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Original Research Article

Diagnostic Performance of Saliva Samples in Asymtomatic COVID-19 Infected Patients in Kakamega Kenya

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Abstract: The pandemic coronavirus SARS-CoV-2 in the world has caused a large infected population suffering from COVID-19. To curb the spreading of the virus, WHO urgently demanded an extension of screening and testing; thus, a rapid and simple diagnostic method is needed which is non-invasive. Use of self-collected saliva can minimize healthcare worker exposure and expand testing capabilities for symptomatic and asymptomatic patients. The main aim of this study was to document the ability of patients to self-collect sufficient saliva specimens for SARS-CoV-2 in the quantitative detection by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) in asymptomatic patients by themselves under observation by a healthcare provider. The researcher recorded whether the patients were confident and the suitability of the specimen for laboratory testing that would inform clinical decision making. Seventy-one patients aged from 13 years and above were included between December 2020 and July, 2021. Saliva samples and Nasopharyngeal samples were taken from each patient. Quantitative PCR was performed to detect SARS-CoV-2 viral load in the nasopharyngeal samples and qualitative Reverse Transcriptase Loop-mediated isothermal amplification (RT-LAMP) was used to detect the presence of the virus in saliva samples. Results of saliva vs. nasopharyngeal samples testing using the two different methods were compared. Statistical analyses were performed. Out of the 350 samples tested, 314 samples were found to be Covid 19 positive. Result of the test was validated by the RT-PCR test. This showed that only 314 samples were tested both by saliva rapid test and PCR test while the rest 36 samples were not tested using RT-PCR method but were tested using saliva test. Thus, the salivary test based on pure oral saliva samples easily obtained by noninvasive techniques using RT-LAMP has the same agreement with the nasopharyngeal technique using RT-PCR one in asymptomatic COVID-19 patients.

Keywords: SARS COV2, Polymerase Chain Reaction, Positivity Rate, Rt-lamp, Saliva.

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INTRODUCTION

A novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, previously provisionally named 2019 novel coronavirus or 2019-nCoV), has been identified as the cause of respiratory infection including severe pneumonia outbreak that started in Wuhan, China in late 2019 [1-6], and has since become a global pandemic. The disease was named the coronavirus disease of 2019 (COVID-19) by the World Health Organization in February 2020. It has been determined that SARS-CoV-2 can be transmitted from person-to-person (symptomatic or asymptomatic) and is more transmissible than SARS-CoV [3-6]. Nasopharyngeal swab (NPS) and oropharyngeal swab (OPS) samples are widely accepted as specimens for the detection of SARS–CoV–2 since the start of the COVID-19 pandemic. However, the collection procedures for NPS and OPS specimens may cause discomfort and, in some people, sneezing and coughing. The latter in turn can generate droplets or aerosol particles that place healthcare workers collecting these specimens at risk [6],

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requiring heavy use of personal protective equipment (PPE). Poor tolerability of NPS and OPS sampling can result in false-negative tests due to inadequate or poor quality of specimen collection [7-10]. Use of oral samples for the isolation of DNA has become a very attractive alternative to isolation from blood or tissue for a number of compelling reasons: oral collection is fast, cost effective and noninvasive and may be performed by individuals with minimal training. In addition, no specialized equipment is required. Human genomic DNA extracted from whole saliva collected using the proprietary SimplOFy™ Whole Saliva DNA Collection Kit can be used in a growing number of applications in the research and life sciences areas. Examples include the use of DNA in PCR-based molecular assays for the detection of disease or the determination of susceptibility to disease, applications in microarray technology, genotyping, personal genomics, genome wide association studies, next generation sequencing, and others. The ease of use of the SimplOFy™ kit makes it an ideal candidate for services requiring home collection and long term DNA stabilization at the point of collection. Recent investigations by Wyllie *et al*., [11] and Hanson *et al*., [12] suggested that saliva is a viable and even more sensitive alternative to NPS specimens, and could also enable at-home self-administered sample collection for large-scale SARS-CoV-2 molecular testing. Other researchers [13] also reported that SARS-CoV-2 was detected in 91.7% (n = 11) of the initial saliva specimens from confirmed COVID-19 patients. All saliva specimens (n = 33) collected from patients whose NPS specimens tested negative for COVID-19 also tested negative. It is apparent that detection of SARS CoV-2 in saliva can be used as a more appealing and cost-effective alternative for the diagnosis of COVID-19. Indeed, a molecular test using saliva samples was first approved for FDA under EUA on May 8, 2020 [14-16].

