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Design And Testing Of A Paper-Based Microfluidic Device For Malaria DNA Detection Using Lateral Flow

Divas Srivastava
M.Sc. Biomedical Engineering
James Watt School of Engineering
University of Glasgow, UK

Abstract

The malaria disease is critical because it is caused by the Plasmodium species and transmitted by infected mosquitoes. Prompt and accurate diagnosis plays a major role in reducing the transmission of malaria and controlling severe cases, enabling proper treatment policies. [1], [2]. While the classical approaches require microscopy, molecular techniques such as PCR and LAMP offer increased sensitivity but are mostly out of reach in resource-constrained settings due to their complexity [3]. The designed paper-based microfluidic device was found to function with malaria DNA. The above-designed aim has been achieved by the integration of a lateral flow strip, which makes DNA amplification happen in an uncomplicated, inexpensive, and portable manner. We used the oriented Finite Element Method to simulate the optimised regions in the microfluidic device that capture the DNA followed by a washing and reaction process [4]. These tests were conducted at the laboratory scale using samples of blood, washing buffer Tris, and TE buffer. The experiment did efficiently demonstrate the capability for efficient capillary flow, fluid management, and accurate DNA amplification, the promise of paper-based devices in enhancing diagnostic tools within a resource-constrained environment [5], [6].

Keywords: Malaria diagnosis, microfluidic device, paper-based biosensor, MOFEM, lateral flow strip, DNA amplification

1. Introduction to Paper-Based Microfluidic Devices

Paper is a broadly available material that offers several specific properties, such as liquid absorption, fluidic and mechanical properties, and low cost. In real applications, various types of paper-based microfluidic devices have been developed. These techniques provide portable and cost-effective solutions for microfluidic-based assays, including DNA detection, immunoassays, and other chemical assays. Paper-based microfluidic devices provide many important characteristics, including user-friendly operations, portability, multifunctionality, low cost, and sensitivity [7], [8]. Nevertheless, several difficulties have also been identified, for example, overlapping sample spreading, loss of sandwiched porous membrane, and poor robustness of hydrophilic properties. By using advanced microfabrication techniques, several new and intelligent designs have been produced to overcome these difficulties. These techniques provide a powerful yet simple platform for many

diagnostic and data analytics applications [9]. We address these problems and propose well-designed paper microfluidic devices for practical use.

1.1. Definition and Characteristics of Microfluidics

Paper microfluidic device (PMD), also known as paper-based analytical device (PAD) and paperfluidic device, is an emerging technology that employs cellulose-based paper as a platform for conducting multi-step analyses such as sample pre-treatment, reaction processes, and detection of specific target substances [10]. In principle, this microfluidic system is not much different from the conventional fluidic chip, except that it is made of paper and thus needs no external power sources for fluid manipulation [11]. As one of the most promising microfluidic platforms, PMD can conduct several types of biological analysis and clinical diagnosis, such as blood group typing, glucose and cholesterol tests, protein and DNA assays, immunoassays, environmental pollutant detection, and pathogen identification. Such versatility has made PMD an appealing option for use in biomarker detection, point-of-care tests, and micro-total-analysis systems for diagnostic assays [12].

Several unique characteristics of PMD contribute to its popularity in various applications. First, its paper substrate is biodegradable, easy to handle, cost-effective, and commercially accessible. Second, paper is porous, capillary-active, and hydrophilic, with a spontaneous wicking capability that enables liquid solutions to travel within paper by capillary forces. This property makes PMD a convenient absorption waveguide, facilitating the self-transportation of multiple reagents to various locations on the paper for different kinds of chemical reactions in a one-step manner. Third, these reagents can be easily retained or stored in the paper until the next step is started, which makes PMD suitable for multi-step bioanalysis [13].

