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Colorimetric reverse transcription loop-mediated isothermal amplification-based approach for early detection of cancer-related pathogens

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ABSTRACT

Sexually transmitted infections are linked to increased risk of some cancers, with chronic inflammation and persistent infections accounting for roughly 20% of cancer cases worldwide. Early detection of these pathogens can help prevent cancer development or progression. This study focused on Human Immunodeficiency Virus-1, Human Papilloma Virus-16, and Epstein-Barr Virus, known to increase cancer risk. A simple colorimetric and reverse transcription Loop-Mediated Isothermal Amplification (LAMP) assay was developed and standardized for rapid detection of these pathogens. The amplification can be visually detected based on color changes. Specifically designed primers targeting conserved genes of HIV-1, EBV, and HPV-16 were analyzed for their specificity and limit of detection. No cross-reactivity was noticed with other pathogenic RNAs and primer sets displayed 100% specificity to their respective pathogenic RNA. We were able to detect as low as 4 RNA copies per reaction within a brief period of 25 minutes. LAMP amplifies a target nucleic acid sequence under isothermal conditions, requiring only basic equipment like a water bath or heating block. Our results show that LAMP's simplicity and reliability make it ideal for point-of-need (PON) use, with its early detection of cancer-related pathogens offering significant potential for cancer prevention, public health education, and interventions.

1. INTRODUCTION

Approximately 20% of human cancers have been found to be associated with inflammation and connected to infection or infectious agents [1–3]. Various sexually transmitted infections (STIs), such as Herpes, gonorrhea, Trichomonas vaginalis, HPV, and EBV have been identified as contributors to both the initiation and progression of cancer [4–6]. Cancer, characterized by abnormal growth of cells, carries an increased risk influenced by multiple factors. Those factors include poor emotional and physical health, high-stress levels, environmental disparities, along with genetics, family history of cancer, and chronic inflammation due to various factors including STIs [7–10].

There has been evidence indicating that the presence of asymptomatic infection contributes to the development of cancers, primarily due to prolonged chronic inflammation [11,12]. This association has been observed to contribute to racial disparities in several cancers including lung, colorectal, liver, prostate, cervical, breast cancer, and so on [13–15]. Studies have demonstrated a significantly higher incidence of prostate cancer among men with a history of exposure to any sexually transmitted infections including gonorrhea, HPV and EBV [16–18]. For cervical cancer,

the fourth most diagnosed cancer in female, HPV infection is one of the most important risk factors [19]. Additional evidence has revealed an association between HPV and EBV, which modify expression patterns in various cellular factors including inflammation, metastasis, and tumor progression, suggesting that coinfection contributes to human malignancies including cervical, nasopharyngeal, and prostate tumorigenesis [20–24]. HIV-1 virus has been linked to an increased risk of developing various types of cancer, primarily due to HIV-1-induced immune suppression [25–27]. For example research indicates that HIV-positive women face an elevated risk of contracting HPV infections and developing cervical cancer [28, 29].

These studies firmly assert that microbial infection plays a significant role in the susceptibility to carcinogenesis and cancer development. In resource-limited communities, infections account for nearly a quarter of all cancer cases, a significant and concerning statistic [30]. Rural communities face a disproportionately higher burden of STIs compared to their counterparts, particularly those with history of certain STIs, facing the higher incidence of cancers and are likely to have more advanced stage cancers compared to other groups [31–38]. Studies showed the prevalence of viral and bacterial infections such as *T. vaginalis*, HPV, gonorrhea as well as prostate inflammation, in African American men than in those of Caucasian men [2, 17, 18, 36–41]. Similarly, The association between HPV infection and cervical cancer is undeniable, with

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African American women being the most likely to die from cervical cancer among all women affected by this disease [42–45]. Evidence showed that chronic infections with these pathogens can lead to sustained inflammation, immune system modulation, and direct genetic damage, all of which can contribute to cancer development. This, in turn, plays a significant role in cancer stage at diagnosis and survival rates. These facts underscore the urgent necessity for the early detection of pathogenic triggers linked to cancer development, particularly within high-risk communities.

