack sensitivity in cases of low parasitemia (Berzosa *et al*., 2018). While mRDTs are more user-friendly and cost effective with a test costing less than 1USD, they primarily detect antigens (Pf. hrp2/3, pLDH, and Aldolase) which may persist in the body and fail to distinguish active and passed infections and might not provide detailed information on the Plasmodium species or resistance profiles (Kumar R et al., 2020).

Malaria diagnosis using Polymerase Chain Reaction (PCR) assays would be the best alternative because they offer a high precision in detection and species identification but are exclusively laboratory-based, expensive, and require sophisticated equipment, making them less feasible in resource-limited settings (Kumar R et al., 2020).

The emergence and spread of drug-resistant strains, as well, poses a formidable challenge to effective malaria treatment. Antimalarial drugs, such as Artemisinin-based Combination Therapies (ACTs) as first line drugs for uncomplicated malaria, intravenous artesunate and quinine for managing severe malaria cases; antifolates and quinolines as prophylaxis in pregnant mothers and visitors to malaria endemic areas. All these medications are crucial in malaria control efforts; however, the rise of resistance threatens the efficacy of all these treatments (Asua et al., 2021). Early detection of drug-resistant strains is essential to adapting treatment strategies promptly and preserving the effectiveness of available antimalarials which is not the case today as drug resistance is only monitored during large epidemiological surveys.

Current diagnostic methods often focus on detecting the presence of the parasite, neglecting detailed information on the Plasmodium species responsible for the infection (RN.Kigozi et al., 2021). A comprehensive diagnostic solution capable of identifying various Plasmodium species is essential for tailored treatment approaches.

The conventional approach to monitoring drug resistance involves periodic surveys, which may not provide timely information on emerging resistance patterns. A real-time monitoring system is crucial for adapting treatment strategies promptly and mitigating the spread of drug resistance as CDC has always recommended since 2023 (CDC, 2023).

In response to these challenges, this project introduces a transformative solution—an integration of mRNA detection for various Plasmodium species and real-time drug resistance monitoring within a single lateral flow device, using antisense oligonucleotides (ASOs). The use of mRNA as a diagnostic marker is pivotal. mRNA, being inherently unstable, serves as a reliable indicator of active malaria infection. This breakthrough ensures that the diagnostic tool not only identifies the specific Plasmodium strain responsible for the infection but also distinguishes between ongoing and past infections with a high degree of accuracy. Simultaneously, the integration of drug resistance monitoring on the same platform eliminates the dependence on periodic surveys for such crucial information.

Moreover, positioning this integrated diagnostic solution as a point-of-care kit adds a critical dimension to its impact. The ability to perform diagnostics at the point of care ensures accessibility, timely decision-making, and the potential for rapid intervention. The real-time nature of this diagnostic tool fundamentally transforms the landscape by mitigating the emergence of antimalarial drug resistance. In summary, this project strives to bridge existing diagnostic gaps, offering a precise, real-time, and point-of-care solution that has the potential to revolutionize malaria diagnostics and treatment.

Problem statement

The current drawbacks in rapid malaria diagnostics stem from the limited capabilities of existing tools to provide detailed insights into both the Plasmodium species and their resistance to antimalarial drugs simultaneously. This dual inadequacy hampers the effective tailoring of treatments, posing a potential threat to overall antimalarial efficacy.

Adding complexity to the issue is the inability of current diagnostic tools to distinguish between past and active infections. Relying on the detection of persistent antigens, even after viable parasites have been cleared, introduces uncertainty. This uncertainty complicates the accurate prescription of targeted treatments and contributes to the development of antimalarial drug resistance.

In essence, the current rapid diagnostic methods struggle to offer a comprehensive understanding of the malaria infection, encompassing the identification of specific Plasmodium species and the assessment of their resistance to antimalarial drugs. The reliance on antigen detection exacerbates the challenge, as these antigens may persist in the body post-parasite clearance, making it difficult to differentiate between ongoing and past infections.

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In response to these challenges, our project seeks to pioneer an advanced diagnostic tool. This innovative tool integrates mRNA detection for various Plasmodium species and real-time drug resistance monitoring within a single lateral flow device. This pioneering approach aims to improve the precision of treatment decisions, mitigate the emergence of drug resistance, and transform malaria diagnostics at the point of care.

Objectives

- To develop a multiplexed lateral flow assay for the sensitive and specific detection of Plasmodium mRNA.

