CANCER

LAMP-enabled diagnosis of Kaposi's sarcoma for sub-Saharan Africa

Duncan McCloskey[1], Aggrey Semeere[2], Racheal Ayanga[2], Miriam Laker-Oketta[2], Robert Lukande[3], Matthew Semakadde[4], Micheal Kanyesigye[5], Megan Wenger[6], Philip LeBoit[7], Timothy McCalmont[7,8], Toby Maurer[9], Andrea Gardner[10], Juan Boza[1], Ethel Cesarman[10], Jeffrey Martin[6], David Erickson[11,12]*

Kaposi's sarcoma (KS) is an endothelial cancer caused by the Kaposi's sarcoma-associated herpesvirus (KSHV) and is one of the most common cancers in sub-Saharan Africa. In limited-resource settings, traditional pathology infrastructure is often insufficient for timely diagnosis, leading to frequent diagnoses at advanced-stage disease where survival is poor. In this study, we investigate molecular diagnosis of KS performed in a point-of-care device to circumvent the limited infrastructure for traditional diagnosis. Using 506 mucocutaneous biopsies collected from patients at three HIV clinics in Uganda, we achieved 97% sensitivity, 92% specificity, and 96% accuracy compared to gold standard U.S.-based pathology. The results presented in this manuscript show that LAMP-based quantification of KSHV DNA extracted from KS-suspected biopsies has the potential to serve as a successful diagnostic for the disease and that diagnosis may be accurately achieved using a point-of-care device, reducing the barriers to obtaining KS diagnosis while increasing diagnostic accuracy.

INTRODUCTION

Kaposi's sarcoma (KS) is a cancer of lymphatic endothelial origin caused by the KS-associated herpesvirus [KSHV; also known as human herpesvirus 8 (HHV-8)] (*1*–*4*). It is one of the most common and deadly cancers in sub-Saharan Africa where its development is frequently driven by immune suppression from HIV infection (*5*, *6*). Treatment for KS entails use of antiretroviral therapy and chemotherapeutics with recovery substantially more likely when a diagnosis is obtained and therapies administered in the early stages of the disease (*7*). Survival, however, is still poor for most patients in sub-Saharan Africa as the disease is often not diagnosed until it is at an advanced stage (*8*–*14*). A population-based estimate found that 82% of newly diagnosed patients with KS are classified as T1-advanced disease by AIDS Clinical Trials Group criteria (*15*). Patients diagnosed at this stage have between a 2.7- and 7.4-fold greater chance of death than those at the T0 stage (*12*, *13*, *16*, *17*).

The reason for persistent late-stage diagnosis of KS is multifactorial but is at least partially due to the lack of local capacity for traditional pathologic diagnosis. KS typically presents with nonspecific dark lesions on the skin or mucous membranes, and clinical diagnosis is often made on the basis of the macroscopic appearance of these lesions, where they can be confused with other common skin conditions. In a study analyzing 739 East African patients referred with lesions suspicious for KS, only 77% of clinical diagnoses were found to be accurate after review by U.S. pathologists (*18*). Therefore, clinical suspicion alone is suboptimal, and a more objective diagnostic test is needed. The typical alternative is pathologic diagnosis, but an assessment of local pathology revealed a sensitivity of 72% and a specificity of 84% when compared to gold standard analysis (*18*, *19*). This challenge is compounded by the scarcity of trained pathologists, as almost all sub-Saharan African countries have fewer than one for every 500,000 people (*20*). Where pathologists are available, patients must return for a second visit to receive results, and turnaround time is often extensive, all resulting in delays in receiving diagnosis and subsequent therapy (*21*).

To address the issue of late-stage diagnosis, we hypothesized that a rapid point-of-care diagnostic test for KS could be developed by taking advantage of the viral nature of the disease and quantifying the amount of KSHV DNA from a KS-suspected mucocutaneous lesion biopsy. Qualitative testing only for the presence of KSHV infection would not work due to the endemic nature of the virus, with between 30 and 60% seroprevalence in sub-Saharan Africa (*22*). However, quantification of the viral load within suspected KS lesions could offer much higher specificity that would be needed for an accurate diagnostic test. Point-of-care tests based on nucleic acid detection have proven successful at decreasing time to diagnosis and improving outcomes for infectious disease (*23*–*26*), but there have been few attempts to apply similar methodologies to cancers. To our knowledge, there is no prior evidence of successful nucleic acid–based testing for the diagnosis of KS (*27*).

