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Suitcase Laboratory for Rapid Detection of SARS-CoV-2 Virus Based on Recombinase Aided Isothermal Amplification Assay.

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Abbreviations/Acronomys

AHRI	Armauer Hansen Research Institute
ARDS	Acute respiratory distress syndrome
AU	African union
℃	Degree Celsius
CCDC	Chinese centre for disease control
CDC	Centre for disease control
COVID	Corona virus disease
DNA	Deoxyribonucleic Acid
EPHI	Ethiopian public health institute
HBV	Hepatitis B virus
IRB	Institution review board
LAMP	Loop mediated isothermal amplification
MERS	Middle east respiratory syndrome
MSL	Mobile Suitcase Laboratory
ORF	Open reading frame
RAA	Recombinase aided amplification
RNA	Ribonucleic Acid
RPA	Recombinase polymerase amplification
RT-PCR	Real time polymerase chain reaction
SARS-COV-2	Severe acute respiratory syndrome
SSB	Single stranded DNA binding protein
VTM	Viral transport medium
WHO	World health organization

Abstract

Introduction: Techniques such as RT-PCR are not only expensive and time-consuming but also require a high level of expertise, limiting their widespread deployment, particularly in resource-constrained regions. Mobile Suitcase Laboratory equipped with Recombinase Aided Isothermal Amplification assay, is an alternative approach which potentially overcomes those challanges by providing a rapid, cost-effective, and user-friendly assay for the diagnosis of COVID-19. Therefore this study aimed to evaluate the diagnostic accuracy of a point of need Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay for diagnosis of SARS-CoV-2 virus.

Methods*:* A combination of retrospective and prospectivecross-sectional study was conducted adhering to established guidelines for the assessment of diagnostic kits, aiming to evaluate the performance of a cost-effective and straightforward isothermal assay in comparison to the standard RT-PCR method for diagnosing SARS-CoV-2. From archived samples, 290 positive samples were selected based on their CT values, along with 86 negative samples for the study. Additionally, 98 positive and 52 negative fresh samples were added. In total, 388 positive samples and 138 negative samples were used. Statistical Packages for Social Sciences (SPSS) version 22.0.

Results: Of the total, 388 individuals tested positive with the RT PCR, 334 (86.0 %) were also positive with tool kit. Additionally, 138 individuals tested negative with the RT PCR, 133 (96.4%) of them also negative with the Tool kit. The Kappa statistic (0.7) and the low P value (<0.0001) suggests highly significant agreement.

Conclusion: This study evaluating the Mobile Suitcase Laboratory (MSL) based on Recombinase Aided Isothermal Amplification (RAA) assay showed its potential utility in resource-limited settings such as Ethiopia. Our findings demonstrate that the MSL-RAA assay offers a promising alternative for decentralized COVID-19 testing, overcoming the infrastructure and logistical challenges associated with laboratory-based methods like RT-PCR.

Key words: Rapid test, diagnosis, RT-PCR, Recombinase, SARS-Cov-2.

1. Introduction

1.1 Background

SARS CoV2 is a virus that causes severe acute respiratory syndrome (SARS), and initially it was identified in individuals who fell ill in China in December 2019. Researchers discovered the virus to have closer resemblance to the SARS-CoV virus, which caused a similar outbreak in 2002-2003. Through genome sequencing and phylogenetic studies, it was determined that SARS-CoV-2 belongs to the Betacornavirus genus, part of the Coronavirinae subfamily in the Coronaviridae family. Because of its significant genetic similarity (about 80%) to the original SARS-CoV, the new virus was named Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), also referred to as 2019-nCoV (1-3).

Subsequently, it was found that transmission between humans significantly contributed to the subsequent outbreak (4). The illness triggered by this virus was labeled Coronavirus Disease 2019 (COVID-19), and it was officially designated as a pandemic by the World Health Organization. COVID-19 has had a profound worldwide influence, spreading to numerous countries and territories, with reported cases exceeding 200 across the globe (5). COVID-19 presents with symptoms resembling influenza, including fever, cough, fatigue, dyspnea, and gastrointestinal symptoms. Early differentiation from other viral infections is challenging, underscoring the urgent need for a rapid and precise diagnostic approach. Such a method would be instrumental in containing the disease spread and informing treatment strategies. While quantitative reverse transcription polymerase chain reaction (RT-PCR) currently serves as the benchmark for molecular diagnostics, its implementation is hindered by its time-demanding nature and the requirement for specialized equipment (6).

Recombinase-polymerase amplification (RPA) is a method of DNA amplification that operates under constant temperature condition, with out requiring for a conventional heat-resistant enzyme or complex thermal cycling equipment. The RAA system comprises three key proteins: recombinase, single-stranded DNA binding protein (SSB), and DNA polymerase. Amplification begins when a primer-recombinase complex homes in on and penetrates the DNA double-strand at locations that match the primer sequences. SSB plays a vital role in stabilizing the reaction and facilitating polymerase extension. The entire process occurs within a short timeframe of 5–20

minutes at a constant temperature of 39°C, making RAA particularly favorable for rapid point-of-care testing applications (7).

A new single-tube assay known as reverse transcription recombinase-aided amplification (RT-RAA) was developed for detecting SARS-CoV-2. This kit was introduced on January 29, 2020, marking its initial release. It underwent external quality evaluations carried out by the National Institute for Viral Disease Control and Prevention (IVDC), the Chinese Centre for Disease Control and Prevention (CCDC), and the Beijing Centre for Disease Control (CDC) (8).

Several promising isothermal methods, such as recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP), have emerged as viable alternatives to PCR, offering potential solutions to the need for extensive SARS-CoV-2 testing. These techniques offer portability and speed, often yielding results within 5-20 minutes, compared to the several hours required by qRT-PCR. This facilitates its applicability in resource-limited settings without the necessity of a thermal cycler. By leveraging a Mobile Suitcase Laboratory equipped with Recombinase Aided Isothermal Amplification assay, these benefits could be extended to remote areas, enhancing accessibility to efficient and rapid COVID-19 testing (9).

