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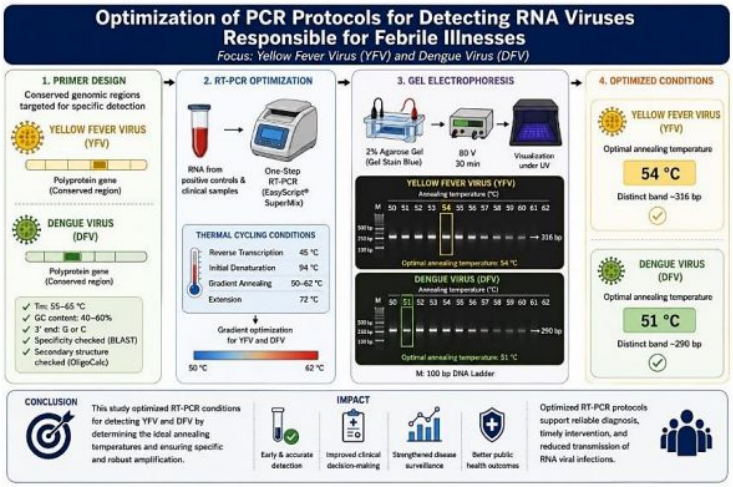
Optimization of PCR Protocols for Detecting RNA Viruses Responsible for Febrile Illnesses

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Graphical Abstract



ABSTRACT

Background: Febrile illnesses, often caused by RNA viruses, remain a significant health challenge in tropical regions, especially in sub-Saharan Africa, where diagnostic infrastructure is inadequate, leading to frequent misdiagnosis. This study aims to optimize RT-PCR protocols to enhance early detection, improve diagnostic accuracy, and strengthen public health surveillance, ultimately reducing morbidity and mortality associated with these infections.

Methods: Primers were designed for selected RNA viruses, including Dengue and Yellow Fever viruses, by targeting conserved genomic regions and optimizing key parameters such as melting temperature, GC content, and binding stability to ensure efficient and specific amplification. Positive controls, including synthetic templates and confirmed positive samples, were used to validate the RT-PCR assays, ensuring consistent results. Reverse transcription and PCR amplification were carried out using the EasyScript® One-Step RT-PCR SuperMix kit, with cDNA synthesis at 45°C, denaturation at 94°C, and gradient annealing (50°C–62°C) followed by extension at 72°C. The PCR products were visualized by gel electrophoresis on a 2% agarose gel prepared with Gel Stain Blue, run at 80 V for 30 minutes, and analyzed under UV light for DNA visualization.

Results: RT-PCR conditions for Yellow Fever Virus (YFV) and Dengue Virus (DFV) were optimized using a gradient of annealing temperatures, with the optimal temperature for YFV determined to be 54°C and for DFV, 51°C. These findings provide the ideal conditions for further diagnostic assays, ensuring distinct and well-defined amplification bands for both viruses.

Conclusion: This study optimized RT-PCR conditions for detecting YFV and DFV, emphasizing the importance of precise annealing temperatures and primer design.

Keywords: Optimization, PCR, RNA viruses, Febrile Illness

1.0 INTRODUCTION

Febrile illnesses pose a persistent health burden in tropical and subtropical regions, particularly in sub-Saharan Africa, where diagnostic infrastructure is often inadequate [1]. These illnesses are commonly caused by a range of pathogens, with RNA viruses representing a significant but frequently overlooked group [2]. Due to their clinical presentation characterized by nonspecific symptoms such as fever, fatigue, and headache RNA viral infections are often misdiagnosed or undiagnosed, especially in settings with limited access to advanced diagnostic tools [3]. Pathogens such as Lassa virus, Yellow Fever Virus (YFV), Chikungunya virus, Human Immunodeficiency Virus (HIV), and Hepatitis C Virus (HCV) are among the most notable RNA viruses implicated in such conditions.