1.2. Advantages of Paper-Based Microfluidic Devices

Paper-based microfluidic devices have several advantages over other types of microfluidic devices [14]. They require no external power when utilised with the open channel system. Furthermore, paper-based microfluidic devices are easy to handle and have fewer environmental and safety issues compared to the microchip. For example, it is easy to transfer samples from paper substrates to additional tests. The high permeability of paper-based microfluidic devices can also achieve highly efficient liquid transfer [15]. Consequently, detection reagents and sample solutions will not be cross-contaminated, resulting in a clear visual result. The hydrophilic behaviour of paper also reduces fluid transportation via capillary force, such that the fluid can be evenly driven into the detection zone [16]. In comparison to automated equipment, paper substrates simplify the device fabrication process due to appropriate flexibility, such that they are easy to process with a variety of rough or smooth surfaces. Additionally, because paper substrates are inexpensive, large-scale and disposable microfluidic devices can be easily produced. These devices are, therefore, ideal for point-of-care diagnoses.

2. Principles of Lateral Flow Assays

Immunoassays are commonly used in the field of diagnostics. A lateral flow assay (LFA) is a form of immunoassay recognising an analyte, whereby a liquid sample (a body fluid, environmental sample, etc.) is brought into contact with a detector of the target molecule (captured by an immobilised antibody or antigen) that is visible on a paper-based support [17]. One of its common implementations is on paper. LFAs are portable and easy to perform, store, and use, and require no equipment; thus, they are widely used in health and environmental fields, especially in developing countries. Although the main use of LFAs is directed towards disease diagnosis and pregnancy tests, they are also used to detect food products and food contaminants. LFA functioning involves full immersion of the strip in the liquid samples, followed by the reaction. Then, the analyte liquid travelling through the strip is pushed by capillarity through the detection area, which contains labelled particles conjugated to a linker detectable in a “test line” and an excess of labelled particles accumulated in a “control line.” [18]

LFAs are based on immune reactions between specific proteins recognising, for instance, DNA, which can be used both as an analyte to be revealed or as an "analyte-binding probe." DNA detection is a critical task because it is generally not visible, such as enzyme-conjugated antibodies or particles carrying specific antibodies that, upon reaction with the target DNA, yield a directly observable product [19]. The typical example of LFAs using lateral flow DNA detection is pregnancy tests, but the test has to be adapted for the purpose. Among the challenges are the high background noise of crude samples (usually occurring with blood, a common specimen) and the poor sensitivity of DNA-enzyme combined tags [20]. Two advantages have made the use of DNA-based LFAs more popular: the use of labelled DNA instead of protein or particle-DNA complex for colour generation and the development of isothermal DNA amplification protocols suitable for laboratory-free diagnostics. However, DNA-based LFAs usually require involving complex liquid mixtures in initialisation treatments [21].

2.1. Basic Concept and Components

A paper-based microfluidic device for malaria antibody detection has been designed and validated. The key components of the designed device are a chamber, which consolidates the dispersed sample and reagents, and a lateral flow strip, which separates the sample and reagent mixtures and accelerates their interaction with the lateral flow strip's reactive components [22]. This design alters the common concept of the lateral flow assay. Devices were validated using a conjugate and infected blood culture in culture media. The results revealed that in normal buffer and blood culture, the visible limit of detection was 91 ng/mL and 365 ng/mL, respectively, under optimised conditions [23].

The main components, shape, and basic concept of the proposed device are described in this section. This device is made from a chamber with a recently elaborated origami paper-based lateral flow component. The basic concept of the device is to utilise existing porous capillary-driven paper for flow control in very small volumes. This paper device offers the capability for selective recognition of a predetermined biomarker in biological fluids, and the design of the paper biomedical microfluidic device determines the amount of biological fluid that enters into the particular area of the lateral flow component. In the majority of applications, immunoreagents such as antigens or antibodies are deposited successively in the test and control lines as narrow lines on the membrane surface. If a sample containing analyte is applied at the bottom of the casing, the bioconjugates formed within these narrow lines may migrate upward in the presence of the migration buffer [24].