The evaluation of Mobile Suitcase Laboratory utilizing Recombinase Aided Isothermal Amplification assay serves as a crucial endeavor in addressing the shortcomings of existing diagnostic methods for COVID-19. Techniques such as RT-PCR are not only expensive and time-consuming but also require a high level of expertise, limiting their widespread deployment, particularly in resource-constrained regions. Additionally, other diagnostic approaches may suffer from pitfalls such as variable sensitivity and specificity, hindering accurate and timely diagnosis. By assessing the effectiveness of the Mobile Suitcase Laboratory equipped with Recombinase Aided Isothermal Amplification assay is an alternative approach which potentially overcomes those challanges by providing a rapid, cost-effective, and user-friendly assay for the diagnosis of COVID-19, thereby facilitating more efficient containment and management of the pandemic on a global scale. Hence, the objective of this study was to evaluate the diagnostic accuracy of a point-of-need Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay for diagnosis of SARS-CoV-2 virus.

1.2. Problem statement

An outbreak of the new Coronavirus Disease (COVID-19) has significantly affected public health and economic progress in China and worldwide. Recognizing the severity of the situation, on March 11th, 2020, the World Health Organization (WHO) formally declared COVID-19 a pandemic, acknowledging its widespread and global impact on populations (10). COVID-19 represents a swiftly advancing global menace, akin to any pandemic, exerting strain on healthcare systems, causing loss of life, and presenting substantial hazards to global economic stability and security. Based on statistics from the World Health Organization (WHO) and Johns Hopkins University, by the conclusion of June 2020, the global tally of COVID-19 cases had surpassed ten million, leading to almost half a million fatalities across approximately 215 nations. As of January 16, 2022, there have been more than 323 million confirmed cases and over 5.5 million deaths reported globally (11).

Egypt confirmed the inaugural COVID-19 case in Africa on February 14, 2020, following two weeks from the World Health Organization's declaration of the outbreak as a global public health emergency. Responding swiftly, the African Union (AU) Commission, under the guidance of the Africa Centers for Disease Control and Prevention (Africa CDC), organized an urgent gathering of health ministers on February 22, 2020. This assembly demonstrated a united front and proactive leadership across the continent, resulting in the formulation and endorsement of a Collective Continental Strategy to address the COVID-19 crisis (12).

In the WHO African Region, a total of 2,789,965 confirmed cases and 71,204 deaths have been reported across all 47 nations, resulting in a case fatality rate of 2.6%. Notably, countries like South Africa, Ethiopia, Nigeria, Algeria, Kenya, Ghana, Zambia, and Mozambique have each reported over 50,000 confirmed cases. South Africa leads with 1,507,448 cases (13).

Following the identification of the first COVID-19 case in Ethiopia on March 13, 2020, the government implemented several measures to control and mitigate infections. These actions included closing schools, suspending sports events and public gatherings, adjusting transportation procedures, mobilizing communities, and enforcing mask mandates. However, many of these measures have since been eased or relaxed (14).

Ethiopia leads among East African nations in both confirmed COVID-19 cases and fatalities as of July 2021, according to EPHI report. The report indicates 277,071 confirmed cases and 4,343 deaths. Despite these figures, the total number of laboratory tests conducted up to July 2021 stands at 2,920,624, suggesting that testing coverage relative to the entire population remains relatively low (15).

While Polymerase Chain Reaction (PCR) stands as the premier and standard method for diagnosing COVID-19, its accessibility in most developing nations remains limited. Even when PCR machines are available, the associated operational costs and infrastructure requirements pose significant challenges. Consequently, many developing countries suffer from inadequate diagnostic coverage. Despite being hailed as the optimal diagnostic tool, PCR exhibits drawbacks due to its cost and complexity. To address these limitations, alternative assays such as Recombinase Aided Amplification (RAA) offer promising solutions, particularly for resource-constrained countries, presenting a more viable option compared to PCR.

1.3. Significance of the study

Assessing a Mobile Suitcase Laboratory (MSL) based on Recombinase Aided Isothermal Amplification (RAA) assay holds significant promise, particularly in resource-limited countries like Ethiopia. This ensures reliable diagnosis even in settings with limited laboratory capabilities, reducing the risk of false negatives or false positives. Community outreach and surveillance, the portability of MSLs allows for community-based testing and surveillance efforts, enabling early detection of outbreaks and targeted intervention strategies. This is essential for monitoring the spread of COVID-19 in resource-limited countries like Ethiopia. Capacity building and sustainability, implementing MSLs with RAA technology not only facilitates COVID-19 diagnosis but also contributes to capacity building and sustainable healthcare infrastructure development. In general, evaluating MSLs based on RAA technology holds immense potential for improving COVID-19 diagnosis and surveillance in resource-constrained countries like Ethiopia.

2. Literature review

The onset of the SARS-CoV-2 outbreak was initially attributed to a zoonotic transmission associated with a seafood market in Wuhan, China. Subsequently, it was revealed that human-to-human transmission significantly contributed to the ensuing spread of the virus (4). The sole effective control strategies include early detection and isolation of infected cases, contact tracing, implementation of social distancing measures, and the use of facial masks in enclosed spaces (16).

Recombinase polymerase amplification (RPA) is conducted at a consistent temperature of 42 °C and typically concludes within 15 minutes or less. Its rapidity is enabled by a highly accelerated amplification process employing a blend of enzymes and proteins, including recombinase, single-stranded DNA-binding protein, and strand-displacing polymerase (16).

Although PCR-based assays are widely acknowledged as the gold standard for molecular detection of viral diseases by governmental and medical authorities, their implementation poses challenges in resource-limited settings. These methods demand well-trained personnel and advanced equipment, rendering them impractical for many developing countries. Moreover, PCR-based protocols are complex and time-consuming, constraining their diagnostic effectiveness during a dynamic pandemic with rapidly escalating case numbers, especially in densely populated areas. Therefore, there is a pressing need for a more accessible and reliable molecular detection method to diagnose and manage COVID-19 (17).

RT-RPA assays have successfully detected other coronaviruses like MERS-CoV and bovine coronavirus (BCoV). With its proven effectiveness in frontline screening for H7N9 avian influenza virus and Ebola virus using portable suitcase laboratories, RT-RPA technology offers significant potential for early diagnosis in rapidly evolving and resource-limited outbreak scenarios (18).