- To integrate synthetic biosensors to enable real-time monitoring of drug resistance markers.
- To achieve simultaneous detection of multiple Plasmodium species

Significance and justification

The integrated diagnostic tool addresses the current limitations in malaria diagnostics, providing precise information about the specific Plasmodium species causing the infection and its resistance profile to antimalarial drugs hence precision in treatment. Precision in treatment is crucial to ensure the right antimalarial drugs are administered, enhancing treatment efficacy and reducing the risk of complications.

The real-time drug resistance monitoring capability aids in promptly identifying emerging resistance patterns, allowing for timely adjustments in treatment strategies which would mitigate antimalarial drugs resistance. Mitigating the emergence of antimalarial drug resistance is critical to preserving the effectiveness of available treatments and ensuring sustained success in malaria control efforts.

The development of a point-of-care diagnostic kit enables swift and accessible testing directly at the patient's location, facilitating timely decision-making which will improve Point-of-Care Diagnostics. Rapid diagnostics at the point of care enhance accessibility, particularly in resource-limited settings, leading to quicker interventions and improved patient outcomes.

The integration of mRNA detection and drug resistance monitoring in a single lateral flow device represents a groundbreaking advancement in malaria diagnostics thus revolutionizing Malaria Management. Revolutionizing malaria diagnostics contributes to a paradigm shift in management approaches, offering a comprehensive tool for healthcare providers to make accurate and informed decisions.

The study leverages synthetic biology components in developing an innovative diagnostic solution, contributing to advancements in the field which will be a research Innovation in Synthetic Biology. This pushing of the boundaries of research in synthetic biology will open new avenues for the developments of novel tools and methodologies in biomedical applications.

The study's outcomes have the potential to impact global health by providing a more effective and accessible diagnostic solution for a prevalent and life-threatening diseases. This will improve diagnostics for malaria as a crucial step in reducing morbidity and mortality rates associated with the disease, particularly in regions with a high prevalence like sub-Saharan Africa.

Study outcomes

The integrated diagnostic device is anticipated to exhibit high diagnostic sensitivity, enabling accurate detection of even low levels of Plasmodium mRNA, ensuring early identification of active malaria infections. Similarly, the device is expected to demonstrate high diagnostic specificity, minimizing the likelihood of false-positive results, and providing precise information about the resistance profile of the malaria parasite to antimalarial.

The study also envisions that the integrated device will possess real-time monitoring capabilities for the malaria parasites, allowing healthcare providers to receive immediate insights into emerging antimalarial drug resistance patterns. This feature is crucial for timely adjustments to treatment strategies, preventing treatment failures, and addressing the challenge of drug-resistant strains.

Lastly, the expected outcome additionally involves the implementation of multiplexing in the diagnostic device, enabling the simultaneous detection of various Plasmodium species and their

resistance to multiple antimalarial drugs in a single test. This multiplexed approach streamlines the diagnostic process, providing comprehensive information in an efficient and resource-effective manner.

LITERATURE REVIEW

Introduction

Malaria, a mosquito-borne infectious disease caused by Plasmodium parasites, continues to pose a significant global health burden and most especially in sub-Saharan Africa accounting for 95.4% deaths globally (*World Malaria Report 2022*, 2022). Effective control of malaria like any other infectious disease is facilitated by informed decisions that require accurate and timely diagnosis of the disease(Tripathi et al., 2023).

The existing methods include; light microscopy, Rapid diagnostic kits and quantitative PCR assay. However, these existing diagnostic methods have limitations, including variable sensitivity, specificity, and accessibility (Mirahmadi et al., 2021)**.**

Over the years, there has been advancement in malaria diagnosis from traditional methods of microscopy to rapid diagnostic kits (RDTs), ELISAs and PCR methods. Light microscopy is the gold standard method for malaria diagnosis and continues to be at the frontline of malaria diagnosis by using stained films of blood for identification and quantification of malaria parasites (CDC (Centers of Disease Control), 2022). However, it is a time-consuming method that presents challenges of false results in cases of low parasitemia and requires a well-trained microscopist (Kahama-Maro et al., 2011).

In the recent years, rapid diagnostic tests have gained prominence over traditional microscopy for malaria diagnosis. Rapid diagnostic tests also called lateral flow assays (LFAs) are rapid chromatographic tests that operate on the principle of capillary action where the sample flows along a membrane strip containing immobilized reagents. This technique is based on an antigen-antibody interaction or DNA-probe hybridization (Bahadır & Sezgintürk, 2016).