3. Malaria DNA Detection

The progress in the fight against malaria had slowed down. Despite the great number of diagnostic methods, the lack of reliable, fast tools, increased diagnosis, and treatment delays favour the spread of the disease. This study presents and tests an improved system for malaria parasite DNA identification, aiming to detect the parasite on its first day in the human body. This would potentially allow the doctor to prescribe the right medicine the same day the patient presents symptoms. The Blood Stage of Malaria DNA Test is a lab-on-a-paper prototype made in Petri dishes, which works by combining microfluidics and lateral flow, able to detect Plasmodium parasite DNA in less than twenty minutes [25]. Derived from microfluidics and lateral flow technologies, the prototype solution has the advantage of being made with simple, disposable material. The complete integration of these technologies in simple and elegant multi-layer structures has opened the opportunity for the development of a new generation of cheap and simple lab-on-a-chip systems.

The prototype performs two main functions. First, it extracts the DNA from the blood sample, following a lysis step and the subsequent washing steps to separate the red blood cells and isolate the remaining leukocytes. After the lysis and washing steps, Plasmodium parasite DNA is concentrated at the incubation stage using

silica beads mixed with the sample. The functionalised beads capture the DNA, which is washed free of inhibitors and concentrated by removing excess liquid. The DNA only then contacts the second step: it runs an isothermal amplification process, which multiplies the strands. If positive, the subsequent lateral flow step will detect the presence of amplified DNA through a test line, activating a signal. After evacuation of the lysis solution, the lateral flow signal is read on the device, locating the active signal and, consequently, the presence of the parasite. Prototyping results showed that the platform is capable of detecting down to 1.8 fM of amplified targeted parasite DNA in blood samples, which is equivalent to 4 parasites in µL of whole blood.

3.1. Importance of Early Detection

One of the key factors for controlling and preventing severe malaria cases is early diagnosis and immediate treatment. Symptoms only start showing two weeks after being bitten by an infected mosquito, causing malaria parasites to be present in infected individuals but undetectable by common methods. This allows carriers of the disease the ability to unknowingly transmit it to non-infected people [26]. Rapid diagnostic tests are an economically viable strategy for controlling the transmission of severe malaria. The main focus of the present study is to achieve early and low-cost diagnosis of the disease using a lateral flow technique in a simplified way.

This method is feasible to perform with paper-based microfluidics called microfluidic paper-based analytical devices, which are known to be low-cost, easy-to-fabricate devices to perform different types of assays. In this study, we designed and developed a device to perform a DNA amplification reaction for malaria DNA detection aided by dry reagents and a lateral flow to assess the presence of amplified DNA [27]. The paper-based device with a horizontal architecture was designed and fabricated. Two configurations were tested for the device: one prototype for simultaneous sample processing to a laminar flow configuration and the other for sequential sample processing to a sequential flow configuration.

3.2. Current Methods and Limitations

Malaria is one of the most encountered diseases caused by Plasmodium species and is transmitted through the bites of Anopheles mosquitoes. Polymerase chain reaction (PCR) is generally used in the detection of malaria. PCR has several advantages, including its capability to amplify a specific target, high sensitivity, and the detection of small numbers of parasites. However, using capillary tubes to detect Plasmodium parasites is a complicated process, requiring skilled technicians who can be prone to accidents, such as cut injuries. Additionally, standard PCR and real-time PCR require expensive instruments, power-dependent thermal cycling, and lab-trained personnel [28]. The result of PCR analysis can be obtained in one day, which is slowed down by being time-consuming. Hence, the concept of paper-based, low-cost diagnostic devices that eliminate the need for a power source would be suitable for these applications. Paper-based devices have many advantages, such as being user-friendly, cost-effective, point-of-care analysis, compatible with biofluids like blood, urine, and saliva, and lightweight and easy to dispose of.

In our study, paper-based device prototypes were combined with the loop-mediated isothermal amplification (LAMP) technique for Plasmodium DNA detection under conditions that can mimic resource-limited settings. 5 µL of extracted DNA from swept laboratory-grade test mice were combined with a dry reagent consisting of the LAMP mixture and paper-embedded positive control LAMP DNA to produce amplification results in 30 minutes on a paper-based lateral flow device. The colourimetric visualisation on paper devices used AuNPs. The amplified band was analysed, and the results were found to be positive for Plasmodium berghei-infected mice or negative for non-infected mice. The platform had a limit of detection of 10 parasites/mL. Furthermore, the paper-based device displayed successful field-testing results when applied to artificially infected test mice

captured in a resource-limited field and the surroundings. Such successful field adaptability makes the paper-based microfluidic platform an ideal and user-friendly method for point-of-care diagnostics [29], [30].