The use of saliva specimens might decrease the risk of nosocomial transmission of COVID-19 and is ideal for situations in which NPS or OPS specimen collection may be impractical [15–21] Collecting saliva is easy and more tolerable to patients, can reduce risk of cross-infection, and can be used in settings where PPE is not readily available. It will also be useful for testing infants and young children in daycare facilities and schools. In this study, we shown that saliva sampling is an adequate alternative to nasopharyngeal and oropharyngeal sampling and can be used for COVID-19 testing using the RT-LAMP test.

METHODOLOGY

Study Area

This scientific work was undertaken in Kakamega District Referral Hospital. It is in Kakamega County is located in former western province of Kenya. County populace is1, 867,579 and an area of 3,033.8 square kilometers. (*Kenya census, 2019)*. Western Kenya block cumulatively had 20,281 confirmed COVID-19 cases with a majority in urban settings (*Cultural Practices Resilience in the Wake of COVID-19 among Communities in Western Kenya| Research Journal in Advanced Humanities, n.d.).* The hospital receives patients from all over western region. Testing and treatment of patients infected with new coronavirus was important.

Study Design

This was a descriptive, analytical and cross-sectional study design was used among patients with Covid 19 attending Kakamega County referral hospital.

Study Population

Patients with COVID 19 that attended Kakamega County Referral Hospital were recruited in this study. They were aged 18years and above who were asymptomatic for COVID-19 with or without co-morbidities. Participants were recruited based on the researcher's understanding on the occurrence of asymptomatic cases of the disease and the diagnostic cues of mild corona virus disease. They were divided into three groups in order to classify severity of the disease into moderate, severe and critical (WHO, 2020).

Clinical Sample Collection

Morning saliva and nasopharyngeal samples were collected from patients with suspected of having infection and analyzed for the presence of COVID-19 RNA/DNA in saliva. Saliva and nasopharyngeal samples were self-collected at the same time. All samples to be tested were stored at room temperature and transported to the laboratory within two hours. The Oasis Diagnostics® Corporation SimplOFy™ Whole Saliva DNA Collection Kit was used for saliva collection, following the kit insert instructions and under the supervision of healthcare providers. The patients were not allowed to eat, drink, smoke, or use oral hygiene products for at least 30 minutes before saliva samples collection process starts. Each saliva sample contains about 2 mL liquid saliva and 2 mL viral transport media. The nasopharyngeal and saliva samples were refrigerated and processed for testing within 24 hours after collection. Also information on host factors (age, gender, patient category,) was obtained from the hospital patient records. Ethical approvals were provided by MMUST EIREC and NACOSTI.

SimplOFy™ Whole Saliva DNA Collection

This study used the Oasis Diagnostics® Corporation SimplOFy™ Whole Saliva DNA Collection Kit (Figure 1 & Figure 2). The kit is intended for the collection and stabilization of whole saliva for subsequent extraction of DNA downstream testing.