Here, we present the results of LAMP (loop-mediated isothermal amplification)–based molecular diagnosis of KS using nucleic acid extracted from skin lesion biopsies collected from 506 patients from three different HIV clinics in Uganda. The analysis is conducted in TINY (Tiny Isothermal Nucleic acid quantification sYstem), a portable and energy-flexible point-of-care device, which we have

[1]Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY 14850, USA. [2]Infectious Diseases Institute, Makerere University College of Health Sciences, Kampala, Uganda. [3]Pathology Department, Makerere University College of Health Sciences, Kampala, Uganda. [4]HIV Clinic, Masaka Regional Referral Hospital, Masaka, Uganda. [5]Immune Suppression Syndrome Clinic, Mbarara Regional Referral Hospital, Mbarara, Uganda. [6]Department of Epidemiology and Biostatistics, University of California, San Francisco, San Francisco, CA 94143, USA. [7]Pathology and Laboratory Medicine, University of California, San Francisco, San Francisco, CA 94143, USA. [8]Golden State Dermatology Dermatopathology, Walnut Creek, CA 94598, USA. [9]Department of Dermatology, Indiana University School of Medicine, Indianapolis, IN 46202, USA. [10]Pathology and Laboratory Medicine, Weill Cornell Medical College; New York, NY, 10021, USA. [11]Sibley School of Mechanical and Aerospace Engineering, Cornell University; Ithaca, NY, 14850, USA. [12]Division of Nutritional Science, Cornell University, Ithaca, NY 14850, USA.
*Corresponding author. Email: de54@cornell.edu

demonstrated (*28*) is compatible with the requirements for operation in limited resource settings (*29*–*32*). This paper represents the first large-scale effort to determine both if LAMP-based nucleic acid quantification may be able to accurately diagnose KS and conduct that analysis in a point-of-care compatible device. For all samples, a portion of the tissue was used for local standard-of-care pathology to guide clinical treatments. Molecular analysis and gold standard pathology were performed in the United States to reduce the number of confounding variables. Further investigations under additional real-world settings are required before the approach could be used clinically.

RESULTS

We consecutively consented and collected 506 patient biopsies across three different clinics in Uganda to test whether the LAMP-based approach could be a feasible alternative to traditional histopathology methods (Fig. 1). Standard care for patients who present with lesions clinically suspected of being KS includes a biopsy that is sent for pathology analysis. This traditional approach has substantial associated costs due to the scarcity of skilled pathologists and limited availability of resources. Our proposed alternative is to apply modern molecular techniques by extracting and purifying viral DNA from the tissue, which can then be quantified using LAMP to offer a more timely and inexpensive diagnosis.

Parallel tissue analysis using histopathology and LAMP

To assess the efficacy of LAMP as a KS diagnostic tool, we analyzed each patient sample using two techniques. Tissue samples received a diagnosis using (i) gold standard U.S.-based histopathology and (ii) molecular analysis using LAMP (Fig. 2). Biopsies were collected in Uganda, with a portion used for local care and the remaining portion shipped to the United States for both pathology and molecular analyses.

Traditional diagnosis begins with hematoxylin and eosin (H&E) staining, which was performed in Uganda. Immunohistochemistry for KSHV latency-associated nuclear antigen (LANA) was performed at Weill Cornell Medicine, where a blinded pathologic assessment for the presence versus absence of KS was conducted. This was followed by a second assessment by a dermatopathologist at the University of California, San Francisco. If the two pathology readings were in agreement, then a consensus diagnosis was rendered positive for KS, negative for KS, or indeterminate. An indeterminate result could result from either insufficient or inadequately processed tissue, as well as tissue with some but not all features of KS in combination with a negative or unconvincing LANA stain. Given a disagreement or indeterminate consensus, a third reading by an additional pathologist was conducted. The consensus diagnosis of positive or negative for KS was then made if two of the three readings agreed, where a result of indeterminate could mean the consensus was indeterminate or there was no consensus.