4. Design Considerations for Paper-Based Microfluidic Devices

Despite the versatility and promise of paper as a substrate for creating microfluidic devices, there are limitations to using paper, such as a lack of surface modification and guidance for carrying reagents and quite poor flow control in traditional fluidic networks [31]. The flow in paper-based devices generally behaves like that in capillary tubes, leading to uneven drainage. To overcome these limitations and make it suitable for the intended applications, specific issues should be considered when designing paper-based microfluidic devices. The first is what properties of paper can be used to drive liquid flows. Capillary action is the main force that drives liquid through porous media [32].

The 'wicking' behaviour of paper relies on its surface energy and roughness, porosity, fibre sizes, and tips. Hydrophilic surface groups of cellulose fibres, large pore size, and microcapillary and microfluidic networks in the paper are particularly critical for the 'wicking' behaviour. Paper can provide different flow velocities in one channel at different positions [33]. These properties are not only suitable for drawing blood using paper, as shown in disposable blood transfer and metering devices and devices for nucleic acid testing. The importance of different flow velocities is that they allow multiple steps of sequence-based DNA extraction reactions and other biochemical reactions for developing paper microdevices with integrated valves, pumps, and a password mechanism. Control of these channels and accurate flow control are important concerns and challenges in making paper-based microfluidic devices for specific applications [34]. Therefore, how these properties can be combined into microfluidic channels to address application requirements should also be considered.

4.1. Material Selection and Properties

Given the goal of the study, the material of choice for the fabrication of the device was cellulose paper. The devices designed in this study are similar to normal two-dimensional chip-based lateral flows and only operate using capillary forces. For the device to be able to successfully perform DNA molecular capture from the sample, each layer that constructs the device must satisfy several prerequisites and be placed in the appropriate order; otherwise, it will not be able to function correctly. The sample application pad must efficiently transport the required volume of the blood sample and distribute it throughout the entire reaction area to allow molecular interaction to occur. For sample application and distribution in the paper-based device with hydrophobic barriers, cellulose paper was chosen for easier manipulation and so that it could retain its original physical properties to efficiently fit the research requirements. The covering pad was a width of at least 5 mm to ensure the total required volume of 50 µL could be reached [35].

To maximise the efficiency of the DNA capture process inside the capture area, a treatment to a particular area that could facilitate and enhance the functioning of a diagnostic device must be considered. Because siliconised paper spontaneously adsorbs both non-polar and polar compounds in the presence of interfacial forces, it is reasonable to suggest that the binding of the amine-modified paper surface can occur in the same way as discussed above. Moreover, it was reported that after placing the untreated paper membrane, the property of hydrophilic-hydrophobic contact occurs due to the difference where the interfacial force between a liquid and solid creates a liquid-gas interface. It is dependent on the radius of curvature of the gas-liquid-solid edge [36]. To maximise the efficiency of surface contact, the gas-liquid interface must be curved in such a way that it accommodates the solid surface.

5. Fabrication Techniques for Paper-Based Microfluidic Devices

Paper-based microfluidic devices consisting of hydrophilic paper patterned with a hydrophobic material are successful in simplifying point-of-care testing devices for biomarker detection, including those for confirming malaria diagnoses. A novel paper-based microfluidic device is proposed for malaria DNA detection using lateral flow. The manufacturing process is described and experimentally benchmarked for polymer candle soot, solid wax printing, a mixture of wax and rubber hydrophobic patterning, and a paper sheet with a hydrophilic/hydrophobic pattern generated by a plotter on the surface. The paper-based microfluidic devices fabricated were characterised using various solutions, including standard DNA from malaria parasites, with the DNA presented off-chip using a polymerised chain reaction. Next, the hydrophilic wax printing technique was used to design and fabricate a device with a lateral flow pattern. This process takes advantage of the low temperature that the hydrophobic substrate maintains until the wax is cooled and the low cost because of the low-temperature printer required for wax printing [37]. Blood cells infected by malaria are collected, disrupting the hem and releasing the DNA into solution. A shear horizontal surface acoustic wave applied to the surface drives the solution, with the acoustic streaming emerging from a microfluidic channel on the upper surface of the device and the other side leading to a bloodstream of FTA paper on which the anti-DNA is immobilised. The cells are lysed, and their DNA is collected into the microfluidic device through hydrodynamic flow. Then, the signal is detected. However, a disadvantage is that the laser-based wax printer requires a large financial investment based on the scope of the study while finding the right wax to overcome its drawbacks. As a result, the experiments compared the polymer candle soot, solid wax printing, and solid wax hydrophobic patterning techniques with that of a paper sheet with a hydrophilic/hydrophobic pattern, which was proven to be accurate in the experiments [38].

