Epidemiology and alternative approaches for SARS-CoV-2 testing within limited resources settings

By

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DECLARATION

I, **Zamathombeni Duma**, hereby declare that I have undertaken the thesis titled **"Epidemiology and alternative approaches for SARS-CoV-2 testing within limited resources settings"** for the award of Doctor of Philosophy (Medicine). I completed this research under the supervision of **Prof Zilungile L. Mkhize-Kwitshana, Prof Anil A. Chuturgoon, and Prof Veron Ramsuran** from the University of KwaZulu Natal's College of Health Sciences.

I also declared that this thesis had not previously been submitted for the award of any degree or other similar titles of recognition in this University or any other University. This thesis does not include any data, pictures, graphs, or other information from other people unless they are specifically acknowledged as coming from them. When I reproduced a publication in which I am an author, I specified which portion of the publication was written entirely by myself and fully referenced such publications.

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LIST OF PUBLICATIONS

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1. **Duma Z**, Ramsuran V, Chuturgoon AA, Edward VA, Naidoo P, Mkhize-Kwitshana ZL (2022). Evaluation of Various Alternative Economical and High Throughput SARS-CoV-2 Testing Methods within Resource-Limited Settings. *International Journal of Molecular Sciences*. 23: 14350. doi.org/10.3390/ijms232214350.

2. **Duma Z**, Chuturgoon AA, Ramsuran V, Edward V, Naidoo P, Mpaka-Mbatha MN, Bhengu KN, Nembe N, Pillay R, Singh R, Mkhize-Kwitshana ZL (2022). The challenges of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) testing in low-middle income countries and possible cost-effective measures in resource-limited settings. *Globalization and Health Journal*. 18(1):5. doi: 10.1186/s12992-022-00796-7.

List of manuscripts under review:

1. **Duma Z**, Chuturgoon AA, Ramsuran V, Edward VA, Naidoo P and Mkhize-Kwitshana ZL. Epidemiology of symptomatic and asymptomatic SARS-CoV-2 infections in a South African population. Submitted to *ACS Infectious Disease Journal* (Under Review). Manuscript ID Number: id-2023-00052e.

List of additional publications during the PhD period:

1. Mpaka-Mbatha MN, Naidoo P, Islam MM, Singh R, Bhengu KN, Nembe-Mafa N, Pillay R, **Duma Z**, Niehaus AJ and Mkhize-Kwitshana ZL (2023). Immunological interaction during helminth and HIV coinfection: Integrative research needs for Sub-Saharan Africa. *South African Journal of Science*. 119 (1/2). doi.org/10.17159/sajs.2023/15108.

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Presentations

1. **Duma Z**. Epidemiology and alternative approaches for SARS-CoV-2 testing within limited resource settings. School of Laboratory Medicine and Medical Science Research Update, Medical Microbiology Discipline, University of KwaZulu-Natal, Durban, South Africa, 19 October 2022, Oral presentation.

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LIST OF ABBREVIATIONS

ACE2	Angiotensin-Converting Enzyme 2
~	Approximately
CDC	Centers for Disease Control and Prevention
$\chi 2$	Chi-squared test
COPD	Chronic Inflammatory Pulmonary Disease
COVID-19	Coronavirus disease
Ct	Cycle threshold value
℃	Degree Celsius
E protein	Envelope protein
GADH	Glyceraldehyde 3-phosphate dehydrogenase
$\times g$	Gravitational force
>	Greater than
HCW	Health Care Workers
HICs	High income countries
h	Hour
IC	Internal Control
<	Less than
LMICs	Low-middle income countries
M protein	Membrane
µL	Microliter
MICs	Middle Income Countries
min	Minutes
NK	Natural killer cells
N protein	Nucleocapsid
n	Numbers
ORF1ab	Open Reading Frames
%	Percentage

PPE	Personal Protective Equipment
PCR	Polymerase Chain Reaction
RT-PCR	Real-Time-Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic Acid
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
s	Seconds
S protein	Spike protein
USD	United States Dollar
WHO	World Health Organization
ZAR	South African Rand

ABSTRACT

Background: In the context of the global battle to contain the rapidly mutating SARS-CoV-2, diagnostic testing for SARS-CoV-2 infection remains a challenge, particularly in low-middle-income countries (LMICs) due to low socioeconomic backgrounds. Concerningly, because less attention is paid to asymptomatic cases, particularly in LMICs with limited resources for SARS-CoV-2 testing, the virus is spreading silently in communities, and the majority of these individuals could be contributing to the resurgence of SARS-CoV-2 infection. This study aimed to determine the epidemiology and alternative approaches for SARS-CoV-2 testing within limited resources settings **Methods:** A total sample size of 1335 residual patient samples from the Global Health Innovation (GHI) laboratory was used for the epidemiology study and methods comparison. **Results and Discussion:** Literature review showed that high income countries (HICs) test more frequently for SARS-CoV-2 infection, with a range of 113% to 146% higher than LMICs (1% to 43%). The present study demonstrated a higher proportion of asymptomatic cases (68%) among SARS-CoV-2 infected patients. Regarding the methods comparison for the detection of SARS-CoV-2, the evaluated alternative methods [three RNA extraction (Lucigen QuickExtract™ RNA Extraction Kit, Bosphore EX-Tract Dry Swab RNA Solution, Sonicator method and four commercial SARS-CoV-2 RT-PCR assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES^{TM} COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit)] were found to be cheaper and faster. **Conclusion:** Notably LMICs are undertesting for SARS-CoV-2 infection compared to HICs, and there was a higher proportion of asymptomatic cases among SARS-CoV-2 infected patients in South Africa. This study suggests that using the above-mentioned cost-effective, quick, and accurate evaluated alternative methods for mass SARS-CoV-2 testing in routine diagnostic laboratories with limited resources can help to increase testing capacity for SARS-CoV-2 infection in LMICs. This means that the sooner SARS-CoV-2 infection control and prevention measures can be implemented to reduce community transmission.

Keywords: SARS-CoV-2, epidemiology, alternative diagnostic testing approaches, resource limited settings

CHAPTER ONE

Introduction

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a highly contagious, rapidly mutating virus that has been identified as the cause of Coronavirus disease 2019 (COVID-19). In December 2019, the World Health Organization (WHO) reported the first pneumonia cases in Wuhan, China (Cascella *et al*., 2022; Lai *et al*., 2020). Since the SARS-CoV-2 pandemic broke out in Wuhan, it has spread throughout the world, and the number of new infections and deaths is still rising. To date, over 668 million COVID-19 cases have been confirmed globally, resulting in over 6.7 million deaths (Worldometer, 2022).

SARS-CoV-2 is a positive-stranded RNA virus that primarily infects the upper respiratory tract and causes lymphopenia, dyspnea, acute respiratory distress syndrome, pneumonia, and sudden cardiac death (Huang *et al*., 2020; Pal *et al*., 2020). SARS-CoV-2 is transmitted through person-to-person contact via small droplets produced by sneezing, coughing, and talking (Jayaweera *et al*., 2020; Stadnytskyi *et al*., 2020; Leung, 2021). Surface transmission is another mode of SARS-CoV-2 transmission because the virus can live on surfaces for up to 96 hours (Greenhalgh *et al*., 2021; Vidia *et al*., 2022; Chu *et al*., 2022). Individuals with symptoms are most likely to spread the SARS-CoV-2 infection. However, there is also a high risk of the virus spreading without any signs or symptoms. This is known as an asymptomatic spread (World Health Organization, 2022; Gao *et al*., 2020). It has been reported that the average incubation period for the virus is approximately 5 days before signs and symptoms appear; however, this varies from person to person (Linton *et al*., 2020).

When the COVID-19 outbreak hit countries around the world in 2020, various governments decided to lock down their countries at different times to slow the spread of SARS-CoV-2 infection globally (Haider *et al*., 2020). The lockdown was effective in reducing virus spread, but it had the unintended consequence of negatively impacting the country's economy. As a result, many people lost their jobs, increasing the rate of unemployment and poverty in these countries (Jain *et al*., 2020; Posel *et al*., 2021; Chackalackal *et al*., 2021). Due to the negative economic impact of the

lockdown, governments in various countries decided to relax lockdown restrictions to help uplift the economy (Han *et al*., 2020). The concern is that there is still no cure for SARS-CoV-2 infection, despite the fact that the vaccine program was supposed to be the solution to this crisis (Schmith *et al*., 2020; Bhuta *et al*., 2022; Shafiee *et al*., 2022). Hence, LMICs must learn from previous waves of SARS-CoV-2 infection to avoid being caught off guard by future waves, while also avoiding overburdening medical laboratories and health systems.

To date, diagnostic testing has been one of the most important aspects of lowering the SARS-CoV-2 infection rate. The polymerase chain reaction (PCR) technique and nasopharyngeal swab sample are the gold standard test and sample for detecting SARS-CoV-2 (Centers for Disease Control and Prevention, 2019; Liu *et al*., 2020; Wang *et al*.2020, Reid *et al*., 2021). The challenge is that using this molecular technique in conjunction with a nasopharyngeal swab to test each individual regularly is extremely expensive, especially for LMICs (Mutesa *et al*., 2021; Bastos *et al*., 2021). This is due to the financial constraints of LMICs and poor resources. As a result, identifying and monitoring SARS-CoV-2 infection cases has been the most difficult task in LMICs (Pasquale *et al*., 2021). It is critical to developing alternative diagnostics methods for SARS-CoV-2 mass testing that are fast and cheaper within a limited resource setting to prevent transmission.

1.1 Study Rationale

Due to the constant emergence of new variants, diagnostic testing for SARS-CoV-2 infection is still necessary even after vaccination. In South Africa and other LMICs, the SARS-CoV-2 outbreak and a lack of resources for mass testing of SARS-CoV-2 infection remain a challenge. With a high prevalence of HIV and tuberculosis, as well as poverty, inequality, and social instability, overcrowding, and limited access to quality medical treatment, water, and sanitation. Mass testing becomes an additional challenge as the number of SARS-CoV-2 infection cases rises. As a result, LMICs should focus on developing cheaper and faster alternative strategies for mass testing of SARS-CoV-2 infection. The reason for this is that any delays in detecting SARS-CoV-2 can be harmful to an individual, family members, and the community.

Given the high prevalence of HIV, tuberculosis, and noncommunicable diseases in South Africa and other African countries, as well as poor socioeconomic factors, an increase in SARS-CoV-2 infection mortality was expected. However, infection rates and case fatality have not met expectations in several African countries, despite the continent's historically fragile healthcare system. On the other hand, Africa has some factors that predispose it to severe and fatal SARS-CoV-2 infection, including diabetes, obesity, and cardiovascular disease. This leads to the question of what is preventing South Africa and other African countries from a high fatality rate, as well as the epidemiological profile of SARS-CoV-2 infected individuals?

1.2 Aims and Objectives

1.2.1 Aim 1:

To survey SARS-CoV-2 testing in low-middle income countries (LMICs) and high-income countries (HICs), and to make recommendations on possible interventions and cost-effective measures to increase testing capability in LMICs.

1.2.1.1 Objective 1:

To collect data on tests performed in LMICs using publicly available data from the online resource, Worldometer COVID-19; and evaluate various methodologies including the use of saliva instead of swabs; pooling of samples and alternative cheaper extraction methods as suitable cost-effective methods that could be adapted to resource-limited settings.

1.2.2 Aim 2:

To retrospectively characterize SARS-CoV-2 infected cases who presented to testing facilities.

1.2.2.1 Objective 2:

To assess the demographic, clinical, symptomatic and asymptomatic profile of the individuals who are infected with SARS-CoV-2 using data from the Global Health Innovation (GHI) laboratory.

1.2.3 Aim 3:

To compare and evaluate alternative methods for the mass testing of SARS-CoV-2 infection in laboratories with limited resources to identify cost-effective, faster, and accurate alternatives to the internationally approved SARS-CoV-2 testing kits.

1.2.3.1 Objective 3:

To compare extraction methods using nasopharyngeal swab samples: Thermo Fisher PureLink™ Kit, lysis buffers (Lucigen Quick Extract™ RNA Extraction Kit and Bosphore EX-Tract Dry Swab RNA Solution) and the Sonicator method on the RT-PCR and to assess whether these extraction methods can be used interchangeably.

1.2.3.2 Objective 4:

To compare different SARS-CoV-2 Assay Kits using nasopharyngeal swab samples: Thermo Fisher TaqPath™ COVID-19 Assay Kit, Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES^{TM} COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit).

1.3 Research questions

1.3.1 What is the testing rate and what are the challenges regarding mass testing of SARS-CoV-2 infection experienced by LMICs?

1.3.2 What are the characteristics of SARS-CoV-2 infection in the South African population that could be useful for future pandemics?

1.3.3 What are the alternative methods that can reduce the costs and workload for SARS-CoV-2 mass testing in laboratories within a limited resource setting?

Outline of the Thesis

This Thesis is written in manuscript format and adheres to the University of KwaZulu Natal's recommended standards for Thesis submissions by publications. The Thesis is divided into five

chapters. Two of the manuscripts have been published, and one has been submitted to a journal and is being reviewed in some of the chapters. The manuscripts are presented as per journal format requirements.

Chapter 1: This chapter includes introduction, research problem, study rationale, aims, objectives, research questions.

Chapter 2: Manuscript 1 title _ "The challenges of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) testing in low-middle income countries and possible cost-effective measures in resource-limited settings" and is published in Globalization and Health Journal. Aim 1 and Objective 1 are covered in the manuscript.

Chapter 3: Manuscript 2 title_ "Epidemiology of symptomatic and asymptomatic SARS-CoV-2 infections in a South African population" and it covers Aim 2 and Objective 2. The manuscript has been submitted to the journal and is being reviewed.

Chapter 4: Manuscript 3 title_ "Evaluation of Various Alternative Economical and High Throughput SARS-CoV-2 Testing Methods within Resource-Limited Settings" has been published by International Journal of Molecular Sciences. The manuscript covers Aim 3 and Objectives 3 and 4.

Chapter 5: This chapter focuses on the study's synthesis and conclusion.

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CHAPTER TWO: LITERATURE REVIEW

Testing for SARS-CoV-2 in Low-Middle Income Countries

There is a severe challenge in terms of testing infrastructures required for the detection of SARS-CoV-2 infection that complies with WHO guidelines, particularly in LMICs. As is well known, laboratory results impact patient care decisions. Alarmingly, when compared to HICs, LMICs undertest for SARS-CoV-2 infection. It means that a large number of people are spreading the virus unknowingly, which suggests that this could be one of the reasons why these countries are still struggling to monitor the SARS-CoV-2 infection. It is critical to develop and implement strategies for SARS-CoV-2 infection that are less expensive and more reliable to increase testing capacity for SARS-CoV-2 mass testing and prevent virus transmission in countries with limited resources. As a result, part of this work discussed the challenges that LMICs face when it comes to mass testing for SARS-CoV-2, as well as suggestions that meet WHO quality standards and could assist in increasing LMICs testing capacity. These are discussed in the review below, which was published in the manuscript titled: **"The challenges of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) testing in low-middle income countries and possible cost-effective measures in resource-limited settings". The published PDF version is shown below under Appendix A.**

Duma Z, Chuturgoon AA, Ramsuran V, Edward V, Naidoo P, Mpaka-Mbatha MN, Bhengu KN, Nembe N, Pillay R, Singh R, Mkhize-Kwitshana ZL. The challenges of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) testing in low-middle income countries and possible cost-effective measures in resource-limited settings. Globalization and Health Journal. 2022 Jan 22;18(1):5. doi: 10.1186/s12992-022-00796-7. **(Impact factor =3.714)**

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Manuscript Title: The challenges of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) testing in low-middle income countries and possible cost-effective measures in resource-limited settings

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Abstract

Diagnostic testing for the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection remains a challenge around the world, especially in low-middle-income countries (LMICs) with poor socio-economic backgrounds. From the beginning of the pandemic in December 2019 to August 2021, a total of approximately 3.4 billion tests were performed globally. The majority of these tests were restricted to high income countries. Reagents for diagnostic testing became a premium, LMICs either cannot afford or find manufacturers unwilling to supply them with expensive analytical reagents and equipment. From March to December 2020 obtaining testing kits for SARS-CoV-2 testing was a challenge. As the number of SARS-CoV-2 infection cases increases globally, large-scale testing still remains a challenge in LMICs**.** The aim of this review paper is to compare the total number and frequencies of SARS-CoV-2 testing in LMICs and high-income countries (HICs) using publicly available data from Worldometer COVID-19, as well as discussing possible interventions and cost-effective measures to increase testing capability in LMICs. In summary, HICs conducted more SARS-CoV-2 testing (USA: 192%, Australia: 146%, Switzerland: 124% and Canada: 113%) compared to middle-income countries (Vietnam: 43%, South Africa: 29%, Brazil: 27% and Venezuela: 12%) and low-income countries (Bangladesh: 6%, Uganda: 4% and Nigeria: 1%). Some of the cost-effective solutions to counteract the aforementioned problems includes using saliva instead of oropharyngeal or nasopharyngeal swabs, sample pooling, and testing high-priority groups to increase the number of mass testing in LMICs.

Keywords: SARS-CoV-2, Diagnostic testing challenges, Low-middle-income countries, Cost-effective strategies, Resource-limited settings

Background

Overview of SARS-CoV-2

In December 2019, the World Health Organization (WHO) reported several pneumonia cases in Wuhan, China [1]. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was confirmed as the cause of Coronavirus disease 2019 (COVID-19) [2]. Severe Acute Respiratory Syndrome Coronavirus 2 is a positive-stranded ribonucleic acid (RNA) virus that primarily infects the upper respiratory tract and is associated with a wide range of complications, including lymphopenia, dyspnea, acute respiratory distress syndrome, pneumonia and acute cardiac arrest [3].

The virus contains four structural proteins namely the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins [4]. SARS-CoV-2 entrance into the host cells is mediated by the spike protein. The receptor-binding domain that binds to the peptidase domain of angiotensin-converting enzyme 2 (ACE2) is located at the S1 subunit of the S protein [4,5]. The M protein is the virus's most abundant structural protein [4,6] because it interacts with all of the other major coronaviral structural proteins, and it is assumed to be the central coordinator of coronavirus assembly [4]. The E protein is a minor component of the membrane. During the replication cycle, the E protein is widely expressed inside the infected cell, but only a small portion of it is integrated into the virion membrane [4,7]. The nucleocapsid is made up of the viral RNA genome, and the N protein is the only protein that binds to it. The N protein is largely involved in viral genome activities, and also plays a role in other aspects of the viral replication cycle as well as the host cellular response to viral infection [4, 8].

Countries worldwide have been struggling to contain the highly contagious and rapidly mutating SARS-CoV-2 for more than a year [9]. As of 30 September 2021, the virus has spread to over a hundred countries, and about 222 million coronavirus cases had been confirmed worldwide, resulting in over 4,6 million deaths [10]. The WHO has declared COVID-19 a Public Health Emergency of International Concern [11, 12].

Transmission of SARS-CoV-2 and incubation period

The main mode of transmission of SARS-CoV-2 is by person-person contact [13]. The virus is spread between people through minute aerosol droplets created by sneezing, coughing and talking during close contact. Another way a person can become infected with the virus is by surface transmission [14]. This is because the virus can live on surfaces for up to 96 hours [13]. The virus is more likely to transmit through people who display symptoms early in the disease; this is known as symptomatic transmission [14]. In addition, there is a high chance of passing the virus without showing any signs and symptoms and this is known as an asymptomatic spread [15].

The virus transmission channel, the amount of virus that enters the host, and the interaction between the virus and the host immune system are all factors that influence the incubation period [16, 17]. According to the WHO and the Centers for Disease Control and Prevention (CDC), the incubation period for SARS-CoV-2 infection is estimated to be 1-14 days, with an average incubation period of around 5-6 days [17]. According to a study conducted in Wuhan, China (January 2020 – February 2020), roughly 97.5% of SARS-CoV-2 infected patients exhibited clinical symptoms after 11.5 days, and the remaining 2.5% in 2.2 days [19]. To note, the incubation period varies from person to person [19]. One of the most serious concerns is that SARS-CoV-2 variants have evolved in huge numbers, causing transmission alterations [20]. However, there is limited information about the incubation duration for specific variants [20]. Therefore, more studies are needed to determine whether novel SARS-CoV-2 mutations affect the incubation period. Furthermore, that is the reason why the WHO still emphasizes the recommended quarantine period of 14 days [21].

Safety measures for SARS-CoV-2 infection in LMICs

According to WHO (2020), the most recommended preventive measures for SARS-CoV-2 infection include social distancing, hand hygiene, using face masks and coughing in the elbow [22]. Implementing the recommended preventive measures in LMICs is a challenge due to unfavorable conditions such as overcrowding in the household, inadequate ventilation in dwellings, ambient and indoor air pollution, lack of clean water supply, refugee settings, the number of persons living on the streets, and poor sanitation [23]. Sanitation is a crucial issue in LMICs because a large number of people, particularly in rural and peri-urban regions, still rely on

surface and groundwater sources for their daily water needs [24]. Pit toilets and groundwater are widely used in LMICs, while open defecation near surface water has also been reported as well [25]. The untreated effluent is dumped into the environment, potentially contaminating groundwater and surface resources [26]. As a result, this might partially contribute to the risk of SARS-CoV-2 transmission. Also, to be noted, a few studies reported detecting SARS-CoV-2 in wastewater which has epidemiologic potential and can be used as a backup technique to monitor viral tracking and circulation in places with limited SARS-CoV-2 testing capacity or highly populated regions where door-to-door tracing is difficult. However, in order to improve sensitivity, special attention must be paid to virus concentration and detection assays [27].

The consequences of lockdown restrictions in LMICs

As a way to curb the spread of the rapidly mutating SARS-CoV-2, countries worldwide enforced strict lockdown restrictions [28]. By April 2020, more than 90 countries were in some form of lockdown. Stay-at-home orders, quarantine, isolation, social distancing, curfews, school and company closures, and travel restrictions are all part of the lockdown regulations [29]. The WHO proposed response to the SARS-CoV-2 outbreak involves personal hygiene, effective contact tracing, and isolation when an individual is infected, to strike a balance between lockdown restrictions and normalcy [30]. If implemented in a timely and comprehensive manner, lockdown can be an effective infection control and prevention mechanism, reducing the risk of virus transmission from person to person and population spread while buying enough time to scale up preventative measures, diagnostic tests, and treatment capability [31]. While the rigorous restrictions associated with lockdowns are effective, they come at a cost: they impose significant social and economic constraints on individuals and groups, particularly in LMICs [31].

Workers in the informal economy are affected the most by the lockdown because they lack social security and access to adequate health care, as well as having lost access to productive assets [32]. Hence, without the means to earn an income during lockdowns, many are unable to feed themselves and their families [33]. Due to border closures, trade barriers, and other restrictions, farmers are unable to access markets, causing a disruption in domestic and international food supply chains as well as limiting access to balanced, healthy, and diverse meals [34]. Therefore, millions of women, children and men's food security have been compromised as a result of

breadwinners losing their jobs due to the lockdown in low-income countries, with vulnerable communities such as small-scale farmers and indigenous people being the hardest hit [34].

Attempting to strengthen the economy of several LMICs, various governments have opted to ease lockdown restrictions. Therefore, lockdown restrictions in various countries were relaxed at various periods [35]. Currently, governments throughout the world are struggling to figure out whether and how to relax restrictions while balancing numerous health, social, and economic issues. Premature lifting of lockdown restrictions by allowing businesses to operate, opening schools and higher education institutions, and allowing traveling are among the key factors contributing to the resurgence of SARS-CoV-2 waves [36]. Hence, LMICs should try to learn from previous waves of SARS-CoV-2 infection and try to avoid being caught off guard by more waves of SARS-CoV-2 infection in the future [36]. This means it is crucial to develop methods that are cheaper, simple, and have a quick diagnostic turnaround time to avoid the medical laboratory staff becoming overwhelmed during the future waves.

Challenges of SARS-CoV-2 testing in LMICs

Mass testing is one of the most significant aspects of lowering the SARS-CoV-2 infection rate through early detection of cases for treatment and subsequent cautionary measures such as isolation to prevent death and further virus transmission, respectively [37]. However, identification and monitoring of the SARS-CoV-2 infection cases have been the greatest challenge in the LMICs [37]. In LMICs, SARS-CoV-2 infection testing is problematic due to financial constraints and other factors [38]. These countries have no domestic capacity to manufacture nasopharyngeal swabs, analytical reagents and COVID 19 kits for SARS-CoV-2 testing [39]. With an increase in the number of SARS-CoV-2 infection cases, mass testing becomes disrupted due to a shortage of nasopharyngeal swabs, analytical reagents and COVID 19 kits. It is because buying all of the materials needed to test for SARS-CoV-2 infection is excessively expensive [40]. Furthermore, the cost of Personal Protective Equipment (PPE) has increased since the SARS-CoV-2 outbreak started, with LMICs bearing the brunt of the burden. The prices of surgical masks have increased sixfold, N95 breathing masks have tripled, and gowns have doubled. The problem is supply delivery could take months, and market manipulation is common, with inventories being sold to the highest bidder. This is concerning since healthcare workers rely on personal protective

equipment to safeguard themselves and their patients from SARS-CoV-2 infections and the spread of infections. Therefore, doctors, nurses, and other frontline workers in LMICs are severely underequipped to care for SARS-CoV-2 patients because of limited access to equipment including gloves, medical masks, respirators, goggles, face shields, gowns, and aprons [41]. In addition, there are fewer laboratory staff trained for SARS-CoV-2 testing in LMICs. As the number of infection cases increases the laboratory staff becomes overwhelmed, and as a result, diagnostic turnaround time and transmission rates will be increased [40, 41].

Obtaining the best effective vaccine program and uneven access to vaccine programs are two other important concerns in LMICs. Vaccine distribution in the world remains highly unequal, with a majority of the existing supply going to high-income countries (HICs) [42]. Hence, it will take months to years for the COVID-19 vaccine to have an impact against the SARS-CoV-2 in LMICs. As a result, this is concerning because the vaccine program was supposed to be the way out of this crisis [43]. In 2021, millions of people in LMICs would be denied access to the COVID-19 vaccine due to wide disparities in COVID-19 vaccine access between HICs and LMICs [43]. As a consequence, the outbreak may be prolonged, increasing the risk of additional mutation and reducing the efficacy of current vaccines. Therefore, LMICs need to come up with innovative approaches to fight this contagious virus [20].

The major concern is how will LMICs deal with the SARS-CoV-2 pandemic? As a result, using publicly available data from Worldometer COVID-19 [10], this review paper will compare the total number and frequencies of SARS-CoV-2 testing in LMICs and HICs, as well as discussing possible interventions and cost-effective measures to increase testing capability in LMICs.