Concurrent to the pathology readings, molecular analysis of a biopsy section was performed by quantifying the amount of KSHV present using LAMP. Total DNA was extracted from the tissue, purified using the QIAGEN DNeasy Blood and Tissue kit, and diluted to 2 ng/μl to maintain consistency between patient samples in the event of different or incomplete extractions. Amplification was performed in the point-of-care LAMP device TINY while targeting the *Orf26* gene of KSHV (fig. S1). Part of our hypothesis is that lesions diagnosed positive for KS should exhibit large amounts of KSHV present within the biopsy. Therefore, strong KSHV amplification would likely indicate a positive KS diagnosis. Conversely, weak or no amplification would likely indicate a negative KS diagnosis. Samples were tested in duplicate and classified according to their mean amplification threshold time. Since LAMP does not operate using temperature cycles like in quantitative polymerase chain reaction (PCR), the amplification threshold time for our LAMP reaction signifies the point, measured in minutes, at which amplification overcomes our threshold for random noise. Samples were tested again in duplicate if their threshold times disagreed—one sample amplified and one did not, or the disparity between replicates was greater than 2 min 40 s (top 5%).

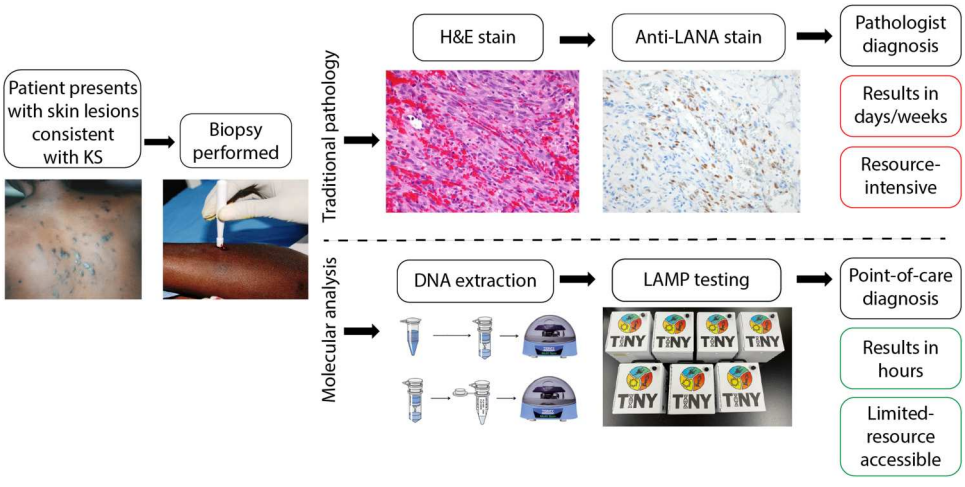

Fig. 1. Flow diagram for traditional and proposed diagnosis techniques. Comparison of traditional histopathologic diagnosis of KS and proposed molecular diagnostic method. After clinical examination and biopsy, traditional diagnosis is performed using a hematoxylin and eosin (H&E) stain and an anti-LANA stain if available. This is considered the gold standard for KS diagnosis yet is resource demanding. Molecular analysis using skin requires DNA extraction and purification before LAMP testing in TINY. This point-of-care approach may provide a diagnosis in only a few hours, as well as be accessible within limited-resource settings.

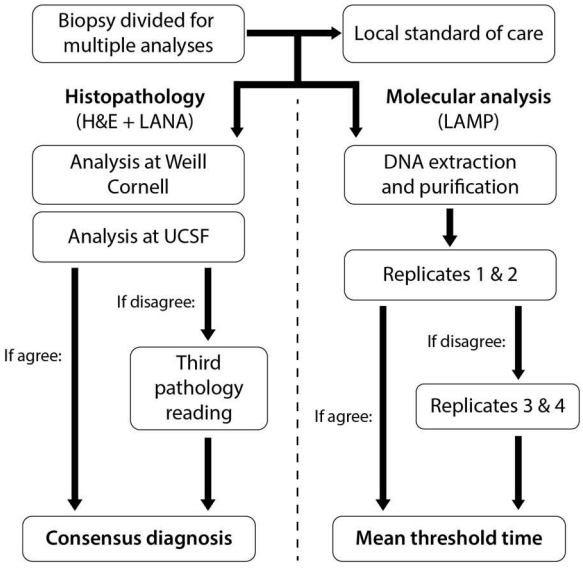

Fig. 2. Study design flow for biopsy analysis. Study design for analysis of LAMP as a diagnostic tool for KS. A section of biopsy is sent for analysis by at least two U.S. pathologists, where a third pathologist is used if a consensus diagnosis is not made. A different portion of the biopsy undergoes DNA extraction and purification before being tested in duplicate in the TINY. If those replicates disagree—one did not amplify and the other did, or they are among the top 5% of disparate threshold times—then additional replicates are performed. The output value used for KS diagnosis is the mean threshold time of all available (two or four total) replicates.