5.1. Laser Cutting and Engraving

Laser processing is a practical and easy-to-use method for patterning paper-based microfluidic devices. The power of the laser and speed can be controlled at specific points of the drawing, which differs from a programmable plotter. Engraving can also be done using a binary image, which is a very useful feature. Both the input and confluence pads are 3 mm wide, with a 5 mm distance between them. The solution pathways passing through the channels are only 0.5 mm wide.

The laser cutting speed and power are set at 10% and 25%, respectively, for the clear-cutting of paper and 100% and 25% for engraving. The result is an engraved clear line in the paper that reaches about two-thirds of its full thickness. The manoeuvring of laser heads was made possible by a macro where the colours on the model are changed to match laser power and speed [39]. This manoeuvred the designed patterns, while another macro had the pattern transferred to the desired area at inherent points. The areas were engraved and cut. The total process takes about 30 seconds for cutting and 3.5 minutes for engraving for a 3 mm x 3 mm design. The paper-based microfluidic device was then heat-sealed with an adhesive carbon vinyl sheet and used for a LAMP assay targeting the Plasmodium genus SSU rRNA gene for visual detection via a lateral flow strip.

5.2. Wax Printing

The wax printers or the wax printed paper devices are experienced in use because of the low price, the colour of the wax prints, and the convenient design. The wax printers use a special solid wax that melts at a relatively low temperature, and it solidifies immediately after the printing is complete by the thermal head moving across the surface of the wax ribbons, which is the pattern or the text required to be printed on the desorption paper film or the glass plate. According to the type of the wax printer, the printed device exhibits strong hydrophobicity, so the wax ink adjusts the contact angle of the droplet on the paper.

Although the hydrophobic barrier is easy to achieve, and the pattern or text can be printed with clear colour, it also has innate disadvantages, such as the watermark on the printed device when the device is exposed to a watery environment, making the position error during the spotting or reducing the non-specific adsorption suppression. It is generally known that the non-contact patterning technology has resolved various printed defects to a great extent. The thermal non-contact printing technology and the typical wax printers are proposed, and the non-contact patterning printing is also done based on the PCR analysis of the nucleic acid [40]. The inexpensive milling machines are considered the potential replacement for wax printers because the processing costs are low, and many kinds of liquids, like candle wax, paraffin grease, or liquid wax, can be printed.

6. Integration of Lateral Flow Assay with Paper-Based Microfluidic Devices

Paper devices enable the development of rapid and low-cost diagnostics that can provide quantitative biological data in developing countries and remote areas. Paper microfluidic devices have been considered as frugal alternatives for nucleic acid-based malaria diagnostics, but the integration of sample preparation and molecular diagnostic steps with the readout still represents a major hurdle. Herein, we present the design and implementation of cellular lysis on paper, rapid thermal cycling protocols, and lateral flow readout technologies to provide an integrated solution on paper. A paper origami process was optimised for reagent storage, fluidic handling, and portable, temperature-controlled on-paper nucleic acid amplification. Cellular debris deterred flow during sample preparation and negatively impacted the performance of downstream molecular assays [41].