Presently, nucleic acid amplification-based detection methods such as conventional polymerase chain reaction/reverse transcription polymerase chain reaction (PCR/RT-PCR) are widely used and golden standard for diagnosis of infectious agents [46,47]. Although sensitive and reliable yet, the reliance on these methodologies involves expensive equipment, reagents, trained technicians, and sample transportation to the laboratories equipped with required instruments. This poses a significant challenge for rural, remote, and underserved communities, where access to laboratories and research infrastructure is often nonexistent. Consequently, there is an urgent need to develop a simple, single-step, ultrasensitive, and cost-effective DNA/RNA amplification-based assay technique, enabling the identification of potential pathogen sources swiftly, whether at the PON or out in the field. The isothermal nucleic acid amplification technique amplifies the DNA/RNA at a constant temperature thus eliminating the need of thermocycler [48]. Strand displacement amplification, nucleic acid sequence-based amplification, Helicase-dependent amplification, rolling circle amplification, multiple strand displacement amplification (MDA), transcription-mediated amplification, signal-mediated amplification of RNA technology, and loop-mediated isothermal amplification (LAMP) are some most frequently used isothermal nucleic acid amplification techniques use for the detection of the infectious agents [48–52]. All these techniques have diverse methods of amplification and detections; therefore, different requirements, strengths, and weaknesses depending on the need. LAMP is a simple, highly sensitive, and cost-effective, nucleic acid amplification technique [53–56]. Due to its capability to rapidly produce large quantities of DNA/RNA and allow naked-eye visualization of the amplified product, this technology has become widely utilized for molecular pathogen detection [52,57–60]. Unlike PCR, it does not require thermocycling and reactions take place at a constant temperature (60°C–65°C) using standard displacement reaction [46]. LAMP uses six different primers to bind six regions of a target sequence, making this technique highly specific for the target sequence and eliminating the problem of non-target amplification, and taking as little as 30 minutes [53,61]. WarmStart RTx Reverse Transcriptase (New England Biolabs, UK) has combined reverse transcription (RT) and LAMP together in a single reaction making it possible to amplify RNA in one step [47,61], shortening the reaction time and allowing the rapid detection of pathogens. In addition, this technique is very simple, and its simplicity makes it practical to be used in remote communities, private clinics, and in the field to detect pathogens. In this work, we developed and standardized a simple colorimetric one-step LAMP assay for rapid detection of HIV-1, HPV-16, and EBV and analyzed them for their specificity and limit of detection. We designed custom primers targeting conserved regions of the respective pathogenic RNA to ensure high specificity and minimize the risk of cross-reactivity. Viral RNA amplification can be confirmed by visualizing a simple color change by the naked eye within as short as 25 minutes. This method can serve as a powerful detection tool for low-intensity

infection due to its sensitivity and selectivity and helps cancer risk reduction and management.

2. MATERIALS AND METHODS

2.1. Virus Source

EBV Verification panel, HIV-1 verification panel, and the HPV Genotype 16 Verification Panel were from Exact Diagnostics. The verification panels (EBV and HIV-1) contain the heat-inactivated whole virus. The HPV Genotype 16 Verification Panel is formulated from human cell lines containing an integrated HPV virus.

2.2. RNA Extraction

Total RNAs were extracted from virus sources using TRI Reagent Solution, according to the manufacturer's protocol {TRI Reagent® Solution Protocol (PN 9738M Rev D)}. The elution volume for RNA extraction was 22 µl.

2.3. LAMP Primer Design

Four different sets of primers were designed for each pathogen using LAMP Designer 1.15 (Premier Biosoft Primer Explorer V5 software). Primer sets were selected based on the guidelines from "A Guide to LAMP Primer Design". Each set of primers comprises the forward inner primer (FIP), backward inner primer (BIP), forward outer primer (F3), backward outer primer (B3), Loop-forward primer (LF), and loop-backward primer (LB). Designed primer sets

Table 1. List of selected set of primers for each pathogen used in this study.