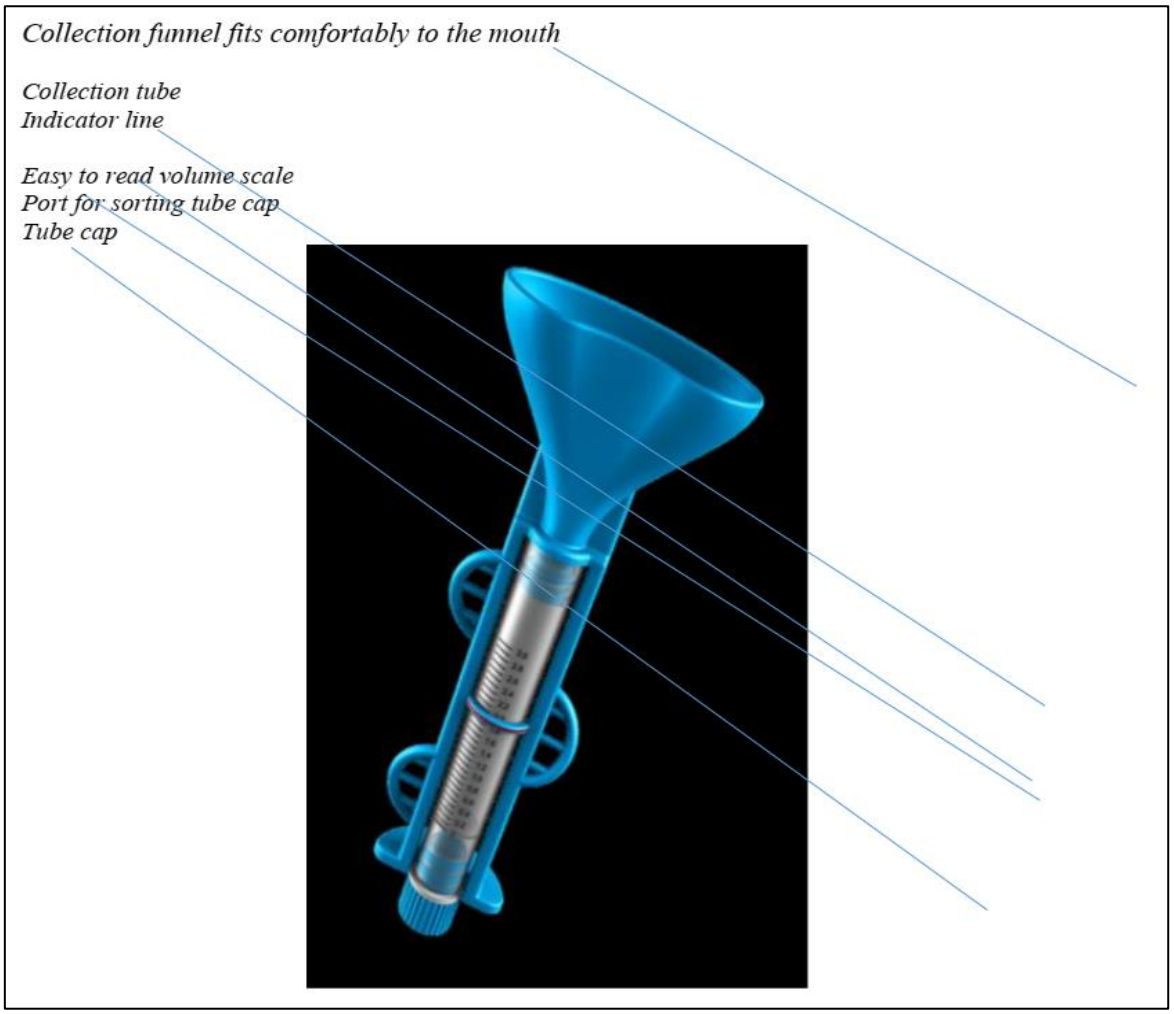


Figure 1: SimplOFy™ Whole DNA Saliva collection Kit

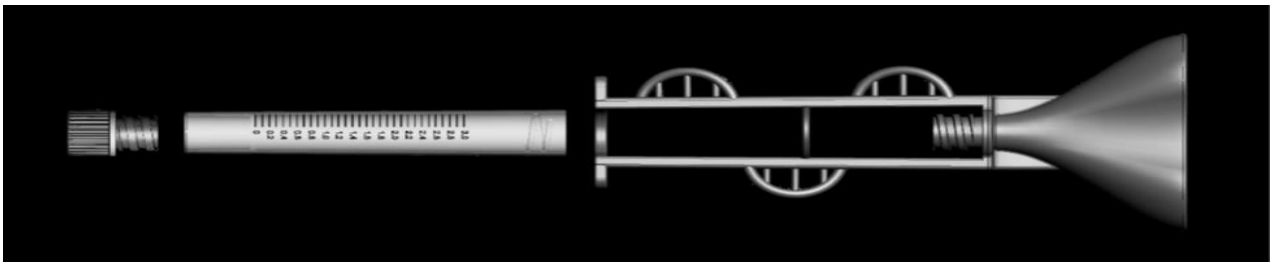


Figure 2: SimplOFy™ Whole Saliva DNA collection Kit

The Oasis Diagnostics® Corporation SimplOFy™ Whole Saliva DNA Collection Kit works under the principles that kit is a proprietary, patented system intended for the collection and stabilization of whole saliva DNA. To collect a specimen, subjects are requested to pool saliva in the mouth and 'expectorate' (spit) into the Collection Funnel until the sample reaches the brightly marked Indicator Line. Once sample collection is complete, the Collection Tube is separated from the Collection Funnel by carefully rotating the Collection Tube until it is completely detached from the Collection Funnel. The Collection Funnel is then discarded and the Tube Cap is removed from the bottom of the Collection Tube and screwed tightly closed to the top of the Collection Tube. The Tube Cap has dried-down stabilizing reagents inside, so the Collection Tube is continually inverted for at least 1 [one] minute, which results in adequate mixing of the stabilizing reagent with the saliva sample. The sample is now ready for transportation to a laboratory or storage for later testing.