In recent times, recombinase polymerase amplification (RPA) assays have surfaced as a promising substitute for real-time PCR in diagnosing infectious diseases. This method offers several advantages over other isothermal amplification technologies like LAMP. RT-RPA is notably swift (10–15 minutes), demands basic equipment, and is easily conducted at room

temperature (37–42 °C). Another recombinase-based assay, known as reverse transcription recombinase-aided amplification (RT-RAA), has also been recently devised for detecting infectious pathogens (19).

Both RT-RPA and RT-RAA rely on isothermal amplification, where a mixture of recombinase enzymes, single-stranded binding proteins, and DNA polymerases facilitates double-stranded DNA denaturation and strand invasion. The sole distinction between the techniques lies in the origin of the recombinase, which is derived from phages for RT-RPA and from bacteria/fungi for RT-RAA (7, 19).

Investigations are underway to detect SARS-CoV-2 in clinical samples within a span of 10 minutes using a reverse-transcription recombinase-aided amplification (RT-RAA) assay. This method, known for its high specificity, sensitivity, and user-friendly nature, holds potential for application in primary care environments. Positive outcomes have been observed with this assay for adenovirus, respiratory syncytial virus, HBV, salmonella, and various other pathogens (6). Accurate diagnosis of COVID-19, resulting from the novel coronavirus SARS-CoV-2, is essential for curtailing the disease's transmission and directing effective patient care strategies. Various diagnostic methods have been developed to assist in detecting SARS-CoV-2 infection, each possessing distinct advantages and constraints(20). Among the primary diagnostic methods are nucleic acid amplification tests (NAATs), such as reverse transcription polymerase chain reaction (RT-PCR), which detect viral genetic material. RT-PCR remains the reference standard due to its high disgnostic performance. Antigen tests, which detect viral proteins, offer rapid results but may have lower sensitivity compared to RT-PCR. Serological tests, including antibody and antigen tests, detect the body's immune response to the virus and can provide information on past infection (21). Additionally, innovative technologies like isothermal amplification assays and CRISPR-based methods are being developed for COVID-19 diagnosis, promising rapid and accurate detection. Understanding the strengths and limitations of these diagnostic tools is essential for effective disease surveillance, outbreak control, and patient care in the ongoing battle against COVID-19 (22). In resource-constrained countries, the diagnosis of COVID-19 is often hindered by challenges associated with implementing complex diagnostic methods like reverse transcription polymerase chain reaction (RT-PCR). RT-PCR requires modernized laboratory infrastructure, skilled personnel, and costly reagents, making it impractical for many underserved regions (9, 23). However, emerging technologies offer

promising alternatives, notably the Mobile Suitcase Laboratory (MSL) based on Recombinase Aided Isothermal Amplification (RAA) assay. The MSL overcomes the limitations of RT-PCR by providing a portable, cost-effective, and user-friendly solution for decentralized testing (24). RAA assays function at a consistent temperature, removing the requirement for complex thermal cycling machinery. Moreover, the MSL can be easily transported to remote areas, enabling on-site testing and rapid diagnosis without relying on centralized laboratory facilities (25). By leveraging the advantages of RAA-based assays within the MSL platform, resource-limited settings can enhance their capacity for timely and accurate detection of COVID-19, ultimately facilitating more effective control measures and patient management strategies.

3. Objectives

3.1 General Objective

- To determine the diagnostic accuracy of a point of need Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay for the diagnosis of SARS-Cov-2 Virus.

3.2 Specific objectives

- To determine the sensitivity and specificity of the RAA-based MSL in detecting SARS-CoV-2 virus by comparing its results with those of reference standard diagnostic tests, such as RT-PCR.
- To evaluate the overall diagnostic performance of the RAA-based MSL, including positive predictive value (PPV), negative predictive value (NPV), and accuracy, in identifying SARS-CoV-2 infection.
- To assess the level of agreement between the RAA-based MSL and reference standard diagnostic methods (e.g., RT-PCR) in detecting SARS-CoV-2 virus, using measures such as Cohen's kappa statistic.

4. Hypothesis

HO; there is no difference between PCR and mobile suitcase laboratory device for the diagnosis of SARS-COV-2 virus.

5. Method and materials

5.1. Study area

The study was conducted at the Armauer Hansen Research Institute (AHRI), founded in 1970 GC, marking its 50th anniversary. Renowned for various health-related research endeavors conducted in collaboration with national and international partners, AHRI boasts a workforce of 400 staff members. Following the initial COVID-19 report, AHRI assumed significant responsibilities alongside the Ethiopian Public Health Institute (EPHI) for COVID-19 diagnosis and related research. AHRI received a substantial influx of nasopharyngeal samples from diverse areas of Addis Ababa, underscoring the critical need to ensure an ample sample size for the study.

5.2. Study design and period

A retrospective cross-sectional study was conducted following established guidelines for evaluating diagnostic kits. Its objective was to assess the performance of an affordable and simple isothermal assay compared to the standard RT-PCR method for diagnosing SARS-CoV-2. Named reverse transcription recombinase aided amplification (RT-RAA), this assay rapidly detects SARS-CoV-2 within 15 minutes at 39°C, using portable instruments after adding extracted RNA. The clinical effectiveness of the RT-RAA assay was assessed using 526 clinical samples alongside an approved commercial fluorescence quantitative real-time PCR (qRT-PCR) kit. Nucleic acid extraction was performed on archived nasal swab samples stored in viral transport medium (VTM). Sensitivity and specificity of the RT-RAA assay were compared with those of RT-PCR. The study was conducted from June 2022 to December 2022..

5.3. Population

5.3.1. Source populations

All nasopharyngeal sample that stored in AHRI COVID-19 Laboratory.

5.3.2. Study populations

388 Positive samples and 138 Negative samples from those stored samples.

5.4. Inclusion and Exclusion Criteria

5.4.1. Inclusion Criteria

All nasopharyngeal samples collected for COVID-19 test, adequate sample volume and sample stored in -80 ^{0}C Freezer.

5.4.2. Exclusion Criteria

The samples that are not correctly labeled, samples that transport incorrect temperature and those samples lost their request paper.