Primer name	Type	Sequence (5'-3')	bp
HIV-GAG	FIP	GGTCTCTTTTAACATTTGCATGGC-TTTAAACACCATGCTAAACACA	46
	BIP	ATGAGGAAGCTGCAGAATGGG-CCCTTGGTTCTCTCATCTG	40
	F3	TCAGCATTATCAGAAGGAGC	20
	B3	AGTTCCTGCTATGTCACTTC	20
	LF	TGCTTGATGTCCCCCCAC	18
	LB	CATGCAGGGCCTATTGCACC	20
HPV-L2	FIP	ATTTGATCAGCAATAGTTTTGCCTT-CATGCAAACAGGCAGGTA	43
	BIP	GGAAGTATGGGTGTATTTTTTGGTG-ATATACCCAGTGCGTCCG	43
	F3	AAAACGTGCATCGGCTAC	18
	B3	GAGGCCTTGTTCCCAATG	18
	LF	CCTTAGGTATAATGTCAGGTGGACA	22
	LB	GGTTAGGAATTGGAACAGGGTC	25
EBV-BGLF	FIP	AGACCGGGTCCCAGTCAACATC-ATTGGAGGAGCACCTGGTAG	42
	BIP	CCGCATTTTCGCTTGGGTCC-GTGGCAGACAGCCTAGCT	38
	F3	GCGGACGTAGTCTCCAACA	19
	B3	TGTCCGTAAGCGCAAACTG	19
	LF	CAATCCACGGCACAACTACTT	21
	LB	TAGACTCCACCCCCCTGT	18

targeted the conserved genes of their respective pathogens. All the primer sets were evaluated for their ability to amplify the target RNA by LAMP. One set of primers for each pathogen was selected and used for further studies (Table 1). Depicts the sequences of selected primers set for HIV-1, EBV, and HPV-16. Primers were named based on the region of the genome in which sequences were derived. Primer sequences were blasted using BLAST: Basic Local Alignment Search Tool (nih.gov) to ensure specificity to its target pathogen.

2.4. One-Step LAMP Reaction

LAMP reactions were prepared to amplify the target pathogenic RNAs. A 10X primer mix for each primer set was prepared beforehand to be used for LAMP reactions. LAMP reactions were carried out

using WarmStartTM Colorimetric LAMP 2X Master Mix from New England Biolabs. A 20 µl reaction mixture (LAMP 2X Master Mix, 10 µl; pathogen-specific primer mix, 2 µl; RNA target, 1 µl; DNase & RNase-free molecular grade water, 7 µl) was mixed and centrifuged for 1 s. LAMP was performed in a thermocycler at 65°C for 15–60 minutes. To ensure the reliability of this assay, negative control reactions were prepared by replacing the target RNA with ultrapure water. Amplification can be directly detected by observing the color change with the naked eye, and gel electrophoresis was performed to confirm the result. All the LAMP experiments were carried out with a minimum of three replicates.

2.5. Gel Electrophoresis

LAMP reaction products were analyzed by using gel electrophoresis to confirm the presence of amplicons. Five microliters of amplified products were run in 1.5% agarose gel containing ethidium bromide, by using 1X TBE buffer at 100V for 30 minutes. The presence of amplicons was confirmed by visualizing the gel under a UV transilluminator system.

2.6. Sensitivity and Specificity

The selected primers were tested for both sensitivity in terms of time and copy numbers, and for specificity. The time sensitivity was determined by performing reactions at time intervals of 15, 25, 30, 45, and 60 minutes. To determine the minimum copy number that can be identified by this assay, 10-fold serial dilutions of HIV-1, HPV-16, and EBV RNA templates ranging from 4 to 40,000 RNA copies per reaction were prepared. An aliquot of each dilution was used as an RNA template to perform the LAMP reaction for a total of 15–60 minutes. The specificity test was performed by using the RNA of non-target pathogens to determine that the assay specifically amplifies only their pathogen of interest. Each set of primers should only be able to amplify its target RNA; to test primer specificity we crossed each primer set with non-target RNA. In particular, HIV-1 and EBV RNAs were used as templates with HPV-L2 primer, while EBV and HPV-16 RNAs were
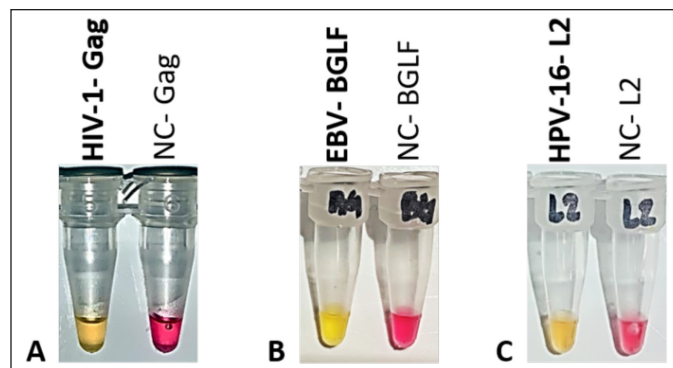