Preparation for Oral fluid (saliva) Collection

In preparation for oral fluid (saliva) collection, place contents on a clean and dry surface (Figure 3). The

patient was then instructed to Pool saliva and spit into the opening of the Collection Funnel (Figure 4). Saliva was then collected until the sample level reached the red Indicator Line (measure fluid level, not foam). In an upright position, the Collection Funnel was unscrewed from the Collection Tube by rotating the Collection Tube in the direction shown until the Collection Tube was completely detached from the Collection Funnel (Figure 5). The Tube Cap was then carefully removed from the bottom of the Collection Tube and the Tube Cap tightly is screwed and closed on the top of the Collection Tube (Figure 6). At this stage, the Collection Funnel was discarded. The vial was then Inverted for 1 minute to stabilize the sample (Figure 7). Sample was then now ready for immediate testing or transportation to a laboratory using standard shipping methods for liquid samples (Figure 8).

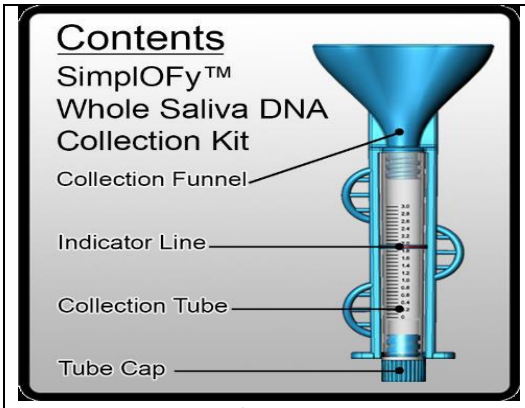
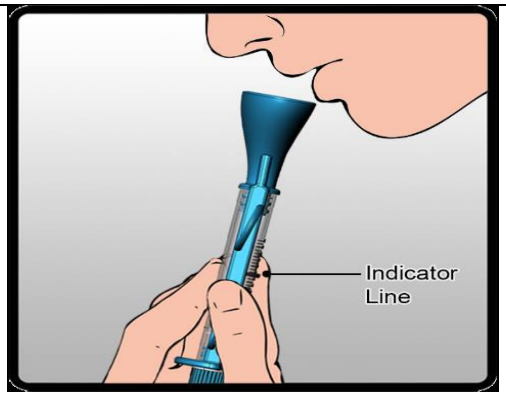


Figure 3



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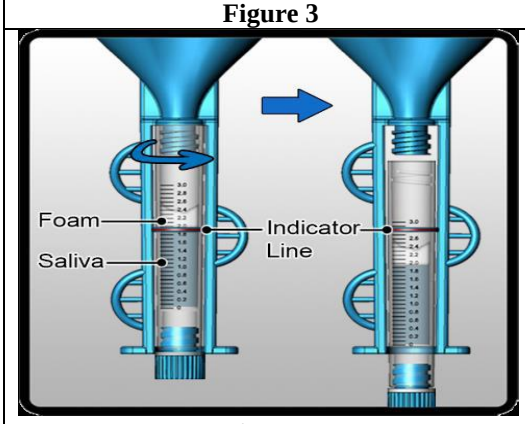

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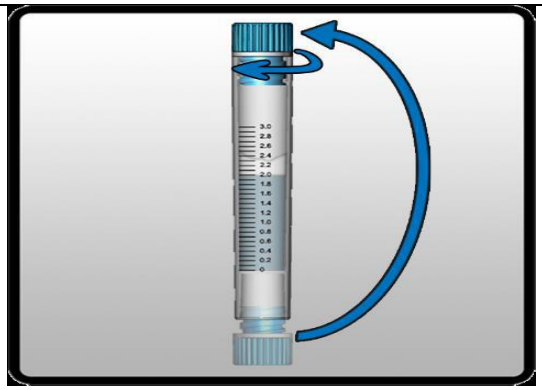

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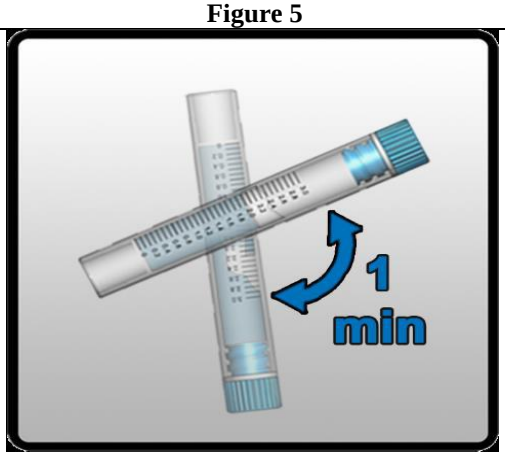