5.5. Study Variables

5.5.1. Dependent variables

Diagnostic Accuracy of RAA Toolkit.

5.5.2. Independent variables

The storage condition of the sample, the storage condition of the kit and cycle of threshold.

5.6. Sample size calculations and sampling method

5.6.1. Sample size calculations

According to CLSI Doc EP09-A3 recommended at least 100 samples for method evaluation based on this used 526 samples for this study (26).

5.6.2. Sampling Method

For both positive and negative samples, was blinded to the primary results. The positive samples were categorized based on their cycle of threshold (CT) values into high, medium, and low CT value groups, resulting in the selection of 388 samples. For those 138 negative samples simply picked from the stored samples.

5.7. Measurement and Data collection

5.7.1. Sample collection procedure

The Nasopharyngeal samples were collected using 3ml viral transport medium (VTM) and transported to the laboratory with a cold chain to maintain the temperature. Upon arrival at the laboratory, RNA extraction was performed. In some instances, extraction could not be immediately carried out, and the samples were stored in a -20°C freezer. After extraction and PCR analysis, the remaining samples were stored for future use in the study. Additionally, fresh

samples were obtained for the study. Socio-demographic data were collected and analyzed from the database.

5.7.2. Laboratory Analysis

5.7.2.1. RNA Extraction

RNA extraction was carried out using the QIAamp (QIAGEN) Viral RNA extraction kit, known for its rapid and user-friendly RNA purification process suitable for amplification technologies. To maintain sample integrity, whether fresh or frozen, samples were thawed for only one cycle. Initially, samples underwent lysis under denaturing conditions to inactivate RNases and ensure isolation of intact viral RNA. Buffer conditions were adjusted to optimize RNA binding to the QIAamp membrane, followed by efficient removal of contaminants through two-step washing with different wash buffers. High-quality RNA was then eluted in a specialized RNase-free buffer, devoid of proteins, nucleases, and other impurities. The unique QIAamp membrane ensured exceptionally high recovery of pure RNA in just 20 minutes, eliminating the need for phenol/chloroform extraction or alcohol precipitation. The use of two distinct wash buffers, AW1 and AW2, notably enhanced RNA purity. Additionally, Buffer AVE, containing RNase-free water with 0.04% sodium azide, was employed to prevent microbial growth and RNase contamination. Given RNA's susceptibility to RNase, careful handling was imperative throughout the process.

5.7.2.2. RT-RAA Nucleic Acid Detection

After RNA extraction was completed, reagent preparation was initiated. Prior to commencing the detection procedure, the ESEQuant TS2 machine was powered on, and the reagents were brought out from the freezer and left at room temperature for at least 30 minutes to equilibrate. The ESEQuant TS2 machine, utilized in this study, represented next-generation technology for point-of-need tests. This compact, user-friendly fluorescence measurement system for isothermal nucleic acid amplification proved to be highly cost-effective. The kit utilized in this investigation relied on recombinase-aided amplification (RAA) technology, specifically targeting the amplification of the ORF1ab gene, which is crucial for the molecular detection of SARS-CoV-2. In the fluorescence RT-RAA process, reverse transcription of SARS-CoV-2 nucleic acid to cDNA occurred at a consistent temperature of 39°C. The formation of a primer/recombinase/single-stranded DNA binding protein (SSB) complex facilitated the complementary pairing with the cDNA. Subsequently, 40 µl of buffer VII was introduced into each reaction tube, along with 5 µl

of Magnesium Acetate I solution added to the tube lid. Following this, 5 µl of the sample was added to the reaction tube, resulting in a total volume of 50 µl, and the detection procedure commenced. This method enabled the amplification of the target sequence, the ORF1ab gene. In this study, RT-PCR served as the reference method, and the researcher remained blinded to the PCR results.

Figure 1 Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay accessories

5.8. Data Quality Assurance

Samples were gathered by proficient personnel trained for the task. All laboratory protocols adhered strictly to established standard operating procedures and guidelines from the manufacturer. Before commencing actual laboratory procedures, the integrity of each reagent, including its lot number, expiration date, and storage conditions, was verified. Quality checks

were implemented as necessary throughout the laboratory process. Both positive and negative controls supplied with the kit were utilized to verify the accuracy of the analysis. Prior to data input, the completeness and coherence of the data were manually verified. It was imperative that all these criteria were satisfied concurrently in every experiment; otherwise, the experiment was considered invalid and necessitated repetition.

5.9. Data Analysis and interpretation

The data underwent analysis using Statistical Packages for Social Sciences (SPSS) version 22.0. Sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy were computed with reference to RT-PCR as the standard. Cohen's kappa was employed to assess the degree of concordance between the test assays and real-time RT-PCR methods.

5.10. Ethical considerations

The study was performed after obtaining approval letters from the departmental research and review committee of the Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University protocol No DRERC/700/22/MLS.

5.11. Dissemination of the Result

The study findings shared with Addis Ababa University's School of Medical Laboratory Sciences as part of the requirements for completing the Master's degree program in diagnostic and public health microbiology. Additionally, the results was shared with the Armauer Hansen Research Institute and submitted to both national and international journals for publication.

6. Result

6.1 Socio-demographic and clinical characteristics of the study participants

The dataset provided contains information about 526 individuals. The primary variable documented is gender, wherein 280 individuals (53.2%) are identified as male, and 246 individuals (46.8%) are identified as female among the total sample. The second variable encompasses COVID-19 symptoms. Among the 526 individuals surveyed, 369 (70.1%) reported experiencing symptoms indicative of COVID-19, while 146 individuals (27.8%) reported no such symptoms. For those individuals who reported experiencing COVID-19 symptoms (n=369), detailed data on various clinical manifestations were recorded. The most prevalent symptom within this subgroup was a cough, reported by 269 individuals (72.8%). Headache was reported by 61 individuals (16.5%), sore throat by 21 individuals (5.7%), loss of smell by 13 individuals (3.5%), and joint pain by 5 individuals (1.3%). Additionally, there were 11 individuals who did not provide specific responses regarding their clinical symptoms. Table 1 Socio-demographic and clinical characteristics of the study participants

Table 1 Socio-demographic and clinical characteristics of the study participants

Variables		N	%
Gender (n=526)	Male	280	53.2 %
	Female	246	46.8 %
COVID Symptoms (n=526)	Yes	380	72.2 %
	No	146	27.8 %
Clinical Symptoms (n=369)	Cough	269	70.8 %
	Headache	61	16.0 %
	Sore throat	21	5.5 %
	Loss of smell	13	3.4 %
	Joint pain	5	1.3 %

6.2 Comparison of detection rate of tool kit against RT PCR

Of the total, 388 individuals tested positive with the RT PCR, 334 (86.0d%) were also positive with tool kit. Additionally, 138 individuals tested negative with the RT PCR, 133 (96.4%) of them also negative with the Tool kit. The Kappa statistic (0.7) indicates substantial agreement between the two methods, and the low P value (<0.0001) suggests highly significant agreement.