Figure 1. Pathogen specific LAMP primer screening. LAMP primers designed for HIV-1, EBV, and HPV-16 pathogens were screened to test sucessful amplification of their respective target pathogens. Pink color indicates no amplification and yellow color indicates sucessful amplification. A. HIV-GAG primer amplified target HIV-1 RNA. B. EBV-BGLF primer amplified target EBV RNA. C. HPV-L2 primer amplified target HPV-16 RNA. Negative Control (NC) for all three reactions stayed pink indicating no reaction.
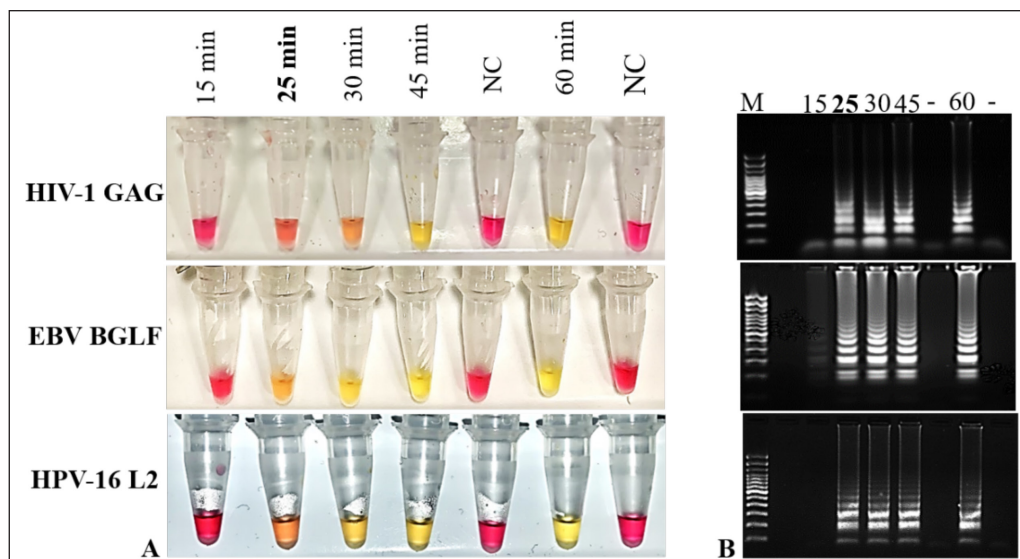