Data Sourcing

Article search strategies, inclusion and exclusion criteria and data sourcing for the study is presented in the Prisma flow diagram (Figure 1)

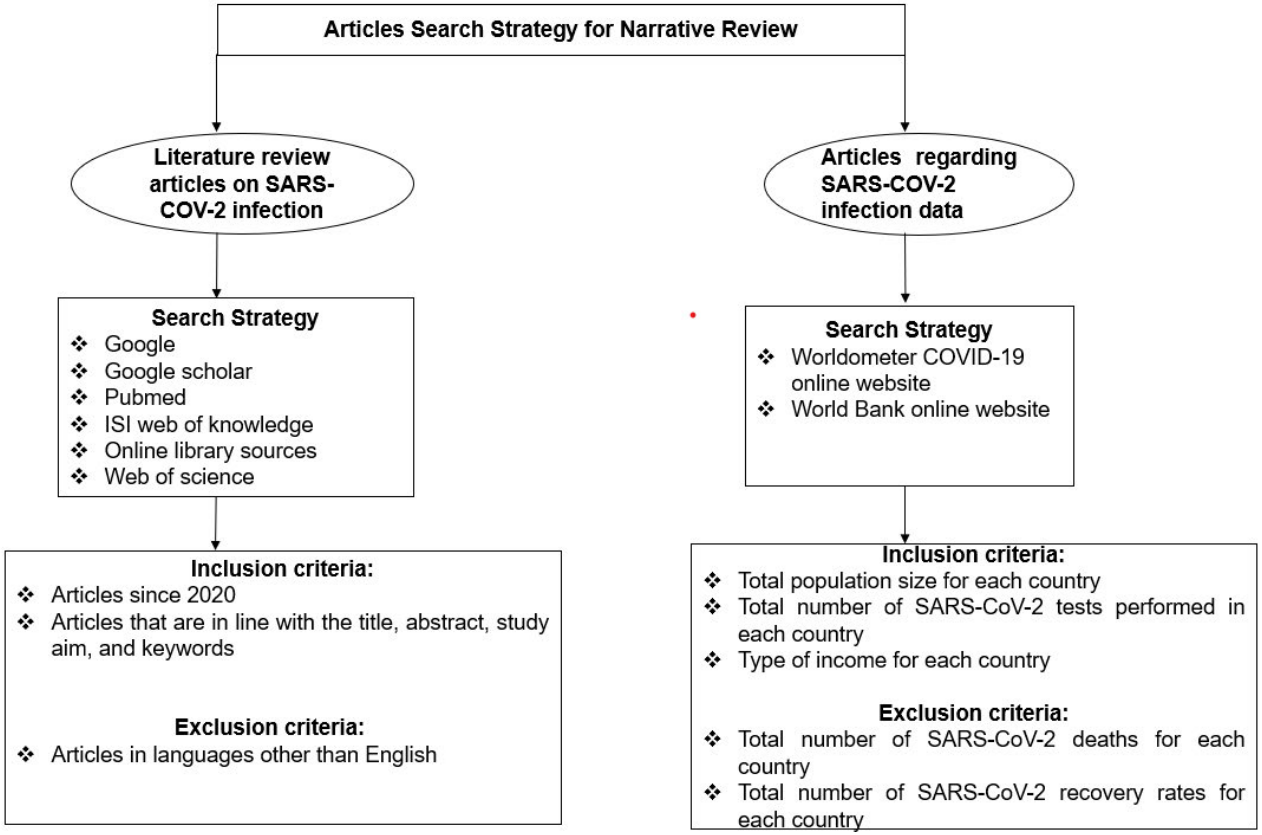

Figure 1: Prisma flow chart for article search of this narrative review paper

Results and overall findings

Table 1, Figures 2 and Figure 3 provide a comparison of the total number and frequencies of SARS-CoV-2 testing in each income group (low, middle, and high) and continent. The data in Table 1, Figures 2 and Figure 3 shows that high-income countries have undertaken 10 times more SARS-CoV-2 testing compared to LMICs [10]. More than 100% of the population in HIC (USA: 192%, Australia: 146%, Switzerland: 124% and Canada: 113%) has been tested for SARS-CoV-2, whereas only 27,5% of the population in middle-income countries (MIC) (Vietnam: 43%, South Africa: 29%, Brazil: 27% and Venezuela: 12%) and approximately 3% of the population in low-income countries (LIC) (Bangladesh: 6%, Uganda: 4% and Nigeria: 1%) has been tested.

The possible reasons for under-testing for SARS-CoV-2 infection in LMICs are probably many people are unable to afford SARS-CoV-2 testing due to financial restrictions, unstable health systems and reliance on global supply chains. As a result, many positive cases are simply missed, putting LMICs at higher risk of spreading the virus [31]. The pandemic will easily shatter the poor health system and overburden hospitals and clinical services if effective prevention is not implemented [44].

Table 1: Comparison between the total number of tests (in million) performed and the total population (in million) in high-income countries and LMICs

Continent	Countries	Income	Total Tests Performed (million)	Total Population (million)	Percentage of Tests performed
North America	USA	High	639 832 856	333 416 037	192
Oceania	Australia	High	37 832 547	25 854 460	146
Europe	Switzerland	High	10 796 404	8 733 303	124
North America	Canada	High	43 215 201	38 153 447	113
Europe	Germany	High	73 348 901	84 117 156	75
Asia	Vietnam	Middle	42 517 091	98 427 082	43
Africa	South Africa	Middle	17 649 727	60 237 549	29
South America	Brazil	Middle	57 282 520	214 437 809	27
South America	Venezuela	Middle	3 359 014	28 335 663	12
Asia	Bangladesh	Low	9 704 722	166 728 314	6
Africa	Uganda	Low	1 680 863	47 529 564	4
Africa	Nigeria	Low	2 997 060	212 473 029	1

Data were retrieved from the Worldometer Covid 19 on 30 September 2021 [10]. % Tests performed = (Total Tests/Total Population) * 100). The data on various types of income for each country was obtained from the World Bank online site [45]. [(1)]

[1] **Table 1: Excessive SARS-CoV-2 testing is indicated by percentage values above 100 (SARS-CoV-2 tests performed more than the actual population)**

[2]

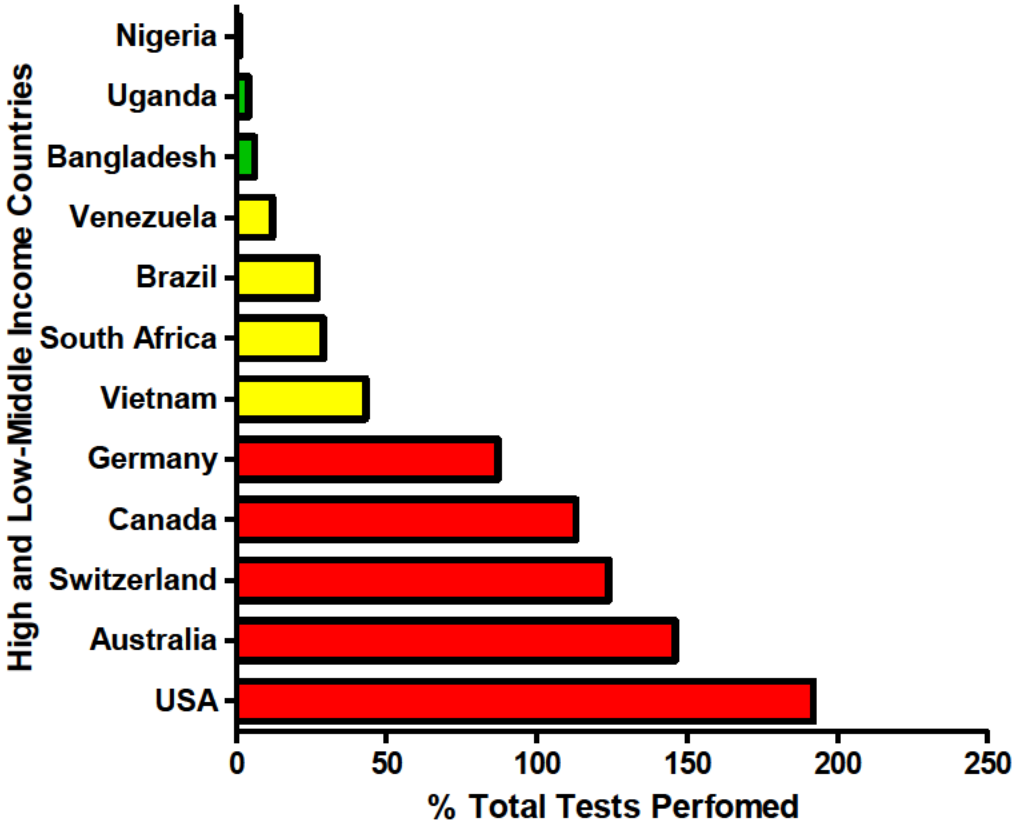

Figure 2: The number of SARS-CoV-2 tests (%) performed in high-income and middle-low-income countries and these samples represents counties of each income group. High-income countries are highlighted in red, middle-income countries are highlighted in yellow, and low-income countries are highlighted in green [10].

[2]**Figure 2: High-income countries (USA, Switzerland, Australia, Canada, Germany). Middle-income countries (Venezuela, Vietnam, South Africa, Brazil). Low-income countries (Bangladesh, Uganda, Nigeria)**

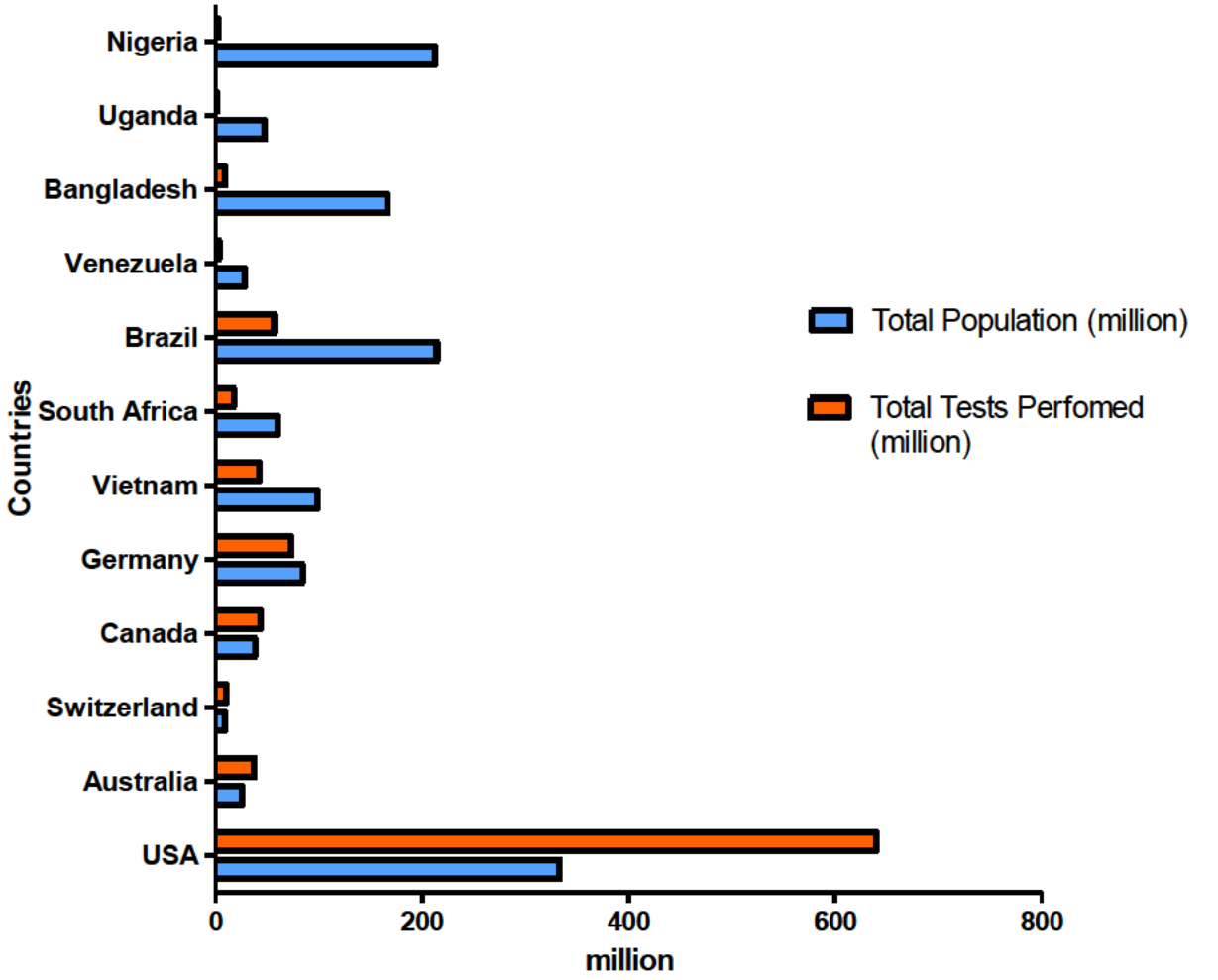

Figure 3: Comparison between the total population size (million) and the total number of SARS-CoV-2 tests performed (million) in each country [10].[3]

[3]**Figure 3: High-income countries (USA, Switzerland, Australia, Canada, Germany). Middle-income countries (Venezuela, Vietnam, South Africa, Brazil). Low-income countries (Bangladesh, Uganda, Nigeria)**

Possible cost-effective strategies to increase testing capability in LMICs

The management of SARS-CoV-2 infection cases entails early detection of the virus and prompt isolation as a result, which will aid in the prevention and control of virus spread [46]. Using cost-effective approaches such as saliva instead of oropharyngeal or nasopharyngeal swabs, sample pooling, testing high-priority groups and using antigen rapid tests can help to increase the number of mass testing in LMICs.

Saliva sample for SARS-CoV-2 infection testing

The recommended sample type for SARS-CoV-2 detection is nasopharyngeal and/ or oropharyngeal swabs [47]. The problem with nasopharyngeal and oropharyngeal swabs is that it makes patients uncomfortable, forcing them to cough during sample collection and exposing health care workers to the high risk of infection. Furthermore, nasopharyngeal and oropharyngeal swabs are expensive because the sample has to be collected by trained health care personnel wearing PPE [48]. Therefore, the nasopharyngeal and oropharyngeal swab is not an ideal sample to utilize for monitoring SARS-CoV-2 load. Therefore, saliva could be used as an alternative sample for SARS-CoV-2 testing and viral load monitoring, due to its numerous advantages [49].

Saliva is a transparent biofluid generated by the salivary glands that clean and protects the oral cavity, has antibacterial properties, and aids in food digestion [50]. Angiotensin-converting enzyme 2 (ACE-2) has been identified as the principal host cell receptor of SARS-CoV-2, and it is thought to play a key role in the virus's entry into the cell and subsequent infection. The ACE-2 receptor is highly expressed in the salivary gland and oral mucosa, [50] ACE2-positive cells in the salivary glands are likely to be SARS-CoV-2 target cells [52]. Furthermore, the presence of SARS-CoV-2 in saliva could be due to the mixing of upper and lower respiratory tract fluid that conveys the virus to the saliva. These findings imply that the salivary gland and oral mucosa could be a high-risk source site for SARS-CoV-2 infection [52]. Hence, this is what makes saliva a suitable specimen for testing SARS-CoV-2 infection.

Saliva could be utilized as a diagnostic sample for detecting SARS-CoV-2 and monitoring viral load [53]. Patients collect their own samples, which has several benefits, including the elimination of health care staff and the elimination of PPE for sample collection. The time, cost, and risk of viral transmission associated with sample collection are reduced, allowing for increased SARS-CoV-2 mass testing [53, 54]. Furthermore, saliva can be utilized efficiently in large organizations such as universities since PPE is not required, and this could help to lower the danger of viral transmission. Hence, the addition of saliva testing for SARS-Cov-2 infection will allow universities to test thousands of students and staff, with the aim that the results will aid in keeping campuses safe. As a result, saliva testing, in addition to wearing a face mask and maintaining social distance, is an innovative option [55]. However, less attention has been given to its potential usefulness in testing and monitoring for SARS-CoV-2 infection [54].

Sample pooling

The gold standard for diagnosing SARS-CoV-2 infection is reverse-transcription polymerase chain reaction (RT–PCR), a molecular method [56]. Real-time PCR is precise, but it is expensive to test each individual regularly [57]. Therefore, high prices limit affordability for many people, particularly in LMICs. The cost savings can be achieved by pooling samples [57, 58].

The principle of sample pooling allows multiple samples to be mixed and tested as a single sample [59]. When using a pooling method and the pooled test result is negative, each batch component is treated as if it were analyzed separately. Individual testing is required only when the pool test results are positive [59]. Sample pooling testing should be recommended for asymptomatic suspected cases, excluding those who are symptomatic [60]. This method is advantageous because it is cost-effective and allows for increased mass testing for SARS-CoV-2 without compromising testing accuracy or wasting consumables such as analytical reagents and extraction kits [61]. As a consequence, this technique improves testing efficiency by reducing the backlog of SARS-CoV-2 pending tests while also enhancing diagnostic turnaround time, which is one of the most important elements in managing and controlling the SARS-CoV-2 outbreak [62]. The pooling technique will be extremely advantageous in a laboratory with limited resources because this type of testing is more feasible and less expensive for mass screening in a large community [63].

Prioritized groups for testing of SARS-CoV-2 infection

It is critical to have a quick and accurate strategy for detecting and controlling SARS-CoV-2 outbreaks in communities and hospitals in LMICs [64]. In LMICs, prioritizing certain individuals for testing of SARS-CoV-2 infection should be considered. This testing strategy will help to accommodate the countries with limited resources by prioritizing individuals according to their categories of urgent clinical need while trying to reduce a backlog of pending testing [65]. Testing becomes the highest priority when it contributes to improving clinical outcomes and decreasing the transmission rate of the virus [66].

When prioritizing groups, the most important factors to consider are the size of each group, the number of tests needed, and the number of tests available. Hence, the most critical groups should be tested first. As testing becomes more generally available, it should be gradually spread to other groups based on their priorities. Additionally, those who tested positive for SARS-CoV-2 infection will need to undergo further testing [65].

A list of priority groups for SARS-CoV-2 testing in the private and public sectors is as follows: (i) Symptomatic patients, regardless of their age or underlying health issues, should be given the highest priority to reduce the risk of nosocomial transmission and protect health care staff and the general public. (ii) People who had contact with people who had tested positive for SARS-CoV-2 infection, whether asymptomatic or symptomatic, in order to quickly identify patients at high risk of complications and ensuring that the required precautions are taken. (iii) SARS-CoV-2 testing should also be prioritized for healthcare workers, frontline responders, essential critical infrastructure workers, miners, travelers, people going for surgery, testing pregnant women who are admitted at the labor ward and post-mortem testing, regardless of whether they are asymptomatic, to prevent a possible spread in the community and at work. (iv) If resources are available, testing for non-essential workers may be permitted [67, 68]. The most important thing to note is that healthy people who have not been tested should continue to practice social distance and wearing masks as recommended by their local and state health authorities [69, 70].

Antigen rapid test as a screening test for SARS-CoV-2 infection in LMIC

As the world continues to wrestle with SARS-CoV-2 infections, the number of cases in LMIC are increasing, causing national economies to lock down and putting further strain on already struggling economies [71]. As a result, the antigen rapid test can be used as alternative strategy for SARS-CoV-2 infection in LMIC. Antigen rapid tests have the advantage of providing results in 15–30 minutes instead of in hours or days, allowing mass testing to be increased, especially in LMICs with limited laboratory facilities or qualified health professionals to do molecular (PCR) tests [72]. The antigen rapid test allows healthcare workers to quickly identify individuals who are infected with SARS-CoV-2, so they be isolated and treated while their contacts are tracked to prevent the virus from spreading to their families and communities. In this case of a SARS-CoV-2 outbreak, where the test turnaround time is crucial, antigen rapid tests play an important role in delivering early results [73].

While governments are increasingly relying on less expensive antigen rapid tests to increase SARS CoV-2 infection testing coverage, however, the test may have low sensitivity [74]. It's critical to confirm an antigen test result with a PCR test, especially if the result of the antigen rapid test contradicts the clinical setting. Therefore, PCR tests remain the gold standard, and their value remains high [73, 75]. To be noted antigen rapid tests are typically used on symptomatic individuals since they perform best in symptomatic individuals and within a particular number of days of symptom onset [74]. By adopting this alternative strategy for mass testing of SARS-CoV-2 infection, LMICs can spend less money on diagnostics and more money on essential medical equipment for hospitals treating SARS-CoV-2 infected patients, resulting in more lives saved [72].

Conclusion and implication for future research

In conclusion, as the number of reported cases rises, the pandemic's long-term effect on individuals and populations in LMICs remains unknown. Moreover, the provision of a specific, effective vaccine to the people in LMICs is still a challenge. With an ongoing, unprecedented outbreak of SARS-CoV-2, the importance of laboratory detection of human coronavirus infections has been emphasized around the world in order to prevent the spread of the infection and properly treat

those individuals who have a serious infection. However, due to weak health systems and poverty, LMICs are finding it difficult to manage the SARS-CoV-2 outbreak. This paper highlights the importance of developing alternative strategies for SARS-CoV-2 mass testing that are simple and cost-effective in a resource-constrained setting, and the summary is illustrated in Figure 4.

For future, the goal is to evaluate alternative methods that are simple, cheaper, with fast turn-around time and have a high throughput for a resource-constrained laboratories, so that they can be implemented to facilitate mass testing for SARS-CoV-2 infection in LMICs. There should be common pricing standard for SARS-CoV-2 kits in LMIC, which should be implemented.

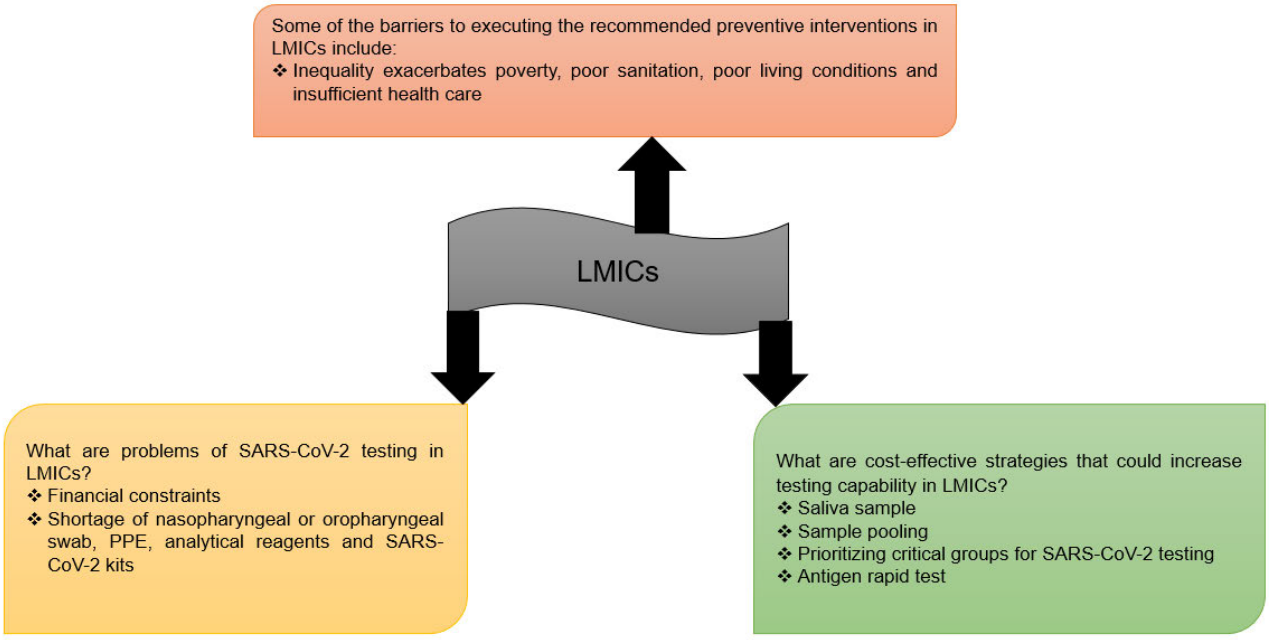

Figure 4: The summary of the review paper highlights the challenges that LMICs have when it comes to SARS-CoV-2 testing, as well as possible cost-effective strategies for increasing mass testing

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

There are no competing interests declared by the authors.

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Availability of data and materials

All the data reported in this review was retrieved from the publicly available original sources.

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Author's contribution

All the authors have contributed to manuscript conceptualization, review and editing in preparation for submission. All the named authors have read and approved the manuscript for submission.

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CHAPTER THREE

Profile of SARS-CoV-2 infected subpopulation of South African individuals

Countries all over the world are struggling with how to contain the SARS-CoV-2 infection because the virus mutates quickly and some variants are highly contagious. This is what makes it difficult for all countries to stop the virus from spreading. Moreover, there is no cure for SARS-CoV-2 infection. To keep everyone up to date on COVID-19 statistics, there is publicly available data from an online resource demonstrating the daily epidemiological trajectory of the COVID-19 pandemic for countries around the world. The problem with this publicly available COVID-19 online statistic system is that it is mainly based on symptomatic screening and the number of asymptomatic cases is not fully captured. Asymptomatic cases are of great concern because they are silent spreaders of the SARS-CoV-2 infection and unfortunately, asymptomatic carriers are given less attention when it comes to testing for SARS-CoV-2 infection. This is quite concerning for LMICs, which are already undertesting for SARS-CoV-2 infection compared to HICs due to financial constraints and limited resources. Furthermore, there is also limited information on the comparison of prevalence and epidemiological data between asymptomatic cases and symptomatic cases, particularly for African countries categorized as LMICs. As a result, determining the frequency of asymptomatic versus symptomatic SARS-CoV-2 cases is critical for an effective public health response to SARS-CoV-2 infection. A South African study aimed at analyzing such data was conducted and is currently under review in an article titled **"Epidemiology of symptomatic and asymptomatic SARS-CoV-2 infections in a South African population"** as presented below.

Duma Z, Ramsuran V, Chuturgoon AA, Edward VA, Naidoo P, Mkhize-Kwitshana ZL. **Epidemiology of symptomatic and asymptomatic SARS-CoV-2 infections in a South African population (Under Review). Manuscript Id No: id-2023-00052e (Impactor factor= 5.084)**

[Presented as per ACS Infectious Disease Journal format requirements]

Manuscript Title: Epidemiology of symptomatic and asymptomatic SARS-CoV-2 infections in a South African population

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Abstract

Background: The SARS-CoV-2 outbreak became a major public health emergency, affecting healthcare systems worldwide. Asymptomatic carriers are thought to be of great concern due to their role in the ongoing pandemic.

Aim: To assess and describe the demographic and clinical profiles of SARS-CoV-2 infected individuals, both asymptomatic and symptomatic, in a South African population.

Method: A retrospective review of electronic clinical records of 1310 patients with SARS-CoV-2 diagnosed during the first (2020) and second (2021) waves, was performed to collect data on demographic, clinical, and laboratory records. After 1310 patient records were evaluated for eligibility, 1146 patients met the requirements for the study.