Reproducibility of LAMP threshold times during KSHV analysis

All 506 patient samples were analyzed according to the study design described in Fig. 2. Initial LAMP analysis produced two threshold times for each patient sample. A Bland-Altman plot was used to visualize the concordance between the first two replicates for each patient when both replicates amplified (Fig. 3). A large majority of the samples had a threshold difference of less than 2 min, especially at earlier time points (n = 460). Lower amounts of KSHV present produced later amplification times, which is less consistent in our LAMP assay (*28*). Samples that were in the top 5% of threshold time difference had a disparity of 2 min 40 s or greater and were retested in duplicate (n = 18). In addition, samples where one replicate did not amplify and one replicate saw positive amplification also had third and fourth replicates completed (n = 28). The Bland-Altman coefficient of repeatability for samples that amplified earlier, with mean threshold times under 18 min 45 s, was 1 min 53 s (n = 315). Including samples that amplified later, using a cutoff of 26 min 10 s, the coefficient of repeatability was 3 min 5 s (n = 344). In general, samples with later threshold times had a larger repeatability coefficient due to lower copy numbers of KSHV causing less consistent amplification.

Performance of LAMP as a KS diagnostic method

Comparison of LAMP threshold times with the consensus pathology result shows that samples with early threshold times are highly likely to be reported KS-present by pathology, while KS-negative samples are most often not amplifying at all (Fig. 4). A large majority of the KS-present samples as indicated by the consensus pathology result had mean threshold times between 12 and 22 min, while KS-absent samples often had threshold times of 50 min, indicating that no amplification was detected throughout the duration of the assay. Overall, KS diagnosis using LAMP shows good agreement with gold standard pathology diagnosis (Table 1) in this dataset. Consensus pathology results were mostly achieved using two pathology readings (n = 437), while a third pathologist reading was required for ~13% of patients (n = 69). Of the 506 samples analyzed, 15 were indeterminate after analysis by three pathologists, largely due to poor quality samples or lacking LANA. Results for the LAMP analysis of these 15 samples were split, with 7 and 8 samples that would be considered negative and positive for KS, respectively.

A receiver operating characteristic (ROC) curve is used to illustrate the efficacy of LAMP as a diagnostic method for KS (Fig. 5). The area under the curve was 0.967 with a 95% confidence interval (CI) of 0.948 to 0.985. Two cutoff times were selected to maximize either sensitivity or specificity of the assay while keeping 90% as the minimum for the nonmaximized value. At 18 min 45 s, specificity is optimized at 94% with a sensitivity of 90%. This cutoff produces an overall accuracy of 91% when using the mean of all available replicates. A cutoff time of 26 min 10 s optimizes the sensitivity of the assay at 97% while maintaining a specificity of 92%, producing an overall accuracy of 96%.

For generating the ROC curve, we introduced a cutoff time for the threshold values (fig. S2). At a given cutoff time t, any samples with a mean threshold time value before the cutoff would be considered a KS-positive diagnosis by the LAMP assay. Similarly, samples with a mean threshold time value after the cutoff would be considered KS negative. Each cutoff value produces a sensitivity and specificity result when compared to the known true diagnosis from the pathology classification. An ROC is generated using the cumulative sensitivity and specificity results as the cutoff time t is moved through the range of available times.