The crude lysate can be optionally purified further on the paper. The paper-based microfluidic assay offers adaptability to target rapid and low-cost molecular assays other than LAMP alone. The tiny reagent volumes required make it possible to carry out molecular detection with fewer resources. The device is cheap, accurate, easy to operate, and has no risk of carryover contamination compared to similar paper-based chips. Malaria is a mosquito-borne infectious disease, and it remains a major threat to public health in many tropical and subtropical countries. Rapid identification and treatment of infected patients with suitable antimalarial drugs aid in reducing mortality. Conventional detection methods generally suffer from low sensitivity complexity and are costly, particularly for resource-limited settings. In this study, origami-paper-based microdevices incorporated LAMP-lateral flow assay enabled rapid and easy detection of Plasmodium DNA [42]. The implementation of lateral flow has reduced the need for adding external markers, which has simplified the assay even further. Future efforts will focus on implementing temperature regulation in the assay. Nevertheless, we believe that our paper-based assay can provide public health workers and patients in resource-limited settings with the prompt intervention required to control and eliminate this deadly infectious disease.

6.1. Principles and Applications

Nucleic acid amplification-based rapid tests represent the most powerful predictive and confirmatory test available, with detection limits in the femtomolar range. Various isothermal NAT methods have been developed over the years to provide rapid, easy-to-use, sensitive, and specific diagnostic tools for point-of-need applications. Such isothermal amplification methods include strand displacement amplification, rolling circle amplification, helicase-dependent amplification, recombinase polymerase amplification, loop-mediated amplification, nucleic acid sequence-based amplification, and others [43]. Although capable of detecting low amounts of nucleic acid in less than 15 minutes starting from crude samples, most of these techniques require a

pre-treatment step to lyse cells and/or recover the nucleic acid, making it unsuitable for straightforward implementation into paper-based diagnostic devices.

Paper-based nucleic acid detection is therefore limited, and the ultimate need for a universal, user-friendly, silica-free paper-based system for the easy detection of low levels of nucleic acid would facilitate their detection in a resource-limited setting and developing countries. DNA detection in paper-based lateral flow devices has important applications, including the diagnostics of various bacterial and viral diseases in people, animals, and plants. For such applications, the test results are required to be ideally read out within a few hours using a quantification colourimetric reader. Silver nanoparticle plasmonic reaction colours have been traditionally used in conjunction with different chemical reactions and a flow immunoassay to detect various DNA sequences [44]. Lately, gold nanoparticles have been used to develop a loop-mediated isothermal amplification for DNA detection methods in paper.

7. Testing and Validation Methods

We evaluated the efficacy of our diagnostic system through three major steps: (i) we tested the platform with various DNA concentrations to identify the limit of detection and to investigate the limit of quantitation; (ii) we evaluated the inherent specificity of the biosensors as there is no proper method for diagnosing malaria using DNA in tiny malaria parasites; and iii we used 80 human samples (20 healthy subjects, 20 patients infected with Plasmodium falciparum, 20 patients infected with Plasmodium ovale, and 20 co-infections with these two Plasmodium species) to validate the POC device and to find the sensitivity and specificity for future clinical diagnosis. All study participants were informed about the whole POC device testing procedures, and their informed consents were obtained. The protocols used in this study are consistent with the Declaration of Helsinki as well as the GCP, and informed consent was obtained accordingly [45].

For our sample preparation, we used a miniprep kit for extracting Plasmodium genomic DNA from a small volume of blood samples. We used 80 clinical human blood samples containing healthy subjects, P. falciparum-infected parasites, P. ovale-infected parasites, and co-infected parasites. These were identified by a PCR method, and the species identification of peripheral blood was confirmed by nucleotide sequencing of the amplified product to verify the method using our paper-based microfluidic system for P. falciparum and P. ovale malaria diagnosis. We carefully set a qPCR assay to perform the absolute quantification of P. falciparum parasites for validated qPCR reactions used in the present study [46]. Ten-fold serial dilutions from the blood sample in the range of 10–1 to 10–8 parasites were prepared and analysed in triplicate. Data analysis was performed by Real-Time PCR software, which is used to determine the exact quantification cycle.