Results: The majority of SARS-CoV-2 infected patients were asymptomatic (68%). Female patients had a higher prevalence of asymptomatic (62.0% versus 37.9%) and symptomatic (56.9% versus 43.0%) infections compared to males. Asymptomatic cases were more prevalent compared to symptomatic cases in the Eastern Cape (67% versus 50%) and Gauteng (8.9% versus 6.7%) ($p<0.0001$). The <18 years group had the lowest rate of infection when compared to the >18 years group (3% vs 97%; $p<0.0001$). The most prevalent comorbidities among infected patients were hypertension (23.4%), diabetes (12.3%), and chronic inflammatory pulmonary disease (10.3%). Cough (56%) and fever (55%) were most common in symptomatic patients.

Conclusion

This study found a higher proportion of asymptomatic SARS-CoV-2 than symptomatic cases. Asymptomatic cases need to be timeously identified to prevent SARS-CoV-2 transmission. One way to accomplish this is by developing cost-effective, faster, and more accurate alternative methods to boost testing capacity for SARS-CoV-2 infection. Most importantly this study represents one of the few COVID-19 epidemiological reports within Southern Africa.

Keywords: SARS-CoV-2, asymptomatic and symptomatic, clinical profile, demographic profile, South Africa

Introduction

The coronavirus disease 2019 (COVID-19) was first detected in Wuhan, China, and has since spread throughout the world.[1] Globally there have been challenges to contain the highly contagious and rapidly mutating Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2).[2] On March 11, 2020, the World Health Organization (WHO) declared COVID-19 a pandemic.[3, 4] The virus has spread to over 180 countries, with over 237 million confirmed coronavirus cases worldwide[5], resulting in over 4.8 million deaths.[6] In sub-Saharan Africa, 8.4 million coronavirus infections were confirmed, resulting in over 213 000 deaths[7], with South Africa having the highest number of SARS-CoV-2 infection cases to date, with a total of 2.9 million people being infected and over 88 000 recorded deaths.[6]

South Africa declared the first confirmed case of SARS-CoV-2 infection on March 5, 2020, with the first known patient being a male citizen who tested positive after returning from Italy.[8] This male patient had symptoms such as fever, headache, fatigue, sore throat, and cough.[8] To date, South Africa has remained among the top 50 countries with the highest rates of SARS-CoV-2 infection worldwide.[6]

Several studies indicated that SARS-CoV-2 transmit through respiratory droplets between humans.[9-11] The estimated average incubation period of SARS-CoV-2 infection is approximately 5 days.[12] The SARS-CoV-2 infection has been shown to have a clinical spectrum ranging from asymptomatic infection to mild upper respiratory tract illness to severe interstitial pneumonia with respiratory failure and even death.[13-15] Alarmingly, asymptomatic and mildly symptomatic COVID-19 infections frequently go unrecognized because the majority of infected individuals are not critically ill enough to seek medical attention, and even screening methods such as temperature checks cannot detect those individuals.[16] As a result, asymptomatic transmission is a major source of concern for the SARS-CoV-2 community spread.[17] Asymptomatic people can spread the virus rapidly, and the rise of these SARS-CoV-2 silent spreaders has complicated the control of the pandemic.[18] Studies have reported that the world has a large number of asymptomatic infections indicating that the potential of SARS-CoV-2 infection spreading asymptomatically may be higher

than expected.[19-21] This study aimed to assess and describe the demographic and clinical profiles of asymptomatic and symptomatic SARS-CoV-2 infected South Africans.

Methods

Study participants

This was a retrospective review of the medical records of 1310 patients from the Eastern Cape, KwaZulu Natal, Gauteng, and Western Cape provinces of South Africa who had SARS-CoV-2 infection confirmed and diagnosed at the Global Health Innovation (GHI) laboratory. The gold standard method of a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay and nasopharyngeal swab samples was used to confirm SARS-CoV-2 positivity in all patients.

Ethical Consideration

The study was conducted after receiving ethical approval from the University of KwaZulu Natal Biomedical Research Ethics Committee (BREC/00003671/2021) and permission from the GHI laboratory to use the medical records of SARS-CoV-2-infected patients.

Data collection

Patient data were collected using standardized collection forms and captured electronically to extract demographic, clinical and laboratory information. The data was collected in 2020-2021, during the first (May to August 2020) and second waves (December to January 2021) of the COVID-19 outbreak. The medical records of SARS-CoV-2-infected patients from the GHI laboratory were used for this study because it was not possible to conduct a community-based survey during this period since the country was in lockdown to curb the spread of the virus. The research assistants had access and collected the electronic medical records of the SARS-CoV-2-infected patients from the GHI laboratory standardized collection forms, which they used to electronically collect patient demographic, clinical, and laboratory data for this epidemiology study. The electronic medical records of the SARS-CoV-2-infected patients were recorded on the

Microsoft Excel spreadsheet. The following categories of variables was extracted from the patient electronic medical record file: (i) demographic information included age, sex, race, ethnicity and the provinces in which the patients lived; (ii) comorbidities conditions; and (iii) signs and symptoms reported at the time of presentation including cough, fever, body aches, sore throat, fatigue/weakness, chills, loss of smell, shortness of breath, redness of the eye, nausea/vomiting and diarrhea. Patients who presented clinical signs and symptoms for SARS-CoV-2 infection were classified as symptomatic cases. Asymptomatic cases were classified as individuals infected with SARS-CoV-2 who had no clinical signs and symptoms. The principal researcher independently verified whether each patient's information on the laboratory standardized data collection form matches that on the electronic medical record to ensure that the study's data was accurate.

Selection criteria

After 1310 patient records were evaluated for eligibility, 1146 patients met the requirements for the study and were included (Figure 1). All patients who were SARS-CoV-2 infected and with readily available and completed demographic, clinical, and laboratory records were included. Patients without complete clinical records or demographic information were excluded from this study.

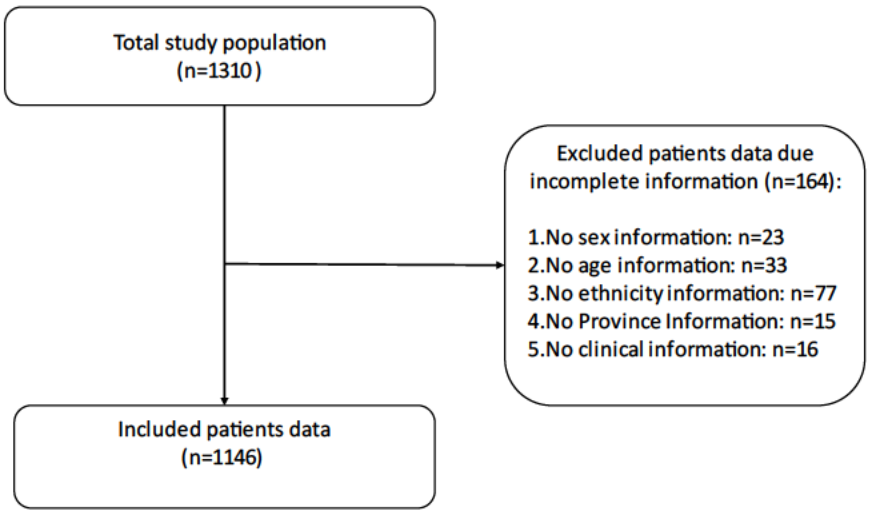

Figure 1: Flow diagram illustrating the eligibility criteria for selecting SARS-CoV-2 patients for the present study.

Statistical Analysis

All patient data were entered into Microsoft Excel. The GraphPad Prism 5 statistical software package (GraphPad Software, Inc., San Diego, CA) was used to analyze all data. All the data were analyzed using the Chi-squared ($\chi 2$) test to compare the demographic and clinical profile of asymptomatic and symptomatic cases by age, province, ethnicity, and comorbidities conditions, with the exception of sex which was analyzed using the Fishers exact test. A $p<0.05$ was considered statistically significant. All descriptive data, including demographic and clinical presentation characteristics, are presented as numbers (n) and percentages (%).

Results

Demographic profiling for asymptomatic and symptomatic SARS-CoV-2 infected patients

A total of 1146 patients who were diagnosed with SARS-CoV-2 infection were enrolled in this study. The majority of SARS-CoV-2 infected patients (68%) were asymptomatic, while (32%) were symptomatic. The median age for asymptomatic and symptomatic cases ranged between 37-

38 years. Furthermore, compared to males, female patients had a higher prevalence of asymptomatic (62.0% versus 37.9%) and symptomatic (56.9% versus 43.0%) infections (Table 1).

The Black African population were overrepresented in both asymptomatic and symptomatic cases, with an overall prevalence 92.5%. There was a significant difference between asymptomatic and symptomatic cases in terms of ethnicity (**p < 0.000**1). The Eastern Cape Province had the highest infection rate (62%), followed by KwaZulu Natal (27.4%), Gauteng (8.2%) and Western Cape (2.7%). More specifically, asymptomatic cases were more prevalent compared to symptomatic cases in the Eastern Cape (67% versus 50%) and Gauteng (8.9% versus 6.7%). The opposite trend was noted for KwaZulu Natal (21.7% versus 39.2%) and Western Cape (2.1% versus 4.0%) (**p<0.0001**) (Table 1).

Table 1: Demographic characteristics of asymptomatic and symptomatic SARS-CoV-2 patients.

Characteristics	**Total population n= 1146 (n, %)**	**Asymptomatic cases n=774 (68%) (n, %)**	**Symptomatic cases n=372 (32%) (n, %)**	**p-value**
Mean age in years		38 (30-48)	37 (30-46)	0.3525
Gender				
Female	692 (60.3%)	480 (62.0%)	212 (56.9%)	0.1033
Male	454 (39.6%)	294 (37.9%)	160 (43.0%)	
Ethnicity				
Black	1061 (92.5%)	734 (94.8%)	327 (87.9%)	**p < 0.0001**
Indian	56 (4.8%)	23 (3.0%)	33 (8.8%)	
White	11 (0.9%)	4 (0.5%)	7 (1.9%)	
Mixed race	14 (1.2%)	9 (1.1%)	5 (1.3%)	
Provinces				

Eastern Cape	707 (62%)	521 (67%)	186 (50%)	**p < 0.0001**
KwaZulu Natal	314 (27.4%)	168 (21.7%)	146 (39.2%)	
Gauteng	94 (8.2%)	69 (8.9%)	25 (6.7%)	
Western Cape	31 (2.7%)	16 (2.1%)	15 (4.0%)	

Notes: A $p<0.05$ was considered statistically significant. Abbreviations: n, number; %, percentage.

Age distribution between symptomatic and asymptomatic patients

The 30-39 year age group had the highest positivity rate for SARS-CoV-2 infections, with an overall infection rate of 33.3%, followed by the 40-49 year age group, with an overall infection rate of 23.4%, and the 18-29 year age group which had an overall infection rate of 19% ($p = 0.0019$). In addition, the prevalence of asymptomatic and symptomatic infections in the aforementioned age groups were similar (Figure 2A). Children under the age of 18 years had the lowest rate of SARS-CoV-2 infection (overall prevalence = 3%; asymptomatic = 5% and symptomatic = 1%) when compared to the >18 years age group (overall prevalence = 97%; asymptomatic = 95% and symptomatic = 99%) (**p<0.0001**). In addition, the majority of cases in the < 18 years age group were asymptomatic, whereas most cases in > 18 years age group were symptomatic (Figure 2B).

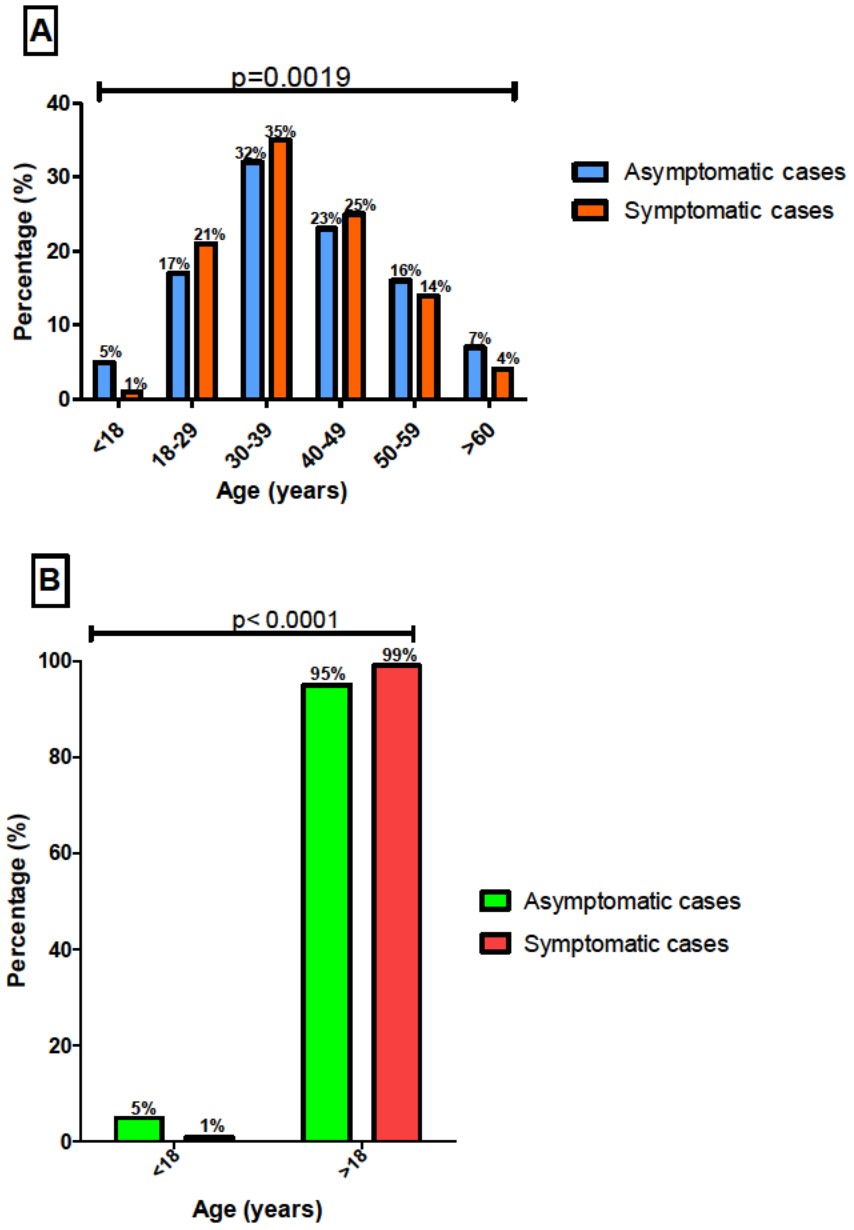

Figure 2: The distribution of asymptomatic and symptomatic SARS-CoV-2 infections between the different categorized age groups (A), and between children (<18 years) and adults (>18 years) (B). With regards to Figure B, irrespective of asymptomatic and symptomatic infection, the overall SARS-CoV-2 prevalence in the <18 years age group was 3% (5% of asymptomatic cases + 1% of symptomatic cases / 2) and 97% (95% of asymptomatic cases + 99% of symptomatic cases / 2) in the >18 years age group.

Comorbidities among asymptomatic and symptomatic patients

In summary, the most prevalent comorbidities among SARS-CoV-2-infected patients were hypertension (23.4%), diabetes (12.3%), and chronic inflammatory pulmonary disease (COPD) (10.3%). Around 5.8% had asthma, while 3.4% and 2.6% were infected with HIV and tuberculosis, respectively. Obesity, sinusitis and renal failure prevalence ranged between 1.0% - 1.6%. Cancer 0.5%, psoriasis 0.08%, and epilepsy 0.08% were among the least prevalent comorbidities identified in this study population. In addition, all the above-mentioned comorbidities were more prevalent in symptomatic patients compared to the asymptomatic group (Table 2).

Table 2: The prevalence of comorbidities among asymptomatic and symptomatic SARS-CoV-2 infected patients

Characteristics	**Total Population n= 1146 (n, %)**	**Asymptomatic cases n=774 (68%) (n, %)**	**Symptomatic cases n=372 (32%) (n, %)**	**p-value**
Comorbidities				
Hypertension	269 (23.4%)	169 (21.8%)	100 (26.8%)	
Diabetes	141 (12.3%)	81 (10.4%)	60 (16.1%)	
Chronic inflammatory lung disease	118 (10.3%)	72 (9.3%)	46 (12.4%)	
Asthma	67 (5.8%)	39 (5.0%)	28 (7.5%)	p=0.0549
HIV	39 (3.4%)	22 (2.8%)	17 (4.5%)	
Tuberculosis	30 (2.6%)	16 (2.1%)	14 (3.7%)	
Obesity	18 (1.6%)	10 (1.2%)	8 (2.2%)	
Sinusitis	12 (1.0%)	5 (0.6%)	7 (1.8%)	
Renal failure	14 (1.2%)	3 (0.4%)	11 (3.0%)	
Cancer	6 (0.5%)	1 (0.1%)	5 (1.3%)	
Epilepsy	1 (0.08%)	0 (0%)	1 (0.3%)	
Psoriasis	1 (0.08%)	0 (0%)	1 (0.3%)	

Clinical symptoms and signs presented by patients infected with SARS-CoV-2

As shown in Figure 3, among the 372 symptomatic patients in the study population, cough 56% and fever 55% were the most SARS-CoV-2 associated common symptoms, followed by body aches 30%, sore throat 24%, and fatigue/weakness 23%, chills (20%), loss of smell (19%), shortness of breath (19%), and redness of eyes (11%). Diarrhea (7%) and nausea (8%), however, were the least common symptoms (Figure 3).

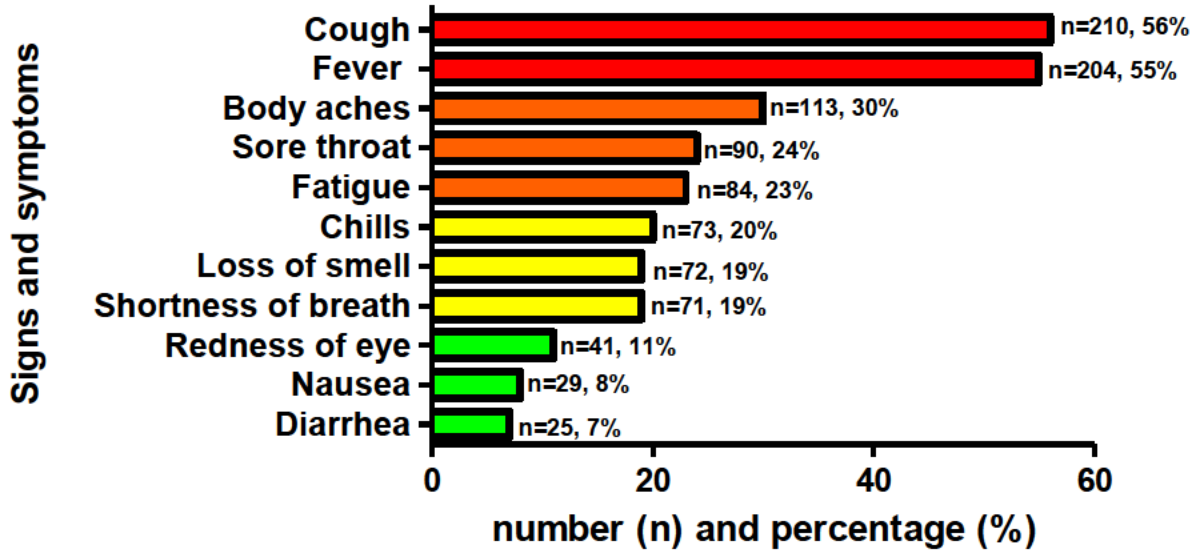

Figure 3: Clinical symptoms and signs among symptomatic SARS-COV-2 infected patients (n=372). The most common signs and symptoms with the highest percentage (≥50%) were presented in red, while the least common signs were presented in orange and yellow (≥ 15% and ≤ 30%) and green (<15%).

Discussion

The present study aimed to describe the demographic and clinical profiles of asymptomatic and symptomatic SARS-CoV-2 infected patients in South Africa. The results showed that more than half of the study population (68%) that were infected with SARS-CoV-2 were asymptomatic. This finding highlight that the risk of SARS-CoV-2 infection spreading asymptomatically within the community may be higher than expected, especially as public life slowly returns to normal. Studies from Wuhan, China[20] and Lusaka, Zambia[22], reported similar findings to the current study, suggesting that asymptomatic cases are a silent spreader of SARS-CoV-2 infection and also significantly contribute to the spread of SARS-CoV-2 infection in the community. As a result, this study emphasizes the importance of increasing testing capacity for increased detection of asymptomatic individuals. This could have a positive impact on the community because mass testing is the only strategy that can help control the spread of this non-curable, contagious, and mutating virus within the community. Consequently, this could be accomplished by developing cheaper, faster, and more accurate alternative testing strategies.

SARS-CoV-2 can infect individuals of any age and sex. Based on the results there was no significant difference in symptomatic and asymptomatic infection among males and females ($p=0.1033$). A higher proportion of the females (60.3%) was infected compared to males (39.6%) in this study. One of the factors which could have contributed to the higher number of females is the general composition of the SA population that is made of 30.48 million females and 29.56 million males.[23] In addition, at the time of the study, the female made up the majority of the health care workers (HCW) and more PCR tests were performed among HCW thus increasing the number of cases detected. Another possible explanation for the higher number of female cases could be that females exhibit higher health-seeking behavior than males, as indicated by one study reporting on sex patterns in health information seeking behavior.[24] Furthermore, females are more likely to participate in caring or tendering activities that expose them to crowded public spaces. For example, females make up a larger proportion of social grant recipients than men, particularly in South Africa, and they could contract SARS-CoV-2 while queuing for monthly payments.[25] On average, women spend more time than men doing unpaid care work such as daycare for children, elders, and sick people at home, and this is particularly the case during the SARS-CoV-2

outbreak.[26-28] The results of the present study are consistence with other studies findings regarding sex differences in SARS-CoV-2-infected patients. The findings suggest that males could be undertested for SARS-CoV-2 infection, which is quite concerning given that males tend to have higher expression of angiotensin-converting enzyme-2 (ACE 2) receptors for SARS-CoV-2, making males more susceptible to SARS-CoV-2 infection than females.[29, 30] As a result, epidemiology studies have identified this as one of the contributing factors to the high mortality rate among male cases.[30] It is critical to teach males to pay attention to their health early on and to address the issue of testing for SARS-CoV-2 infection earlier, as this may help to reduce transmission and death rates in the community.

The distribution frequency of SARS-CoV-2 infection was determined across the age groups of the study patients. The SARS-CoV-2 infection rate was significantly low among children (<18 years old), however, the majority of children infected with SARS-CoV-2 were asymptomatic. The low positive rate of SARS-CoV-2 infection among children is in line with previous reports that children are less susceptible to SARS-CoV-2 infection.[31] One of the reasons suggested is that children have a stronger innate immune response, with a higher number of natural killer (NK) cells, which serves as the first-line defense against SARS-CoV-2.[32] The infection rate of SARS-CoV-2 was low among older adults (>60 years old), and the majority of SARS-CoV-2 infected older adults were asymptomatic. The overall positivity rates were highest among those aged 30-39 years (33.3%) and 40-49 years (23.4%), followed by those aged 18-29 years (17.8%) and 50-59 years (15.3%). Infected people between the ages of 18 and 49 were mostly symptomatic, while those between the ages of 50 and 59 were mostly asymptomatic. This finding could be reflective of South Africa population being young adults (18-59 years) and this age group accounts for a large percentage of working age with high mobility and social interactions which could be a possible reason for the higher positivity rate in South Africa.[33] Previous studies reported a rapid increase in SARS-CoV-2 cases among young adults worldwide.[34, 35] Furthermore, the use of diagnostic tests for SARS-CoV-2 has been found to be significantly higher in this population between the ages of 18 and 65 years, which makes up about 75% of those infected globally.[36] These results demonstrate the necessity of early detection and infection control of SARS-CoV-2 in this working class for a positive economic impact. However, although the population of South Africa is generally young,

older age is still regarded as a risk factor for SARS-CoV-2 severity and death rate. More attention should be paid to this older age group by continuing to prioritize diagnostic testing for SARS-CoV-2 infection in this age group.

As shown in Table 1, for demographic profiling among ethnic groups for both asymptomatic and symptomatic cases, the Black African group had the highest positivity rate for SARS-CoV-2 infection (92.5%) compared to other ethnic groups, with the White group (0.9%) having the lowest positive rate. The following factors may have contributed to the highest positive rate in the Black ethnic group: most people in this ethnic group live in multigenerational households with poor conditions such as clustered and crowded houses and communities, limited access to quality water, and sanitation which may have resulted in an increase in viral transmission within families and the community.[37] When the SARS-CoV-2 infection rate in the four South African provinces was analyzed for this study. Among the four provinces in South Africa, the Eastern Cape had the highest overall infection rate (62%). The Eastern Cape province may have been hit harder by the SARS-CoV-2 outbreak than the other provinces because it is one of the poorest provinces in South Africa, with the majority of the population living in extreme poverty.[38]

It has been reported that people with underlying comorbidities are more susceptible to SARS-CoV-2 infection, and the prognosis for people with underlying comorbidities who are infected with SARS-CoV-2 is extremely poor. Based on the clinical profile results of SARS-CoV-2 infected individuals with comorbidities coexisting conditions, the most common comorbidities in this study population for both asymptomatic and symptomatic cases were hypertension (23.4%), diabetes (12.3%), and COPD (10.3%). Taken into consideration the frequency of symptomatic cases of SARS-CoV-2 was higher than that of asymptomatic cases among patients with comorbidities (Table 2). These findings are consistence with the most recent data reported by the Center for Disease Control in January 2022, in which the most common underlying comorbidities associated with an increased risk of being infected with SARS-CoV-2 and increasing the severity of COVID-19 are heart conditions, lung conditions, diabetes or obesity, and a weakened immune system.[39] Although widespread vaccination has reduced the severity and mortality rate of SARS-CoV-2 infection, underlying comorbidities remain to be the most significant risk factor for SARS-CoV-2

infection because vaccine effectiveness wears out quickly in these populations.[40, 41] Therefore, individuals with comorbidities should take extra precautions to avoid contracting SARS-CoV-2.

Based on the clinical symptoms and signs presented by patients infected with SARS-CoV-2, cough and fever were the most common signs and symptoms in this study population of 372 symptomatic patients infected with SARS-CoV-2 infection (56% and 55%, respectively). The most common signs and symptoms observed in this South African population matched those reported in other studies worldwide.[42, 43] The least common signs and symptoms were diarrhea 25 (7%) and nausea 29 (8%). Patients in Wuhan, China and Europe with SARS-CoV-2 had a low diarrhea rate of less than 10%, according to Guan *et al*. 2020; Haung *et al*. 2020; Xu *et al*. 2020; Chen *et al*. 2020; and Spiteri *et al*. 2020; and the current study found similar results.[44-48] According to studies that investigated the association between COVID-19 and nausea, these symptoms were not common for SARS-CoV-2 infection [47] and the present study had a similar finding.