Figure 7

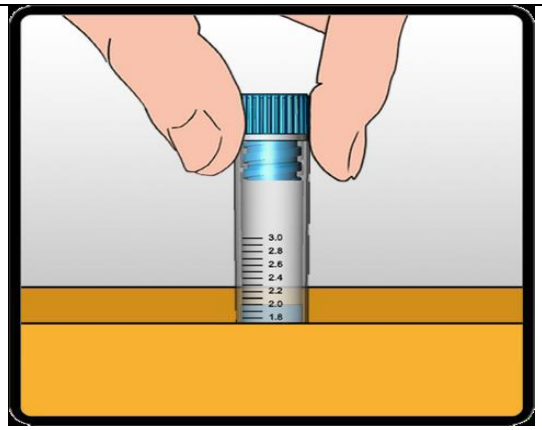

Figure 8

Viral RNA extraction for RT-PCR

MGI's automatic RNA/DNA extraction instrument MGISP-960 (MGI Tech Co., Ltd, China) was used for the SARS-CoV-2 viral RNA extraction according to the manufacturer's instructions, for which 200 µL of each NPS VTM or saliva sample was used. For each batch of clinical samples to be tested, an extraction control (EC) was included (spike 20 µL of EC from the

QuantiVirus™ SARS-CoV-2 kit into 180 µL sterile RNase-free water). The clinical samples and spiked EC were processed and extracted on the MGI platform. The extraction output is RNA in 30–50 µL RNase-free water, 5.5 µL of which was used for the PCR reaction per test. The turnaround time from sample extraction to PCR final report was around 4 hrs. Precautions were taken while handling extracted RNA samples to avoid RNA degradation. Extracted RNA samples were stored at -80˚C if not immediately used for RT-PCR.

Viral RNA extraction for RT-LAMP

The RT-LAMP primers targeting the N gene of COVID-19 (GenBank accession No. MN997409.1) were designed using PrimerExplorer V5 (http://primerexplorer.jp/e/) and Oligo 7 (Molecular Biology Insights, Inc. Colorado Springs, CO, USA) software packages. The primer sequences are GCC AAA AGG CTT CTA CGC A (F3), TTT GGC CTT GTT GTT GTT GG (B3), TCC CCT ACT GCT GCC TGG AGT TTT CGG CAG TCA AGC CTC TTC (FIP), TCC TGC TAG AAT GGC TGG CAA TTT TTT TTG CTC TCA AGC TGG TTC A (BIP), CGA CTA CGT GAT GAG GAA CGA (LF) and GCG GTG ATG CTG CTC T (LB), Table 1, and the length of the targeted sequence was 233 bp.

The N gene (GenBank accession No. MN997409.1) of COVID was chemically synthesized and cloned into pUC57 plasmid (herein referred to as pUC57-N DNA) by General Biosystems (Anhui) Co., Ltd, the pUC57-N DNA was used as the template for optimization of the RT-LAMP system, as well as for determination of sensitivity.

The real-time RT-LAMP assay with above designed RT-LAMP primers was performed in a 50-µL reaction mixture containing 0.8 mM each of forward inner primer (FIP) and backward inner primer (BIP), 0.2 mM each of forward outer primer (F3) and backward outer primer (B3), 0.4 mM of forward loop primer (LF) and backward loop primer (LB), 1.2 mM dNTPs, 1× Bst DNA Polymerase Buffer (Zhengzhou Shenxiang Industrial Co., Ltd, China), 1 × EvaGreen, 1 × Rox, 1 pg pUC57-N DNA, and 25 U Bst DNA/RNA Polymerase 3.0 (New England Biolabs, Inc., MA, USA) 8, 9. The reaction mixtures were heated at 55°C, 57°C, 59°C and 61°C for 50 min (30 s per cycle), individually. The amplification plot and melt curve were obtained using a StepOneTM System (Applied Biosystems, Foster City, CA, USA).