Table 2 Comparison of detection rate of tool kit against RT PCR

Table 2 Comparison of detection rate of tool kit against RT PCR

		RT PCR			Kappa	P value
		Positive	Negative	Total		
Tool Kit	Positive	334	5	339	0.7	<0.0001
	Negative	54	133	187		
	Total	388	138	526		

6.3 Diagnostic performance of tool kit against RT PCR

The tool kit has shown a sensitivity and specificity of 86% (95% CI (82.6 - 89.5)) and 96.4% (95% CI (93.2 - 99.5)) respectively. The positive predictive value was shown to be 98.5% (95% CI (93 – 99.5)). Table 3 Diagnostic performance of the tool kit

Table 3 Diagnostic performance of the tool kit against the gold standard RT PCT

Performance indicators	%age	95% CI	P value
Sensitivity	86	82.6 - 89.5	<0.0001
Specificity	96.4	93.2 - 99.5	
Positive predictive value	98.5	93 - 99.5	
Negative predictive value	71	64.6 - 77.6	
Positive likely hood ratio	23.7	10-56	
Negative likely hood ratio	0.14	0.1 - 0.18	

6.4 Detection rate comparison at different Ct values of RT PCR results

The performance of the Tool Kit was assessed in comparison to RT-PCR across different cycle threshold (Ct) values. Notably, when the Ct value is less than 30, the Tool Kit exhibited a 100% detection rate. However, as the Ct value increased further, particularly above 35, there was a noticeable rise in the number of negative results, accounting for 47 out of 65 cases (72.3%). Consequently, the sensitivity of the Tool Kit decreased substantially to 27.7% in this subset of samples with higher Ct values.

Table 4 Detection rate comparison at different Ct values of RT PCR results

Table 4 Detection rate comparison at different Ct values of RT PCR results

		RT PCR Ct values					P value
		<20 (n=12)	20-30 (n=179)	31-35 (n=139)	>35 (n=65)	Negative (n=131)	
		Positive	Positive	Positive	Positive		
Tool kit	Positive	12	179	126	18	4	<0.0001
	Negative	0	0	13	47	127	
	Total	12	179	139	65	131	
	Sensitivity	100%	100%	90.60%	27.70%	N/A	
	Specificity	N/A	N/A	N/A	N/A	96.90%	

6.5 Diagnostic performance of tool kit using area under the curve (AUC)

The receiver operating characteristic (ROC) curve analysis has shown a good diagnostic predictor with AUC of 0.956 for the tool kit (Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay). Table 5 Diagnostic performance of Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay (Tool kit) through calculating AUC & Figure 2 ROC curve for Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay (Tool kit) with RT PCR as a reference standard

Table 5 Diagnostic performance of Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay (Tool kit) through calculating AUC.

Area	SE	Asymptotic significance	Asymptotic normal
			[95% conf. interval]
0.956	0.011	0.0001	0.934 - 0.979

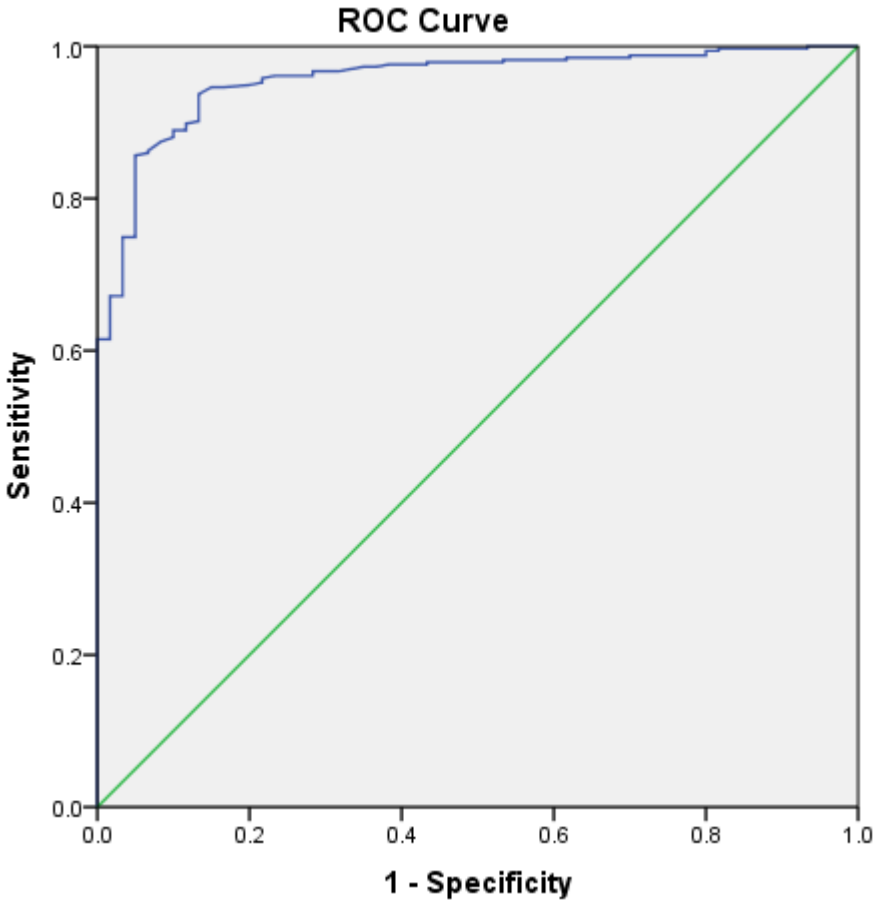

Figure 2 ROC curve for Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay (Tool kit) with RT PCR as a reference standard.