Limitation

This study had a number of limitations. Firstly, our data lacked information on the severity of the COVID-19 disease for patients with comorbidities and clinical outcomes for all patients, which is why it was excluded from the analysis. Second, this retrospective study was biased because data was collected from patients who presented to a testing facility and not representative of the entire South African population. As a result, the generalizability of our findings was limited. Lastly, there was a lack of information regarding the time point of the infection for asymptomatic patients, including whether they developed symptoms later on or stayed asymptomatic the entire time.

Conclusion

The current study found a high proportion of asymptomatic SARS-CoV-2 cases compared to symptomatic cases, prompting concern because this suggest that a significant number of people are unknowingly spreading the uncurable SARS-CoV-2 infection to the community. The findings highlight the need for policymakers and health programmers to develop interventions and strategies for the early detection of asymptomatic cases to prevent SARS-CoV-2 transmission.

Therefore, this study emphasizes the importance of developing cost-effective, faster, and more accurate alternative methods for mass-testing for SARS-CoV-2 infection in the community, because testing is still the most important factor in controlling virus transmission in the community. Most importantly, our data showed that the findings consistent with data from other parts of the world in terms of the frequency of male to female, most common comorbidities, and clinical symptoms. The importance of this study is in its contribution to scientific knowledge as one of the few COVID-19 epidemiological reports from Southern Africa.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions: Z.L.M.-K., A.A.C. and V.R.—supervision; Z.D., Z.L.M.-K., A.A.C., V.R., V.A.E., and P.N.—manuscript conceptualization; Z.D., Z.L.M.-K., A.A.C., V.R., V.A.E., and P.N.—visualization; Z.L.M.-K., A.A.C., V.R., and Z.D.—methodology; Z.D., Z.L.M.-K., A.A.C., V.R., V.A.E., and P.N.—formal analysis; Z.D.—original draft preparation; V.A.E—granted permission for the use of COVID-19 patients data from the GHI laboratory; Z.L.M.-K., A.A.C., V.R., V.A.E., and P.N.—writing: review and editing. All authors have read and agreed to the published version of the manuscript.

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CHAPTER FOUR

Testing methodologies for SARS-CoV-2 for resource-limited settings

Limited resources in the LMICs have hindered mass testing for SARS-CoV-2 infection. As a result, the above could be one of the factors contributing to LMICs undertesting when compared to high-income countries (HICs). To date, diagnostic testing remains critical for SARS-CoV-2 pandemic monitoring. Therefore, the routine testing of people for SASR-CoV-2 infection using accurate, fast and cost-effecting methods is important. The challenge is that more frequent mass testing for SARS-CoV-2 infection will incur enormous economic costs that most LMICs will be unable to bear, especially given their already facing serious financial constraints. The burden of mass SARS-CoV-2 testing can be reduced by developing more cost-effective, accurate, and faster diagnostic methods. Most importantly, these low-cost alternatives must meet WHO quality standards. The rationale is that increasing detection capacity while limiting the risk of reagent shortages, as well as detecting SARS-CoV-2 infected individuals early, is the only effective way to prevent the virus from spreading in the community, especially now that most governments in various countries have decided to ease lockdown restrictions and reopen the economy. This prompted the analysis of various testing methods and the work has been published as presented below in the manuscript titled: **"Evaluation of Various Alternative Economical and High Throughput SARS-CoV-2 Testing Methods within Resource-Limited Settings". The published PDF version is shown below under Appendix B.**

Duma Z, Ramsuran V, Chuturgoon AA, Edward VA, Naidoo P, Mkhize-Kwitshana ZL. Evaluation of Various Alternative Economical and High Throughput SARS-CoV-2 Testing Methods within Resource-Limited Settings. Int. J. Mol. Sci. 2022, 23, 14350. https://doi.org/10.3390/ijms232214350. **(Impact factor = 6.208)**

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Manuscript title: Evaluation of various alternative economical and high throughput SARS-CoV-2 testing methods within resource-limited settings

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Abstract: The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak posed a challenge for diagnostic laboratories worldwide, with low-middle income countries (LMICs) being the most affected. The polymerase chain reaction (PCR) is the gold standard method for detecting SARS-CoV-2 infection. However, the challenge with this method is that it is expensive, which has resulted in under-testing for SARS-CoV-2 infection in many LMICs. Hence, this study aimed to compare and evaluate alternative methods for the mass testing of SARS-CoV-2 infection in laboratories with limited resources to identify cost-effective, faster, and accurate alternatives to the internationally approved kits. A total of 50 residual nasopharyngeal swab samples were used for evaluation and comparison between internationally approved kits (Thermo Fisher PureLink™ RNA Isolation Kit and Thermo Fisher TaqPath™ COVID-19 Assay Kit) and alternative methods (three RNA extraction and four commercial SARS-CoV-2 RT-PCR assay kits) in terms of the cost analysis, diagnostic accuracy, and turnaround time. In terms of performance, all of the alternative RNA extraction methods evaluated were comparable to the internationally approved kits but were more cost-effective (Lucigen QuickExtract™ RNA Extraction Kit, Bosphore EX-Tract Dry Swab RNA Solution and Sonicator method) and four commercial SARS-CoV-2 RT-PCR assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES^{TM} COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit) with a sensitivity range of 76–100% and specificity of 96–100%. The cost per sample was reduced by more than 50% when compared to internationally approved kits. When compared to the Thermo Fisher PureLink™ Kit and Thermo Fisher TaqPath™ COVID-19 Assay Kit, the alternative methods had a faster turnaround time, indicating that laboratories with limited resources may be able to process more samples in a day. The above-mentioned cost-effective, fast, and accurate evaluated alternative methods can be used in routine diagnostic laboratories with limited resources for mass testing for SARS-CoV-2 because these were comparable to the internationally approved kits, Thermo Fisher PureLink™ Kit and Thermo Fisher TaqPath™ COVID-19 Assay Kit. The implementation of alternative methods will be the most cost-effective option for testing SARS-CoV-2 infection in LMICs**.**

Keywords: SARS-CoV-2; diagnostic testing; low-middle income countries; resource-limited settings; alternative cost-effective and high throughput testing approaches

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (COVID-19) [1], has spread globally since its first rec-orded outbreak in Wuhan, China in December 2019 [2, 3]. As of 08 August 2022, there have been over 589 million confirmed cases and 5.4 million deaths worldwide [4].

Diagnostic testing remains critical in controlling the SARS-CoV-2 outbreaks, allowing patients to be cared for while also simultaneously providing decision-makers with critical information for test-trace isolation programs [5, 6]. Most countries experienced increased demand for SARS-CoV-2 diagnostic testing, with some countries unable to meet the demand. This is one of the major challenges especially in low-middle income countries (LMICs), where unstable health systems and reliance on global supply chains have frequently prevented people from accessing critical tests for detecting SARS-CoV-2 infection [7, 8]. In many LMICs, insufficient testing may have resulted in an underestimation of SARS-CoV-2 infections. What is concerning is that the pandemic's long-term impact on individuals and communities in LMICs remains uncertain as the number of confirmed cases continues to increase [9].

SARS-CoV-2 is an enveloped virus with a single positive-sense RNA genome [1]. Furthermore, the SARS-CoV-2 genome contains open reading frames (ORF1ab), which serve as the primary sites for viral transcription and replication [10-12]. It also contains four structural proteins namely the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins which contribute to the SARS-CoV-2 overall structure [13, 14]. SARS-CoV-2 infection can be detected using two different types of tests: real-time reverse transcription polymerase chain reaction (RT-PCR) and antigen rapid tests [15, 16]. Anti-gen rapid tests are less expensive and provide results faster than RT-PCR. However, anti-gen rapid tests, on the other hand, are less accurate in detecting SARS-CoV-2 infection, especially in asymptomatic individuals and those with low SARS-CoV-2 viral load [17-19]. According to a Cochrane systematic review of 22 antigen rapid test trials for detecting SARS-CoV-2 infection, the antigen rapid test showed an average sensitivity of 56.2% [20]. Furthermore, antigen rapid testing was found to have a higher risk of false negatives than molecular RT-PCR tests, with some evidence indicating false negative rates as high as 50%, therefore a confirmatory RT-PCR test is still recommended [20, 21].

The RT-PCR assay is the most accurate test for detecting SARS-CoV-2, hence it is regarded as a gold standard diagnostic procedure for diagnosing SARS-CoV-2 infection [22, 23]. The N gene, S gene, E gene and ORF1ab gene are the most tested target genes for SARS-CoV-2 infection using the RT-PCR assay [24, 25]. The RT-PCR assay has superior sensitivity and specificity in comparison to antigen and antibody rapid tests however it is highly specialized and expensive, especially for LMICs. This is partly due to a lack of local capacity in these countries to produce their analytical instrument and reagents for RT-PCR-based SARS-CoV-2 testing [8, 23]. As a result, mass testing for SARS-CoV-2 infection in LMICs poses a challenge. To note, high-income countries (HICs) test more samples daily to control the spread of the SARS-CoV-2 virus, whereas LMICs test fewer samples due to financial constraints [26]. It is, therefore, essential to evaluate the alternative methods for SARS-CoV-2 testing that are simple, cost-effective, and produce high throughput results in a short period of time in a laboratory within a resource-limited set-ting.

This study aimed to compare and evaluate whether alternative RNA extraction methods (Lucigen QuickExtract™ RNA Extraction kit, Bosphore EX-Tract Dry Swab RNA solution and Sonicator method) are cost-effective, faster and have clinical accuracy com-parable to an international approved kit (Thermo Fisher PureLink™ kit) and to also assess whether these extraction methods can be used interchangeably. Furthermore, the performance of commercially available RT-PCR SARS-CoV-2 assay kits (Nucleic Acid COVID-19 Test kit (SARS-CoV-2), abTES^{TM} COVID-19 qPCR I kit, PCL COVID19 Speedy RT-PCR kit and PCLMD nCoV one-step RT-PCR kit) was compared to the international approved kit (Thermo Fisher TaqPath™COVID-19 assay kit) in terms of sensitivity, specificity, costs, and turnaround times.

2. Results

In the first part of this study, the Thermo Fisher PureLink™ Kit was compared to three alternative RNA extraction methods. In the second part of this study, the Thermo Fisher TaqPath™ COVID-19 Assay Kit was compared to four alternative commercially available RT-PCR SARS-CoV-2 assay kits. The overall aim of this study was to assess and compare the clinical performance of

these methods as well as determine whether they could be used interchangeably to help increase the testing capacity for SARS-CoV-2 infection in LMICs.

2.1 Comparison of RNA Extraction Methods

The multiplex Thermo Fisher TaqPath™ COVID-19 Assay Kit was used to assess the RNA extraction efficiency of each extraction method. For the positive (infected) group, the efficiency of the RNA extraction methods was assessed by comparing the mean Ct values of the SARS-CoV-2 targeting genes (N, S, ORF1ab), and the internal control (MS2) between the alternative RNA extraction methods and the Thermo Fisher PureLink™ Kit. The efficiency of the RNA extraction methods was assessed for the control (uninfected) group by comparing the mean Ct values of the internal control (MS2) between the alternative RNA extraction methods and the Thermo Fisher PureLink™ Kit. The PCR was run in triplication for each sample for the purpose of method comparison, and the average Ct value result was used for each sample. The results were considered positive if the cycle threshold value (Ct value) for all three target genes for SARS-CoV-2 and the internal control were less than 40 ($Ct \leq 40$). When all of the SARS-CoV-2 target genes were negative and the internal control was positive ($Ct \leq 40$), the results were considered negative. When the internal control was negative, the results were considered invalid due to the inefficacy of the RNA extraction method.

The current results showed that all the RNA extraction methods could be used to extract high-quality RNA for the testing of SARS-CoV-2 infection because the Ct values of SARS-CoV-2 targeting genes (N, S, ORF1ab), and the internal control (MS2) for all RNA extraction methods were less than 35 ($Ct \leq 35$) (Table 1). There was also no significant difference in the mean Ct values ($p \leq 0.05$ **) between the alternative RNA extraction methods (Lucigen QuickExtract™ RNA Extraction Kit (Parmenter St Middleton, WI, USA), Bosphore EX-Tract Dry Swab RNA (New Ash Green Longfield, England) and Sonicator method (Europe) and the internationally approved kit (Thermo Fisher PureLink™ Kit) for the SARS-CoV-2 targeting genes (N, S, ORF1ab) and internal control (MS2) (Table 1 and Figure 1A–D). However, the results of one of the alternate RNA extraction methods, the Sonicator method, showed that there was a statistically significant difference ($p \leq 0.05$) in Ct values between the Sonicator method and the Thermo Fisher PureLink™ Kit for the mean Ct value of the ORF1ab gene (Table 1 and Figure 1C).

2.2 Comparison of Sensitivity and Specificity for RNA Extraction Methods

Based on the small sample size, caution should be taken when using this study's sensitivity and specificity results for the method comparison. The accuracy of each RNA extraction method used in testing SARS-CoV-2 infection was assessed by comparing the calculated sensitivity and specificity of alternative RNA extraction methods to the internationally approved kit. In a total of 25 nasopharyngeal swab samples for the positive (infected) group, the results of 25 positive samples extracted using two alternative extraction methods (Bosphore EX-Tract Dry Swab RNA Solution and Lucigen QuickExtract™ RNA Extraction Kit) matched the results of 25 positive samples extracted using the Thermo Fisher PureLink™ Kit. However, only 24 of the 25 positive nasopharyngeal swab sample results extracted using the Sonicator method (alternative RNA extraction method) matched the 25 positive sample results extracted using the Thermo Fisher PureLink™ Kit, with one sample result being invalid.

Table 1. Performance of the RNA extraction methods in testing SARS-CoV-2 infection for the positive (infected) group.

Target Genes for SARS-CoV-2 and Internal Control	Name of Extraction Method	N	Ct Mean Value (SD)	Difference between Means	*p*-Value
***N* gene**	Thermo Fisher PureLink™ Kit	25	21.32 (±3.966)		
	Sonicator method	24	23.16 (±3.716)	−1.840	$p = 0.075$
	Bosphore EX-Tract Dry Swab RNA Solution	25	22.04 (±3.963)	−0.720	$p = 0.501$
	Lucigen QuickExtract™ RNA Extraction Kit	25	21.88 (±5.036)	−0.560	$p = 0.625$
***S* gene**	Thermo Fisher PureLink™ Kit	25	19.96 (±5.111)		
	Sonicator method	24	20.84 (±3.197)	−0.880	$p = 0.198$
	Bosphore EX-Tract Dry Swab RNA Solution	25	21.44 (±4.647)	−1.480	$p = 0.135$
	Lucigen QuickExtract™ RNA Extraction Kit	25	21.16 (±5.421)	−1.200	$p = 0.417$
***ORF1ab* gene**	Thermo Fisher PureLink™ Kit	25	20.12 (±4.702)		
	Sonicator method	24	23.40 (±2.646)	−3.280	$p = 0.006$**
	Bosphore EX-Tract Dry Swab RNA Solution	25	22.16 (±4.249)	−2.040	$p = 0.064$
	Lucigen QuickExtract™ RNA Extraction Kit	25	22.36 (±5.469)	−2.240	$p = 0.067$
***MS2* (Internal control)**	Thermo Fisher PureLink™ Kit	25	23.72 (±4.912)		
	Sonicator method	24	25.76 (±2.818)	−2.040	$p = 0.125$

Bosphore EX-Tract Dry Swab RNA Solution	25	25.56 (±2.043)	−1.840	$p = 0.098$
Lucigen QuickExtract™ RNA Extraction Kit	25	22.92 (±3.290)	+0.800	$p = 0.340$

Difference between means = mean Ct value of the Thermo Fisher PureLink™ Kit—mean Ct value of each alternative extraction method. ** It showed a statistically significant difference in method comparison.

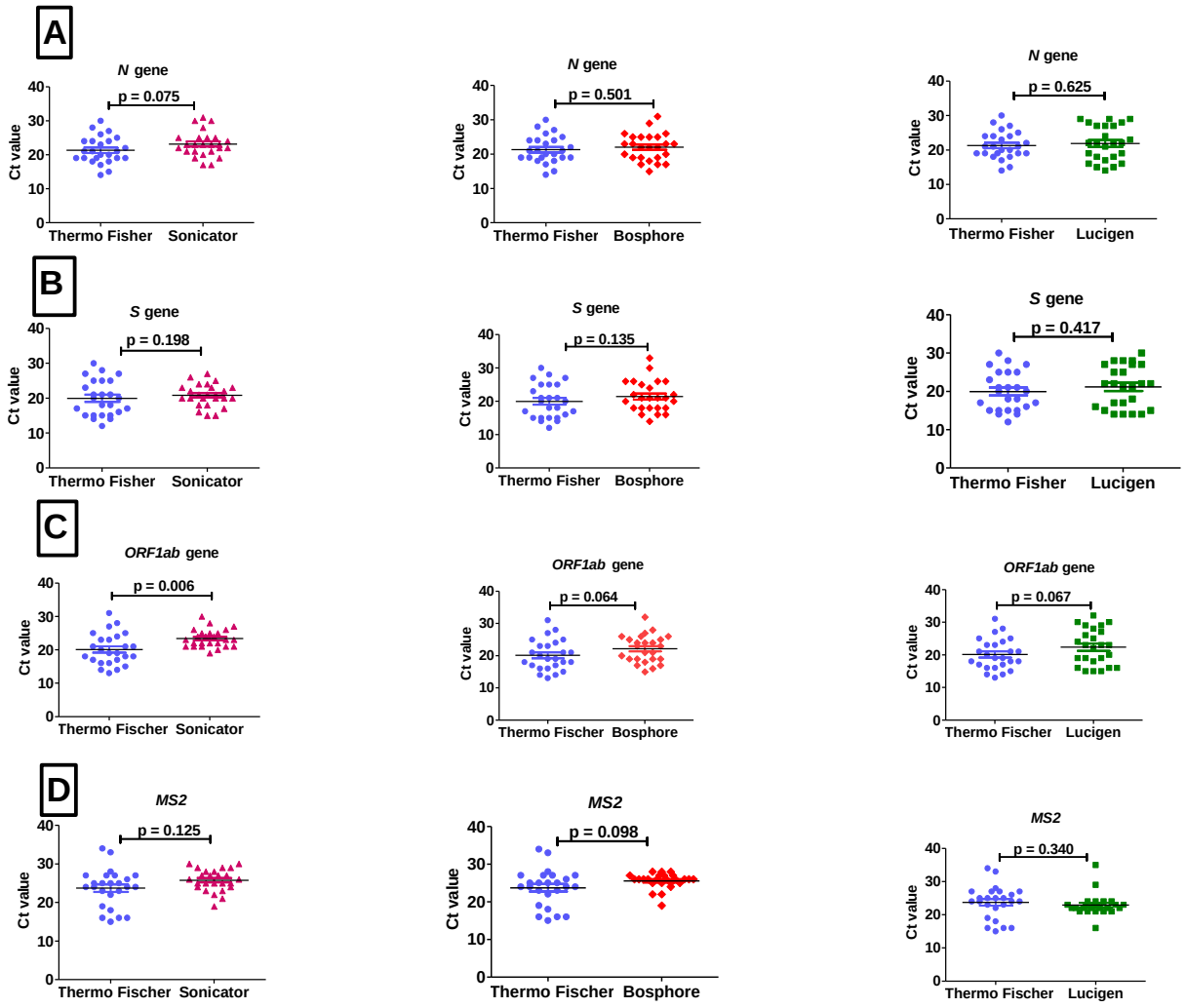

Figure 1. The Ct values were compared between the internationally approved kit (Thermo Fisher PureLink™ Kit) and alternative extraction methods (Sonicator method, Bosphore EX-Tract Dry Swab RNA Solution, and Lucigen QuickExtract™ RNA Extraction Kit) for the SARS-CoV-2 target genes: *N* gene (**A**), *S* gene (**B**), *Orf* gene (**C**), and MS2 internal control (**D).** The results with a level of $p \leq 0.05$** were considered significant. Thermo Fisher PureLink™ Kit is represented in blue, Sonicator method in pink, Bosphore EX-Tract Dry Swab RNA Solution in red, and Lucigen QuickExtract™ RNA Extraction Kit in green.

Additionally, in a total of 25 nasopharyngeal swab samples for a control group, all 25 negative samples extracted using alternative RNA extraction methods matched the 25 negative sample results that were extracted using the Thermo Fisher PureLink™ Kit. The sensitivity ranged from 96 to 100% for all alternative RNA extraction methods, while the specificity was 100% (Table 2).

Table 2. The sensitivity and specificity of alternative RNA extraction methods.

Name of Extraction Method	Sensitivity (*n*=25)	Specificity (*n* = 25)
Sonicator method	24 (96%)	25 (100%)
Bosphore EX-Tract Dry Swab RNA Solution	25 (100%)	25 (100%)
Lucigen QuickExtract™ RNA Extraction Kit	25 (100%)	25 100%)

2.3 Overview and Comparison of the Commercially Available SARS-CoV-2 RT-PCR Assay Kits

According to the manufacturer's guidelines for each kit, the test results were considered positive when all of the SARS-CoV-2 target genes and internal control used in the particular kit were detected at the same time ($Ct \leq 40$ or $Ct < 35$). When all of the SARS-CoV-2 target genes were negative ($Ct > 40$) and the internal control was positive ($Ct \leq 40$ or $Ct < 35$), the results were considered negative. None of the manufacturers were involved in the analysis and interpretation of the results. Table 3 summarizes the requirements for all of the commercially available RT-PCR SARS-CoV-2 test kits used to detect the SARS-CoV-2 target genes, as specified in the documentation for each RT-PCR SARS-CoV-2 assay kit.

Table 3. Overview of the commercially available RT-PCR assay kits used to detect the SARS-CoV-2 target genes.

SARS-CoV-2 Assay Kit	Catalog Number	Target Genes for Detection of SARS-CoV-2	Internal Control	Results Interpretation
Thermo Fisher TaqPath™ COVID-19 Assay Kit	A51738	*ORF1ab* *N* gene *S* gene	*MS2*	Ct ≤40 Positive result
Nucleic Acid COVID-19 Test Kit (SARS-CoV-2)	1006524-T	*N* gene *ORF1ab*	*RNase P*	Ct ≤40 Positive result
abTES™ COVID-19 qPCR I Kit	BN300142	*NS1* *NS2*	*GAPDH*	Ct ≤40 Positive result
PCL COVID-19 Speedy RT-PCR kit	MD02	*N* gene *E* gene	*RNase P*	Ct <35 Positive result
PCLMD nCoV One-Step RT-PCR Kit	MD01E	*N* gene	IC	Ct <35 Positive result

This study compared the calculated sensitivity and specificity of the alternative commercial RT-PCR assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES™ COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit) to the internationally approved kit (Thermo Fisher TaqPath™ COVID-19 As-say Kit). The sensitivity and specificity of the evaluated commercially available RT-PCR SARS-CoV-2 assay kits varied, with the PCLMD nCoV One-Step RT-PCR Kit having the highest sensitivity and specificity (96% and 100%, respectively) and the abTES™ COVID-19 qPCR I Kit having the lowest sensitivity and specificity (76% and 96%, respectively). The results are presented in Table 4.

Table 4. Comparison of the sensitivity and specificity of the commercial SARS-CoV-2 RT-PCR assay kits.

Name of the Kits	Number of Positive Samples Detected (n = 25)	False Negative	Sensitivity (%)	Number of negative Samples Detected (n = 25)	False Positive	Specificity (%)
Nucleic Acid COVID-19 Test Kit (SARS-CoV-2)	22	3	88	25	0	100
abTES^{TM} COVID-19 qPCR I Kit	19	6	76	24	1	96
PCL COVID-19 Speedy RT-PCR Kit	23	2	92	25	0	100
PCLMD nCoV One-Step RT-PCR Kit	24	1	96	25	0	100

2.4 Cost Analysis, Simplicity and the Turnaround Time for Each Evaluated Method and Internationally Approved Kits

The costs of the RNA extraction methods and commercially available RT-PCR SARS-CoV-2 assay kits were calculated and compared using the pricing of the reagents, consumables, and equipment. The processing time for each RNA extraction method and the observed run time for each commercial RT-PCR test kit were calculated to determine the turnaround time for each RNA extraction method and the commercially available RT-PCR SARS-CoV-2 assay kits. The prices for the RNA extraction methods and commercially available RT-PCR SARS-CoV-2 assay kits are specific to South Africa. The results in Table 5 show that the RNA extraction methods varied in terms of cost, processing time, and procedure simplicity. The Thermo Fisher PureLink™ Kit was the most expensive and had a per sample cost of USD ~2.96. The Sonicator method (an alternative RNA extraction method) was the least expensive, with a cost per sample of USD ~0.18. The processing time results for each RNA extraction method were as follows: Thermo Fisher PureLink™ Kit (~1 h); Sonicator method (~30 min); Bosphore EX-Tract Dry Swab RNA Solution (~15 min); Lucigen QuickExtract™ RNA Extraction Kit (~15 min). The Bosphore EX-Tract Dry Swab RNA Solution and Lucigen QuickExtract™ RNA Extraction Kit were considered to be the two simplest methods (three steps each) for extracting RNA nucleic acid used for testing SARS-CoV-2 infection.

Table 5. Comparison of RNA extraction methods based on their simplicity cost and observed run time for each method.

Name of extraction method	Simplicity	Approximate (~) observe run time	Cost per sample
Thermo Fisher PureLink™ kit	**5 steps:** Vortexing Sample lysis (Proteinase K buffer) Binding beads Washing (1st, 2nd, 3rd) Elution	~1 hour	~2.96USD
Sonicator method	**4 steps**: Vortexing Heating at 65℃ Sonicate at 65 ℃ Centrifuge	~30 min	~0.18USD
Bosphore EX-Tract Dry Swab RNA solution	**3 steps:** Vortexing Adding sample to buffer Heating sample at 95℃	~15 min	~0.89USD
Lucigen QuickExtract™ RNA Extraction kit	**3 steps:** Vortexing Adding sample to buffer Heating sample at 95℃	~15 min	~0.59USD

*The cost per sample for RNA extraction methods includes extraction kit, reagents, consumables and exclude equipment. The ZAR to USD exchange rate: 1 ZAR = 0.0592 USD [30].

The results of the cost analysis for each commercially available RT-PCR SARS-CoV-2 assay kit showed that all alternative commercial RT-PCR SARS-CoV-2 assay kits were the least expensive when compared to the Thermo Fisher TaqPath™ COVID-19 Assay Kit. The results of the cost of the commercially available RT-PCR SARS-CoV-2 assay kits are presented in Table 6. Additionally, the results indicated that there was a slight difference in the observed run time between the Thermo Fisher TaqPath™ COVID-19 Assay Kit and the three commercial SARS-CoV-2 RT-PCR assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES™ COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit). The observed run times between the Thermo Fisher TaqPath™ COVID-19 Assay Kit (64 min) and the PCLMD nCoV One-Step RT-PCR Kit (137 min), one of the alternative commercial SARS-CoV-2 RT-PCR assay kits, were, however, significantly different (Table 6).