A major challenge in managing febrile illnesses caused by RNA viruses lies in the difficulty of early and accurate diagnosis [4]. Misdiagnosis not only delays appropriate treatment but also hampers public health surveillance efforts [5]. Although Reverse Transcription Polymerase Chain Reaction (RT-PCR) is a powerful tool for detecting RNA viruses due to its high sensitivity and rapid turnaround, its effectiveness can be compromised by several technical factors [6]. These include poor primer design, suboptimal reaction conditions, degraded or contaminated samples, and the presence of PCR inhibitors [7]. Furthermore, the absence of standardized diagnostic protocols in resource-limited settings leads to variability in test performance and undermines the reliability of results [6]. This study is designed to optimize RT-PCR protocols for the detection of RNA viruses associated with febrile illnesses. By improving primer specificity, enhancing amplification efficiency, and fine-tuning reaction conditions, the goal is to develop more robust and reliable molecular diagnostics. The optimized protocols are expected to improve early detection, support timely clinical decision-making, and strengthen disease monitoring efforts. It serves as the foundation for the development of a multiplex assay. Establishing efficient and standardized RT-PCR methods tailored to low-resource settings will have a direct impact on public health by enabling faster diagnosis and reducing the risk of disease progression or outbreaks.

2.0 METHODOLOGY

2.1 Primer Design and Verification

Primers were designed based on conserved genomic regions of selected RNA viruses included in this study: Dengue Virus (DFV; polyprotein gene), and Yellow Fever Virus (YFV; polyprotein gene) as shown in table 1. Reference sequences for each virus were retrieved from the NCBI GenBank database. Multiple sequence alignments were performed using tools such as Clustal Omega to identify conserved regions suitable for primer design.

Primers were designed using PrimerQuest, optimizing for melting temperatures (Tm) between 55–65 °C, GC content ranging from 40–60%, and ensuring the last three bases contained a guanine (G) or cytosine (C) for enhanced binding stability. OligoCalc was used to assess primer quality by checking for the absence of dimers, hairpins, and secondary structures. Primer specificity was validated using NCBI BLAST to ensure unique alignment to the target viral genomes.

Table 1: Below summarizes the primer sequences and expected amplicon sizes for each virus:

S/N	Primer	Forward	Reverse	Band size (bp)
1	DFV	TTATCATCGACGGCCCAAAC	CCAGAACTCCATTGCTCCATAG	315
2	YFV	TTGGAAAGGCGAAGGGAAG	GACATGCTGGTGGTGTATCA	512

2.2 Positive Controls for RT-PCR Optimization and Validation

Positive controls for validating and optimizing the RT-PCR assays for detecting RNA viruses was sourced from various laboratories. Some of which are synthetic positive template (SPT), confirmed positive samples or live attenuated vaccines. By using these positive controls in each RT-PCR run, we can ensure the accuracy and consistency of the assay results, promptly identifying any issues related to reagent performance or potential contamination.

2.3 Reverse Transcription and PCR Amplification

Reverse transcription and PCR amplification were performed using the EasyScript® One-Step RT-PCR SuperMix kit (AE411), following the manufacturer's instructions. Each 20 µl reaction mixture contained RNA template (1 pg to 1 µg), 0.4 µl of 10 µM forward gene-specific primer (GSP), 0.4 µl of 10 µM reverse GSP, 10 µl of 2× ES One-Step Reaction Mix, 0.4 µl of EasyScript® One-Step Enzyme Mix, and RNase-free water to make up the final volume. Thermal cycling was conducted using

a [PCR machine model] under the following conditions: cDNA synthesis at 45°C for 15–30 minutes, initial denaturation at 94°C for 2–5 minutes, followed by 35–40 cycles of denaturation at 94°C for 30 seconds, annealing with a gradient from 50°C to 62°C for 30 seconds, and extension at 72°C at a rate of 1–2 kb per minute. A final extension was carried out at 72°C for 5–10 minutes.

2.4 Gel Electrophoresis:
2% agarose gel was prepared by dissolving 2 g of agarose powder in 100 ml of 1× TAE (Tris-Acetate-EDTA) buffer. The mixture was heated in a microwave until the agarose was completely dissolved, then allowed to cool to approximately 60°C. After cooling, 2 µl of Gel Stain Blue was added to the gel solution for DNA visualization. The gel solution was then poured into a gel casting tray with a comb inserted to create wells. The gel was left to solidify at room temperature for 30-45 minutes. Once solidified, the gel was placed in the electrophoresis chamber, and the chamber was filled with 1× TAE buffer to submerge the gel. DNA samples were loaded into the wells, and electrophoresis was performed at 80 V for 30 minutes. After the run, the gel was visualized under UV light, and images were captured for analysis.