Figure 2. Reaction time optimization for LAMP assay. A. To determine the time sensitivity of selected primers LAMP reactions were carried out at different time intervals from 15 to 60 minutes. The shortest time of detection. was recorded at 25minutes for all three HIV1-GAG, EBV-BGLF, and HPV-16 L2 primer sets. B. Gel electrophoresis results corresponded with colorimetric color detection results. Pink color reaction and absence of bands in gel of Negative control reactions (NC) indicate no reactivity.
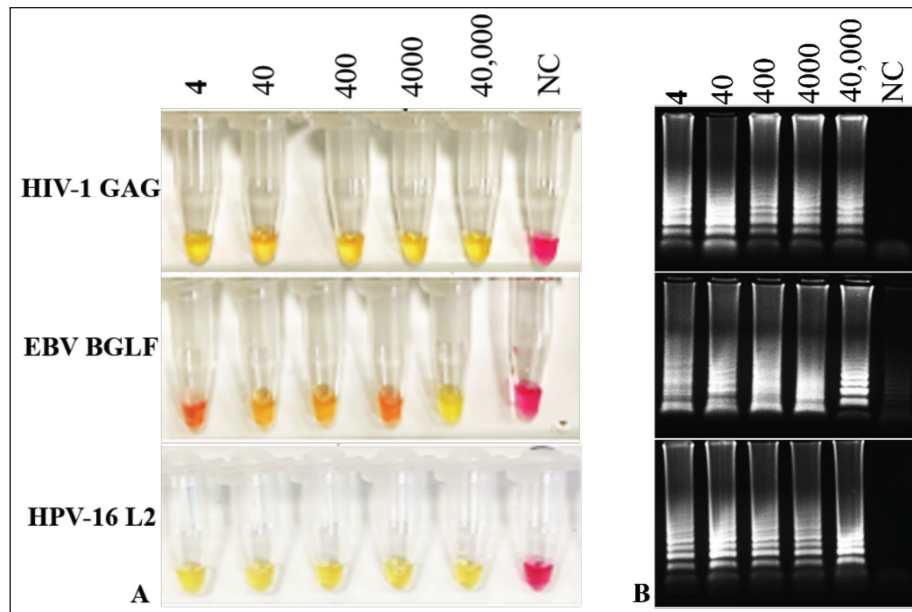
Figure 3. Lowest limit of RNA copy number detection by LAMP. A. RNA detection limit of each primer set was examined by using 10-fold serial dilution of their respective pathogenic RNA ranging from 40,000 to 4 RNA copies per reaction. Each primer set was able detect as low as 4 RNA copy number reaction by visual color change to yellow. B. Results of gel electrophoresis correlate with colorimetric results. No reaction observed in negative control reactions (pink color, and no band in gel).
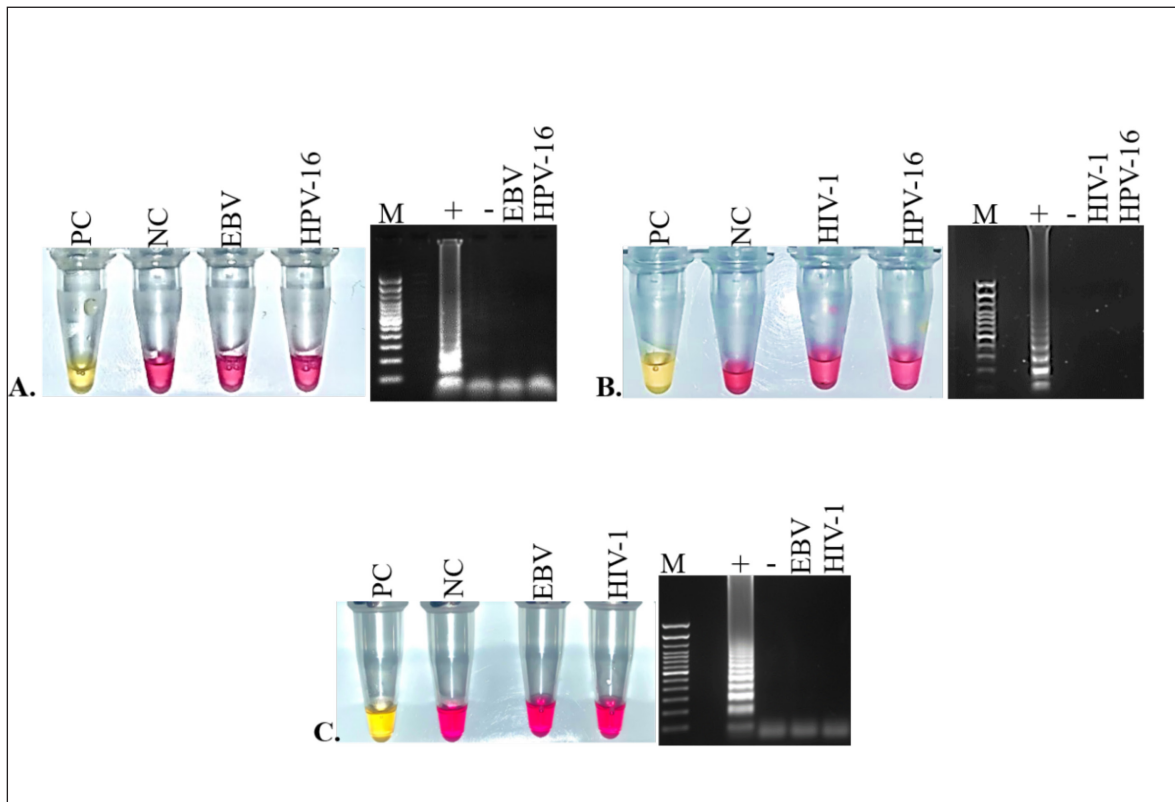

Figure 4. Cross reactivity assay to determine the specificity of LAMP primers. Specificity of each primer set was tested by cross reacting the primer sets with non-target RNAs as template. Every positive control (PC) has primer and it's respective target RNAs in reaction. Target RNAs were replaced by the distilled water in each negative control (NC) reaction. A. HIV-1 specific Gag primer cross-checked with EBV, and HPV-16 non-target RNAs. B. EBV specific BGLF primer cross-checked with HPV-16, and HIV-1 non-target RNAs. C. HPV 16 specific L2 primer cross-checked with EBP, and HIV1 non-target RNAs. No cross reactivity was observed with any primer set. Each primer set demonstrated 100% specificity to their respective target RNAs. Positive controls showed amplification (yellow color) and no reaction was observed in negative controls (pink color).

used as templates with HIV-GAG primer, and HIV-1 and HPV-16 RNAs were used as templates with EBV-BGLF primer. The LAMP reactions were run for a total of 15–60 minutes, followed by gel electrophoresis.