Table 6. Overview of the commercially available RT-PCR assay kits that were evaluated in terms of the running time, costs, and the number of SARS-CoV-2 target genes.

Name of SARS-CoV-2 Assay Kits	Running Time PCR (min)	* Cost per Sample
Thermo Fisher TaqPath™ COVID-19 Assay Kit	~64 min	USD ~14.80
Nucleic Acid COVID-19 Test Kit (SARS-CoV-2)	~83 min	USD ~4.44
abTES^{TM} COVID-19 qPCR I Kit	~79 min	USD ~9.83
PCL COVID19 Speedy RT-PCR Kit	~ 62 min	USD ~7.11
PCLMD nCoV One-Step RT-PCR Kit	~137 min	USD ~8.88

*The cost per sample includes commercially available RT-PCR SARS-CoV-2 assay kits reagents, consumables, and sample processing. Excludes equipment. The ZAR to USD exchange rate: 1 ZAR = 0.0592 USD [30]. Overall running time PCR includes the ramp time and run cycles for each RT-PCR assay kits.

3.Discussion

As countries around the world continue to search for effective treatment and eradication of the SARS-CoV-2 virus, diagnostic testing is still one of the most effective ways to track the spread of the virus and subsequently implement appropriate preventative measures [28,29]. Many factors, particularly financial and infrastructural resources, limit the quantum of testing in LMICs. The main goal of this study was to evaluate the cost-effective, accurate, and faster alternative methods that could assist in increasing the testing capacity for SARS-CoV-2 infection in laboratories within limited-resource settings. Results showed that the alternative RNA extraction methods (Sonicator method, Lucigen QuickExtract™ RNA Extraction Kit, and Bosphore EX-Tract Dry Swab RNA Solution) were qualitatively comparable to the internationally approved kit Thermo Fisher PureLink™ Kit. Likewise, the performance characteristics of the alternative commercial RT-PCR SARS-CoV-2 assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES^{TM} COVID19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit) were as follows: (i) sensitivity (76–96%); (ii) specificity (96–100%); (iii) negative predictive value (4–24%); and (iv) positive predictive value (4%).

The efficiency of each RNA extraction method was assessed by comparing the performance of alternative RNA extraction methods to the Thermo Fisher PureLink™ Kit (internationally approved kit). In the present study results, there was no statistically significant difference in the mean Ct value between the Thermo Fisher PureLink™ Kit and the alternative extraction methods (Ct value ≤ 35. All of the alternative RNA extraction methods had high specificity (100%) and sensitivity (96–100%). The statistically significant difference in the Ct value for the ORF1ab gene between the Sonicator extraction method and the Thermo Fisher PureLink™ Kit had no appreciable effect on the Sonicator extraction method's high sensitivity. The results showed that the efficiency and recovery rates of the alternative RNA extraction methods were satisfactory to be used for RNA extraction in testing for the SARS-CoV-2 infection.

When the cost-effectiveness, processing time, and simplicity of each RNA extraction method were compared, it was found that both the Bosphore EX-Tract Dry Swab RNA Solution and Lucigen QuickExtract™ RNA Extraction Kit were simpler, faster, accurate, and cheaper, despite the fact that neither of these extraction methods had any inhibitor removal. These two alternative methods had three procedural steps and took approximately 15 min to extract RNA from 25 nasopharyngeal samples compared to the Thermo Fisher PureLink™ Kit, which had five procedural steps and took approximately an hour to process 25 nasopharyngeal samples. The Thermo Fisher PureLink™ Kit was the most expensive (Table 5). Despite being more time-consuming, the Sonicator method was the cheapest of the two alternative RNA extraction methods (Bosphore EX-Tract Dry Swab RNA Solution and Lucigen QuickExtract™ RNA Extraction Kit) and the internationally approved kit (Thermo Fisher PureLink™ Kit). When comparing the cost of the Sonicator method (USD ~0.18 per sample) to the Thermo Fisher PureLink™ Kit (USD ~2.96 per sample), there was more than a 94% price reduction with Sonicator methods, making the Sonicator method the cheapest method. Hence, the Sonicator extraction method may be a good choice, especially considering that laboratories in low-income countries (LICs) have limited resources for the mass testing of SARS-CoV-2 infection, and setting up a laboratory for testing SARS-CoV-2 infection in these countries is a challenge. This study recommends that LICs use the Sonicator method. Consequently, SARS-CoV-2 mass testing will be improved, and viral transmission will be optimally monitored for control and reduction.

Viral RNA extraction is necessary for the RT-PCR tests to be performed [30]. The Lucigen QuickExtract™ RNA Extraction Kit was the method of choice for RNA extraction in this study. The extracted RNA was required for the evaluation and comparison of commercial RT-PCR SARS-CoV-2 kits. The Lucigen QuickExtract™ RNA Extraction Kit was chosen due to its simplicity, speed, and low cost. When the clinical accuracy of the alternative commercial RT-PCR SARS-CoV-2 assay kits was compared to the Thermo Fisher TaqPath™ COVID-19 Assay Kit, the study found that all four alternative commercially available RT-PCR SARS-CoV-2 assay kits had good diagnostic sensitivity ranging from 76–96% and a specificity of 96–100% (Table 5), with the PCLMD nCoV One-Step RT-PCR Kit having superior sensitivity compared to other the three alternative commercially available RT-PCR SARS-CoV-2 assay kits. The high specificity of 100% reported on the manufacturer's package insert for all RT-PCR SARS-CoV-2 assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES^{TM} COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, PCLMD nCoV One-Step RT-PCR Kit, and an internationally approved kit (Thermo Fisher TaqPath™ COVID-19 Assay Kit) matched the present study's high specificity results (96–100%). All of the evaluated alternative RT-PCR SARS-CoV-2 assay kits and the internationally approved kit had a sensitivity of 92.89–100%, as specified on the manufacturer's package insert, which matched the present study's high sensitivity results (76–96%).

Interestingly, the inclusion of the N target gene on these purchased three alternative commercial RT-PCR SARS-CoV-2 assay kits as well as the Thermo Fisher TaqPath™ COVID19 Assay Kit could be one of the main contributions that helped improve the sensitivity of these assay kits. According to published studies, the N gene is the most sensitive target gene for detecting SARS-CoV-2 because it contains a greater number of subgenomic N gene messenger RNAs compared to other target genes. [31,32]. Furthermore, the ORF1ab gene has been identified as the target gene with the highest contribution to specificity in the RTPCR assay kits for the detection of SARS-CoV-2 infection. This is because the ORF1ab gene is the most conserved compared to other target genes such as the N or E genes [33,34]. The results demonstrated a good match between all four alternative commercial RT-PCR SARSCoV-2 assays kits and the internationally approved Thermo Fisher TaqPath™ COVID-19 Assay Kit (100% sensitivity and 100%

specificity) and can therefore be recommended for use interchangeably in routine diagnostic laboratories.

When comparing the costs of the Thermo Fisher TaqPath™ COVID-19 Assay Kit and alternative commercial RT-PCR SARS-CoV-2 assay kits, the Thermo Fisher TaqPath™ COVID-19 Assay Kit (USD ~14.80 per sample) was the most expensive of all of the commercial RT-PCR SARS-CoV-2 assay kits, with alternative commercial RT-PCR SARS-CoV-2 assay kit prices ranging between a USD ~4.44 and USD ~9.83 cost per sample. In terms of the observed run time, there seemed to be little difference between the Thermo Fisher TaqPath™ COVID-19 Assay Kit and the other three commercial RT-PCR SARS-CoV-2 kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit), and the turn-around time was shorter for all these methods. Despite having the longest run time (137 min) of all the evaluated commercial RT-PCR SARS-CoV-2 assay kits, the PCMLD nCov One-Step RT-PCR Kit had some advantages, being cheaper and having a higher diagnostic sensitivity and specificity. For these reasons, the use of a Thermo Fisher TaqPath™ COVID-19 Assay Kit or low-cost alternative commercial RT-PCR SARS-CoV-2 assay kits may help to increase the mass testing for SARS-CoV-2 infection within a limited resource laboratory setting.

The sample size was one of the study's limitations due to the challenge of a limited cost budget. Therefore, a larger sample size is required for future studies in order to validate the current study results. Nonetheless, we believe that useful information has been established. Furthermore, this work is not only relevant for SARS-CoV-2 testing in LMICs, but also to the fact that scientists predict more pandemics as there is increased interaction between the environment, wildlife, and humans [35,36]. This then warrants that LMICs prepare for such, and one of the requisites for preparedness is cost-effective laboratory testing capabilities.

4. Material and Methods

This was a retrospective study to compare various methods to identify cost-effective methods that can be used by low-middle income countries. This study used residual nasopharyngeal swab samples from adult (over the age of 18) male and female participants who had SARS-CoV-2 symptoms or not. The study was approved by the University of KwaZulu Natal Biomedical

Research Ethics Committee (BREC/00003671/2021) with permission to use residual nasopharyngeal samples with blinding to protect patient identity from both the BREC and the Global Health Innovation (GHI) laboratory, a subsidiary of the Aurum Institute, Johannesburg, South Africa.

4.1 Clinical Specimens

A total of 50 residual nasopharyngeal swab samples were used, which were initially collected from South African patients to test for SARS-CoV-2 infection by trained health care workers. Dry sterile nasopharyngeal swabs were used to test for SARS-CoV-2 infection, and the samples were transported in a cooler bag with ice to the GHI laboratory. Furthermore, for the comparison of methods in this study, the participants' residual nasopharyngeal swab samples that were in deionized water and first tested for SARS-CoV-2 infection by the GHI laboratory were used. The GHI laboratory is accredited by the South African National Accreditation System (SANAS) for diagnostic testing. The 50 residual samples were subdivided into two groups: (i) Group 1: 25 nasopharyngeal residual samples from the SARS-CoV-2 infected patients (positive group), and (ii) Group 2: 25 nasopharyngeal residual samples from the SARS-CoV-2 uninfected patients (control group). All specimens were processed in a biosafety cabinet level 3 (BSL 3) facility with full personal protective equipment (PPE).

This study compared the RNA extraction methods and commercially available SARS-CoV-2 RT-PCR assay kits using the same residual nasopharyngeal swab samples. The standard operating procedure in this study was normalized by using the same residual nasopharyngeal swab samples stored under the same conditions. In addition, the residual nasopharyngeal swabs used for method comparison were chosen from participants with almost identical Ct values for the SARS-CoV-2 target genes (N, S, ORF1ab) and internal control (MS2) as well as the high viral load for the positive group. Participants with almost identical Ct values for MS2 (internal control) were also chosen for the negative group. The residual nasopharyngeal swab samples were stored in a locked −80 ◦C bio-freezer to ensure the stability and integrity of the samples.

4.2 Comparison of SARS-CoV-2 RNA Extraction Methods

The Thermo Fisher PureLinkTM Kit was chosen in this study as the gold standard method for the comparison of RNA extraction methods (Lucigen QuickExtract™ RNA Extraction Kit, Bosphore EX-Tract Dry Swab RNA Solution, and Sonicator method) because of its superior clinical accuracy and it is an internationally approved kit. Furthermore, the Thermo Fisher TaqPathTM COVID-19 Assay Kit was also chosen as the gold standard assay kit for assessing the efficacy of each RNA extraction method and as a comparison method for assessing the clinical performance of alternative commercially available SARS-CoV-2 RT- PCR assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES^{TM} COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit) because of the following advantages: (i) The TaqPath™ COVID-19 Assay Kit can detect SARS-CoV-2 infection by identifying the presence of three gene targets from the virus's S, N, and ORF1ab regions [37]; (ii) even if one of the targets is altered by a mutation, the test can provide reliable results, and (iii) the World Health Organization (WHO), Food and Drug Administration (FDA, Centers for Disease Control and Prevention (CDC), and European Centers for Disease Control (ECDC) have acknowledged the Thermo Fisher TaqPath Assay for using the S-gene target failure (SGTF) of PCR assays as a proxy for the variation aided in the diagnosis of Omicron [38,39]. Therefore, it is an internationally approved kit.

The RNA extraction methods used for comparison purposes include the Thermo Fisher PureLink™ Kit, an internationally approved kit, and the alternative extraction methods (Lucigen QuickExtract™ RNA Extraction Kit, Bosphore EX-Tract Dry Swab RNA Solution, and Sonicator method). Initially, 5 µL MS2 was added as an internal control to all of the Eppendorf microtubes (Merck, Darmstadt, Germany) containing nasopharyngeal swab samples in 300 µL deionized water. Then, the mixture was vortexed (Scientific Industries Inc., Bohemia, NY, USA) for 2 min to homogenize the samples. Thereafter, the homogenized samples were further used for the comparison of the methods (RNA extraction methods and commercially available SARS-CoV-2 RT-PCR assay kits).

4.2.1 Thermo Fisher PureLink™ Kit

RNA was extracted according to the manufacturer's instructions using the Thermo Fisher PureLink™ Kit (Thermo Fischer Scientific, Pleasanton, CA, USA, Cat No. A47813 and A47814). A total of 200 µL of the homogenized samples were used for manual RNA extraction. The procedures for RNA extraction with this kit included proteinase K buffer digestion, the addition of the binding bead solution, washing of the beads three times, and elution of the nucleic acid (RNA). The working sample volume of the extracted and eluted RNA was 50 µL. The Applied Biosystems Real-Time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA, USA) was used for the amplification and detection of SARS-CoV-2 target genes (S, N, and ORF1ab).

4.2.2 Lucigen QuickExtract™ RNA Extraction Kit

Following the manufacturer's instructions, RNA was extracted using the Lucigen, QuickExtract™ RNA Extraction Kit (LGC Biosearch Technologies, Parmenter St Middleton, WI, USA, Cat No. QER090150) with the minor protocol modification described below: 20 µL of the homogenized nasopharyngeal swab samples was added to MicroAmp 8-tube strips with 20 µL of the Lucigen, QuickExtract™ RNA Extraction solution to extract RNA. To inactivate the virus, the extracted sample was placed on the Applied Biosystems heat cycler for 5 min at 95 ◦C. The Applied Biosystems real-time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA USA) was used for the amplification and detection of SARS-CoV-2 target genes from the extracted RNA samples.

4.2.3 Bosphore EX-Tract Dry Swab RNA Solution

RNA was extracted from the sample using the Bosphore EX-Tract Dry Swab RNA solution as per the manufacturer's instructions, with the following protocol modification: 20 µL of the homogenized nasopharyngeal swab samples was added to MicroAmp 8-tube strips with 20 µL Bosphore lysis buffer solution (Anatolia Geneworks, New Ash Green Longfield, England, Cat No. CS-003) to extract the RNA. The extracted sample was placed on the Applied Biosystems thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA) at 95 ◦C (5 min) to inactivate the virus.

The Applied Biosystems real-time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA, USA) was used for the amplification and detection of SARS-CoV-2 target genes from the extracted RNA samples.

4.2.4 Sonicator Method

The Sonicator method was used to extract the RNA from the homogenized nasopharyngeal swab sample. The procedures described below were used to obtain a high-quality RNA extract using this extraction method: Eppendorf microtubes (Merck, Darmstadt, Germany) containing homogenized nasopharyngeal swab samples were placed on a dry heating block (Thermo Fisher Scientific, Waltham, MA, USA) at 65 ◦C for 10 min to inactivate the virus. Thereafter, the samples were processed for 15 min at 65 ◦C (40 kHz) with an Ultra Bath Sonicator (RS PRO, Europe) to lyse the cells and extract RNA. The Sonicator temperature was maintained by using a thermometer. The sonicated samples were centrifuged at 179× g for 1 min (Eppendorf, Fisher Scientific, USA). Approximately 20 µL of the extracted RNA supernatant was transferred into another empty MicroAmp 8-tube strip (Applied Biosystem, Thermo Fischer Scientific, China), and the SARS-CoV-2 target genes were amplified and detected using a real-time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA, USA).

4.3 Detection of SARS-CoV-2 Genes Using RT-PCR

The Thermo Fisher TaqPath™ COVID-19 Assay Kit (Thermo Fischer Scientific, Pleasanton, CA, USA, Cat No. A47813 and A47814), an internationally approved kit, Applied Biosystemsreal-time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA USA), and Quant-Studio Design & Analysis Software (Thermo Fisher Scientific, Waltham, MA, USA) were used to assess the RNA yield from each extraction method for the testing of SARS-CoV-2 infection. The Thermo Fisher TaqPath™ COVID-19 Assay Kit is a multiplex diagnostic solution that contains both the assays and controls needed for the RT-PCR detection of SARS-CoV-2 viral RNA. The Thermo Fisher TaqPath™ COVID-19 Assay Kit targets the S gene, N gene, and ORF1ab gene of SARS-CoV-2 and MS2 (internal control). Approximately, 6.3 µL of extracted RNA was added to 2 µL of 4X Taqpath 1-step multiplex Master Mixture (mix) (Thermo Fischer Scientific, Pleasanton,

CA, USA), 0.3 µL of the probe, 21.4 µL nuclease-free water, and 2 µL MS2 (added only to the housekeeping control). The 4× Taqpath master mix (7.5 µL for 30 µL) already contains probes. The total volume used per reaction was 30 µL. Conditions for the Applied Biosystems real-time thermal cycler included one cycle of 2 min at 25 ◦C (incubation), 10 min at 53 ◦C (reverse transcription), 2 min at 95 ◦C (activation of the Taq DNA polymerase), followed by 40 cycles of 3 s at 95 ◦C (denaturation) and 30 s at 60 ◦C (anneal/extension). The results were analyzed using Quant-Studio Design & Analysis Software (Madison, WI, USA).

4.4 Comparison of the Five Commercially Available SARS-CoV-2 Real-Time PCR Assay Kits

4.4.1 Selection Criteria for RT-PCR Assay Kits

The following criteria were used to select the commercially available SARS-CoV-2 RTPCR kits used in this study: (a) the assay kits could use RNA samples extracted using any manual nucleic acid extraction methods; (b) the assay kits could be performed on a Applied Biosystems real-time thermal cycler; (c) the assay kits were available on the market and could be obtained in less than 4 weeks; (d) diagnostic laboratories in LMICs should be able to afford the assay kits; and (e) the assay kits had already obtained CE-IVD certification.

4.4.2 RT-PCR Laboratory Procedure

In the method comparison of commercially available RT-PCR SARS-CoV-2 assay kits, the evaluated Lucigen QuickExtract™ RNA Extraction Kit was chosen as the preferred method for RNA extraction. This choice was made after it was discovered in this study (Section 4.2.2) that this extraction was cheaper and faster. Five commercially available RT-PCR SARS-CoV-2 assay kits from different manufacturers were selected in this study for the method comparison including the Thermo Fisher TaqPath™ COVID-19 Assay Kit (Thermo Fischer Scientific, Pleasanton, CA, USA), an internationally approved kit, and the four alternative RT-PCR SARS-CoV-2 assay kits: Nucleic Acid COVID-19 Test Kit (SARS-CoV-2) (Wuhan Easy-diagnosis Biomedicine, Wuhan, China), abTESTM COVID-19 qPCR I Kit (Anatech Instrument (PTY) LTD, Meadowbrook,

Business Estate, Sloane Park, Gauteng, South Africa), PCL COVID19 Speedy RT-PCR Kit (PCL Inc. Multiplex In Vitro Diagnostic Global Leader, Seoul, South Korea), and the PCLMD nCoV One-Step RT-PCR Kit (PCL Inc. Multiplex In Vitro Diagnostic Global Leader, Seoul, South Korea). Positive and negative controls were included in each test run to ensure that the results were accurate and reliable. All of the commercial RT-PCR SARS-CoV-2 assay kits were compatible with the Applied Biosystems real-time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA, USA).

Thermo Fisher TaqPath™ COVID-19 Assay Kit

The Thermo Fisher TaqPath™ COVID-19 Assay Kit (Thermo Fischer Scientific, Pleasanton, CA, USA) methodology is described in full detail in Section 2.3.

Nucleic Acid COVID-19 Test Kit (SARS-CoV-2)

This is a reverse transcription, multiplex, one-step RT-PCR assay kit designed to detect different SARS-CoV-2 specific target genes in a single tube well. A total of 25 µL of the reaction mixture was tested, with 20 µL of Master mix and 5 µL of extracted RNA sample or SARS-CoV-2 positive control or negative control. The Applied Biosystems real-time thermal cycler was used for the amplification and detection of the SARS-CoV-2 target genes. The condition of the PCR instrument included one cycle of 15 min at 50 ◦C for reverse transcription, 30 s at 95 ◦C for pre-degeneration; 45 cycles of 3 s at 95 ◦C for degeneration, and 45 s at 60 ◦C for annealing and extension.

abTES™ COVID-19 qPCR I Kit

This commercially available test kit is a qualitative, multiplex real-time polymerase chain reaction (qPCR) kit that allows for the simultaneous detection of two SARS-CoV-2 specific targeted genes in a single reaction. Sample reagents required for the preparation of a 20 µL reaction mixture included 10 µL of 2× RT-PCR Master mix, 1 µL of RT/ Taq enzyme mix, 2 µL of Primer/Probe mix, 2 µL of nuclease-free water, and 5 µL of RNA Template from the patient sample or negative control or the SARS-CoV-2 positive control. The SARS-CoV-2 target genes were amplified and

detected using an Applied Biosystems real-time thermal cycler. The RT-PCR instruments were set up as follows: One cycle of 10 min at 59 ◦C for cDNA synthesis, 2 min at 95 ◦C for initial denaturation, and 45 cycles of 10 s at 95 ◦C and 30s at 57.5 ◦C for amplification and extension.

PCL COVID-19 Speedy RT-PCR Kit

The assay kit is a one-step multiplex RT-PCR kit designed to identify two SARS-CoV2 target genes simultaneously in a single tube. The reaction mixture volume of 20 µL contained 5 µL of Master mix, 2 µL of Primer + Probe mix, 8 µL of nuclease-free water, and 5 µL extracted RNA sample or the negative control and positive control. Using an Applied Biosystems real-time thermal cycler, the SARS-CoV-2 target genes were amplified and identified. The RT-PCR instruments were programmed as one cycle of 5 min of cDNA synthesis at 50 ◦C, 2 min of initial denaturation at 95 ◦C, and 40 cycles of 5 s at 95 ◦C and 30 s at 55 ◦C for amplification and extension.

PCLMD nCoV One-Step RT-PCR Kit

The assay kit is a one-step, qualitative RT-PCR kit for the detection of SARS-CoV-2. This assay kit requires three types of master mixture for each sample being tested (one test tube for one gene). (i) PCR tube 1: The 20 µL reaction mixture consisted of 5 µL of Master mix, 2 µL Primer + Probe Mixture 1 (confirmatory target gene for SARS-CoV-2 infection), 8 µL nuclease-free water, and 5 µL RNA sample; (ii) PCR tube 2: The 20 µL reaction mixture consisted of 5 µL of Master mix, 2 µL Primer + Probe Mixture 2 (for screening), 8 µL nuclease-free water, and 5 µL RNA sample; (iii) PCR tube 3: The 20 µL reaction mixture consisted of 5 µL of Master mix, 2 µL of IC Primer + Probe mix (internal control), 8 µL of nuclease-free water, and 5 µL RNA sample. The PCR reaction was performed using an Applied Biosystems real-time thermal cycler under the following conditions: One cycle of 30 min at 50 ◦C (cDNA synthesis), 10 min at 95 ◦C (initial denaturation) and 40 cycles of 15 s at 95 ◦C and 1 min at 55◦C (amplification and extension).

4.5 Statistical Analysis

The data generated by the RT-PCR Quant-Studio Design & Analysis Software were analyzed using GraphPad Prism 5. Since the data had a normal distribution, the continuous variables were presented as the mean and standard deviation. The categorical variables were presented in percentages and numbers. The paired t-test was used to assess whether there was a statistically significant difference between the alternative RNA extraction methods and the gold standard RNA extraction method by comparing the mean Ct value of the SARS-CoV-2 target genes (N, S, ORF1ab) and internal control (MS2). The calculated sensitivity and specificity for each method were used to assess and compare the clinical diagnosis between the alternative methods and the internationally approved kits for testing SARS-CoV-2 infection. A $p < 0.05$ was considered as statistically significant.

5. Conclusions

In conclusion, the present study found that alternative methods were cheaper, simpler, and faster and that they could be used interchangeably with internationally approved kits (Thermo Fisher PureLink™ Kit and Thermo Fisher TaqPath™ COVID-19 Assay Kit). All alternative RNA extraction methods (Lucigen QuickExtract™ RNA Extraction Kit, Bosphore EX-Tract Dry Swab RNA Solution, and Sonicator method) and all four commercial RT-PCR SARS-CoV-2 assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES^{TM} COVID-19 qPCR I Kit, PCL COVID-19 (Speedy RT PCR), and PCMLD nCov One-Step RTPCR) can be recommended for routine diagnostic use because of their excellent performance in identifying positive samples. Furthermore, implementing these cost-effective alternative methods in LMIC laboratories will help to expand the testing capacity for the mass testing of SARS-CoV-2 infection. This will allow for the early detection of infected individuals in the community. As a result, controlling and preventative measures can be implemented sooner to avoid the spread of SARS-CoV-2 infection.

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and P.N.—visualization; Z.L.M.-K., A.A.C., V.R. and Z.D.—methodology; Z.D., Z.L.M.-K., A.A.C., V.R., V.A.E. and P.N.—formal analysis; Z.D.—performed the experiment and original draft preparation; VE—granted permission for the use of COVID-19 residual samples from the GHI laboratory; Z.L.M.-K., A.A.C., V.R., V.A.E. and P.N.—writing: review and editing. All authors have read and agreed to the published version of the manuscript.

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CHAPTER FIVE

Synthesis and Conclusion

SARS-CoV-2 symptomatic and asymptomatic testing are still important components of virus control. The resources and equipment needed to identify SARS-CoV-2 infection per WHO guidelines, on the other hand, are in limited supply, particularly in LMICs. The unfortunate part is that currently there is still no cure for SARS-CoV-2 infection, and the number of cases and death rate from the virus have increased exponentially since the pandemic emerged in 2020. In the current study findings, comparison of the total number and frequency of SARS-CoV-2 testing between LIMCs and HICs using publicly available Worldometer COVID-19 data showed that HICs tested for SARS-CoV-2 at a higher rate, with a range of 113%-192%, than MICs (12%-43%), and LMICs (1%-6%). Due to LMICs financial constraints and reliance on global chains supply for reagents, PPE, and equipment, this has resulted in a shortage of testing reagents and consumables such as swabs, PPE, kits for SARS-CoV-2 RNA extraction, and RT-PCR assay kits, which limits testing capabilities in many LMICs. LMICs undertesting is concerning because it puts these countries at high risk of silently transmitting the virus within the community (Chapter Two). It is critical to developing methods to expedite diagnostic procedures in LMICs so that the health system does not collapse or become overwhelmed in the event of future SARS-CoV-2 waves, or any pandemic, particularly now that the lockdown restrictions have eased and the global economy is open.