3.0 RESULTS

Optimization of RT-PCR conditions for Yellow Fever Virus (YFV) and Dengue Fever Virus (DFV) was successfully achieved using a gradient of annealing temperatures. For YFV, distinct amplification bands were observed at 50°C, 51°C, 52°C, 54°C, 55°C, and 56°C, with the most intense and well-defined band appearing at 54°C, indicating it as the optimal annealing temperature as shown in figure 1. For DFV, bands were visible at 50°C, 51°C, and 52°C, with the strongest amplification observed at 51°C as shown in figure 2, suggesting it as the optimal condition for further diagnostic assays.

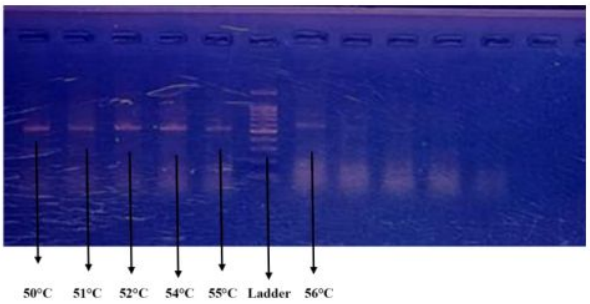


Figure 1: Image of gel view for YFV optimization

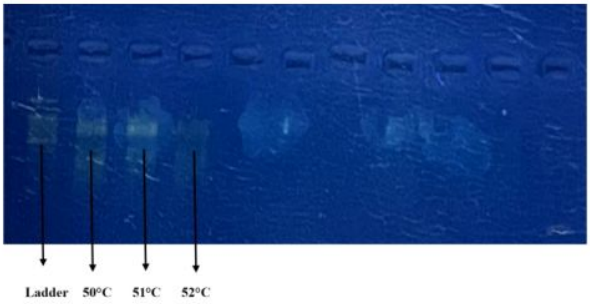


Figure 2: Image of gel view for DFV optimization

4.0 DISCUSSION

The significance of choosing exact annealing temperatures to achieve dependable and specific amplification of viral RNA was highlighted by this study's successful establishment of ideal RT-PCR conditions for the detection of YFV and DFV. At various temperatures, distinct amplification bands were seen; the most pronounced and specific results were obtained at 54°C for YFV and 51°C for DFV. These results demonstrate how minor adjustments to the annealing temperature can have a substantial impact on RT-PCR performance, corroborating previous research by Ishii and Fukui [8] on the role of primer design and thermocycling parameters in enhancing molecular diagnostics.

The availability of optimized, effective diagnostic techniques is crucial in many sub-Saharan African regions where laboratory capabilities are still limited [9]. This study shows that even in low-resource settings, highly effective molecular diagnostics can be developed by carefully adjusting RT-PCR conditions, improving clinical management and early detection. In endemic areas, febrile illnesses caused by RNA viruses such as YFV and DFV are frequently misdiagnosed, owing to clinical similarities to other infections such as malaria and bacterial sepsis [10]. By improving the amplification conditions for these pathogens, the current study helps to develop more accurate diagnostic tools, allowing for better differentiation and timely intervention.

Furthermore, using positive controls at every stage of the RT-PCR procedure including optimization decreased the possibility of false-negative results and helped to guarantee consistency. This approach, which used synthetic sequences and attenuated viral vaccines provide a workable validation method for labs without access to high-containment facilities.
To sum up, this study highlights the importance and feasibility of tailoring RT-PCR protocols to identify particular viral targets. During outbreaks, these

improved assays have great potential to strengthen surveillance networks and increase the precision of diagnoses. Significantly, this work lays the groundwork for the development of multiplex RT-PCR assays, which would enable the simultaneous detection of several RNA viruses in a single reaction. This development would be particularly beneficial for differential diagnosis in high-burden, resource-constrained environments. The applicability of these protocols will be further extended across a wider range of febrile illnesses in future research that includes clinical specimens and additional viral targets.

5.0 CONCLUSION

This study successfully optimized RT-PCR conditions for the detection of YFV and DFV, highlighting the critical role of precise annealing temperatures in ensuring accurate and specific amplification. By employing well-designed primers and validated positive controls, the assay maintained high accuracy, even in resource-limited settings. These findings support the development of multiplex RT-PCR protocols capable of detecting multiple RNA viruses in a single reaction enhancing diagnostic precision, facilitating early detection of febrile illnesses, and strengthening public health surveillance and outbreak response efforts in endemic regions.

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