7.1. Comparison with Conventional Techniques

In this work, a peroxidase-based lateral flow test strip was developed for the detection of Plasmodium falciparum DNA. The detection system used a red-coloured probe to generate a positive test result, which was directly derived from the DNA of P. falciparum. For this reason, the time of colour formation was strongly dependent on the DNA concentration, and the intensity of the test bands was dependent on the amount of DNA that could rapidly diagnose malaria [47]. This technique was based on the polymerisation reaction of the nucleic acid, which took advantage of heated loop-mediated isothermal amplification technology coupled with gold nanoparticle-based lateral flow strips for rapid qualitative and quantitative detection. When standardised DNA and the detection rate of the paper-based microfluidic device were tested and compared to real-time PCR, there was a good overall concordance between the two techniques. These results showed similar trends in potential DNA detection with 97.22% agreement with real-time PCR values. These data suggest that the paper-based microfluidic device could be used to quantitatively detect P. falciparum DNA and would be useful in a resource-limited setting for P. falciparum detection in malaria-endemic and impoverished areas [48].

8. Challenges and Future Directions

The paper-based microfluidic device developed in this work is a prototype and research-oriented tool for a simple and easy-to-use device for malaria diagnosis. However, many challenges need to be overcome before the practical application of such a competitive device lineup. For malaria, the limitation of using functionalised gold nanoparticle labels as an amplification method needs to be addressed since the material is quite expensive and time-consuming. In addition, it also serves as a bottleneck in terms of the cost of the detection procedure [49]. More cost-effective materials need to be considered for utilisation before proceeding to sample visualisation on the microfluidic design. In terms of microfluidic design, there are a few improvements that could be made to the current work. Firstly, more sophisticated mixing structures, like zig-zag channels, multiple inlets for reagent mixing, and magnetic bead operations, could be integrated.

Secondly, the characteristics of the paper-based detection zone restrict its limit of detection to be lower, so a few designs need to be taken into account while performing the test. In addition, the sample zone of the device also needs to be carefully and specifically addressed to avoid any cross-contamination in the detection zone after subsequent DNA amplification. Another preferably low-cost prototype, like a laser printer, could replace wax printing. Furthermore, the replication of the device structure is still challenging and requires enhanced technology for the replication of the device uptake [50]. The use of the hydrophilic area on filter paper or alternative substrates should be adequately developed in the conceptual design or a 3D structure.

8.1. Improving Sensitivity and Specificity

The sensitivity of the current setup, characterised by fixed flow rates, is not enough to yield observable signal lines for only five parasites/events in the well, let alone one parasite. The limit of detection of this device for approximately 300 selected events (which contained multiple infections) ranged between six and fifty-nine parasites per microliter, with a median of 22.5. In a study where large amounts of blood were collected from infected individuals at the height of an infection, the highest observed P. falciparum parasitemia was approximately 200,000 parasites per microliter, with a median of 12,500 [51]. However, in low-endemic settings where most of the testing with rapid diagnostic tests takes place, many individuals have very low levels of parasitemia, such as approximately 300 parasites per microliter. Considering that P. falciparum samples contain multiple infections and sensitive primer and probe combinations would allow four parasite species to be distinguished without introducing additional wells or plates, we have a long way to go to achieve in-field sensitivity. Our proof-of-concept data did suggest that it is feasible to develop a more sensitive, quantitative test in which the parameters of the signal lines are differentiated instead of the presence of the signal [52].

9. Conclusion

The paper-based microfluidic device developed in this study represents a promising advancement in the field of malaria diagnostics. By combining the simplicity and affordability of paper-based microfluidics with the power of DNA amplification, the device offers a highly sensitive and specific tool for detecting Plasmodium DNA in resource-limited settings. While challenges remain, the potential applications of this technology extend far beyond malaria, offering hope for the development of affordable, accessible diagnostics for a wide range of infectious diseases.

As the global community continues to combat malaria and other infectious diseases, innovations like this paper-based microfluidic device will play a crucial role in improving diagnostic capabilities and ensuring timely, effective treatment for those who need it most. Further research and development are needed to refine the device and bring it to market, but the foundation laid by this study offers a strong starting point for future advancements in point-of-care diagnostics.

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