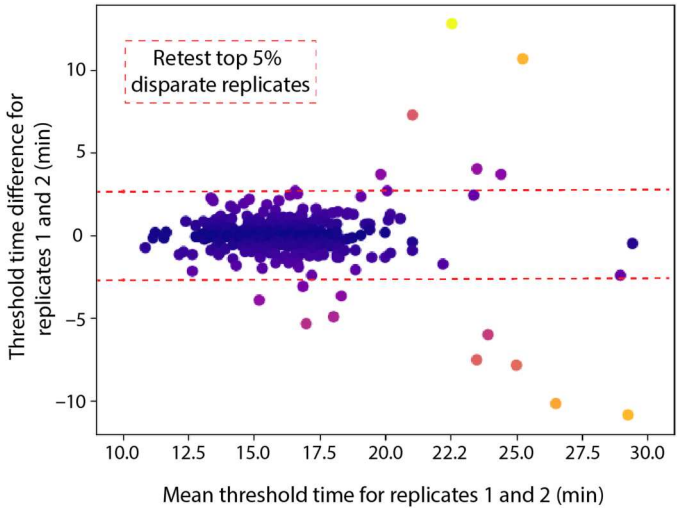

Fig. 3. Multireplicate KSHV-LAMP reproducibility analysis. A Bland-Altman plot (n = 356) of LAMP replicates 1 and 2 when both amplified. Dashed lines exclude replicates (n = 18) with top 5% largest difference (absolute threshold time difference of 2 min 40 s and greater) to be retested in duplicate. Darker data point color indicates a difference closer to zero.

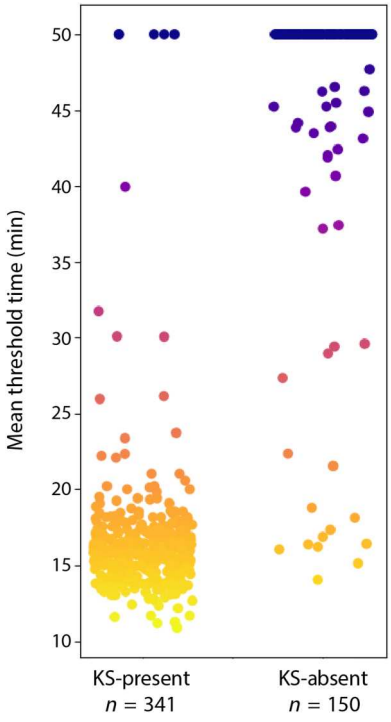

Fig. 4. Threshold time comparison of KS-present and KS-absent samples. Mean KS-LAMP threshold time value for all available replicates organized by consensus pathology result; mean of two replicates used for $n = 448$ and mean of four replicates used for $n = 43$. Excluding samples indeterminate by pathology ($n = 15$). Darker color indicates later threshold time.

DISCUSSION

KS has a high disease burden in sub-Saharan Africa where there is limited infrastructure for traditional pathology, making timely diagnosis difficult. Our goal was to assess the performance of LAMP-based biopsy analysis as an approach to diagnose KS at the point of care. With biopsies collected from Ugandan patients suspected of having KS, we compared our molecular approach to gold standard U.S.-based histopathology. Analysis of these 506 biopsies shows that we may be able to diagnose KS with an accuracy of 96%, a sensitivity of 97%, and a specificity of 92%, exceeding the accuracy of clinical suspicion and local pathology (*18*, *19*). The impact of our approach is further amplified by the fact that diagnosis is accomplished using a point-of-care device that can give accurate results with minimal training, equipment, and consumables required (*28*).

With the preliminary success of LAMP and the TINY in our U.S.-based laboratory setting, the continuation of this effort will include assessment of the accuracy of our approach in an external validation sample of specimens and expanding the dataset for more cases through deployment and testing at several sites across sub-Saharan Africa. One of the limitations of this study is the fact that all molecular testing was performed in pristine laboratory conditions with a highly trained U.S. operator, without assessing the real-world performance of our approach with local testing in real time, where issues like contamination and power interruptions could affect overall diagnostic performance (*33*). The robustness of the assay could also be improved through additional measures such as implementing a cellular DNA control using the housekeeping gene, GAPDH, and the potential inclusion of deoxyuridine triphosphate and uracyl-DNA glyclosylase to prevent carryover contamination. Improved software algorithms can also be implemented to improve the ability of our approach in identifying contamination and other problems with amplification.