7. Discussion

In this study, a male gender preponderance was shown. An inconsistent with this study was reported by Mahmoodi *et al* which showed female preponderance accounting for 61% of the total participants (27). Most of the participants, 72%, were symptomatic and majority of them, 70.8%, presented with a cough. Joint pain and loss of smell were shown to be the least presented symptoms. Similarly an increased number of participants presenting with cough has been shown by a study conducted in Ethiopia (28). On a similar note, another report from Ethiopia has also shown an increased proportion of participant presenting with cough (29). On another r note, a low level of loss of smell has been reported by one study (30). This might be due to the fact that patients with lower cycle threshold (Ct) values upon RT-PCR examination, indicating a higher viral load, often exhibit more pronounced symptoms of COVID-19, such as coughing. This heightened viral load may lead to increased viral shedding and replication in the respiratory tract, resulting in more severe respiratory symptoms like coughing. Additionally, joint pain is a common symptom associated with COVID-19, albeit typically milder in individuals with lower viral loads. The exact mechanisms underlying joint pain in COVID-19 are not fully understood but may involve inflammatory responses triggered by the virus. However, individuals with lower Ct values may experience less severe joint pain due to a relatively lower level of systemic inflammation compared to those with higher viral loads. Overall, the severity and type of symptoms experienced by COVID-19 patients can vary based on various factors, including viral load, immune response, and individual susceptibility.

In this study, the detection rate of RT PCR, 73.8%%, was superior to that of the Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay (Tool kit). Similarly, Sisay et al has also reported an increased yield of RT PCR (31). A higher level of positive case detection rate by RT PCR has also been reported by Ashagre et al from their study conducted in Ethiopia (32). This might be due to the combination of sensitivity, specificity, established methodology, wide availability, and regulatory approval contributing to the high detection rate of RT-PCR in the diagnosis of COVID-19.

The detection rate of the positive cases of the Mobile suitcase laboratory based on the Recombinase Aided isothermal amplification assay (Tool kit) in our study was shown to be 64%.

Consistently with this study, reports have shown a higher detection rate of Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay (16, 19).

The Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay (Tool kit) in this study has shown a sensitivity of 86%. A multi country phase 2 study has reported a varying degree of sensitivity ranging from 91-100% (33). Its higher sensitivity was also witnessed by another study (24). Similarly an increased sensitivity of the suit case tool kit was also reported by one study (18).On the other hand, the specificity of Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay (Tool kit) was 96.4%.

The Mobile suitcase laboratory based on Recombinase Aided isothermal amplification assay (Tool kit) sensitivity was excellent in those sample with lower Ct values. But the sensitivity drops much lower for those samples with a Ct value of >35. A similar trends of a drop in sensitivity as a Ct value gets high was reported by one study (16). The observed decrease in sensitivity might be associated with various factors such as detection threshold, RAA assays may have a higher detection threshold compared to RT-PCR, meaning they require a higher viral load to produce a positive result. Samples with lower viral loads may fall below this threshold, resulting in false-negative results. Amplification efficiency, RAA assays may exhibit reduced amplification efficiency for samples with lower viral loads. This could be due to factors such as suboptimal reaction conditions or inhibition of the amplification process by contaminants in the sample. Target sequence variation, the RAA assay targets specific regions of the viral genome for amplification. If the viral load is low or the target sequence is mutated or absent in the sample, the assay may fail to amplify the target sequence, leading to false-negative results. Or might be associated with sample quality in that samples with lower viral loads may also have lower RNA quality, which can affect the performance of the RAA assay. Poor RNA quality may result in decreased amplification efficiency and reduced sensitivity of the assay.

8. Conclusion

This study underscores the potential of the Mobile Suitcase Laboratory (MSL) utilizing the Recombinase Aided Isothermal Amplification (RAA) assay in resource-limited settings like Ethiopia. The findings reveal that the MSL-RAA assay is a viable alternative to traditional laboratory-based methods, such as RT-PCR, for decentralized COVID-19 testing. Its portability, user-friendliness, and rapid results make it particularly beneficial for remote and underserved regions where centralized laboratory facilities are scarce.

Despite its slightly lower sensitivity compared to RT-PCR, the MSL-RAA assay offers significant advantages in terms of accessibility and speed, making it a valuable tool for timely COVID-19 diagnosis and monitoring. This can lead to quicker public health responses and better patient management strategies in areas with limited resources.

Further research and investment are essential to scale up the deployment of MSL-RAA technology in resource-limited countries like Ethiopia. Enhancing the capacity for effective pandemic response and control through widespread adoption of such innovative technologies will be crucial in addressing future public health challenges.

9. Recommendation

Our study recommends scaling up and deploying Mobile Suitcase Laboratory (MSL) technology utilizing the Recombinase Aided Isothermal Amplification (RAA) assay in resource-limited countries such as Ethiopia. The MSL-RAA assay presents a promising decentralized testing solution for COVID-19, particularly in remote and underserved regions.

To maximize its impact, future efforts should concentrate on the following:

- **Refinement and Optimization**: Focus on enhancing the sensitivity of the MSL-RAA assay while maintaining its portability and ease of use.
- **Strategic Investment**: Invest in training programs for healthcare workers and infrastructure development to facilitate the successful integration of MSL-RAA technology into existing healthcare systems.
- **Research and Development**: Encourage continuous research to improve the assay's performance and adapt it to various testing environments.

By prioritizing the deployment and integration of MSL-RAA technology, Ethiopia and other resource-limited countries can significantly enhance their capacity for timely and effective pandemic response. This strategic approach will contribute to global efforts in combating COVID-19 and improving public health outcomes in underserved regions.