Studies that have compared the performance of RDTs and microscopy have shown that RDTs are more sensitive than microscopy hence their effectiveness in malaria diagnosis (Azikiwe et al., 2012).

Despite significant progress, gaps still remain in the use of rapid diagnostic tests due to the increasing number of plasmodium falciparum mutations especially those exhibiting HRP2 and HRP3 gene deletions that cannot be detected using the available histidine rich protein-based RDTs (Kiemde et al., 2022).

Meta-analysis of several studies estimate the prevalence of hrp2 and hrp3 gene deletions to be 21.3 and 34.5 % respectively globally with a higher deletion status in South America followed by Africa[1].

Nucleic Acid Multiplexed Lateral Flow Methods

Research shows that the usage of hrp2 (antigen-antibody) based interaction for malaria diagnosis is limited by false results which are a result of; a mutation in hrp2 gene responsible for hrp2 antigen formation, presence of non-falciparum plasmodium species. Therefore, a better approach would be the

[1]

use of nucleic acid that is constant across all LFAs and can simultaneously detect multiple nucleic acid targets for the different plasmodium species.

Nucleic acid-based assays offer significant advantages that include; Species Differentiation by targeting specific nucleic acid markers, multiplexed assays can differentiate Plasmodium species (e.g., P. falciparum, P. vivax, P. ovale, P. malariae) and high Sensitivity: Amplification techniques (e.g., PCR) enhance sensitivity, detecting even low parasite loads. Point-of-Care Testing: Lateral flow platforms allow rapid, on-site testing without the need for specialized equipment.

METHODOLOGY

The aim of this project is to develop an innovative 2 in 1 malaria diagnostic tool that can integrate Plasmodium mRNA for plasmodium falciparum detection alongside real-time monitoring of drug resistance using molecular bicons . The project will consist of the following steps:

1. Identifying of all the mRNA targets for plasmodium falciparum detection and identifying of the relevant drug resistance markers towards the routinely used antimalarials, ensuring diversity in targets covering various genes of interest.
2. For the detection of plasmodium falciparum, we shall target the mRNA form the HRP2 gene. Gene: PF33D7_0831800, RNA title: mRNA-histidine-rich protein II (XM_002808697.2). The mRNA will be detected by a molecular beacon which contains a complementary sequence to the that of a specific region within the mRNA by binding of the to the specific region in the mRNA causing the beacon to unfold giving off a fluorescence.

3. **Design and synthesis of specific mRNA probes for detection of the different Plasmodium mRNA targets and synthetic biosensors.**

Based on the literature review and the available Plasmodium genome sequences, we will design and synthesize specific mRNA probes that can hybridize to the target transcripts of the different plasmodium species. We will also design and synthesize synthetic biosensors that can sense and report the presence of drug resistance markers in Plasmodium parasites, such as mutations or gene expression changes that confer resistance to commonly used antimalarial drugs majorly for Single Nucleotide Polymorphism.The mRNA probes and synthetic biosensors will be labeled with different fluorescent or colorimetric reporters that can be detected by a lateral flow device.

The designing of appropriate probes and synthetic biosensors will be done using specialized tools and software like Primer-BLAST on the National Center for Biotechnology Information (NCBI) or Primer Quest by Integrated DNA Technologies (IDT).

4. **Optimization and validation of Plasmodium mRNA detection method and synthetic biosensors used for resistance marker identification.**

We will optimize and validate the performance of the mRNA probes and synthetic biosensors in terms of sensitivity, specificity, dynamic range, and stability using various Plasmodium strains and clinical samples.We will compare the results of the mRNA probes and synthetic biosensors with the conventional methods of malaria diagnosis and drug resistance monitoring, such as microscopy, rapid diagnostic tests (RDTs), MAD4HATTER Library preparation and Next Generation Sequencing technique and polymerase chain reaction (PCR) methods .

5. **Designing of the lateral flow strip.**

Basing on the sample to be used and type of reactions that will take place a suitable membrane e.g., nylon will be selected for the immobilizing of the nucleic acid probes. The lateral flow will be designed with multiple test lines, each corresponding to a specific mRNA target. A control line will be incorporated into the kit for the validation purposes.