3. RESULTS

3.1. Screening of LAMP Primers for Each Pathogen

Four different sets of LAMP primers for HIV-1, HPV-16, and EBV targeting the conserved genes of each pathogenic RNA, respectively, were designed using Primer Explorer_V5 software. Each set has six primers FIP, BIP, F3, B3, LF, and LB. To determine the ability of each set of primers to successfully amplify their target RNA, the LAMP reaction was carried out with the use of colorimetric pH-sensitive detection dye. Results demonstrated that HIV-GAG (Fig. 1A left panel), EBV-BGLF (Fig. 1B left panel), and HPV-L2 (Fig. 1C left panel) successfully amplified their target RNA by LAMP reaction. Each reaction was carried out with three replicates and results were consistent showcasing the efficacy of these set of primers in amplification of their target RNA. Therefore, HIV-GAG, EBV-BGLF, and HPV-L2 primer sets were selected as optimal primers for detecting HIV-1, EBV, and HPV-16 viruses, respectively, and used for further studies. No amplification was detected in any negative control sample using any primer set (Fig. 1 A–C, right panels). The color change was noted with the naked eyes, positive samples turned pink to yellow while negative samples with no template remained pink in color.

3.2. Sensitivity of LAMP Assay

The selected LAMP primer sets were next tested to determine the lowest time limit of detection and the minimum copy number of RNA detectable under the reaction conditions described previously in section 2.6. To determine the earliest time point of detection, amplification reactions were carried out in triplicate at various time intervals from 15 to 60 minutes. As shown in Fig 2. A. all three primers HIV-GAG, HPV-L2, and EBV-BGLF primer were able to detect their respective RNAs within 25 minutes. The colorimetric result corresponds to the gel electrophoresis. Positive reactions with amplified product produced many bands of different sizes, similar to a typical ladder-like pattern on a 1.5% agarose gel, whereas no bands were observed from negative control reactions (Fig. 2B).

The sensitivity of the assay in terms of RNA copy number was analyzed using 10-fold serial dilutions ranging from 40,000–4 RNA copies per reaction in triplicates. As shown in Figure 3A the detection limit for all three primers HIV-GAG, EBV-BGLF, and HPV-L2 was as low as 4 RNA copies per reaction. The colorimetric color change (Fig. 3A) readings were in complete accordance with the results of gel electrophoresis (Fig.3B).

3.3. Specificity of Pathogen-Specific LAMP Primers

To evaluate the pathogenic specificities of each primer, cross reaction assays with non-target RNAs as templates were performed. HIV-GAG primer was cross tested with HPV-16 and EBV RNAs, EBV-BGLF primers were cross tested with HIV-1 and HPV-16 RNAs, and HPV-L2 primer was cross tested with HIV-1 and EBV RNAs. Results revealed that all three primers HIV-GAG (Fig. 4A, left panel), EBV-BGLF (Fig. 4B, left panel), and HPV-L2 (Fig. 4C, left panel) are highly specific and only produced the amplicons when reacted with their respective pathogenic RNA. No cross reactivity was observed with non-target RNA templates (Fig. 4A–C, left panel). Indicating that HIV- GAG, HPV- L2, and

EBV- BGLF primers are specific only to HIV-1, HPV-16, and EBV sequences, respectively. The same results were observed with all triplicate and gel electrophoresis results corresponding with color changes (Fig. 4A–C, right panels).