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Annexes

1. RNA Extraction kit

Principle

The QIAamp Viral RNA Mini Kit offers a rapid and simple method for purifying viral RNA, suitable for use in various amplification technologies. It can isolate viral RNA from plasma (treated with anticoagulants other than heparin), serum, and other cell-free body fluids, whether fresh or frozen. However, if samples are frozen, they should undergo no more than one thawing cycle to maintain optimal sensitivity. Repeated freeze-thaw cycles can reduce viral titers and lead to cryoprecipitate accumulation, potentially causing clogging of the QIAamp membrane when using the vacuum protocol. While the kit is designed for general use and can isolate viral RNA from a wide range of viruses, its performance may vary for different viruses, and complete efficacy cannot be guaranteed for every virus.

The QIAamp Viral RNA Mini Kit is a widely recognized technology for preparing viral RNA suitable for various applications. It utilizes a silica-based membrane that selectively binds RNA and offers rapid processing of multiple samples simultaneously using microspin or vacuum technology. Initially, samples are lysed under denaturing conditions to inactivate RNases and ensure intact RNA isolation. Buffering conditions are then adjusted for optimal RNA binding to the QIAamp membrane, followed by loading onto the QIAamp Mini spin column. RNA binds to the membrane, while contaminants are removed in two steps using different wash buffers. High-quality RNA is eluted in an RNase-free buffer, ready for immediate use or safe storage, devoid of protein, nucleases, and other inhibitors. The specialized QIAamp membrane ensures efficient recovery of pure RNA in just 20 minutes without the need for phenol/chloroform extraction or alcohol precipitation. Adjusting lysate buffering conditions is crucial for optimal viral RNA binding before loading onto the QIAamp Mini column. Viral RNA binds to the QIAamp silica membrane through brief centrifugation steps or vacuum, while salt and pH conditions in the lysate prevent retention of contaminants that may inhibit downstream enzymatic reactions. For sample volumes exceeding 140 µl, loading the lysate onto the QIAamp Mini column may require multiple steps

Elimination of residual contaminants: Viral RNA, which adheres to the QIAamp membrane, undergoes two brief centrifugation or vacuum steps to eliminate contaminants. The employment of two distinct wash buffers, AW1 and AW2, substantially enhances the purity of the eluted RNA. Carefully optimized wash conditions guarantee thorough elimination of any lingering contaminants without compromising RNA binding.

Elution using Buffer AVE: Buffer AVE is composed of RNase-free water with 0.04% sodium azide to inhibit microbial growth and prevent RNase contamination. Sodium azide may interfere with absorbance readings at wavelengths between 220 and 280 nm but does not affect downstream applications like RT-PCR. It is advisable to elute RNA with RNase-free water rather than AVE buffer for assessing RNA purity. Given the high sensitivity of RNA to RNase, it is crucial to handle it with care, as contamination sources such as hands and dust particles often harbor bacteria and molds that carry RNases.

PCR must adhere to stringent laboratory protocols. Therefore, a PCR facility should be segregated into three zones: one for reagent preparation, another for sample handling, and a third for amplification and analysis. Given PCR's high sensitivity, maintaining the purity of all reagents is paramount and requires regular vigilance. Any contaminated reagents must be promptly disposed of to ensure the integrity of the results.

Sample Volumes

QIAamp Mini columns have the capacity to capture RNA molecules exceeding 200 nucleotides in length. The actual yield depends on factors such as sample volume, storage conditions, and viral concentration. While the protocol is optimized for 140 µl samples, volumes up to 280 µl are acceptable. Smaller samples should be adjusted to 140 µl using phosphate-buffered saline (PBS) before loading, and samples with low viral concentrations should be concentrated to 140 µl prior to processing. For larger volumes, the amount of lysis buffer and other reagents added to the sample should be scaled accordingly, although the volumes of Buffers AW1 and AW2 used in the wash steps typically remain unchanged. When the initial sample volume exceeds the recommended capacity, multiple loading steps may be required onto the QIAamp Mini column without risking overload or compromising RNA purity. For volumes exceeding 560 µl, sample concentration is advised.

Lysis

Under the stringent conditions facilitated by Buffer AVL, the sample is initially lysed to deactivate RNases, ensuring the preservation of intact viral RNA. Additionally, the inclusion of carrier RNA in Buffer AVL enhances the adherence of viral RNA to the QIAamp membrane, particularly beneficial for samples with low titers, while also mitigating the risk of RNA degradation caused by any remaining RNase activity.

Carrier RNA

Carrier RNA serves two primary purposes. Firstly, it enhances the attachment of viral nucleic acids to the QIAamp Mini membrane, particularly beneficial when the sample contains a low concentration of target molecules. Secondly, the presence of abundant carrier RNA diminishes the risk of viral RNA degradation, offering protection against residual RNase activity that may persist despite denaturation by chaotropic salts and detergent in Buffer AVL. The absence of carrier RNA in Buffer AVL may result in diminished recovery of viral RNA. The quantity of lyophilized carrier RNA provided is tailored to the volume of Buffer AVL included in the kit. The concentration of carrier RNA has been optimized to ensure compatibility with various amplification systems, rendering the QIAamp Viral RNA Mini Kit suitable for purifying RNA from a diverse array of RNA viruses.

The efficiency of different amplification systems can vary based on the total nucleic acid content in the reaction. Eluates from this kit contain both viral nucleic acids and carrier RNA, with carrier RNA levels significantly surpassing those of viral nucleic acids. Consequently, calculations for downstream amplifications should be determined based on the quantity of carrier RNA added. Achieving optimal sensitivity in amplification reactions may necessitate adjustments to the amount of carrier RNA supplemented to Buffer AVL.

Spin and vacuum procedures

The QIAamp Viral RNA Mini purification procedure involves three stages using QIAamp Mini columns, which can be performed in a standard microcentrifuge, on a vacuum manifold, or on QIAcube Connect. QIAamp Mini columns are compatible with most standard microcentrifuge tubes. For the spin protocol, 2 ml collection tubes (provided) are necessary to support the

QIAamp Mini column during loading and wash steps due to the volume of filtrate. Eluted RNA can be collected in standard 1.5 ml microcentrifuge tubes (not provided), which must be RNase-free to prevent degradation of viral RNA by RNases.

Equipment and Reagents

When handling chemicals, ensure to wear appropriate protective gear including a lab coat, disposable gloves, and protective goggles. For further guidance, refer to the relevant safety data sheets (SDSs) provided by the product supplier.