6. **Validation and optimization of the diagnostic tool.**

Validation of the tool will be done using synthesized mRNA targets and control samples. Optimization of the kit to ensure proper flow of the sample and optimization to determine the appropriate sample type to use that is compatible with the kit and the optimization assay conditions will be based on sensitivity, specificity and reproducibility. We will also evaluate the feasibility and usability of the mRNA probes and synthetic biosensors in a lateral flow format, which is a simple, rapid, and low-cost diagnostic platform .

7. **Field testing and evaluation of the multiplexed malaria diagnostic tool.**

We will conduct a field testing and evaluation of the multiplexed malaria diagnostic tool in a malaria endemic area, where we will recruit and enroll patients with suspected malaria infection and collect their blood samples .

We will use the multiplexed malaria diagnostic tool to identify the presence and species of Plasmodium parasites, as well as their drug resistance status, in the blood samples, and compare the results with the conventional methods .

We will also assess the impact of the multiplexed malaria diagnostic tool on the timely intervention and personalized treatment strategies for malaria patients, as well as on the malaria control and elimination efforts.

ETHICAL CONSIDERATIONS

The development of the proposed kit raises several ethical considerations. Addressing these concerns is crucial for ensuring the responsible and ethical advancement of scientific research and technology.

Informed Consent:

Individuals participating in any trials or sample collection shall be fully informed about the nature of the study, potential risks, and benefits.

Informed consent will be obtained from individuals providing biological samples, making it clear that their samples will be used for research purposes.

Privacy and Confidentiality:

The privacy of individuals whose samples are used in the study shall be respected. Any identifiable information shall be protected and adherence to data protection regulations will be enforced.

Secure data storage and transmission protocols shall be implemented to prevent unauthorized access to sensitive information relevant to the study.

Benefit Sharing:

The benefits of the development of the assay will be shared with the communities involved, especially in regions where malaria is prevalent, ensuring that the communities have access to the benefits of the technology.

Humanitarian Concerns:

The potential impact of the technology on public health will be assessed, taking into account the specific needs and vulnerabilities of the population and the potential consequences of false positives or false negatives.

Regulatory Compliance:

The compliance of the proposed kit development with relevant national and international regulations, including ethical guidelines for clinical research and the development of medical devices shall be assessed. IRB approval shall be sought prior to commencement of the kit development.

Long-Term Impact:

The potential long-term impact of the technology on malaria control, drug resistance monitoring, and public health outcomes shall be assessed, in order to determine whether any modifications to the kit need be made to maintain its efficiency.

POTENTIAL OUTCOMES

Understanding the potential outcomes of the development and deployment of a nucleic acid-based lateral flow device for detecting Plasmodium falciparum, including the use of antisense oligonucleotides and identification of drug resistance markers, is crucial for ethical and responsible research planning. These are some potential outcomes to be considered:

Improved Malaria Diagnosis:

Successful development of the lateral flow device could lead to improved and rapid diagnosis of Plasmodium falciparum infections. This could enhance early detection and timely treatment, potentially reducing the morbidity and mortality associated with malaria in Uganda. The assay's sensitivity and specificity will be tested against the currently most utilised diagnostic methods of microscopy and Antibody-Antigen based RDTs in order to prove that it can be utilised in low-resource settings as an alternative diagnostic method, with the added advantage of being more suitable in diagnosis of active malaria due to detection of the ephemeral Plasmodium falciparum mRNA.

Drug Resistance Monitoring:

Detection of drug resistance markers can contribute to ongoing efforts to monitor and understand the emergence of resistance to antimalarial drugs. This information is critical for adapting treatment strategies and controlling the spread of drug-resistant strains. The use of anti-sense oligonucleotides in a nucleic-acid-based lateral flow device would enable cheaper detection of drug resistance markers than the currently used qPCR and next-generation sequencing methods that have proven to be costly and arduous.

Enhanced Public Health Interventions:

Accurate and timely diagnosis, along with drug resistance information, can inform public health interventions. This may include targeted treatment, vector control measures, and strategies to prevent the spread of drug-resistant parasites.

Research Advancements:

The research associated with developing the nucleic acid-based lateral flow device may contribute to a deeper understanding of Plasmodium falciparum biology, drug resistance mechanisms, and the design of nucleic acid-based diagnostics. This knowledge could have broader implications for malaria research and related fields, including applications in diagnosis and drug-resistance monitoring of other Plasmodium species.

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