4. DISCUSSION

The early detection and timely identification of cancer-related pathogens such as HPV, EBV, and HIV can play a crucial role in understanding and managing illnesses, especially in asymptomatic patients. For rapid detection of viral infections directly from the source, PCR/RT-PCR and immunoassay methods are among the most popular approaches today [62]. PCR/RT-PCR is typically highly sensitive but can be expensive, time-consuming, and require costly thermocyclers and trained personnel to operate [63]. On the other hand, immunoassay-based detection is cheaper, more robust, and accessible to untrained users. However, antibody-based detection does not achieve the same level of sensitivity as nucleic acid amplification methods [63,64]. When choosing a method for pathogen detection, it is crucial to consider several factors, including whether the assay is intended for laboratory-based use or point-of-care (POC)/PON testing, the urgency of the diagnosis, and the associated costs. A POC test is conducted at or near the location where a patient first engages with the healthcare system. It offers rapid turnaround times and delivers reliable information that can be used to develop patient management plans. The immediacy of PON testing and the swift receipt of results reduce the need for multiple patient visits and enable timely treatment. In this study, a simple, rapid, and sensitive nucleic acid amplification-based assay was developed to detect three cancer-related pathogen HPV-16, EBV, and HIV-1 by using one-step LAMP colorimetric method. Four different sets of primers were designed for each pathogen and after screening HIV-GAG, EBV-BGLF, and HPV-L2 primer sets targeting HIV-1, EBV, and HPV-16 viruses, respectively, were selected. In consideration of the specificity and accuracy of pathogen detection, the assay was developed to specifically amplify a consered region of each pathogenic RNA and results were monitored by visual inspection of color change from pink to yellow. All positive samples identified by color change were also detected by gel electrophoresis. The result of gel electrophoresis was in complete accordance with visual color change detection. It eliminates the need of postamplification procedures like ethidium bromide staining and gel electrophoresis saving time, reducing the need of trained people, and making it suitable for PON use.

The LAMP assay exhibited a noteworthy amplification sensitivity both in terms of time and template copy number. All three pathogenic RNAs were amplified within 25 minutes by LAMP assay (Fig. 2). The LAMP assay successfully detected 4 RNA copies per reaction for all three pathogens HIV-1, EBV, and HPV-16 (Fig. 3) proving the high sensitivity of this method and making it possible to detect low concentration of pathogen present in the earlier stage of infections. Cross reactions with non-target RNAs were performed to further confirm the specificity of HIV-GAG, EBV-BGLF, and HPV-L2 primer sets. No false positive reactions were observed and none of the primers showed any cross reactivity with non-target RNAs (Fig. 4). These results demonstrated that all three primers showed 100% specificity and only amplified their target RNA. The 100% specificity of these primers presents a promising opportunity to develop a multiplex detection assay based on LAMP. This assay would enable the simultaneous detection of all three target pathogens by introducing distinct sets of primers to amplify specific DNA/RNA regions within a single reaction. We have

developed a test for three cancer-related pathogens, highlighting the promising potential of the LAMP assay in clinically diagnosing various cancer-associated pathogens. Nonetheless, the reliability of this assay warrants additional scrutiny through comprehensive testing using a large-scale clinical sample pool. LAMP can be designed for multiplexing, enabling the simultaneous detection of multiple pathogens. The comparison of pathogen detection methods highlights the distinct advantages and limitations of each approach. Most used traditional PCR/Real-time PCR requires 2–4 hours to deliver results, incurs a cost of approximately $5–$10 per reaction, and requires an expensive thermocycler ($3,000–$25,000). Antigen–antibody detection methods, such as ELISA or lateral flow assays, provide results from 15 minutes to 2 hours at a lower cost of $1–$5 per reaction. However, these methods often lack the sensitivity required to detect low pathogen concentrations. In contrast, the LAMP assay is highly sensitive, and offers a rapid result within 25–30 minutes at a cost of $1–$2 per reaction, making it particularly suitable for PON testing and resource-limited settings. LAMP could serve as a powerful detection tool for low-intensity infection due to its sensitivity and selectivity and help cancer risk management and STI treatment decisions. Rural communities would benefit from this highly sensitive diagnostic to inform the timely treatment of infectious or even chronic diseases.

5. CONCLUSION

In conclusion, this study successfully developed a one-step colorimetric LAMP assay for the rapid and precise detection of HIV-1, HPV-16, and EBV. The assay exhibits outstanding sensitivity, enabling the identification of even minimal traces of these pathogens. Furthermore, it holds great potential for simultaneous and multiplexed detection of multiple human pathogens in a high-throughput format, ensuring highly accurate results even at very low pathogen concentrations.

6. ACKNOWLEDGMENTS

7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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