For SARS-CoV-2 pandemic control and preparedness, widespread testing and rapid diagnosis are crucial. This seems to be true for SARS-CoV-2, as asymptomatic individuals contribute significantly to virus spread. Understanding the epidemiology of SARS-CoV-2 in each part of the world is critical for policy guidance; however, due to limited access to tests, inadequate laboratory infrastructure, insufficient personnel, and strained health systems, particularly in LMICs, this is not always possible (Arons *et al*., 2020). Notably, COVID-19-related deaths have been reported to be lower on the African continent, where all of the countries are classified as LMICs (Wamai *et al*., 2021). This has prompted speculation about the epidemiological profile of people infected with SARS-CoV-2 in Africa, where the healthcare system is already known to be fragile. As a result, electronic clinical records of SARS-CoV-2 infected patients living in South Africa were reviewed

retrospectively in this study. More than 60% of SARS-CoV-2 infected patients in this study were asymptomatic. This is a major concern because South Africa, as a middle-income country, is already undertesting for SARS-CoV-2 infection when compared to high-income countries (Chapter Three). The above-mentioned challenge could put more vulnerable members of the community, such as the elderly and those with comorbidities, at risk of developing severe COVID-19 symptoms and dying if infected with SARS-CoV-2. In addition, having a high proportion of asymptomatic cases in LMICs is risky because people in these countries already live in poor conditions, such as not having access to running water for hand washing or adequate sanitation; living in crowded or multigenerational households; homeschooling is difficult in these countries due to poor or no internet access; and people struggling to buy basic items such as soap (Levison, 2020; Gibson & Rush, 2020). The high proportion of asymptomatic cases in this study population reflects an agency for developing alternative methods that are fast, accurate, and less expensive for mass testing of SARS-CoV-2 in LMICs with limited resources. This would help to increase testing capacity for SARS-CoV-2 infection while also controlling virus transmission in the community (Chapter Three). Our study was unable to report on SARS-CoV-2 fatality rates because our data lacked information on clinical outcomes for all SARS-CoV-2 infected patients. After observing that Africa had fewer COVID-19-related deaths than Western countries, the current study discovered that COVID-19 epidemiology in African countries is similar to that of other Western countries (Chapter Three).

Despite the development of numerous immunologic assays (antigen and antibodies) for the detection of SARS-CoV-2, the gold standard for diagnosing SARS-CoV-2 infection continues to remain real-time-reverse transcriptase polymerase chain reaction (RT-PCR) and nasopharyngeal swab sample (Drame *et al*., 2020; Lui *et al*., 2020; Chau *et al*., 2020; Walker *et al*., 2020; Sharma *et al*., 2021; Dutta *et al*., 2022). However, the reverse transcription quantitative PCR method is costly, particularly for LMICs with limited resources. Cheaper and faster testing strategies must be considered in resource-constrained countries, based on the fact that early and accurate COVID-19 detection is critical for countries to track and control the virus's spread (Chapter Four). Until the majority of the LMICs are protected from SARS-CoV-2, countries' diagnostic testing needs will remain extremely high. Attempting to overcome the scarcity of SARS-CoV-2 detection

reagents, consumables, and assay kits while improving testing capacity and turnaround time in laboratories with limited resources, various alternative PCR methods were evaluated in terms of cost, accuracy, and turnaround time as part of this study. Based on our experimental and analytical findings, policymakers should consider implementing these evaluated, cheaper and faster alternative PCR methods for mass SARS-CoV-2 testing in resource-poor settings (Chapter Four).

In conclusion, the true burden of the SARS-CoV-2 global epidemic appears to be underestimated in LMICs due to limited testing resources. According to the current study findings, the high proportion of asymptomatic individuals suggests that screening based solely on signs and symptoms may miss a large proportion of SARS-CoV-2 infected individuals in the community, resulting in rapid virus spread. In countries with limited laboratory resources, using cheaper, faster, and more accurate alternative testing to detect and isolate positive cases early on can be more effective in controlling the SARS-CoV-2 pandemic. As a result, this study recommends that our evaluated alternative strategies be implemented in LMICs because it has the potential to increase testing capacity for SARS-CoV-2 infection diagnosis and monitoring within poor communities.

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APPENDIX A

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Globalization and Health

REVIEW Open Access

The challenges of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) testing in low-middle income countries and possible cost-effective measures in resource-limited settings

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Abstract

Diagnostic testing for the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection remains a challenge around the world, especially in low-middle-income countries (LMICs) with poor socio-economic backgrounds. From the beginning of the pandemic in December 2019 to August 2021, a total of approximately 3.4 billion tests were performed globally. The majority of these tests were restricted to high income countries. Reagents for diagnostic testing became a premium, LMICs either cannot afford or find manufacturers unwilling to supply them with expensive analytical reagents and equipment. From March to December 2020 obtaining testing kits for SARS-CoV-2 testing was a challenge. As the number of SARS-CoV-2 infection cases increases globally, large-scale testing still remains a challenge in LMICs. The aim of this review paper is to compare the total number and frequencies of SARS-CoV-2 testing in LMICs and high-income countries (HICs) using publicly available data from Worldometer COVID-19, as well as discussing possible interventions and cost-effective measures to increase testing capability in LMICs. In summary, HICs conducted more SARS-CoV-2 testing (USA: 192%, Australia: 146%, Switzerland: 124% and Canada: 113%) compared to middle-income countries (MICs) (Vietnam: 43%, South Africa: 29%, Brazil: 27% and Venezuela: 12%) and low-income countries (LICs) (Bangladesh: 6%, Uganda: 4% and Nigeria: 1%). Some of the cost-effective solutions to counteract the aforementioned problems includes using saliva instead of oropharyngeal or nasopharyngeal swabs, sample pooling, and testing high-priority groups to increase the number of mass testing in LMICs.

Keywords: SARS-CoV-2, Low-middle-income countries, Diagnostic testing challenges, Cost-effective strategies, Resource-limited settings

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Background

Overview of SARS-CoV-2

In December 2019, the World Health Organization (WHO) reported several pneumonia cases in Wuhan, China [1]. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was confirmed as the cause of Coronavirus disease 2019 (COVID-19) [2]. Severe Acute Respiratory Syndrome Coronavirus 2 is a positive-stranded ribonucleic acid (RNA) virus that primarily infects the upper respiratory tract and is associated with a wide range of complications, including lymphopenia, dyspnea, acute respiratory distress syndrome, pneumonia and acute cardiac arrest [3].

The virus contains four structural proteins namely the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins [4]. SARS-CoV-2 entrance into the host cells is mediated by the spike protein. The receptor-binding domain that binds to the peptidase domain of angiotensin-converting enzyme 2 (ACE2) is located at the S1 subunit of the S protein [4, 5]. The M protein is the virus's most abundant structural protein [4, 6] because it interacts with all of the other major coronaviral structural proteins, and it is assumed to be the central coordinator of coronavirus assembly [4]. The E protein is a minor component of the membrane. During the replication cycle, the E protein is widely expressed inside the infected cell, but only a small portion of it is integrated into the virion membrane [4, 7]. The nucleocapsid is made up of the viral RNA genome, and the N protein is the only protein that binds to it. The N protein is largely involved in viral genome activities, and also plays a role in other aspects of the viral replication cycle as well as the host cellular response to viral infection [4, 8].

Countries worldwide have been struggling to contain the highly contagious and rapidly mutating SARS-CoV-2 for more than a year [9]. As of 30 September 2021, the virus has spread to over a hundred countries, and about 222 million coronavirus cases had been confirmed worldwide, resulting in over 4,6 million deaths [10]. The WHO has declared COVID-19 a Public Health Emergency of International Concern [11, 12].

Transmission of SARS-CoV-2 and incubation period

The main mode of transmission of SARS-CoV-2 is by person-person contact [13]. The virus is spread between people through minute aerosol droplets created by sneezing, coughing and talking during close contact. Another way a person can become infected with the virus is by surface transmission [14]. This is because the virus can live on surfaces for up to 96 h [13]. The virus is more likely to transmit through people who display symptoms early in the disease; this is known as symptomatic transmission [14]. In addition, there is a high chance of passing the virus without showing any signs and symptoms and this is known as an asymptomatic spread [15].

The virus transmission channel, the amount of virus that enters the host, and the interaction between the virus and the host immune system are all factors that influence the incubation period [16, 17]. According to the WHO and the Centers for Disease Control and Prevention (CDC), the incubation period for SARS-CoV-2 infection is estimated to be 1–14 days, with an average incubation period of around 5–6 days [17, 18]. According to a study conducted in Wuhan, China (January 2020 – February 2020), roughly 97.5% of SARS-CoV-2 infected patients exhibited clinical symptoms after 11.5 days, and the remaining 2.5% in 2.2 days [19]. To note, the incubation period varies from person to person [19]. One of the most serious concerns is that SARS-CoV-2 variants have evolved in huge numbers, causing transmission alterations [20]. However, there is limited information about the incubation duration for specific variants [20]. Therefore, more studies are needed to determine whether novel SARS-CoV-2 mutations affect the incubation period. Furthermore, that is the reason why the WHO still emphasizes the recommended quarantine period of 14 days [21].

Safety measures for SARS-CoV-2 infection in LMICs

According to WHO (2020), the most recommended preventive measures for SARS-CoV-2 infection include social distancing, hand hygiene, using face masks and coughing in the elbow [22]. Implementing the recommended preventive measures in LMICs is a challenge due to unfavorable conditions such as overcrowding in the household, inadequate ventilation in dwellings, ambient and indoor air pollution, lack of clean water supply, refugee settings, the number of persons living on the streets, and poor sanitation [23]. Sanitation is a crucial issue in LMICs because a large number of people, particularly in rural and peri-urban regions, still rely on surface and groundwater sources for their daily water needs [24]. Pit toilets and groundwater are widely used in LMICs, while open defecation near surface water has also been reported as well [25]. The untreated effluent is dumped into the environment, potentially contaminating groundwater and surface resources [26]. As a result, this might partially contribute to the risk of SARS-CoV-2 transmission. Also, to be noted, a few studies reported detecting SARS-CoV-2 in wastewater which has epidemiologic potential and can be used as a backup technique to monitor viral tracking and circulation in places with limited SARS-CoV-2 testing capacity or highly populated regions where door-to-door tracing is difficult. However, in order to improve sensitivity, special

attention must be paid to virus concentration and detection assays [27].

The consequences of lockdown restrictions in LMICs

As a way to curb the spread of the rapidly mutating SARS-CoV-2, countries worldwide enforced strict lockdown restrictions [28]. By April 2020, more than 90 countries were in some form of lockdown. Stay-at-home orders, quarantine, isolation, social distancing, curfews, school and company closures, and travel restrictions are all part of the lockdown regulations [29]. The WHO proposed response to the SARS-CoV-2 outbreak involves personal hygiene, effective contact tracing, and isolation when an individual is infected, to strike a balance between lockdown restrictions and normalcy [30]. If implemented in a timely and comprehensive manner, lockdown can be an effective infection control and prevention mechanism, reducing the risk of virus transmission from person to person and population spread while buying enough time to scale up preventative measures, diagnostic tests, and treatment capability [31]. While the rigorous restrictions associated with lockdowns are effective, they come at a cost: they impose significant social and economic constraints on individuals and groups, particularly in LMICs [31].

Workers in the informal economy are affected the most by the lockdown because they lack social security and access to adequate health care, as well as having lost access to productive assets [32]. Hence, without the means to earn an income during lockdowns, many are unable to feed themselves and their families [33]. Due to border closures, trade barriers, and other restrictions, farmers are unable to access markets, causing a disruption in domestic and international food supply chains as well as limiting access to balanced, healthy, and diverse meals [34]. Therefore, millions of women, children and men's food security have been compromised as a result of breadwinners losing their jobs due to the lockdown in low-income countries, with vulnerable communities such as small-scale farmers and indigenous people being the hardest hit [34].

Attempting to strengthen the economy of several LMICs, various governments have opted to ease lockdown restrictions. Therefore, lockdown restrictions in various countries were relaxed at various periods [35]. Currently, governments throughout the world are struggling to figure out whether and how to relax restrictions while balancing numerous health, social, and economic issues. Premature lifting of lockdown restrictions by allowing businesses to operate, opening schools and higher education institutions, and allowing traveling are among the key factors contributing to the resurgence of SARS-CoV-2 waves [36]. Hence, LMICs should try to learn from previous waves of SARS-CoV-2 infection and try to avoid being caught off guard by more waves of SARS-CoV-2 infection in the future [36]. This means it is crucial to develop methods that are cheaper, simple, and have a quick diagnostic turnaround time to avoid the medical laboratory staff becoming overwhelmed during the future waves.

Challenges of SARS-CoV-2 testing in LMICs

Mass testing is one of the most significant aspects of lowering the SARS-CoV-2 infection rate through early detection of cases for treatment and subsequent cautionary measures such as isolation to prevent death and further virus transmission, respectively [37]. However, identification and monitoring of the SARS-CoV-2 infection cases have been the greatest challenge in the LMICs [37]. In LMICs, SARS-CoV-2 infection testing is problematic due to financial constraints and other factors [38]. These countries have no domestic capacity to manufacture nasopharyngeal swabs, analytical reagents and COVID 19 kits for SARS-CoV-2 testing [39]. With an increase in the number of SARS-CoV-2 infection cases, mass testing becomes disrupted due to a shortage of nasopharyngeal swabs, analytical reagents and COVID 19 kits. It is because buying all of the materials needed to test for SARS-CoV-2 infection is excessively expensive [40]. Furthermore, the cost of Personal Protective Equipment (PPE) has increased since the SARS-CoV-2 outbreak started, with LMICs bearing the brunt of the burden. The prices of surgical masks have increased sixfold, N95 breathing masks have tripled, and gowns have doubled. The problem is supply delivery could take months, and market manipulation is common, with inventories being sold to the highest bidder. This is concerning since healthcare workers rely on personal protective equipment to safeguard themselves and their patients from SARS-CoV-2 infections and the spread of infections. Therefore, doctors, nurses, and other frontline workers in LMICs are severely underequipped to care for SARS-CoV-2 patients because of limited access to equipment including gloves, medical masks, respirators, goggles, face shields, gowns, and aprons [41]. In addition, there are fewer laboratory staff trained for SARS-CoV-2 testing in LMICs. As the number of infection cases increases the laboratory staff becomes overwhelmed, and as a result, diagnostic turnaround time and transmission rates will be increased [40, 41].

Obtaining the best effective vaccine program and uneven access to vaccine programs are two other important concerns in LMICs. Vaccine distribution in the world remains highly unequal, with a majority of the existing supply going to high-income countries (HICs) [42]. Hence, it will take months to years for the COVID-19 vaccine to have an impact against the SARS-CoV-2 in LMICs. As a result, this is concerning because the

vaccine program was supposed to be the way out of this crisis [43]. In 2021, millions of people in LMICs would be denied access to the COVID-19 vaccine due to wide disparities in COVID-19 vaccine access between HICs and LMICs [43]. As a consequence, the outbreak may be prolonged, increasing the risk of additional mutation and reducing the efficacy of current vaccines. Therefore, LMICs need to come up with innovative approaches to fight this contagious virus [20].

The major concern is how will LMICs deal with the SARS-CoV-2 pandemic? As a result, using publicly available data from Worldometer COVID-19 [10], this review paper will compare the total number and frequencies of SARS-CoV-2 testing in LMICs and HICs, as well as discussing possible interventions and cost-effective measures to increase testing capability in LMICs.

Literature review and data sourcing

Article search strategies, inclusion and exclusion criteria and data sourcing for the study is presented in the Prisma flow diagram (Fig. 1).

Results and overall findings

Table 1, Figs. 2 and 3 provide a comparison of the total number and frequencies of SARS-CoV-2 testing in each income group (low, middle, and high) and continent. The data in Table 1, Figs. 2 and 3 shows that high-income countries have undertaken 10 times more SARS-CoV-2 testing compared to LMICs [10]. More than 100% of the population in HIC (USA: 192%, Australia: 146%, Switzerland: 124% and Canada: 113%) has been tested for SARS-CoV-2, whereas only 27,5% of the population in middle-income countries (MIC) (Vietnam: 43%, South Africa: 29%, Brazil: 27% and Venezuela: 12%) and approximately 3% of the population in low-income countries (LIC) (Bangladesh: 6%, Uganda: 4% and Nigeria: 1%) has been tested.

The possible reasons for under-testing for SARS-CoV-2 infection in LMICs are probably many people are unable to afford SARS-CoV-2 testing due to financial restrictions, unstable health systems and reliance on global supply chains. As a result, many positive cases are simply missed, putting LMICs at higher risk of spreading the virus [31]. The pandemic will easily shatter the poor health system and overburden hospitals and clinical services if effective prevention is not implemented [45].

Possible cost-effective strategies to increase testing capability in LMICs

The management of SARS-CoV-2 infection cases entails early detection of the virus and prompt isolation as a result, which will aid in the prevention and control of virus spread [46]. Using cost-effective approaches such as saliva instead of oropharyngeal or nasopharyngeal swabs, sample pooling, testing high-priority groups and using antigen rapid tests can help to increase the number of mass testing in LMICs.

Saliva sample for SARS-CoV-2 infection testing

The recommended sample type for SARS-CoV-2 detection is nasopharyngeal and/ or oropharyngeal swabs [47]. The problem with nasopharyngeal and oropharyngeal swabs is that it makes patients uncomfortable, forcing them to cough during sample collection and

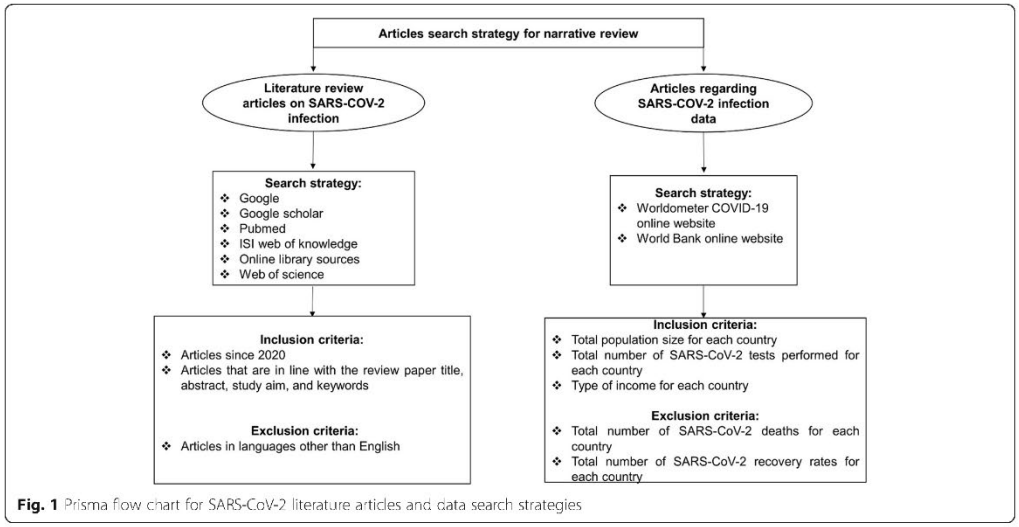

Fig. 1 Prisma flow chart for SARS-CoV-2 literature articles and data search strategies

Table 1 Comparison between the total number of tests (in million) performed and the total population (in million) in high-income countries and LMICs

Continent	Countries	Income	Total Tests Performed (million)	Total Population (million)	Percentage of Tests performed (%)
North America	USA	High	639,832,856	333,416,037	192[1]
Oceania	Australia	High	37,832,547	25,854,460	146[1]
Europe	Switzerland	High	10,796,404	8,733,303	124[1]
North America	Canada	High	43,215,201	38,153,447	113[1]
Europe	Germany	High	73,348,901	84,117,156	75
Asia	Vietnam	Middle	42,517,091	98,427,082	43
Africa	South Africa	Middle	17,649,727	60,237,549	29
South America	Brazil	Middle	57,282,520	214,437,809	27
South America	Venezuela	Middle	3,359,014	28,335,663	12
Asia	Bangladesh	Low	9,704,722	166,728,314	6
Africa	Uganda	Low	1,680,863	47,529,564	4
Africa	Nigeria	Low	2,997,060	212,473,029	1

Data were retrieved from the Worldometer Covid 19 on 30 September 2021 [10]. % Tests performed = (Total Tests/Total Population) * 100). The data on various types of income for each country was obtained from the World Bank online site [44]. [1] Excessive SARS-CoV-2 testing is indicated by percentage values above 100 (SARS-CoV-2 tests performed more than the actual population)

exposing health care workers to the high risk of infection. Furthermore, nasopharyngeal and oropharyngeal swabs are expensive because the sample has to be collected by trained health care personnel wearing PPE [48]. Therefore, the nasopharyngeal and oropharyngeal swab is not an ideal sample to utilize for monitoring SARS-CoV-2 load. Therefore, saliva could be used as an alternative sample for SARS-CoV-2 testing and viral load monitoring, due to its numerous advantages [49].

Fig. 2 The number of SARS-CoV-2 tests (%) performed in high-income and middle-low-income countries and these samples represents counties of each income group. High-income countries are highlighted in red, middle-income countries are highlighted in yellow, and low-income countries are highlighted in green [10]. High-income countries (USA, Switzerland, Australia, Canada, Germany). Middle-income countries (Venezuela, Vietnam, South Africa, Brazil). Low-income countries (Bangladesh, Uganda, Nigeria)

Saliva is a transparent biofluid generated by the salivary glands that clean and protects the oral cavity, has antibacterial properties, and aids in food digestion [50]. Angiotensin-converting enzyme 2 (ACE-2) has been identified as the principal host cell receptor of SARS-CoV-2, and it is thought to play a key role in the virus's entry into the cell and subsequent infection. The ACE-2 receptor is highly expressed in the salivary gland and oral mucosa, [51] ACE2-positive cells in the salivary glands are likely to be SARS-CoV-2 target cells [52]. Furthermore, the presence of SARS-CoV-2 in saliva could be due to the mixing of upper and lower respiratory tract fluid that conveys the virus to the saliva. These findings imply that the salivary gland and oral mucosa could be a high-risk source site for SARS-CoV-2 infection [52]. Hence, this is what makes saliva a suitable specimen for testing SARS-CoV-2 infection.

Saliva could be utilized as a diagnostic sample for detecting SARS-CoV-2 and monitoring viral load [53]. Patients collect their own samples, which has several benefits, including the elimination of health care staff and the elimination of PPE for sample collection. The time, cost, and risk of viral transmission associated with sample collection are reduced, allowing for increased SARS-CoV-2 mass testing [53, 54]. Furthermore, saliva can be utilized efficiently in large organizations such as universities since PPE is not required, and this could help to lower the danger of viral transmission. Hence, the addition of saliva testing for SARS-Cov-2 infection will allow universities to test thousands of students and staff, with the aim that the results will aid in keeping campuses safe. As a result, saliva testing, in addition to wearing a face mask and maintaining social distance, is an innovative option [55]. However, less attention has

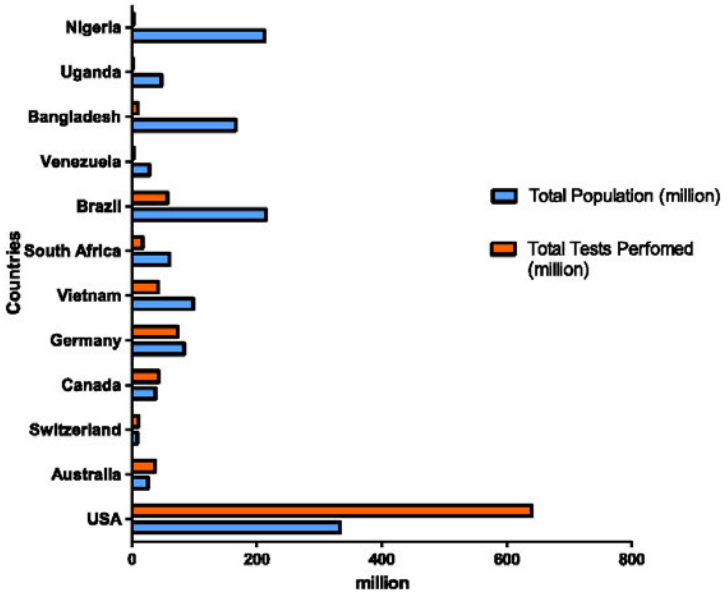

Fig. 3 Comparison between the total population size (million) and the total number of SARS-CoV-2 tests performed (million) in each country [10]. High-income countries (USA, Switzerland, Australia, Canada, Germany). Middle-income countries (Venezuela, Vietnam, South Africa, Brazil). Low-income countries (Bangladesh, Uganda, Nigeria)

been given to its potential usefulness in testing and monitoring for SARS-CoV-2 infection [54].

Sample pooling

The gold standard for diagnosing SARS-CoV-2 infection is reverse-transcription polymerase chain reaction (RT–PCR), a molecular method [56]. Real-time PCR is precise, but it is expensive to test each individual regularly [57]. Therefore, high prices limit affordability for many people, particularly in LMICs. The cost savings can be achieved by pooling samples [57, 58].

The principle of sample pooling allows multiple samples to be mixed and tested as a single sample [59]. When using a pooling method and the pooled test result is negative, each batch component is treated as if it were analyzed separately. Individual testing is required only when the pool test results are positive [59]. Sample pooling testing should be recommended for asymptomatic suspected cases, excluding those who are symptomatic [60]. This method is advantageous because it is cost-effective and allows for increased mass testing for SARS-CoV-2 without compromising testing accuracy or wasting consumables such as analytical reagents and extraction kits [61]. As a consequence, this technique improves testing efficiency by reducing the backlog of SARS-CoV-2 pending tests while also enhancing diagnostic turnaround time, which is one of the most important elements in managing and controlling the SARS-CoV-2 outbreak [62]. The pooling technique will be extremely advantageous in a laboratory with limited resources because this type of testing is more feasible and less expensive for mass screening in a large community [63].

Prioritized groups for testing of SARS-CoV-2 infection

It is critical to have a quick and accurate strategy for detecting and controlling SARS-CoV-2 outbreaks in communities and hospitals in LMICs [64]. In LMICs, prioritizing certain individuals for testing of SARS-CoV-2 infection should be considered. This testing strategy will help to accommodate the countries with limited resources by prioritizing individuals according to their categories of urgent clinical need while trying to reduce a backlog of pending testing [65]. Testing becomes the highest priority when it contributes to improving clinical outcomes and decreasing the transmission rate of the virus [66].

When prioritizing groups, the most important factors to consider are the size of each group, the number of tests needed, and the number of tests available. Hence,

the most critical groups should be tested first. As testing becomes more generally available, it should be gradually spread to other groups based on their priorities. Additionally, those who tested positive for SARS-CoV-2 infection will need to undergo further testing [65].