- Ethanol (96–100%)
- 1.5 ml microcentrifuge tubes
- Sterile, RNase-free pipette tips (pipette tips with aerosol barriers for preventing crosscontamination are recommended)
- Microcentrifuge (with rotor for 1.5 ml and 2 ml tubes)

Preparation of reagents

Mix Buffer AVE with the lyophilized carrier RNA to achieve a concentration of 1 µg/µl (e.g., add 310 µl Buffer AVE to 310 µg lyophilized carrier RNA, or 1550 µl Buffer AVE to 1550 µg lyophilized carrier RNA; refer to the tube label for the content). Ensure thorough dissolution of the carrier RNA, then divide it into suitable aliquots and store them at temperatures between -30 to -15°C. Avoid subjecting the aliquots to more than three freeze-thaw cycles.

To incorporate the dissolved carrier RNA into Buffer AVL:

- Check Buffer AVL for any precipitates and, if present, incubate at 80°C until the precipitate dissolves
- Determine the volume of Buffer AVL–carrier RNA mixture required for each batch of samples based on the number of samples to be processed simultaneously. For larger sample sizes, use the following calculation to determine the volumes required:

For larger numbers of samples, volumes can be calculated using the following sample calculation:

n x 0.56 ml = y ml

y ml x 10 µl/ml = z µl

Where: n = number of samples to be processed simultaneously

y = calculated volume of Buffer AVL

z = volume of carrier RNA–Buffer AVE to add to Buffer AVL

Gently mix by inverting the tube 10 times. To avoid foaming, do not vortex.

Table1. Volumes of Buffer AVL and carrier RNA–Buffer AVE mix required for the QIAamp Viral RNA Mini procedure

No. samples	Buffer AVL (ml)	Carrier RNA–AVE (µl)
1	.56	5.6
2	1.12	11.2
3	1.68	16.8
4	2.24	22.4
5	2.80	28.0
6	3.36	33.6
7	3.92	39.2
8	4.48	44.8
9	5.04	50.4
10	5.60	56.0
11	6.16	61.6
12	6.72	67.2
13	7.28	72.8
14	7.84	78.4
15	8.4	84
16	8.96	89.6
17	9.52	95.2
18	10.08	100.8
19	10.64	106.4
20	11.20	112.0
21	11.76	117.6
22	12.32	123.2
23	12.88	128.8
24	13.44	134.4

Prepare Buffer AVL–carrier RNA solution freshly, and store it at 2–8°C for a maximum of 48 hours. If a precipitate forms during storage at 2–8°C, warm the solution at 80°C to redissolve the precipitate before use. Avoid warming the Buffer AVL–carrier RNA solution more than six times. Additionally, do not incubate at 80°C for more than 5 minutes, as frequent warming and

prolonged incubation can degrade the carrier RNA, resulting in reduced recovery of viral RNA and potentially leading to false negative RT-PCR results, especially with low-titer samples.

Buffer AW1 and Buffer AW2 are both provided as concentrates. Before initial use, ethanol (96–100%) should be added to each according to the instructions on the bottle. Buffer AW1 and Buffer AW2 are stable for up to one year when stored in a closed container at room temperature, but this stability extends only until the expiration date of the kit

Procedure

1. Transfer 560 µl of the prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube. If the sample volume exceeds 140 µl, adjust the amount of Buffer AVL–carrier RNA accordingly in proportion to the sample volume (for instance, a 280 µl sample will necessitate 1120 µl of Buffer AVL–carrier RNA) and utilize a larger tube.
2. Combine 140 µl of the nasopharyngeal swab sample with the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix thoroughly by pulse-vortexing for 15 seconds. It is crucial to ensure thorough mixing to achieve efficient lysis. Frozen samples that have undergone only one thawing cycle are also acceptable for use.
3. Let stand at room temperature for 10 min for incubation.
4. Spin the tube to remove drops from the inside of surface of the lid.
5. Add 560 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
   Note: Use only ethanol, since other alcohols may result in reduced RNA yield and purity. Do not use denatured alcohol, which contains other substances, such as methanol or methylethylketone. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280 µl sample will require 1120 µl ethanol). To ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.
6. Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.
   Note: Centrifugation is performed at 6000 x g (8000 rpm) to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the

solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

7. Gently open the QIAamp Mini column, then repeat step 6. If the sample volume exceeded 140 µl, continue repeating this step until all of the lysate has been loaded onto the spin column.
8. Carefully open the QIAamp Mini column, and add 500 µl Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
   Note: It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl.
9. Open the QIAamp Mini column carefully and add 500 µl of Buffer AW2. Close the cap securely and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 minutes. Proceed directly to step 11, or to prevent potential carryover of Buffer AW2, perform step 10 before continuing with step 11. Note: Buffer AW2 residue in the eluate might affect subsequent applications. Some centrifuge rotors may cause vibration during deceleration, leading to flow-through containing Buffer AW2 to contact the QIAamp Mini column. Similarly, removing the QIAamp Mini column and collection tube from the rotor could also result in contact between the flow-through and the column. In such cases, consider performing the optional step 10.
10. It is recommended to transfer the QIAamp Mini column to a fresh 2 ml collection tube (not included) and discard the previous collection tube along with the filtrate. Centrifuge at maximum speed for 1 minute.
11. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.
12. Centrifuge at 6000 x g (8000 rpm) for 1 min. A single elution with 60 µl Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp Mini column. Performing a double elution using 2 x 40 µl Buffer AVE will increase yield by up to 10%. Elution with volumes of less than 30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate. Viral RNA is stable for up to 1 year when stored at −30 to −15°C or at –90 to −65°C.

Declaration

I, the undersigned, hereby accept responsibility for the scientific, ethical, and technical conduct of the research project, and I commit to providing the necessary progress reports in accordance with the terms and conditions set forth by the research publications office.

M.Sc. candidate: **Daniel Mussa(B.Sc.)**

Signature: _____

Date: _____

This thesis has been submitted with our approval as advisors.

Advisor: **AndargahewMulu(PhD)**

Signature: _____

Date: _____

Advisor: **Kassu Desta (PhD)**

Signature: _____

Date: _____