Molecular approaches for screening of cancer and other skin conditions can offer the possibility of earlier diagnosis compared to traditional approaches (*34*, *35*), especially in limited resource settings (*36*, *37*). Our LAMP-based approach for KS may provide an accurate diagnosis after only a few hours compared to the days to weeks that patients wait for a pathology diagnosis (*28*). This reduced time to result could enable faster initiation of accurate treatment, improving patient outcomes. Eliminating the long wait time for a biopsy result could also eliminate the risk of inaccurate treatment, such as prescribing dangerous chemotherapeutics to a KS-negative patient. Further investigation can also be performed on repeat testing to be used for patients with a borderline LAMP result, where a patient follow-up LAMP analysis could be performed after a certain period of time or repeat biopsy of a different lesion. With continued improvement, molecular analysis could be the first step for future KS diagnosis, where assessment by a pathologist is only needed in borderline or unclear cases. In addition, advancements in point-of-care technologies should continue to reduce testing costs, increase testing capacity, and simplify the user training required (*38*).

While our immediate focus is on KS diagnosis, the use of LAMP and other forms of isothermal amplification can be adapted for similar dermatological diseases (*39*, *40*). Skin-associated infections of various organisms—viral, mycobacterial, and fungal—can all cause conditions where immunohistochemistry or microbiologic culture is needed for diagnosis (*39*, *41*, *42*). Even in settings with plentiful resources, diagnosis of certain conditions can be difficult (*43*), making a definitive diagnosis extremely rare in limited resource settings. It is also unlikely that sufficient immunohistochemistry and culture capability will become common in developing nations before nucleic acid testing devices become widely available. Clinical adoption of nucleic acid testing in limited resource countries has already been successful with certain diseases, such as using the GeneXpert for tuberculosis testing (*23*). Future advancements in sample processing technologies could also reduce the time and processing steps required before molecular analysis (*44*). Hence, point-of-care devices with broad applications in this testing domain

Table 1. Pathology and KS-LAMP cumulative results. Results for consensus pathology and both KS-LAMP cutoff times show overall good agreement. For the pathology consensus, 437 results were determined with two pathology readings, with 69 needing a third interpretation to reach consensus.

	Pathology consensus $n = 506$	KS-LAMP (26 min)* $n = 491$	KS-LAMP (19 min)* $n = 491$
KS-present	341	345	318
KS-absent	150	146	173
Indeterminate	15		

*Not including $n = 15$ indeterminate by pathology.

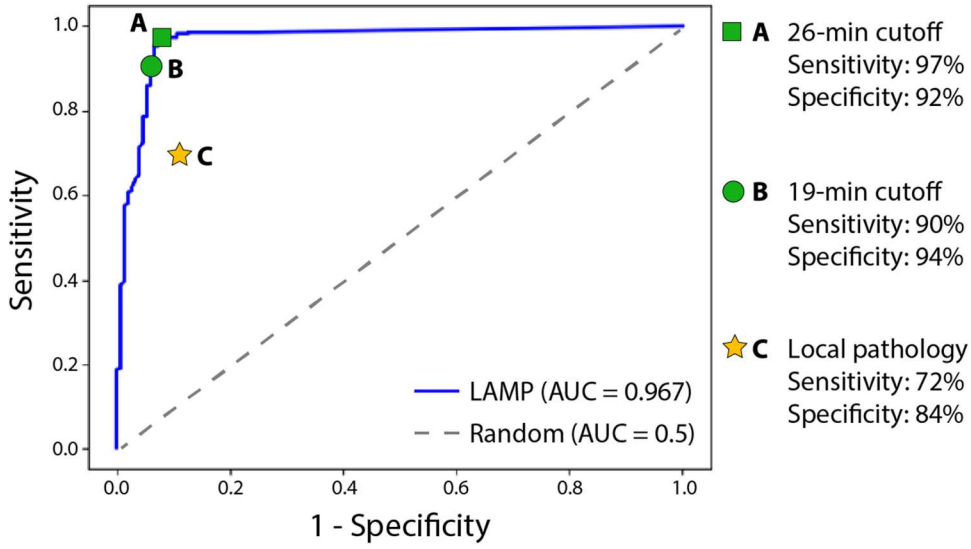

Fig. 5. Diagnostic performance of KSHV-LAMP. An ROC curve was generated from the sensitivity and specificity results for each cutoff time through the range of all possible values. The area under the curve (AUC) was 0.967 (95% CI: 0.948 to 0.985) with two cutoff times shown at maximum sensitivity or specificity while keeping the other above 90%. Local pathology had a reported 72% sensitivity and 84% specificity (*19*).

should become exceedingly useful as assays are developed for more skin diseases.