A list of priority groups for SARS-CoV-2 testing in the private and public sectors is as follows: (i) Symptomatic patients, regardless of their age or underlying health issues, should be given the highest priority to reduce the risk of nosocomial transmission and protect health care staff and the general public. (ii) People who had contact with people who had tested positive for SARS-CoV-2 infection, whether asymptomatic or symptomatic, in order to quickly identify patients at high risk of complications and ensuring that the required precautions are taken. (iii) SARS-CoV-2 testing should also be prioritized for healthcare workers, frontline responders, essential critical infrastructure workers, miners, travelers, people going for surgery, testing pregnant women who are admitted at the labor ward and post-mortem testing, regardless of whether they are asymptomatic, to prevent a possible spread in the community and at work. (iv) If resources are available, testing for non-essential workers may be permitted [67, 68]. The most important thing to note is that healthy people who have not been tested should continue to practice social distance and wearing masks as recommended by their local and state health authorities [69, 70].

Antigen rapid test as a screening test for SARS-CoV-2 infection in LMIC

As the world continues to wrestle with SARS-CoV-2 infections, the number of cases in LMIC are increasing, causing national economies to lock down and putting further strain on already struggling economies [71]. As a result, the antigen rapid test can be used as alternative strategy for SARS-CoV-2 infection in LMIC. Antigen rapid tests have the advantage of providing results in 15–30 min instead of in hours or days, allowing mass testing to be increased, especially in LMICs with limited laboratory facilities or qualified health professionals to do molecular (PCR) tests [72]. The antigen rapid test allows healthcare workers to quickly identify individuals who are infected with SARS-CoV-2, so they be isolated and treated while their contacts are tracked to prevent the virus from spreading to their families and communities. In this case of a SARS-CoV-2 outbreak, where the test turnaround time is crucial, antigen rapid tests play an important role in delivering early results [73].

While governments are increasingly relying on less expensive antigen rapid tests to increase SARS CoV-2 infection testing coverage, however, the test may have low sensitivity [74]. It's critical to confirm an antigen test result with a PCR test, especially if the result of the antigen rapid test contradicts the clinical setting. Therefore, PCR tests remain the gold standard, and their value remains high [73, 75]. To be noted antigen rapid tests are typically used on symptomatic individuals since they perform best in symptomatic individuals and within a particular number of days of symptom onset [74]. By adopting this alternative strategy for mass testing of SARS-CoV-2 infection, LMICs can spend less money on diagnostics and more money on essential medical equipment for hospitals treating SARS-CoV-2 infected patients, resulting in more lives saved [72].

Conclusion and implication for future research

In conclusion, as the number of reported cases rises, the pandemic's long-term effect on individuals and

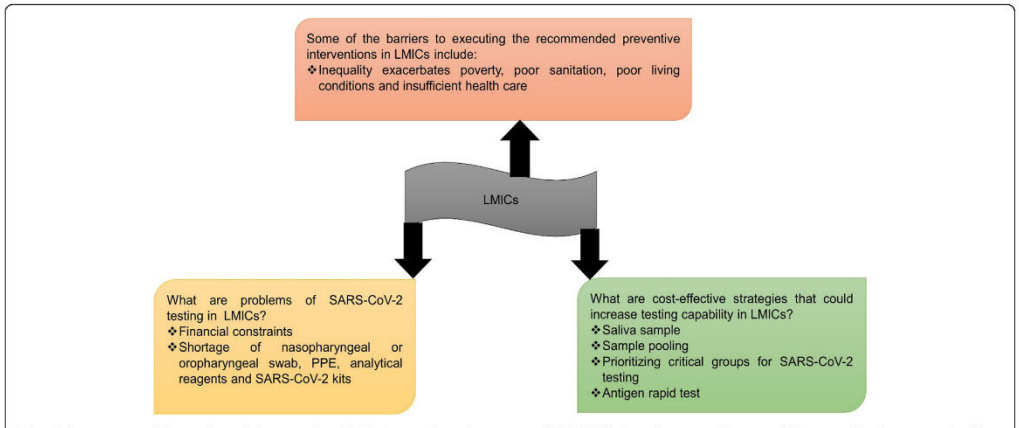

Fig. 4 A summary of the major challenges that LMICs have when it comes to SARS-CoV-2 testing, as well as possible cost-effective strategies for increasing mass testing

populations in LMICs remains unknown. Moreover, the provision of a specific, effective vaccine to the people in LMICs is still a challenge. With an ongoing, unprecedented outbreak of SARS-CoV-2, the importance of laboratory detection of human coronavirus infections has been emphasized around the world in order to prevent the spread of the infection and properly treat those individuals who have a serious infection. However, due to weak health systems and poverty, LMICs are finding it difficult to manage the SARS-CoV-2 outbreak. This paper highlights the importance of developing alternative strategies for SARS-CoV-2 mass testing that are simple and cost-effective in a resource-constrained setting, and the summary is illustrated in Fig. 4.

For future research, the goal is to evaluate alternative methods that are simple and cheaper, with fast turnaround time and have a high throughput for a resource-constrained laboratory, so that they can be implemented to facilitate mass testing for SARS-CoV-2 infection in LMICs. Furthermore, such research will have a good impact on the development of a common pricing standard for SARS-CoV-2 kits in LMICs.

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Authors' contributions
ZD, AAC, VR and ZLMK have contributed to manuscript conceptualization, review and editing in preparation for submission. ZD wrote the manuscript. VE, PN, MNMM, KNB, NN, RP and RS edited the manuscript in preparation for submission. All the named authors have read and approved the manuscript for submission.

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Availability of data and materials
All the data reported in this review was retrieved from the publicly available original sources.

Declarations

Ethics approval and consent to participate
Not applicable.

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Publisher's Note

APPENDIX B

Published manuscript from Chapter Four

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Article

Evaluation of Various Alternative Economical and High Throughput SARS-CoV-2 Testing Methods within Resource-Limited Settings

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Abstract: The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak posed a challenge for diagnostic laboratories worldwide, with low-middle income countries (LMICs) being the most affected. The polymerase chain reaction (PCR) is the gold standard method for detecting SARS-CoV-2 infection. However, the challenge with this method is that it is expensive, which has resulted in under-testing for SARS-CoV-2 infection in many LMICs. Hence, this study aimed to compare and evaluate alternative methods for the mass testing of SARS-CoV-2 infection in laboratories with limited resources to identify cost-effective, faster, and accurate alternatives to the internationally approved kits. A total of 50 residual nasopharyngeal swab samples were used for evaluation and comparison between internationally approved kits (Thermo Fisher PureLink™ RNA Isolation Kit and Thermo Fisher TaqPath™ COVID-19 Assay Kit) and alternative methods (three RNA extraction and four commercial SARS-CoV-2 RT-PCR assay kits) in terms of the cost analysis, diagnostic accuracy, and turnaround time. In terms of performance, all of the alternative RNA extraction methods evaluated were comparable to the internationally approved kits but were more cost-effective (Lucigen QuickExtract™ RNA Extraction Kit, Bosphore EX-Tract Dry Swab RNA Solution and Sonicator method) and four commercial SARS-CoV-2 RT-PCR assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES™ COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit) with a sensitivity range of 76–100% and specificity of 96–100%. The cost per sample was reduced by more than 50% when compared to internationally approved kits. When compared to the Thermo Fisher PureLink™ Kit and Thermo Fisher TaqPath™ COVID-19 Assay Kit, the alternative methods had a faster turnaround time, indicating that laboratories with limited resources may be able to process more samples in a day. The above-mentioned cost-effective, fast, and accurate evaluated alternative methods can be used in routine diagnostic laboratories with limited resources for mass testing for SARS-CoV-2 because these were comparable to the internationally approved kits, Thermo Fisher PureLink™ Kit and Thermo Fisher TaqPath™ COVID-19 Assay Kit. The implementation of alternative methods will be the most cost-effective option for testing SARS-CoV-2 infection in LMICs.

Keywords: SARS-CoV-2; diagnostic testing; low-middle income countries; resource-limited settings; alternative cost-effective and high throughput testing approaches

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1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (COVID-19) [1], has spread globally since its first recorded outbreak in Wuhan, China in December 2019 [2,3]. As of 8 August 2022, there have been over 589 million confirmed cases and 5.4 million deaths worldwide [4].

Diagnostic testing remains critical in controlling the SARS-CoV-2 outbreaks, allowing patients to be cared for while also simultaneously providing decision-makers with critical information for test-trace isolation programs [5,6]. Most countries have experienced increased demand for SARS-CoV-2 diagnostic testing, with some countries unable to meet the demand. This is one of the major challenges, especially in low-middle income countries (LMICs) where unstable health systems and reliance on global supply chains have frequently prevented people from accessing critical tests for detecting SARS-CoV-2 infection [7,8]. In many LMICs, insufficient testing may have resulted in an underestimation of SARS-CoV-2 infections. What is concerning is that the pandemic's long-term impact on individuals and communities in LMICs remains uncertain as the number of confirmed cases continues to increase [9].

SARS-CoV-2 is an enveloped virus with a single positive-sense RNA genome [1]. Furthermore, the SARS-CoV-2 genome contains non-structural open reading frames (ORF1ab), which are polypeptide coding genes that are translated from genomic RNA [10–12]. It also contains four structural proteins, namely, the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins that contribute to the SARS-CoV-2 overall structure [13,14]. SARS-CoV-2 infection can be detected using two different types of tests: real-time reverse transcription polymerase chain reaction (RT-PCR) and antigen rapid tests [15,16]. Antigen rapid tests are less expensive and provide results faster than RT-PCR. However, antigen rapid tests, on the other hand, are less accurate in detecting SARS-CoV-2 infection, especially in asymptomatic individuals and those with low SARS-CoV-2 viral load [17–19]. According to a Cochrane systematic review of 22 antigen rapid test trials for detecting SARS-CoV-2 infection, the antigen rapid test showed an average sensitivity of 56.2% [20]. Furthermore, antigen rapid testing was found to have a higher risk of false negatives than molecular RT-PCR tests, with some evidence indicating false negative rates as high as 50%, therefore, a confirmatory RT-PCR test is still recommended [20,21].

The RT-PCR assay is the most accurate test for detecting SARS-CoV-2, hence it is regarded as a gold standard diagnostic procedure for diagnosing SARS-CoV-2 infection [22,23]. The *N* gene, *S* gene, *E* gene, and *ORF1ab* gene are the most tested target genes for SARS-CoV-2 infection using the RT-PCR assay [24,25]. The RT-PCR assay has superior sensitivity and specificity in comparison to antigen and antibody rapid tests, however, it is highly specialized and expensive, especially for LMICs. This is partly due to a lack of local capacity in these countries to produce their analytical instrument and reagents for RT-PCR-based SARS-CoV-2 testing [8,23]. As a result, mass testing for SARS-CoV-2 infection in LMICs poses a challenge. Of note, high-income countries (HICs) test more samples daily to control the spread of the SARS-CoV-2 virus, whereas LMICs test fewer samples due to financial constraints [26]. It is therefore essential to evaluate the alternative methods for SARS-CoV-2 testing that are simple, cost-effective, and produce high throughput results in a short period of time in a laboratory within a resource-limited setting.

This study aimed to compare and evaluate whether alternative RNA extraction methods (Lucigen QuickExtract™ RNA Extraction Kit, Bosphore EX-Tract Dry Swab RNA Solution, and Sonicator method) are cost-effective, faster, and have clinical accuracy comparable to an internationally approved kit (Thermo Fisher PureLink™ Kit, Pleasanton, CA, USA) and to also assess whether these extraction methods can be used interchangeably. Furthermore, the performance of commercially available RT-PCR SARS-CoV-2 assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTESTM COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit) was compared to the internationally approved kit (Thermo Fisher TaqPath™ COVID-19 Assay Kit) in terms of the sensitivity, specificity, costs, and turnaround times.

2. Results

In the first part of this study, the Thermo Fisher PureLink™ Kit was compared to three alternative RNA extraction methods. In the second part of this study, the Thermo Fisher TaqPath™ COVID-19 Assay Kit was compared to four alternative commercially available RT-PCR SARS-CoV-2 assay kits. The overall aim of this study was to assess and compare the clinical performance of these methods as well as determine whether they could be used interchangeably to help increase the testing capacity for SARS-CoV-2 infection in LMICs.

2.1. Comparison of RNA Extraction Methods

The multiplex Thermo Fisher TaqPath™ COVID-19 Assay Kit was used to assess the RNA extraction efficiency of each extraction method. For the positive (infected) group, the efficiency of the RNA extraction methods was assessed by comparing the mean Ct values of the SARS-CoV-2 targeting genes (*N*, *S*, *ORF1ab*), and the internal control (*MS2*) between the alternative RNA extraction methods and the Thermo Fisher PureLink™ Kit. The efficiency of the RNA extraction methods was assessed for the control (uninfected) group by comparing the mean Ct values of the internal control (*MS2*) between the alternative RNA extraction methods and the Thermo Fisher PureLink™ Kit. The PCR was run in triplication for each sample for the purpose of method comparison, and the average Ct value result was used for each sample. The results were considered positive if the cycle threshold value (Ct value) for all three target genes for SARS-CoV-2 and the internal control were less than 40 ($Ct \leq 40$). When all of the SARS-CoV-2 target genes were negative and the internal control was positive ($Ct \leq 40$), the results were considered negative. When the internal control was negative, the results were considered invalid due to the inefficacy of the RNA extraction method.

The current results showed that all the RNA extraction methods could be used to extract high-quality RNA for the testing of SARS-CoV-2 infection because the Ct values of SARS-CoV-2 targeting genes (*N*, *S*, *ORF1ab*), and the internal control (*MS2*) for all RNA extraction methods were less than 35 ($Ct \leq 35$) (Table 1). There was also no significant difference in the mean Ct values ($p \leq 0.05$ **) between the alternative RNA extraction methods (Lucigen QuickExtract™ RNA Extraction Kit (Parmenter St Middleton, WI, USA), Bosphore EX-Tract Dry Swab RNA (New Ash Green Longfield, England) and Sonicator method (Europe) and the internationally approved kit (Thermo Fisher PureLink™ Kit) for the SARS-CoV-2 targeting genes (*N*, *S*, *ORF1ab*) and internal control (*MS2*) (Table 1 and Figure 1A–D). However, the results of one of the alternate RNA extraction methods, the Sonicator method, showed that there was a statistically significant difference ($p \leq 0.05$) in Ct values between the Sonicator method and the Thermo Fisher PureLink™ Kit for the mean Ct value of the *ORF1ab* gene (Table 1 and Figure 1C).

2.2. Comparison of Sensitivity and Specificity for RNA Extraction Methods

Based on the small sample size, caution should be taken when using this study's sensitivity and specificity results for the method comparison. The accuracy of each RNA extraction method used in testing SARS-CoV-2 infection was assessed by comparing the calculated sensitivity and specificity of alternative RNA extraction methods to the internationally approved kit. In a total of 25 nasopharyngeal swab samples for the positive (infected) group, the results of 25 positive samples extracted using two alternative extraction methods (Bosphore EX-Tract Dry Swab RNA Solution and Lucigen QuickExtract™ RNA Extraction Kit) matched the results of 25 positive samples extracted using the Thermo Fisher PureLink™ Kit. However, only 24 of the 25 positive nasopharyngeal swab sample results extracted using the Sonicator method (alternative RNA extraction method) matched the 25 positive sample results extracted using the Thermo Fisher PureLink™ Kit, with one sample result being invalid.

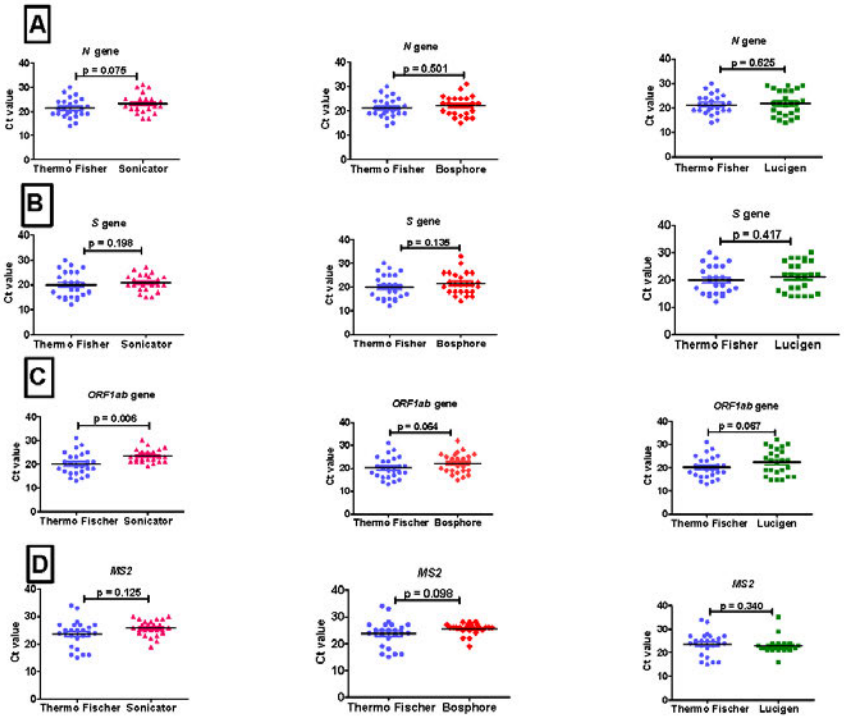

Figure 1. The Ct values were compared between the internationally approved kit (Thermo Fisher PureLink™ Kit) and alternative extraction methods (Sonicator method, Bosphore EX-Tract Dry Swab RNA Solution, and Lucigen QuickExtract™ RNA Extraction Kit) for the SARS-CoV-2 target genes: *N* gene (**A**), *S* gene (**B**), *Orf* gene (**C**), and MS2 internal control (**D**). The results with a level of $p \leq 0.05$ were considered significant. Thermo Fisher PureLink™ Kit is represented in blue, Sonicator method in pink, Bosphore EX-Tract Dry Swab RNA Solution in red, and Lucigen QuickExtract™ RNA Extraction Kit in green.

Additionally, in a total of 25 nasopharyngeal swab samples for a control group, all 25 negative samples extracted using alternative RNA extraction methods matched the 25 negative sample results that were extracted using the Thermo Fisher PureLink™ Kit. The sensitivity ranged from 96 to 100% for all alternative RNA extraction methods, while the specificity was 100% (Table 2).

Table 1. Performance of the RNA extraction methods in testing SARS-CoV-2 infection for the positive (infected) group.

Target Genes for SARS-CoV-2 and Internal Control	Name of Extraction Method	N	Ct Mean Value (SD)	Difference between Means	*p*-Value
N gene	Thermo Fisher PureLink™ Kit	25	21.32 (±3.966)		
	Sonicator method	24	23.16 (±3.716)	−1.840	$p = 0.075$
	Bosphore EX-Tract Dry Swab RNA Solution	25	22.04 (±3.963)	−0.720	$p = 0.501$
	Lucigen QuickExtract™ RNA Extraction Kit	25	21.88 (±5.036)	−0.560	$p = 0.625$
S gene	Thermo Fisher PureLink™ Kit	25	19.96 (±5.111)		
	Sonicator method	24	20.84 (±3.197)	−0.880	$p = 0.198$
	Bosphore EX-Tract Dry Swab RNA Solution	25	21.44 (±4.647)	−1.480	$p = 0.135$
	Lucigen QuickExtract™ RNA Extraction Kit	25	21.16 (±5.421)	−1.200	$p = 0.417$
ORF1ab gene	Thermo Fisher PureLink™ Kit	25	20.12 (±4.702)		
	Sonicator method	24	23.40 (±2.646)	−3.280	$p = 0.006$ **
	Bosphore EX-Tract Dry Swab RNA Solution	25	22.16 (±4.249)	−2.040	$p = 0.064$
	Lucigen QuickExtract™ RNA Extraction Kit	25	22.36 (±5.469)	−2.240	$p = 0.067$
MS2 (Internal control)	Thermo Fisher PureLink™ Kit	25	23.72 (±4.912)		
	Sonicator method	24	25.76 (±2.818)	−2.040	$p = 0.125$
	Bosphore EX-Tract Dry Swab RNA Solution	25	25.56 (±2.043)	−1.840	$p = 0.098$
	Lucigen QuickExtract™ RNA Extraction Kit	25	22.92 (±3.290)	+0.800	$p = 0.340$

Difference between means = mean Ct value of the Thermo Fisher PureLink™ Kit—mean Ct value of each alternative extraction method. ** It showed a statistically significant difference in method comparison.

Table 2. The sensitivity and specificity of alternative RNA extraction methods.

Name of Extraction Method	Sensitivity ($n = 25$)	Specificity ($n = 25$)
Sonicator method	24 (96%)	25 (100%)
Bosphore EX-Tract Dry Swab RNA Solution	25 (100%)	25 (100%)
Lucigen QuickExtract™ RNA Extraction Kit	25 (100%)	25 (100%)

2.3. Overview and Comparison of the Commercially Available SARS-CoV-2 RT-PCR Assay Kits

According to the manufacturer's guidelines for each kit, the test results were considered positive when all of the SARS-CoV-2 target genes and internal control used in the particular kit were detected at the same time ($Ct \leq 40$ or $Ct < 35$). When all of the SARS-CoV-2 target genes were negative ($Ct > 40$) and the internal control was positive ($Ct \leq 40$ or $Ct < 35$), the results were considered negative. None of the manufacturers were involved in the analysis and interpretation of the results. Table 3 summarizes the requirements for all of the commercially available RT-PCR SARS-CoV-2 test kits used to detect the SARS-CoV-2 target genes, as specified in the documentation for each RT-PCR SARS-CoV-2 assay kit.

Table 3. Overview of the commercially available RT-PCR assay kits used to detect the SARS-CoV-2 target genes.

SARS-CoV-2 Assay Kit	Catalog Number	Target Genes for Detection of SARS-CoV-2	Internal Control	Results Interpretation
Thermo Fisher TaqPath™ COVID-19 Assay Kit	A51738	*ORF1ab* *N* gene *S* gene	*MS2*	Ct ≤ 40 Positive result
Nucleic Acid COVID-19 Test Kit (SARS-CoV-2)	1006524-T	*N* gene *ORF1ab*	*RNase P*	Ct ≤ 40 Positive result
abTES™ COVID-19 qPCR I Kit	BN300142	*NS1* *NS2*	*GAPDH*	Ct ≤ 40 Positive result
PCL COVID-19 Speedy RT-PCR kit	MD02	*N* gene *E* gene	*RNase P*	Ct < 35 Positive result
PCLMD nCoV One-Step RT-PCR Kit	MD01E	*N* gene	IC	Ct < 35 Positive result

This study compared the calculated sensitivity and specificity of the alternative commercial RT-PCR assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES™ COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit) to the internationally approved kit (Thermo Fisher TaqPath™ COVID-19 Assay Kit). The sensitivity and specificity of the evaluated commercially available RT-PCR SARS-CoV-2 assay kits varied, with the PCLMD nCoV One-Step RT-PCR Kit having the highest sensitivity and specificity (96% and 100%, respectively) and the abTES™ COVID-19 qPCR I Kit having the lowest sensitivity and specificity (76% and 96%, respectively). The results are presented in Table 4.

Table 4. Comparison of the sensitivity and specificity of the commercial SARS-CoV-2 RT-PCR assay kits.

Name of the Kits	Number of Positive Samples Detected (n = 25)	False Negative	Sensitivity (%)	Number of negative Samples Detected (n = 25)	False Positive	Specificity (%)
Nucleic Acid COVID-19 Test Kit (SARS-CoV-2)	22	3	88	25	0	100
abTES™ COVID-19 qPCR I Kit	19	6	76	24	1	96
PCL COVID-19 Speedy RT-PCR Kit	23	2	92	25	0	100
PCLMD nCoV One-Step RT-PCR Kit	24	1	96	25	0	100

2.4. Cost Analysis, Simplicity and the Turnaround Time for Each Evaluated Method and Internationally Approved Kits

The costs of the RNA extraction methods and commercially available RT-PCR SARS-CoV-2 assay kits were calculated and compared using the pricing of the reagents, consumables, and equipment. The processing time for each RNA extraction method and the observed run time for each commercial RT-PCR test kit were calculated to determine the turnaround time for each RNA extraction method and the commercially available RT-PCR SARS-CoV-2 assay kits. The prices for the RNA extraction methods and commercially available RT-PCR SARS-CoV-2 assay kits are specific to South Africa.

The results in Table 5 show that the RNA extraction methods varied in terms of cost, processing time, and procedure simplicity. The Thermo Fisher PureLink™ Kit was the most expensive and had a per sample cost of USD ~2.96. The Sonicator method (an alternative RNA extraction method) was the least expensive, with a cost per sample of USD ~0.18. The

processing time results for each RNA extraction method were as follows: Thermo Fisher PureLink™ Kit (~1 h); Sonicator method (~30 min); Bosphore EX-Tract Dry Swab RNA Solution (~15 min); Lucigen QuickExtract™ RNA Extraction Kit (~15 min). The Bosphore EX-Tract Dry Swab RNA Solution and Lucigen QuickExtract™ RNA Extraction Kit were considered to be the two simplest methods (three steps each) for extracting RNA nucleic acid used for testing SARS-CoV-2 infection.

Table 5. Comparison of the RNA extraction methods based on their simplicity cost and the observed run time for each method.

Name of Extraction Method	Simplicity	Approximate (~) Observe Run Time	* Cost per Sample
Thermo Fisher PureLink™ Kit	5 steps: Vortexing Sample lysis (Proteinase K buffer) Binding beads Washing (1st, 2nd, 3rd) Elution	~1 h	USD ~2.96
Sonicator method	4 steps: Vortexing Heating at 65 °C Sonicate at 65 °C Centrifuge	~30 min	USD ~0.18
Bosphore EX-Tract Dry Swab RNA Solution	3 steps: Vortexing Adding sample to buffer Heating sample at 95 °C	~15 min	USD ~0.89
Lucigen QuickExtract™ RNA Extraction Kit	3 steps: Vortexing Adding sample to buffer Heating sample at 95 °C	~15 min	USD ~0.59

* The cost per sample for the RNA extraction methods includes the extraction kit, reagents, consumables and excludes equipment. The ZAR to USD exchange rate: ZAR 1 = USD 0.0592 [27].

The results of the cost analysis for each commercially available RT-PCR SARS-CoV-2 assay kit showed that all alternative commercial RT-PCR SARS-CoV-2 assay kits were the least expensive when compared to the Thermo Fisher TaqPath™ COVID-19 Assay Kit. The results of the cost of the commercially available RT-PCR SARS-CoV-2 assay kits are presented in Table 6. Additionally, the results indicated that there was a slight difference in the observed run time between the Thermo Fisher TaqPath™ COVID-19 Assay Kit and the three commercial SARS-CoV-2 RT-PCR assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES™ COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit). The observed run times between the Thermo Fisher TaqPath™ COVID-19 Assay Kit (64 min) and the PCLMD nCoV One-Step RT-PCR Kit (137 min), one of the alternative commercial SARS-CoV-2 RT-PCR assay kits, were, however, significantly different (Table 6).