RESULTS

The study had sought to evaluate Performance of saliva test. Out of the 350 samples tested, 314 samples were found to be Covid 19 positive. Result of the test was validated by the RT-PCR test. This showed that only 314 samples were tested both by saliva rapid test and PCR test while the rest 36 samples were not tested using RT-PCR method but were tested using saliva test. The results are shown in table 1 below:

Table 1: Performance of the saliva test method

Covid-19 Diagnosis	Covid-19 Positive samples	Covid-19 PCR confirmed Positive	Performance (%)
Saliva test	130	117	90.0%

This study team was also able to observe that some patients had difficulties in doing the self-swab, due to fear associated with uneasiness of swabbing themselves. This lead to superficial swabbing. Some patients were not able to remove the swab stick from the kit; others had difficulty breaking the swab stick despite the video instruction and a pictorial guide. All the patients reported that saliva self-collection was the easiest, most comfortable in-terms of sample collection hence all participants expressed confident in it and that it is user friendly.

DISCUSSION

Diagnostic testing is a key element in detection of outbreaks and emergency response [22]. Initially during the coronavirus outbreak globally, there was pressure to expand to an effective response to the testing capacity for the novel corona virus. In some countries it was mandatory to obtain clearance prior to shipping and use of diagnostic test kits [23]. In our study as part of the scaling up it was important to use best practices for timely detection of the virus causing the pandemic. The gold standard for coronavirus detection is RT-PCR, but however, increasing the testing capacity while maintaining a good TAT, required incorporation of rapid test kits which in projection could be adopted to increase coverage.

Based on the results of this study, the overall impression shows that participants had more confidence in doing saliva sample collection as opposed to the nose and throat swab. The subjects were also able to provide qualitative feedback on their experience. When it comes to nasopharyngeal sample collection through the assistance of the health care worker and the self-saliva collection sampling. When it comes to self-saliva sample collection, the participants were comfortable as they could collect sample without any stress and with a lot of easiness. The collection of nasopharyngeal by the assistance of a health care worker, majority were unsure on how deep the health care worker should put the swab in, and they found it uncomfortable and very invasive.

The issue of how deep the nasopharyngeal swab should go, could be improved by putting a marking on the swab stick to guide how deep to go. For the saliva sample component, there was general difficulty in generating saliva and there was uncertainty as to when enough saliva is collected. While the saliva collection process is a very attractive option for COVID-19 testing, as there is minimal discomfort to the person undergoing the test, the collection process ought to be simplified to minimize the risk of error. Based on the user feedback on both collection methods, it would seem that the self-collection is more suitable to be carried out in a highly motivated population; one example would be persons working in an environment with high-risk infectious exposure. The saliva testing performance is likely more consistent, and less prone to differences in dexterity and test motivation.

CONCLUSION

The conclusion is that the relationship between the COVID status of a patient is not statistically significantly different across the different samples. This means that saliva can be used in place of the nasopharyngeal samples since they all give the same results and that Rt-lamp is cheaper and saliva is easily obtained using non-invasive method. In comparison, when collecting COVID-19 samples using nasopharyngeal
swabs, a healthcare professional must administer the test for the donor; therefore, bringing them into close contact with each donor they test, increasing the risk of spread. This clinical process increases transmission risk. By using self-collecting of saliva samples coupling with RT-LAMP, it is a major speedbump for COVID-19 diagnostic testing and a solution to improve the current COVID-19 sample collection method and process. The focus is on providing testing options to populations that are currently underserved by the testing options available today. Self-collection of saliva samples will enable testing for people that do not have the ability to get to a collection center or are at home because they are sick, quarantined at increased risk for infection or simply concerned about exposing themselves by traveling to a collection site.

RECOMMENDATION

There is an urgent need for rapid diagnosis of SARS-CoV-2 infected COVID-19 patients even before an immune response can occur and for asymptomatic carriers. This is critical in making decisions on public health measures, such as movement restrictions, and quarantine duration. noted that validation of sample self-collection methods holds great promise for broad testing strategies that would mitigate infection risk and PPE resource utilization. "The current 'test, track, and trace' public health approach to surveillance relies heavily on testing for both diagnosis and surveillance. The use of self-collected saliva provides a cheaper and less invasive option for viable sample collection. It's certainly easier to spit in a cup twice a week than undergoing frequent nasopharyngeal swabs. This can improve patient compliance and satisfaction particularly for surveillance testing, which requires frequent sample collection. Since we also showed that the virus was stable at room temperature for at least 24 hours, saliva collection has potential for use at home."

Another study in the community with a larger sample is recommended.

Declarations: This study was not funded

Conflict of Interest: There was no conflict of interest reported.

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