MATERIALS AND METHODS

Experimental design

We consented and enrolled Ugandan patients presenting to clinics with skin lesions that were clinically consistent with KS. These patients were enrolled from various clinics in Uganda that included The Infectious Diseases Institute (IDI) clinic in Kampala; all Kampala City Council Authority clinics that provide HIV care, which are supported by the IDI; Mbarara University Immune Suppression Syndrome (ISS) Clinic in Mbarara; and the Uganda Cares HIV clinic located at the Masaka Regional Referral Hospital in Masaka. Informed consent was obtained after the nature, and possible consequences of the study were explained. All research was granted Institutional Review Board (IRB) regulatory approval in the United States and Uganda.

Biopsy removal and preparation

Longitudinal skin punch biopsies were taken using a 5-mm cylindrical punch biopsy tool on skin lesions consistent with KS morphology. Following removal, biopsies were longitudinally sectioned into two pieces. One half of the biopsy was immediately placed in 10% neutral-buffered formalin (NBF) and processed for histopathology analysis, while the other half was stored in RNAlater for KSHV DNA analysis and subsequently frozen at −80°C.

Histopathology preparation and diagnosis

The portion of the biopsy designated for histopathology was placed in 10% NBF for no more than 24 hours and processed using routine procedures for paraffin embedding. This was performed at the Pathology Department of Makerere University, Kampala, Uganda. For each patient, blocks were sent to Weill Cornell Medicine Department of Pathology and Laboratory Medicine, where immunohistochemistry for KSHV LANA was performed on a Leica Bond III system. Sections were pretreated using heat-mediated antigen retrieval with sodium-citrate buffer (pH 6, epitope retrieval solution 1) for 30 min. The sections were then incubated with anti-LANA rat monoclonal HHV-8 ORF72 clone LN53 (Abcam) for 15 min at room temperature and detected using a horseradish peroxidase–conjugated compact polymer system. 3,3′-Diaminobenzidine was used as the chromogen. Sections were then counterstained with hematoxylin and mounted with micromount. Histology (H&E) and immunohistochemistry were reviewed in a blinded fashion by two pathologists.

DNA extraction and purification

One quarter of the stored biopsy tissue in RNAlater underwent DNA extraction and purification using the DNeasy Blood and Tissue kit (QIAGEN). Samples weighing around 10 mg, on average, were incubated with 20 μl of proteinase K and 180 μl of ATL buffer until completely clear according to protocol, often taking 1 to 3 hours with intermittent vortexing. Once clear, 200 μl of AL buffer was added, and samples were vortexed and then incubated for 10 min at 56°C. After incubation, 200 μl of 200 proof ethanol was added, and samples were vortexed. Two wash steps were performed using the DNeasy spin columns and AW1/AW2 buffers to remove any potential remaining contaminants. The DNA was eluted into 75 μl of AE buffer after 1 min of the buffer soaking the membrane at room temperature. Purified DNA was then measured using NanoDrop and diluted to a final concentration of 2 ng/μl for analysis in TINY to standardize the total amount of DNA per reaction.

LAMP assay for KSHV

Mastermix was created using Isothermal Amplification Buffer, deoxynucleotide triphosphate mix, and $MgSO_4$ (all from New England Biolabs), as well as six LAMP primers for the target *Orf26* of KSHV. Approximately 101 μl of this mastermix was

aliquoted into individual 1.5-μl Eppendorf tubes, enough for one test in TINY using all six wells (after addition of water, enzyme, and DNA). The exact composition of this mastermix can be found in our previous publication (*28*). Before running a test in TINY, 104 μl of deoxyribonuclease/ribonuclease-free water was added to the 101 μl of mastermix. Then, 9.8 μl of Bst 2.0 Warmstart polymerase (New England Biolabs) is added and gently vortexed to homogenize the complete mixture. Thirty-five microliters of this final mixture is distributed to six 200-μl PCR tubes, and 5 μl of DNA is added to each. Two tubes are reserved for positive and negative control DNA, while the other four tubes are used for testing sample DNA from two patients in duplicate.

Statistical analysis

A Bland-Altman plot was used to determine the reproducibility of TINY replicates (*45*). Obtaining sensitivity and specificity values, as well as ROC curve generation and CI determination, was performed using scikit-learn with Python 3.6.

Supplementary Materials

This PDF file includes:

Figs. S1 and S2

View/request a protocol for this paper from *Bio-protocol*.

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