Table 6. Overview of the commercially available RT-PCR assay kits that were evaluated in terms of the running time, costs, and the number of SARS-CoV-2 target genes.

Name of SARS-CoV-2 Assay Kits	Running Time PCR (min)	* Cost per Sample
Thermo Fisher TaqPath™ COVID-19 Assay Kit	~64 min	USD ~14.80
Nucleic Acid COVID-19 Test Kit (SARS-CoV-2)	~83 min	USD ~4.44
abTES™ COVID-19 qPCR I Kit	~79 min	USD ~9.83
PCL COVID19 Speedy RT-PCR Kit	~ 62 min	USD ~7.11
PCLMD nCoV One-Step RT-PCR Kit	~137 min	USD ~8.88

* The cost per sample includes the commercially available RT-PCR SARS-CoV-2 assay kits reagents, consumables, and sample processing and excludes equipment. The ZAR to USD exchange rate: ZAR 1 = USD 0.0592 [27]. Overall running time PCR includes the ramp time and run cycles for each RT-PCR assay kit.

3. Discussion

As countries around the world continue to search for effective treatment and eradication of the SARS-CoV-2 virus, diagnostic testing is still one of the most effective ways to track the spread of the virus and subsequently implement appropriate preventative measures [28,29]. Many factors, particularly financial and infrastructural resources, limit the quantum of testing in LMICs. The main goal of this study was to evaluate the cost-effective, accurate, and faster alternative methods that could assist in increasing the testing capacity for SARS-CoV-2 infection in laboratories within limited-resource settings. Results showed that the alternative RNA extraction methods (Sonicator method, Lucigen QuickExtract™ RNA Extraction Kit, and Bosphore EX-Tract Dry Swab RNA Solution) were qualitatively comparable to the internationally approved kit Thermo Fisher PureLink™ Kit. Likewise, the performance characteristics of the alternative commercial RT-PCR SARS-CoV-2 assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES™ COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit) were as follows: (i) sensitivity (76–96%); (ii) specificity (96–100%); (iii) negative predictive value (4–24%); and (iv) positive predictive value (4%).

The efficiency of each RNA extraction method was assessed by comparing the performance of alternative RNA extraction methods to the Thermo Fisher PureLink™ Kit (internationally approved kit). In the present study results, there was no statistically significant difference in the mean Ct value between the Thermo Fisher PureLink™ Kit and the alternative extraction methods (Ct value $\leq$ 35. All of the alternative RNA extraction methods had high specificity (100%) and sensitivity (96–100%). The statistically significant difference in the Ct value for the *ORF1ab* gene between the Sonicator extraction method and the Thermo Fisher PureLink™ Kit had no appreciable effect on the Sonicator extraction method's high sensitivity. The results showed that the efficiency and recovery rates of the alternative RNA extraction methods were satisfactory to be used for RNA extraction in testing for the SARS-CoV-2 infection.

When the cost-effectiveness, processing time, and simplicity of each RNA extraction method were compared, it was found that both the Bosphore EX-Tract Dry Swab RNA Solution and Lucigen QuickExtract™ RNA Extraction Kit were simpler, faster, accurate, and cheaper, despite the fact that neither of these extraction methods had any inhibitor removal. These two alternative methods had three procedural steps and took approximately 15 min to extract RNA from 25 nasopharyngeal samples compared to the Thermo Fisher PureLink™ Kit, which had five procedural steps and took approximately an hour to process 25 nasopharyngeal samples. The Thermo Fisher PureLink™ Kit was the most expensive (Table 5). Despite being more time-consuming, the Sonicator method was the cheapest of the two alternative RNA extraction methods (Bosphore EX-Tract Dry Swab RNA Solution and Lucigen QuickExtract™ RNA Extraction Kit) and the internationally approved kit (Thermo Fisher PureLink™ Kit). When comparing the cost of the Sonicator method (USD ~0.18 per sample) to the Thermo Fisher PureLink™ Kit (USD ~2.96 per sample), there was more than a 94% price reduction with Sonicator methods, making the Sonicator method the cheapest method. Hence, the Sonicator extraction method may be a good choice, especially considering that laboratories in low-income countries (LICs) have limited resources for the mass testing of SARS-CoV-2 infection, and setting up a laboratory for testing SARS-CoV-2 infection in these countries is a challenge. This study recommends that LICs use the Sonicator method. Consequently, SARS-CoV-2 mass testing will be improved, and viral transmission will be optimally monitored for control and reduction.

Viral RNA extraction is necessary for the RT-PCR tests to be performed [30]. The Lucigen QuickExtract™ RNA Extraction Kit was the method of choice for RNA extraction in this study. The extracted RNA was required for the evaluation and comparison of commercial RT-PCR SARS-CoV-2 kits. The Lucigen QuickExtract™ RNA Extraction Kit was chosen due to its simplicity, speed, and low cost. When the clinical accuracy of the alternative commercial RT-PCR SARS-CoV-2 assay kits was compared to the Thermo Fisher TaqPath™ COVID-19 Assay Kit, the study found that all four alternative commercially

available RT-PCR SARS-CoV-2 assay kits had good diagnostic sensitivity ranging from 76–96% and a specificity of 96–100% (Table 5), with the PCLMD nCoV One-Step RT-PCR Kit having superior sensitivity compared to other the three alternative commercially available RT-PCR SARS-CoV-2 assay kits. The high specificity of 100% reported on the manufacturer's package insert for all RT-PCR SARS-CoV-2 assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES™ COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, PCLMD nCoV One-Step RT-PCR Kit, and an internationally approved kit (Thermo Fisher TaqPath™ COVID-19 Assay Kit) matched the present study's high specificity results (96–100%). All of the evaluated alternative RT-PCR SARS-CoV-2 assay kits and the internationally approved kit had a sensitivity of 92.89–100%, as specified on the manufacturer's package insert, which matched the present study's high sensitivity results (76–96%).

Interestingly, the inclusion of the *N* target gene on these purchased three alternative commercial RT-PCR SARS-CoV-2 assay kits as well as the Thermo Fisher TaqPath™ COVID-19 Assay Kit could be one of the main contributions that helped improve the sensitivity of these assay kits. According to published studies, the *N* gene is the most sensitive target gene for detecting SARS-CoV-2 because it contains a greater number of subgenomic *N* gene messenger RNAs compared to other target genes. [31,32]. Furthermore, the *ORF1ab* gene has been identified as the target gene with the highest contribution to specificity in the RT-PCR assay kits for the detection of SARS-CoV-2 infection. This is because the *ORF1ab* gene is the most conserved compared to other target genes such as the *N* or *E* genes [33,34]. The results demonstrated a good match between all four alternative commercial RT-PCR SARS-CoV-2 assays kits and the internationally approved Thermo Fisher TaqPath™ COVID-19 Assay Kit (100% sensitivity and 100% specificity) and can therefore be recommended for use interchangeably in routine diagnostic laboratories.

When comparing the costs of the Thermo Fisher TaqPath™ COVID-19 Assay Kit and alternative commercial RT-PCR SARS-CoV-2 assay kits, the Thermo Fisher TaqPath™ COVID-19 Assay Kit (USD ~14.80 per sample) was the most expensive of all of the commercial RT-PCR SARS-CoV-2 assay kits, with alternative commercial RT-PCR SARS-CoV-2 assay kit prices ranging between a USD ~4.44 and USD ~9.83 cost per sample. In terms of the observed run time, there seemed to be little difference between the Thermo Fisher TaqPath™ COVID-19 Assay Kit and the other three commercial RT-PCR SARS-CoV-2 kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit), and the turn-around time was shorter for all these methods. Despite having the longest run time (137 min) of all the evaluated commercial RT-PCR SARS-CoV-2 assay kits, the PCMLD nCov One-Step RT-PCR Kit had some advantages, being cheaper and having a higher diagnostic sensitivity and specificity. For these reasons, the use of a Thermo Fisher TaqPath™ COVID-19 Assay Kit or low-cost alternative commercial RT-PCR SARS-CoV-2 assay kits may help to increase the mass testing for SARS-CoV-2 infection within a limited resource laboratory setting.

The sample size was one of the study's limitations due to the challenge of a limited cost budget. Therefore, a larger sample size is required for future studies in order to validate the current study results. Nonetheless, we believe that useful information has been established. Furthermore, this work is not only relevant for SARS-CoV-2 testing in LMICs, but also to the fact that scientists predict more pandemics as there is increased interaction between the environment, wildlife, and humans [35,36]. This then warrants that LMICs prepare for such, and one of the requisites for preparedness is cost-effective laboratory testing capabilities.

4. Material and Methods

This was a retrospective study to compare various methods to identify cost-effective methods that can be used by low-middle income countries. This study used residual nasopharyngeal swab samples from adult (over the age of 18) male and female participants who had SARS-CoV-2 symptoms or not. The study was approved by the University of KwaZulu Natal Biomedical Research Ethics Committee (BREC/00003671/2021) with permission to use residual nasopharyngeal samples with blinding to protect patient identity

from both the BREC and the Global Health Innovation (GHI) laboratory, a subsidiary of the Aurum Institute, Johannesburg, South Africa.

4.1. Clinical Specimens

A total of 50 residual nasopharyngeal swab samples were used, which were initially collected from South African patients to test for SARS-CoV-2 infection by trained health care workers. Dry sterile nasopharyngeal swabs were used to test for SARS-CoV-2 infection, and the samples were transported in a cooler bag with ice to the GHI laboratory. Furthermore, for the comparison of methods in this study, the participants' residual nasopharyngeal swab samples that were in deionized water and first tested for SARS-CoV-2 infection by the GHI laboratory were used. The GHI laboratory is accredited by the South African National Accreditation System (SANAS) for diagnostic testing. The 50 residual samples were subdivided into two groups: (i) Group 1: 25 nasopharyngeal residual samples from the SARS-CoV-2 infected patients (positive group), and (ii) Group 2: 25 nasopharyngeal residual samples from the SARS-CoV-2 uninfected patients (control group). All specimens were processed in a biosafety cabinet level 3 (BSL 3) facility with full personal protective equipment (PPE).

This study compared the RNA extraction methods and commercially available SARS-CoV-2 RT-PCR assay kits using the same residual nasopharyngeal swab samples. The standard operating procedure in this study was normalized by using the same residual nasopharyngeal swab samples stored under the same conditions. In addition, the residual nasopharyngeal swabs used for method comparison were chosen from participants with almost identical Ct values for the SARS-CoV-2 target genes (*N*, *S*, *ORF1ab*) and internal control (MS2) as well as the high viral load for the positive group. Participants with almost identical Ct values for MS2 (internal control) were also chosen for the negative group. The residual nasopharyngeal swab samples were stored in a locked −80 °C bio-freezer to ensure the stability and integrity of the samples.

4.2. Comparison of SARS-CoV-2 RNA Extraction Methods

The Thermo Fisher PureLink™ Kit was chosen in this study as the gold standard method for the comparison of RNA extraction methods (Lucigen QuickExtract™ RNA Extraction Kit, Bosphore EX-Tract Dry Swab RNA Solution, and Sonicator method) because of its superior clinical accuracy and it is an internationally approved kit. Furthermore, the Thermo Fisher TaqPath™ COVID-19 Assay Kit was also chosen as the gold standard assay kit for assessing the efficacy of each RNA extraction method and as a comparison method for assessing the clinical performance of alternative commercially available SARS-CoV-2 RT- PCR assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES™ COVID-19 qPCR I Kit, PCL COVID19 Speedy RT-PCR Kit, and PCLMD nCoV One-Step RT-PCR Kit) because of the following advantages: (i) The TaqPath™ COVID-19 Assay Kit can detect SARS-CoV-2 infection by identifying the presence of three gene targets from the virus's *S*, *N*, and *ORF1ab* regions [37]; (ii) even if one of the targets is altered by a mutation, the test can provide reliable results, and (iii) the World Health Organization (WHO), Food and Drug Administration (FDA, Centers for Disease Control and Prevention (CDC), and European Centers for Disease Control (ECDC) have acknowledged the Thermo Fisher TaqPath Assay for using the S-gene target failure (SGTF) of PCR assays as a proxy for the variation aided in the diagnosis of Omicron [38,39]. Therefore, it is an internationally approved kit.

The RNA extraction methods used for comparison purposes include the Thermo Fisher PureLink™ Kit, an internationally approved kit, and the alternative extraction methods (Lucigen QuickExtract™ RNA Extraction Kit, Bosphore EX-Tract Dry Swab RNA Solution, and Sonicator method). Initially, 5 µL MS2 was added as an internal control to all of the Eppendorf microtubes (Merck, Darmstadt, Germany) containing nasopharyngeal swab samples in 300 µL deionized water. Then, the mixture was vortexed (Scientific Industries Inc., Bohemia, NY, USA) for 2 min to homogenize the samples. Thereafter, the homogenized

samples were further used for the comparison of the methods (RNA extraction methods and commercially available SARS-CoV-2 RT-PCR assay kits).

4.2.1. Thermo Fisher PureLink™ Kit

RNA was extracted according to the manufacturer's instructions using the Thermo Fisher PureLink™ Kit (Thermo Fischer Scientific, Pleasanton, CA, USA, Cat No. A47813 and A47814). A total of 200 µL of the homogenized samples were used for manual RNA extraction. The procedures for RNA extraction with this kit included proteinase K buffer digestion, the addition of the binding bead solution, washing of the beads three times, and elution of the nucleic acid (RNA). The working sample volume of the extracted and eluted RNA was 50 µL. The Applied Biosystems Real-Time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA, USA) was used for the amplification and detection of SARS-CoV-2 target genes (*S*, *N*, and *ORF1ab*).

4.2.2. Lucigen QuickExtract™ RNA Extraction Kit

Following the manufacturer's instructions, RNA was extracted using the Lucigen, QuickExtract™ RNA Extraction Kit (LGC Biosearch Technologies, Parmenter St Middleton, WI, USA, Cat No. QER090150) with the minor protocol modification described below: 20 µL of the homogenized nasopharyngeal swab samples was added to MicroAmp 8-tube strips with 20 µL of the Lucigen, QuickExtract™ RNA Extraction solution to extract RNA. To inactivate the virus, the extracted sample was placed on the Applied Biosystems heat cycler for 5 min at 95 °C. The Applied Biosystems real-time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA USA) was used for the amplification and detection of SARS-CoV-2 target genes from the extracted RNA samples.

4.2.3. Bosphore EX-Tract Dry Swab RNA Solution

RNA was extracted from the sample using the Bosphore EX-Tract Dry Swab RNA solution as per the manufacturer's instructions, with the following protocol modification: 20 µL of the homogenized nasopharyngeal swab samples was added to MicroAmp 8-tube strips with 20 µL Bosphore lysis buffer solution (Anatolia Geneworks, New Ash Green Longfield, England, Cat No. CS-003) to extract the RNA. The extracted sample was placed on the Applied Biosystems thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA) at 95 °C (5 min) to inactivate the virus. The Applied Biosystems real-time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA, USA) was used for the amplification and detection of SARS-CoV-2 target genes from the extracted RNA samples.

4.2.4. Sonicator Method

The Sonicator method was used to extract the RNA from the homogenized nasopharyngeal swab sample. The procedures described below were used to obtain a high-quality RNA extract using this extraction method: Eppendorf microtubes (Merck, Darmstadt, Germany) containing homogenized nasopharyngeal swab samples were placed on a dry heating block (Thermo Fisher Scientific, Waltham, MA, USA) at 65 °C for 10 min to inactivate the virus. Thereafter, the samples were processed for 15 min at 65 °C (40 kHz) with an Ultra Bath Sonicator (RS PRO, Europe) to lyse the cells and extract RNA. The Sonicator temperature was maintained by using a thermometer. The sonicated samples were centrifuged at 179× *g* for 1 min (Eppendorf, Fisher Scientific, USA). Approximately 20 µL of the extracted RNA supernatant was transferred into another empty MicroAmp 8-tube strip (Applied Biosystem, Thermo Fischer Scientific, China), and the SARS-CoV-2 target genes were amplified and detected using a real-time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA, USA).

4.3. Detection of SARS-CoV-2 Genes Using RT-PCR

The Thermo Fisher TaqPath™ COVID-19 Assay Kit (Thermo Fischer Scientific, Pleasanton, CA, USA, Cat No. A47813 and A47814), an internationally approved kit, Applied Biosystems

real-time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA USA), and Quant-Studio Design & Analysis Software (Thermo Fisher Scientific, Waltham, MA, USA) were used to assess the RNA yield from each extraction method for the testing of SARS-CoV-2 infection. The Thermo Fisher TaqPath™ COVID-19 Assay Kit is a multiplex diagnostic solution that contains both the assays and controls needed for the RT-PCR detection of SARS-CoV-2 viral RNA. The Thermo Fisher TaqPath™ COVID-19 Assay Kit targets the *S* gene, *N* gene, and *ORF1ab* gene of SARS-CoV-2 and *MS2* (internal control). Approximately, 6.3 µL of extracted RNA was added to 2 µL of 4X Taqpath 1-step multiplex Master Mixture (mix) (Thermo Fischer Scientific, Pleasanton, CA, USA), 0.3 µL of the probe, 21.4 µL nuclease-free water, and 2 µL MS2 (added only to the housekeeping control). The 4× Taqpath master mix (7.5 µL for 30 µL) already contains probes. The total volume used per reaction was 30 µL. Conditions for the Applied Biosystems real-time thermal cycler included one cycle of 2 min at 25 °C (incubation), 10 min at 53 °C (reverse transcription), 2 min at 95 °C (activation of the Taq DNA polymerase), followed by 40 cycles of 3 s at 95 °C (denaturation) and 30 s at 60 °C (anneal/extension). The results were analyzed using Quant-Studio Design & Analysis Software (Madison, WI, USA).

4.4. Comparison of the Five Commercially Available SARS-CoV-2 Real-Time PCR Assay Kits

4.4.1. Selection Criteria for RT-PCR Assay Kits

The following criteria were used to select the commercially available SARS-CoV-2 RT-PCR kits used in this study: (a) the assay kits could use RNA samples extracted using any manual nucleic acid extraction methods; (b) the assay kits could be performed on a Applied Biosystems real-time thermal cycler; (c) the assay kits were available on the market and could be obtained in less than 4 weeks; (d) diagnostic laboratories in LMICs should be able to afford the assay kits; and (e) the assay kits had already obtained CE-IVD certification.

4.4.2. RT-PCR Laboratory Procedure

In the method comparison of commercially available RT-PCR SARS-CoV-2 assay kits, the evaluated Lucigen QuickExtract™ RNA Extraction Kit was chosen as the preferred method for RNA extraction. This choice was made after it was discovered in this study (Section 4.2.2) that this extraction was cheaper and faster. Five commercially available RT-PCR SARS-CoV-2 assay kits from different manufacturers were selected in this study for the method comparison including the Thermo Fisher TaqPath™ COVID-19 Assay Kit (Thermo Fischer Scientific, Pleasanton, CA, USA), an internationally approved kit, and the four alternative RT-PCR SARS-CoV-2 assay kits: Nucleic Acid COVID-19 Test Kit (SARS-CoV-2) (Wuhan Easy-diagnosis Biomedicine, Wuhan, China), abTES™ COVID-19 qPCR I Kit (Anatech Instrument (PTY) LTD, Meadowbrook, Business Estate, Sloane Park, Gauteng, South Africa), PCL COVID19 Speedy RT-PCR Kit (PCL Inc. Multiplex In Vitro Diagnostic Global Leader, Seoul, South Korea), and the PCLMD nCoV One-Step RT-PCR Kit (PCL Inc. Multiplex In Vitro Diagnostic Global Leader, Seoul, South Korea). Positive and negative controls were included in each test run to ensure that the results were accurate and reliable. All of the commercial RT-PCR SARS-CoV-2 assay kits were compatible with the Applied Biosystems real-time thermal cycler (RT-PCR) instrument (Thermo Fisher Scientific, Waltham, MA, USA).

Thermo Fisher TaqPath™ COVID-19 Assay Kit

The Thermo Fisher TaqPath™ COVID-19 Assay Kit (Thermo Fischer Scientific, Pleasanton, CA, USA) methodology is described in full detail in Section 2.3.

Nucleic Acid COVID-19 Test Kit (SARS-CoV-2)

This is a reverse transcription, multiplex, one-step RT-PCR assay kit designed to detect different SARS-CoV-2 specific target genes in a single tube well. A total of 25 µL of the reaction mixture was tested, with 20 µL of Master mix and 5 µL of extracted RNA sample or SARS-CoV-2 positive control or negative control. The Applied Biosystems real-time thermal cycler was used for the amplification and detection of the SARS-CoV-2 target genes.

The condition of the PCR instrument included one cycle of 15 min at 50 °C for reverse transcription, 30 s at 95 °C for pre-degeneration; 45 cycles of 3 s at 95 °C for degeneration, and 45 s at 60 °C for annealing and extension.

abTES™ COVID-19 qPCR I Kit

This commercially available test kit is a qualitative, multiplex real-time polymerase chain reaction (qPCR) kit that allows for the simultaneous detection of two SARS-CoV-2 specific targeted genes in a single reaction. Sample reagents required for the preparation of a 20 µL reaction mixture included 10 µL of 2× RT-PCR Master mix, 1 µL of RT/ Taq enzyme mix, 2 µL of Primer/Probe mix, 2 µL of nuclease-free water, and 5 µL of RNA Template from the patient sample or negative control or the SARS-CoV-2 positive control. The SARS-CoV-2 target genes were amplified and detected using an Applied Biosystems real-time thermal cycler. The RT-PCR instruments were set up as follows: One cycle of 10 min at 59 °C for cDNA synthesis, 2 min at 95 °C for initial denaturation, and 45 cycles of 10 s at 95 °C and 30 s at 57.5 °C for amplification and extension.

PCL COVID-19 Speedy RT-PCR Kit

The assay kit is a one-step multiplex RT-PCR kit designed to identify two SARS-CoV-2 target genes simultaneously in a single tube. The reaction mixture volume of 20 µL contained 5 µL of Master mix, 2 µL of Primer + Probe mix, 8 µL of nuclease-free water, and 5 µL extracted RNA sample or the negative control and positive control. Using an Applied Biosystems real-time thermal cycler, the SARS-CoV-2 target genes were amplified and identified. The RT-PCR instruments were programmed as one cycle of 5 min of cDNA synthesis at 50 °C, 2 min of initial denaturation at 95 °C, and 40 cycles of 5 s at 95 °C and 30 s at 55 °C for amplification and extension.

PCLMD nCoV One-Step RT-PCR Kit

The assay kit is a one-step, qualitative RT-PCR kit for the detection of SARS-CoV-2. This assay kit requires three types of master mixture for each sample being tested (one test tube for one gene). (i) PCR tube 1: The 20 µL reaction mixture consisted of 5 µL of Master mix, 2 µL Primer + Probe Mixture 1 (confirmatory target gene for SARS-CoV-2 infection), 8 µL nuclease-free water, and 5 µL RNA sample; (ii) PCR tube 2: The 20 µL reaction mixture consisted of 5 µL of Master mix, 2 µL Primer + Probe Mixture 2 (for screening), 8 µL nuclease-free water, and 5 µL RNA sample; (iii) PCR tube 3: The 20 µL reaction mixture consisted of 5 µL of Master mix, 2 µL of IC Primer + Probe mix (internal control), 8 µL of nuclease-free water, and 5 µL RNA sample. The PCR reaction was performed using an Applied Biosystems real-time thermal cycler under the following conditions: One cycle of 30 min at 50 °C (cDNA synthesis), 10 min at 95 °C (initial denaturation) and 40 cycles of 15 s at 95 °C and 1 min at 55 °C (amplification and extension).

4.5. Statistical Analysis

The data generated by the RT-PCR Quant-Studio Design & Analysis Software were analyzed using GraphPad Prism 5. Since the data had a normal distribution, the continuous variables were presented as the mean and standard deviation. The categorical variables were presented in percentages and numbers. The paired *t*-test was used to assess whether there was a statistically significant difference between the alternative RNA extraction methods and the gold standard RNA extraction method by comparing the mean Ct value of the SARS-CoV-2 target genes (*N*, *S*, *ORF1ab*) and internal control (MS2). The calculated sensitivity and specificity for each method were used to assess and compare the clinical diagnosis between the alternative methods and the internationally approved kits for testing SARS-CoV-2 infection. A $p < 0.05$ was considered as statistically significant.

5. Conclusions

In conclusion, the present study found that alternative methods were cheaper, simpler, and faster and that they could be used interchangeably with internationally approved kits (Thermo Fisher PureLink™ Kit and Thermo Fisher TaqPath™ COVID-19 Assay Kit). All alternative RNA extraction methods (Lucigen QuickExtract™ RNA Extraction Kit, Bosphore EX-Tract Dry Swab RNA Solution, and Sonicator method) and all four commercial RT-PCR SARS-CoV-2 assay kits (Nucleic Acid COVID-19 Test Kit (SARS-CoV-2), abTES™ COVID-19 qPCR I Kit, PCL COVID-19 (Speedy RT PCR), and PCMLD nCov One-Step RT-PCR) can be recommended for routine diagnostic use because of their excellent performance in identifying positive samples. Furthermore, implementing these cost-effective alternative methods in LMIC laboratories will help to expand the testing capacity for the mass testing of SARS-CoV-2 infection. This will allow for the early detection of infected individuals in the community. As a result, controlling and preventative measures can be implemented sooner to avoid the spread of SARS-CoV-2 infection.

Author Contributions: Z.L.M.-K., A.A.C. and V.R.—supervision; Z.D., Z.L.M.-K., A.A.C., V.R., V.A.E. and P.N.—manuscript conceptualization; Z.D., Z.L.M.-K., A.A.C., V.R., V.A.E. and P.N.—visualization; Z.L.M.-K., A.A.C., V.R. and Z.D.—methodology; Z.D., Z.L.M.-K., A.A.C., V.R., V.A.E. and P.N.—formal analysis; Z.D.—performed the experiment and original draft preparation; VE—granted permission for the use of COVID-19 residual samples from the GHI laboratory; Z.L.M.-K., A.A.C., V.R., V.A.E. and P.N.—writing: review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was approved by the University of KwaZulu Natal Biomedical Research Ethics Committee (Protocol number: BREC/00003671/2021) on the 7 April 2022.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Furthermore, we had no affiliation with the manufacturers of the RNA extraction kits/reagents and SARS-CoV-2 assay kits that we purchased.

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APPENDIX C

Approved BREC Ethical Clearance Certificate

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

07 April 2022

Ms Zamathombeni Duma (204000765)
School of Lab Med & Medical Sc
Medical School

Dear Ms Duma,

Protocol reference number: BREC/00003671/2021
Project title: Epidemiology and alternative approaches for SARS-Cov-2 testing within limited resources settings
Degree: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 07 April 2022. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Amended_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 07 April 2022. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 10 May 2022.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
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